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APRIL 1, 1902.

NO. I.

ON THE EFFECTS OF SOLUTIONS OF VARIOUS ELECTROLYTES AND NON-CONDUCTORS UPON RIGOR MORTIS AND HEAT RIGOR.

BY ANNE MOORE.

[From the Hull Physiological Laboratory of the University of Chicago, Ill.]

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INTRODUCTION.

SINCE the classic work of Kühne connected the occurrence of rigor mortis with the coagulation of proteids found in muscle plasma, the specific proteids present there have been subjected to repeated examination.¹ As a result of the adoption by Halliburton and

¹ HALLIBURTON, W. D.: Journal of physiology: 1884, v, p. 152; 1885, vi, p. 300; 1887, viii, p. 133; 1888, ix, p. 229; 1894, xv, p. 90; STEWART and SOLLMAN: Journal of physiology, 1899, xxiv, p. 427; v. FÜRTH, O.: Archiv für experimentelle Pathologie und Pharmakologie, 1895, xxxvi, p. 231; 1896, xxxvii, p. 389; Zeitschrift für physiologische Chemie, 1900, xxxi, 3 and 4, p. 338.

others of the method of fractional heat precipitation, which presupposes that each proteid has a definite coagulation temperature, the possibility of altering the supposed specific temperatures by the addition of salts of different kinds and concentrations to a solution containing the proteids in question has received more or less attention.

Although investigators do not agree as to the cause of coagulation,¹ it is generally accorded that the coagulation temperatures of the different proteids may be modified considerably by the presence of a salt and by the reaction of the solution.

The present work was undertaken at Dr. Loeb's suggestion and carried out under his direction to ascertain the effects of certain solutions upon (a) the temperature for the sudden coagulation of proteids in the muscle *in situ*, (b) the time necessary for coagulation at room temperature.

ON THE EFFECTS OF SOLUTIONS UPON THE COAGULATION TEMPERATURE OF MUSCLE SUBSTANCE.

Method.—The gastrocnemius of the frog was used throughout the experiments. The muscle was placed in a covered dish containing 50 c.c. of the solution to be tested and was allowed to stand one hour at room temperature. It was then arranged for graphic registration upon the kymograph. A glass rod bent at right angles was screwed into the support holding the lever. The lower end of the rod drawn out to a fine point passed through the tendon of Achilles. The upper end of the muscle was attached to the short arm of the lever by means of a silk thread tied to a copper hook, which passed through the tendon covering the end of the femur. The solution was transferred to a beaker and arranged so that it surrounded the glass rod with it, attached muscle without touching the copper hook. A source of error pointed out by Vernon² was thus avoided. He found that if a muscle were attached by a thread which was wet by the solution

¹ Hardy regards coagulation as an affair of physical conditions due to the separation of the solid particles in a colloidal solution from the liquid. The influence of electrolytes which cause the solid particles to increase in size by a process of clumping he explains on an electrical basis. *Journal of physiology*, 1899, xxiv, p. 158. Pauli, on the other hand, explains coagulation on a chemical basis. Coagulation caused by the addition of a salt he thinks due to the formation of an insoluble double salt by the union of the proteid and the salt molecule. *Archiv für die gesammte Physiologie*, 1899, lxxviii, p. 315.

² VERNON, H. M., *Journal of physiology*, 1899, xxiv, p. 239.

variations in the length of the thread might easily be taken for muscular contraction. The lever magnified seven times; very small changes in the length of the muscle could, therefore, be determined. The beaker containing the solution was immersed in a water bath heated by a Bunsen burner. Half an hour was allowed for the temperature to rise from 15° C. to 65° C. A thermometer was fastened with its bulb as near as possible to the muscle and the rise in temperature was marked by hand upon the drum, which revolved once during an hour. When the muscle was first attached, the lever remained stationary if an equilibrium had been produced between the muscle and the solution, if not, slight changes in the level occurred, depending upon the changes which were being produced by the solution. When heat was first applied, this action of the lever continued until the critical temperature was reached, when it began to rise.

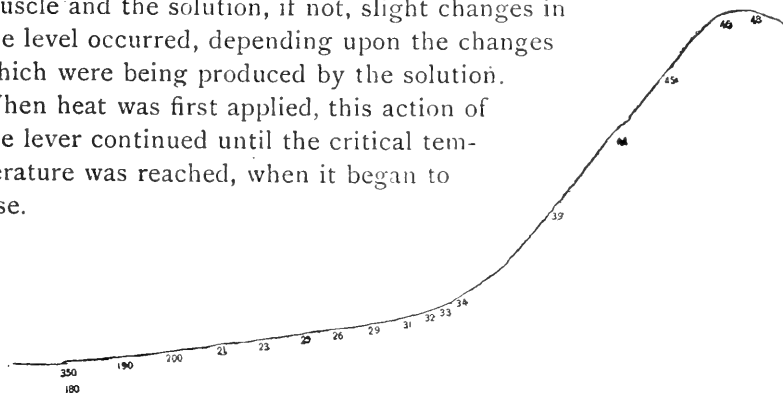


FIGURE 1. — Curve of heat rigor obtained in $\frac{2}{50}$ H₂SO₄.

The interpretation of curves.—The point at which the lever first began to rise varied from 15° to 41°. At about 47° it ceased to rise, at least for a time. In certain solutions it did not rise beyond this point (Fig. 1).

As a rule, after a short interval, a second rise began about 50° and continued to about 65° (Figs. 2 and 3). Occasionally a third elevation¹ appeared, but it was always slight when compared with the other two.

While the form of the curves is fairly constant in the same strengths of one solution, in differing solutions or in different strengths of the same solution much variation occurs.

In some solutions the rise was at first very gradual. This is interpreted as an indication of the stage described by workers on muscle plasma as the cloudy or opalescent stage which occurs before a defi-

¹ VERNON, working by the same method, also distinguishes three such contractions.

nite coagulum is formed.¹ Coagulation proper is marked by a sudden change in the angle of the curve. Figs. 2 and 3 serve as an illustration. In Fig. 3 coagulation occurs suddenly without a preliminary cloudy stage; in Fig. 2 the curve rises gradually, indicating a pronounced cloudy stage. At 33° it turns sharply, marking the occurrence of coag-

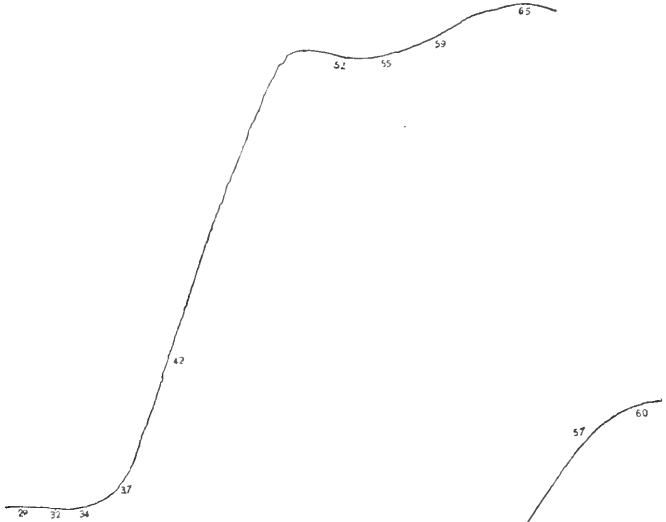


FIGURE 2.—Curve of heat rigor obtained in $\frac{27}{1000}$ trichloroacetic acid.

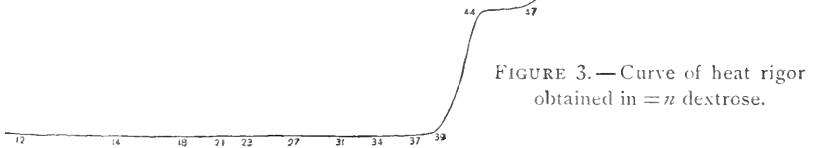


FIGURE 3.—Curve of heat rigor obtained in 22 dextrose.

ulation. A short time after the culminating point was reached the lever dropped suddenly. Strong salt solutions and solutions of acids and alkalis have a decidedly deleterious effect upon the tendon of Achilles, so that its connection with the muscle becomes impaired to such an extent that the two abruptly part as the temperature in-

¹ HAYCRAFT and DUGGAN, *British medical journal*, 1890, i, p. 167. CHITEND and WYCKOFF-CUMMINS, *Centralblatt für Physiologie*, 1890, iii, p. 16. RINGER and SAINSBURY, *Journal of physiology*, 1890, xi, p. 369; 1891, xii, p. 170.

creases. In this case the gradual rise occurred in a very weak solution; it might be argued therefore that it indicates not opalescence, but merely absorption of water. This gradual rise is, however, not a distinguishing characteristic of weak solutions. In addition to this variation in the curve due to the occurrence or the suppression of the opalescent stage, other variations occur due chiefly to the relative

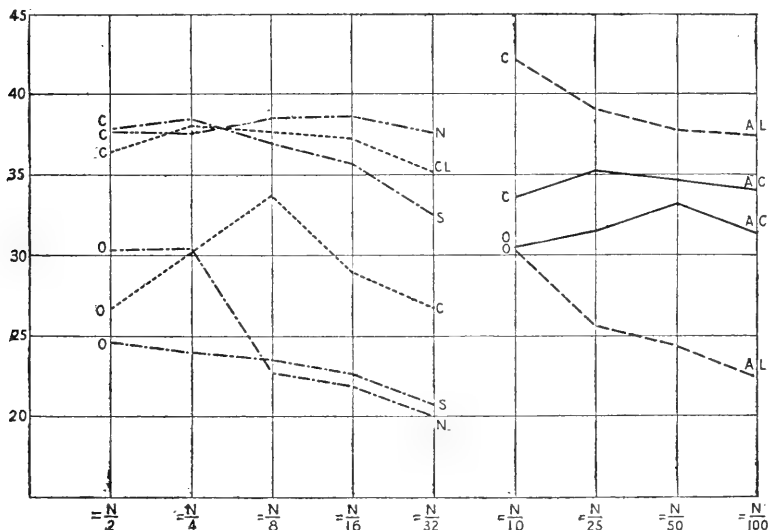


FIGURE 4.—Plotted curve showing average temperature of opalescence and coagulation in the various solutions. The temperature is indicated on the ordinate, the concentration on the abscissa.

- C ————— AC = coagulation temperature of acids.
- C - - - - - AL = coagulation temperature of alkalies.
- C - S = coagulation temperature of sulphates.
- C CL = coagulation temperature of chlorides.
- C - - - - - N = coagulation temperature of non-conductors.
- O - - - - - S = opalescence temperature of sulphates.
- O C = opalescence temperature of chlorides.
- O - - - - - N = opalescence temperature of non-conductors.
- O ————— AC = opalescence temperature of acids.
- O - - - - - AL = opalescence temperature of alkalies.

height of the two contractions and to the interval separating them. All gradations were present between those curves which showed only one elevation and those which showed two. Where two elevations were present, they were sometimes approximately equal, sometimes the second was very slight in comparison with the first, and sometimes this relation was exactly reversed.¹

¹ While the relative height of the sections of the curves cannot be taken as an accurate expression of the quantitative relations of the coagulated substances, at the

According to the accepted interpretation, each elevation in the curve corresponds to the coagulation of a definite proteid. If this is true, the variation in the number of elevations shown by my curves indicates a curious inconsistency. It is possible, however, that (1) a proteid may be so affected after a certain temperature is reached, that coagulation may be arrested for a time and then begin again, *i. e.*, it may be discontinuous; (2) the coagulation temperature of the second proteid may be so changed by the solution that it falls within the temperature limits of coagulation of the first. The results of the two coagulations are then added to each other algebraically, but the lever does not show where one ends and the other begins. This is suggested especially by a comparison of curves which are transitional between Figs. 1 and 2, and of curves obtained with different strengths of the same solution. The third contraction I find to be so very irregular, both as regards occurrence and temperature, and to be so insignificant as a rule in comparison with the other two that I am inclined to agree with Stewart and v. Fürth in believing that this contraction is due to the coagulation not of a third proteid but merely of a small remnant of uncoagulated myosinogen, and that there are only two proteids in muscle capable of coagulation.¹ In those curves which show only one contraction the coagulation temperature of the second proteid falls within the limits of the first. In those curves which show more than two contractions, the contraction of one or of both proteids has been made discontinuous. Hereafter these proteids will be referred to as the first and second proteid, the first coagulating at the lower temperature.

Results. — Pauli points out that the effects of salt solutions upon the coagulation temperature of proteids is a function of the physico-chemical peculiarities of both the salt solution and the colloid. They are not due merely to a general relation of colloids and crystalloids. He bases this opinion upon the fact that solutions of different salts

same time the comparison is interesting when taken in connection with the quantitative results of Stewart and v. Fürth. Stewart finds that the ratio of the amount of paramyosinogen to the amount of myosinogen present in muscles is 2.53, while v. Fürth finds that it is 0.3.

¹ Halliburton believes he has obtained four proteids from muscle plasma, paramyosinogen coagulating at 47° C., myosinogen at 56°, myoglobulin at 63°, muscle albumin at 73°, and myoalbumose not coagulable by heat.

Von Fürth and Stewart think that only two proteids have been isolated with certainty from muscle plasma, paramyosinogen, a typical globulin coagulable at 45°-50°, and myosinogen, an atypical globulin coagulating at 50°-65°.

having the same number of molecules in a given volume, or solutions of those which have the same degree of dissociation, do not affect similarly the coagulation temperature of egg-white, while two dilutions of the same salt may have the same effect upon the temperature. My results are in accord with this.

Pauli finds that an increase in the concentration of a dilute solution always causes an increase up to a certain point in the coagulation temperature. After the maximum point is reached the further addition of the salt causes no change, or an immediate fall in the coagulation temperature, or a gradual fall after a stationary period. Although I find this to be true in many solutions, I do not find it to be universal. In some dilute solutions, especially of acids, I found an actual decrease in the coagulation temperature with increasing strength; in other cases, the temperatures remained constant with increasing strength.

In general the entire height of the curve and also the height of the separate sections of the curve increase as the strength is decreased. This is true of sugar, urea, NaCl, LiCl, NH_4Cl , CaCl_2 , MgSO_4 , K_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$, NH_4OH , NaOH, KOH, LiOH, KHSO_4 , NaHSO_4 , tartaric, trichloroacetic, acetic, oxalic, valerianic, and formic acids. In the strongest solutions coagulation may already have begun, leaving less substance to coagulate on being heated, but it is more probable that in dilute solutions an increase in the amount of H_2O present renders the coagulum more compact, so that it occupies less space. In certain of the solutions variations from this general rule occur. In $\frac{1}{10}$ $\text{Ba}(\text{OH})_2$, for example, the first contraction is slight, the second very marked. As the strength is decreased, the first steadily increases in height as compared with the second. This is true also of $\text{Sr}(\text{OH})_2$, KOH, and certain strengths of Na_2SO_4 and Li_2SO_4 . In a few solutions, *e. g.*, HNO_3 , H_2SO_4 and HCl, a decrease in the height of the curve appears as the strength is decreased. In NH_4Cl the height of the curve remains the same.

The effect of non-conductors. — If a perfectly fresh muscle be heated in a moist chamber, contraction will begin gradually at 29° and will continue to 47° , a sharp rise of the lever occurring at 37° . Later a second contraction begins at 51° and continues to 65° . After a muscle has remained in H_2O one hour at room temperature, the first contraction begins at 22° and continues to 46° , a sharp rise of the lever occurring at 32° ; the second begins at 56° and continues to 62° . Considering the first contraction alone, which is all we shall attempt

to do in this paper, it seems that H_2O distinctly lowers both the temperature of opalescence and the coagulation temperature of the first proteid in muscle. This is what would naturally be expected, as coagulation occurs so readily in H_2O . Pauli, however, states that there is no simple relation between the coagulating power of a solution and the change in the coagulation temperature.

Pauli finds that urea and sugar have an effect similar to that of distilled water. This would naturally be true of a substance like urea where the molecules pass as freely in one direction as the other. The sugar molecule does not pass so readily, therefore somewhat different results might be expected. And, indeed, I find some difference in the action of the two substances. Glycerine and urea in all concentrations lower the temperature of opalescence. Weak solutions of dextrose and cane sugar also lower it, but strong solutions of dextrose and cane sugar raise it. Increase in concentration causes a sudden increase in the temperature of opalescence in urea, dextrose, and sugar, while the effect of glycerine is practically unaffected by the strength of the solution. The temperature of coagulation is slightly lowered by urea, and slightly raised by glycerine, dextrose, and sugar. A change of concentration in the various solutions produces very little effect on the temperature of coagulation.

The effect of electrolytes: Acids. — As a rule acids cause an increase in the temperature of opalescence. $\frac{n}{10}$ trichloroacetic, $\frac{n}{10}$ tartaric, $\frac{n}{100}$ HCl and various strengths of HNO_3 lower it, however. In some of the acids, *c. g.*, valerianic, acetic, formic, H_2SO_4 , very little difference results from a change in concentration; in others, however, there is a decided variation. In most cases, increase in concentration causes at first a slight increase in the opalescence temperature, and later a decided decrease. With the exception of certain strengths of acetic, trichloroacetic, valerianic and formic acids, the temperature of coagulation was lowered by acids. The effect of increasing concentration upon the temperature of coagulation varied greatly. A comparison of the relative effects of acids shows that the change in temperature produced by them is largely dependent upon ionization. The greater the degree of dissociation of the acid, the more the temperature is lowered. At a dilution of 110, Dr. Loeb¹ gives the degree of dissociation of the acids used as follows:

¹ LOEB, J.: Archiv für die gesammte Physiologie, 1897, lxi, p. 1; 1898, lxxi, p. 457.

Valerianic	0.04	H ₂ SO ₄	0.80
Acetic	0.04	Oxalic	0.92
Formic	0.14	Trichloroacetic	0.94
Malic	0.23	HCl	0.95
Tartaric	0.27	HNO ₃	0.95

A comparison with Table II shows how closely this order is followed in the lowering of temperature. Apparently, therefore, the H-ion is responsible for the lowering of temperature. Dr. Loeb found, how-

TABLE I.

The temperature C. of opalescence (column *a*) and of coagulation (column *b*) after the muscle has remained in the given solution one hour at room temperature.

$= \frac{5}{4} n^1$		$= n$		$= \frac{n}{2}$		$= \frac{n}{4}$		
	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>
Cane sugar	40°	40°	Cane sugar	37° 38°	Dextrose .	38° 40°	Dextrose .	35° 39°
Urea . . .	22°	33°	Dextrose .	36° 39°	Cane sugar	36° 37°	Cane sugar	36° 39°
			Urea . . .	26° 35°	Urea . . .	26° 36°	Urea . . .	27° 35°
					Glycerine .	21° 38°	Glycerine .	21° 38°
$= \frac{n}{8}$			$= \frac{n}{16}$			$= \frac{n}{32}$		
	<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>
Dextrose . .	28°	40°	Dextrose . .	25°	?	Cane sugar .	21°	37°
Cane sugar .	22°	40°	Cane sugar .	23°	41°	Urea	19°	38°
Glycerine . .	22°	38°	Glycerine . .	21°	38°			
Urea	19°	36°	Urea	19°	37°			

¹ The sign = is used here and throughout the paper to indicate solutions isosmotic with NaCl of the given strength.

ever, that the addition of H-ions to NaCl caused an increase in the taking up of water. This fact taken in connection with the power of H₂O to lower the coagulation temperature indicates that the effect of acids, though largely determined by the H-ion, may not be entirely independent of water absorption.

Alkalies.—The temperature of opalescence was much lowered by the alkalies used, except in the concentrations $\frac{n}{10}$, $\frac{n}{4}$, $\frac{n}{2}$, where it was raised. As the concentration is increased the temperature in-

creases until $\frac{n}{10}$ is reached, after which further increase in concentration causes a decrease in the temperature. The temperature of coagulation is raised by alkalis except in certain strengths of NaOH, KOH and Ba(OH)₂. Much variation in the temperature of coagulation was caused by increase in concentration. In order to further test the effects of acids and alkalis, 3 c.c. of $\frac{n}{5}$ NaOH and of $\frac{n}{5}$ HNO₃ were added to 100 c.c. $\frac{n}{8}$ NaCl and the results obtained in these solutions were compared with those obtained in $\frac{n}{8}$ NaCl. It was

TABLE II.

The temperature C. of opalescence (column *a*) and of coagulation (column *b*) after the muscle has remained in the given solution one hour at room temperature.

ACIDS.											
$\frac{2n}{10}$			n				$\frac{n}{2}$		$\frac{n}{4}$		
	<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>	
HNO ₃	22°	27°	Acetic	36°	38°	Acetic	37°	37°	Acetic	37°	37°
			H ₂ SO ₄	31°	35°	H ₂ SO ₄	30°	31°	H ₂ SO ₄	31°	31°
			HNO ₃	28°	31°	HNO ₃	26°	31°	HNO ₃	25°	27°
$\frac{n}{10}$			$\frac{n}{25}$				$\frac{n}{50}$		$\frac{n}{100}$		
	<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>	<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>	
Valerianic	38°	42°	Valerianic	37°	40°	Valerianic	37°	39°	Valerianic	36°	37°
Acetic	36°	39°	Acetic	36°	38°	Malic	36°	37°	Trichloracetic	35°	35°
Formic	34°	39°	Formic	35°	40°	Acetic	35°	35°	Formic	34°	37°
Malic	33°	38°	Malic	34°	37°	Formic	35°	36°	Oxalic	34°	36°
H ₂ SO ₄	30°	31°	Oxalic	31°	33°	Oxalic	34°	37°	Acetic	33°	39°
NaHSO ₄	30°	30°	Tartaric	30°	34°	Tartaric	34°	36°	HNO ₃	32°	32°
KHSO ₄	30°	30°	K ₂ SO ₄	30°	32°	HCl	33°	36°	KHSO ₄	31°	31°
Oxalic	29°	30°	NaHSO ₄	30°	30°	H ₂ SO ₄	32°	35°	NaHSO ₄	31°	31°
HCl	29°	31°	KHSO ₄	30°	30°	Trichloracetic	32°	32°	H ₂ SO ₄	30°	32°
Trichloracetic	27°	29°	Trichloracetic	29°	47°	NaHSO ₄	31°	31°	Malic	29°	29°
HNO ₃	27°	27°	HCl	29°	33°	KHSO ₄	31°	31°	Tartaric	29°	36°
Tartaric	24°	36°	HNO ₃	27°	28°	HNO ₃	29°	32°	HCl	24°	35°

found that in the acid solution the temperature of both coagulation and opalescence was lowered; in the alkaline solution it was raised. The effects of alkalis upon temperature are thus seen to be exactly the opposite of those of acids. The effect of alkalis may be further shown by a comparison of the action of $\frac{n}{10}$ NaOH with that of the Na salts used isosmotic with $\frac{n}{8}$ NaCl. The OH ion elevates the coagulation temperature. This result indicates that the effects of the OH

TABLE III.

The temperature C. of opalescence (column *a*) and of coagulation (column *b*) after the muscle has remained in the given solution one hour at room temperature.

ALKALIES.											
$2n$		n				$\frac{n}{2}$		$\frac{n}{4}$			
	<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>
NaOH	26°	27°	NaOH	30°	33°	NaOH	34°	37°	KOH	35°	38°
KOH	24°	26°	KOH	27°	30°	KOH	31°	33°	NaOH	32°	38°
$\frac{n}{10}$		$\frac{n}{25}$				$\frac{n}{50}$		$\frac{n}{100}$			
	<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>
Sr(OH) ₂	39°	45°	KOH	29°	39°	LiOH	28°	39°	Ba(OH) ₂	26°	38°
NaOH	37°	41°	LiOH	28°	39°	NaOH	28°	39°	NaOH	24°	32°
KOH	34°	40°	NaOH	27°	36°	Ba(OH) ₂	25°	32°	LiOH	22°	39°
Ba(OH) ₂	32°	44°	Sr(OH) ₂	26°	39°	KOH	23°	41°	KOH	21°	?
LiOH	30°	41°	Ba(OH) ₂	22°	38°	NH ₄ OH	22°	37°	Sr(OH) ₂	21°	39°
			NH ₄ OH	22°	44°	Sr(OH) ₂	20°	39°	NH ₄ OH	21°	39°

ion are specific effects independent of the entrance of water, for the presence of the OH ion as well as that of the H-ion causes increased absorption of H₂O.

Salts. — The chlorides as a rule lower the opalescence temperature, except when used in a strength isosmotic with $\frac{n}{8}$ NaCl, when the temperature is raised. CaCl₂ is a notable exception to this. It causes decided elevation of the temperature of opalescence. This is somewhat surprising as the tendency seems to be for good coagulants to lower the temperature. It seems to support the opinion of Pauli referred to above, p. 7. The difference may be due to the fact that

in Ca solutions little water is absorbed, while in solutions which lower the temperature much water is absorbed. With a few exceptions the temperature of coagulation is raised. The chlorides are good illustrations of Pauli's suggestion that osmosis is insufficient to account for

TABLE IV.

The temperature C. of opalescence (column *a*) and of coagulation (column *b*) after the muscle has remained in the given solution one hour at room temperature.

CHLORIDES.														
$= \frac{5}{4} n$			$= n$				$= \frac{n}{2}$							
		<i>a</i>	<i>b</i>			<i>a</i>	<i>b</i>			<i>a</i>	<i>b</i>			
NH ₄ Cl . . .	43°	48°	NH ₄ Cl . . .	38°	40°	CaCl ₂ . . .	33°	37°						
MgCl ₂ . . .	39°	41°	MgCl ₂ . . .	37°	38°	KCl	29°	35°						
CaCl ₂ . . .	32°	40°	CaCl ₂ . . .	34°	39°	NH ₄ Cl . . .	27°	36°						
KCl	28°	54°	KCl	25°	57°	MgCl ₂ . . .	24°	36°						
NaCl	?	41°	NaCl	21°	58°	NaCl	19°	39°						
		<i>a</i>	<i>b</i>			<i>a</i>	<i>b</i>			<i>a</i>	<i>b</i>			
$= \frac{n}{4}$			$= \frac{n}{8}$				$= \frac{n}{16}$				$= \frac{n}{32}$			
KCl	37°	39°	MgCl ₂	36°	38°	CaCl ₂	35°	40°	CaCl ₂	33°	40			
CaCl ₂	35°	37°	NaCl	36°	38°	NaCl	33°	36°	NaCl	28°	35°			
MgCl ₂	31°	36°	LiCl	35°	37°	KCl	29°	36°	NH ₄ Cl	25°	30°			
NH ₄ Cl	27°	38°	KCl	35°	39°	MgCl ₂	27°	33°	MgCl ₂	24°	32°			
NaCl	21°	40°	CaCl ₂	33°	37°	NH ₄ Cl	27°	39°	LiCl	22°	38°			
			NH ₄ Cl	27°	38°	LiCl	22°	39°	KCl	21°	36°			

coagulation phenomena. As concentration increases, increase of the opalescence temperature occurs until $= \frac{n}{8}$ is reached. Beyond this point, further increase in concentration causes first an increase, then a decrease in temperature.

With the exception of MgSO₄, in concentrations isosmotic with $\frac{n}{8}$ and $\frac{n}{4}$, NaCl, the sulphates decidedly lower the opalescence temperature. Weak solutions with the exception of CaSO₄ lower also the coagulation temperature. Strong solutions with the exception of certain strengths of K₂SO₄ raise it. Other Ca precipitating solutions

also lower the opalescence temperature, and with the exception of NaF they lower the coagulation temperature. As Ca is supposed to play so important a rôle in coagulation phenomena, it is interesting to compare these results with those obtained in CaCl₂. The opalescence

TABLE V.

The temperature C. of opalescence (column *a*) and of coagulation (column *b*) after the muscle has remained in the given solution one hour at room temperature.

SULPHATES.										
$= \frac{5}{4} \mu$		$= \mu$				$= \frac{\mu}{2}$		$= \frac{\mu}{4}$		
	<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>		
MgSO ₄	21°	39°	MgSO ₄	21°	39°	Na ₂ SO ₄	29°	38°	MgSO ₄	31° 38°
Li ₂ SO ₄	21°	38°	Li ₂ SO ₄	21°	37°	MgSO ₄	26°	38°	Na ₂ SO ₄	26° 42°
Na ₂ SO ₄	21°	39°	K ₂ SO ₄	20°	33°	K ₂ SO ₄	25°	37°	K ₂ SO ₄	22° 36°
K ₂ SO ₄	19°	39°	Na ₂ SO ₄	19°	38°	Li ₂ SO ₄	22°	39°	Li ₂ SO ₄	21° 38°
(NH ₄) ₂ SO ₄	17°	39°	(NH ₄) ₂ SO ₄	19°	38°	(NH ₄) ₂ SO ₄	21°	37°	(NH ₄) ₂ SO ₄	20° 39°

$= \frac{\mu}{8}$		$= \frac{\mu}{16}$				$= \frac{\mu}{32}$		
	<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>
MgSO ₄	31°	37°	MgSO ₄	27°	33°	CaSO ₄	25°	40°
Li ₂ SO ₄	23°	37°	(NH ₄) ₂ SO ₄	23°	36°	K ₂ SO ₄	22°	36°
K ₂ SO ₄	22°	35°	K ₂ SO ₄	22°	37°	MgSO ₄	20°	27°
(NH ₄) ₂ SO ₄	21°	37°	Li ₂ SO ₄	22°	36°	Na ₂ SO ₄	20°	33°
Na ₂ SO ₄	20°	39°	Na ₂ SO ₄	20°	37°	(NH ₄) ₂ SO ₄	19°	24°
						Li ₂ SO ₄	18°	35°

OTHER CA-PRECIPIATING SOLUTIONS.										
$= \frac{\mu}{2}$		$= \frac{\mu}{4}$				$= \frac{\mu}{8}$		$= \frac{\mu}{16}$		
	<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>		<i>a</i>	<i>b</i>		
Na ₂ C ₂ O ₄	24°	35°	Na ₂ C ₂ O ₄	22°	35°	Na ₂ C ₂ O ₄	22°	32°	Na ₂ C ₂ O ₄	23° 34°
Na ₂ CO ₃	21°	43°	Na ₂ CO ₃	19°	?	NaF	20°	56°	NaF	22° 37°
NaF	19°	61°	NaF	19°	56°	Na ₂ CO ₃	19°	?	Na ₂ CO ₃	21° 32°

temperature is raised in CaCl_2 , lowered in Ca precipitating solutions. The coagulation temperature is little affected in CaCl_2 ; in most cases it is lowered in Ca precipitating solutions.

In determining the effect of external agents on heat rigor Vernon found that the temperature of onset of the initial heat contraction was lowered by hypotonic solutions and raised by hypertonic solutions. He thus makes the modification of the coagulation temperature of muscle proteids an affair of osmotic pressure. His opinion is based upon the use of NaCl alone. His experiments were too limited to allow the conclusion that osmosis is responsible for the results obtained. Undoubtedly the effects of osmosis are important. In those solutions in which much water is absorbed, there is a decided lowering in temperature, but in very strong solutions in which water is withdrawn the same results are often obtained. Osmosis therefore does not entirely explain the facts. In some cases the absorption of water seems to be a primary factor, and ionic effects secondary factors, merely governing the absorption of water, but the work of Hardy and Pauli has shown clearly the importance of electrolytes in coagulation phenomena.

ON THE EFFECT OF SOLUTIONS UPON THE TIME TAKEN BY
MUSCLE PROTEIDS TO UNDERGO COAGULATION AT
ROOM TEMPERATURE.

Method.—The muscles were fixed in position as described above and left immersed in 100 c.c. of the fluid to be tested. The room temperature remained fairly constant at about 20° C. during the day; at night it was somewhat lower than this. The changes produced by the solutions were registered upon a drum which was turned by hand from time to time. The graphic representation of the muscular contraction was not to be depended upon as the sole test of coagulation, for contraction may be a specific effect of certain of the solutions. A heat test was therefore adopted. It is well known that normal muscles of the frog are thrown at once into rigor if they be heated to 40° , becoming white and opaque and firm to the touch. If a muscle is in rigor, sudden increase of the temperature to 40° causes no further contraction; if it is not in rigor, contraction occurs. After a muscle had remained in a solution for a certain length of time the solution was heated to 40° . If further contraction occurred, the muscle was evidently not in rigor; if no contraction occurred, there

were two possibilities, either coagulation of the first proteid at least had taken place, and the muscle was in rigor, or the solution had prevented the occurrence of rigor. The point was determined by the registration on the drum and the appearance of the muscle. Very often no contraction occurred at 40°, but on heating to 60° decided contraction occurred, showing that the coagulation of the second proteid takes place with much difficulty at room temperature, and suggesting that probably the second proteid is not necessarily concerned in normal rigor. The loss of irritability was used to indicate when to apply heat. So long as a muscle is irritable, rigor is not complete. The time of completion is determined by (1) the character of the solution, (2) its concentration, (3) its temperature. In those solutions in which rigor occurred in all concentrations it occurred more quickly in hypotonic and hypertonic solutions than in isosmotic solutions.

Results. — *The effect of non-conductors: Cane Sugar.* — In strong solutions the muscle becomes candied, *i. e.*, it is hard and transparent. No rigor can be said to be produced. In lower strengths rigor seems to occur. A slight contraction takes place and the muscle has the opaque appearance of natural rigor. In all the solutions except = $\frac{2}{32}$ rhythmical contractions occurred lasting from twenty minutes to an hour. It is quite probable that the sugar contained a small amount of Na, but it is not likely that the contractions were due to this. An impure solution of cane-sugar isosmotic with $\frac{2}{8}$ NaCl, would contain a very small amount of NaCl compared with a pure $\frac{2}{8}$ NaCl solution. The contractions in the sugar solution of that strength began at once, while in the pure $\frac{2}{8}$ NaCl they did not begin for an hour. These experiments were performed in the spring. The experiments upon which Dr. Loeb based his conclusions concerning rhythmical contractions were made in the autumn. It is a well-known fact that the spring frogs differ in their behavior from the autumn frogs. Whether this difference is indicative of a chemical difference which would account for such a phenomenon as rhythmical contractions in a non-conductor is yet to be determined. However this may be, it is not probable that the non-conductor as such is responsible for the contractions; it is more likely, as Howell¹ recently suggested, that the contractions are caused by the establishment of the necessary balanced proportion of ions as a result of a difference in the relative rapidity of diffusion of the dissociable ions.

¹ HOWELL, W. II.: This journal, 1901, vi, p. 181.

Dextrose. — In $=\frac{n}{2}$ dextrose contraction began at once and increased gradually. While it seemed to be a better coagulant than cane-sugar, no marked difference such as that noted by Locke¹ was noticed in its action. However, it was used in only two strengths.

Urea. — In all strengths rigor occurs. The height of the curve indicates that the coagulum is of a compact character. Remarkable absorption of H_2O occurs in urea. Its power to produce rigor may therefore be due to this quality. The marked similarity in the plotted time curve of urea and that of calcium was interesting. Rhythmical contractions were noted in strengths above $=\frac{n}{8}$. As in the case of sugar, the contractions began at once instead of after an interval as in $\frac{n}{8}$ NaCl. They could hardly be attributed therefore to the small amount of NaCl which might have been present in an impure solution.

Glycerine. — In $=\frac{n}{2}$ glycerine contraction began at once and continued gradually. After twenty-two hours no effect was produced on heating to either 40° or 60° . The effect of $=\frac{n}{16}$ was similar, the length of time, however, was increased to fifty-two hours.

The effect of salts: NaCl. — A strong solution causes a violent contraction of the muscle. This is followed by partial relaxation and twitching. When the twitching ceases, which occurs in a few moments, the lever retains its level. This contraction is not rigor and must be distinguished from it. The muscle remains irritable for some time and if suddenly heated to 40° contracts further. The rigor contraction may therefore be added to the first contraction algebraically. This fact is opposed to Hermann's idea that muscular contraction is a coagulation. The longer the muscle is left in the solution the less does it contract when the temperature is raised to 40° until finally no effect is produced by heating. By some sort of readjustment the first contraction seems to have passed over into rigor. The approach of rigor is not marked by further elevation of the lever, it may even be marked by a slight relaxation. Howell² notes a similar occurrence in oxalate solutions. If before rigor begins the muscle is changed to a weaker solution, *e. g.*, $\frac{n}{8}$ rapid relaxation takes place, *i. e.*, the first contraction is reversed and rigor may not occur. If rigor has begun but is not complete, the change does not interfere with further contraction but the process of completion will be slow. Water is withdrawn by the strong solution and re-enters from the weak solution: This in part explains the first case. In the second

¹ LOCKE, F. S.: Centralblatt für Physiologie, 1901, xiv, p. 670.

² HOWELL, W. H.: Journal of physiology, 1894, xvi, p. 476.

case, the entrance of water probably causes the formation of a more compact coagulum. If heat be applied some time after rigor is set, the lever may drop. This is not to be taken as evidence of reversibility, however, for the lowering of the lever may be due to various causes, *e. g.*, the giving up of water, the giving away of the tendon, or the solution of the clot. I have found no case of true reversibility after rigor is complete. Pauli finds that reversibility may occur in early stages in the heat coagulation of egg-white solutions, but it is impossible in later stages.

In weaker solutions, NaCl seems to lose the power of causing rigor. In $\frac{n}{2}$ the contraction is very slight, and solutions weaker than this cause no contraction except that which may take place in hypotonic solutions from the absorption of water.

KCl. — In strong solutions an immediate strong contraction with occasional twitching occurs. This is followed by a slight relaxation which sometimes amounts to one half the original contraction. Contraction then begins again and continues until rigor is complete. After rigor is complete relaxation again occurs. This is probably due to the dissolving of the coagulum. In weaker solutions the first strong contraction may not take place and the vigor with which the relaxation and further contraction take place is reduced. In $=\frac{n}{3\ 2}$, however, the rigor contraction is very marked, indicating a compact coagulum. The muscle appears more or less transparent, not firm and opaque as after heat rigor.

CaCl₂. — A strong solution has at first a relaxing effect, but after a period varying from half an hour to one hour and a quarter, contraction begins, the lever reaching its maximum height in from three quarters to four hours. If the solution is heated immediately to 40° further contraction takes place, showing that rigor is not completely set. Half an hour afterwards heat either produces no effect or causes a slight relaxation. In weaker solutions, $=\frac{n}{2}$, $=\frac{n}{4}$, contraction is slower in beginning and slower in setting, but as the strength of the solution is further decreased, rigor takes place more quickly.

In the case of blood and milk many authors suppose that Ca is essential to the formation of a clot. Starke asserts that no coagulation occurs without Ca. Hammarsten,¹ however, shows that coagulation occurs in the absence of all Ca salts precipitable by oxalates, and Duclaux² states that it is not proved that milk will not coagulate in

¹ HAMMARSTEN, O.: A text-book of physiological chemistry.

² DUCLAUX: *Traité de microbiologie*, 1899, ii.

the absence of Ca. In the case of the muscle, Ca is possibly the most efficacious coagulant, but it is not essential to coagulation. Howell has shown this in his work on muscles treated with oxalate solutions.

MgCl₂.—The effect of MgCl₂ is relaxing. It not only does not cause rigor, but actually prevents it. A very slight contraction may take place at first. This is, however, followed by a very marked relaxation. Contrary to the above statement, however, rigor seems to be produced in the strength = $\frac{1}{2}$; the first relaxation is slight, lasting from three quarters of an hour to one hour and a half. This is followed by a decided contraction, lasting from five to twelve hours. After this no further contraction occurs on heating to 40°. Mg seems to have an especially deleterious effect upon the tendon. In strong solutions the tendon often breaks loose from the muscle on heating. After heat rigor has been produced in MgCl₂, its relaxing effect is still in evidence. On heating again contraction occurs. Apparently the coagulum is dissolved in MgCl₂ and is re-coagulated on the application of heat. This could not be repeated more than once on the same muscle because putrefaction occurs in a few days.

Na₂SO₄.—As in the case of NaCl, strong solutions cause a marked initial contraction and a few rhythmical twitches. This is followed by relaxation lasting several hours, at the end of which contraction begins. This is the true rigor contraction. Unlike NaCl, it is separated from the initial contraction by a relaxation. The process is much slower than in NaCl. In isosmotic solutions the Na content is nearly the same; the difference is probably due to a difference between the action of SO₄ and Cl. In solutions weaker than $\frac{1}{2}$, rigor is not produced until = $\frac{1}{3\frac{1}{2}}$ is reached. Rigor caused in this strength is undoubtedly due to the absorption of H₂O.

MgSO₄.—No immediate effect was produced on the muscles immersed in the solution. A gradual rise began soon, however, indicating the approach of rigor, and the muscle assumed the usual rigor appearance. Rigor was noted in solutions stronger than = $\frac{1}{4}$. In = $\frac{1}{3\frac{1}{2}}$ it occurred as the result of water absorption. This result is in accord with that of Halliburton, who finds that strong and very weak solutions cause coagulation, while 5 per cent does not. According to J. R. Green¹ and Halliburton, both Na₂SO₄ and MgSO₄ when used alone restrain blood coagulation. Schäfer,² however, finds that MgSO₄ is a very good precipitant of serum globulin. In the case of muscle, I

¹ GREEN, J. R. : *Journal of physiology*, 1887, viii, p. 354.

² SCHÄFER, E. A. : *Ibid.*, 1880-82, iii, p. 181.

find that both are effective in certain strengths. When used together Na_2SO_4 and MgSO_4 are excellent precipitants of serum albumin. The difference between the action of MgCl_2 and MgSO_4 also indicates a difference in the action of the Cl and the SO_4 ion.

$\frac{N}{32} \text{CaSO}_4$.— CaSO_4 is so insoluble that it could only be used in the strength $\frac{N}{32}$. Coagulation begins at once and proceeds gradually.

TABLE VI.

Time in hours after which no contraction occurred on raising the temperature to 40°C .

	$= 5 \frac{N}{4}$	$= N$	$= \frac{N}{2}$	$= \frac{N}{4}$	$= \frac{N}{8}$	$= \frac{N}{16}$	$= \frac{N}{32}$	H_2O
CaCl_2 . . .	3	3	18	29	22	6	$5\frac{3}{4}$	22
NaCl	1	$1\frac{1}{4}$	7N	27N	75N	100N	72N	
MgCl_2 . . .	$3N^1$	6N	12	57N	..	46N	54N	
KCl	3	$3\frac{1}{4}$	24	30	70	36	47	
Na_2SO_4 . . .	12	18N	24	72N	72	46	30	
$(\text{NH}_4)_2\text{SO}_4$	7	24	48	..	120N	75	60	
MgSO_4 . . .	12	16	16	18	40N	50N	40	
Cane sugar	60N	22N	22	18	30	40	48	
Urea	8	9	28	30				
NaF	4					
$\text{Na}_2\text{C}_2\text{O}_4$	$5\frac{1}{2}$					
Na_2CO_3	6					
Dextrose	7	24		
Glycerine	22	52		
	$15^\circ-20^\circ$	$20^\circ-25^\circ$	$25^\circ-27^\circ$	$30^\circ-34^\circ$	$35^\circ-37^\circ$	40°		
H_2O	22	7-12	6	3	$1\frac{1}{4}$	22 min.		
$\text{CaCl}_2 = N$	2	1	..	$\frac{3}{4}$		
$\text{NaF} = \frac{N}{2}$	$1\frac{1}{2}$				
$\text{CaSO}_4 \frac{N}{32}$	4			
$\text{MgSO}_4 = 5 \frac{N}{4}$	$1\frac{1}{2}$			

¹ In the cases marked N no rigor occurred, or its occurrence was doubtful.

Coagulation would naturally occur in this strength as the result of water absorption. The marked contraction, however, indicates a specific Ca effect.

$(NH_4)_2SO_4$. — Coagulation occurs in all the concentrations used except $=\frac{n}{4}$ and $=\frac{n}{8}$. Pauli states that NH_4 compounds are not good coagulants, but Halliburton and v. Fürth have found them extremely effective. Rhythmical contractions are very pronounced in certain strengths.

NaF . — In $=\frac{n}{2}$ NaF immediate contraction occurs followed by a slight relaxation and an additional contraction. After from two hours and one half to four hours and one half no further contraction was produced, even on heating to 50° . NaF precipitates Ca. It would seem from this result that Ca is not essential to the coagulation of muscle proteids.

$Na_2C_2O_4$. — As is the case with NaF immediate contraction followed by relaxation and further contraction occurs in $=\frac{n}{2}$ $Na_2C_2O_4$. After five hours and one quarter no effect followed on heating to 40° . In one case a slight contraction occurred at the end of twenty-four hours on heating to 60° . Locke¹ states that muscle does not go into rigor after treatment with $Na_2C_2O_4$. Howell,² on the contrary, finds that oxalated muscle does go into rigor. From this he draws the conclusion that rigor is different from ordinary contraction, because contraction requires the presence of Ca while rigor does not.

Na_2CO_3 . — In $=\frac{n}{2}$ Na_2CO_3 a contraction occurs followed by relaxation and further contraction. The effect is similar to that of NaF and $Na_2C_2O_4$; but the alkali influence appears in the height of the contraction, which is much more marked in this than in either of the other two solutions. After heating to 40° a slight relaxation is caused. Remarkable contraction occurs on raising the temperature to 60° .

Effect of acids and alkalis. — HCl, H_2SO_4 , HNO_3 , acetic, malic, tartaric, oxalic, valerianic, and trichloroacetic acids were used in the strength $\frac{n}{10}$. n solutions were also used of H_2SO_4 , HNO_3 , and acetic acid. The immediate effect of all the acids was similar. They caused a sudden contraction of the muscle which jerked the lever magnifying fifteen times, to a height of four or five inches. Irritability lasted a half hour or more after this. The first contraction was followed at first by a slight relaxation, later by a slow, steady contraction. In HNO_3 , H_2SO_4 , and trichloroacetic acid, the muscle assumed

¹ LOCKE, F. S.: *Journal of physiology*, 1894, xv, p. 119.

² HOWELL: *Loc. cit.*

every appearance of rigor. The heat test was, however, in every case followed by further contraction in $\frac{11}{2}$, $\frac{11}{10}$, $\frac{11}{50}$ H_2SO_4 at the end of two, five, sixteen, forty-seven hours, in $\frac{11}{10}$ HNO_3 , forty hours. This result was somewhat puzzling. It was supposed that the outside of the muscle became coagulated, preventing the rapid penetration of the acid, and that the contraction was due to a sudden penetration of the acid to the inmost fibres of the muscle. It was found, however, that the contraction occurred in $\frac{11}{10}$ trichloroacetic even after the muscle had been left in the solution three days. The only plausible explanation seemed to be that some one (or more) of the coagulated substances of the muscle was dissolved by the acid and was capable of re-coagulating. And, indeed, it was found that the lever regained its level after a time. A muscle placed in trichloroacetic acid and left three days was heated to 40° ; the lever was raised four inches. The next morning it had sunk four inches. This process was repeated on three successive days. It was then supposed that a muscle thrown into heat rigor would be relaxed by acids. HNO_3 , HCl , and trichloroacetic acids were used, in each of which the process was as described above.

This is in accord with the experience of other observers. Hermann states that acids hasten rigor, but it is not complete, for myosin is dissolved. Stewart, Halliburton, and others also mention the power of acids to dissolve myosin.

The organic acids and HCl had a decided digesting effect upon the muscles. After being left in the solution for a time, the muscle assumed a transparent appearance and was easily macerated. In HNO_3 , H_2SO_4 , and trichloroacetic acid, however, the appearance was entirely different. The coagulating effect of trichloroacetic acid was at once evident and the acid soon reached the inside, rendering the muscle opaque and fibrous. In HNO_3 and H_2SO_4 the muscle was tough and resistant.

Hammarsten states that the additions of acids and alkalis retard the coagulation of blood, — the opposite of what I find in the case of muscle proteids. Halliburton, however, shows that in any coagulable fluid the greater the acidity up to a certain point the earlier coagulation occurs. In the case of milk this effect is well known. A certain similarity between the effects of acids and alkalis is noticeable. That the H and OH ions should have similar action is not surprising, for Loeb has shown that they act similarly in increasing the absorption of H_2O and in accelerating rhythmical contractions. As in the case

of acids immersion in an alkali is followed by a strong contraction in which the lever reaches greater height than in any of the neutral salt solutions, except possibly CaSO_4 . Possibly a more compact coagulum is formed in acids and alkalies, or possibly, coagulation may be incomplete in salt solutions. Later relaxation occurs. The appearance of a muscle placed in an alkali is very different from its appearance when placed in HCl , H_2SO_4 or trichloroacetic acid. It is more like that occurring in acetic acid. The muscle is more or less transparent and slimy. When it is heated, however, it retains its transparent quality and becomes decidedly elastic. It can be stretched, and will again resume its shape. $\frac{n}{10}$ NaOH , KOH , and $\text{Sr}(\text{OH})_2$ were used. At the end of three hours and twenty-four hours it was found that heating to 40° produced no change. A strong contraction, however, occurred on heating to 60° . This indicates that coagulation of the first proteid had occurred, but not of the second. Both in the time and the temperature experiments the alkalies were found to exert a marked influence on the second proteid, the second contraction being both actually and comparatively much greater than in any other solution. Apparently some transformation occurs in this substance. This would accord with Starke's¹ idea. He believes that the dissolving and coagulating reactions of the globulins can only be explained on the supposition that the globulins exist in animal fluids only as alkali albumin compounds. The neutral salts are important in keeping them in solution, because they increase the alkalinity. In accord with Starke's idea that the neutral salts are of importance in increasing alkalinity it has been found that dialysis increases the acidity of muscle extracts and that after coagulation acidity increases.

A few experiments were tried in which acid and alkali in small quantities were added to various salts. The result was very striking in the case of $=n$ CaCl_2 , and $\frac{n}{8}$ NaCl . The muscles from the same frog were put, the one into $=n$ CaCl_2 , the other into 100 c.c. $=n$ CaCl_2 + 2 c.c. $\frac{n}{5}$ HNO_3 . In the first, rigor was complete in three and one half hours; in the second a greater contraction occurred and at the end of one hour and a half no change took place on heating. In another experiment the muscles from a frog were put, the one into $=n$ CaCl_2 , the other into 100 c.c. $=n$ CaCl_2 + 2 c.c. $\frac{n}{5}$ NaOH . In each contraction occurred almost at once. Four hours afterward the first contracted both at 40° and at 60° , the second did not contract at 40° , but contracted strongly at 60° . Similar results were obtained

¹ STARKE, J.: Zeitschrift für Biologie, 1900, x, pp. 419-446.

with NaCl. The solutions used were 100 c.c. $\frac{2}{8}$ NaCl + 3 c.c. $\frac{2}{5}$ HNO₃; 100 c.c. $\frac{2}{8}$ NaCl + 3 c.c. $\frac{2}{5}$ NaOH; 100 c.c. $\frac{2}{8}$ NaCl + 3 c.c. = $\frac{2}{8}$ cane sugar; $\frac{2}{8}$ NaCl. In the first two solutions an immediate contraction took place. At the end of two hours and three quarters no change occurred on heating to 40°. At 60° the tendon of the muscle in the acid solution gave way; the muscle in the alkaline solution contracted strongly. The muscles showed the characteristic acid and alkaline appearance. In the third and fourth solutions relaxation occurred. After thirty hours and three quarters contraction took place at both 40° and 60°.

If it has not been clearly proved that the formation of acid is responsible for both the onset and the solution of rigor as C. Schipiloff¹ has suggested, at any rate the increased formation of acid in fatigue is quite sufficient to account for the earlier onset of rigor in fatigued animals, for acids hasten the onset of rigor at ordinary temperatures and also lower the heat coagulation temperature. As acids and certain salt solutions have this effect there is no reason for adhering to the idea of a radical distinction between heat rigor and rigor mortis. They are both due in all probability to the coagulation in the muscle of substances corresponding to the proteids obtained from muscle plasma. In rigor mortis the coagulation temperature of muscle proteids has been lowered through the unwonted presence or juxtaposition of certain salts of H₂O or of acid. Either one or both proteids may be involved. The difficulty with which the coagulation of the second proteid occurs at room temperature suggests that this substance, either not at all or only in part, enters into the ordinary rigor mortis contraction.

SUMMARY.

1. Stewart's and v. Fürth's conclusion that two proteids exist in muscle capable of coagulation receives support.

2. In general, hypotonic solutions, and other solutions in which much water is absorbed, lower the temperature of coagulation and shorten the time of completion of rigor.

3. Acids increase the temperature of opalescence and lower the temperature of coagulation, the amount of lowering increasing with the degree of dissociation.

¹ Schipiloff, C.: *Centralblatt für die medicinischen Wissenschaften*, 1882, p. 291.

4. Alkalies lower the temperature of opalescence and increase the temperature of coagulation. In alkalies a specific influence is present which may find its explanation in the formation of some such alkali compound as Starke suggests.

5. Rigor occurs readily in acids and alkalies at room temperature, but it is incomplete, for the coagulum tends to dissolve in these substances and also to some extent in some salt solutions. The dissolution is not a case of reversibility, for when once completely coagulated, the proteids of muscles do not resume their original character.

6. The chlorides in solutions isosmotic with $\frac{n}{8}$ NaCl show practical uniformity; the temperature of opalescence is raised, that of coagulation is only slightly affected. In other concentrations, variation occurs within wide limits; with the exception of CaCl_2 , the temperature of opalescence in them is lowered, while the temperature of coagulation is lowered in weak solutions, raised in strong solutions.

7. Rigor occurs readily at room temperature in strong solutions of NaCl, Na_2SO_4 , and MgSO_4 , in CaSO_4 , in all strengths of CaCl_2 and $(\text{NH}_4)_2\text{SO}_4$, and in other Ca precipitating solutions. It also occurs in KCl, but this substance is not a good coagulant. The effect of MgCl_2 is relaxing; rigor occurs only in $= \frac{n}{2}$ concentration.

8. Ca precipitating solutions affect the coagulation temperature somewhat differently from Ca solutions. Ca, however, is not essential for the coagulation of muscle proteids, unless the small amount retained in the tissues is sufficient, for coagulation takes place in Ca precipitating solutions and also in non-conductors.

9. Although the entrance of water favors coagulation and lowers the coagulation temperature, coagulation phenomena cannot be explained entirely on the basis of osmosis. For after a certain concentration is reached, further increase in concentration may cause either increase or decrease in temperature. The nature of the ions present is a determining factor.

10. Normal rigor is not comparable to ordinary muscular contraction, for (1) heat rigor may be added algebraically to contraction; (2) rigor is not reversible.

11. Heat rigor is not essentially different from normal rigor. Normal rigor is probably due to the lowering of the coagulation temperature of muscle proteids caused by the unwonted presence or juxtaposition in the tissue of acid, of H_2O , or of certain salts.

ON THE EFFECTS OF VARIOUS SOLUTIONS ON CILIARY AND MUSCULAR MOVEMENT IN THE LARVÆ OF ARENICOLA AND POLYGORDIUS. II.

BY RALPH S. LILLIE.

[From the Marine Biological Laboratory, Wood's Holl, Mass.]

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I. INTRODUCTION.

DURING the past summer at Wood's Holl I have continued the study of the action of simple solutions of inorganic salts on the larvæ of Arenicola, and have been able to confirm and materially to extend the results obtained with the same organism in the previous summer.¹ I have also tested the action of such solutions on the larvæ of Polygordius, another marine annelid of a more primitive type, with results essentially similar to those gained with Arenicola. The facts about to be presented deal chiefly with the action of solutions containing three chlorides, and with the effects following the transfer of larvæ from one solution to another of different composition. In addition to solutions of inorganic salts, I have employed solutions of non-electrolytes (cane-sugar, glycerine), and mixtures of these with salt-solutions.

The chief general problem considered is briefly as follows: what relation exists between the salts of the medium and the inorganic substances present in the living and active tissues? Do the salts form chemical combinations (possibly of the nature of "ion-proteid

¹ R. LILLIE: This journal, 1901, v, p. 56.

compounds")¹ with the proteids and in this way influence the properties of the tissues? or is it simply that the tissues require for normal activity the presence of certain inorganic substances which, however, need not necessarily enter into such combinations but play a passive and purely physical part (as was formerly supposed of the sodium chloride of blood plasma), such as the maintenance of osmotic pressure, or the promotion of the solubility of certain constituents (*e. g.*, globulins)?

It seems probable that the salts do in reality form such compounds, although concerning the chemical nature of these very little is known at present. Certain of the facts which I have to present, for instance those relating to the comparative reversibility of the changes induced by different salts (especially $MgCl_2$ and KCl) seem to indicate not only that such compounds are formed but that they possess varying degrees of dissociability according to the chemical nature of the combined salt. It is certain that the properties of contractile tissues may be made to undergo constant alterations by the use of definite salt solutions, and that the properties induced by one salt solution may be replaced by other and different properties upon transfer to media containing other salts. If we provisionally accept the hypothesis that the salts form chemical combinations with the proteids, we are justified in assuming that a condition of chemical equilibrium exists between the salts in the medium and the salt-proteid compounds in the tissues. The nature and relative amounts of these compounds and, in consequence, the properties of the tissues, would then intimately depend upon the nature and relative amounts of the salts in the medium and would vary with variations in the composition and relative proportions of these. Such an assumption would be in conformity with the ascertained rules of chemical equilibrium and mass-action, besides being in close accord with the actual results of experimental study on living tissues.

II. GENERAL EFFECTS OF SOLUTIONS OF THREE CHLORIDES.²

It is now a familiar and well established fact that salt solutions containing only one of the several metallic chlorides normally present in

¹ Cf. LOEB, J.: This journal, 1899, iii, p. 327; and PAULI, W.: Archiv für die gesammte Physiologie, 1899, lxxviii, S. 315.

² The means for securing solutions of absolutely standard strength were wanting at Wood's Holl at the time of most of these experiments, and the denominations $\frac{1}{2}$ *n*, etc., as used in this and the preceding paper must be regarded as only approxi-

the natural media of living tissues are highly injurious to the vital activities of such tissues. The addition of a second chloride to such solutions greatly diminishes the poisonous effect; but for normal and long-continued activity the presence of at least three such chlorides (usually NaCl, KCl, and CaCl₂) has been found to be essential. This has been proved for the skeletal and heart muscles of vertebrates by Ringer,¹ Howell,² Greene,³ Loeb,⁴ Lingle⁵ and others, for the lymph-hearts of the frog by Miss Moore,⁶ for smooth muscle by Stiles,⁷ for cell-division and the contraction of the swimming-bell of Medusæ by Loeb.⁸ My own observations on the larvæ of Arenicola and Polygordius accord fully in most essential respects with the descriptions of these authors. It will therefore be unnecessary to reproduce in full the records of my experiments on solutions of three chlorides, and I shall merely give a summarized account of the chief facts ascertained, together with a few details respecting the action of the more favorable of such solutions.

I have already shown for Arenicola (*loc. cit.*) that the most favorable solutions of two chlorides—NaCl + trace CaCl₂, 90 parts NaCl + 10 parts MgCl₂, the former of which especially favors muscular, the latter ciliary movement—are unable to preserve these activities for longer periods than from sixty to seventy hours. Other solutions of two chlorides (NaCl + KCl, KCl + CaCl₂, MgCl₂ + CaCl₂, MgCl₂ + KCl) are less favorable. It can be shown, however, (1) that addition of a favorable quantity of a third chloride to such solutions markedly prolongs the period during which activity continues, and (2) that the specifically injurious effect due to the pre-

mately exact in the chemical sense. I have ascertained that very considerable variations in the concentration of a particular salt solution (*e. g.* from $\frac{8}{8}$ *n* to $\frac{4}{8}$ *n* in the case of MgCl₂) have no essential influence except in accelerating or retarding its specific action. It is, however, of some importance that solutions regarded as equimolecular should be actually so, as far as possible, and this I believe to have been very nearly the case in the experiments recorded below.

¹ RINGER, S.: Various papers, especially in Journal of physiology, beginning 1883, iv, p. 29.

² HOWELL: This journal, 1898, ii, p. 47; also *Ibid.*, 1901, vi, p. 181.

³ GREENE: This journal, 1898, ii, p. 126.

⁴ LOEB, J.: Festschrift für Fick, Braunschweig, 1899, p. 101; Archiv für die gesammte Physiologie, 1900, lxxx, p. 229.

⁵ LINGLE, D.: This journal, 1900, iv, p. 265.

⁶ MOORE, ANNE: This journal, 1901, v, p. 87.

⁷ STILES: This journal, 1901, v, p. 338.

⁸ LOEB, J.: This journal, 1900, iii, p. 383.

ponderance of a particular ion may be partially counteracted by the addition of a third ion favorable to the particular form of activity in question. The following will illustrate: KCl has a specifically injurious action on muscular contractility; a $\frac{5}{8} n$ NaCl solution containing one part of $\frac{5}{8} n$ KCl in twenty (95 vols. $\frac{5}{8} n$ NaCl + 5 vols. $\frac{5}{8} n$ KCl) completely arrests both muscular and ciliary activity within four or five hours. If we compare the action of this solution with that of two others, similar in the proportions of Na and K but containing in addition Ca and Mg respectively, we find the following: Solution A (90 NaCl + 5 KCl + 5 CaCl₂) enables muscular movement, and to a less extent ciliary movement, to continue for a period of sixty or seventy hours. It is, however, noticeable that the muscular contractions in this solution and others of similar composition (see below, page 30) are jerky and irregular, showing plainly that the K still exercises an impeding influence; the presence of the Ca, however, renders possible the continuance of contractions although in a modified and imperfect form. On the other hand, Solution B (90 NaCl + 5 KCl + 5 MgCl₂), while decidedly more favorable than the Ca-containing mixture toward *ciliary* movement has a relatively slight influence in prolonging muscular movement, which is as a rule brought to rest within the comparatively brief space of three or four hours.

Thus the addition of a small amount of Ca — whose presence, unless in excess, always favors muscular movement — prevents the injurious influence of the K¹ from becoming predominant; while the Mg-ion, which has no such specific influence on muscular movement, is relatively without effect. Its influence, however, is seen in the marked extension of the period during which ciliary activity continues.

The examination of a large number of such solutions, presenting nearly all possible variations in the proportions of the constituent salts, has shown that a remarkably close relation exists between composition and physiological action. Indeed, it may be said that each of the above ions exercises upon the properties of the tissues

¹ That the K still exercises its specific influence remains, however, evident in the abnormal character of the muscular contractions. Replacement of the K by Mg, which is without specifically injurious influence on muscular contractility (see below, page 42), results in a solution — 90 NaCl + 5 MgCl₂ + 5 CaCl₂ — in which muscular contractility is at first completely normal (as shown by the typical heliotoxic swarming) and may continue actively for several days.

a specific influence which, though modified by the presence of other ions, is to a very considerable degree of an independent character. Thus the presence of the Na-ion is essential to continued muscular movement; K, on the contrary, is specifically injurious to such movement; Mg furthers ciliary movement to a marked degree — a specific peculiarity which may be demonstrated in a variety of other organisms; while Ca, especially in conjunction with Na, favors muscular movement, and to a less degree ciliary movement as well. A curious specific property of Ca and one apparently peculiar to this ion is that of conferring upon various protoplasmic structures a marked adhesive or sticky consistency. In all solutions containing more than a small proportion of CaCl_2 the larvæ exhibit a strong tendency to cohere in masses or to adhere firmly to the sides of the dish — a tendency which is almost entirely lacking in solutions from which Ca is absent.¹

Each constituent thus exercises a characteristic influence, peculiar to itself, on the properties of the tissues. Accordingly, while each solution of three chlorides possesses as a whole distinctive properties of its own, apart from the properties of any one of the component ions, it is always possible on closer examination to detect the specific action of each one of these, however modified by the presence of the others. An extract from my note-book giving a portion of the records of two solutions of related composition will illustrate this:

LARVÆ PLACED IN SOLUTIONS AT 1.45-2.00 P. M., JULY 11, 1900.

Solution 1. — 60 c.c. $\frac{3}{8} n$ NaCl + 30 c.c. $\frac{5}{8} n$ MgCl_2 + 10 c.c. $\frac{1}{8} n$ KCl. July 11, 5.13 P. M. Larvæ are rigid and without muscular movement. Cilia are active in all; larvæ are slowly swimming at bottom of vessel. No adhesion to walls of vessel. 9.33 P. M. As before; cilia are still active.

¹ This property appears to be widespread; attention has been called to it by LOEB (This journal, 1900, iv, p. 446), who has observed it in the ova of echinoderms and annelids (*Chætopterus*). It appears in fact that in segmenting ova the coherence of the blastomeres is largely dependent upon the calcium salts contained in the sea-water, for if such ova are introduced into Ca-free sea-water the blastomeres readily fall apart, a fact which has been turned to practical advantage by HERBST (*Cf. Archiv für Entwicklungsmechanik*, 1900, ix, p. 424), as a means of isolating the blastomeres of sea-urchin eggs. I may add that I have found, in experiments as yet unpublished, that the cells of the ciliated epithelium of the frog's œsophagus are readily isolated when portions of mucous membrane are transferred to certain Ca-free media. Isolation is prevented by the presence of a trace of CaCl_2 .

July 12, 11.17 A. M. Larvæ are rigid and somewhat shrunken, but cilia are still active in all. 9.28 P. M. Larvæ are dead and shrunken.

Solution 2. — 60 c.c. NaCl + 30 c.c. CaCl₂ + 10 c.c. KCl. July 11, 5.23 P. M. Cilia have been mostly dissolved; are slowly active where they remain. Jerky and irregular muscular movements are present in all. Larvæ adhere strongly to sides and bottom of dish. 9.40 P. M. Cilia are dissolved; no ciliary movement; jerky muscular movements are present in all.

July 12, 11.24 A. M. Almost all larvæ are dead and shrunken; twitching movements persist in a few. 9.35 P. M. Larvæ are dead and shrunken.

The most noticeable difference between these two solutions — persistence of ciliary movement in 1, of muscular contractility in 2 — depends chiefly on the characteristic difference in the respective physiological effects of the Mg- and Ca-ions. The retarding influence of the K-ion on muscular movement is evident in both, particularly in Solution 2, where, however, sufficient Na and Ca are present to sustain contractions for a period of nearly two days. Thus the respective specific actions of Mg, Ca, K, and Na are apparent in the above solutions. Each solution, acting as a whole, preserves the larvæ alive for about two days.

Proceeding on this principle, it is possible to predict with a very close approach to accuracy the physiological action of any given solution containing any of the above salts in known proportions. In other words, the properties of the tissues in any solution depend in large part upon a combination or summation of the separate physiological effects of the several component ions. Such a result is in perfect accordance with the theory to which reference is made above, which regards the properties of the tissues at any given time as largely determined by the nature and relative proportions of the contained ion-proteid compounds.

It is, however, essential for normal activity that these compounds should be present in definite "physiologically balanced"¹ proportions, since the presence of an excess of any one ion will disturb the normal physiological balance and act injuriously; hence the well-known injurious action of pure solutions of one salt, and solutions containing three salts in unfavorable proportions are similarly injurious. Thus a solution of the composition 80 vols. $\frac{8}{8}$ n NaCl + 15 vols. $\frac{8}{8}$ n MgCl₂ + 5 vols. $\frac{8}{8}$ n CaCl₂ appears to contain these three

¹ LOEB, J.: This journal, 1900, iii, p. 445.

salts in nearly the best possible proportions, for it is found to be capable of preserving life and normal activities, together with power of growth and development, for very considerable periods (two to three weeks in favorable instances. See below, pp. 32, 37). If, however, the relative proportions of Mg and Ca are reversed, the resulting solution — 80 NaCl + 5 MgCl₂ + 15 CaCl₂ — is found to be greatly inferior to the first in preservative properties: heliotropic swarming is less active and ceases relatively soon, the larvæ tend to adhere strongly to the glass, and within three or four days at most practically all are dead and maceration is well advanced. An excess of Ca and an insufficiency of Mg are plainly indicated.

The most favorable solutions of three chlorides are those containing the three chief chlorides of the natural medium, namely, NaCl, MgCl₂, and CaCl₂. As in sea-water, NaCl should greatly preponderate, and the amount of MgCl₂ should exceed by several times that of CaCl₂. The relative proportions of the four chief chlorides of sea-water are approximately preserved in a solution of the following composition per 100 volumes:¹— 85 volumes $\frac{4}{8} n$ NaCl + 11 vols. $\frac{8}{8} n$ MgCl₂ + 2.2 vols. $\frac{8}{8} n$ CaCl₂ + 1.8 vols. $\frac{4}{8} n$ KCl. Mg-atoms are thus approximately five times as numerous as Ca-atoms and six times as numerous as K-atoms. In artificial solutions of the above three chlorides, the most favorable ratio seems to be approximately Mg:Ca::3:1, the increased proportion of Ca apparently compensating for the deficiency in K. It is, however, not necessary to adhere strictly to this ratio, although it is indispensable that MgCl₂ molecules should considerably outnumber CaCl₂ molecules.

Partial records of the following five solutions are given in illustration. In the first four, MgCl₂ and CaCl₂ are in the proportion of 3 to 1, while the quantity of NaCl shows a regular decrease. In the last solution, the proportion of MgCl₂ is increased.

Solution 1.	96 c.c. $\frac{4}{8} n$ NaCl + 3 c.c. $\frac{8}{8} n$ MgCl ₂ + 1 c.c. $\frac{8}{8} n$ CaCl ₂
Solution 2.	80 c.c. $\frac{4}{8} n$ NaCl + 15 c.c. $\frac{8}{8} n$ MgCl ₂ + 5 c.c. $\frac{8}{8} n$ CaCl ₂
Solution 3.	60 c.c. $\frac{4}{8} n$ NaCl + 30 c.c. $\frac{8}{8} n$ MgCl ₂ + 10 c.c. $\frac{8}{8} n$ CaCl ₂
Solution 4.	40 c.c. $\frac{4}{8} n$ NaCl + 45 c.c. $\frac{8}{8} n$ MgCl ₂ + 15 c.c. $\frac{8}{8} n$ CaCl ₂
Solution 5.	40 c.c. $\frac{4}{8} n$ NaCl + 55 c.c. $\frac{8}{8} n$ MgCl ₂ + 5 c.c. $\frac{8}{8} n$ CaCl ₂

July 9, 1901. Larvæ are placed in the above solutions at 4.05 P. M. Heliotropic swarming immediately appears in all, but lasts for only a short period in Solutions 4 and 5 in which the larvæ quickly collect in clumps, indicat-

¹ Calculated from Dittmar's figures in "Challenger" Report.

ing an early loss of muscular contractility. At 4.30 Solutions 2 and 3 are decidedly the most favorable; in these the larvæ are swimming actively and still largely exhibit positive heliotropism. In Solution 3, however, a much larger proportion than in 2 swim irregularly without orientation. The larvæ in Solutions 4 and 5 are largely in clumps; they are still capable of swimming movements, but these are irregular and without orientation; the larvæ are rigid and almost entirely without muscular movement. In Solution 1 the larvæ are still active and largely heliotropic.

Further records: —

1. 96 NaCl + 3 MgCl₂ + 1 CaCl₂.

- July 10. — 8.10 A. M. Lively muscular movement. Ciliary movement in most; ciliary movements are slow, and absent in a large proportion.
 July 11. — 8.25 A. M. Muscular and ciliary movement are present in most; ciliary movements are slow, and absent in a large proportion.
 July 12. — 12.25 P. M. Larvæ are dead and motionless.

2. 80 NaCl + 15 MgCl₂ + 5 CaCl₂.

- July 10. — 8.10 A. M. Active swimming movements and strong heliotropism in most.
 July 11. — 8.30 A. M. Active and normal movements. Comparatively few are swimming; of these a fair proportion are still heliotropic.
 July 18. — 8.45 A. M. Fair number living and contractile; some have 5 sets of setæ.

3. 60 NaCl + 30 MgCl₂ + 10 CaCl₂.

- July 10. — 8.15 A. M. Active swimming movements. A few still show positive heliotropism. Muscular movements are slight, and larvæ are noticeably stiff. Ciliary movements are very active, and swimming is fully as rapid as normal though mostly unoriented.
 July 11. — 8.35 A. M. Ciliary activity and slow swimming movements in practically all larvæ. No heliotropism; all exhibit muscular movements but these are stiff and limited.
 July 18. — 8.48 A. M. Most larvæ are dead. A fair number are still living and contractile although feebly so. Elongation slight; many with 4 sets of setæ; a few with 5.

4. 40 NaCl + 45 MgCl₂ + 15 CaCl₂.

- July 10. — 8.20 A. M. Larvæ are rigid and somewhat shrunken. Ciliary and slow swimming movements are present in all. Stiff muscular movements.
 July 11. — 8.38 A. M. Ciliary movements are present in a fair proportion of larvæ, but wanting in many; larvæ are rigid and shrunken; practically no muscular movements; one or two stiff movements seen.
 July 18. — 8.55 A. M. All are dead and macerated.

5. 40 NaCl + 55 MgCl₂ + 5 CaCl₂.

July 10. — 8.25 A. M. Larvæ are rigid and shrunken; most are swimming slowly. Practically no muscular movement persists; one or two feeble jerks are seen.

July 11. — 8.40 A. M. Slow ciliary movements persist in most; no muscular movement.

July 18. — 8.58 A. M. All are dead and macerated.

Of the above five solutions No. 2 is clearly the most favorable. In Solution 1 Mg and Ca are present in insufficient quantity, as shown by the comparatively early cessation of both muscular and ciliary movement, and the early death of the larvæ. In Solution 3 the proportion of Na has already considerably fallen below the optimum; heliotropic response disappears at a much earlier period than in Solution 2 and muscular contractions soon become stiff and limited. In the two succeeding solutions (4 and 5), the deficiency of Na is still more evident; both are manifestly less favorable than Solution 3; the more rapid loss of muscular contractility in Solution 5 (as compared with Solution 4) evidently follows from its relative deficiency in Ca-ions. In Solution 2, on the other hand, normal activity continues for a very considerable period; furthermore, growth and development, although slower than normal, are still possible in this solution, as shown by the elongation of the trunk region and the appearance of new somites with setæ. Growth is also possible, under favorable conditions, in Solution 3, and even in Solutions 4 and 5, but to a much less degree in these than in Solutions 2 and 3.

In all these solutions growth is distinctly favored by the addition of a trace of alkali (see below). In a parallel series, similar in composition to the above and differing only in the presence in each solution of a trace of NaOH, a decided increase in the rate of growth was evident, especially in Solution 2, in which a considerable proportion of larvæ were found to have reached a stage of 6 somites by July 17. In Solution 5, a few larvæ had attained 5 somites by July 17; and in Solutions 4 and 5 many larvæ had attained a length of four somites by July 13, but very little further growth took place in the last two solutions.

Growth and development are therefore possible in media containing only the above three chlorides in certain proportions. They are *not* possible, according to my observation, in even the most favorable solutions of two chlorides, nor in solutions in which any one of the

above chlorides is replaced by KCl. The addition, however, of a little KCl (4 c.c. $\frac{4}{8}$ *n* KCl to 100 c.c. solution) to Solution 1 proved favorable and within eight days a fair proportion of larvæ had attained a length of 4 somites, while a few showed 5 somites; but with the four other solutions a similar addition of KCl effected no improvement, and indeed seemed rather detrimental than otherwise.

A slight decrease in the osmotic pressure of the solution also appears to favor growth and development. In a series of five solutions of the same composition and proportions as the above, to each of which had been added one third its volume of distilled water (making the composition 96 c.c. $\frac{3}{8}$ *n* NaCl + 3 c.c. $\frac{6}{8}$ *n* MgCl₂ + 1 c.c. $\frac{6}{8}$ *n* CaCl₂, etc.) the following was observed. Relative favorability was the same as above; growth, however, was noticeably more rapid than in the more concentrated solutions, and was exhibited by a larger proportion of larvæ. In Solution 1, all larvæ died within four days; but in all four of the remaining solutions growth and development were possible. The order of favorability was 2, 3, 4, 5; comparatively few larvæ in Solutions 4 and 5 attained a length of 5 somites; but in Solution 2 (the most favorable) a fair number attained a length of 6 somites within six days; in Solution 3 the sixth setæ appeared in fewer larvæ and were not apparent until after eight days had elapsed; while in Solution 4 very few larvæ attained this length, and in Solution 5 none.

Development is slower in all of the above solutions than in normal sea-water and a length of six somites is very rarely exceeded. The limit to the possible development in such solutions must be set by the available food supply, and since in pure aqueous solutions this is confined to the yolk contained in the organism itself, it is clear that development must cease when the yolk has been entirely consumed. It is in fact readily observable that as growth proceeds the opacity characteristic of earlier stages gives way to a gradually increasing transparency, while at the same time the yolk-laden intestine, which in swarming larvæ fills almost the entire body cavity, gradually diminishes in size until finally it is reduced to the condition of a slender and almost transparent band widely separated from the body-wall. Such larvæ, while active and to all appearance healthy, are almost perfectly transparent and as a rule exhibit no further growth. The observed changes indicate a progressive consumption and translocation of the yolk-material, which is evidently being put to use in the constructive processes of development. The fact that these

processes can take place in a medium containing only Na-, Mg-, Ca- and Cl-ions is of interest in view of the statements made by Herbst and others that certain inorganic substances absent in the above solutions are necessary for development (Herbst's¹ "nothwendige Substanzen"). It is true that the presence of such substances may accelerate or further development; but that their presence is a necessity is disproved by such facts as those cited above, as Loeb has already pointed out. It is of course possible to assume that the remaining necessary substances are present in the body-fluids or tissues of the organism, although in view of the very general diffusibility of such substances even this is doubtful; whatever the internal conditions may be, the fact remains that development is possible in a medium from which many of the substances in question are lacking.

III. INFLUENCE OF ACID AND ALKALI ON DEVELOPMENT.

It has been shown that the oxidative capacity of various animal tissues or the extracts of these tissues is very generally increased by the presence of a trace of alkali and lessened by corresponding amounts of acid. Spitzer² observed that the presence of a small amount of alkali furthered the glycolytic and other oxidative properties of tissues; also that the oxidative action of the nucleo-proteid (nucleo-histon) isolated from the liver was similarly accelerated by alkali. The presence of a small amount of acid, on the contrary, retarded or prevented oxidation in both instances. Other authors have made similar observations³ in experiments on the oxidative capacities of different tissues. The liberation of oxygen from solutions of hydrogen peroxide by the catalytic action of various ferments — pancreatic extract, malt extract, emulsin (Jacobson⁴) — or even of colloidal platinum (Bredig and v. Berneck⁵), an action essentially oxidative in nature, is also furthered by the presence of small amounts of alkali and hindered by acids.

¹ HERBST: *Archiv für Entwicklungsmechanik*, 1897, v, p. 649, and 1901, xi, p. 617.

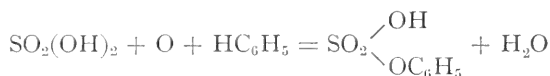
² SPITZER, W.: *Archiv für die gesammte Physiologie*, 1895, lx, p. 303, and 1897, lxxvii, p. 615.

³ Cf. ABELOUS et BIARNÈS, various papers in *Archives de physiologie normale et pathologique*, (5) vi-x incl., 1894-8.

⁴ JACOBSON, J.: *Zeitschrift für physiologische Chemie*, 1892, xvi, S. 340.

⁵ BREDIG u. VON BERNECK, *Zeitschrift für physikalische Chemie*, 1899, xxxi, S. 258.

Schmiedeberg,¹ who also observed that weak alkali furthered the oxidations of various organic substances through the action of animal tissues, has attempted to show that an important connection exists between processes of oxidation and the synthetic formation of organic compounds in the living organism. For example, in the oxidation of benzol by the tissues to form phenol, he considers that the O-atom is not directly introduced into the molecule but that the primary reaction is probably one of combination with an acid and an atom of oxygen to form an ethereal compound, thus :



The resulting compound, in this instance phenyl-sulphuric acid, would yield free phenol on hydrolysis. Similarly, he assumes that in the living organism oxidation and compound-formation go hand in hand, the oxidation taking place in connection with a combination, probably as a rule with water-elimination. Such reactions he calls "synthetic oxidations." On this theory, it is clear that conditions favorable to oxidative processes will also favor those synthetic processes upon which we may suppose growth and development largely to depend. Development should then be favored by the presence of small amounts of alkali and hindered by acid; this has, in fact, been found to be the case by Loeb,² who has ascertained that the development of *Arbacia*- and *Fundulus*-eggs is accelerated by a slight increase in the alkalinity of the sea-water and retarded by the addition of a trace of acid.

The addition of a trace of acid or alkali to sea-water was not found to have any appreciable influence on the rate of growth of *Arenicola* larvæ. In artificial solutions, however (*e. g.*, 80 NaCl + 15 MgCl₂ + 5 CaCl₂), growth is very perceptibly accelerated by the presence of alkali and retarded or prevented by acid. Only a slight increase of alkalinity is possible in solutions containing MgCl₂ since Mg(OH)₂ is almost insoluble in water; yet even this slight increase has a well marked influence. One characteristic effect is an immediate liquefaction of the cilia; this occurs very constantly in all alkaline solutions,

¹ SCHMIEDEBERG: Archiv für experimentelle Pathologie und Pharmakologie, 1881, xiv, p. 288.

² LOEB, J.: Archiv für Entwicklungsmechanik der Organismen, 1898, vii, p. 631.

even in alkaline sea-water; swarming is therefore at once arrested in such solutions. Muscular contractility, on the contrary, appears to be favored rather than hindered by the presence of a trace of alkali, and the same is more evidently true of growth and development. On the other hand, the presence of a trace of acid is highly injurious to all forms of activity; even 0.25 c.c. $\frac{1}{16}$ HCl in 100 c.c. of solution is fatal within a few hours. Smaller quantities while not entirely preventing growth retard it very considerably.

The following records will illustrate:

Solution used:— 80 c.c. $\frac{4}{8}$ n NaCl + 15 c.c. $\frac{8}{8}$ n MgCl₂ + 5 c.c. $\frac{8}{8}$ n CaCl₂.

To this solution small quantities of $\frac{1}{16}$ HCl and $\frac{1}{16}$ NaHO were added as below. Larvæ were added to these solutions at 4.30 P. M., July 20, 1901.

Solutions 1-4: 100 c.c. solution + 2, 1.5, 1 and 0.5 c.c. $\frac{1}{16}$ HCl respectively.

Solution 5, neutral; 6, 7, and 8, with 0.5, 1 and 1.5 c.c. $\frac{1}{16}$ NaOH respectively.

1-4.

July 21. — 11.00 A. M. Larvæ are all dead and motionless.

5 (neutral).

July 21. — 11.07 A. M. Larvæ are active, and many are still heliotropic. 4th setæ are just visible in a fair proportion.

July 22. — 11.00 A. M. Larvæ are still active; 4th setæ are beginning to appear in a large proportion.

July 24. — 11.45 A. M. Larvæ are active; largely show 5th setæ.

July 27. — 9.45 A. M. No further elongation since 24th.

August 1. — 2.32 P. M. Many larvæ are still living and active, most with 5 setæ.

6 (+ 0.5% $\frac{1}{16}$ NaOH).

July 21. — 11.12 A. M. A fair proportion of larvæ show 4 setæ.

July 22. — 11.01 A. M. Larvæ have decidedly increased in length since July 21st; but are not distinctly more advanced than in Solution 5.

July 24. — 11.50 A. M. Practically all larvæ have 5 sets of setæ.

July 27. — 9.59 A. M. As on 24th. Larvæ are active and elongated.

August 1. — 2.40 P. M. As in solution 5. One larva seen with 6 setæ.

7 (+ 1.0% $\frac{1}{16}$ NaOH).

July 21. — 11.14 a. m. Largely with 4th setæ.

July 22. — 11.04 A. M. 4th setæ are visible in a large proportion.

July 24. — 11.55 A. M. All are active and normal in appearance, mostly with 5 setæ. More favorable than Solution 5 or 6.

July 27. — 10.02 A. M. Larvæ are more active than in solutions 5 and 6 and a number have acquired a 6th set of setæ. A large proportion remain living and these are more transparent than in Solutions 5 and 6 (indicating a greater absorption of yolk).

August 1. — 2.45 P. M. A fair number of larvæ exhibit the 6th setæ. Larvæ are transparent and intestine is reduced to a slender band. A larger proportion remain alive than in Solutions 5 and 6.

8 (+ 1.5% $\frac{n}{10}$ NaOH).

July 21. — 11.18 A. M. Larvæ adhere closely to the glass. Cilia are largely liquefied.

July 22. — 11.07 A. M. 4 setæ are present in a fair number of larvæ. Less favorable than Solution 7.

July 24. — 12.00 noon. Less favorable than Solution 7; a fair proportion of larvæ are dead. Elongation in general is less than in Solution 7; larvæ largely show 5 setæ.

July 27. — 10.05 A. M. Elongation in general is less than in Solution 7; larvæ are otherwise similar.

August 1. — Larvæ are essentially as in Solution 7, — transparent, 5 and 6 setæ, intestine reduced. Somewhat less favorable than Solution 7.

In the presence of a mere trace of acid, larvæ may live for several days, but growth is retarded or even altogether prevented. The following will illustrate:

Solution used: 80 c.c. $\frac{5}{8} n$ NaCl + 15 c.c. $\frac{1}{8} n$ MgCl₂ + 5 c.c. $\frac{1}{8} n$ CaCl₂.
Larvæ were added to solutions at 12.15 A. M., July 29, 1901.

Solution 1 (100 c.c. solution + .125 cc. $\frac{n}{10}$ HCl [$\frac{1}{8}$ %]).

July 29. — 3.30 P. M. Larvæ are slowly swimming; many are still heliotropic; muscular movement is slow and feeble.

July 30. — 10.30 A. M. Slow ciliary and muscular movements persist in most; a fair number are slowly swimming.

August 1. — 10.47 A. M. Largely dead; many are still living and contractile, and a few are slowly swimming; none of the living larvæ show perceptible elongation. 4th setæ are visible in none.

August 4. — 2.35 P. M. Most are dead; the few living larvæ are *opaque* and have undergone no elongation.

Solution 2 (100 c.c. solution + .0625 c.c. $\frac{n}{10}$ HCl [$\frac{1}{16}$ %]).

July 29. — 3.35 P. M. Larvæ are swimming actively, and many are strongly heliotropic.

July 30. — 10.17 A. M. Many are still swimming actively; and some are still heliotropic.

August 1. — 10.50 A. M. Almost all are living and active; elongation is decidedly greater than in Solution 1 and in a few the 4th setæ have appeared. In most, however, only three setæ are as yet visible.

August 4. — 2.40 P. M. Larvæ are active and show mostly 4 setæ; a few have 5 setæ. In contrast to Solution 1, many larvæ have become quite transparent, especially those with 5 setæ.

Solution 3 (neutral).

July 29. — 3.38 P. M. Larvæ exhibit active swimming movements and strong positive heliotropism. Decidedly more favorable than Solution 2.

July 30. — 10.19 A. M. Active swimming; many are still heliotropic.

August 1. — 10.55 A. M. Larvæ are decidedly more elongated than in Solution 2. 4th setæ are visible in a large proportion and conspicuous in many.

August 4. — 2.44 P. M. A large proportion are elongated and transparent.

Solution 4 (100 c.c. solution + .5 c.c. NaOH [$\frac{1}{2}$ %]).

July 29. — 3.44 P. M. Larvæ adhere to glass; cilia are mostly dissolved.

July 30. — 10.21 A. M. Larvæ remain living and contractile. Ciliary movement is slight.

August 1. — 10.59 A. M. Larvæ are active and elongated; elongation is unmistakably greater than in Solution 3; larvæ are more transparent and more uniformly elongated; the majority have 4 setæ. Muscular movements are also more active than in Solution 3.

A slight degree of alkalinity is therefore favorable to growth and development in the above solutions, while acidity has the reverse effect. It should be said, however, that in none of the artificial solutions hitherto examined is growth so rapid as in sea-water. Under natural conditions at normal summer temperature, larvæ may grow from the free-swimming stage (3 setæ) to a stage of 6 setæ within three or four days, whereas in the most favorable of the above artificial solutions at least six days are required. It is therefore clear that solutions of the above simple composition do not furnish all of the conditions needed for normal activity. What these further conditions are is a subject for future study. The rôle played by the anions has been provisionally neglected in the above experiments; and it is more than probable that solutions in which other anions than chlorine and a trace of hydroxyl are present would prove much more efficacious in promoting growth and development than solutions containing chlorides alone.

IV. EXPERIMENTS ON THE LARVÆ OF POLYGORDIUS.

Polygordius larvæ usually make their appearance at Wood's Holl towards the middle of June, when they are found in considerable numbers in the tow, especially in the early morning and evening. They disappear, as a rule, within a week or ten days, and it has therefore not proved possible to make more than a relatively limited number of experiments, which, however, so far as they have been carried, have led to results essentially identical with those described above for Arenicola.

The organism presents a familiar and characteristic appearance with the large transparent umbrella-shaped head-region, frequently 0.5 mm. or more in diameter, which is bordered by a double row of long cilia. From the head hangs down the slender cylindrical segmented body-region; this varies in length and number of somites according to the stage of development. The larvæ, as obtained in the tow, are thus not completely uniform in size, behavior, and stage of development (like Arenicola in the swarming stage), but show considerable variability in all these respects. They are heliotropic, though less strikingly so than Arenicola; and locomotion is effected by a similar combination of muscular and ciliary activity.

Pure $\frac{5}{8} n$ NaCl solutions are rapidly injurious to these larvæ. The following record will illustrate:

Solution: $\frac{5}{8} n$ NaCl. Larvæ were added to this solution at 2.45 P.M., June 12, 1901.

Observations: 2.45 P.M. — Ciliary movement is largely arrested and cilia liquefy; in some larvæ ciliary movement continues at 2.48, although enfeebled. At 2.48 *a number of cilia have become detached from the edges of the umbrella and are vibrating freely in the solution*; such cilia are terminated by enlarged basal knob-like bodies. At 2.50, the larvæ are bent and almost motionless; the majority, however, show feeble muscular movements. Cilia are still active in a fair number. 3.05. Ciliary activity has almost entirely ceased, and cilia are for the most part dissolved. Most larvæ show feeble muscular movements. Disorganization is already apparent; detached portions of larvæ are floating freely in the solution. 3.17. Muscular movement is feeble and largely absent; cilia are almost entirely dissolved, but a few remain feebly active. 4.00. A fair number show feeble muscular movement; ciliary movement persists in very few. 4.55. A good many show feeble muscular movements; no ciliary movement. 8.15 P.M. Practically all larvæ are dead, motionless, and macerated; one feeble muscular twitch seen.

The action of the pure NaCl solution on *Polygordius* is thus, in general, the same as on *Arenicola*. The liquefaction of the cilia is, however, less prompt in *Polygordius*, probably on account of the different structure of the individual cilium, which is long and lash-like — rather a flagellum than a cilium — and, apparently on account of its more considerable dimensions and relatively smaller surface extent, somewhat less quickly affected by reagents. Qualitatively, however, the effect of the pure $\frac{5}{8}n$ NaCl solution is exactly as in *Arenicola*. Incidentally the interesting fact may be noted that the contractility of the cilium is inherent in the structure itself and independent of its connection with the cell-body. Isolated cilia will continue their movements for a considerable time, alternately bending and straightening, in a variety of solutions. The enlarged knob-like basal termination gives each cilium when thus isolated a striking superficial resemblance to a spermatozoon.

Liquefaction of the cilia is prevented, as in *Arenicola*, by the addition of a small quantity of $MgCl_2$. In solutions of the composition 95 NaCl + 5 $MgCl_2$, and 80 NaCl + 20 $MgCl_2$ ciliary movement continues actively for many hours. The second of these solutions illustrates somewhat better than the first the specific action of Mg in sustaining ciliary movement. On the addition of this solution, the larvæ at once show jerky, irregular, and trembling muscular movements; these last for a brief period only, and within fifteen minutes muscular contractions have almost entirely ceased. The effect on ciliary movement is peculiar; the cilia are at first arrested and stand out straight, motionless, and apparently rigid from the margin of the umbrella. Within a minute or less, slight vibratory movements reappear and gradually regain their former rate and amplitude. The larvæ may then swim actively for several hours, and slow movements of the cilia may persist for thirty or forty hours.

The addition of $CaCl_2$ to the pure NaCl solution is much less favorable to ciliary movement, but decidedly prolongs the period of muscular activity. The addition of KCl in small quantities to $\frac{5}{8}n$ NaCl appears to preserve both muscular and ciliary movements; if present in larger quantity (80 NaCl + 20 KCl), K causes a complete loss of muscular contractility within a few minutes.

Solutions of three chlorides have essentially the same effect on *Polygordius* as on *Arenicola*. With *Polygordius*, also, the most favorable solutions proved to be mixtures of NaCl, $MgCl_2$ and $CaCl_2$. Solu-

tions containing only 60 volumes of $\frac{5}{8}$ NaCl (60 NaCl + 30 MgCl₂ + 10 CaCl₂, etc.) proved injuriously deficient in Na-ions, as shown by the early cessation of muscular movement and the occurrence of death within two days or less. Solutions with 80 or 90 volumes NaCl-solution proved on the contrary surprisingly favorable when MgCl₂ and CaCl₂ were present in suitable proportions. Of a series of four such solutions of the following composition (1) 90 NaCl + 5 MgCl₂ + 5 CaCl₂, (2) 80 NaCl + 10 MgCl₂ + 10 CaCl₂, (3) 80 NaCl + 5 MgCl₂ + 15 CaCl₂, (4) 80 NaCl + 15 MgCl₂ + 5 CaCl₂, Solution 4 was the most favorable and Solution 3 (with an excess of Ca) the least. In Solutions 1 and 2 a fair number of larvæ remained living and capable of both muscular and ciliary movement after an interval of ten days; in Solution 3 all larvæ were found dead after two days. In Solution 4, however, many larvæ were found swimming actively after an interval of ten days; in a few of these the rudiments of the two tentacles were visible, showing that development could proceed in such a solution. Even after sixteen days, many larvæ were found still living and contractile in this solution, and a few remained capable of swimming movements. Thus the solution 80 NaCl + 15 MgCl₂ + 5 CaCl₂ proved with *Polygordius*, as with *Arenicola*, the most favorable of the artificial combinations of three chlorides. Experiments with alkaline and acid solutions were not tried.

V. EFFECTS OF TRANSFER FROM ONE SOLUTION TO ANOTHER OF DIFFERENT COMPOSITION.

The first experiment was the transfer of *Arenicola* larvæ from MgCl₂-solutions to sea-water. As I have already described, muscular contractility is almost immediately lost in pure Mg-solutions, while ciliary movement continues actively and causes the larvæ to collect in groups or "clumps" at the bottom of the vessel. Larvæ that have remained for only a few minutes in $\frac{5}{8} n$ MgCl₂ completely recover normal activity on a return to sea-water and exhibit again the usual behavior (active heliotropic swarming, in-and-out movements of the setæ, etc.). If a longer period (one hour) is allowed to elapse before the transfer is made, recovery is less complete; muscular contractions invariably reappear in all larvæ immediately after the transfer, but comparatively few exhibit a return of heliotropic swarming, though many show irregular and unoriented swimming motions. After a still longer stay in $\frac{5}{8} n$ MgCl₂ (two to six hours), muscular con-

tractility is only imperfectly restored by sea-water, and the resulting movements are stiff and limited. Nevertheless, even after sixteen hours in the Mg-solution — treatment that produces marked shrinkage and a partial loss of ciliary movement — muscular contraction, though limited, will still reappear in practically all larvæ on addition of sea-water. Longer immersion induces a rigidity from which recovery is impossible.

With pure CaCl_2 solutions recovery is less complete than with MgCl_2 . Sea-water will produce reappearance of heliotropism after a few minutes in $\frac{6}{8}n$ CaCl_2 , but the stickiness (among other changes) induced by the solution seems unfavorable to complete recovery. Pure CaCl_2 also acts more rapidly than MgCl_2 in producing a general disorganization from which recovery is impossible. Nevertheless larvæ that have been subjected to its action for twelve to fourteen hours — after which interval they are shrunken and badly disorganized — will in some instances show feeble muscular twitches on transfer to sea-water.

KCl exhibits special peculiarities of action which will be considered more in detail below.

The restorative action of sea-water on muscular movement depends chiefly on the contained Na-ions, which by its action are restored to the tissues. I have performed a large number of experiments on transfer and re-transfer between pure solutions of MgCl_2 and NaCl. The method of procedure in such experiments is as follows. Larvæ are treated in watch-glasses with pure $\frac{8}{8}n$ MgCl_2 ; after muscular contractions have entirely ceased the MgCl_2 is drawn off and $\frac{4}{8}n$ NaCl is substituted. Muscular contractions are always found promptly to reappear; but heliotropic swarming does not return, as in sea-water, since pure NaCl-solution is injurious to ciliary movement. On re-transference to MgCl_2 muscular contractions at once cease, while ciliary movements are revived. This process of transfer and re-transfer may be continued with the same larvæ for hours at a time, and at each change of solution the changes described appear with striking regularity. A partial record of one series of experiments is given for illustration:

June 24, 1901. — 10.54 A. M. Larvæ are placed in $\frac{8}{8}n$ MgCl_2 ; they immediately become rigid and collect in clumps at the bottom of the watch-glass.

10.58. 5, $\frac{8}{8}n$ MgCl_2 is replaced by $\frac{4}{8}n$ NaCl. Immediate reappearance

ance of muscular movements. Active squirming appears. Ciliary movements are checked.

11.01, $MgCl_2$. Immediate cessation of muscular movements.

11.03. 5, $NaCl$. Muscular movements reappear.

11.05. 5, $MgCl_2$. Muscular movements are arrested. Partial revival of ciliary movements.

11.08, $NaCl$. Revival of muscular contractions.

- 11.11.5, $MgCl_2$. Immediate stiffening of larvæ. Partial revival of ciliary movements.

11.14, $NaCl$. The revival of muscular movement is somewhat more gradual than before. At 11.20 larvæ show active squirming movements; ciliary movement is mostly feeble and largely absent.

11.22, $MgCl_2$. Larvæ immediately become rigid. Gradual revival of ciliary movement is observed in a good many larvæ.

11.27, $NaCl$. Revival of muscular movements. These are most active within one minute after the addition of $NaCl$; at 11.30 they are noticeably stiffer. 11.33. Ciliary movements are feeble and have ceased in many larvæ.

11.35.5, $MgCl_2$. Instant stiffening appears. Distinct revival of ciliary movement is seen in many; rapid trembling movements appear immediately after the addition of $MgCl_2$; these movements continue until the addition of $NaCl$, when they become slow and sluggish.

11.45, $NaCl$. Return of muscular movement as before. Ciliary movement becomes sluggish.

It is unnecessary to continue this record. The transfer back and forth from solution to solution was continued throughout the afternoon; at 5.25 P. M., the addition of $NaCl$ still produced marked muscular contractions. By this time the larvæ had been transferred back and forth twelve times (24 single transfers); the experiment was then discontinued. At each transfer the described change takes place almost with the certitude and invariability of a simple chemical reaction. This statement applies more especially to muscular movement; ciliary movement does not so readily withstand repeated change of solution. This latter peculiarity, however, becomes intelligible on considering the destructive influence of pure $NaCl$ -solutions on cilia; it is clear that after the process of liquefaction has proceeded to a certain limit the mere addition of $MgCl_2$ cannot restore all of the conditions requisite for activity. For this something more is needed than a certain state of physical consistency, which is apparently all that the action of $MgCl_2$ can confer. In the experiment described above, ciliary movement was not perceptible after 12.31 P. M.

In the case of the cilia the change in physical consistency following transfer is indicated by definite alterations in the character of the movement, especially after this has become considerably weakened. In $MgCl_2$ -solutions the cilia show rapid trembling or vibrating movements, indicating a certain stiffness in the individual cilium; in $NaCl$ -solutions, on the contrary, the movement is sluggish and wavy, and the cilia are plainly of a much more flexible consistency. Under the conditions of the above experiments, complete liquefaction does not as a rule follow transfer to $\frac{1}{3}n$ $NaCl$ since the removal of the $MgCl_2$ is necessarily incomplete.

In the case of muscular movement, repeated transfer is less injurious. With each successive transfer to $NaCl$, however, it is in general observable that the contractions become less energetic until finally their reappearance is very slight; eventually they entirely fail to appear. Shortly before this final stage is reached, it is noticeable that contractions occur only for a brief period during the first minute after transfer, after which time all the larvæ again become rigid and remain so. This appears to indicate that during the progressive replacement of Mg -ions in the muscle by Na -ions, contractions are possible only while the two ions are present in the most favorable proportions. Eventually, the action of the pure $NaCl$ solution results in an excess of Na -ions which render contraction impossible.

Similar experiments have been performed with solutions of $CaCl_2$ and have led to essentially similar results, with the exception that ciliary movement is more quickly arrested than in $MgCl_2$ -solutions. In pure $CaCl_2$ -solutions, muscular movement continues longer than in pure $MgCl_2$ -solutions; once arrested, however, a transfer to $NaCl$ produces an immediate revival precisely as with $MgCl_2$. On one occasion the transfer back and forth between $CaCl_2$ and $NaCl$ was made seven times (15 changes of solution) within two and one half hours. In each instance a revival of contractions took place on addition of $NaCl$, even in larvæ which at the end showed marked shrinkage and general disorganization.

The reappearance of muscular contractions on transfer to sea-water or $NaCl$ -solution after even prolonged action of $MgCl_2$ or $CaCl_2$, takes place promptly and without a delay of more than a few seconds. On the other hand, after the action of KCl -solutions, *the revival of contractions is incomplete and appears only after the lapse of a certain interval whose duration is often considerable and bears a direct proportion to the previous time of action of the KCl .*

The following record will illustrate :

June 27, 1901. Larvæ are transferred to $\frac{1}{2}n$ KCl at 2.30 P.M. Initial contraction, clumping, etc., appear as usual. Larvæ are completely rigid within one minute or less.

2.36. A portion of larvæ are transferred to sea-water. Contractions slowly reappear ; at 2.37 they first become evident ; at 2.40 all larvæ show somewhat stiff muscular contractions ; ciliary movement is slow.

2.

2.49.5. Larvæ in original KCl₂ are rigid and actively swimming. Sea-water is added at 2.49, 5 ; a marked checking of ciliary movements results ; larvæ sink to bottom and cease swimming. 2.53. Larvæ are still rigid and motionless ; cilia are partly dissolved. 2.56. Larvæ are nearly all rigid ; one or two feeble twitches are seen. 2.59. A few feeble twitches and movements of setæ are seen. 3.01. Most show twitches and feeble movements of setæ. 3.08. Well defined contractions are present in all, though stiff and limited.

3.

3.14. Ciliary movement in original KCl is slower than at 2.49, but is still active. Sea-water is added at 3.14. At 3.21 the larvæ are rigid and motionless ; cilia are largely dissolved, but the ventral band is active in most. 3.25. Muscular twitches are beginning to appear in a few ; most larvæ are still motionless. 3.38. A fair number of larvæ show twitches. 3.48. All show well defined muscular contractions, though somewhat limited. 4.04. Active contractions in all.

4.

4.15. Ciliary action in original KCl is much enfeebled and largely wanting. Sea-water is added at 4.15.5. 4.40. All larvæ are still motionless and without muscular movement. Ventral band of cilia is feebly active in a few. At 4.50 twitches are beginning to appear. 4.55. Almost all larvæ show feeble contractions. 5.05. Well-marked contractions in all. 7.00 P. M. Well-marked contractions in all.

5.

7.02 P. M. Larvæ in original KCl are motionless ; no ciliary movement. At 7.03 sea-water is added. 8.20 ; larvæ are beginning to show muscular twitches. 8.25. Most show slight but well-marked twitches. 9.05. Well-marked contractions are present in practically all ; contractions are jerky and appear at intervals.

6

June 28.—6.55 A. M. Larvæ in original KCl are apparently dead ; all are motionless and somewhat swollen. At 6.57 A. M. sea-water is added. 8.55 A. M. All are still motionless. At 9.00 A. M., one or two feeble contractions are noticed. 9.15. Contractions are better marked ; a fair number of larvæ show feeble contractions. 9.40. A large proportion of larvæ show feeble contractions at intervals.

On tabulating the results of the above experiments, the following appears :

Time of Action of KCl-solution.	Interval after which contrac- tions appear on return to sea-water.
1. 6 minutes	• About 1 minute.
2. 20 minutes	• From 6 to 9 minutes.
3. 44 minutes	• From 11 to 24 minutes.
4. 105 minutes	• About 35 minutes.
5. 270 minutes	• About 70 minutes.
6. 16 hours	• About 120 minutes.

Similar results appear on transferring alternately from KCl to sea-water and back. This change can be made several times in succession, but at each successive transfer from KCl to sea-water the interval that elapses before the contractions reappear is measurably greater than before. In one experiment with four such transfers, the respective intervals were 3, 10, 12 and 19 minutes.

What is the explanation of this difference of behavior between KCl on the one hand and MgCl₂ and CaCl₂ on the other? Muscular contractions reappear *immediately* on transfer from solutions of the last two salts to a favorable salt solution, whereas in the case of KCl a considerable time must elapse before they appear. Is it not probable that this peculiarity of K-salts is to be brought into relation with their specifically injurious influence on muscular contractility? If we assume that the K-ion forms with the proteids of the muscle-fibre compounds that are dissociable only slowly and with difficulty, the phenomena become somewhat more intelligible. In that case we may infer that a prolonged exposure to the action of KCl results in the formation of such compounds in correspondingly large quantity, so that an equivalent time is required for their replacement by the Na- and Ca-compounds necessary for contractility. The presence of even small quantities of the K-compounds seems sufficient to hinder contractility. This is in accordance with

the fact that no immediate result appears on return to sea-water after two or three hours in KCl. It is only after remaining perfectly motionless for at least half an hour that a few larvæ begin to show slight muscular twitches; soon after this first indication of revival, however, such movements appear in a large number and gradually become well defined in all. It is of interest to note that these contractions appear almost simultaneously in all larvæ, so that on the appearance of the first twitches in a few individuals it may be confidently expected that in a short time all the others will exhibit well marked movements.

The Mg- and Ca-compounds, on the contrary, seem readily dissociable. Neither of these ions is specifically injurious to muscular contractility; each acts injuriously only when present in such excess as to interfere with the presence of the proper proportions of the other necessary ions (see above). The question, then, may be put thus: Why should the presence of a substance (K) that has a tendency to form difficultly dissociable compounds with the proteids of the contractile elements tend to deprive muscular tissue of the power of contractility? It is clear that a tissue whose efficiency depends upon the labile condition of its constituents is likely to be injuriously affected by such a substance. It may reasonably be assumed that muscle is such a tissue. Its well-known susceptibility to changes in the composition and proportions of its inorganic constituents suggests that its activity may depend, to a very considerable degree, upon changes in the relative proportions of its ion-proteid compounds. The appearance of rhythmical contractions in skeletal muscle under the action of pure NaCl solutions¹ shows that a change in the relative proportion of ions will in itself act as a stimulus to contraction. So also, the well-known fact that an increase in the proportion of the calcium salts will act as a stimulus to a heart which has come to rest in a Ringer's solution (Howell,² Greene,³ Walden⁴). The presence of K, by forming difficultly dissociable compounds, will tend to limit such changes, and hence to retard or limit the activity of the associated contractions.

In this manner it may be possible to explain certain characteristic physiological properties of the salts of this element. The KCl of the blood has for some time been regarded as influential in causing relax-

¹ LOEB, J. : Festschrift für Fick, Braunschweig, 1899, p. 101.

² HOWELL : *Loc. cit.*

³ GREENE : *Loc. cit.*

⁴ WALDEN : This journal, 1899, iii, p. 123.

ation of the heart-muscle and in neutralizing the stimulating action of the Ca-salts (Howell). The above suggestions, although as yet perhaps insufficiently supported by facts, may help to explain this action among other characteristic peculiarities of potassium salts. Thus the action of the K-ion on nerve seems strikingly similar to its action on muscle (*cf.* Mathews¹), K-salts having a marked effect (more marked than Mg-, Ca-, Sr-, or Ba-salts) in lowering conductivity.² This fact, together with those given above, throws much light on the general pharmacological action of K-compounds. As is well known, these are poisonous when administered in large amounts, the characteristic symptoms being depression of the central nervous system and of the heart,³ combined with great muscular weakness and apathy (*cf.* Cushny⁴). A moderate lowering of nervous and muscular irritability is produced by smaller doses, hence the familiar use of these compounds, especially KBr, as sedatives. It seems probable from the above experiments that these effects depend ultimately upon the production of difficultly dissociable and therefore non-reactive salt-proteid compounds within the living tissues.

Pure $\frac{4}{8}$ *n* NaCl is much less effective than sea-water in renewing contractions in KCl-larvæ. Even after a stay of fifteen minutes or even less in KCl-solution, transfer to NaCl produces at best merely a momentary and incomplete recovery. Mixtures of NaCl and CaCl₂, however, effect a partial recovery. The following record will illustrate these statements:

Larvæ are added to $\frac{4}{8}$ *n* KCl at 10.40 A. M., June 28, 1901. At 2.31 P. M. KCl is replaced in dish "A" by sea-water and in "B" by $\frac{4}{8}$ *n* NaCl. At

¹ MATHEWS: Journal of the Boston Society of Medical Sciences, 1901, v. p. 349.

² Conductivity thus lost may be restored by the action of NaCl, which thus seems essential to the maintenance of nervous as well as of muscular irritability. It is noteworthy that muscle and nerve, although widely different in physiological rôle, are alike in this one characteristic, — that their activity is by its very nature *intermittent* and called forth by slight changes in the surroundings (stimuli). The prime characteristic of each is irritability, for which an especially labile condition of the protoplasm is indispensable. Hence K is specifically injurious to muscle and nerve, whereas processes that are essentially continuous and uninterrupted in their activity, as cell-division and ciliary motion, do not seem to be injuriously affected by its action.

³ *Cf.* BOTTAZZI: Archives de physiologie normale et pathologique, 1896, (5) viii, p. 882.

⁴ CUSHNY: A text-book of pharmacology and therapeutics. Second edition, 1901, p. 481.

2.42 KCl is replaced in "C" by a mixture of 13 vols. $\frac{2}{3} n$ NaCl + 2 vols. $\frac{1}{3} n$ CaCl₂.

In "A" twitches begin at 3.10, and at 3.25 most larvæ show well-defined contractions. In "B" no movements appear. In "C" twitches begin at 3.38 and are well defined at 3.40.

The NaCl and CaCl₂ may be added separately and in either order. As instances the following are given:

June 29. — 10.42 A. M. $\frac{2}{3} n$ KCl is added to larvæ. In 20 minutes (11.02) KCl is replaced by $\frac{2}{3} n$ NaCl. No immediate result; larvæ remain motionless at 11.06. At 11.07 NaCl is withdrawn and $\frac{2}{3} n$ CaCl₂ is added. *Immediate* contractions appear momentarily in all larvæ.

As an example of the reverse experiment, the following is a typical instance.

Larvæ are placed in KCl at 11.45.5 A. M., June 28, 1901. At 12.06 P. M. KCl is replaced by $\frac{2}{3} n$ CaCl₂ in a portion of the larvæ (A). No contractions are seen. At 12.12 the CaCl₂ is replaced by NaCl. At the same time, as a control, KCl is replaced in a part of the original larvæ (B) directly by NaCl.

At 12.14.5 well-marked contractions appear in A while the larvæ in B are still rigid and motionless.

12.35. A. Well-marked contractions in all.

B. Entirely without movement.

Both of the above experiments were repeated a number of times with varying times of exposure to the action of the different salts, and with results identical with those above set down. Very generally, however, momentary contractions appear immediately after the transfer from KCl to CaCl₂. It appears, therefore, that for resumption of contractions after prolonged action of KCl, the presence of both Na- and Ca-ions is necessary. If the previous action of CaCl₂ has supplied the necessary Ca-ions, the addition of NaCl will call forth immediate contractions; similarly if Ca is added after previous action of NaCl. But in the latter instance the contractions are only momentary, since the action of the pure CaCl₂ quickly results in an injurious excess of Ca-ions, rendering further contraction impossible.

VI. THE ACTION OF SOLUTIONS OF NON-ELECTROLYTES AND OF MIXTURES OF THESE WITH SALT-SOLUTIONS.

From the above experiments on the effects of transfer, it is clear that the ions present at any time in the tissues may be readily re-

placed by others according to the nature of the medium in which the tissues are immersed. This fact plainly supports the view that the relation between the medium and the tissues is one of chemical equilibrium between the salts in the one and the ion-proteid compounds in the other. If this is true, the endeavor to establish equilibrium in a medium free from electrolytes should lead to a continued diffusion of ions outward from the tissues, and hence to an eventual loss of the active properties of these. If, however, a certain proportion of an isotonic salt-solution be added to the non-electrolyte solution, the tissues should tend less rapidly to lose the properties that depend upon the presence of this particular salt, since, when equilibrium is established, the corresponding ion-proteid compound will still remain present to a greater or less degree. It should also be found that restoration of the proper saline constituents to the medium within a reasonable time after the loss of the characteristic activities in a non-electrolyte solution will be followed by a return of these activities. All of these expectations are very accurately realized by the results of actual experiment.

Pure $\frac{7}{8} n$ glycerine and cane-sugar solutions (approximately isotonic with $\frac{4}{8} n$ NaCl, etc.), were first used, followed by mixtures of these with solutions of a single salt, as follows:

1. Pure $\frac{7}{8} n$ glycerine.
 2. 80 vols. $\frac{7}{8} n$ glycerine + 20 vols. $\frac{4}{8} n$ NaCl.
 3. 50 vols. $\frac{7}{8} n$ glycerine + 50 vols. $\frac{4}{8} n$ NaCl.
 4. 20 vols. $\frac{7}{8} n$ glycerine + 80 vols. $\frac{4}{8} n$ NaCl.
 5. Pure $\frac{4}{8} n$ NaCl.
- 6-9 inclusive. Like 2 to 5 with $\frac{4}{8} n$ KCl instead of NaCl.
 10-13, inclusive. Glycerine and $\frac{8}{8} n$ MgCl₂ as above.
 14-17, inclusive. Glycerine and $\frac{8}{8} n$ CaCl₂ as above.

The second series was in all respects similar to the first except that $\frac{7}{8} n$ cane-sugar was used in place of glycerine.

In pure solutions of sugar and glycerine there is seen a *gradual* loss of both ciliary and muscular contractility. The following record will illustrate:

July 5, 1901 — 11.08 A. M. Larvæ are transferred to $\frac{7}{8} n$ cane-sugar solution.

Muscular movements are at once perceptibly checked; so are ciliary movements, though less markedly than muscular movements. 11.13. Most larvæ show slow muscular and ciliary movements; a tendency to collect in clumps is also observable. 11.22. Feeble muscular and ciliary movements are still present in most. 11.34. Larvæ are rigid, and show only slight contractions; slow swimming movements continue in a fair number.

12.02. Larvæ are rigid and without muscular movement: shrinkage is well marked. Slow ciliary movements continue. . 1.22. Larvæ are rigid and shrunken; slow ciliary movements continue in many.

Ciliary movement is thus retained longer than muscular movement in pure sugar-solutions. The action of pure glycerine solutions is essentially similar; glycerine, however, has in itself a specifically injurious influence, and in its solutions all activities cease sooner than in the corresponding sugar-solutions.

It is unnecessary to give complete records of the action of the various combinations of salt and non-electrolyte, since the effect of such solutions is almost identical with that of the pure salt-solutions in various grades of dilution. In general, dilution of a pure salt-solution with an isotonic sugar-solution markedly diminishes the injurious action characteristic of the pure salt. Thus while in pure $\frac{4}{3}n$ NaCl muscular movements entirely cease within three or four hours, in the sugar-mixtures contractions continue for a period whose length is within certain limits inversely proportional to the concentration of the NaCl. Thus in Solutions 2 and 3 above, contractions were found after almost ten hours, long after they had ceased in Solutions 4 and 5. Similarly, of the four Na-containing solutions ciliary movement continued longest in Solutions 2 and 3, while in Solutions 4 and 5 the characteristic liquefaction induced by pure NaCl appeared early (sooner in Solution 5) and destroyed activity.

With mixtures of KCl and sugar, the peculiar action of K becomes immediately evident, as seen in the rapid cessation of muscular movement. Ciliary movement, however, continues unchecked in such solutions for a considerable space of time, longest in Solutions 6 and 7 (longer in 6 than in 7) where it may continue for so long as eighteen hours, and ceasing relatively soon in Solutions 8 and 9.

The Mg-mixtures also exhibit essentially similar conditions. Ciliary movement remains active longest in Solutions 10 and 11. These solutions seem at first even to have a stimulating influence; when first added the larvæ exhibit excessively active ciliary movement and rapid heliotropic swarming; heliotropism ceases within a few seconds, however (sooner in Solution 11 than in 10), and muscular contractility is quickly lost. Solutions 12 and 13, with a higher concentration of Mg, arrest muscular contractions more quickly, as indicated by the non-appearance of heliotropic swarming. Ciliary movement is long-continued in all these solutions: in one instance it continued

in all four for a period of eighteen hours — a fact which strikingly illustrates the specific influence of Mg on this form of activity.

In the mixtures of sugar and CaCl₂ heliotropism also appears in the first two solutions but not in the last two. Muscular movement, on the other hand, although limited, continues much longer than in the KCl- and Mg-mixtures, while ciliary movement ceases relatively soon.

Mixtures of *n*-cane-sugar solution and sea-water yield analogous results. The following five solutions were tried on larvæ of a more advanced stage than the above (mostly of six somites):

1. 100 volumes *n*-cane-sugar.
2. 75 volumes *n*-cane-sugar + 25 vols. sea-water.
3. 50 volumes *n*-cane-sugar + 50 vols. sea-water.
4. 25 volumes *n*-cane-sugar + 75 vols. sea-water.
5. 100 volumes pure sea-water.

In the pure cane-sugar-solution, muscular movements were found to have completely ceased within less than an hour, and the larvæ were extended and rigid. In Solution 2, contractions continued for more than fifty-six hours, while in Solutions 3, 4, and 5 active movements continued for many days. After ten days infusoria and bacteria had become abundant and most larvæ were dead; the experiment was then discontinued. In Solution 2 insufficient salts seem to be present. Yet even a solution containing ninety volumes *n*-cane-sugar and only ten volumes sea-water was found capable of sustaining contractions in many larvæ for more than twenty-four hours; and a few slight muscular twitches were still visible after forty hours.

Solutions containing seventy-five volumes *n*-sugar-solution and twenty-five volumes of various mixtures of two and three chlorides were also tested; but, as might have been expected, even the most favorable of these were decidedly more injurious than Solution 2 of the above series.

Finally, brief mention should be made of those experiments in which larvæ were transferred to sea-water after the complete cessation of contractions in *n*-sugar-solution. The following record will illustrate:

August 15, 1901. Active and elongated larvæ of mostly six somites were added to *n*-sugar-solution at 3.00 P. M. By 3.23 practically all larvæ were perfectly rigid and motionless. A number of larvæ were transferred from sugar-solution to sea-water at 3.24, 4.00 and 5.50 P. M., respectively. In each instance well marked contractions appeared after a brief interval;

recovery, however, was less perfect after prolonged action of the sugar-solution. At 8.35 A. M., August 16, transfer to sea-water produced no contractions.

Experiments on the effects of transfer from sugar-solutions to various artificial solutions have not yet been made.

These experiments taken in conjunction with those described above, justify the inference that the inability of solutions of non-electrolytes to support muscular contractions for any length of time (as pointed out by Loeb, Greene, Lingle, and others) is not due to any specifically injurious action of the dissolved substance itself, but solely to an exclusion of the necessary salts, resulting from the replacement of the natural media of the tissues by such solutions. Apparently in the absence of the salts, the dissociation of the ion-proteid compounds and the outward diffusion of the ions in a short time so alter physical and chemical conditions as to render continued contractions impossible.

VII. SUMMARY AND CONCLUSIONS.

1. Each of the chlorides NaCl , MgCl_2 , CaCl_2 and KCl exercises a definite specific influence on the properties of the contractile tissues; this influence is evident even in the presence of other salts. The inference is that each salt forms within the tissues a salt-proteid compound (ion-proteid) possessing definite physical properties.

2. For normal activity these various compounds must be present in the tissues in certain definite proportions. Hence the favorable action of mixtures containing three chlorides in certain proportions, and the unfavorable effect of a change in these proportions (*e. g.*, a reversal of the relative proportions of Ca and Mg).

3. The salt-proteid compounds are dissociable, and the salts are thus readily replaced by one another in the tissues with corresponding changes in the properties of these. Hence, *e. g.*, the loss of muscular contractility in solutions containing an insufficiency of Na -ions, and the revival of contractions on transfer to pure NaCl -solution or sea-water.

4. K -salts are peculiar in forming combinations that are not readily dissociable; hence their specifically injurious influence on muscular contractility.

5. In non-electrolyte solutions the active properties of the tissues

are gradually lost; the addition of small quantities of isotonic salt-solutions to the non-electrolyte solution prevents the immediate loss of the properties that are favored by the presence of those salts.

6. Development may proceed in favorable artificial mixtures of NaCl, MgCl₂, and CaCl₂; it is hindered by the presence of a trace of acid and furthered by the presence of a trace of alkali.

EFFECTS OF POTASSIUM CYANIDE AND OF LACK OF OXYGEN UPON THE FERTILIZED EGGS AND THE EMBRYOS OF THE SEA-URCHIN (*ARBACIA PUNCTULATA*).

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INTRODUCTION.

IN 1895 Professor Loeb¹ published the results of observations on the effects of lack of oxygen on the development of certain marine eggs. He found that the fertilized eggs of *Ctenolabrus*, a bony fish, cannot segment in sea-water freed from oxygen. Moreover, if eggs in the two, four, or eight cell stage be placed under conditions of lack of oxygen, the cell walls will liquefy. But neither the unsegmented eggs nor those with liquefied cell walls are dead. They may recover and go on developing if brought back into aerated sea-water.

He found also that the eggs of the sea-urchin, *Arbacia*, cannot segment in absence of oxygen, although in this organism the solution of already formed membranes does not occur. On the contrary the eggs of another bony fish, *Fundulus*, as Loeb² showed in an earlier paper, can go on segmenting for several hours in total absence of

¹ LOEB: *Archiv für die gesammte Physiologie*, 1895, lxii, p. 249.

² LOEB: *Archiv für die gesammte Physiologie*, 1893, lv, p. 530.

oxygen. It thus appears that there are striking differences in the behavior of the eggs of different species of animals.

Loeb studied also the effects of lack of oxygen upon the embryos of *Ctenolabrus* and *Fundulus*. The heart of the former, he found, stops beating very soon. That of the latter beats for many hours. Thus again the differences in the protoplasm of different species of animals are emphasized.

In his experiments Loeb used a stream of hydrogen to displace the oxygen in an Engelmann chamber containing the eggs or embryos. To prevent segmentation before the air had all been driven out of the chamber, he made use of ice. The eggs were fertilized, placed immediately in the chamber and cooled nearly to the freezing point of water. Meanwhile the stream of gas was started, and continued for about two hours. Then the eggs were allowed to assume room temperature and were observed from time to time, the stream of hydrogen being continued slowly throughout the experiment.

It was partly with the hope of finding an easier method of experimenting with lack of oxygen that Dr. Loeb suggested that I try potassium cyanide on sea-urchin eggs. Furthermore he desired that I ascertain whether the degree of resistance to this poison remained constant or varied during the progress of development. In other words, is oxygen equally essential at all stages of development? Other questions came up during the progress of the experiments.

METHODS.

A titrated solution of KNC in distilled water had been brought to Woods Hole from the laboratory in Chicago. This was a 0.95 Mol¹ (= *m*) solution. This solution was diluted with filtered sea-water to make a solution $\frac{m}{10}$; and from this, with sea-water as the diluent, the other solutions were made up as needed. A slight precipitate, probably of magnesium and calcium hydrates, follows the addition of strong KNC to sea-water. This was filtered off from the $\frac{m}{10}$ solution, but in subsequent dilutions was not sufficient to be noticeable or troublesome.

The eggs of the sea-urchins used in these experiments were fertilized in the laboratory in the usual way. After the proper time they

¹ The Mol is a solution made by dissolving a number of grams of substance numerically equal to its molecular weight and then diluting to one litre. The *m* of KNC contains 65 grams in 1 litre and is therefore a 6½% solution.

were brought into measured quantities (usually 50 c.c.) of the solutions contained in flasks of about 75 c.c. capacity. A slight change of concentration was unavoidable when the eggs were added. This change was kept constant and as small as possible by always introducing, by means of a marked pipette, the same amount of sea-water (about one c.c.).

In the early experiments the flasks were kept covered with loose fitting glass caps, the idea being that plenty of air should be admitted. But it was found that the solution rapidly weakens, chiefly, no doubt, through the evaporation of HNC. In most of the experiments, therefore, the flasks were kept tightly corked. Since the constant effect of the cyanide in these experiments, as well as in those on higher animals, seems to be to paralyze the power of oxygen consumption in the cells, no complications were introduced by thus closing the flasks.

When it was desired to remove eggs from the cyanide solution, they were drawn up by a pipette and carefully dropped into the top of a test tube full of filtered sea-water. The eggs sank slowly through this, leaving most of the poison in the upper part of the tube, whence it was drawn off by means of a bulb syringe. If necessary the process was repeated until the eggs were thoroughly washed. Finally they were placed in watch-glasses filled with sea-water. After a convenient interval, usually several hours, the eggs were examined. Further examinations were made later if there was still a chance that the eggs might develop or if interesting features presented themselves.

All the experiments were carried on at room temperature, which averaged about 20° C.

RELATIVE RESISTANCE AT DIFFERENT PERIODS OF DEVELOPMENT.

Professor Loeb¹ has shown that the egg differs strikingly from advanced stages in its resistance to certain ions. For example, potassium is much less poisonous to the egg and to embryonic tissue than to the heart or skeletal muscles in later stages. I find that the egg is quite resistant to cyanide, and that there is a progressive loss of resistance as development proceeds. For example, after immersion in $\frac{1}{1500}$ solution of KNC during forty-eight hours or more some unsegmented fertilized eggs are still able, when brought back into

¹ LOEB: This journal, 1900, iii, p. 383.

normal sea-water, to segment and produce swimming larvæ. Embryos (plutei) two days old, from the same culture, would, on the contrary, be killed in five or six hours in the above strength of cyanide, while a one-day embryo might survive eighteen or twenty hours.

I give below in tabulated form the results of one experiment designed to test resistance at different ages. The first of the two numbers in any space indicates the number of hours in the poison after which some of the eggs developed to swimming larvæ; or in case of embryos, the number of hours after which some recovered from the poison. The second number denotes the time of immersion after which no development or no recovery took place. In other words, the organisms were killed somewhere between the two times mentioned.

TABLE I.

Time after fertilization eggs were placed in solutions.	Strength of KNC solutions.						
	$\frac{m}{100}$	$\frac{1}{250}$	$\frac{m}{500}$	$\frac{m}{750}$	$\frac{m}{1000}$	$\frac{m}{1500}$	$\frac{m}{2000}$
Before first cleavage. (15 minutes after fertilization?) . . .	5-6 $\frac{1}{2}$ *	5-18	6 $\frac{1}{2}$ -18	24-44	24-44	48-72	68-72
7 hours. Early blastula . . .	-11	-11	-11	14-18	71-83	42-83	71-83
19 hours. Gastrula	1 $\frac{1}{4}$ -1 $\frac{1}{2}$	2-4	4-6	8-	8-24	8-24	8-24
27 hours. Advanced gastrula	-1 $\frac{1}{2}$	2-	2-5	5-18	5-18	18-24	18-24
48 hours. Pluteus	$\frac{1}{4}$ - $\frac{1}{2}$	1 $\frac{1}{2}$ -2	1 $\frac{1}{2}$ -2	3-5	3-5	5-7	5-7

* Figures indicate number of hours of two observations between which death occurred.

It is understood that the above are extreme limits. Often only one or two eggs would develop out of several hundred. Many might begin to segment but fail to reach the blastula stage. Others might reach that stage, only to disintegrate immediately after ciliary motion began. It is practically impossible, therefore, to get average lethal periods. The above figures express the truth in a broad way but not in detail.

In one particular, however, further experiments are desirable. The table would seem to indicate that the early blastula (seven hour embryo) may resist the poison longer than the unsegmented egg. Some of the other experiments contain inconclusive evidence to the same effect, while in others it does not come out at all. It is certain

that the cilia of the *Arbacia* embryo are very resistant to KCN. As will be shown later, plutei often recover the power of ciliary motion after treatment with cyanide, only to go to pieces immediately after. It seems probable from my experiments that, in the blastula stage, it may be possible by a proper period of treatment to arrest development forever, while the power of motion may be recovered and the embryo swim about for days. In other words the functions of cell-division, differentiation, and growth may be stopped, while ciliary motion continues. It is certain that, in some of my experiments, blastulas, after being taken from the cyanide solution, recovered ciliary activity and swam about actively for three or four days without showing any trace of further development. At the end of that time some of them went on into the gastrula and pluteus stages, but I think that others never did.

In this connection it is interesting to compare my results for the fertilized egg with those of Loeb and Lewis¹ for the unfertilized. It will be seen that fertilization considerably diminishes the period which an egg can live in KCN. In $\frac{m}{750}$ KCN no fertilized egg developed after forty-four hours. Even after only twenty-four hours very few became swimming larvæ, and most of these were unnatural ciliated forms that never became plutei. Loeb and Lewis found, however, that "many unfertilized eggs segment and develop into swimming larvæ" after seventy-five hours or even longer in $\frac{m}{750}$ KCN. My solutions being in flasks probably did not lose their strength so fast as those of Loeb and Lewis, which were in loosely covered finger bowls. But that this is not the cause of the whole difference is clear from their experiments with renewed solutions and corked flasks. For example, a few unfertilized eggs were able to begin development after sixty-six hours in $\frac{m}{1000}$ KCN in a corked flask. No fertilized eggs developed or even began cleavage after forty-four hours in $\frac{m}{1000}$ KCN contained in a loosely capped flask.

It should be said, however, that all my experiments on the relative resistance of eggs in solutions of strengths comparable with those of Loeb and Lewis were made before I found that there are successive stages of relatively high and low resistance in each cleavage. In these early experiments I usually waited about fifteen minutes after fertilization before placing the eggs in the solutions, the supposition being that I should wait until union of the pronuclei had occurred, in order to avoid killing the sperm. The condition fifteen minutes after

¹ LOEB and LEWIS: This journal, 1902, vi, p. 305.

fertilization is, as it turns out, one of comparatively slight resistance. It is probable that if I had known this fact and had waited for the period of greatest resistance (say thirty-five or forty minutes after fertilization), all my lethal periods for the unsegmented eggs would, on the one hand, have been higher than those for the blastula and, on the other hand, have more nearly approached the periods found for unfertilized eggs by Loeb and Lewis.

IN HOW STRONG SOLUTIONS IS SEGMENTATION POSSIBLE?

There seems to be much variation in different cultures of eggs regarding the strength of solution needed to stop segmentation. In some cases one or two divisions took place in $\frac{m}{10000}$ KNC. In $\frac{m}{15000}$ some eggs went to the sixteen cell stage, but many stopped in two or four cells. In $\frac{m}{20000}$ few stop at two cells and at $\frac{m}{30000}$ some may develop to the blastula stage and swim about. The above results were exceptional. In other cases solutions as weak as $\frac{m}{100000}$ were perhaps sufficiently strong to stop segmentation in the two or four cell stage. These differences, again, are perhaps partially explainable on the basis that the eggs were not all placed in the solutions at similar periods of resistance.

HASTENING OF SEGMENTATION AND GROWTH BY WEAK SOLUTIONS OF KNC.

An unexpected phenomenon which seems fairly established by these experiments is the actually advanced condition of cultures raised in very weak KNC solutions, over the control raised in sea-water. In solutions of less than $\frac{m}{100000}$ segmentation and development usually went on to the pluteus stage, and the embryos remained alive as long as the control. If the solution was as strong as $\frac{3m}{1000000}$ or $\frac{4m}{1000000}$, the embryos developed about as fast as the control. If the solution was stronger, they fell behind. But if the solution was weaker, say $\frac{m}{1000000}$ or $\frac{2m}{1000000}$, numerous individuals developed faster than the control. This could be especially well noticed when the embryos went into the pluteus stage. The difference was not great, but well enough marked to be noted by all whom I asked to compare the cultures. I may add here that I have since found that paramœcia lived longer in very weak KNC solution than in distilled water (ordinary, not distilled in glass) of the same quality as that used in making up the solution.

Loeb¹ has found that the addition of a small amount of OH ions to sea-water hastens the development of *Arbacia* eggs. Since KNC solutions, at least strong ones, are alkaline, it might be supposed that we had here to do with the same phenomenon. But a comparison of the added alkalinity in the two cases shows that this is not the explanation. For example Loeb added 2 c.c. of $\frac{m}{10}$ NaOH to 100 c.c. of sea-water in one of his experiments. Titration showed that about 1.4 c.c. remained in solution. In other words, there was an added alkalinity amounting to about 0.0014 *m*. In my experiments the added KNC was only 0.000001 to 0.000002 *m*, and the added alkalinity much less, if indeed so weak a solution contains any free OH ions.

Loeb noted further, in his work on *Fundulus*, *Ctenolabrus*, and *Arbacia* eggs already cited, that specimens in a current of hydrogen (the chamber not being, however, as yet entirely free from oxygen), might segment a little before the control. Often the difference was from six to ten per cent in favor of the eggs in the lessened amount of oxygen. Whether my experiments are an expression of the same fact remains for further investigations to determine.

CILIARY MOTION AND DISINTEGRATION OF EMBRYOS.

I have mentioned that plutei placed in KNC solutions went to pieces very soon after being returned to sea-water. Examined under the microscope, the cilia which had been paralyzed in such cases were seen to start up. Then at some exposed part, usually on one of the arms, cells would begin to break loose. The fragments were generally single cells; occasionally, a small number of united cells. These loose cells looked like minute flagellates. They swam for an instant, then settled down and were not seen to move again. A dozen or more might leave inside of two minutes. Meanwhile the pluteus continued to swim about, gradually losing cells until the skeleton was exposed and finally very little else was left. The disintegration became complete.

It was interesting to note that disintegration did not occur unless the cilia recovered from the poison. If the plutei were placed in very strong KNC or were kept for a long time in a weak one, there was no recovery of the cilia on return to sea-water, and the organism remained intact until putrefaction set in. If, however, the cilia re-

¹ LOEB: *Archiv für Entwicklungsmechanik der Organismen*, 1898, vii, p. 632.

covered, the whole mass of cells might swim away from the skeleton within a few minutes. It would seem, therefore, that the poison weakens the union between the cells. They drop apart at the slightest shock, for example, the pull of the cilia.

The same thing could be demonstrated at any stage after ciliary motion had begun. Eggs placed in cyanide just at the beginning of the blastula stage and with the vitelline membrane still present might recover after being placed in sea-water. Then cilia could be seen in motion, and cells would swim away through the ruptured membrane. The whole embryo would quickly go to pieces.

This weakened coherence of the cells was seen also in embryos which came from eggs which had been treated with cyanide before cleavage began. Suppose a large number of eggs to be placed, immediately after fertilization, in $\frac{m}{100}$ KNC and taken out after two hours, washed, and placed in sea-water. Probably ninety-nine per cent of the eggs would segment and continue development to the blastula stage. As soon as they acquired cilia, the cells would fall apart and die. Perhaps not one per cent would keep together and go on developing. This was noticed so often that the presence of numbers of loose cells became a sure sign that swimming larvæ had developed, even though not one could be found at the time of examination.

It is interesting to note that this disintegration by ciliary action, while apparently a secondary effect of the poison, really goes back to lack of oxygen or lack of the power of using it. This is shown by the fact that the same kind of disintegration follows prolonged exposure to pure hydrogen gas. Examination of the tables which will accompany the part of this paper devoted to the varied resistance seen at different periods in each cleavage will indicate to how great an extent this occurs.

From experiments on the *Tradescantia* cell De Moor¹ concluded that the oxygen was needed for the formation of the cell walls. Loeb² found that the cell walls of *Ctenolabrus* eggs when in the two to eight cell stages were liquefied in an oxygen vacuum and reformed on readmitting air. He noted that the membranes in *Arbacia* are not so liquefied. But though not visibly different, the membranes, as my experiments show, are apparently in some way so changed that the cells do not cling tightly together. Moreover if the eggs are put in KNC or under lack of O, they are in some way

¹ DE MOOR : Archives de biologie, 1895, xiii, p. 163.

² LOEB : Archiv für die gesammte Physiologie, 1895, lxii, p. 249.

affected so that membranes not to be formed for hours to come are nevertheless weakened. And for this reason alone, apparently, the majority of eggs treated with cyanide for any length of time cannot develop beyond the blastula stage.

IMMUNITY TO KNC.

So far as I know, no experiments have heretofore been made on immunity to this poison. My own investigations are not extensive, but sufficient to show that a considerable degree of immunity can be developed. The results of two experiments will be given:—

Experiment K.—Eggs of *Arbacia* were allowed to develop for three days in $\frac{6\text{ }m}{1000000}$, $\frac{8\text{ }m}{1000000}$ and $\frac{10\text{ }m}{1000000}$ KNC. All were by that time in the pluteus stage, and those in the first two solutions were as far along as the control in sea-water. Embryos from each solution and from the control were then drawn off and placed in solutions of $\frac{10\text{ }m}{1000000}$, $\frac{20\text{ }m}{1000000}$ and $\frac{30\text{ }m}{1000000}$. In less than ten minutes the embryos from the control were precipitated in all these solutions, although still moving feebly at the bottom of the tubes. In twenty minutes the control embryos placed in $\frac{30\text{ }m}{1000000}$ were dead and, soon after, those in $\frac{20\text{ }m}{1000000}$. Meanwhile all those which had been raised in KNC solutions were swimming actively in the stronger solutions. After four hours, those in the $\frac{30\text{ }m}{1000000}$ solution were precipitated; but those in $\frac{20\text{ }m}{1000000}$ and, of course, $\frac{10\text{ }m}{1000000}$ were still active twenty-four hours later. Some of the embryos removed from the control to $\frac{10\text{ }m}{1000000}$ recovered. It appears, then, that embryos raised in a weak solution of KNC are able to live in solutions two or three times as strong as embryos of the same culture raised in sea-water.

Experiment F.—Fertilized eggs were placed in $\frac{3\text{ }m}{1000000}$, $\frac{5\text{ }m}{1000000}$ and $\frac{10\text{ }m}{1000000}$ KNC. Twelve hours later the $\frac{5\text{ }m}{1000000}$ solution was full of active blastulas. Some of these were transferred to $\frac{10\text{ }m}{1000000}$. They soon fell to the bottom but later recovered and went on developing. But the eggs originally placed in $\frac{10\text{ }m}{1000000}$ went to pieces in advanced cleavage or in the blastula stage.

Two days after the eggs were placed in the solutions the embryos in $\frac{3\text{ }m}{1000000}$ KNC were found as far advanced as the control raised in sea-water. Samples of each were transferred to $\frac{20\text{ }m}{1000000}$ KNC. Those raised in sea-water were all precipitated in less than an hour and a half. Those raised in $\frac{3\text{ }m}{1000000}$ did not begin to go down till much later. A few were still swimming two days later.

VARIED RESISTANCE TO KNC DURING CLEAVAGE.

In investigating the time which eggs could live in cyanide solutions, I found considerable variation among different cultures. As I had not been careful to always place the eggs in solutions exactly the same number of minutes after fertilization, it occurred to me that the eggs might not be equally resistant throughout the first cleavage, and that the variations noted might be due to this fact. Experiments were made to test this point. The eggs from the ovaries of a single ripe female were fertilized with sperm from a single male. This procedure was followed in order to obtain a culture in which the first and each succeeding cleavage in all the eggs might be as nearly simultaneous as possible. Very soon after fertilization (say three minutes after adding the sperm, by which time the eggs would have settled to the bottom and examination of a drop would have shown the formation of the membrane), a portion of the eggs was transferred to KNC solution. Strong solutions of the poison (usually $\frac{m}{50}$ or $\frac{m}{100}$) were used in order that development might be stopped very quickly. Ten minutes after fertilization another portion of eggs was placed in cyanide, and so on. As the first cleavage approached, I usually proceeded at five-minute intervals and continued this through the more rapid second and third cleavages. Farther than the third division I did not study; indeed, it would be difficult to get satisfactory results, because the cleavages cease to be even approximately simultaneous.

After the first eggs (those placed in the cyanide immediately after fertilization) had been in the solution a certain time, say one hour, part of them were drawn off, washed in sea-water in the manner already described, and left in sea-water in a watch-glass to develop. Ten minutes after the first sample was taken out, some eggs were drawn off from the second flask and similarly treated. This was continued until samples were drawn off from all the flasks—samples which had developed for different lengths of time after fertilization before they were put in the poison, but which had all been in the cyanide for the same interval; *e. g.*, one hour. This lot of samples was called the one-hour series. In the same way one and one half hour, two-hour, two and one half-hour series, etc. were prepared. Next day the samples were examined for swimming larvæ and for disintegrated cells, which, as I have said, constituted a sure sign that blastulas had developed and then fallen to pieces. There were

usually two or three hundred eggs in each sample. Before discussing the results of these experiments, I will give in convenient form the notes taken in some of them.

TABLE II.
Experiment D. — $\frac{m}{100}$ KNC.

Mins. after fertilization eggs were placed in solution.	1½-hour series.	2½-hour series.	3½-hour series.
0 (3)	Several swimmers. Much disintegration. All eggs to morulas, or farther.	Several blastulas. Much disintegration. No unsegmented eggs.	4-cell stages abundant. Some morulas. Two or three swimmers. Some disintegration.
10 (13)	About like above. Less disintegration.	Two or three blastulas. Less disintegration than above. Mostly unsegmented eggs.	<i>All unsegmented eggs.</i>
20 (23)	Decidedly more blastulas.	More morulas than at 10 min., but no blastulas.	Many morulas.
30 (33)	About same as 20-min. sample.	10% or more swim.	
50 (53)	Very lively lot of blastulas. Dozens to one in any of preceding.	Large number of blastulas. No unsegmented eggs. All morulas, or farther.	
55 (58)	Fewer blastulas than in 50-min. sample.	<i>Practically all dead in 2-cell stage; "as perfect as if fixed."</i>	<i>Practically all dead in 2-cell stage.</i>
60 (63)	Many blastulas.	Thirty blastulas. Some dead 2-cells, but mostly morulas, or farther.	
65 (68)	Many blastulas.	A few swim. Mostly morulas, or 2-celled.	
70 (73)	A good many blastulas.	Several swimmers; all advanced cleavage. Not a 2-cell in lot.	Morulas and 2-cell. One swimmer.
75 (78)	No blastulas. <i>Mostly dead in 4-cell stage.</i>	Several blastulas. Many morulas. <i>Many 4-cell stages.</i>	<i>Mostly 4-celled.</i>

Experiment D. — Eggs fertilized August 20, 3.12 P. M. First sample placed in $\frac{m}{100}$ KNC at 3.15. At 3.25, 3.35, 3.45, 3.55,¹ 4.05, 4.10, 4.15, 4.20, 4.25, and 4.30 other samples placed in the solution. Samples taken from each lot one and one-half, two, two and one-half, three, and three and one-half hours after it was put in. (The notes on the two and three

¹ No further record of this lot found.

hour series are not published). At 4.00 a few eggs in the control were in the two-cell stage. At 4.05 the control was mostly in two-cell stage. At 4.15 a very few of the control were in four-cell stage. At 4.20 not many in four-cell stage as yet. 4.30, many four-cell in control. Numbers in parentheses indicate number of minutes after addition of sperm. Three minutes were allowed for fertilization and for the eggs to settle, before the first lot (those marked "o") was placed in cyanide.

TABLE III.
Experiment D'. — $\frac{m}{200}$ KCN.

Mins. after fertilization eggs were placed in solution.	3-hour series.	4-hour series.	5-hour series.
0 (3)	A few swimmers (blastulas). Many morulas and disintegrated cells. Some unsegmented eggs.	One swimmer. Segmentation common and always to morulas.	Over half of eggs segmented, and always to large number of cells.
10 (13)	No blastulas. Many morulas and disintegrated masses of cells. Many unsegmented eggs.	Practically no segmentation.	No segmentation at all.
20 (23)	Numerous blastulas. No unsegmented eggs.		
30 (33)	Many blastulas.	Many blastulas. Many morulas.	Large number of blastulas.
50 (53)	Literally alive with blastulas. Practically all eggs have developed.		
55 (58)	Like 50.		
60 (63)	Hundreds of dead 2-cell stage. Three swimmers.	Practically all 2-cell. Rarely a 4, and nothing higher.	
65 (68)	Mostly 2-cell. One swimmer and a few morulas.		
70 (73)	No 2-cell. All morulas, or farther. Several blastulas.		
75	Many blastulas. All rest morulas. No 2 or 4-cell stage.		

Experiment D'. — Like Exp. D, except that eggs were placed in $\frac{m}{200}$ KCN, and samples taken out after three, four, and five hours.

TABLE IV.
Experiment XX.

Mins. after fertilization eggs were placed in solutions	$\frac{m}{50}$ KNC, 3-hour series.	$\frac{m}{100}$ KNC, 2½-hour series.	$\frac{m}{100}$ KNC, 3-hour series.
0 (3)	All eggs to morulas, or farther. Many fallen apart. A few blastulas.	20% swim. Rest morulas or disintegrated.	Numerous swimmers. Much disintegration.
10 (13)	95% not segmented. No blastulas and almost no disintegration.	A very few swim but not one to 25 in above. Mostly unsegmented.	Mostly unsegmented. A few swim but much less than in above.
30 (33)	Many good morulas. One blastula. Few unsegmented eggs.	Several blastulas (more than in 10-minute sample). Advanced cleavage common.	Several blastulas and much advanced cleavage.
45 (48)	Many morulas and much disintegration.	More swimmers than in 30-min. sample.	Many good blastulas. Better than 30-min. sample.
50 (53)	Like 45-min. sample, except many 2-cell stage present.	Only one or two blastulas. Advanced cleavage.	90% dead in 2-cell stage. None in advanced cleavage.
55 (58)	Less 2-cell than 50-min. sample and more advanced cleavage.	Many more blastulas than in 50-min. sample.	Numerous blastulas and all in advanced cleavage or disintegrated.
60 (63)	Like 55-min. sample.	Like 55-min. sample.	Like 55-min. sample.

Experiment XX. Eggs fertilized Sept. 7, 4:57 P. M. Eggs put into $\frac{m}{100}$ and $\frac{m}{50}$ KNC at 5.00, 5.10, 5.30, 5.45, 5.50, 5.55, 6.00. Samples taken out after two, two and one-half, three, three and one-half and four hours, washed and left till next day, when these notes taken. (Only three series published.)

A study of these experiments and of many others that I made leaves no doubt that there is a stage about ten or fifteen minutes after fertilization (at the temperature of my experiments), when the egg is especially susceptible to KNC. Again soon after the first cleavage comes a second stage of small resistance. A third follows the second division. Further than this the experiments did not go.

From experiments in which samples were taken out at five-minute intervals through the first cleavage period it appears that the resistance of the egg to KNC diminishes steadily from the moment of fertilization to the above mentioned minimum. From this there is a progressive increase in resistance. Twenty-five or thirty minutes after fertilization eggs possess about the same degree of resistance as at the

time of fertilization. Forty minutes after fertilization they are more resistant than when they were fertilized; and the increase goes on, apparently, up to the time of separation into two cells. A great fall in resistance appears soon after the first cleavage, and then a rise again. The question arises, therefore, whether the changes here follow exactly the same course as those preceding the first cleavage. But the second cleavage follows so quickly after the first that the problem could be solved only by taking samples at much shorter intervals than I did, say every minute. It is probable, however, that in the susceptible period after the first division the egg is less resistant than after the corresponding period which comes soon after fertilization. Similarly after the second cleavage there is a stage of great susceptibility, — greater, it would appear, than the preceding similar stage. It seems, too, that the resistance never again rises so high as just before the first division. These probabilities are indicated in Experiment D. In the seventy-five minute sample (time of the second cleavage), of the one and one half hour series most of the eggs are "dead in the four-cell stage," and no blastulas are present. At the preceding susceptible periods (fifty-five and ten minutes) in the same series there are only slight indications of the weakened resistance. The fifty-five minute sample, for example, simply has fewer blastulas than the fifty-minute sample. In the two and one half hour series, however, we find that the fifty-five minute sample is composed wholly of eggs killed in the two-cell stage — "as perfect as if fixed," as one of the men in the laboratory remarked. But in this series the first susceptible period is not marked by a total lack of segmentation, although a majority of the eggs have failed. Only in the three and one half hour series do we find a complete lack of development in the first susceptible stage.

Unfortunately I have little further evidence of this interesting probability than that contained in Experiment D. The experiments already detailed, showing loss of resistance from stage to stage of development, do not necessarily prove a loss at each cleavage, although rendering it probable. And in experiments like Experiment D success in this regard evidently depends, for one thing, on getting a lot of eggs which are practically simultaneous in cleavage. Success also depends on hitting the proper periods for the action of the poison and proper intervals for taking out samples. Therefore one can expect but few experiments as clear as Experiment D.

Assuming as a fact a progressive loss of resistance at each cleav-

age, the curve of resistance to KNC during cleavage may be diagrammatically represented by Fig. 1. Of course the exact rise and fall of the curve relative to its average height above the base line are wholly assumed; and the characters of the curve itself, for the present, are largely guesswork.

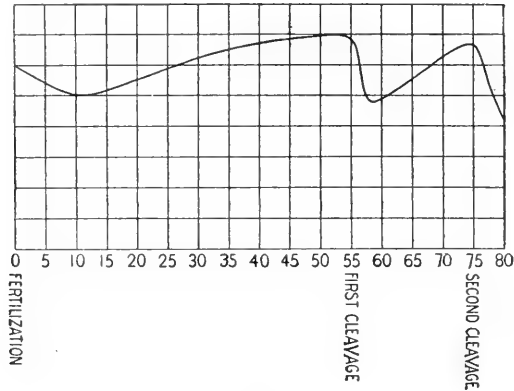


FIGURE 1.

The ordinates indicate the degree of resistance, — the figures, the number of minutes after fertilization.

SUBSTITUTION OF HYDROGEN FOR AIR.

After I had secured these results with KNC, Professor Loeb suggested that I try lack of oxygen brought about in other ways. I used hydrogen. The breeding season for *Arbacia* was nearly over, and eggs were hard to get and more difficult still to fertilize. However, I succeeded in getting three good experiments, each consisting of several series. All point to the same result. There is a stage when the eggs are very susceptible to lack of oxygen, and this period is about ten or twelve minutes after fertilization. For these experiments, hydrogen gas was generated from sulphuric acid and zinc and purified by passing through wash bottles containing successively potassium hydrate, potassium permanganate and distilled water. The gas was then passed through four small rubber-stoppered wash bottles, each of about 40 c.c. capacity. The bottles were arranged "two in series, and two in parallel;" that is, the stream of gas was divided into two parts and each part passed through two bottles. The ideal plan would have been to give each bottle an independent supply of gas, thus insuring that no air which might get into the one

nearest the generator at the time of taking out a sample could be carried over into the next bottle. But my generator was too small to supply gas for so many outlets.

The experiments were conducted as follows: Sea-water was boiled to drive off the oxygen, then diluted with recently boiled distilled water to its former volume. (I had previously found that the eggs develop as well in such water, when aerated, as in the natural sea-water.) About 25 c.c. of this water was placed in each bottle and the hydrogen started through it. The current of gas was continued for five or six hours. Then at proper intervals and with a powerful stream of gas going, the bottles were opened very slightly and a few drops of water containing fertilized *Arbacia* eggs put in. The stoppers were immediately made tight, and the stream of hydrogen kept up until next day. The eggs were placed in the bottles at convenient intervals after fertilization; *c. g.*, 0, 5, 10, and 30 minutes.

From experiments made by Miss Emerson (not yet published), I knew about how long the eggs would live in hydrogen and therefore took out my first sample after sixteen hours. To take out eggs without admitting air to the remaining portion, a very powerful stream of gas was kept up through the bottle, the stopper was opened just enough to admit a fine pipette, some eggs were drawn out and the bottle immediately recorked. The method was not elegant, but that it was effective in keeping oxygen from the eggs is indicated by the total absence of cleavage in any of the bottles, to the end of the experiments. The samples taken out were placed in sea-water and observed after a proper interval to allow the development of swimming larvæ, usually next day.

The notes on two of these experiments are given in the tables on page 72.

These notes need little comment. It is plain that there is a close parallel between the results with H and those with KNC. Starting at the moment of fertilization with a certain degree of resistance to lack of oxygen, there is a gradual loss of this power during the next ten or fifteen minutes. Then the resistance begins to increase. That the differences are striking is seen by comparing the sixteen hour and the forty-two hour series in Experiment I. After sixteen hours there is "little or no segmentation" in the ten minute sample. But even after forty-two hours some of the eggs which were placed in H immediately after fertilization are able to develop.

Nothing can be more striking than the comparison of two samples,

TABLE V.

Experiment I. — With Hydrogen, September 6, 1901.

Mins. after fertilization eggs were placed in hydrogen.	16-hour series.	21-hour series.	42-hour series.
0 (3)	Numerous swimmers (later formed perfectly normal gastrulas). Few eggs failed to develop.	No blastulas, but some good morulas. Much disintegration; <i>i. e.</i> , blastulas have gone to pieces.	Much segmentation with and without disintegration. Some morulas may swim later.
5 (8)	Good blastulas abundant.	A few blastulas. Many good morulas.	Some segmentation and disintegration.
10 (13)	No blastulas. Little or no segmentation.	No segmentation.	No segmentation.
30 (33)	Blastulas abundant. Practically every egg to morulas, or further. Much disintegration.	Most of eggs segmented.	Many 2 and 4 cell stages.

TABLE VI.

Experiment K. — With Hydrogen, September 9, 1901.

Mins. after fertilization eggs were placed in hydrogen.	16-hour series.	18-hour series.	21-hour series.
0 (3)	95% swimming larvæ.	Numerous swimmers. Very few unsegmented eggs.	Numerous swimmers. Much disintegration.
5 (8)	Perhaps 5% swimmers.	Much advanced cleavage.	Little or no segmentation.
10 (13)	No swimmers though hundreds of eggs in the sample. Mostly unsegmented.	99% unsegmented.	No segmentation.
20 (23)	One or two swimmers and considerable disintegration.	No swimmers. Some disintegration. Mostly unsegmented.	Some segmentation and disintegration. No swimmers.

say of an eighteen hour series. The eggs which were placed in an oxygen vacuum just after fertilization, left eighteen hours, then removed to aerated sea-water and left over night, all developed. The dish was full of beautiful blastulas. The eggs which were treated in exactly the same way except that they were not placed in the hydrogen till ten minutes after fertilization were unsegmented and dead.

It seems, therefore, both from the experiments with KNC and also from those with hydrogen that oxygen is essential for some process taking place about ten or fifteen minutes after fertilization. Not only is oxygen necessary for this process; but if it is withheld for a comparatively short time, the egg cannot recover. But if we ask what the particular process is, we are unable to state with certainty. My first idea was that the susceptibility to KNC was connected essentially with the union of the sperm and egg cells. It might be that the sperm was killed by the cyanide. But the fact that the susceptible period recurs at each segmentation put an end to such speculations. If we consider the processes going on during division that might need oxygen, the active parts of karyokinesis, the splitting and separation of chromosomes, first attract our attention. Yet time considerations throw these out of court. In *Toxopneustes*, according to Wilson,¹ the nuclear membrane is dissolved about twenty-five or thirty minutes and the chromosomes separate about forty-two minutes after fertilization. Mathews,¹ who described the fertilization and cleavage of *Arbacia*, did not state exact times, but asserted the close similarity to *Toxopneustes*. In conversation with me he states his remembrance that there is a long pause after the union of the pronuclei before solution of the nuclear membrane and the division and migration of the chromosomes. The solution of the membrane, he thinks, occurs thirty or thirty-five minutes after fertilization. These processes, therefore, seem to come in the period most resistant to KNC and lack of oxygen. The further fact that the second susceptible period comes immediately after the first cleavage instead of just before the second cleavage shows again that the period when oxygen is most needed is not that of active nuclear division but rather the resting stage. We must look, therefore, for processes occurring about ten or fifteen minutes after fertilization.

In their description of the cleavage of *Toxopneustes* and *Arbacia*, Wilson and Mathews mention two processes which occur sufficiently

¹ WILSON and MATHEWS: *Journal of morphology*, 1895, x. p. 319.

near to the susceptible stage to be worthy of consideration. One is the growth and division of the sperm aster. The other is the growth of the nucleus, that of *Arbacia*, according to Mathews, increasing in bulk over eight times. But whether either of these processes is especially dependent on free oxygen and concerned in my results remains for further experimentation to determine. Nor can any general conclusions as to the necessity of oxygen in cleavage be drawn without experiments on many other kinds of cells.

In this connection I have been especially interested in De Moor's¹ work on *Tradescantia* and leucocytes. It will be remembered that he showed that the nucleus can divide in an atmosphere of hydrogen (or at least, in an atmosphere so free from oxygen that visible protoplasmic streaming had ceased), and that division takes place in the normal time and manner. If it were true that the nucleus in *Tradescantia* could go through its entire series of changes without oxygen it would seem that my susceptible period must be concerned with the cytoplasm only, or else that no general rule regarding the necessity of oxygen for the nucleus could be established. (Indeed there is support for the latter idea in the observation of Loeb, already mentioned, that *Fundulus* eggs can continue segmentation for several hours in an atmosphere of hydrogen.) It is possible, however, that the nuclei that De Moor saw divide were ready or nearly ready for that process before they were deprived of oxygen. In other words, they had passed into that stage which my experiments show to be the most resistant to lack of oxygen. There are several things in his paper that make me incline to this belief. De Moor never saw more than one division of the same nucleus. He usually speaks of the nucleus as being in the spireme stage at the beginning of his observations. In the case which he describes to show that the entire series of nuclear changes can be gone through without oxygen he says he selected cells which appeared to have divided recently. But he did not wait for a cell to divide, then immediately turn on the hydrogen and see whether the nucleus could divide again. My experiments indicate that oxygen is especially needed by the egg very shortly after division and for a comparatively short time only. It may therefore have happened that De Moor missed this stage, and that my work is in no sense contradictory to his.

I am glad to express my indebtedness to Professor Loeb for suggesting this line of investigation.

¹ DE MOOR: *Archives de biologie*, 1895, xiii, p. 163.

SUMMARY.

1. There is a loss in resistance to potassium cyanide during the development of the sea-urchin egg. Probably each cleavage from the very first increases the susceptibility.

2. Development is slightly hastened in very weak solutions of KNC.

3. By prolonged exposure of *Arbacia* embryos to the poison the union of the cells is weakened. Then on transference to sea-water, the cilia may recover and the cells under the influence of ciliary motion may swim away from the embryo. The whole organism thus collapses. Unsegmented eggs placed in cyanide for a time and then returned to sea-water may go on developing. But the poison has so affected them that as soon as cilia are developed, the embryos disintegrate. Lack of oxygen brought about by a current of hydrogen produces the same result.

4. Some degree of immunity to KNC can be induced by raising embryos from the start in very weak solutions of the poison.

5. From fertilization to a point about ten or fifteen minutes after fertilization the resistance of the egg to KNC diminishes. From the most susceptible stage there is an increase in resistance up to the time of the first cleavage. Very soon after division there is another susceptible period followed by another increase in resistance. Similar variations have been found in the interval between the second and third cleavage.

6. Similar changes in resistance are found when the eggs are subjected to hydrogen and consequent lack of oxygen. There is a susceptible period about ten minutes after fertilization, followed by a much more resistant period.

ERGOGRAPHIC STUDIES IN MUSCULAR SORENESS.

By THEODORE HOUGH.

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IN my paper on neuro-muscular fatigue published in this journal for May, 1901, I made but slight reference to the subject of muscular soreness beyond the statement that "when an untrained muscle makes a series of contractions against a strong spring, a soreness frequently results which cannot be regarded as a phenomenon of pure fatigue." Further discussion of the matter was postponed to await the completion of the experiments which form the basis of the present paper.

The use of the word "trained" at that time was somewhat indefinite, and it may be well at the outset to define my use of it then and now, in order to prevent possible misconception. By a trained muscle or a muscle in training is meant one which has been making regular ergographic experiments for some time previously without resulting soreness or lameness. The muscle may have been accustomed to ergographic work and may be very strong; but if a considerable period of time have elapsed since the last experiment, such a muscle would be classified as untrained or out of training. I am unable to state what length of time must elapse to put a muscle out of training; but I have never classified a muscle as trained unless it had made an experiment within the preceding three weeks.

My attention was first attracted to the subject of muscular soreness in the latter part of November, 1899. Six months before a series of almost daily ergographic tracings from the flexors of the middle finger had been brought to a conclusion.¹ The muscle was in a high degree of training, the curve of work always falling asymptotically to a constant fatigue level, from which recovery took place rapidly on passing to a slower rhythm. No soreness had been noticed.

The experiments were resumed in November, after the lapse of six

¹ HOUGH: This journal, 1901, v, pp. 258-259. The ergographic methods used in the present series of experiments are the same as those given in the above paper.

months, during which no ergographic tracings were taken nor any work done which involved hard work on the part of the muscles in question. The first tracing (November 30) was recorded without discomfort at the time of experiment, but it was noticed that it failed to show a fatigue level. This is shown in Fig. 1, which will enable the reader to compare the result of this experiment with that of one taken during the previous period of tracing. It was also observed

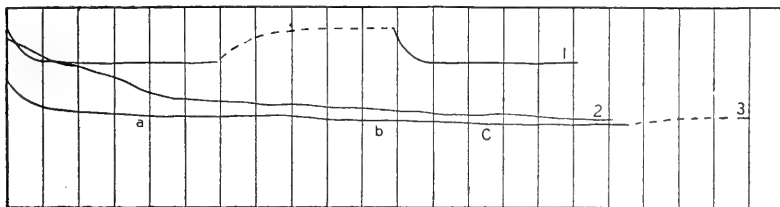


FIGURE 1.—1. Curve plotted from a tracing given by the trained muscle, May 5, 1899. 2. Curve plotted from the initial tracing of the same muscle out of training, Nov. 30, 1899. 3. Curve plotted from the tracing of the same muscle on the following day (Dec. 1, 1899) while in a condition of soreness. In this curve, the contractions were very painful at *a*; much less so at *b*; at *c* the pain had entirely disappeared, or was noticed only at the height of contraction and then was slight,

$$\text{———} = \text{Rhythm, } \frac{C}{R} = \frac{\frac{1}{2} \text{ sec.}}{\frac{2}{3} \text{ sec.}} \quad \text{-----} = \text{Rhythm, } \frac{C}{R} = \frac{\frac{1}{2} \text{ sec.}}{\frac{1.9}{2} \text{ sec.}}$$

Abscissæ give time in minutes.

that, not only did the curve in the former case continue to fall to the end of the experiment, but that the fall was not uniform; on the contrary sudden diminutions were frequently noticed in the height of contractions, as shown somewhat imperfectly in the figure. The same thing has been more or less marked in the nine other initial tracings taken from the untrained muscle during the past two years.

As already stated, no discomfort accompanied this tracing, nor was any noticed for some hours afterward. Eight or ten hours later, however, the muscle began to show signs of soreness, and this soreness increased to its height twelve or more hours after the tracing. In other subjects this soreness has appeared much later, reaching its maximum as late as forty-eight or even sixty hours after the tracing.

On the following day, while the muscle was still sore, a second experiment was made. For the first two or three minutes the act of contraction against the resistance of the spring was excessively painful, as if countless needles were being driven into the arm.

This pain, however, gradually wore away as the experiment continued, and in the course of five or ten minutes had entirely disappeared, or was noticed only at the height of contraction.

The curves obtained on the first and second days may be compared by reference to Fig. 1. It will be noticed that the disappearance of pain was without effect on the height of contraction, which, under the conditions of our experiments, is equivalent to saying that it was not accompanied by improvement in the strength of the muscle. It will also be noticed that the rather striking level of work maintained throughout the second experiment is approximately that to which the curve of the previous day had fallen.

The soreness, which thus disappeared during the tracing and which was not noticed for some hours afterward, returned in ten or twelve hours and ran the same general course as that observed after the tracing of the first day. On the following (third) day a similar experiment gave a curve almost identical with that of the second day. Indeed, no improvement whatever was noticed in the working capacity of the muscle so long as daily experiments were made; each tracing gave the same reduced level of work; the soreness, at first very intense, passed off in from five to ten minutes, to return some hours later. Such daily experiments were made for six days.

I have previously shown that on changing from the rhythm $\frac{C}{R} = \frac{\frac{1}{2} \text{ sec.}}{\frac{3}{2} \text{ sec.}}$ to the rhythm $\frac{C}{R} = \frac{\frac{1}{2} \text{ sec.}}{\frac{1}{2} \text{ sec.}}$ practically complete recovery occurs in the trained muscle within some ten minutes. It is interesting to note that neither in the first nor in subsequent tracings of this and similar series was any marked recovery observed in the sore muscle. A somewhat greater recovery may be obtained by massaging the muscle between contractions, but even this improvement is comparatively insignificant.

Finally the soreness gradually passed away when periods of from four to seven days were allowed between tracings. In the course of a month and a half the working capacity of the muscle, as indicated by the height of the initial contraction and of the fatigue level improved step by step until it was somewhat better than that observed eight months before. The third series of experiments given in my former paper¹ were from this period, after the muscle was again in training.

¹ HOUGH: This journal, 1901, v, p. 260.

The experiments of this series were made with the rhythm $\frac{C}{R} = \frac{\frac{1}{2} \text{ sec.}}{\frac{3}{2} \text{ sec.}}$. Later, when soreness no longer accompanied or followed work, an experiment was made with sustained volitional tetanus which brought out a new phase of the matter. I have elsewhere commented on the frequently painful character of these tracings, and this one was no exception. Five contractions of from four to two minutes' duration were made, with intermissions of two minutes' rest. The pain may be described as beginning with a dull ache which increased in intensity until it was almost unbearable. It began in the first tetanus during the third minute, in later tracings after the first thirty seconds, becoming more intense with each succeeding tracing. Immediately after the close of the experiment no marked discomfort was noticed; within fifteen minutes, however, the flexor muscle¹ felt lame, was sore to the touch, and contraction against even slight resistance was uncomfortable or even painful. Although I have no record of the exact time at which this condition was most marked, it is safe to place it about one half hour after the end of the experiment. From this time it gradually wore off, disappearing entirely within four hours. It will be remembered that, in the experiments described at the beginning of this paper, no discomfort whatever was experienced during the experiment nor for several hours afterward. On the following day the usual rhythmic tracing was taken to test the working capacity of the muscle, which was found to be in a perfectly normal condition, showing no diminution in the height of the initial contraction or of the fatigue level, and suffering no soreness either during or after the experiment.

These two experiences, thus described in some detail because subsequent experience has shown me that they are quite typical, suggested the advisability of studying the whole phenomenon of muscular soreness (resulting from work) on a large number of individuals. Observations have accordingly been made, under my direction, by members of the senior class of the Boston Normal School of Gymnastics, partly in the physiological laboratory of the Massachusetts Institute of Technology, but mostly at the Normal School of Gymnastics. The subjects of experiment were in all cases students or instructors of the latter school. Speaking for those who have

¹ It will be understood that the pain, soreness, lameness, stiffness, etc., mentioned in this paper always refers to the condition of the belly of the muscle, and in no case to the carpal portion of its tendon or to the fingers.

worked with me in the study of this problem it gives me pleasure to thank those who have served as subjects of experiment and for myself to acknowledge my own indebtedness to those who have so carefully and efficiently conducted the observations.

During the spring of 1900 experiments were conducted by Miss Mabel D. Cherry, Miss Susanne A. Craig, Miss Marian D. Richards, and Miss Gertrude L. Farmer. A preliminary account of their results¹ has already appeared. In order to increase the number of observations and to settle, if possible, certain questions suggested by their results, a second and more extensive series of observations was undertaken in the spring of 1901 by Miss Harriet Wilde, Miss Grace L. Shepardson, and Miss Lilian Gleason.

The results of these two series of observations are given in Table I. Except in the case of one subject (T. H.), the adjustable hook of the finger splint was attached at approximately $1\frac{1}{8}$ inches (28 mm.) from the joint. Owing, however, to the inexperience of some subjects in adjusting the splint, it should be said that it is not safe to assume that this distance was always accurately determined; in a few cases the adjustment was not made at all. This error is, however, of little importance in the present investigation, since our study does not involve comparison of daily records from the same individual; it is mentioned here, because the experiments have been arranged in the table in the order of the height of contractions. While this no doubt gives approximately the relative strength of the same muscle in different subjects, the classification is not absolutely accurate.

Study of the results will, I think, bear out the following conclusions:

1. There are two kinds of muscular soreness or lameness. The first is very marked during work and may be noticeable for three or four hours afterward; it then passes away entirely. The second kind, on the contrary, is not noticed at all at the time of the tracing nor for some time afterwards; it usually begins about eight hours after the work, increases to a maximum which may occur from ten to twenty-four, or even more hours later; indeed this soreness may not make its appearance until the second day after the tracing; it gradually passes away, but may be noticeable for four or more days, when the muscle is contracted against resistance or is over-extended.

¹ HOUGH: *Journal of the Boston Society of Medical Sciences*, 1900, v, pp. 81-92.

TABLE I.

Sustained volitional tetanus.							
Subject.	Date.	Observer.	Height of initial contraction. cm.	Height at 3 min. cm.	Soreness.		
					During work.	1-3 hours after work.	12 hours after work.
A. L. R.	1890. —	M. D. C.	8.5	2.4	Yes	No	No
F. Y.	—	“	8.3	5.6	Yes!	No	Very slight
B. W. H.	—	“	6.9	2.8	No	No	No
M. G. C.	—	“	6.9	1.9	Slight	No	No
G. E. F.	—	“	6.6	1.7	Yes!	Yes	Yes
M. F. S.	—	“	6.0	2.6	?	?	Yes
M. B.	—	“	5.6	2.1	Yes	No	No
B. B. W.	—	“	5.5	1.7	Yes	No	No
A. L. D.	—	“	5.4	1.4	No	No	Yes
A. F. H.	—	“	5.0	1.6	?	No	No
J. A. B.	—	“	5.0	1.3	No	No	No
J. T.	—	“	4.5	0.8	Yes	Yes	Yes
M. D. C.	—	“	4.2	1.4	No	No	No
M. P.	—	“	3.5	1.4	Slight	No	No
T. H.	1901. Mar. 15	H. W.	8.5	2.9	Yes!	Yes	Yes
G. L. S.	“ 22	“	5.7	—	No	No	No
“	“ 29	“	5.6	1.4	Yes	No	No
C. A. B.	Apr. 11	“	5.4	1.3	Yes	No	No?
M. M. T.	“ 23	“	5.3	2.2	Yes	No	Yes
M. J. S.	“ 10	“	4.7	2.0	Yes	Slight	No
F. C.	Mar. 25	“	4.6	—	Yes	Yes	No
H. W.	“ 29	“	4.6	1.5	Yes	No	No
S. L. S.	“ 25	“	4.2	—	Yes	Slight	No
E. M. S.	“ 28	“	4.1	1.2	No	No	No

TABLE I (continued).

Subject.	Date.	Observer.	Height of initial contraction.	Height at 3 min. cm.	Soreness.		
					During work.	1-3 hours after work.	12 hours after work.
H. W.	1901. Apr. 1	H. W.	3.9	1.4	Yes	No	No
"	Mar. 22	"	3.9	1.2	Yes	No	No
E. P.	Apr. 8	"	3.8	0.8	Yes!	Yes	Yes
Rhythm $\frac{C}{R} = \frac{1 \text{ sec.}}{1 \text{ sec.}}$							
Subject.	Date.	Observer.	Height of initial contraction.	Height of fatigue level.	Soreness.		
					During work.	1-3 hours after work.	12 hours after work.
M. F. S.	1900. —	S. A. C.	7.8	5.5	No	No	Yes
H. M. M.	—	"	7.5	4.3	Yes	No	No
J. I. G.	—	"	5.5	3.5	No	No	Yes!
M. Bd.	—	"	5.5	2.5	No	No	No
A. L. D.	—	"	4.8	2.8	No	Slight	No
S. A. C.	—	"	4.5	1.8	No	No	No
C. N. R.	—	"	4.0	2.5	No	Slight	Yes!
M. H. H.	—	"	3.0	2.0	No	No	No
T. H.	1901. Apr. 26	C. L. G.	6.2	3.0	No	No	Yes
C. S. M.	" 17	"	8.4	5.1	No	No	Yes
A. L. R.	" 17	"	5.7	3.7	No	No	Slight
G. G. W.	Mar. 27	"	5.4	2.8	No	No	Yes
G. L. S.	" 25	"	5.2	2.7	No	No	No
E. L. S.	Apr. 13	"	4.7	3.0	No	No	No
G. L. S.	May 8	"	4.5	2.1	Yes	No	Slight
H. S. M.	Mar. 27	"	4.4	2.8	No	?	?

TABLE I (continued).

Subject.	Date.	Observer.	Height of initial contraction.	Height of fatigue level.	Soreness.		
					During work.	1-3 hours after work.	12 hours after work.
B. P. E.	1901. Apr. 9	C. L. G.	4.4	2.3	No	No	No
M. Bd.	" 9	"	4.1	2.2	No	No	Very slight
C. A. B.	" 30	"	3.8	2.1	No	No	Slight
A. W. P.	Mar. 28	"	3.4	2.3	No	No	No
O. P.	Apr. 9	"	3.4	1.5	No	No	No
M. M. F.	" 17	"	2.4	1.8	No	No	No
Rhythm $\frac{C}{R} = \frac{1 \text{ sec.}}{9 \text{ sec.}}$							
A. L. R.	1900. —	G. L. F.	9.3	8.0	No	No	Yes
F. Y.	—	"	8.0	6.6	No	No	Yes
A. B.	—	"	7.9	6.9	No	No	Yes
C. S. M.	—	"	6.9	6.8	No	No	Yes
G. L. F.	—	"	No	No	Yes
A. O. B.	—	"	5.6	3.0	Yes	No	Slight
M. G.	—	"	5.5	5.0	No	No	No
A. C. H.	—	"	4.5	3.5	Slight	Yes	Slight
E. M. M.	—	"	4.5	3.2	No	No	No
E. B. S.	—	"	4.3	2.9	No	No	No
M. H. H.	—	"	3.2	2.4	Yes	Yes	Yes
M. K. K.	1901. Apr. 26	C. L. G.	No	No	Slight
C. L. G.	" 30	"	No	No	Yes!
L. N.	" 26	"	No	No	No

TABLE I (continued).

Rhythm $\frac{C}{R} = \frac{\frac{1}{2} \text{ sec.}}{\frac{2}{3} \text{ sec.}}$							
Subject.	Date.	Observer.	Height of initial contraction.	Height of fatigue level.	Soreness.		
					During work.	1-3 hours after work.	12 hours after work.
T. H.	1900. —	T. H.	No	No	Yes
M. D. R.	—	M. D. R.	5.3	2.0	No	No	Yes
M. D. R.	—	"	4.7	2.9	No	No	Yes
E. B. S.	—	"	4.4	1.7	No	?	Yes
M. D. C.	—	"	4.4	1.4	No	No	Yes
N. G. D.	—	"	4.3	2.4	No	No	No
C. N. R.	—	"	3.8	1.9	No	No	Slight
T. H.	1901. Apr. 15	G. L. S.	6.4	4.1	No	No	Yes
A. L. R.	May 8	"	8.5	5.4	No	No	Yes
C. S. M.	Apr. 15	"	8.0	6.1	No	No	Yes
B. W. H.	Mar. 29	"	6.0	4.3	No	No	Yes
G. L. S.	" 15	"	6.0	2.3	No	No	Yes
E. L. S.	Apr. 8	"	5.8	4.5	No	No	Slight
C. L. G.	May 6	"	5.6	3.5	No	No	No
H. W.	Mar. 29	"	5.5	3.4	No	No	Slight
E. L. S.	" 22	"	5.3	3.4	No	No	Yes!
S. L. S.	Apr. 8	"	5.3	3.0	No	No	Yes
H. W.	Mar. 25	"	5.2	3.5	No	No	No
G. L. S.	" 25	"	5.0	2.5	No	No	Yes
C. L. W.	" 28	"	4.9	3.1	No	No	Very slight
G. L. S.	Apr. 18	"	4.6	2.3	No	No	Yes
C. A. B.	" 22	"	4.5	2.6	No	No	Yes
G. L. S.	May 6	"	4.4	2.3	No	No	Slight

TABLE I (concluded).

Subject.	Date.	Observer.	Height of initial contraction.	Height of fatigue level.	Soreness.		
					During work.	1-3 hours after work.	12 hours after work.
A. W. P.	1901. Mar. 29	G. L. S.	4.3	2.9	No	No	Slight
B. P. E.	Apr. 11	"	3.0	2.4	No	No	No
E. P.	" 22	"	3.0	2.0	No	No	Yes
H. D.	" 11	"	2.1	1.5	No	No	Yes

2. The first kind of soreness is especially characteristic of sustained tetanic contractions and rarely occurs with the rhythmic contractions used in these experiments.

3. The second kind of soreness is comparatively rare after tetanic contractions, but very frequently occurs after rhythmic contractions.

4. It is especially noteworthy that, while the first kind of soreness is most marked where fatigue is greatest (sustained tetanus), the second kind not only follows the less fatiguing work of the usual rhythmic ergographic experiments, but may appear in very intense form after experiments with the slow rhythm ($\frac{C}{R} = \frac{1 \text{ sec.}}{9 \text{ sec.}}$) which show relatively slight diminution in the height of contraction, as the experiment proceeds. To this point we shall return; for the present it is desired to emphasize the fact that the intensity of the second kind of soreness not only does not vary with the intensity of fatigue, but seems, on the contrary, to be an entirely independent phenomenon.

5. Both kinds of soreness are, in general, much more marked with those muscles which give the strongest (highest) contractions.

6. While the second kind of soreness followed experiments of the rhythm $\frac{C}{R} = \frac{\frac{1}{2} \text{ sec.}}{\frac{3}{2} \text{ sec.}}$ in 85 per cent of the cases, it followed those of the rhythm $\frac{C}{R} = \frac{1 \text{ sec.}}{1 \text{ sec.}}$ in only 45 per cent.

In certain subjects of experiment the same muscle was used at different times with different forms of contraction. Table II gives the results in these cases for convenience of comparison and will be found to support the above conclusions in a very striking manner.

Each "yes," "no," or "slight," indicates a separate experiment. The subjects are arranged in general in the order of the strength of pull exerted.

TABLE II.

Showing the occurrence of soreness after ten or more hours in those muscles which worked with more than one rhythm. Where marked soreness followed all kinds of contraction the results are given in italics.

Subject.	Tetanus.	$\frac{1 \text{ sec.}}{1 \text{ sec.}}$	$\frac{1 \text{ sec.}}{9 \text{ sec.}}$	$\frac{\frac{1}{2} \text{ sec.}}{\frac{2}{3} \text{ sec.}}$
T. H.	<i>Yes</i>	<i>Yes</i>	<i>Yes</i>	<i>Yes</i>
C. S. M.	<i>Yes</i>	<i>Yes</i>	<i>Yes</i>
F. Y.	Very slight	Yes	
A. T. R.	No	Slight	Yes	Yes
B. W. H.	No	Yes
G. L. F.	<i>Yes</i>	<i>Yes</i>	
M. F. S.	Yes	Slight	
G. L. G.	No	Yes!	No
A. L. D.	Yes	Slight		
G. L. S.	No, No	No, Slight	Yes, Yes, Yes, Yes
E. L. S.	No	Slight, Yes!
H. W.	No, No, No	Slight?
C. A. B.	Slight	Yes
C. N. R.	Yes	Slight
S. L. S.	No	Yes
A. W. P.	No	Slight
E. P.	<i>Yes</i>	<i>Yes</i>	<i>Yes</i>
B. P. E.	No	No	
M. H. H.	No	Yes	

DISCUSSION OF RESULTS. THE NATURE OF THE TWO KINDS OF SORENESS.

The above results are worthy of attention because of the light which they throw on the nature of muscular soreness, and from their bearing upon the technique of ergographic work in general. It will be convenient to discuss these two phases of the matter separately.

The muscular soreness which for the want of a better term we have simply called "the first kind" and which so frequently appears during sustained tetanus would seem to be closely associated with the phenomena of fatigue. Upon this basis it presents little difficulty of explanation. We know that waste products accumulate, first within the sarcolemma and subsequently in the interstitial lymph spaces of the muscle. Loeb¹ has shown, moreover, that when an excised muscle has been thrown into activity the osmotic tension within the muscle fibre increases, as is shown by its increase in weight when placed in a solution of sodium chloride isotonic with the contents of a resting muscle. It would seem, in view of these facts, that until the blood and lymph currents are able to remove the diffusible wastes from the muscle, there must be more or less œdema in the organ. Whether the pressure of the swollen muscle fibres upon the sensory nerve endings is to be regarded as the cause of the soreness, or whether we are dealing with chemical stimulation of these nerve endings, or whether both causes take part in the soreness must for the present be left undecided; if the former is the only cause, the thickness of the muscle fibre must obviously be greater than it is in a maximal contraction, where no such soreness is felt.

In the experiments made in 1901, measurements were made of the girth of the forearm before and after the experiment. Four marks were made on the skin over the belly of the flexor muscle so as to get the measurements at the same places. The results on the whole have been unsatisfactory; it is, however, of interest to note that whereas the measurements following rhythmic contractions showed at times an increase, and at other times no change at all, sustained tetanus was always followed by an increase of girth, varying from 3 to 6 mm. On the other hand, equally large increases were sometimes observed after rhythmical work and when no soreness whatever was present.

¹ LOEB: *Archiv für die gesammte Physiologie*, 1894, lvi, p. 270.

If this soreness, then, be caused by the same conditions which produce fatigue, namely the presence of the diffusible waste products of activity we should expect to find, as we do find, that it takes the course described above, passing away within very few hours of the work itself. One other peculiarity of this soreness may also be urged in support of our explanation of its cause. It will be remembered that it was a common experience to find that the pain accompanying the work of sustained tetanus ceased altogether with the end of contraction, while lameness developed to a marked degree during the following half hour or more. This would mean that the pain felt during contraction is partly due to the pressure of the contracted and now somewhat swollen muscle fibres on sensory nerve endings; the release of this element of pressure by the cessation of contraction brings temporary relief; the greatly increased blood-flow, however, which follows the relaxation of the muscle now gives for a time the osmotic conditions favorable for the absorption of water by the fibres, with the return of pressure on their nerve endings. This alone may be enough to produce soreness, which is greatly increased, of course, should the muscle be contracted while in this condition.

In any case, we should expect the lameness to pass away, as it actually does with the removal of the diffusible waste products by the blood current. Attention may also be called to the fact that we should expect the soreness to be greatest in the stronger muscles.

Passing now to the second kind of soreness, I think that the facts already given show clearly that it is not a phenomenon of fatigue. It occurs least frequently where fatigue is greatest, that is to say, in sustained tetanus; it rarely follows rhythmic contractions which show marked fatigue, that is to say in those tracings in which the fall to the fatigue level was rapid and the fatigue level itself was low; it has appeared in a most severe form with slow rhythms where the fall in the height of contraction was least of all; and it makes its appearance at a time when we should expect the conditions of fatigue to be entirely removed. The two processes, in other words, run an entirely independent course.

On the other hand, it may appear after any kind of muscular work, especially if the height of contraction be very great, thus subjecting the muscle to great tension.¹ It follows some kinds of work, however more readily than others. It almost always appears to a greater, or

¹ This statement, of course, refers to the height of contraction in our ergographic experiments, which involves correspondingly increased tension.

less degree after experiments with the rhythm $\frac{C}{R} = \frac{\frac{1}{2} \text{ sec.}}{\frac{3}{2} \text{ sec.}}$; in a smaller but still large per cent after experiments with the rhythm $\frac{C}{R} = \frac{1 \text{ sec.}}{9 \text{ sec.}}$; in a much smaller percentage after experiments with the rhythm $\frac{C}{R} = \frac{1 \text{ sec.}}{1 \text{ sec.}}$; and least frequently after sustained tetanus.

It varies directly with a combination of two factors; the suddenness and the height of the contraction. The relation between the height of contraction and the frequency of soreness has already been pointed out. As to the suddenness of contraction, it will be seen that soreness is more frequent with the rhythm $\frac{1}{2} \text{ sec.} : \frac{3}{2} \text{ sec.}$ than with the rhythm $1 \text{ sec.} : 1 \text{ sec.}$, where the number of contractions in a unit of time is the same but where more time is allowed for shortening. Any one who has used these two rhythms is acquainted with the entirely different psychical effect they produce; in the former the subject feels somewhat hurried, in the latter, that there is ample time; the result is that with the former rhythm the contraction inevitably assumes the form of a jerk, — with the latter it is slower and more deliberate.

It will be at once suggested that the results with the slow rhythm $\frac{C}{R} = \frac{1 \text{ sec.}}{9 \text{ sec.}}$ do not bear out this view of the case. Experience, however, will show that this is not the case. The subject seems to be unconsciously afraid that when the tenth second comes, he will not make the best of his opportunity, and he accordingly puts so much effort into the contraction, that he gives it in almost all cases the form of a jerk. I am sure, from watching the experiment in some cases where the resulting soreness was very marked, that this is true; moreover, in one experiment which I have myself made with this rhythm upon an untrained muscle and in which special care was taken to avoid the jerk form of contraction, the resulting soreness was comparatively slight. It must also be remembered that this rhythm adds the other factor which we have seen to be so closely associated with the production of soreness, namely the higher contraction, or greater tension.

It seems that not only are these facts all explained on the assumption that the second kind of soreness described in this paper has its origin in some sort of rupture within the muscle itself, but that this assumption explains other things which have been observed. It

will be remembered that the initial experiment with an untrained muscle showed a continuous fall in the height of contraction, but that this fall presented frequent sudden diminutions in the height of contraction, from which there was little or no subsequent recovery on passing to a slower rhythm. This is in striking contrast to the result with trained muscles; their curves are strikingly regular under favorable conditions and on passing to the slower rhythm the contractions reach the height of the initial contraction in some ten minutes; indeed, they almost reach this level in two minutes. If now in the untrained muscle, owing to the rupture either of the muscle fibres themselves, or of the connective tissue which transmits the pull of the fibre to the tendon, certain fibres are thrown out of play, the working cross section of the muscle will be to that extent diminished; the height of the later contractions against a spring will be determined not only by the conditions of fatigue but by the diminished number of effective fibres; as these ruptures take place there will be sudden falls in the height of contraction; the slight recovery noticed upon passing to the slower rhythm is due to the amelioration of fatigue conditions, but complete recovery is impossible. This view of the case is further borne out by the facts that the height of the initial contraction on the following day is much lower than in the first experiment of the series, and that the fatigue level is no higher, indeed is practically the same as the final contractions of the first experiment. (See Fig. 1.)

The history of the soreness itself, its appearance only some hours after the tracing, the very painful character of maximal contractions while the muscle is in this condition, the disappearance of the soreness upon continuing these contractions, and its subsequent reappearance some hours later, all present the familiar picture of the formation of adhesions in the process of repair and the painful breaking up of the same adhesions by movement of the affected part. Similarly the recovery observed when rest is given point in the same direction, and suggest analogous experiences with a sprained joint. It gives me pleasure to acknowledge my indebtedness to Dr. P. M. Dawson, of Johns Hopkins University, for useful suggestions in this connection.

Special attention may also be directed to the fact that the first few contractions of the muscle which is "out of training" show but little diminution in working capacity as compared with that of the same muscle in training. (See Fig. 1.) The fibres of the untrained

muscle are not weaker, so far as our evidence goes, than those of the trained muscle.

Why the muscle not in training should act as it does, suffering rupture of its structure, if our explanation be correct, is not clear. It may be suggested, however, that the efficiency of the muscle as a working machine depends, not only on its cross section and on the strength of the individual fibres, but also on the perfection in the coördination of their work; they must work together to exert the greatest pull. This, in turn, depends on the coördination of their innervation, and suggests that the rupture is due to the uneven action of the stimulating neurones; if, for example, the group of fibres innervated by one motor nerve fibre receive their maximal stimulation while the neighboring fibres, innervated by a second neuron, fail of stimulation or receive a decidedly weaker stimulus, it is probable that the strong contraction of the former group would rupture their connective tissue attachment to the second group and thus initiate the phenomenon of soreness. A muscle out of training would thus differ from one in training not in the condition of the muscle itself but in that of its innervating mechanism. Many facts which I have noticed, and some of which I have already given, might be urged in support of this view; but to discuss the matter at length would lead us too far into regions of speculation for the pages of this journal.

The bearing of these results upon the technique of ergographic work is obvious. The first kind of soreness (that which accompanies or immediately follows work) is a disturbing factor where the ergograph is used for the study of fatigue only in so far as it interferes with the continuance of maximal volitional innervation. The second kind, which we have explained as involving actual rupture within the muscle, is much more serious. Even if we trace it to the fatigue of nerve cells, the tracing gives no intelligible record of such fatigue. A more important matter is the fact that the diminution in the working capacity of the muscle is due not to fatigue alone but to other causes as well; the tracing becomes a record, partly of fatigue, partly of the results of muscular lesions; or, without insisting upon our explanation of the cause of soreness, the experiments given above leave no doubt that it must be sharply distinguished from fatigue. No ergographic experiment, therefore, which is followed by the least soreness (of the second kind) is trustworthy in the study of simple neuro-muscular fatigue. Such experiments must be made

on trained muscles, that is to say, upon muscles which can be depended upon to give typical fatigue curves with the establishment of a fatigue level; and in testing the influence of any agent such as temperature changes, barometric pressure, humidity, smoking, stimulants, special food-stuffs, etc., upon simple neuro-muscular fatigue those experiments which are complicated by soreness should be rigorously excluded. Not only this, but the muscle should in all cases be tested for soreness in the period beginning twelve hours after the completion of the experiment. This is all the more important in view of the fact that the abnormal condition of the muscle frequently escapes notice, unless attention is specially directed to it by making it work or by over extension.

SUMMARY OF RESULTS AND CONCLUSIONS.

In addition to the results already given on pages 80 and 85, we may add :

1. The first kind of soreness would seem to be due to the same causes as those which produce fatigue, namely the presence of the diffusible waste products of activity.

2. There are reasons for thinking that the second kind of soreness is fundamentally the result of ruptures within the muscle.

3. When the ergograph is used to study fatigue, all experiments which are followed by soreness of the second kind should be excluded; and in all such work the muscle should be tested twelve or more hours later for soreness by contracting it against resistance and by over extension.

CHEMICAL STUDIES OF ELASTIN, MUROID, AND OTHER PROTEIDS IN ELASTIC TISSUE, WITH SOME NOTES ON LIGAMENT EXTRACTIVES.¹

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COMPREHENSION of function is dependent on knowledge of structure and composition. The influence of any tissue on the other parts of the body is more easily understood as our appreciation of the varieties and relations of its constituent elements increases. Elastic tissues have received little analytic attention. They have been overlooked by reason, apparently, of their seeming metabolic passivity and because they serve mainly mechanical functions.

The earlier observers regarded the cervical ligament as an extravascular tissue, for the most part, with practically no special chemical

¹ Some of the results of this research have already been given in the Proceedings of the American Physiological Society: This journal, 1900, iii, p. v.; 1901, v, p. xi.

activity and believed that it consisted almost wholly of the albuminoid elastin. Recently, however, it has been found in this laboratory¹ that the ligamentum nuchæ of the ox contains not only the large percentage of water and elastin, and the slight amounts of inorganic matter, collagen, and fat assumed to be present by the earlier investigators, but also appreciable quantities of mucoid,² coagulable proteid and crystalline extractives. These later results indicate that the production of elastin is the feature of ligament metabolism, and they indicate, further, that the chemical changes normally occurring in yellow elastic tissue are greater than had been supposed.

We have recently subjected the various constituents of elastic tissue to a somewhat detailed study. The particular form of tissue from which the constituents were prepared in all our experiments was the ligamentum nuchæ of the ox.

I. LIGAMENT ELASTIN.

Preparation. *Historical.* — Tilanus³ was probably the first to analyze elastic tissue. In his earlier preparations of "pure tissue" small pieces of the cervical ligament of the cow were first extracted in cold water to remove traces of blood and inorganic matter, and then dehydrated (and fat eliminated) with alcohol and ether. This product was hardly anything better than "prepared" ligament. In a second preparation he extracted in boiling dilute acetic acid in addition. Extraction with the acid doubtless removed all of the coagulable proteid and most of the collagen, but probably left behind most, or at least much, of the mucoid. The residue prepared in this way (after thorough removal of acid by washing in water and then dehydrating), unlike the product obtained by the first method, was said to be free of sulphur. Tilanus assumed it to be a pure chemical substance — elastin — and gave it the formula $C_{52}H_{80}N_{14}O_{14}$. In both of the methods used by Tilanus the tissue extractives were doubtless completely eliminated.

W. Müller⁴ improved Tilanus's methods by adding treatment in boiling dilute alkali and cold dilute mineral acid to the preparation

¹ VANDEGRIFT and GIES: This journal, 1901, v, p. 287.

² We use the word "mucoid" in the sense first suggested by COHNHEIM. See CUTTER and GIES: This journal, 1901, vi, p. 155 (foot-note).

³ TILANUS: See MULDER, Versuch einer allgemeinen physiologischen Chemie, Zweite Hälfte, 1844-51, p. 595.

⁴ W. MÜLLER: Zeitschrift für rationelle Medicin, dritte Reihe, 1861, x, p. 173.

process. He alternately boiled finely divided ligamentum nuchæ from the horse and ox in dilute acetic acid and in dilute potassium hydroxide, and then extracted in cold dilute hydrochloric acid.¹ Such treatment tended to remove the residual collagen and all of the mucoïd, but also favored decomposition of the elastin. Müller states that his purified product was fibrous in microscopic appearance and seemed to be unaffected by the alkali treatment.

Horbaczewski² made still further modification of the method used by Müller by introducing repeated extraction of the cervical ligament of the ox in boiling water. The treatment in boiling water thoroughly transformed insoluble collagen into soluble gelatin although it made subsequent extraction of coagulated proteïd more difficult. Horbaczewski continued all of his extractions for longer periods than any of his predecessors. Subsequently, Chittenden and Hart,³ commenting on Horbaczewski's work and the method of elastin preparation used by him wrote as follows: "So vigorous is the method of treatment, that it appears almost questionable whether a body belonging to a group noted for ease of decomposition might not suffer some change in such a long process of preparation."

Chittenden and Hart⁴ compared elastin made from the ligamentum nuchæ of the ox by Horbaczewski's method with that obtained in their own process, which was the same except that the substance was not extracted in alkali. The chief difference noted was that the elastin which had been treated with potassium hydroxide contained no sulphur, whereas that which had not been extracted with alkali contained 0.3 per cent. For the first time the danger in the use of hot alkali was appreciated and pointed out.⁵ At the same time the probable presence of mucoïd was overlooked. There is no reason for believing that the mucoïd could have been completely removed from the tissue pieces without the aid of alkali.

Bergh⁶ recently obtained elastin from the cervical ligament by Horbaczewski's method, but added, also, digestion in pepsin-hydro-

¹ This was the method then commonly used for the preparation of resistant tissue elements like cellulose and chitin.

² HORBACZEWSKI: *Zeitschrift für physiologische Chemie*, 1882, vi, p. 330.

³ CHITTENDEN and HART: *Studies from the Laboratory of Physiological Chemistry*, Yale University, 1887-88, iii, p. 19.

⁴ CHITTENDEN and HART: *Loc. cit.*

⁵ Objections had also been raised from another standpoint by ZOLLIKOFER: *Annalen der Chemie und Pharmacie*, 1852, lxxxii, p. 169.

⁶ BERGH: *Zeitschrift für physiologische Chemie*, 1898, xxv, p. 337.

chloric acid. Elastin is readily digestible in gastric juice,¹ however, so that this modification of treatment could hardly have dissolved very much that the acid and alkali did not remove, except elastin itself. Aside from determining the presence of sulphur in his own preparation of elastin and in Grüber's (a commercial product from the same source and prepared by Horbaczewski's method), Bergh made no attempt to ascertain the elementary composition of ligament elastin.

The ligament elastins made in various studies of this albuminoid by other observers (whose analytic results are referred to below), were all from the same source—ligamentum nuchæ of the ox. Morochowetz² made his products by Müller's method. Stohmann and Langbein³ obtained theirs by the Horbaczewski process. Zoja,⁴ Mann,⁵ and Eustis⁶ each used the method of Chittenden and Hart.

The following summary gives the average analytic results for percentage composition of the ash-free products prepared from ligament by the above methods.⁷

	Method of preparation.	C	H	N	S	O
TILANUS:						
	(a) Not extracted with acid	54.65	7.26	17.41	0.34	20.34
	(b) Extracted with acid	55.65	7.41	17.74	19.20
MÜLLER ⁸	Extracted with hot alkali	55.46	7.41	16.19	20.94
HORBACZEWSKI ⁹	Extracted with hot alkali	54.32	6.99	16.75	21.94

¹ See page III.

² MOROCHOWETZ: St. Petersburger medicinische Wochenschrift, neue Folge, 1886, iii, p. 135.

³ STOHMANN und LANGBEIN: Journal für praktische Chemie, neue Folge, 1891, lxiv, p. 353.

⁴ ZOJA: Zeitschrift für physiologische Chemie, 1897, xxiii, p. 236.

⁵ MANN: Archiv für Hygiene, 1899, xxxvi, p. 166.

⁶ CHITTENDEN (for EUSTIS): Proceedings of the American Physiological Society, 1899, This journal 1900, iii, p. xxxi.

⁷ For the composition of elastins from other sources than ligament see VANDEGRIFT and GIES, *loc. cit.*, also COHNHEIM: Chemie der Eiweisskörper, 1900, p. 293. We have no occasion in this review to refer to elastins which were not analyzed. Various observers have engaged in chemical studies of elastin without satisfying themselves of the purity of their products.

⁸ MÜLLER found 0.08 per cent of sulphur in his elastin, but assumed it to be due to accidental impurity.

⁹ The analytic results credited to ETZINGER by CHARLES in his "Elements of Physiological and Pathological Chemistry" (1884, p. 129), were those obtained by HORBACZEWSKI. ETZINGER made no analyses of ligament elastin. See Zeitschrift für Biologie, 1874, x, p. 84.

	Method of preparation	C	H	N	S	O
MOROCHOWETZ ¹	Extracted with hot alkali	?	?	?	0.63	?
CHITTENDEN and HART:						
	(a) Prepared by Horbaczewski's method	54.24	7.27	16.70	21.79
	(b) Their own, without extraction in alkali	54.08	7.20	16.85	0.30	21.57
STOHMANN and LANGBEIN:						
	Extracted with hot alkali	55.03	7.20	16.91	0.18	20.68
ZOJA	Not extracted with hot alkali	?	?	16.96	0.28	?
MANN	Not extracted with hot alkali	?	?	16.52	?	?
EUSTIS ²	Not extracted with hot alkali	54.42	7.40	16.65	0.14	21.39

It will be observed, from the preceding statements and summary, that as a general rule extraction with hot alkali resulted in the preparation of a sulphur-free product. On the other hand, methods which did not include alkali extraction gave elastins containing sulphur. The exceptions resulted, probably, when the alkali treatment was not as prolonged or thorough as customarily.

That treatment in hot alkali is apt to cause decomposition is now almost self-evident. Referring to this subject, Chittenden and Hart stated that "treatment with acid of the alkaline solution obtained in preparing elastin by Horbaczewski's method, plainly showed the presence of hydrogen sulphide." Did this sulphur come from the elastin and is elastin a sulphur-containing body, or did it arise from another substance originally in the ligament?³

The only constituents of elastic tissue which seem to require

¹ It has been shown by CHITTENDEN and HART that in elastoses there is a diminution of the content of carbon, and an increase in the content of oxygen, proportional to the extent of proteolysis. In spite of this fact, MOROCHOWETZ's analyses of elastose gave the following results:

C	H	N	S	O
55.90	7.29	16.68	0.62	19.50

He did not completely analyze the original elastin — only sulphur was determined as given above. It seems necessary to conclude that the elastin used by MOROCHOWETZ was an impure product and that the figures above for sulphur are inaccurate.

² EUSTIS made only a partial analysis. We are greatly indebted to him for a sample of his product, from which we obtained the remaining results. The individual ash-free determinations, by the methods we used farther on, were as follows:

C	H	N	S
54.52	7.47	16.64	0.12
54.32	7.32	16.66	0.15

³ See page 105.

treatment with alkali, in addition to acid, in order to effect their complete solution are mucoid, and traces of nucleoproteid. These bodies as they are situated, resist the action of acids, the former particularly, and their removal from compact tissue particles is easy only when alkali is used. They are readily soluble in cold dilute lime-water, which has no effect on the elastin.¹

Improved method.—Our improved method of preparing ligament elastin includes extraction in cold lime-water instead of destructive treatment with boiling potassium hydroxide, and may be given briefly as follows: Ligamentum nuchæ was cut into strips, these very finely minced in a meat chopper² and the resultant hash thoroughly washed in cold running water for from twenty-four to forty-eight hours. Traces of blood, lymph, and much coagulable proteid, with extractives, were removed in this process. The finely divided tissue was then thoroughly extracted for from forty-eight to seventy-two hours in large excess of cold half-saturated lime-water, renewed occasionally, for complete removal of residual simple proteid,³ and also mucoid and nucleoproteid. After the alkali had been thoroughly removed by washing in water, the minced substance was boiled in water, with repeated renewals, until only traces of dissolved proteid (elastoses) could be detected in the washings. The tissue was then boiled in ten per cent acetic acid for a few hours, treated with five per cent hydrochloric acid at room temperature for a similar period, again extracted in hot acetic acid and in cold hydrochloric acid, finally washed free of acid in water, and then kept in boiling alcohol and

¹ It will be remembered that half-saturated lime-water and very weak alkaline fluids of approximately the same strength have been repeatedly used for extractive purposes in the past because they manifest no destructive action on compound proteids and albuminoids at room temperature.

² The ordinary hashing machine can be very advantageously used for this purpose. It not only finely divides the tissue but also tends to loosen the fibres in all of the particles, and thus greatly favors extraction of interfibrillar substance. Previous observers make no mention of the use of special mincing apparatus. In some of the preceding work the ligament was merely cut into small pieces with a knife.

³ Our results with the simple proteids of ligament (page 118) suggest that in the preparation of elastin due regard must be paid to the fact that the fresh ligament contains at least 0.6 per cent of soluble and coagulable proteid. It certainly cannot be an easy matter to remove all this from the fibrous meshwork, particularly *after* the tissue has been boiled in water, and possibly some of the variations in the figures reported for the composition of elastin and the nature of its decomposition products may be due to such impurity not completely eliminated.

ether until dehydration was complete, and all fat and extractive substance had been removed.¹

The elastin particles prepared in this way were soft and porous and could easily be ground in a mortar to a cream-colored, very light powder.² Under the microscope the particles were seen to consist uniformly of typical elastic fibres. No extraneous matter was held in the meshes of these.

In order to study the effect of the above modified method of preparation, as well as to obtain further information on the composition of elastin, we made several samples of elastin both by the Chittenden and Hart method and our own and subjected the products to comparative analysis. The analytic methods employed were the same as those recently described in detail in a paper from this laboratory.³

Elementary composition. Preparation No. 1. — Preparations 1-4 were made by the Chittenden and Hart process as follows: Finely minced tissue (100 grams) was boiled in water until practically nothing more dissolved.⁴ This process required about ten changes of 1 litre of water and a total of seventy-five hours for completion. The substance was next warmed in 1 litre of ten per cent acetic acid for one and one-half hours on a water bath. It was kept in the same fluid eighteen hours longer at room temperature and then boiled for four hours directly over a flame. The acid was then thoroughly washed out and the substance kept in five per cent hydrochloric acid for eighteen hours at room temperature. After the mineral acid had been thoroughly removed the treatment in the acids, with appropriate washing, was repeated. Finally, dehydration and removal of fat and extractive matter were effected in boiling alcohol-ether in the usual manner. The analytic results follow:

Carbon and Hydrogen. 0.2909 gm. substance gave 0.5752 gm. CO₂ and 0.1906 gm. H₂O = 53.93 per cent C and 7.28 per cent H; 0.2538 gm. substance gave 0.5078 gm. CO₂ and 0.1659 gm. H₂O = 54.56 (?) per

¹ Further details will be found with each preparation under records of analysis, pages 99 and 101. See also page 111.

² Compare with the experience of HORBACZEWSKI and of CHITTENDEN and HART, who evidently had not succeeded in completely dehydrating.

³ HAWK and GIES: This journal, 1901, v, p. 387.

⁴ One variation here from the CHITTENDEN and HART process is to be noted. We put the cleaned ligament through a *hashing machine*. The tissue used by CHITTENDEN and HART was "chopped quite fine." The more finely divided the tissue the easier and more complete the extraction, of course. See pages 98 and 104.

Preparation No. 3.

Carbon and Hydrogen. 0.2562 gm. substance gave 0.5101 gm. CO₂ and 0.1694 gm. H₂O = 54.30 per cent C and 7.35 per cent H.

Nitrogen. 0.3305 gm. substance gave 0.0550 gm. N = 16.64 per cent N ; 0.3577 gm. substance gave 0.0596 gm. N = 16.67 per cent N.

Sulphur. 1.1549 gm. substance gave 0.0128 gm. BaSO₄ = 0.15 per cent S ; 0.7953 gm. substance gave 0.0100 gm. BaSO₄ = 0.17 per cent S.

Ash. 0.6690 gm. substance gave 0.0045 gm. Ash = 0.67 per cent Ash ; 0.5782 gm. substance gave 0.0038 gm. Ash = 0.66 per cent Ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

						Average.
C	54.67	54.67
H	7.40	7.40
N	16.75	16.78	16.76
S	0.15	0.17	0.16
O	21.01

Preparation No. 4.

Carbon and Hydrogen. 0.2571 gm. substance gave 0.5084 gm. CO₂ and 0.1671 gm. H₂O = 53.93 per cent C and 7.22 per cent H.

Nitrogen. 0.3386 gm. substance gave 0.0562 gm. N = 16.59 per cent N ; 0.2545 gm. substance gave 0.0426 gm. N = 16.72 per cent N.

Sulphur. 0.9068 gm. substance gave 0.0163 gm. BaSO₄ = 0.25 per cent S ; 1.0077 gm. substance gave 0.0163 gm. BaSO₄ = 0.22 per cent S.

Ash. 0.4931 gm. substance gave 0.0052 gm. Ash = 1.05 per cent Ash ; 0.4412 gm. substance gave 0.0050 gm. Ash = 1.13 per cent Ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

						Average.
C	54.52	54.52
H	7.30	7.30
N	16.77	16.90	16.83
S	0.25	0.22	0.24
O	21.11

Preparation No. 5. — Preparations 5–8 were made by our own method. 100 grams of ligament strips were washed in cold running water 24–48 hours. The strips were next run through a hashing machine and the hash thoroughly extracted several times (for 3 days) in half-saturated lime-water. The last extract did not become turbid on acidification. The alkali was completely washed out of the hash

Preparation No. 7.

Carbon and Hydrogen. 0.2584 gm. substance gave 0.5120 gm. CO₂ and 0.1685 gm. H₂O = 54.04 per cent C and 7.25 per cent H.

Nitrogen. 0.4656 gm. substance gave 0.0764 gm. N = 16.42 per cent N; 0.4482 gm. substance gave 0.0744 gm. N = 16.60 per cent N.

Sulphur. 0.8678 gm. substance gave 0.0096 gm. BaSO₄ = 0.15 per cent S; 0.8896 gm. substance gave 0.0080 gm. BaSO₄ = 0.12 per cent S.

Ash. 0.5082 gm. substance gave 0.0038 gm. Ash = 0.75 per cent Ash; 0.3540 gm. substance gave 0.0030 gm. Ash = 0.85 per cent Ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

						Average.
C	54.47	54.47
H	7.30	7.30
N	16.55	16.73	16.64
S	0.15	0.12	0.14
O	21.45

Preparation No. 8.

Carbon and Hydrogen. 0.2552 gm. substance gave 0.5000 gm. CO₂ and 0.1666 gm. H₂O = 53.43 per cent C and 7.25 per cent H.

Nitrogen. 0.3169 gm. substance gave 0.0536 gm. N = 16.90 per cent N; 0.4482 gm. substance gave 0.0431 gm. N = 16.84 per cent N.

Sulphur. 0.8235 gm. substance gave 0.0087 gm. BaSO₄ = 0.15 per cent S; 0.5679 gm. substance gave 0.0059 gm. BaSO₄ = 0.14 per cent S.

Ash. 0.4533 gm. substance gave 0.0032 gm. Ash = 0.71 per cent Ash; 0.3851 gm. substance gave 0.0031 gm. Ash = 0.81 per cent Ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

						Average.
C	53.84	53.84
H	7.31	7.31
N	17.03	16.96	17.00
S	0.15	0.14	0.14
O	21.71

The results for elementary composition of our eight preparations are brought together in the appended general summary. No great differences in the average composition of the preparations of each group are to be found. In fact the general analytic harmony is very striking and rather unexpected. The significant feature is to be seen in the figures for sulphur. The quantity is slight throughout, with

the content of sulphur in preparations 5-8 regularly lower than that of preparations 1-4.¹

GENERAL SUMMARY OF ELEMENTARY COMPOSITION.

Elements.	Preparations 1-4. Made by the method of CHITTENDEN and HART.					Preparations 5-8. Made by the method of RICHARDS and GIES.					Gen'l av.
	1	2	3	4	Av.	5	6	7	8	Av.	
C	54.38	54.15	54.67	54.52	54.43	53.90	54.32	54.47	53.84	54.14	54.29
H	7.32	7.26	7.40	7.30	7.32	7.42	7.30	7.30	7.31	7.33	7.33
N	16.19	16.82	16.76	16.83	16.65	16.74	17.11	16.64	17.00	16.87	16.76
S	0.21	0.21	0.16	0.24	0.21	0.16	0.14	0.14	0.14	0.14	0.18
O	21.90	21.56	21.01	21.11	21.39	21.78	21.13	21.45	21.71	21.52	21.44

The following summary affords ready comparison in this connection with related results for average elementary composition:—

Ligament elastin:	C	H	N	S	O
HORBACZEWSKI	54.32	6.99	16.75	21.94
CHITTENDEN and HART	54.08	7.20	16.85	0.30	21.57
RICHARDS and GIES	54.14	7.33	16.87	0.14	21.52
Aorta elastin:					
SCHWARZ ²	54.34	7.08	16.79	0.38	21.41
BERGH	53.99	7.54	15.20	0.60	22.67

Reactions.— We have little to add in this connection to what has already been noted. We have found, however, that elastin is not as resistant to acids and alkalis as it is generally considered to be. When the original tissue is very finely and thoroughly divided with a meat chopper, as was the case for the first time in our experiments, the particles undergo some solution in the acids used in the extraction process. The *purified powdered* substance is slightly soluble even in cold 0.2 per cent hydrochloric acid on long standing and dissolves very quickly and completely in 1 per cent potassium hydroxide on warming. These results suggest that the state of division of the tissue in preparation of elastin greatly influences solubility and thereby also purification. We believe that the agreement in composition between the two groups of our products was

¹ See references under "Sulphur content," page 105.

² SCHWARZ: Zeitschrift für physiologische Chemie, 1894, xviii, p. 487.

dependent largely on the particularly fine division of the tissue employed. The acids used for extractive purposes were given an excellent opportunity to decompose and completely dissolve inter-fibrillar extraneous matter.

Sulphur content. — It will be recalled that in the older methods of elastin preparation extraction of the elastic tissue by boiling in dilute alkali for several hours was a part of the process and that, although the resultant substance varied somewhat in composition, it was free from sulphur in a majority of cases. Chittenden and Hart were the first, as we have already pointed out, to call attention to the probability that sulphur is really an integral part of elastin, and that on boiling with alkali the constituent sulphur is removed. By avoiding the use of alkali Chittenden and Hart prepared elastin with a content of sulphur amounting to 0.3 per cent. They said in this connection, "Whether pure elastin does contain sulphur or whether the 0.3 per cent present in preparation B (made by their own method) is a constituent of some adhering proteïd, removable by alkali, we are not at present prepared to say, but deem it probable that elastin does contain a small amount of sulphur."

Zoja and Eustis have recently confirmed the Chittenden and Hart result. Schwarz lately found about the same amount of sulphur in elastin from the aorta, but states that all was removable on boiling with 1 per cent potassium hydroxide and that the residual product was identical with the original body. Bergh has also obtained as much as 0.55 per cent of sulphur in aorta elastin prepared by the old alkali extraction method.

The results for sulphur content of all our preparations are given on page 106.

It will be seen that the average sulphur content of the five preparations made according to the older method was 0.20 per cent, whereas the elastin made by our own process, from which we had positively excluded the presence of mucoïd and coagulable proteïd, shows a percentage of sulphur amounting to 0.15 per cent, an average difference of 0.05 per cent in favor of the improved method. This difference, slight though it is, is fairly constant throughout. The analyses were made with the very greatest care. Our results seem to show conclusively that sulphur, in minute quantity at least, is a component part of pure ligament elastin.

Schwarz, it will be remembered, found that after treatment of aorta elastin with boiling one per cent potassium hydroxide for four hours

all of the sulphur (0.38 per cent) was split off in a form which could be precipitated as lead sulphide, leaving a sulphur-free, insoluble elastin having all of the properties of the original substance. Ligament elastin seems to be a different substance. On decomposing samples of our eight preparations in one per cent potassium hydroxide as Schwarz did, no sulphur in the form of sulphide could be detected

Elastin made by the CHITTENDEN and HART method.			Elastin made by the RICHARDS and GIES method.		
Number of prep- aration.	Percentage of sulphur. ¹		Number of prep- aration.	Percentage of sulphur. ¹	
	Direct deter- minations.	Average.		Direct deter- minations.	Average.
1	0.17 0.25 0.20	0.21	5	0.16 0.17	0.16
2	0.22 0.20	0.21	6	0.13 0.15	0.14
3	0.15 0.17	0.16	7	0.15 0.12	0.14
4	0.25 0.22	0.24	8	0.15 0.14	0.15
9 ²	0.16 0.18	0.17			
General average . . . 0.20			General average . . . 0.15		
<p>¹ The ash of each preparation was slight in amount. The ash contained an appreciable proportion of sulphur—an average of 0.11 per cent of the proteid of each group of preparations. This was doubtless derived in great part from the organic sulphur during incineration.</p> <p>² This preparation was not completely analyzed, and therefore was not included in the series under elementary composition, page 104. It contained only 0.54 per cent ash.</p>					

in any of them, even when the whole volume of alkaline fluid was used for the test. A sample of the elastin prepared by Eustis, by the older method, however, which did not include preliminary treatment with lime-water for removal of mucoids, etc., gave decided sulphide reaction under similar circumstances. Our preparations completely dissolved in the warm alkali.

These facts indicate that the small amount of sulphur contained in

pure elastin is held in a form of combination not convertible into sulphide by treatment with boiling alkali.

Distribution of nitrogen.— The nitrogen of the proteïds appears to exist in various amino forms, none of it being in nitro or nitroso combination. Some of it is easily split off in the form of ammonia by acid and by alkali. Usually, however, the largest quantity is obtainable on decomposition in the form of monamido acids and a considerable proportion is frequently separable in diamido combination.

No attempts to ascertain the distribution of nitrogen in the elastin molecule were made until very recently.¹ Soon after Kossel² had stated his belief that all proteïds would yield hexone bases on decomposition Bergh³ attempted to isolate lysin and arginin from among the cleavage products obtained from elastins of the cervical ligament and the aorta. His attempts resulted negatively.⁴

Hedin⁵ by essentially the same methods came to the same negative result. He was unable, also, to identify histidin. These results would imply that elastin does not contain a protamin radicle.

Kossel and Kutscher,⁶ however, by an improved method, subsequently isolated arginin from among the decomposition products of ligament elastin and thus directly contradicted the conclusions of Bergh and Hedin. The quantity of arginin isolated by them was unusually small — much less than that for most of the other proteïds. Not long ago these same observers⁷ were able to separate and identify lysin also among the bases obtainable from elastin.

The lack of agreement between Bergh and Hedin on the one side and Schwarz and Kossel and Kutscher on the other led to the study made by Eustis,⁸ under Chittenden's direction, of the proportion of basic nitrogen split off from elastin on decomposition with hydrochloric acid and stannous chloride. Following the method adopted

¹ HORBACZEWSKI studied some of the decomposition products from a different standpoint: *Jahresbericht der Thier-Chemie*, 1885, xv, p. 37. SCHWARZ made a study of aorta elastin similar in this respect to that of HORBACZEWSKI: SCHWARZ, *loc. cit.*

² KOSSEL: *Zeitschrift für physiologische Chemie*, 1896-97, xxii, p. 176.

³ BERGH: *Loc. cit.*

⁴ SCHWARZ had previously found "lysatinin" (lysin + arginin) among the cleavage products of aorta elastin. *Loc. cit.*, p. 497.

⁵ HEDIN: *Zeitschrift für physiologische Chemie*, 1898, xxv, p. 344.

⁶ KOSSEL and KUTSCHER: *Zeitschrift für physiologische Chemie*, *Ibid.*, p. 551.

⁷ KOSSEL and KUTSCHER: *Ibid.*, 1900-01, xxxi, p. 165.

⁸ CHITTENDEN (for EUSTIS): *Loc. cit.*

by Schulze¹ and numerous other investigators, Eustis, in five experiments on the same preparation of elastin, obtained the following divergent results for percentage of nitrogen in the form of organic bases:

1	2	3	4	5
0.86	17.69	15.57	6.50	15.14

These discordant data, while they indicated that elastin does yield hexone bases, led to the conclusion that the method then in use for the separation of the hexone bases by phosphotungstic acid and determination of the nitrogen therein, was unreliable for quantitative purposes.

The divergence of the results obtained by Eustis made it seem desirable for us in this work to repeat his experiments, with elastin made by the method of preparation he used and also with products purified by our own method.

The preparations used for this particular purpose were Nos. 1, 3, and 7. (See page 104). The method of cleavage was the same as that used by Eustis. Decomposition took place (in the presence of one gram of stannous chloride) in 20 per cent hydrochloric acid in quantity equal to 4 c.c. per gram of substance used. The mixture was boiled each time in a reflux condenser for ninety-six hours. Separation of tin, precipitation with phosphotungstic acid and the other steps to quantitative determination were the same in detail as those taken in this connection by Schulze. The substance which remained in the acid mixture as an insoluble residue, very slight in quantity (possibly melanin), contained a mere trace of nitrogen.

In addition to a study by Schulze's procedure, we also made a similar decomposition of preparation No. 6 by the method of Kossel and Kutscher.² 100 grams of substance was boiled in a solution of 300 grams of pure concentrated sulphuric acid in 600 grams of water for fourteen hours in a reflux condenser. Further details of the separations were the same as those in the experiments of Kossel and Kutscher.

In the following table we give the essential data obtained by both methods, the figures expressing averages of closely agreeing results:

¹ SCHULZE: *Zeitschrift für physiologische Chemie*, 1898, xxiv, p. 276.

² KOSSEL und KUTSCHER: *Zeitschrift für physiologische Chemie*, 1900-01, xxxi, p. 165. The authors show that this method gives more abundant yield of hexone bases than any other.

Number of preparation.	Weight of ash-free elastin used.	Nitrogen.						
		Grams.				Percentage of total.		
		Grams.	Total.	Ammonia.	Mon-amido acids.	Bases.	Ammonia.	Mon-amido acids.
A-3	13.4361	2.2371	0.0507	2.1351	0.0666	2.26	95.44	2.98
1	11.4472	1.8533	0.0434	1.8238	0.0420	2.34	98.42	2.26
7	11.5549	1.9227	0.0333	1.8311	0.0593	1.73	95.23	3.08
B-6	105 2000	18.1012	0.2572	17.0081	0.9153	1.42	93.96	5.06
Average						1.94	95.76	3.34

It will be noted that although there is some variation in the percentage figures, there is yet a striking agreement among them. The latter fact is true even though two methods were employed and very different quantities of elastin were taken for each series of determinations. It is to be noticed also that the nitrogen in the bases was greatest for preparation No. 6 as determined by the Kossel and Kutscher method, a result in harmony with the claim of these observers that their process furnishes the most abundant supply of hexone bases. The uniformity of our results is in striking contrast to the disagreement of those obtained by Eustis.

Although the strictest quantitative accuracy cannot be claimed for the methods employed,¹ it does seem warrantable to conclude from our results in this connection that elastin contains an appreciable amount of nitrogen which on proper decomposition may be identified as nitrogen in the form of hexone bases.

In addition to the above results somewhat more specific data as to hexone bases were obtained with preparation No. 6 in a continuation of the Kossel and Kutscher method previously used. These afford the direct comparison made with similar results obtained by

¹ See papers in the *Zeitschrift für physiologische Chemie*, 1898-1901 (vols. xxv-xxx) by HEDIN, GULEWITSCH, HENDERSON, FRIEDMANN, KUTSCHER, and SCHULZE and WINTERSTEIN.

Kossel and Kutscher on histon, salmin, zein and gelatin, among other products,¹ in the appended summary:

Substance.	Percentage of total nitrogen.				Percentage of weight of original substance.			
	Histidin.	Arginin.	Lysin.	Ammonia.	Histidin.	Arginin.	Lysin.	Ammonia.
Ligament elastin	0.170	1.380	— ¹	1.375	0.027	0.197	— ¹	0.287
Commercial gelatin	?	16.600	?	1.400	?	9.300	5-6 ²	0.300
Zein	1.410	3.760	0	13.530	0.810	1.820	0	2.560
Histon (thymus)	1.790	25.170	8.040	7.460	1.210	14.360	7.700	1.660
Salmin	0	87.800	0	0	0	84.300	0	0

¹ Unusual difficulty was experienced in our attempts to separate lysin quantitatively. By difference our figures for nitrogen of lysin were 6.65 per cent of the total. We do not include them in the above table, because we have no confidence in their accuracy. The microscopic appearance of the histidin dichloride and arginin nitrate prepared by us was typical. The quantities obtained were too slight for satisfactory analysis.

² Approximate value.

Elastin appears to be characterized by containing a comparatively small quantity of hexone radicles. Our results indicate that histidin as well as the lysin and arginin found by Kossel and Kutscher may be split off from this albuminoid on appropriate treatment.

Is elastin a "fat-proteid compound?"—Nerking² has very recently found that various proteids as they are commonly prepared, contain fatty or fatty acid radicles. He did not examine elastin in this connection. We have done so, with entirely negative result.

Samples of preparations Nos. 5 and 6 were used for the purpose. The amounts of substance taken were 5.6747 gms. of No. 5 and 8.7429 gms. of No. 6. After two weeks' continuous extraction in anhydrous ether in a Soxhlet extractor, only 0.0015 gm. of extractive substance (dried in vacuo) could be obtained from No. 5; only 0.0013 gm. from No. 6. After digesting each preparation in pepsin-

¹ Their work on elastin in this connection was only qualitative. Lysin was isolated and identified. *Loc. cit.*, p. 205.

² NERKING: *Archiv für die gesammte Physiologie*, 1901, lxxxv, p. 330.

hydrochloric acid, in continuation of Dormeyer's method, as was done by Nerking in his work, and then thoroughly extracting the digestive mixture in the customary manner with ether, only 0.0017 gm. of ether-soluble matter was obtained from No. 5, only 0.0013 gm. from No. 6. Thus, in the double extraction process only 0.0032 gm. of ether-soluble matter (0.056 per cent) was obtained from No. 5; only 0.0026 gm. (0.03 per cent) from No. 6. These amounts are too minute, however, to mean anything positive — are, in fact, within the limits of unavoidable errors of extractive work. The pepsin used in the digestive process contained 0.5 to 1 mgm. of ether-soluble matter, which should be subtracted from the above totals in each case. At most the merest trace of soluble matter could have existed in either of the preparations. Thus it is certain that elastin as prepared by the method we employed does not contain fat or fatty acid, either in ordinary molecular combination or as an admixture.¹ These experiments have nothing to do, of course, with the question of fatty radicles contained *within* the proteïd molecule.

Digestibility. — In the preceding determinations of possible fat admixture it was necessary to digest the elastin. Our preparations were readily digested in gastric juice, a result quite in harmony with the earliest observations of Etzinger.² Thus samples of preparations Nos. 5 and 6, weighing respectively 5.6747 gms. and 8.7429 gms., completely dissolved, in twenty-four hours, in mixtures of 300 c.c. of 0.2 per cent hydrochloric acid and 0.2 gm. of commercial pepsin scales (very active preparation) kept at 40°C. Cumulative power of combining with the acid was manifested by the products as is the case with other proteoses and peptones.³ At the end of twenty-four hours only the merest turbidity remained in the fluid, showing that only a very slight amount of antialbumid had formed.

In the work of Chittenden and Hart on elastin and elastoses, elastin peptone could not be detected among the products of pepsin and trypsin proteolysis, even though zymolysis continued under

¹ POSNER and GIES: Proceedings of the American Physiological Society, 1901, This journal, vi, p. xxix. This result indicates that the difficulty experienced by HORBACZEWSKI and CHITTENDEN and HART in completely removing "fat-like matter" from their elastins was due to the compact character of the pieces of their preparations. Dehydration was complete in our own (page 99), with the result that fat extraction in purifying was comparatively easy. See CHITTENDEN and HART, *Loc. cit.*, p. 21.

² ETZINGER: Zeitschrift für Biologie, 1874, x, p. 84.

³ CHITTENDEN: Digestive proteolysis, 1894, p. 52.

favorable conditions for several days. Peptone was absent also from the products formed on hydration of their elastin with very dilute acid. Although they were unable to detect peptone among the proteolytic products of elastin, Chittenden and Hart seem to have assumed its probable formation under favorable conditions, however, for toward the end of their paper they say: "Under the conditions of our experiments, no appreciable amount of true peptone was formed in any of the digestions; at least, nothing approaching a peptone in reactions was to be found in any of the digestive fluids, after saturation with ammonium sulphate. We propose, later, to attempt a study of the elastin peptone, using for this purpose the elastoses just described as well as elastin itself, and more vigorous digestive fluids, both peptic and tryptic."¹ Chittenden and Hart found that Horbaczewski's "elastin peptone" was in reality deutero-elastose and that his "hemi-elastin" is the same as proto-elastose.

After our digestive mixtures had been duly extracted with ether, in accordance with the original aim of the experiments immediately preceding, we allowed proteolysis to continue for about six weeks. Ether was added to the acid mixture occasionally to prevent bacterial changes. At the end of that period the elastose precipitate obtained on saturating the boiling neutral fluid with ammonium sulphate was surprisingly large. The filtrate was also made alternately acid and alkaline and thoroughly boiled each time while saturated with ammonium sulphate.² Only very slight additional proteose precipitates were obtained in this way. Ammonium sulphate was removed from the filtrate with alcohol and barium carbonate in the customary manner. The final filtrate gave a strong biuret reaction with a *slight* amount of cupric sulphate and an *excess* of potassium hydroxide. The amount of peptone precipitable by absolute alcohol was comparatively slight, although more than could have arisen, directly or by auto-digestion, from the pepsin preparation used at the outset.

In a special experiment in this connection 8.15 gms. of preparation No. 6 were digested in a solution of 900 c.c. of 0.4 per cent hydrochloric acid and 2 gms. of the very active commercial pepsin used above. Toluol was added to the mixture as a preservative, although the acid would have prevented bacterial action. Complete solution

¹ CHITTENDEN and HART: *Loc. cit.*, p. 36. See also CHITTENDEN: Digestive proteolysis, 1894, p. 72.

² KÜHNE: *Zeitschrift für Biologie*, 1892, xxix, p. 1.

of the elastin occurred within twenty-four hours. At the end of seventeen days a large proportion of elastose was separated by saturation of the neutral, acid and alkaline fluid with ammonium sulphate. Separated quantitatively in absolute alcohol containing ether the ash-free substance recovered as elastose (albuminate and antialbumid were absent at this stage of the digestion) was 7.43 gms., showing that at least 0.7 gm. of the original elastin had been transformed into peptone.¹ The final ammonium sulphate filtrate gave a strong biuret reaction when large excess of potassium hydroxide was present in the fluid. Some of the peptone contributing to this biuret reaction must, however, have arisen from the pepsin preparation.

These experiments show that elastoses are particularly resistant to progressive proteolysis through the action of pepsin, although they demonstrate that a small proportion of true peptone is formed from them during prolonged periods of favorable contact with the enzyme.²

The precipitate obtained from the above digestive mixtures on saturation with ammonium sulphate contained both primary and secondary elastoses. It retained the color of the original elastin. Judging from the reactions of solutions of the mixed proteoses, the amount of proto-elastose was relatively large. Such solutions, when concentrated, became heavily turbid on warming, as Horbaczewski³ and subsequently Chittenden and Hart observed. Turbidity was decided even when tubes containing the clear concentrated fluid were immersed in water at 38°C or held under the tongue. Such turbid solutions cleared up again on cooling. The clear *concentrated* solution gave heavy precipitates with small quantities of concentrated nitric acid, picric acid, potassio-mercuric iodide, and other proteid precipitants, but such precipitates were only partially, if at all, soluble on warming. When these reagents were added to *dilute* solutions, however, the precipitates which were formed at once *dissolved* on

¹ All weights were made of substance dried to constant weight at 100°-105° C.

² In similar experiments, concluded after this paper had gone to the editor, 8 grams of elastin yielded only 1.38 gram of crude elastose when the digestion had proceeded for forty-six days. After digesting for seventy days 10 grams of elastin yielded less than 1 gram of elastose. A small proportion of proto-elastose was contained in the latter mixture. Large proportions of peptone were formed. These results harmonize with, and emphasize the conclusions above.

³ HORBACZEWSKI, CHITTENDEN, and HART: *Loc. cit.* See also, MOROCHOWETZ, SCHWARZ: *Loc. cit.*

warming and *reappeared* on cooling, just as in the case of other proteoses. Addition of excess of concentrated sodium hydroxide to the concentrated proteose solution was followed by heavy precipitation of some of the proteid, the precipitate persisting even when the solution was boiled.

The above reactions appear to have been due to proto-elastose, which seems to be a peculiar member of the proteose family.

Heat of combustion. — The potential energy of the proteids, expressed in calories, varies from about 5,000 to 6,000 small calories per gram of substance. Proteids such as peptone and osseomuroid, with comparatively small content of carbon, have the lowest combustion equivalents, whereas bodies like hæmoglobin, with relatively large proportion of carbon, have the very highest. The heat of combustion of any albuminous substance depends largely on the amounts and combinations of carbon and oxygen contained in it. The figures for composition of elastin suggest that its heat of combustion is relatively great.

The only previous observations on elastin made in this connection were those published by Stohmann and Langbein.¹ These observers worked with elastin made by Horbaczewski's method. The combustion equivalent was determined by the improved Berthelot method, and averaged 5,961.3 small calories per gram of ash-free substance — the highest equivalent for animal proteid.

Last June, while enjoying the freedom of Professor Atwater's laboratory, we made a thermochemical study of some of our products.² We wish here to express our thanks to Professor Atwater for his help and encouragement in this work and to acknowledge, also, our indebtedness to his assistants, Messrs. E. M. Swett and Emil Osterberg, for experimental aid.

The following table summarizes our results for the preparation of ligament elastin made by Mr. Eustis by the Chittenden and Hart process, for one made by us by the same method (No. 2), and for two preparations made by our own method (Nos. 5 and 6); it also includes the results obtained by Stohmann and Langbein:

¹ STOHMANN and LANGBEIN: *Loc. cit.*

² The apparatus used and method employed were the same as those previously described in *This journal*: 1901, v, p. 419. Quantities of 0.7–0.8 gram were burned at a time. The figures in the table are for substance dried to constant weight at 105°–110° C.

Preparation.	Direct deter- minations.			Averages: Calculated for ash-free substance. ¹						
	Heat of combus- tion. Small cal- ories per gram.			Percentage composition.					Heat of combustion. Small calories.	
	I	II	Av.	C	H	N	S	O	Per gm.	For substance containing 1 gm. of carbon.
EUSTIS . . .	5933	5947	5940	54.42	7.40	16.65	0.14	21.39	5960	10952
RICHARDS and GIES . . .										
Prep. No. 2.	5849	5821	5835	54.15	7.26	16.82	0.21	21.56	5870	10840
Prep. No. 5.	5840	5871	5855	53.90	7.42	16.74	0.16	21.78	5904	10954
Prep. No. 6.	5923	5909	5916	54.32	7.30	17.11	0.14	21.13	5967	10985
Average			5886	54.20	7.34	16.83	0.16	21.47	5925	10933
STOHMANN and LANGBEIN . .				55.03	7.20	16.91	0.18	20.68	5961	10832

¹ The percentage of ash in EUSTIS' preparation was 0.34; in our own it varied between 0.08 and 0.83. The STOHMANN and LANGBEIN preparation contained 0.07 per cent ash.

The general relation of the above results to those for other proteïds, is seen at a glance in the following summary:

Substance.	Average percentage composition.					Heat of combustion. Small calories.	
	C	H	N	S	O	Per gram.	For substance containing 1 gram of carbon.
Ligament elastin ¹ . . .	54.36	7.32	16.85	0.17	21.31	5932	10912
Various animal and veg- etable proteïds, not in- cluding glucoproteïds ² . .	52.64	7.08	16.00	1.03	23.20	5711	10849
Connective tissue mucoids ³	47.43	6.63	12.22	2.32	31.40	4981	10505

¹ The figures for ligament elastin are the averages of the results obtained by STOHMANN and LANGBEIN and in our own experiments.

² Averages obtained by STOHMANN and LANGBEIN.

³ Averages obtained by HAWK and GIES: This journal, 1901, v, p. 423.

II. MUCOID.

Although a few indefinite statements regarding mucoïd in ligament¹ were made shortly after Rollett's detection of this substance among the proteids of tendon, no attempts to separate and identify such a substance in elastic tissue were recorded before this work was begun. It seems that its presence had been inferred, not shown. Vandegrift and Gies have lately found that the quantity of mucoïd in the ligamentum nuchæ of the ox averages 0.525 per cent of the fresh and 1.237 per cent of the dry tissue.² The quantity of mucoïd in ligament is considerably less than in tendon.³

Our mucoïd preparations were made by the method used by Chittenden and Gies.⁴ Quantities of ligament hash varying from three to nine kilos were employed at a time. Much of the mucoïd was lost mechanically in the purification process. Special care was taken to reprecipitate from solution in potassium hydroxide (0.05 per cent) or half-saturated lime-water several times; also, to wash thoroughly and to dehydrate and purify in boiling alcohol-ether.

We have not made an extended analytic study of ligament mucoïd, but the following facts show its near relationship to the other connective tissue mucoïds.⁵

In physical appearance the purified product is practically the same as tendomucoïd or osseomucoïd, although the latter substances can be dehydrated more easily. It gives the proteid color reactions very distinctly. It yields reducing substance and ethereal sulphate on decomposition with two per cent hydrochloric acid. The reducing substance forms dextrosazone-like crystals with phenylhydrazine, a fact indicating the presence of glucosamine among the hydration products. Among the other products resulting from its hydration in dilute acid are an antialbumid-like body, acid albuminate, proteoses and peptone. It is digestible in pepsin-hydrochloric acid and leaves a residue containing considerable reducing substance. Its sulphur may be obtained both as sulphate and sulphide.

¹ KÜHNE: Lehrbuch der physiologischen Chemie, 1868, p. 363.

² VANDEGRIFT and GIES: *Loc. cit.*

³ BUERGER and GIES: This journal, 1901, vi, p. 219.

⁴ CHITTENDEN and GIES: Journal of experimental medicine, 1896, i, p. 186.

⁵ MEAD and GIES: Proceedings of the American Physiological Society, 1901 This journal, 1902, vi, p. xxviii.

Ligament mucoïd is soluble in 0.05 per cent solution of sodium carbonate, half-saturated lime-water and 5 per cent sodium chloride. It is insoluble in 0.1 per cent hydrochloric acid, but is somewhat soluble in 0.2 per cent solution of the same. It is less resistant to acid than the mucoïd from tendon or bone and somewhat more difficult to precipitate completely from its solution. The pure substance does not contain phosphorus. It is acid to litmus, neutralizes dilute alkali and has the same general precipitation reactions as the other connective tissue mucoïds. None of our preparations contained chlorine.

The percentage amounts of nitrogen and sulphur in mucoïds furnish favorable data for general comparisons of composition. The summary below gives our results in this connection, together with the proportion of ethereal sulphur. In the analyses the usual amounts of substance, dried to constant weight at 100°-110° C were taken. The quantity of ash in the preparations varied between 1.04 per cent and 1.90 per cent. The ash consisted mostly of calcium and of phosphoric acid. The quantity of total phosphorus in preparation B (the only one analyzed in this connection) was 0.18 per cent. The phosphorus of the ash of preparation B amounted to 0.16 per cent of the proteïd.

Preparations.	A	B	C	D	E	General Averages.
Nitrogen	12.80	13.40	13.74	13.90	13.27	13.44
	13.01	13.64	13.66	13.82	13.22	
	12.90	13.52	13.70	13.86	13.25	
Total sulphur	2.05	1.77	1.49	1.37	1.45	1.61
	2.09	1.68	—	1.27	1.40	
	2.07	1.73	1.49	1.32	1.42	
Sulphur as SO ₃	1.32	1.02	0.90			1.06
	1.17	—	—			
	1.25	1.02	0.90	

The percentage content of nitrogen appears to be uniformly higher in ligament mucoïd than in related connective tissue glucoproteïds. The content of sulphur is somewhat lower. It is to be noted, how-

ever, that experiments recently completed in this laboratory¹ indicate that there is more than one mucoid in tendon and bone, some of the glucoproteid separable from these tissues having as much as fourteen to fifteen per cent of nitrogen. We are inclined to believe, from the above results, that the same deduction regarding variability of general composition may be made with respect to mucoid substance in ligament also. It is possible, of course, that our preparations have been contaminated somewhat with coagulable proteid or other impurity we failed to remove. At the same time we used every precaution to prevent admixture.

III. COAGULABLE PROTEIDS.

The simple proteids of the connective tissues have received very little attention. Those who have worked with the albuminoid constituents have usually confined their studies to those particular substances, and the various papers on the mucoids have made only incidental reference to the albumins and globulins.

We were surprised at the outset of these studies by the comparatively large amount of coagulable proteid present in ligament. In two preliminary quantitative determinations with the ligamentum nuchæ of the ox we found that the coagulable proteid was equal on an average to 0.64 per cent of the fresh tissue.² The quantities of coagulable proteid in tendon and cartilage, we found, were much less, and, moreover, were very difficult to separate and determine satisfactorily.³

¹ CUTTER and GIES, HAWK and GIES: *Loc. cit.*

² Additional results are given by VANDEGRIFT and GIES: *Loc. cit.*

³ Using the methods employed with ligament (to be described on page 119), we found that aqueous extracts of the tendo Achillis of the ox contained only two coagulable proteids—one separated at 54°–57° C., corresponding to “(2)” in ligament: the other at 73° C, apparently the same as “(4)” in ligament. (See page 120). LOEBISCH, touching on this matter incidentally in his preparation of tendomucoid, referred to what he called serum globulin and a proteid coagulating at 78° C. He took no special pains, however, to remove the blood completely before making the extraction in water. See, *Zeitschrift für physiologische Chemie*, 1886, x, p. 43, foot-note.

Extracts of hyaline cartilage, in the few experiments we tried, gave negative results. On boiling, the extracts became opalescent. Flocks did not form, even with a fairly strong acidity. Chondromucoid and chondroitin sulphuric acid were present, of course. These bodies doubtless interfered with coagulation of such albumin or globulin as may have been contained. VON MERING obtained merely

In order to determine, if possible, the number and character of the simple proteïds present in ligament we made use of various common methods, among them the process of fractional coagulation. For this purpose several extracts were made — aqueous and saline. Five per cent solution of magnesium sulphate was used generally for the latter type.

In the preparation of these extracts only such ligaments were used as seemed to be free from blood in all parts. The tissue was freed of extraneous matter and at first cut into narrow strips, which were kept in running water for from twelve to twenty-four hours. This treatment removed blood and lymph. The strips were then run through a meat chopper and the finely minced substance treated with enough extractive fluid to just cover it. At the end of from twelve to twenty-four hours, after repeated stirring, the fluid was strained through cloth and filtered. Each extract obtained in this way was always free from hæmoglobin, as examination with the spectroscope demonstrated, — a result implying also the absence of most, if not all, lymph proteïds as well. Such extracts were either practically neutral in reaction or weakly alkaline to litmus. On heating, the solutions became very turbid and after addition of a trace of acid, flocculent separation in a water-clear fluid took place. All extracts contained such saline matter in solution as was found by us previously in ligament ash.

In determining the temperatures of coagulation the apparatus recommended by Gamgee¹ and commonly used in such work was employed, and 20–40 c.c. of the extract, made very faintly acid with acetic acid, was taken for each series of observations. The temperature was raised very gradually, and as soon as turbidity ensued the flame was removed and the solution kept at that temperature, or raised very slowly until the precipitate became flocculent. At this point the temperature was kept constant for from one-half to three-quarters of an hour, and then the solution filtered. The filtrates in each case were as clear as water. Upon raising the temperature beyond the previous maximal point the fluid remained clear until it had reached a temperature several degrees higher, when suddenly the next turbidity ensued.

Working in this way we obtained separations at the following temperatures :

the same opalescence on boiling. See, *Ein Beitrag zur Chemie des Knorpels*, 1873, p. 7. (Inaugural-Dissertation, Strassburg.)

¹ GAMGEE: *Text-book of the physiological chemistry of the animal body*, 1880, i, p. 15.

No.	Extremes of temperature. ¹	Average temperature.
1.	31°-49° C.	40° C.
2.	51°-61° C.	56° C.
3.	60°-70° C.	65° C.
4.	74°-76° C.	75° C.
5.	77°-85° C.	82° C.

All of these were obtained from each of the above types of extracts; (1), (4), and (5) were comparatively slight in amount.

The question naturally arose whether the precipitates separating at the above temperatures represented individual proteids in the tissue. Direct elementary chemical analysis would not have sufficed to answer this question definitely, for only very minor differences in composition exist among the albumins and globulins. Nor would a study of the decomposition products of these coagula have afforded any more definite conclusions. (See pages 126 and 127.)

We have sought the solution of the problem in fractional separation experiments by the methods repeatedly used by Hofmeister, Kauder, and others, particularly for the differentiation of albumins and globulins. Our results in this connection, on extracts made by the method previously detailed, are briefly summarized below :

A. Aqueous extracts treated with $(\text{NH}_4)_2\text{SO}_4$ in substance.

(a) When the aqueous solutions were *half-saturated* with $(\text{NH}_4)_2\text{SO}_4$, a fairly heavy precipitate was obtained, which consisted theoretically in whole or for the most part of globulin, albumin not being precipitated by this proportion of $(\text{NH}_4)_2\text{SO}_4$ (see page 124). The MgSO_4 solution of this precipitate contained bodies (1), (2), and (4) in the table above.

(b) In the aqueous solution of this same precipitate (a), bodies (1), (3), (4), and (5) were thrown down on heating. Precipitates (1) and (3) were comparatively heavy, the others were slight. Diluted with an equal volume of water, this aqueous solution of precipitate (a) gave bodies (1), (3), (4), and (5).

(c) The filtrate from precipitate (a) was *saturated* with $(\text{NH}_4)_2\text{SO}_4$. The substance thrown out of solution in this way was dissolved in water and the solution heated. It gave precipitates (2), (3), (4), and (5).

B. MgSO_4 extracts treated with MgSO_4 in substance.

When the MgSO_4 extracts were saturated with MgSO_4 , a heavy pre-

¹ The extremes represent the limits of *all* our observations. As a rule the separations occurred at or about the mean temperature, with comparatively long intervals.

cipitate was obtained, which, dissolved in 5% $MgSO_4$ solution, contained products (1) and (2). The filtrate from the $MgSO_4$ precipitate, on heating, gave bodies (2), (3), (4), and (5).

Comparison of the figures for coagulated products under **A** and **B** will show that of the total number of bodies in the aqueous and saline extracts of ligament only one can be completely separated by saturation with $MgSO_4$ or by half-saturation with $(NH_4)_2SO_4$, viz. — the one which separates at or about 40° C. (1). All the other substances are to be found in the filtrates from the precipitates formed on addition of $MgSO_4$ to saturation or of $(NH_4)_2SO_4$ to half-saturation.

C. *Continuous fractional precipitation of aqueous and $MgSO_4$ extracts with $MgSO_4$ and $(NH_4)_2SO_4$ in substance, and with saturated solution of $(NH_4)_2SO_4$.*

We have attempted to make a closer differentiation of the coagulable proteïds contained in ligament extracts than was possible by the methods under A and B. The extracts for these experiments were made by the method outlined on page 119. The extract to be tested was accurately neutralized. To a measured portion of it was added, a few grams at a time, the salt used for precipitative purposes. As soon as a flocculent precipitate had formed it was filtered off and washed with a solution of the precipitating substance of a strength equivalent to that of the mother liquid. To the filtrate, plus enough of the washings to make it up to the original volume, were again added weighed quantities of the salt. When a second precipitate had appeared it was treated in a manner exactly similar to that to which the first was subjected. This process was continued till the solution was saturated or until all proteïd had been removed. The precipitates were then dissolved in a small quantity of water with the aid of the saline matter adhering to them, and subjected to fractional coagulation in the usual manner. The results for the globulins are appended:

(a) 5% $MgSO_4$ extract. Volume 100 c.c. Solid substance used to precipitate was $MgSO_4$.

Results: Precip. I. 5 gms. = turbidity; 25 gms. = heavy flocculent precipitate.

Precip. II. 35 gms. = turbidity; 53 gms. to saturation = flocks.

Coagulations: Solution of Precip. I. 44°–47° C. (1)

Solution of Precip. II. 64° C. (3)

Nothing more from either I or II on boiling.

(b) Aqueous extract was treated with an equal volume of saturated solution of $(NH_4)_2SO_4$. The resultant precipitate (globulin?) was dis-

solved in water and treated with a very slight amount of dilute acetic acid. A precipitate corresponding to separation No. 1 in the coagulation series formed as a result of this treatment. The same was filtered off and the filtrate carefully neutralized. This neutral filtrate was used below in (c) and (d).

(c) Neutral filtrate obtained in (b). Volume 100 c.c. Precipitating salt $MgSO_4$.

Results: Precip. I. 20 gms. = turbidity; 42 gms. = flocculent precipitate.

Precip. II. 43 gms. = turbidity; 50 gms. = flocculent precipitate.

Precip. III. 56 gms. = turbidity; 63 gms. = flocculent precipitate.

Precip. IV. 73 gms. = turbidity; saturation + acid = final precipitate.

Coagulations: Solution of Precip. I. 51° - 58° C. (2);
 65° - 67° C. (3).
 Solution of Precip. II. 68° - 69° C. (3).
 Solution of Precip. III. 66° - 67° C. (3).
 Solution of Precip. IV. 54° - 56° C. (2);
 67° - 70° C. (3).

If this method gives evidence of the presence of distinct proteids in a solution, as various observers believe, we seem to have dealt in this instance with at least two substances.

(d) Neutral filtrate obtained in (b), previously used in (c). Volume 100 c.c. For precipitation purposes, instead of $MgSO_4$ in substance, saturated solution of $(NH_4)_2SO_4$ was used.¹

Results: Precip. I. 100 c.c. of the original filtrate + 65 c.c. saturated solution of $(NH_4)_2SO_4$ = turbidity; on standing, flocks separate.

Precip. II. 100 c.c. original filtrate + 82 c.c. saturated solution $(NH_4)_2SO_4$ = precipitate.

Precip. III. 100 c.c. original filtrate + 91 c.c. saturated solution $(NH_4)_2SO_4$ = precipitate.

Precip. IV. 100 c.c. original filtrate + 100 c.c. saturated solution $(NH_4)_2SO_4$ = precipitate.

¹ In this series addition of $(NH_4)_2SO_4$ solution was made cautiously until turbidity began. On standing, the precipitate became flocculent. This was filtered off and the total volume made up to the original amount with an appropriate quantity of $(NH_4)_2SO_4$ solution of equal strength. This fluid was then treated carefully with more saturated solution until further precipitation occurred. The intervals between initial turbidities were quite marked, though less so than in the experiments under (a) and (c).

At this point, according to the theoretical differences between albumins and globulins, all the globulin-like substance ought to have been removed from the solution (half-saturated with $(\text{NH}_4)_2\text{SO}_4$). The addition of larger proportions of $(\text{NH}_4)_2\text{SO}_4$ to the solution gave further precipitates as follows :

Results (continued) :

- Precip. V. 100 c.c. original filtrate + 125 c.c. saturated solution $(\text{NH}_4)_2\text{SO}_4$ = precipitate.
- Precip. VI. 100 c.c. original filtrate + 142 c.c. saturated solution $(\text{NH}_4)_2\text{SO}_4$ = precipitate.
- Precip. VII. 100 c.c. original filtrate + 150 c.c. saturated solution $(\text{NH}_4)_2\text{SO}_4$ = precipitate.
- No further precipitation was obtainable, either with more $(\text{NH}_4)_2\text{SO}_4$, by the addition of acid or on boiling.
- Coagulations : Solution of Precip. I. $61^\circ-63^\circ$ C. (3)
Solution of Precip. II. $66^\circ-67^\circ$ C. (3)
Solution of Precip. III. $66^\circ-67^\circ$ C. (3)
Solution of Precip. IV. $56^\circ-58^\circ$ C. (2)
Solution of Precip. V. $53^\circ-59^\circ$ C. (2)
Solution of Precip. VI. $56^\circ-57^\circ$ C. (2);
 $64^\circ-68^\circ$ C. (3)
Solution of Precip. VII. $58^\circ-60^\circ$ C. (2);
 $67^\circ-70^\circ$ C. (3)

A study of the results under **C** shows that among the substances extractable from ligament by MgSO_4 solution or water is one which is precipitable from MgSO_4 extract by addition of 25 gms. of MgSO_4 to 100 c.c. of extract, or from a dilute saline solution by trace of acid at about 40° C. (1), or by larger amount of acid at room temperature.

A second substance, presumably a globulin, was precipitated by 53 gms. of MgSO_4 from MgSO_4 extracts and coagulated at about 65° C. (3). This substance, apparently, may also be separated from the aqueous solution of the precipitate obtained on half-saturation of aqueous extract with $(\text{NH}_4)_2\text{SO}_4$ or by the addition of MgSO_4 in quantities varying from 20 gms. per 100 c.c. of extract to the saturation quantity for the same volume. It was also obtained from such solution by additions of from 65 to 150 c.c. of saturated solution of $(\text{NH}_4)_2\text{SO}_4$ per 100 c.c. of proteid solution.¹

¹ This substance appears to be comparable to fibrinoglobulin, also to serum albumin. See COHNHEIM, *Loc. cit.*, pp. 143 and 161.

There is apparently another substance, separating at about 56° C. (2) and precipitable from solution in water by 42 gms. of MgSO_4 per 100 c.c. of proteid solution; also by from 73 gms. of MgSO_4 to the saturation equivalent for the same volume of proteid fluid. It is precipitated also by 100–150 c.c. of saturated solution of $(\text{NH}_4)_2\text{SO}_4$ per 100 c.c. of proteid extract. From its coagulation temperature it would seem to be comparable to fibrinogen.¹

The two other proteids in the extracts of **A** and **B** coagulated at about 75° C. (4) and 82° C. (5). Like (1) they occurred in only very small amounts. They correspond to the albumins ("serins") found in ox-serum, by Halliburton, coagulating at 77° C. and 84° C. respectively.²

Of these five products the one separating at the lowest temperature is not a coagulum. (See page 125). The proteid which separates at about 65° C. is also peculiar. It begins to separate from its solution when 82 c.c. of saturated solution of $(\text{NH}_4)_2\text{SO}_4$ are added per 100 c.c. of its own, and is not completely precipitated till the amount of admixed saturated $(\text{NH}_4)_2\text{SO}_4$ solution reaches 150 c.c. per 100 c.c. of proteid fluid. According to the generally accepted observations of Hofmeister, Kauder, and others on the proteids of serum, globulins are precipitated by the addition of 92 c.c. of saturated $(\text{NH}_4)_2\text{SO}_4$ solution to 100 c.c. of proteid fluid, whereas the precipitation of albumins does not begin until more than 128 c.c. have been added. This substance, in respect to its behavior toward ammonium sulphate partakes, therefore, of the characteristics of both globulin and albumin.³ The fractional precipitation and coagulation methods are not of sufficient definiteness in result for us to contend that the precipitates we have obtained are not mixtures of albumins and globulins.⁴

¹ Compare with the serum albumins studied by MICHEL: *Jahresbericht der Thier-Chemie*, 1895, xxv, p. 11. See also HAMMARSTEN: *Lehrbuch der physiologischen Chemie*, 1899, p. 132.

² HALLIBURTON: *Jahresbericht der Thier-Chemie*, 1884, xiv, p. 126; 1886, xvi, p. 344. The first of these also corresponds to serum globulin in coagulation temperature, but serum globulin is precipitated on half-saturation with ammonium sulphate, the above bodies were not.

³ This solution was completely saturated. Our analytic results showed the presence of 53.67 per cent of $(\text{NH}_4)_2\text{SO}_4$. KAUDER'S results for the same were 52.42 per cent. See, *Archiv für experimentelle Pathologie und Pharmakologie*, 1886, xx, p. 411.

⁴ The boundary line between albumins and globulins, never very definitely marked, has been growing less and less distinct. See STARKE: *Zeitschrift für Biologie*, 1900, xl, p. 494.

These various proteïds do not appear to come wholly from residues of serum — the quantity in which they may be obtained seems to be too great to permit of such an assumption. We believe, however, that it is impossible to remove every trace of serum from such a tissue without modifying the chemical character of the contents.

We are not unmindful, in considering the character of these products, of the known influences exerted on the coagulation temperature of proteïds by the reaction of the fluid, its degree of acidity, the proportion and character of saline matter in solution, rapidity of heating, presence of foreign soluble organic bodies, concentration, etc. All of these conditioning factors were carefully governed, however, to prevent erroneous deductions.

IV. NUCLEOPROTEID.

We believe that the substance separating at 40° C. (1) in nearly all of the preceding coagulation experiments is, in great part at least, nucleoproteid. That the substance was directly precipitated at that temperature, not coagulated, was apparent from the fact that when the various extracts employed were treated with a slight amount of acetic acid and then allowed to stand over night, a light flocculent precipitate settled out. After its removal only precipitate (2) and the higher bodies previously obtained separated from the filtrate on heating. That this acid precipitate was not a true coagulum was further evidenced by the fact that it dissolved readily in 0.5 per cent sodium carbonate, from which solution it was easily precipitated by slight excess of dilute acid.

When 100 c.c. of the aqueous extract of ligamentum nuchæ was treated with 0.5 c.c. of 36 per cent acetic acid, a bulky flocculent precipitate was obtained which dissolved easily in dilute alkali. This precipitate was not coagulable either in acid or alkaline fluid and after fusion with alkali gave a good phosphate reaction with molybdc solution. Further, after a very large quantity of the aqueous extract of the tissue had been evaporated to a small bulk on the water bath and the heavy precipitate of coagulated proteïd filtered off, the viscid filtrate gave an abundant precipitate on the addition of but a few drops of 36 per cent acetic acid. This precipitate dissolved readily in 5 per cent sodium chloride and was reprecipitated on saturation with the same substance. Its solutions would not coagulate in any

medium. The substance so obtained contained phosphorus in organic combination.

Various proteids are precipitable from their solutions on acidification. Those of special interest for us in this connection are glucoproteids, nucleoproteids and globulins. When carefully tested as to its solubility in dilute acid the substance obtained in these experiments was found to be precipitated by moderate excess of 0.2 per cent acetic or hydrochloric acid. Serum globulin and fibrinogen may be precipitated from their solutions by minute quantities of acids. They are readily soluble, however, in moderate excess of the acids just mentioned — in the proportion which was favorable to the precipitation of the substance above. The same would be true of small quantities of albuminate. Furthermore, as has already been pointed out, our acid precipitate, unlike the other bodies just mentioned, contains phosphorus and was non-coagulable.

Connective tissue mucoïd has much the same characteristics as this substance. Mucoïd, however, is a phosphorus-free glucoproteid, and on boiling with acids yields reducing substances. When our acid-precipitated product was boiled for several hours with 2 per cent hydrochloric acid, the fluid neutralized, and tested with Fehling's solution, only a trace of a reduction occurred. Our substance could not, therefore, be mucoïd, although the slight reduction suggests that a trace of mucoïd might have been admixed with it.¹

A special preparation of this acid precipitate was made as follows: Aqueous extract of 8 kilos of ligamentum nuchæ was obtained as in the method given on page 119, and to it was added 0.5 c.c. of 36 per cent acetic acid per 100 c.c. of extract. The flocculent precipitate which formed on standing was dissolved in 0.3 per cent solution of sodium carbonate. This fluid was neutralized and then acetic acid added until precipitation occurred. 1 to 1.3 c.c. of 36 per cent acetic acid was required per 100 c.c. of fluid to effect the same — a total acidity which would have dissolved globulins readily. This precipitate was again dissolved and was reprecipitated in the same manner, after which it was washed free of acid and dehydrated, and purified as usual in alcohol and ether. 4.5 gms. (0.056 per cent of the fresh tissue) were obtained.

¹ Aqueous extracts of the tissue are in reality extracts in dilute saline solution, the salts of the tissue contributing their solvent power. Mucoïd is somewhat soluble in such extracts. Possibly, however, the reducing substance was derived from the nucleoproteid.

Analysis of this product gave the following results for percentage content of phosphorus in the ash-free substance: ¹ (1) 0.49, (2) 0.45; average, 0.47.

These figures for phosphorus content are somewhat lower than they are for most nucleoproteïds. Mucoïd impurity, as we have already suggested, may have partially accounted for this lowering of phosphorus content.

That the substance was not a "cell nucleo-albumin" ² was shown by the results of the following experiment: About 2 gms. of the substance was decomposed with acid in the usual way and a test made for nucleïn bases among the cleavage products, with positive result. "Ammoniacal silver solution" gave the typical flocculent brown precipitate. No precipitate formed, on cooling, in the solution of this precipitate in nitric acid (1.1 specific gravity). On neutralizing however, and rendering slightly alkaline with ammonia, xanthin silver in quantity practically equal to the original precipitate was obtained. Tested with Fischer's modification of Weidel's reaction this precipitate gave positive results for xanthin. ³

That the substance is nucleoproteïd, or at least contains a large proportion of this compound albuminous substance, we feel confident. Although we are not accustomed to associate nucleoproteïds with any but glandular tissues, the fact remains that nucleoproteïds are to be found in every cell, and therefore must exist in every tissue. Pekelharing ⁴ has lately found that 0.37 per cent of fresh muscle — a comparable tissue in this connection — consists of a nucleoproteïd containing 0.7 per cent of phosphorus.

V. COLLAGEN (GELATIN).

All forms of connective tissues contain collagenous fibres. Eulenberg ⁵ first demonstrated the presence of collagen in ligamentum nuchæ by obtaining gelatin from it. Recently the quantity was

¹ The merest trace of phosphorus was present in the ash, 4-6 per cent of the total quantity. This was deducted from the figures for total phosphorus. The ash amounted to 0.75 and 0.89 per cent — average, 0.82. 0.5-0.6 gram of substance was used in each of the determinations by the usual methods.

² See COHNHEIM: *Loc. cit.*, pp. 181-183.

³ FISCHER: *Berichte der deutschen chemischen Gesellschaft*, 1897, xxx, p. 2236.

⁴ PEKELHARING: *Zeitschrift für physiologische Chemie*, 1896-97, xxii, p. 245. See also, KOSSEL, *Ibid.*, 1882-83, vii, p. 7.

⁵ EULENBERG: See SCHULTZE, *Annalen der Chemie und Pharmacie*, 1894, lxxi, p. 277.

accurately determined and was found to be 7.23 per cent of the fresh and 17.04 per cent of the dry tissue — equal, roughly, to one-fourth the amount of contained elastin.¹

The presence of so much elastin in ligament makes it impracticable to separate the collagen as such, by the Ewald and Kühne process of digestion with trypsin in alkaline medium.² In order to obtain some idea of its character, however, we transformed it into gelatin and then separated and studied the latter.

Preparation of ligament gelatin. — After the cleaned ligament had been put through a meat chopper the hash was thoroughly washed in running water and later thoroughly extracted in half-saturated lime-water. After the alkali had been completely removed with water, the residual tissue was boiled for a short time in distilled water. The filtrate was concentrated somewhat on the water bath and then the gelatin precipitated from it by pouring it into a large excess of alcohol. The typical fibrous precipitate of gelatin was obtained in this way. This was redissolved in water and reprecipitated in alcohol several times, then dehydrated and completely purified in alcohol-ether.

Elementary composition. — The following data were obtained in elementary analysis of one preparation by the methods previously used in this connection for elastin.

Carbon and Hydrogen. 0.2324 gm. substance gave 0.1372 gm. H₂O = 6.56 per cent H; 0.3773 gm. substance gave 0.6860 gm. CO₂ and 0.2250 gm. H₂O = 49.59 per cent C and 6.63 per cent H; 0.3681 gm. substance gave 0.6705 gm. CO₂ and 0.2194 gm. H₂O = 49.68 per cent C and 6.62 per cent H.

Nitrogen. 0.2867 gm. substance gave 0.0501 gm. N. = 17.47 per cent N; 0.3578 gm. substance gave 0.0634 gm. N = 17.72 per cent N.

Sulphur. 0.7370 gm. substance gave 0.03050 gm. BaSO₄ = 0.568 per cent S; 0.9417 gm. substance gave 0.03734 gm. BaSO₄ = 0.544 per cent S.

Ash. 0.3503 gm. substance gave 0.0058 gm. Ash = 1.65 per cent Ash; 0.2746 gm. substance gave 0.0047 gm. Ash = 1.71 per cent Ash.

¹ VANDEGRIFT and GIES: *Loc. cit.*

² EWALD und KÜHNE: *Jahresbericht der Thier-Chemie*, 1877, vii, p. 281.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.¹

								Average.
C	50.44	50.53	50.49
H	6.67	6.74	6.73	6.71
N	17.77	18.02	17.90
S	0.58	0.56	0.57
O	24.33

The following summary of percentage elementary composition shows the relation of ligament gelatin to bone and tendon gelatin and to purified commercial gelatin, the latter consisting of a mixture of gelatins from bone and other connective tissues :

	C	H	N	S	O
Ligament gelatin . . .	50.49	6.71	17.90	0.57	24.33
Tendon gelatin ² . . .	50.11	6.56	17.81	0.26	25.24
Commercial gelatin ³ . .	49.38	6.81	17.97	0.71	25.13
Bone gelatin ⁴	50.40	6.64	18.34	24.64

Recent studies of the composition of connective tissues indicate that there are perhaps three groups of collagens. These appear to be characterized by appreciable differences in elementary composition. Thus the collagens in tendon⁵ and bone⁶ yield gelatins containing approximately 18 per cent of nitrogen. Corneal collagen⁷ contains about 17 per cent of nitrogen. Cartilage collagen yields a gelatin containing little more than 16 per cent of nitrogen.⁸ Our results in this connection indicate that the collagen of ligamentum nuchæ is essentially the same as that in tendon and bone.

Heat of combustion. — In two determinations of the heat of combustion of ligament gelatin we obtained an average of 5276 small calories (5261, 5291) as the combustion equivalent. These figures accord very well with those previously obtained by other observers for different gelatins, as will be seen from the following summary,

¹ The sulphur of the ash amounted to 0.17 per cent of the dry proteïd. This was not subtracted from the above figures — much of it doubtless arose during incineration.

² VAN NAME: Journal of experimental medicine, 1897, ii, p. 124.

³ CHITTENDEN and SOLLEY: See CHITTENDEN, Digestive proteolysis, 1894, p. 32.

⁴ MULDER: See HOPPE-SEYLER, Physiologische Chemie, 1881, p. 100.

⁵ VAN NAME: *Loc. cit.*

⁶ HOPPE-SEYLER: Physiologische Chemie, 1881, p. 100.

⁷ C. TH. MÖRNER: Zeitschrift für physiologische Chemie, 1894, xviii, p. 224.

⁸ C. TH. MÖRNER: Jahresbericht der Thier-Chemie, 1888, xviii, p. 221.

which gives also the combustion equivalents of two proteids having equivalents among the very lowest for albuminous substances:

Substance. Dried at 100°-110° C.	Heat of combustion. Small calories.		Percentage composition.	
	Per gram.	For substance containing 1 gm. of carbon.	Carbon.	Oxygen.
Ligament gelatin . . .	5276	10450	50.49	24.33
Fish gelatin ¹	5242	10800	48.53	25.54
Commercial gelatin ² .	5270
Fibrin pepton ³	5299	10577	50.10	25.79
Tendomucoid ⁴	5003	10415	48.04	30.62

¹ BERTHELOT ET ANDRE: Centralblatt für Physiologie, 1890, iv, p. 611.
² ATWATER: Report of the Storrs (Conn.) Agricultural Experiment Station, 1899, p. 92.
³ STOHMANN UND LANGBEIN: Journal für praktische Chemie, neue Folge, 1891, xliii, p. 375.
⁴ CUTTER and GIES: *Loc. cit.*

CRYSTALLINE EXTRACTIVES.

In our first report of this work¹ we called attention to the fact that ox ligament contains an appreciable quantity of crystalline extractives. The only crystalline substance whose identity we had definitely determined at that time was creatin, although the general method of detecting nuclein bases had shown the presence of one or more of these bodies also. A continuation of this work has given us more definite results.

Preparation of extract. — The "extract" containing the crystalline substances was obtained in the following manner: 15-20 pounds of ligamenta nuchæ, which were perfectly fresh and which had only mere traces of blood in them, were finely minced in a meat-chopper. The hash was extracted in a moderate amount of water at 40° C. for 12-24 hours — ether or powdered thymol preventing putrefaction. The extract was strained through cloth, then heated to boiling, after which sufficient acid was added to completely separate coagulable

¹ RICHARDS and GIES: *Loc. cit.*

proteid and contained mucoïd.¹ That practically no hæmoglobin was present was shown by the fact that the precipitate at this point was entirely white.

The slightly acid filtrate was then neutralized and evaporated on a water bath to a thin syrup. This concentrated extract had all of the physical properties of ordinary "meat extract." It contained traces of proteïd (derived gelatin and albuminate probably) but no reducing substance could be detected in it.² Chloride and phosphate of sodium and calcium were present in comparative abundance. Sulphate was also detected.

Creatin. — The concentrated extract was diluted with several volumes of water and treated with lead acetate for the removal of inorganic radicles. The excess of lead was precipitated with hydrogen sulphide and the filtrate evaporated to a thin syrup on the water bath. On standing thirty-six hours, typical crystals of creatin formed in good quantity. After filtering and evaporating to greater concentration occasionally a new but smaller crop of crystals was obtained each time.

The fluid concentrated in this way was treated with moderate excess of 90 per cent alcohol and the solid matter tested, together with the separated crystals, for creatin. The crystals and the alcohol precipitate were readily soluble in water. On hydration with acid in the usual way, the fluid gave the typical crystals of creatinin zinc chloride with an alcoholic solution of zinc chloride, and also responded to Weyl's reaction.

Hypoxanthin. — The alcoholic filtrate from the precipitated creatin was next evaporated nearly to dryness to get rid of alcohol, a little water added, the fluid made alkaline, filtered, and then treated with an appropriate quantity of "ammoniacal silver solution." The resultant heavy brown precipitate of nucleïn bases, on decomposition with hot nitric acid of 1.1 specific gravity, gave a yellowish filtrate, which, on cooling, deposited a large proportion of crystalline substance, mostly needles of hypoxanthin silver nitrate. The mixture was allowed to

¹ A slight amount of mucoïd is always contained in the aqueous extract of ligament. The salts present in the extract exert solvent action on it.

² Leucin and tyrosin were detected at this point in microscopic examination of one sample of our extracts. We have assumed that these were formed from proteïd by hydration in the process of heating to boiling and subsequent evaporation. Some creatinin was also detected several times. This probably arose from the creatin by hydration.

stand for twenty-four hours for complete precipitation of the crystalline matter.

The filtrate from the crystals still contained nuclein base (doubtless xanthin, which may have been formed from the hypoxanthin), as was shown by the brown precipitate which appeared in small quantity when the fluid was again rendered slightly alkaline.

The crystalline precipitate containing hypoxanthin silver nitrate was decomposed in a warm mixture of water and ammonium sulphide on the water bath, the mixture filtered hot, concentrated on a water bath, there saturated with ammonia and again filtered hot. A comparatively large amount of hypoxanthin could be detected in this filtrate.

Guanin. — The substance insoluble in the ammoniacal fluid yielded beautiful crystals of guanin. These were obtained by Horbaczewski's¹ method of solution in alkali, and treatment with alcohol and acetic acid. The crystals were large and they very closely resembled those of creatinin zinc chloride.

The bulk of the crystalline extractives consisted of creatin, hypoxanthin and guanin. We were unable to prove the presence of adenin and carnin, although we occasionally obtained results by the customary qualitative methods indicating the presence of these substances. No tests were made for other extractives.²

It is interesting to note in this connection that guanin has been found to occur in the ligaments of pigs with guanin gout.³

The amount of nuclein bases found in these extracts was too great to allow of the assumption that they were derived from the small quantity of blood and lymph remaining in the tissue when the separation was begun. Normal blood contains only traces of nuclein bases⁴ and the tissue itself contained at the outset only traces of blood. In tissues, such as muscle, which contain relatively few nuclei, nuclein bases are found in the uncombined state, and in this condition undoubtedly represent late stages in the catabolism of nuclear proteids. Our data show a similar catabolism in ligament, thus leading us to a conclusion which would hardly be suggested by the "passive mechanical functions" of the tissue — a conclusion

¹ HORBACZEWSKI : *Zeitschrift für physiologische Chemie*, 1897, xxiii, p. 229.

² We obtained essentially the same results as those above in continuance of the work on tendon extract already referred to by BUERGER and GIES : *Loc. cit.*

³ HAMMARSTEN : *Lehrbuch der physiologischen Chemie*, 1899, p. 119.

⁴ KOSSEL : *Zeitschrift für physiologische Chemie*, 1882-83, vii, p. 22.

which harmonizes, however, with the fact that this tissue contains a variety of substances which represent intermediate stages of chemical differentiation.

SUMMARY OF CONCLUSIONS.

1. By improved method of preparation several samples of ligament elastin were made, having the following average percentage composition:

C	H	N	S	O
54.14	7.33	16.87	0.14	21.52

All of these preparations contained sulphur. None of it could be split off as sulphide on boiling with caustic alkali.

Only very small proportions of elastin nitrogen could be split off in the form of ammonia and hexone bases on decomposition with acid. Arginin, lysin, and histidin have been identified among the basic bodies separated in this way.

Elastin is not a "fat-proteid compound." No extractive material could be separated from our analyzed preparations by Nerking's process.

Our purified powdered elastin readily digested in pepsin-hydrochloric acid. Elastoses and true peptone were formed, proto-elastose predominating in quantity. The amount of true peptone formed was comparatively small even after long periods of favorable contact of the elastin and elastoses with the enzyme in acid solution, showing that elastoses are particularly resistant to progressive zymolysis.

The average combustion equivalent of four preparations of elastin, determinations in duplicate, was 5925 small calories.

2. Ligament contains mucoïd having the general qualities of other connective tissue glucoproteïds. Analysis of five preparations gave the following average percentage results:

N	S	S as SO ₂
13.44	1.61	1.06

3. Extracts of ligament contain proteïd coagulating at 56° C., 65° C., 75° C., and 82° C. Although these figures indicate identity with some of the albuminous substances of the blood, the coagulable proteïds of our extracts do not appear to have arisen wholly from contained serum.

4. A slight amount of nucleoproteid is contained in ligament and was detected in aqueous and saline extracts.

5. The gelatin obtained from ligament had the following percentage composition:

C	H	N	S	O
50.49	6.71	17.90	0.57	24.33

These results indicate that the collagen of ligament is identical with that of bone and tendon.

The heat of combustion of ligament gelatin was found to be equal to 5276 small calories.

6. Among the crystalline extractives obtainable from ligamentum nuchæ were creatin, hypoxanthin, and guanin.

ON PHOSPHATE METABOLISM.

BY OTTO FOLIN AND PHILIP A. SHAFFER.

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THE investigations here described were begun as a general chemical study of the metabolism of one of the patients in the McLean Hospital for the insane. The results of these investigations will be published as a whole elsewhere, but our observations on the phosphoric acid metabolism of the patient would seem to be of sufficient general importance to warrant publication in a journal devoted to general physiology.

Except in cases of advanced hunger where there is a rapid wasting away of bone tissue, it seems to be regarded as a settled fact that the elimination of phosphoric acid through the kidneys is without any tangible significance whatever as regards the body metabolism. The reason why this is so, is of course the enormous differences in the phosphoric acid contents of the food and the equally great variations in the extent to which the different phosphoric acid combinations of the food are absorbed from the intestinal tract. The phosphoric acid values involved in the building up or the breaking down of even considerable amounts of living tissue other than bone are so small when compared with the above phosphoric acid values of the food that it is only by means of very prolonged and carefully executed experiments, or because of some quite unusual conditions, that it is possible to get any certain information in regard to the phosphoric acid metabolism involved in the activities of living tissues.¹

The subject of these investigations was diagnosed by Dr. A. Hoch as a case of manic depressive insanity. Dr. Hoch gives us the following summary of his description of the case :

“The patient’s condition alternated with absolute regularity from day to day, being one day little removed from the normal, while quite disturbed on the

¹ Fortschritte der Medicin, 1898, p. 1, contains a review by PETER BERGELL of about eighty different papers on various phases of the question of phosphoric acid metabolism. A further review here of the literature bearing on this subject seems therefore unnecessary.

other. These two states presented the following pictures: On the disturbed days the patient was often restless, walking aimlessly about with short steps, talking in the manner described below, or he handled things about him in an aimless way or took off his clothes, etc. His talk indicated that his ability to guide voluntarily his train of thought was much diminished. The different thoughts he uttered were but superficially connected, or his talk was constantly deflected by external happenings (great distractibility). Sometimes when less voluble he repeated the same question many times in a senseless manner or made remarks which were superficially suggested but which had no internal connection with what was spoken or what the circumstances demanded. Consequently when the abnormality was least marked, the main feature was a certain irrelevancy in the patient's remarks. The facial expression was almost always dull and immobile, the mood rather apathetic, but at times an irritability and occasionally a striking exhilaration were noted. He never lost his bearings, probably because the good days were constant correctives, but he had no clear appreciation of things or occurrences about him and on the good days he remembered poorly the sequence of events of the previous bad day. On the good days he often varied but little from the normal, but appeared somewhat dull without perfect appreciation of his condition and at times an irrelevant remark betrayed slight traits of the bad days."¹

This condition, which has now lasted uninterruptedly for several months, seemed to offer an unusual opportunity for trying to discover some alternation in the metabolism of the patient corresponding to the alternation in the mental symptoms. Any sufficiently characteristic alternation in the patient's metabolism was deemed important, because we can scarcely be said to have as yet the proof of the existence of any abnormal metabolism whatever which is characteristically associated with any of the mental diseases.

The existence of an unmistakable periodicity in the elimination of phosphoric acid through the kidneys corresponding to the periodicity in the mental condition of this patient is, we think, proved by the analyses and experiments recorded below.

EXPERIMENTAL PART.

Preliminary analyses. — Owing to the difficulty so frequently met with in the study of the insane, namely, that of obtaining all the urine, our first series of experiments (from October 8 to October 16, 1901) is very incomplete. Each day of this series was divided into

¹ DR. HOCH'S psychiatric studies and our own general metabolism experiments on this case will be published later elsewhere.

three periods (from 6.30 A. M. to 2 P. M., from 2 P. M. to 9 P. M., and from 9 P. M. to 6.30 the following morning), and the urine of each period was separately analyzed. A comparative study of the different periods showed at once a remarkable difference in the phosphate elimination of the two sets of days. October 11 and October 14, the former representing a "good" day, the latter a "nervous" day, are the most completely recorded days of this series.¹

TABLE I.

Date.	Time.	Volume. c.c.	Sp. gr. ¹ 1.0—	N ₂ . Gm.	P ₂ O ₅ . Gm.	100 N ₂ : P ₂ O ₅ .
Oct. 11	6.30 A. M.—2.00 P. M.	240	26	4.22	.77	18.3
" 11	2.00 P. M.—9.00 P. M.	280	28	5.56	.89	16.0
" 11	9.00 P. M.—6.30 A. M.	715	20	8.53	1.59	18.8
Totals		1245	..	18.31	3.25	17.7
Oct. 14	6.30 A. M.—2.00 P. M.	400	25	4.40	1.22	27.7
" 14	2.00 P. M.—9.00 P. M.	280	31	4.54	1.64	36.1
" 14	9.00 P. M.—6.30 A. M.	340	24	5.39	1.37	25.4
Totals		1020	..	14.33	4.23	30.0

At the end of the above series of experiments the patient was given a special nurse, and it is largely due to the intelligence and untiring efforts of this nurse, Mr. Max Huwyler, that we have been able to continue this work uninterruptedly, and to our own satisfaction for a period of over three months.

The second series of experiments extending from October 21 to November 2 is recorded in Table II. The following analytical methods have been used exclusively: the phosphates were de-

¹ Throughout this paper the two sets of days of the patient are referred to as the "good" days and the "nervous" days, and it will be noted that in any given month the "good" days come on the odd dates and the "nervous" days on the even dates or vice versa. Thus through October the good days come on the odd dates, while through November and December they come on the even dates. The day corresponding to any given date is counted from 6.30 in the morning till 6.30 on the following morning.

terminated by titration with uranium salt solutions which had been standardized against a solution of pure crystallized mono-potassium phosphate. The latter salt was prepared from pure phosphoric acid and potassium hydrate (Kahlbaums). Potassium ferrocyanide was used as indicator. The nitrogen was determined according to Kjeldahl, and the sulphates were determined gravimetrically as barium-sulphate. (See the following paper, page 152.)

TABLE II.

Date.	Time.	Vol. c.c.	Sp. gr. 1.0—	N ₂ . Gm.	P ₂ O ₅ . Gm.	SO ₃ . Gm.	100 N ₂ :		100 SO ₃ : P ₂ O ₅ .
							P ₂ O ₅ .	SO ₃ .	
Oct. 21	6.30 A. M.—2.00 P. M.	325	27.5	4.65	0.72	1.19	15.5	25.5	60.5
“ 21	2.00 P. M.—9.00 P. M.	160	27.0	2.45	0.42	0.65	17.1	26.5	64.6
“ 21	9.00 P. M.—6.30 A. M.	625	24.0	8.37	1.26	1.70	15.0	20.3	74.1
Totals		1110	15.47	2.40	3.54	15.5	22.9	68.0
Oct. 22	6.30 A. M.—2.00 P. M.	325	23.0	3.10	0.56	0.42	18.1	13.5	133.3
“ 22	2.00 P. M.—9.00 P. M.	355	31.0	5.00	1.26	1.27	25.2	22.4	99.2
“ 22	9.00 P. M.—6.30 A. M.	305	28.0	5.30	1.09	1.21	20.6	20.6	90.0
Totals		985	13.40	2.91	2.90	21.7	21.7	100.0
Oct. 23	6.30 A. M.—2.00 P. M.	225	26.0	3.52	0.49	0.60	13.9	17.0	81.7
“ 23	2.00 P. M.—9.00 P. M.	225	32.0	4.43	0.59	1.00	13.3	22.6	59.0
“ 23	9.00 P. M.—6.30 A. M.	720	20.0	7.92	1.18	1.21	14.9	16.5	97.5
Totals		1170	15.87	2.26	2.81	14.2	17.7	80.4
Oct. 24	6.30 A. M.—2.00 P. M.	600	13.0	2.97	0.44	0.29	14.8	9.8	151.6
“ 24	2.00 P. M.—9.00 P. M.	135	25.0	1.37	0.27	0.20	19.7	14.6	135.0
“ 24	9.00 P. M.—6.30 A. M.	700	14.0	5.16	1.07	0.73	20.7	14.1	146.0
Totals		1435	9.49	1.78	1.22	18.7	12.9	146.0

TABLE II (continued).

Date.	Time.	Vol. c.c.	Sp. gr. 1.0—	N ₂ . Gm.	P ₂ O ₅ . Gm.	SO ₃ . Gm.	100 N ₂ :		100SO ₃ . P ₂ O ₅ .
							P ₂ O ₅ .	SO ₃ .	
Oct. 25	6.30 A. M.—2.00 P. M.	310	20.0	3.31	0.51	0.61	15.4	18.4	83.6
“ 25	2.00 P. M.—9.00 P. M.	325	25.0	4.10	0.52	0.85	12.7	20.7	61.2
“ 25	9.00 P. M.—6.30 A. M.	800	19.0	7.20	1.08	1.09	15.0	15.1	100.0
	Totals	1435	14.61	2.11	2.55	14.4	17.3	83.0
Oct. 26	6.30 A. M.—2.00 P. M.	310	23.0	3.18	0.48	0.51	15.1	16.0	94.1
“ 26	2.00 P. M.—9.00 P. M.	225	29.0	2.96	0.63	0.58	21.3	19.6	108.6
“ 26	9.00 P. M.—6.30 A. M.	790	08.5	4.03	0.79	0.30	19.6	7.4	256.3
	Totals	1325	10.17	1.90	1.39	18.6	13.7	137.0
Oct. 27	6.30 A. M.—2.00 P. M.	220	22.0	1.83	0.27	0.30	14.7	16.4	90.7
“ 27	2.00 P. M.—9.00 P. M.								
“ 27	9.00 P. M.—6.30 A. M.	620	22.0	7.73	0.98	1.37	12.7	17.7	71.5
Oct. 28	6.30 A. M.—2.00 P. M.	625	17.0	3.94	0.68	0.51	17.2	12.9	133.3
“ 28	2.00 P. M.—9.00 P. M.	195	29.0	3.57	0.76	0.74	21.3	20.7	102.7
“ 28	9.00 P. M.—6.30 A. M.	425	27.5	5.33	1.33	1.30	25.0	24.4	102.3
	Totals	1245	12.84	2.77	2.55	21.5	18.3	108.5
Oct. 29	6.30 A. M.—2.00 P. M.	375	25.0	4.22	0.59	0.83	14.0	19.7	71.1
“ 29	2.00 P. M.—9.00 P. M.	340	27.0	5.16	0.57	1.12	11.0	21.7	50.9
“ 29	9.00 P. M.—6.30 A. M.	800	22.0	8.77	1.13	1.38	12.9	15.7	82.0
	Totals	1515	18.15	2.29	3.33	12.6	18.3	69.0

TABLE II (concluded).

Date.	Time.	Vol. c.c.	Sp. gr. 1.0—	N ₂ . Gm.	P ₂ O ₅ . Gm.	SO ₃ . Gm.	100 N ₂ :		100 SO ₃ : P ₂ O ₅ .
							P ₂ O ₅ .	SO ₃ .	
Oct. 30	6.30 A. M.—2.00 P. M.	450	23.0	4.23	0.67	0.68	15.8	16.0	98.5
“ 30	2.00 P. M.—9.00 P. M.	340	26.0	4.40	0.98	1.12	22.3	25.5	87.5
“ 30	9.00 P. M.—6.30 A. M.	595	16.0	6.19	0.86	1.08	13.9	17.4	80.0
Totals		1385	14.82	2.51	2.82	17.0	19.2	90.0
Oct. 31	6.30 A. M.—2.00 P. M.	175	29.0	3.27	0.30	0.61	9.2	18.6	49.2
“ 31	2.00 P. M.—9.00 P. M.	240	31.0	5.38	0.54	1.15	10.0	21.4	47.0
“ 31	9.00 P. M.—6.30 A. M.	890	14.0	7.48	0.87	0.99	11.6	13.2	87.8
Totals		1305	16.13	1.71	2.75	10.6	17.1	62.5
Nov. 1	6.30 A. M.—2.00 P. M.	420	20.0	3.57	0.39	0.54	10.9	15.1	72.2
“ 1	2.00 P. M.—9.00 P. M.	400	28.0	4.53	1.03	1.05	22.7	23.2	98.1
“ 1	9.00 P. M.—6.30 A. M.	250	27.0	4.07	0.72	0.72	17.7	17.7	100.0
Totals		1070	10.88	2.14	2.31	20.0	21.2	92.6
Nov. 2	6.30 A. M.—2.00 P. M.	335	24.0	3.92	0.40	0.63	10.2	16.1	63.3
“ 2	2.00 P. M.—9.00 P. M.	345	16.0	4.60	0.52	0.56	11.3	12.2	93.0
“ 2	9.00 P. M.—6.30 A. M.	790	12.0	4.79	0.82	0.74	17.1	15.5	110.8
Totals		1470	13.31	1.74	1.93	13.1 ¹	14.5	90.0 ¹

¹ Some error has evidently crept into the analyses of this day, probably into the sulphate analyses, since the N₂ : SO₃ ratio is much lower and the SO₃ : P₂O₅ ratio much higher than on any previous corresponding day. This error was not discovered until it was too late to repeat the analyses.

Table II explains itself. Each day's urine has as before been divided into three parts, and each part analyzed by itself. The fourth set of figures for each day gives the total or average of the three periods.

It will be noted that the absolute amount of P_2O_5 eliminated during this period is not always greater but may even be less on some nervous than on the adjacent good days. The absolute amount of phosphate eliminated in any given twenty-four hours would of course vary greatly with the amount and character of the food eaten; and the diet list kept by the nurse as well as our own nitrogen and sulphate determinations show that the patient almost invariably took more food on his good days. The relative amount of P_2O_5 , *i. e.*, number of parts of P_2O_5 corresponding to 100 parts of nitrogen and to 100 parts of SO_3 , is however invariably greater on the nervous days.

Phosphate metabolism of patient and control person.—The unbroken daily alternations in the relative amounts of phosphoric acid eliminated through the kidneys during the thirteen days covered by Table II could scarcely be accidental, but neither can the figures obtained be considered as rigidly proving that we have here a case of abnormal phosphate metabolism. The changes in the ratios N_2 : P_2O_5 and SO_3 : P_2O_5 might possibly be due to an increased consumption of nitrogenous material on the good days.

The only way to get an approximate control of the figures obtained from an individual living on a mixed and irregular diet would be to have one or more normal persons living under the same conditions and eating the same diet. To use more than one person as control in this case was, however, out of the question, because of the great amount of analytical work involved.¹

Mr. Huwyler, the nurse, undertook at our request to serve as control person, and for six days this program was carried out, Mr. Huwyler always sitting at the same table with the patient and eating as nearly as possible the same kind and amount of food. At the end of the sixth day the patient took such a hearty meal that both he and the nurse were somewhat upset during the night and the experiment was discontinued.

The analytical results obtained from this experiment are recorded in Table III. The odd dates are the patient's nervous days. The relative phosphoric acid values expressed by the column 100 N_2 : P_2O_5 are certainly unmistakably different in the two cases. The ratios in the case of the control person are rather high when compared with what some investigators have considered normal, *i. e.*, 17 to 20 P_2O_5

¹ Besides the determinations here given others, such as acidity, ammonia, urea and chlorides were made.

TABLE

Date.	Time.	Volume.		Sp. Gr. 1.0—.		N ₂ Gm.	
		Patient.	Control.	Patient.	Control.	Patient.	Control.
Nov. 3	6.30 A. M.—2.00 P. M.	615	255	18.0	28.0	6.87	3.86
“ 3	2.00 P. M.—9.00 P. M.	465	315	26.5	30.5	5.58	6.55
“ 3	9.00 P. M.—6.30 A. M.	580	320	20.0	3.15	4.00	5.79
	Totals . . .	1660	890	16.45	16.20
Nov. 4	6.30 A. M.—2.00 P. M.	490	160	23.0	32.0	5.13	2.81
“ 4	2.00 P. M.—9.00 P. M.	375	275	25.0	35.0	6.07	5.28
“ 4	9.00 P. M.—6.30 A. M.	950	430	17.0	34.5	9.06	9.37
	Totals . . .	1815	865	20.26	17.46
Nov. 5	6.30 A. M.—2.00 P. M.	475	365	22.5	32.5	4.01	5.62
“ 5	2.00 P. M.—9.00 P. M.	480	350	26.5	33.0	5.89	6.30
“ 5	9.00 P. M.—6.30 A. M.	570	280	15.0	36.0	5.32	6.17
	Totals . . .	1525	995	15.22	18.09
Nov. 6	6.30 A. M.—2.00 P. M.	665	275	22.0	34.0	6.89	4.98
“ 6	2.00 P. M.—9.00 P. M.	440	375	23.0	27.0	4.37	5.13
“ 6	9.00 P. M.—6.30 A. M.	850	375	20.0	29.0	7.26	6.54
	Totals . . .	1955	1025	18.52	16.65
Nov. 7	6.30 A. M.—2.00 P. M.	450	310	23.0	31.0	4.54	4.86
“ 7	2.00 P. M.—9.00 P. M.	325	700	25.0	13.0	4.14	5.59
“ 7	9.00 P. M.—6.30 A. M.	525	340	19.0	28.0	6.91	6.33
	Totals . . .	1300	1350	15.59	16.78
Nov. 8	6.30 A. M.—2.00 P. M.	300	280	25.0	30.0	4.45	4.85
“ 8	2.00 P. M.—9.00 P. M.	395	375	32.5	29.0	6.86	7.12
“ 8	9.00 P. M.—6.30 A. M.	580	590	21.0	25.0	7.55	9.79
	Totals . . .	1275	1245	18.86	21.76

III.

P ₂ O ₅ . Gm.		SO ₃ . Gm.		100 N ₂ : P ₂ O ₅ .		100 N ₂ : SO ₃ .		100 SO ₃ : P ₂ O ₅ .	
Patient.	Control.	Patient.	Control.	Patient.	Control.	Patient.	Control.	Patient.	Control.
1.50	0.64	1.21	0.84	21.8	16.6	17.6	21.8	124.0	76.2
1.83	1.52	1.29	1.34	32.8	23.2	23.1	20.5	141.8	113.4
0.80	1.50	1.05	1.15	21.5	25.8	26.2	20.0	81.9	130.4
4.19	3.66	3.55	3.33	26.0	22.6	22.2	20.5	118.0	110.0
0.80	0.52	1.00	0.48	15.6	18.5	19.5	17.1	80.0	108.3
0.67	1.11	1.22	0.89	11.0	21.0	20.1	16.8	55.0	124.7
1.23	2.04	1.00	1.66	13.5	21.7	11.0	17.7	123.0	123.0
2.70	3.67	3.22	3.03	13.3	21.0	15.9	17.3	83.9	121.1
0.78	1.15	0.59	0.82	19.4	20.4	14.7	14.6	132.2	140.2
1.51	1.25	1.15	1.38	25.6	20.0	19.5	22.0	122.0	90.6
1.12	1.36	0.92	1.03	21.0	22.0	17.3	16.7	121.7	132.0
3.41	3.76	2.66	3.23	22.4	20.8	17.4	17.8	128.2	116.0
1.05	0.82	1.12	0.89	15.2	16.5	16.3	17.9	93.7	92.1
0.62	0.93	0.86	1.01	14.2	18.1	19.7	20.0	72.1	92.1
1.10	1.40	1.16	1.19	15.1	21.4	16.0	18.2	94.8	117.7
2.77	3.15	3.14	3.09	15.0	18.9	17.0	18.6	88.2	102.0
0.98	0.89	0.73	0.99	21.6	18.3	16.1	20.4	134.3	90.0
1.25	1.01	0.85	1.00	30.2	18.1	20.5	18.0	147.0	101.0
1.81	1.28	1.10	1.34	26.2	20.2	15.9	21.1	164.5	95.5
4.04	3.18	2.68	3.33	25.9	19.8	17.2	19.9	151.0	95.5
0.56	0.83	0.85	0.91	12.6	17.1	19.1	18.8	65.9	91.2
1.18	1.33	1.16	1.57	17.2	18.6	17.0	22.0	101.0	85.0
1.17	2.14	1.13	1.48	15.5	21.8	15.0	15.2	103.5	144.6
2.91	4.30	3.14	3.96	15.4	19.8	16.7	18.2	92.7	109.0

per 100 N_2 . It will be seen, however, that while the absolute values vary considerably from day to day in both patient and control person, the relative values in the case of the latter are fairly constant, varying from 19 to 22.6 while the corresponding values in the case of the patient vary from 13.3 to 26, and show again a 48-hour periodicity as pronounced as is the periodicity in the condition of the patient. The same difference may be observed in the relative phosphate values obtained with reference to the total sulphates. The control figures are here not particularly uniform, probably on account of the varying proportions of sulphur in the food. The variations in the control (from 102 to 121) are, however, again entirely different from the alternating values shown by the patient (from 84 to 150).

One striking feature noticeable in the above table is the increase in the 100 N_2 : P_2O_5 ratio of the afternoon periods above that of the morning periods of the nervous days as against the corresponding values of the good days or as against the simultaneous values of the control person. Different investigators have reached different conclusions in regard to the question whether there is normally a relative increase in the elimination of phosphates in the afternoon above that of the forenoon.¹ However this may be, the interesting fact is that it is only on the nervous days that the increase occurs. Moreover, the change in the mental condition of the patient on the nervous days is not noticeable in the early morning but begins quite suddenly about ten or eleven o'clock, and from that time on continues unabated, and sometimes with increasing intensity, until evening.

Uniform diet. — The results shown in Table III taken together with the results of Tables I and II would seem to leave very little room for doubt that the peculiar periodicity in the phosphate metabolism of the patient must be due to some peculiar condition in the patient and not to the character of the food eaten. To prove conclusively that this is so we decided to put the patient on a uniform diet. This diet may seem somewhat lacking in variety, but the patient could not be depended upon to take any solid food except bread and butter after breakfast on his nervous days and we had to select a diet that suited his needs, and that could be duplicated with some accuracy from day to day, or at any future time.

¹ The preponderance of evidence seems, however, to show that there is more or less of an increase, and a partial explanation of such a phenomenon might perhaps be found in the fact that nucleins which are rich in phosphoric acid are much more difficultly digestible than the other proteids.

TABLE IV.

Date.	Volume. c.c.	Sp. Gr. ¹ 10-	N ₂ Gm.	P ₂ O ₅ Gm.	SO ₃ Gm.	100 N ₂ :		100 SO ₃ :		N ₂ in fæces. Gm.
						P ₂ O ₅	SO ₃	P ₂ O ₅	SO ₃	
Nov. 13	1265	22.5	16.1	3.71	3.26	23.0	20.3	114.0		
" 14	930	29.0	17.0	3.24	3.39	19.0	20.0	95.6		
" 15	960	32.0	17.6	3.81	3.37	21.6	19.1	113.0	3.21	
" 16	1030	29.5	18.2	3.20	3.36	17.5	18.5	95.2		
" 17	925	32.5	17.0	3.90	3.34	22.9	19.6	116.7	0.55	
" 18	900	33.0	17.5	3.34	3.23	19.1	18.4	103.0	6.38	
" 19	850	34.0	16.9	3.89	3.25	23.0	19.2	120.0	1.30	
" 20	820	31.5	16.8	3.02	3.32	18.0	20.0	90.0		
" 21	1055	30.0	17.3	4.34	3.15	25.1	18.2	138.0	2.40	
" 22	960	27.5	16.6	3.09	3.21	18.6	19.3	96.0		
" 23	935	29.0	16.1	3.54	3.18	22.0	19.7	111.0	0.98	
" 24	995	25.0	17.0	3.14	3.01	18.5	17.7	104.0	2.16	
" 25	915	30.0	15.8	3.59	3.12	22.7	19.7	115.0	1.13	

¹ Throughout these experiments the specific gravity has been taken by means of ordinary clinical urinometers. A later examination showed, however, that only one of four urinometers on hand was approximately accurate.

Text books usually state that urinometers should be tested by immersing them in distilled water. If they show the specific gravity of the water to be 1.000 they are supposed to be serviceable. This is entirely misleading. Practically all urinometers in the market will be found to be tolerably correct at 1.000 while between 1.015 and 1.040 where they most need to be accurate they may show an error of from 2 to 5 points.

The diet consisted of the following :

Eggs (with shells)	535 g.
Fresh milk	800 c.c.
Breast of broiled chicken	30 g.
Bread	200 g.
Butter	75 g.
Sugar	80 g.
Salt	5 g.
Water (Dec. 13-18)	600 c.c.
Water (Dec. 18-24)	1000 c.c.

These food products were all obtained from the hospital store rooms and were weighed out in the laboratory on the morning of each day. They have also been repeatedly analyzed but since the uniformity of the food is the only point which has any bearing on the subject of this paper the analytical data may be omitted here.

In this series of experiments each 24-hour quantity of urine was collected together. The nitrogen and at times the phosphates of the faeces were also determined, but as they have no direct bearing on the subject of this paper they are omitted.

The results of the experiments (Table IV) show conclusively that the periodicity in the $N_2 : P_2O_5$ and $SO_3 : P_2O_5$ ratios is due to some kind of periodicity in the metabolism of the patient. Here we have, as is clearly shown by the figures of the table, no increased nitrogen or sulphate elimination on the good days. The nitrogen and sulphate elimination is in fact remarkably constant, as is shown both by the absolute amounts eliminated and by the uniformity of the $N_2 : SO_3$ ratio. The alternation in the phosphate elimination is on the other hand as pronounced as ever.

DISCUSSION OF RESULTS.

Having thus proven the existence of this 48-hour periodicity in the phosphoric acid metabolism of the patient the question presents itself as to what the explanation may be.

It is thought that the periodicity might possibly be due to a corresponding increase in the secretion of hydrochloric acid in the stomach, and that this excess of acid by acting on the less soluble inorganic phosphates of the food might cause an increased absorption and subsequent elimination of phosphoric acid on the nervous days. If this were the case we should expect that the addition of hydrochloric acid to the food on the good days or the addition of an alkali to the food on the nervous days should so change the phosphate elimination as to make the nitrogen-phosphate ratios or the sulphate-phosphate ratios more nearly alike on the two sets of days. At the end of the last series of experiments (on November 26) the patient received accordingly together with his food 150 c.c. tenth-normal hydrochloric acid and on the following nervous day (November 27) he was given four grams of sodium bicarbonate. The results obtained from this experiment seem to show conclusively that the

relative amount of phosphoric acid eliminated is not dependent on changes in the hydrochloric acid secretion of the stomach.

TABLE V.

Date.	Vol. c.c.	Sp. gr. 1.0—	N ₂ Gm.	P ₂ O ₅ Gm.	SO ₃ Gm.	100 N ₂ :		100SO ₃ : P ₂ O ₅	Remarks.
						P ₂ O ₅	SO ₃		
Nov. 26	1155	26.5	18.7	3.60	3.67	19.2	19.6	98	150 c.c. $\frac{1}{10}$ HCl. 50 c.c. after each meal.
" 27	1200	25.0	16.4	3.99	3.17	24.3	19.3	125	+ gm. HNaCO ₃ .

It will be seen (Table V) that the amount of phosphoric acid eliminated on the good day, November 26, is indeed increased by the addition of hydrochloric acid to just about the average amount obtained on the preceding nervous days from the same diet. Together with this increase we find, however, a corresponding increase in the elimination of nitrogen and sulphuric acid, thus leaving the nitrogen-sulphate-phosphate ratios practically unchanged. Since there is no such increase of nitrogen and sulphates on the preceding nervous days of the uniform diet series we can consider the above hydrochloric acid hypothesis disproven. The same conclusion is to be drawn from the results of the sodium carbonate experiment. The amount of phosphate eliminated on this day is rather increased than diminished.

If the periodicity in the phosphate elimination is not due to a periodicity in the digestion it might be supposed to be due to a periodicity in the absorption. Such a selective periodicity in the absorption of phosphates alone and independent of the digestion would have to be confined to the inorganic phosphates. The absorption of inorganic phosphates, could, however, scarcely be independent of the chemical reaction of the digestion mixture. The results obtained from the addition of hydrochloric acid and sodium carbonate to the food seemed to indicate that the phosphates absorbed must be largely organic. In order to gain some idea of how much of the absorbed and eliminated phosphoric acid is due to the inorganic phosphates of the food, we attempted to reduce this factor by adding to the food on a nervous day 15 grams magnesium chloride. The absolute as well as the relative amount of phosphate eliminated was not appreciably diminished by the addition of magnesium chlor-

ide. We are therefore inclined to think that in this case at least the inorganic phosphates of the food form a much smaller proportion of the absorbed phosphates than is generally supposed.¹

It certainly did not seem at all probable that the excess of phosphates eliminated on the nervous days above that eliminated on the good days could be due to a periodically increased disintegration of bone tissue. In order to be certain that this was not the case the percentage of alkali phosphates was determined on four consecutive days, November 14-17. The percentages found were 76, 77, 80, 75, in the order named. There is therefore no increase of the earthy phosphates on the nervous days, and any increased disintegration of bone tissue is thereby excluded.

Early investigators, especially Zuelzer and Mendel, did considerable work which was intended to prove that an increased elimination of phosphates, especially an increase in the relative amount of eliminated phosphate, is due to an increased nerve metabolism. Feder² showed definitely in 1881 that such views are untenable, and his arguments have since been quoted by almost every succeeding writer on the subject. Feder's argument in brief is that to explain the varying $N_2 : P_2O_5$ ratios for different parts of the same day by an increase in the nerve metabolism it would be necessary to assume

¹ In this connection we wish to refer to the experiments made by RIESELL under HOPPE-SEYLER'S direction on the effect on the phosphoric acid absorption of converting the phosphates of the digestive tract into earthy phosphates. (See HOPPE-SEYLER'S *Medicinischem-chemische Untersuchungen*, 1868, p. 319.) RIESELL found that by adding 30 grams calcium carbonate to the food per day he could reduce the amount of phosphate appearing in the urine to one-half or less of what was eliminated before adding the carbonate. These experiments, as it seems to us, do not show that which the investigators meant to show. By taking 10 grams calcium carbonate with each meal it is clear that the acidity of the stomach juice would be more or less completely neutralized and the pepsin digestion would stop; moreover the sodium carbonate of the chyle would react with the neutral calcium chloride and calcium carbonate mixture coming from the stomach and give calcium carbonate and sodium chloride. This would therefore again result in a neutral solution and the pancreatic digestion also would thus in all probability be diminished. That under such circumstances the phosphates of the urine should be much diminished is self-evident, but it seems equally self-evident that the nitrogen elimination would be diminished and if RIESELL and the later investigators who repeated the work had also made some nitrogen determinations it seems as if they must have found that they were simply making partial starvation experiments. In view of these considerations RIESELL'S (or HOPPE-SEYLER'S) discussion of the results obtained seems quite irrelevant.

² FEDER: *Zeitschrift für Biologie*, 1881, xvii, p. 331.

that one quarter of the entire brain must at times be disintegrated in less than two hours; that these varying ratios during the different hours of the day are due to the difference in time required for the absorption and elimination of the nitrogen and the phosphates, and that the differences disappear when twenty-four hour quantities of urine are taken for comparison.

Feder's arguments, to which nothing of consequence has been added by later investigators, however, cannot explain the phosphate variations recorded in this paper. We have here a remarkable coincidence of a purely mental disorder and an abnormal phosphate metabolism, and the conditions are not obscured by any other noticeable physical disorder of any kind. The old question of a noticeably changed or abnormal nerve metabolism, therefore, again presents itself. The nervous system in general does contain certain characteristic phosphorus compounds in notable quantities, and why should these not be essential to at least a part of the specific metabolic processes of the nerve tissues, and why should they not also be subject to noticeable disorders connected in one way or another with some mental disorders? It is not necessary to assume that an increased elimination of phosphates in such cases must necessarily be due to a corresponding disintegration of nerve tissues. Elimination of large quantities of sugar in the urine of diabetic patients or the elimination of albumin in cases of albuminuria is not supposed to represent a corresponding disintegration of living tissue. Similarly it is in this case at least not at all necessary to assume that the half a gram of phosphoric anhydride which is eliminated on the nervous days in excess of that eliminated on the good days must represent a disintegration of a corresponding amount of brain or nerve tissue in order that the difference may be said to be due to a faulty nerve metabolism. The absolute amount of P_2O_5 eliminated is moreover not increased above that of the control in Table III when several consecutive days are taken into consideration, nor is it excessive when compared with the phosphoric acid contents of the uniform diet of November 15-25 (Table IV). We have fed other patients on exactly the same diet, and in the case of Mr. B. (general paralysis), where the nurse succeeded in collecting all the urine, the values shown in Table VI were obtained.

On examining the total phosphate, nitrogen, and sulphate values obtained during the period between November 13 and November 25 (Table V) we find that during the first six nervous days the patient

eliminated through the urine 23 grams P_2O_5 , 101g. N_2 and 19.5 grams SO_3 . On the six alternating good days the patient eliminated 19 grams P_2O_5 , 105g. N_2 and 19.5 grams SO_3 . The average for six consecutive days is therefore 21 grams P_2O_5 , 103g. N_2 and 19.5 grams SO_3 . It will be seen that these values agree very closely with the corresponding total values obtained from Mr. B. (see Table VI).

TABLE VI.

Date.	Volume. c.c.	Sp. Gr. 1.0—	N_2 Gm.	P_2O_5 Gm.	SO_3 Gm.	100 N_2 :		100 SO_3 :
						P_2O_5	SO_3	P_2O_5
Jan. 8	1190	25.5	16.5	3.09	3.36	18.7	20.4	92
" 9	800	29.0	15.0	3.15	2.99	20.8	20.0	105
" 10	1150	26.0	17.3	3.43	3.37	20.0	19.5	101
" 11	1140	..	17.2	3.92	3.44	22.9	20.0	114
" 12	1800	17.0	18.4	3.78	3.61	20.5	20.0	105
" 13	1830	18.0	19.5	3.93	3.67	20.1	19.0	107
Total for 6 days . .			103.9	21.3	20.4

It is clear therefore that in the case of the patient here investigated as in all others the total phosphoric acid elimination is first of all dependent on the amount and kind of food digested and absorbed. It is equally clear, however, that the excess of three grams phosphoric anhydride eliminated on the six nervous days above that eliminated on the six alternating days is not dependent on the phosphates of the food.

To explain therefore the peculiar daily variations in the phosphoric acid excretion which this case presents we venture to advance the following hypothesis: There exists in this patient on every second day a condition somewhat analogous to diabetes, in virtue of which the system or some part of it is unable to assimilate (organize) a part of the phosphate absorbed from the digestive tract. The non-assimilated phosphate is eliminated on the same day, and the total amount of phosphate eliminated on the "nervous" days is therefore greater than the amount eliminated by a normal person absorbing the same

amount of phosphoric acid from the digestive tract. On the alternating days on the contrary a correspondingly less amount of phosphate than the normal is eliminated because on those days the system repairs the loss sustained on the preceding days.¹

Since the nerve tissues contain considerable quantities of organic phosphorus compounds and since the abnormal phosphate metabolism here described is associated only with peculiar mental symptoms which must in turn be dependent on abnormal metabolic processes in the nerve tissues, these are the ones which are subject to an abnormal periodicity in their ability to assimilate "circulating" phosphates. This view is of course advanced only as a working hypothesis which it is hoped may lead to the finding of more facts in connection with the practically unknown field of phosphate metabolism.

¹ This view would to some extent involve the question of the old Voit-Pfänger controversy as to whether the food constituents must be combined with living protoplasm before being oxydized or split up and eliminated. The observed facts would indeed seem to indicate that a considerable fraction at least of the phosphoric acid constituents of the absorbed food is normally retained for some time before being eliminated and such retention could scarcely mean anything else than that intermediate synthetic reactions take place whereby the amount of "circulating" phosphate is diminished.

The question of how far the constructive metabolism of the food products absorbed from the intestinal tract precedes their oxidation would seem to offer an excellent field for renewed investigations in view of COHNHEIM's recent interesting discovery of the new hydrolytic ferment "Erepsin" in the mucous membrane of the intestines (HOPPE-SEYLER'S *Zeitschrift für physiologische Chemie*, 1901, xxxiii, p. 451), and LOEWI's supplementary proof that a dog could be kept in nitrogen equilibrium by means of KUTSCHER'S crystallizable end products of the trypsin digestion containing no peptones or albumoses (*Centralblatt für Physiologie*, 1902, xv, p. 590).

ON THE QUANTITATIVE DETERMINATION OF TOTAL SULPHATES IN URINE.

By OTTO FOLIN.

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TO make total sulphate determinations in urine according to the ordinary gravimetric method is a tedious operation, and yet it is the only method which gives reliable results. This is undoubtedly the chief reason why we have so few complete series of sulphate determinations in connection with metabolism experiments. Total sulphate determinations are, however, exceedingly useful in checking up metabolism experiments similar to those recorded in the preceding paper. Errors in the work or anomalies in the excretion are almost unfaillingly detected by a comparison of the three ratios $N_2 : P_2O_5$, $N_2 : SO_3$, and $SO_3 : P_2O_5$.

The method here described for making such gravimetric sulphate determinations in urine is the result of considerable experience and will be found to greatly facilitate the work:

Fifty c.c. of urine is measured into a small Erlenmeyer flask (capacity 200 c.c.) and after adding a small pinch, about 0.2 gm., pure potassium chlorate and 4 c.c. chemically pure hydrochloric acid (sp. g. 1.20) the mixture is gently boiled for from fifteen to twenty minutes. At the end of this time the contents of the flask should be almost as clear as water. If the contents are not colorless, or if they have been clear but have begun to grow dark again, the flask may be taken off the fire and a few more grains of potassium chlorate added which will clear the urine at once. (It is important that no chlorate should be added to the mixture while it is still boiling, because the contents of the flask will then invariably boil over.) Twenty-five c.c. of barium chloride solution (60 gm. crystallized barium chloride to the litre) is next added, and the mixture is kept hot, but not boiling, for about forty-five minutes. The mixture can then be filtered, and an absolutely clear filtrate obtained. (An excellent filter paper for this work is Schleicher and Schull's black ribbon No. 589, diameter 9 cm.)

The presence of potassium chlorate interferes according to Fre-

The above method for determining total sulphates in urine should give at least as accurate results as the ordinary Baumann-Salkowski method described in most text-books on physiological chemistry. The Baumann-Salkowski method is open to two sources of error, both of which are eliminated in the above procedure. In the first place, the barium sulphate precipitate obtained by the usual method is contaminated by much organic matter, and especially by large quantities of uric acid. Since uric acid cannot be precipitated free from mineral matter by means of acids it is clear that the uric acid remaining with the barium sulphate will leave a certain amount of ash on ignition. The other source of error, of which Baumann as well as Salkowski seems to have taken no notice, is the fact that moist barium sulphate cannot be washed with alcohol, because the first addition of this reagent will invariably carry some of the precipitate through the filter paper.

It may be added that the method suggested above for decolorizing the urine is also applicable when the total sulphates are to be determined volumetrically. The end point of the titration is thus much more readily distinguished, first, because of the transparency of the solution, and secondly, because the barium precipitate settles much more rapidly and completely in the presence of the chlorine formed from the chlorate and hydrochloric acid. The volumetric method is, however, not so accurate as the gravimetric method.

SOME PHYSIOLOGICAL CHARACTERISTICS OF ANNELID MUSCLE.

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and Surgeons, New York.]

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REFERENCES TO PREVIOUS WORK.

RESARCHES into the physiology of muscle in the lower forms of animal life are meagre as compared with those in the vertebrate group. Nevertheless important and conclusive work has been done on numerous representatives of the invertebrates. Prominent instances of such researches are those of Richet¹ ('82), Luchsinger ('82), Biedermann ('87), Rollet ('89), and Piotrowski ('93) on Arthropoda; molluscan muscle has been studied by Coutance ('78), Pawlow ('85), Biedermann ('86), Fuchs ('94), von Uexkull ('92), and Bottazzi ('98); Biedermann ('89) has experimented with the muscles of Echinus and Holothuria; and Bottazzi ('98) has taken graphic records of the contractions of the tube feet of *Asteropecten*.

¹ The number in parenthesis refers to the bibliography (page 178) and consists of the last two figures of the year of publication.

Annelid muscle and nerve have been dealt with by physiologists in various ways. As early as 1880, Krukenberg ('80) studied the normal movements and coördination. Friedländer ('88) extended these observations to experimentation on removal of portions of the nerve cord. At the same time Biedermann ('89) and his pupil Fürst ('89), using earthworms, *Arenicola* and *Hirudo*, made numerous observations on changes in the superficial appearance of the cuticle of intact, alcoholized animals, during stimulation with a constant electric current. Loeb ('94) has made a study of the brain functions, and of movements due to irritation of the surface of intact and decapitated earthworms. Later in the same year the same line of experimentation was taken up and published upon at some length by Friedländer ('94). The retractor and proboscis muscles of *Sipunculus nudus* have been investigated with constant, induced and tetanizing currents by von Uexkull ('96); Bottazzi ('98) more recently studied the same objects, though he made special note only of spontaneous contractions. The only consecutive experiments, carried along lines comparable to those adopted in the present paper, were those published by Straub ('00), and comparison with his results will be made wherever procedure was sufficiently similar to warrant it.

MATERIAL AND METHODS.

The following is a record of a series of experiments, the object of which has been to ascertain, by methods entirely comparable with those used in investigating the skeletal muscle of a vertebrate, the behavior of muscle of such a nature as is presented by an annelid, when subjected to stimuli of different sorts and degrees. Such experiments, then, would come under the general head of smooth muscle study, although, as is well known, annelid muscle differs considerably in its histology from ordinary smooth muscle, such as the muscle of the alimentary canal and urino-genital organs in vertebrates.¹

The form employed in the present investigation was the common earthworm. For convenience in manipulation the largest obtainable specimens were used.

¹ For the distribution of smooth and striated muscle among the different animal phyla, see MARSHALL: *Quarterly journal of microscopical science*, 1887-88, xxviii, p. 75; and for an article on the histology of the earthworm, see CLAPAREDE: *Zeitschrift für wissenschaftliche Zoologie*, 1869, iv, p. 563.

In such an animal, the presence of more than one set of musculatures complicates the experimental results, where the purpose is to ascertain the action of a simple muscle band. For instance, while each system in the portion of the worm from behind the crop region to the posterior extremity has uniform relations with other parts, a piece consisting of a single segment or more furnishes two sets of muscles which work in directions at right angles to one another. In practice ten to fifteen segments was found to be the most convenient length. On stimulation of such a piece, there would result a contraction of the circular musculature, tending to lengthen the piece; and of the longitudinal, tending to shorten the piece. To do away with either set, then, it is necessary merely to cut it in such a way as to render its contractions of no effect. Thus, in attempting to get the effect of contraction of the longitudinal muscles alone, the piece of the worm cylinder should be slit lengthwise on one side; several facts, which will be referred to later, indicate sufficiently that the circular muscles so treated may practically be left out of account, and the piece may be considered as a single longitudinal muscle preparation.

In connecting such a preparation with a recording lever, the segments show a tendency to break apart. If the muscle be grasped in a clamp, it is almost sure to break in two from mechanical reasons; this can largely be avoided by suspending the muscle and connecting it with the lever by ligature threads tied tightly around it. Fine copper wires at either end served as electrodes. Further methods were identical with those used in ordinary experiments with skeletal muscle, *e. g.* that of a frog, the contractions being isotonic and the lever weighted lightly.

SPONTANEOUS CONTRACTIONS.

The more recent demonstrators of spontaneous movements in smooth muscle are: of the bladder, Langley and Anderson ('94), Griffiths ('95), and Stewart ('00); of the retractor penis, Sertoli ('83), Langley and Anderson ('95), Schultz ('97), and de Zilwa ('01); of other preparations, Duchesi ('97), Barbera ('98), Bottazzi ('97), and Woodworth ('99). These investigators are about evenly divided in opinion as to the ultimate cause of this spontaneous activity, whether such movements are the responses to impulses from some nervous centre or tissue, or the expression of a normal characteristic of the muscle itself.

In the case of *Lumbricus*, Friedländer ('94) found that, notwithstanding the fact that he had removed the nerve-cord from five to ten segments in the middle of the worm, the parts of the worm anterior and posterior to that area did not move as separate individuals, but the impulses were carried across the nerveless portion in such a way that coördinated movements still resulted. The relation of this to spontaneous movements, however, is not very close. More apropos to the point in question than this are the results described by Straub ('00), who found spontaneous contractions, even though he had removed the nerve-cord from the entire preparation.

This finding is at complete variance with what has come into evi-

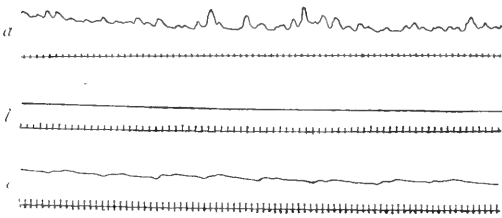


FIGURE 1. — (a) Tracing showing spontaneous movements in fresh preparation, which contained parts of nerve-cord. (b) Showing absence of spontaneity in muscle from same animal as in (a), the nerve-cord removed. (c) Compound rhythm appearing after arrival of partial fatigue. Same muscle as in tracing (a). Time intervals, $\frac{1}{4}$ seconds. One half the original size.

dence in the present work, during which hundreds of experiments have seemed to show conclusively that spontaneous movements are not present after complete removal of the cord.

Straub removed the cord by careful dissection with needles and forceps, and this fact may offer a clue to the cause of difference in our results. In all ex-

periments connected with this research, save in the tracings of spontaneous contractions immediately following, the cord was removed by cutting out completely the tissues of the mid-ventral line with scissors. Thus there would seem to be no chance of any part of the cord, or of the central ends of large nerves issuing from it being left in the preparation. In numerous cases where even a very little of the cord remained, spontaneity was evident, but never when the cutting was carefully done. Lest spontaneity might have been inhibited by the shock of cutting, control preparations, before being used, were left in moist chambers for an hour after the operation, but in these also no automatic contractions appeared. On the other hand, and in agreement with Straub, a piece of an animal in which a cut is made along the side, or dorsal surface, the cord thus being preserved, shows spontaneous contractions for a considerable time. Fig. 1 shows tracings taken from the same animal, illustrating these observations.

Bowditch ('97), Woodworth ('99), and Stewart ('00) in particular have called attention to a very marked regular rhythm in the spontaneous contractions of smooth muscle. It happens in a majority of cases also that while a simple irregular rhythmical character is given to the curve which is recorded when the preparation is fresh, after partial fatigue has been induced, a compound rhythm appears; an instance of this is to be seen in tracing (*c*) of Fig. 1. This complex activity on the part of the muscle may, however, assert itself from the beginning, and Fig. 2 is offered as illustrating representative instances of this nature. Tracing (*a*) shows a distinct difference in the character of the two rhythms: the curves designated by dots have a rather broken, unsteady ascent, but a quite free and rapid descent; those marked by crosses have a more symmetrical shape, with rounded, instead of sharp, apices. These two sets of contractions have time intervals which are more or less inconstant, and a fusion of the two occasionally results.

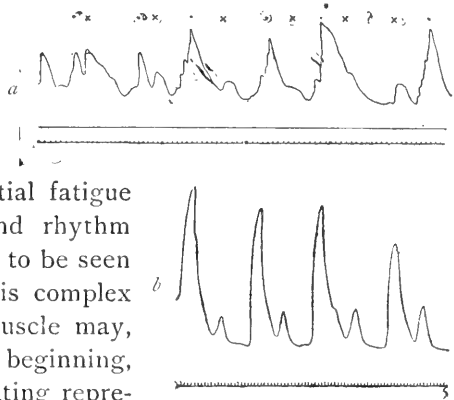


FIGURE 2. — (*a*) Automatic movements in fresh muscle containing the nerve chord. Two rhythms, as indicated by dots and crosses, their rates differing slightly. (*b*) Another tracing of characteristic movements in preparations containing the nerve cord. Time intervals, 2 seconds. One half the original size.

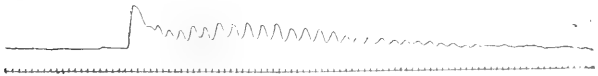


FIGURE 3.—Record of spontaneous contractions induced to return in quiet piece of muscle containing parts of the nerve chord, by mechanically stimulating with a drop of normal saline solution. Chronograph record, 4 second intervals. One half the original size.

Such is the case in the next to the last curve of the dotted series, as is evidenced by its unusual height, the suddenness with which it reaches its apex, and the gradual, broken relaxation period; a further effect of the summation seems to have been to abolish the succeeding contraction of the dotted series type. Fig. 2 (*b*) is a further example of the complex activities of the muscle showing two more or less regular, simultaneous rhythms.

The above described voluntary contractions cease after a time, but

within a limited period are readily induced to return, for a short interval, by a single stimulus. This fact is illustrated by Fig. 3, in which the muscle was mechanically stimulated by the mere falling on it of a drop of normal saline solution. It should be noted in passing also, that these movements, performed after the muscle is past its period of more energetic activity, have a regular, symmetrical character not noticed in the fresher preparations generally. A similar difference in the nature of spontaneous contractions is noted by Stewart ('00) in the cat's bladder, when just excised, and when retained for some hours.

It should be added that the above tracings were not recorded with a Pflüger's vertical writing lever, but with a tangentially writing lever of the third class; however, that fact does not interfere with their value in the connection used. All other figures in the paper were made with the first named pattern.

RESPONSE TO SINGLE INDUCTION SHOCKS.

a. **Latent period.** — On provoking a contraction from this preparation, there is present a very appreciable interval after the stimulus is given, before contraction commences. Straub ('00) states the maximum value of the latent period as 0.01 second. Numerous experiments have seemed to point to a much greater interval; *i. e.*, although the variation is considerable, an average period equals 0.024 seconds. This is scarcely more than one-tenth that described by Stewart ('00) for the bladder of the cat, and by de Zilwa ('01) for the retractor penis of the dog.

b. **Contraction.** — In his account of single contractions of the earthworm muscle, Straub notes a great deal of difference in the character of the curves obtainable, and the same phenomenon has been evident throughout the present investigation. Two quite distinct forms of curve, and a third combining elements of the first two, can frequently be obtained at will, by a very slight variation of the stimulus, such as is effected by moving the secondary coil of an induction apparatus one-half a centimetre toward or away from the primary. The direct relation between strength of stimulus and height of contraction which is characteristic of ordinary skeletal and smooth muscle as distinguished from cardiac, is also present here, but in connection with other constant results which seem worth consideration.

The three sorts of responses referred to are illustrated in Fig. 4 (a), where the different forms of curve were obtained with the secondary coil distant from the primary 13, 12.5, and 12 centimetres, respectively. The method of "after-loading" was followed here, thus avoiding any error which might arise by the muscle becoming progressively more relaxed, and consequently lifting the load while its own degree of contraction differed in one case from that in another. In this record, too, the coils, having been approximated as described, until the difference in behavior of the muscle occurred, were gradually

drawn apart again by the same interval; in this way there would seem to be ruled out the possibility that the change in form of the curve might be due to fatigue, or some deterioration in the preparation.

A better example of this difference in response to a single stimulus is shown in (b)

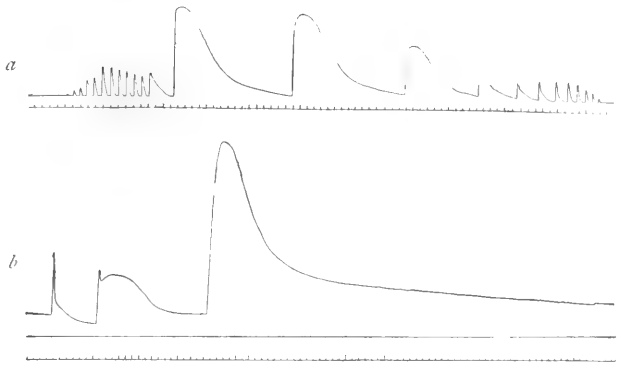


FIGURE 4.—(a) Showing contractions of *Lumbricus* muscle in response to single induction shocks. Secondary coil at 11.5 cm. when second of large contractions was made; approach to, and recession from this point were made with difference in position of coil of 0.5 cm. between each two contractions. Time intervals, 10 seconds. (b) Single contractions, the coil being at 9, 7, and 6 cm. respectively. Time intervals, 4 seconds. One half the original size.

of the same figure. The difference in position of the coils was two centimetres and one respectively. The first curve shows immediate and almost complete relaxation, after a still more rapid contraction. The third contraction, taken with the secondary coil three centimetres nearer the primary, is almost an exact parallel to the curves figured by Stewart ('00) for the cat's bladder, differing only in the latter part of its relaxation period. At seven centimetres a contraction appears showing two periods of contraction and two of relaxation; the first rise is, like any other, comparatively very rapid; relaxation commences at the same rapid rate as in the curve taken at nine centimetres, but this soon changes to further contraction, so that the lever again rises slowly, and for a short

distance; these movements are then followed by a comparatively long and slow relaxation as in the case of the curve taken at six centimetres. With regard to the form of the curve resulting from stimulation when the secondary was at seven centimetres, it is interesting to note in passing that it is exactly similar to what has been figured as the action of skeletal muscle after being poisoned with veratria. Such a form of curve, then, is not unique. The different behavior of white and red muscles of higher forms may also be recalled here. The curve given by an annelid muscle when stimulated with a weak shock has numerous points in common with that given by a white muscle: while a strong shock provokes activity similar to that of a red muscle.

Straub ('00) gives no very detailed discussion of the cause of these two sorts of contraction, but thinks a reason for the phenomena can be found in the condition of the muscle respecting fatigue. Aside from this theory, he finds evidence which would seem to indicate that the contractions giving tracings with sharp apices and fast relaxation curves are characteristic of muscle in a condition of regeneration. The rôle which fatigue may play in this matter can be better discussed in the section of this paper dealing with fatigue; in a word, however, that factor does not seem a sufficient explanation. An observation which appears to strengthen Straub's conclusions as to regenerating muscle is noted in a later paragraph, and is that a preparation from the posterior end of the worm is, as a rule, much quicker in action than a piece from the middle or anterior regions. The piece from the posterior end is, of course, from the area in which new segments are being formed and consequently muscle from this region is legitimately comparable with that produced in regenerating animals. However, while regenerating muscle may react only in the manner described by Straub, that circumstance would not account for the phenomena in normal, fully developed tissues. Rather than from a consideration of the condition of the muscle respecting fatigue, or incomplete formation, it has seemed that one could not safely describe a curve as typical or characteristic, unless at the same time something be said with regard to the strength of stimulus employed; and even then, the results will vary widely with the length and thickness of the preparation.

To make this point of view appear more justifiable, it will be advantageous to give an account of one or two related experiments, which further indicate the necessity for considering the influence of

strength of stimulus. In the course of the experiments, the stimulus was varied in some cases by moving the secondary coil from the point of minimal shock resulting in contraction, toward the primary by certain constant intervals. At other times the secondary was left stationary, and the primary current varied by a rheochord, but the result was the same in either case. The action of the muscle was as follows: after the point of minimal response is reached, as the severity of the shocks is increased, the contractions resulting show a gradual increase in

height during the first few (4-6), after which

time, though the stimulus is strengthened by a constant moving of the secondary coil toward the primary, or, leaving the secondary in a constant position, by lessening the resistance shunt in the rheochord, nevertheless, there appears no proportionate increase in the height of the contraction responses.

At the arrival of a certain intensity of stimulus, however, the contractions increase in height to what has been called a "secondary maximum," beyond which no further increase is obtainable. This result was first figured by Fick ('64) for skeletal muscle.

In Fig. 5 are given two curves taken from annelid muscle, showing the above described action. Record (a) is one taken on a moving drum, on which were recorded contractions made in response to a double (make plus break) stimulus, while the secondary was moved a uniform distance nearer the primary each time, between the contractions. After the first two or three contractions, no difference in the curves is prominent until the last three are reached, and a "secondary maximum" is obtained. It should be observed here that

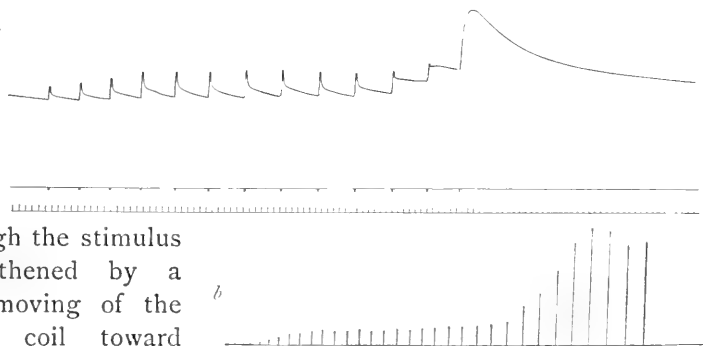


FIGURE 5. — (a) A tracing showing responses to single shocks; the first contraction made with the coil at 11.1 cm.; it was moved 0.3 cm. between each two of the succeeding curves. Time interval, 4 seconds. (b); Record of same experiment as in (a), but the stimulus varied by a rheocord, the drum turned by hand, and muscle allowed to relax with lever in same line as during contraction. One half the original size.

the contractions constituting this "secondary maximum" give the sort of curves represented by the third contraction in (b) of Fig. 4. Tracing (b) was taken with the drum turned by hand; the lever was here adjusted so that it rested on a support when the muscle showed complete relaxation, and to this support the lever was allowed to return each time, thus assuring relaxation between each two contractions. The stimulus was increased, in this case, by a rheochord, the secondary coil remaining stationary. A "secondary maximum" is again a character of the record, and beyond its height no increase is to be gotten.

Lest it might be contended that the extent of contraction of the muscle, *i. e.* its compactness after so vigorous a contraction, brought

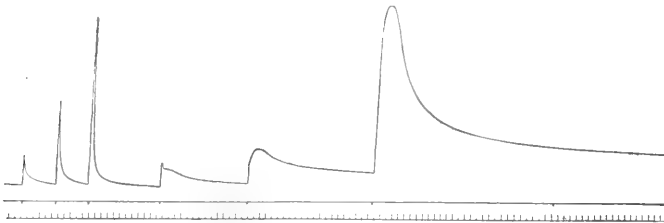


FIGURE 6. Single and summated contractions. First three curves, taken with the coil at 9 cm., are results of 2, 4, and 6 stimuli respectively. Last three contractions obtained with the coil at 8, 7, and 6 cm. respectively. Time intervals, 4 seconds. One half the original size.

about a condition from which it could not relax as readily as from a weak contraction, Fig. 6 is added, in which the secondary coil was at 9 cms. from the primary during the first three stimulations. The first curve was due to two shocks (*i. e.*, make plus break); the second, to four; the third, to six. The last three curves in the tracing were taken with the secondary at 8, 7, and 6 cms., respectively; with this stronger stimulation we obtain a curve of height equal to that gotten by a summation of six stimuli of lesser intensity; but the difference in relaxation is most evident. This seems to show quite clearly that, not so much the compactness of the muscle resulting from a strong or extensive contraction, but the *strength of the stimulus* is the potent influence in determining the rate of relaxation, and the consequent character of the muscle curve.

Approximate times of contraction and relaxation for the three forms of curves noted may be tabulated as follows, the ratio only being preserved and not an actual record in seconds, since this varies with

the height of contractions, and this, in turn, is dependent on the length of the preparation used.

	Short Curve (weak stim.).	Medium Curve (medium stim.).	Long Curve (strong stim.).
Contraction	1	1	10
Relaxation	16	48	300

RESPONSE TO MULTIPLE INDUCTION SHOCKS.

a. Summation. — The result of repetition of stimuli in the muscle of *Lumbricus* is similar in almost every respect to the response of striped and of other smooth muscle. A graphic record of an experiment in summation is inserted as Fig. 7. In (a) the first curve is the result of a single stimulus; this is followed by a contraction, provoked by double the stimulus;

this in turn by stimuli at intervals apart, as denoted by the seconds line. It is noticeable that the second contraction, which is due to double the stimulus of the first, shows also double the height. Within certain limits, this relation has been found to be the rule; *i. e.*, if the stimulus is strong enough to produce only a moderately high contraction, a repetition of the stimulus, whether or not delayed until the muscle has partially relaxed from its first ascent, provokes a response nearly, if not quite, equal to the first contraction. This is indicated in tracing (b) of the same figure, first curve. It is still better shown in the first three contractions of Fig. 6, where the second contraction is due to twice the number of stimuli in the first, and the third to three times as many. Similarly the height of the muscle curve, when provoked by a Faradic current, varies with, though not quite in direct proportion to the length of time of stimulation.

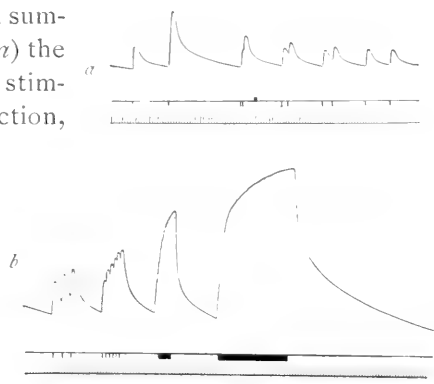


FIGURE 7.—(a) A tracing in which was recorded the effect of a single stimulus, followed by a series showing response of the muscle to two stimuli given at intervals of varying length as shown by the seconds time curve. (b) Record of the development of tetanus. Time in seconds. One half the original size.

When looking for the cause of such a result, one is tempted to sus-

pect that some segments of the worm contract in response to the first stimulus, while other parts respond to the further shocks, especially since the former segments are already in a state of contraction. Such an explanation does not appeal very strongly, however, since at each stimulation the current must pass from one end of the preparation to the other. A close watch of the muscle, also, furnishes no indication of such being the case; but rather indicates that further contraction of the same segments, which responded to the first shock takes place upon repetition of shocks.

b. **Tetanus.** Fig. 7 (*b*) shows the development of tetanus in this muscle. It is found that about four stimuli per second are necessary to produce a "plateau;" this is to be compared to three in the crayfish muscle (Richet, '82), seven in the frog's stomach (Woodworth, '99), one in the cat's bladder (Stewart, '00), and thirty to one hundred in the frog's gastrocnemius muscle (Sanderson, '00).

INFLUENCE OF CONSTANT CURRENTS.

a. **Contraction phenomena.**—A preparation of the longitudinal muscle of *Lumbricus* responds with a contraction at both making and breaking of a current. Such a result is characteristic of smooth muscle in general. The constant current also acts as a stimulus during its flow, but, contrary to the accounts of some writers working on other forms of muscle, the break

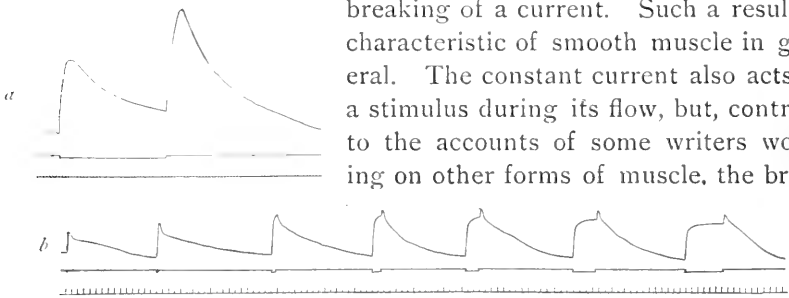


FIGURE 8.—(*a*) Record of the effect of make, flow, and break of a constant current of medium strength, on annelid muscle. A fall in the stimulation line indicates make; rise denotes point of breaking the current. Time is marked in two seconds intervals. (*b*) Another record showing the stimulating effect of a strong constant current when allowed to flow for different lengths of time. Time intervals, 4 seconds. One half the original size.

effect does not seem to be seriously lessened by the flow; in fact it often exceeds the make contraction even after some considerable duration of the current.

Winkler ('98) found no contraction response to a break of the current in the frog's stomach preparation, while Woodworth's ('99)

experiments with the same muscle show the response at the break to be greater than at the make of the current. Stewart ('00) states that with the cat's bladder "the response to the make is generally of much greater extent than that following the break." Fig. 8 furnishes illustration of what obtains in annelid muscle. Record (a) shows the make contractions followed by the flow, and the break contractions. The relaxation during the flow is observed to be slower than that following the break, while the latter contraction is seen to be more extended than the former. The duration of the flow, as Woodworth and Stewart contend, is here also a definite factor in determining the reaction of the muscle. Neither of these workers obtain any response when the make and break stimulations come close together. This result has not been obtainable as a rule in the annelid preparation, but there is always a response of some degree.

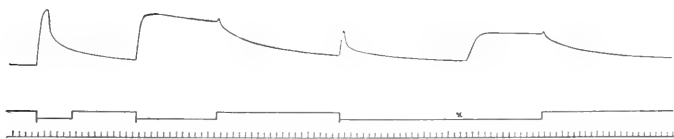


FIGURE 9.—A record showing response of earthworm muscle to (a) the make, (b) the make, flow and break, (c) the make, diminishing of, increasing of, and break of the constant strong current. Time intervals, 4 seconds. One half the original size.

Fig. 8 (b) shows the conduct of this muscle when treated with currents for different lengths of time, as indicated by the four seconds time curve; this tracing also serves to confirm, in a striking way, Biedermann's opinion that each of the three factors' make, flow, and break of the current acts as a stimulus; and also indicates that, within limits, the longer the flow of the current, the more pronounced is the break effect. A separate response to make and break of current is not noticed unless about two seconds elapses between them. With longer intervals of flow, the break effect becomes more pronounced. Since the make and break, coming near together, result in a much reduced curve, the idea, for which Woodworth ('99) contends, that there is an antagonism between the effects of the make and break of the constant current, *seems* to be indorsed here. Such evidence, however, can be accepted as affording but the slightest support to so important a principle.

While the last mentioned tracings furnish good proof of the stimulation attending the passage of the constant current, Fig. 9 is given

in addition as more clearly showing this point. The first contraction is due to the making of the current; this was followed by a gradual turning off of the current with a rheonome; then the current is made again and allowed to flow as indicated, a slight contraction following the break. After relaxation, the current is made again, but immediately turned off with a rheonome, the break effect thus being avoided. At the point marked X the current was again gradually turned on with the rheonome and under the influence of sufficient intensity of current a contraction results. It was found to be impossible to turn on the current so slowly that no contraction would result.

The current employed during the preceding experiments was furnished by two small storage cells, together having an E. M. F. of 2.5-3.5 volts.

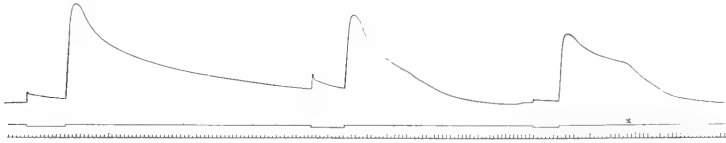


FIGURE 10.—A tracing, the first part of which shows contractions following make and break in the induction apparatus; the second part indicates the make and break induction effects, followed by a weak constant current; in the last curve of the tracing the muscle was allowed to relax at its natural rate for a time, after which a weak constant current was turned on. Time intervals, 4 seconds. One third the original size.

b. Relaxation phenomena. Inhibition of muscular contraction has been recorded by Langley and Anderson ('95); by Fletcher ('98) in the retractor of the hedgehog; by Pawlow ('85) in the abductor muscles of *Anodonta*, and by Richet ('79), Biedermann ('98), and Piotrowski ('93) in the claw muscles of the crayfish. But these results followed single or repeated stimuli, or were obtained by stimulation of nerves. Under the effects of the constant current, Luchsinger ('82) describes relaxation in the muscles of crabs, while Sertoli ('83) notes relaxation as a result of using weak currents, and contraction as the result of strong currents on smooth muscle. Winkler ('98) notes relaxation with the constant current, in the frog's stomach preparation, but Woodworth ('99) holds that this is due merely to the make effect, and that the current has no influence of itself. De Zilwa ('01) shows relaxation in a tonically contracted muscle. Stewart ('00) finds no such effect in the bladder muscle, as a result of current stimulation.

The Lumbricus muscle seems to add positive evidence on the side of a relaxing, inhibiting influence of the constant current, *when this is weak, and when the muscle is in a state of continuous or temporary tonic contraction*; the latter state nearly always follows a very strong shock from an induction coil. Fig. 10 is one of numerous tracings taken to verify this conclusion. The first two contractions are due to make and break stimuli from an induction coil. The slow relaxation is apparent. The next curves show contractions due to shocks of exactly the same intensity as those used in provoking the first contractions, but immediately after breaking the primary circuit, a weak constant current was turned through the preparation, the resulting relaxation being comparatively sudden and complete. In the case of the third curve, the muscle was allowed to relax at its own rate for a time and then the weak current turned on with a rheonome at the point marked X. The response is so immediate as to produce a distinct



FIGURE 11.— Showing the relaxation of a tonically contracted muscle when treated with a weak constant current. Time intervals, 2 seconds. One half the original size.

shoulder in the line of descent. Fully as marked as this is the result shown in Fig. 11, where a very prominent tonically contracted condition of the muscle gives way to a weak current: when this is suspended for a little time, tonic contraction is the condition again; and this in turn becomes inhibited by the introduction of a weak current. Here *the make itself causes a contraction; but the flow is surely accompanied by relaxation*, and the break excites contraction again. In this instance it seems altogether evident that make and break effects are not unlike in their influence on the muscle; and also that the flow of the constant current causes relaxation.

It should be added that the exact strength of current having the influence described was not determined, since any figure obtained would be applicable only to the particular case in hand. The current would necessarily be varied with the length and size in general of the piece of muscle employed; and the different temperament (if such a term may be used) of different annelids, even of the same species, would have to be reckoned with as a determining factor.

THE INFLUENCE OF TEMPERATURE.

a. **On the muscle tone.** — A curve, as Fig. 12, showing the change of tone in a Lumbricus muscle during a rise in temperature from a low degree, possesses features approximately identical with those figured or described in literature for smooth muscle of higher forms. An earthworm preparation passes through but little change, regularly, at temperatures below 15° C. At about 20° , a very slow relaxation begins and continues till the neighborhood of 30° C. is reached. In experiments where a piece of the whole worm was used, or a piece of the longitudinal muscle layer containing the nerve-cord, the presence of a temperature of 30° to 34° C. induced a change in the spontaneous movements from slow, short, contractions, to quicker and comparatively high ones. This observation agrees with Woodworth's ('99) results as to increase in activity of a muscle under these conditions,

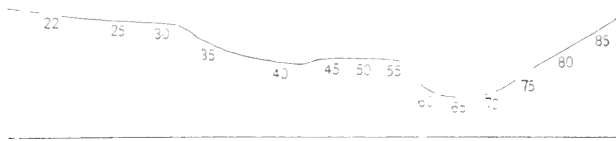


FIGURE 12. A curve showing change of tone in the muscle during a rise in temperature from 22° to 85° C. Time intervals, 2 seconds. One third the original size.

but differs in the fact that, with the frog's stomach preparation, the contractions become smaller, instead of more extensive. The temperature limits found by Woodworth also agree quite closely with those governing an earthworm muscle; *i. e.*, the spontaneity of the preparation ceases at a temperature of 38° to 40° C. In the smooth muscle from warm-blooded animals, spontaneous movements persist to a temperature of 50° C. or thereabouts (Stewart, '00, de Zilwa, '01).

Coincident with this excitation of spontaneous activity, at about 32° a rapid loss of tone sets in, continuing till 42° is reached, when the tone improves again; at 55° it falls rapidly, and the muscle dies. Coagulation and heat rigor appear at about 65° C., a temperature again agreeing closely with records of experimenters on other forms of smooth muscle.

It is worth while to notice that, though pieces of an animal in which the nerve-cord as a whole or in part is still present exhibit spontaneity not previously shown, or an increase of activity already

in progress, yet in pieces from which the nerve-cord and tissues near by have been thoroughly removed, no spontaneous contractions occur. This seems a more precise test of the question of automaticity than any to which allusion has hitherto been made. As Woodworth ('99) found, in the frog's stomach a sudden increase in temperature of itself may excite contractions, which are, therefore, due, not to any intrinsic impulses, but to external irritation.

b. On the character of the contraction.—The correlation of changes in the form of muscle curves with changes in temperature has been worked out in various preparations of vertebrate smooth muscle, but in very few, if any, of the invertebrate class. Temperatures at which maximum contractions are obtained vary widely, as would be expected, when the different normal temperatures and the very different natural environments of the animals, or tissues, are recalled.¹

The extremes of temperature at which *Lumbricus* muscle will respond to stimulation are both within comparatively low temperatures. Furthermore the variations in tone of the muscle at different temperatures make it difficult to obtain a record of experiments in a single series which will represent accurately the real behavior of this type of tissue. Again the strength of stimulus decides to such a marked degree the shape of the contraction curve, and a given strength of stimulus has such different effects at different temperatures, that composite tracings are obtained with the greatest difficulty, and a verbal description will be more desirable than a figure. A curve of slow contraction and relaxation is obtainable at 4°. The times occupied by contraction and relaxation both diminish, as also the latent period, and the contractions become higher, from 4° up to 15°–22°. It is between the latter temperatures that the most vigorous contractions are gotten. When 25° is reached, the muscle loses its irritability very rapidly, and beyond 34° C. it cannot be excited by the strongest induction shocks. So low a temperature is in prominent contrast to the results obtained by Morgen ('90) with the toad's œsophagus, in which this stage is delayed till 50° C. is reached; and for other preparations irritability is retained at yet higher temperatures.²

¹ GRUNHAGEN'S *Lehrbuch*, 1886, ii, p. 121; MORGEN: *Untersuchungen aus der physiologischen Institut zu Halle*, 1890, p. 165; BOTTAZZI and GRUNBAUM: *Journal of physiology*, 1899, xxiv, p. 63; STEWART: *This journal*, 1900, iv, p. 185; DE ZILWA: *Journal of physiology*, 1901, xxvii, p. 200.

² STEWART and DE ZILWA note loss of irritability at about 57°.

FATIGUE AND DURATION OF IRRITABILITY.

Friedländer ('94) and Straub ('00) have both made some observations on fatigue in *Lumbricus* muscle, resulting from repeated stimulation; but their accounts are too brief to allow of much discussion or comparison. Each, however, noticed that fatigue took place early in the experiments, when the muscle was made to contract frequently. In the present investigation, it has become apparent that a great deal of difference in the working ability of the preparations obtains, according as they are taken from the anterior

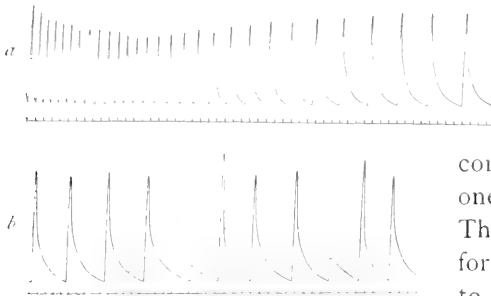


FIGURE 13.—(a) A record showing correlation between the height of muscle contraction and length of the preceding relaxation period. (b) Indicates a similar relation between height of one contraction and its predecessor. Time intervals in both tracings, 4 seconds. One half the original size.

or from the posterior regions of the animal. As a rule, a muscle from the posterior end shows a much shorter period of contraction and relaxation than one taken from the anterior end. The consequence is, that if the former preparation is stimulated to a fresh contraction as often as it fully relaxes from a previous one, fatigue results in a much shorter interval of time than in the case of the latter when submitted to the same test. The total number of con-

tractions of which the posterior piece is capable is also less than in the case of the anterior piece; but a brief consideration of the matter furnishes at least a partial explanation of this difference.

It has just been remarked that the posterior end of an earthworm performs quicker movements, and there comes in here the possibility that the more numerous contractions of which the anterior end is capable may be due to the resuscitation of that muscle during its longer relaxation periods, and consequently a longer retention of power to contract. A very striking correlation between the height and vigor of a contraction, and the nature of the preceding contraction is brought out in results like that reproduced in Fig. 13. In (a), as the muscle fatigued rapidly in consequence of rapid action, the partial exhaustion shows itself in the longer relaxation periods which soon appear. But the outcome of these longer intervals is

that the muscle regains force and therefore the ability to make higher contractions; roughly, the height of the contraction is in direct proportion to the length of the preceding relaxation period.

The second tracing in Fig. 13 shows the beneficial result of a small contraction on the one following it. The cause of the small curve is uncertain, but the same principle involved in tracing (a) is thus brought out in another way.

Fig. 14 is a reproduction of portions of a series of contraction curves taken from a single preparation, showing the effects of partial fatigue on the contraction of annelid muscle. This record was made by a preparation of eighteen segments taken from just back of the crop region, the apparatus being so arranged that the muscle was stimulated anew each time as soon as it had become nearly totally relaxed.

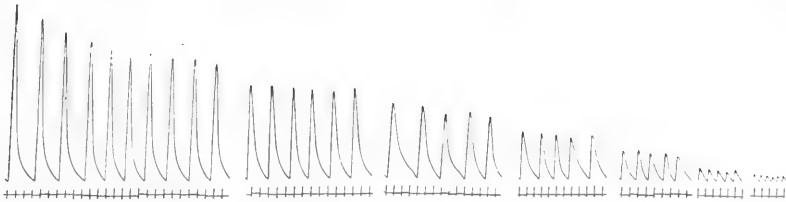


FIGURE 14.— Fatigue curve, in which are reproduced contractions 2-11, 20-25, 40-44, 60-64, 80-84, 106-110, 120-126. Time intervals, 4 seconds. One half the original size.

While changes in the contraction phase are easily noted, the main effect of the fatigued condition is greatly to lengthen the relaxation period. The later curves in the series show relaxation to begin with increasing hesitancy, and to take place at a nearly uniform rate from the apex of the curve to the base line. Since the drum was moving at a uniform rate, we may note the relation between the distance over which the drum moved while the muscle was contracting and relaxing, and the height of the contraction. This relation, in the case of the second contraction is represented by the ratio 7:47; the corresponding ratio for the sixtieth contraction is 5 : 13; and for the one hundred and twentieth, 2 : 2.

A second tracing, Fig. 15, is inserted to further illustrate differences in the form of contraction curves as the muscle becomes progressively fatigued. Changes in the contraction phases are particularly noticeable here.

Pertaining to Straub's suggestion that the muscle curves with sharp apices may be due to a fatigued condition of the muscle, it should be said here, that, while preparations showing very slow

relaxation when fresh, often come to relax much more quickly after having made a number of contractions, and thus give the sharp curves, yet the opposite is as often true also. These facts, together with Fig. 14, which is representative of many tracings taken from perfectly fresh preparations, offer convincing evidence, it would seem, that a fatigued condition alone cannot be regarded as indispensable to an explanation of the dimorphic character of single contraction curves.

In connection with the study of fatigue in the annelid muscle, it has been possible to add another instance of temporary "recovery"

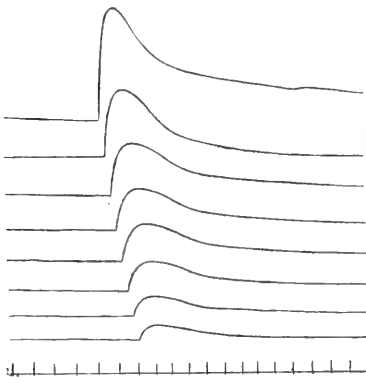


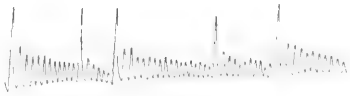

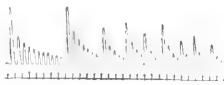
FIGURE 15.—The effect of fatigue on the form of the muscle curve. Contractions 2, 14, 29, 45, 61, 77, 95, and 114 are given in this figure. Time curve, 1 second intervals.

of the preparation on reversal of the constant current used as a stimulus, a phenomenon described several times before this.¹ The facts noticed have been as follows: in the case of a muscle which has become fatigued as a result of repeated contractions, provoked by constant current stimuli of short duration, an apparent resuscitation is effected, without any pause, by a mere reversing of the direction in which the current passes through the preparation. Engelmann's ('70) explanation of the matter was, that different parts of the muscle contracted, some with the current passing in one direction,

and other parts on reversal of the current. This theory has not been upheld, however. If it were true, it would seem that it could be proven by the use of a preparation like that of an annelid muscle, which, more than almost any other, is closely connected with a segmented condition of the other systems of the animal, and which consequently has been accustomed to contract in some parts of its length while remaining quiescent in others. In order to make way for this difference in contracting areas to become evident, the muscle was clamped in the middle, and the ends attached to separate recording levers. The result of the treatment of a muscle so arranged with constant current stimuli, however, merely tended to make the theory of Engelmann more improbable, for each end re-

¹ See ENGELMANN ('70), MORGEN ('90) and STEWART ('00).

sponded equally well to a current in either direction, and showed the same phenomenon of apparent recovery. Stewart ('00) has shown the same facts to hold in the bladder muscle of the cat.

Fig. 16 (*a*) shows the record of one end of a muscle held in a clamp as described, and stimulated several times with an ascending, then with a descending current, again with an ascending, and so on. "Recovery" is of very short duration, and the record leads one to question the applicability of that term to the reaction so demonstrated. Tracing (*b*) of the same   

Both of the tracings to which reference has just been made show a rapidity in the progress of "fatigue" which is characteristic of all experiments in which contraction is produced by stimulation with brief constant currents. It would not seem that the terms fatigue and recovery can be strictly applicable, for the reason that a muscle responding with a good degree of vigor to one hundred or more induction stimuli, will, in response to stimulations with brief constant currents give not more than six strong contractions. Were Engelmann's theory true, this wide difference would still be unexplained. That the condition in which the muscle is left after six contractions is identical with its condition after one hundred contractions is certainly not what would be expected. Again, true recovery from true fatigue, without a resting period, is extremely exceptional, if indeed it ever occurs.

That a change in the direction of the current does act as a stimulus to more vigorous contraction is entirely apparent; and in seeking an explanation for any physiological phenomenon, the action of a gross tissue under consideration is found ultimately by ascertaining the activities of its component parts, or cells. The effect of these brief currents on the individual cell cannot be postulated, save from a process of inference; it does not act independently, but in colla-

FIGURE 16. — (*a*) Tracing showing recovery on reversal of the current. The record shows five series taken alternately with ascending and descending currents, commencing with the former, the preparation being clamped at the middle. (*b*) Same as (*a*), except that the muscle was suspended by one end. Time intervals in both, 4 seconds. One half the original size.

boration with those confining it on all sides. These restrictions, however, cannot be supposed to prevent the protoplasm of each cell from undergoing a process, which may be analogous, at least, to polarization, during the passage of a constant current. If we assume that this be so, the effect of a sudden reversal of the current would be considerable, and, we may fairly imagine, would act as a decided stimulus; contraction being the only form of activity of these cells, this change in electrical conditions manifests itself in that way. As soon as the repolarization of the cell is accomplished, however, repetitions of brief currents in the same direction would be less excitatory, and the contractions provoked be less strong. Such is found to be the case, as is most evident in Fig. 16 (*a*). It is noticed there that after a single high contraction, the curves become of very moderate extent, and do not diminish rapidly thereafter. Assuming that polarization of the cells does come in as a factor in these phenomena, these weaker contractions are accounted for as being merely the responses to make and break of the current.

That the above paragraphs at all accurately describe what takes place in a muscle showing these phenomena of fatigue and resuscitation, is perhaps improbable; but in lieu of no explanation at all, they may be suggestive.

The length of time during which annelid muscle remains irritable, after its excision from the worm, compares favorably with that noted for other smooth muscle preparations.¹ While for reasons of fatigue, or other causes, it becomes unfit for use after a half hour of experimentation, when kept at a temperature in the neighborhood of six degrees, a piece with nerve cord cut out, ready for use, will respond to rapidly interrupted induction shocks after a period of four days. Straub ('00) notes perfect vitality in nerveless preparations after eight days. There has been apparent in the course of these experiments an interesting connection between the presence of the nerve-cord and the duration of life; but at the present time data have not been collected sufficiently to warrant further attention to the point.

SUMMARY OF CONCLUSIONS.

Spontaneous movements are present in all preparations of Lumbricus muscle containing the ventral nerve-cord, or remnants of it,

¹ See references previously given on work of SERTOLI, BOTTAZZI, STEWART, and DE ZILWA.

but are not seen in those where it has been thoroughly cut out. These movements may appear as simple or compound rhythms.

Make and break induction currents produce contractions, which vary in character according to strength of stimulus. Response does not appear to be in direct proportion to strength of stimulus. No refractory period is present.

Repeated stimuli result in summated contractions, which, within limits, have amplitudes in proportion to the number of stimuli. Tetanus is obtained on repetition of stimuli at the rate of about four per second.

The muscle contracts at both make and break of the constant current. The response to the break of the current is greater, the longer the current has been flowing, at least, within limits. To obtain responses of make and break separately, at least two seconds must elapse between them.

A weak constant current produces relaxation in a muscle already in a state of tonic contraction, and hastens relaxation after an induction shock. Strong constant currents produce a state of contraction resembling tetanus. No antagonism between make and break effects of the constant current is apparent.

With increasing temperature, *Lumbricus* muscle shows little constancy in changes of tone below 15° C., and the first uniform result is a gradual loss in tone beginning at 20° C. Rapid loss of tone occurs between 30° and 40°: an improvement from 42° to about 55°, when rapid relaxation sets in; this changes to final heat rigor at about 65° C.

In the case of pieces of muscle containing the nerve cord; automatic movements appear, during rising temperature, between 28° and 38° C. Loss of irritability to artificial stimulation takes place at about 34° C., responses having been obtainable during the previous 30°. Maximum contractions to induction currents are given at about 18° C.

Lumbricus muscle fatigues rapidly, when the contraction and relaxation periods are short and the stimuli frequent. Rapid action is more characteristic of muscle from the posterior region of the animal, than of that from the anterior.

The extent of any one contraction has a very definite dependence on the extent of the preceding contraction, and also on the length of the preceding relaxation period.

A muscle which has been fatigued by constant currents of short

duration, shows marked "recovery" upon reversal of the direction in which the current is applied; and this "recovery" is confined to no particular part of the preparation, *i. e.*, to neither anodic nor cathodic regions.

Recovery of the muscle under ordinary conditions is quite complete, but of short persistence.

While an unused preparation, kept at low temperatures, may remain irritable for upwards of four days, constant experimentation for fifteen minutes unfits the muscle for further use.

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A CONTRIBUTION TO THE PHYSIOLOGY OF THE NER-
VOUS SYSTEM OF THE MEDUSA GONIONEMA¹
MURBACHII. PART II.—THE PHYSIOLOGY OF THE
CENTRAL NERVOUS SYSTEM.

By ROBERT M. YERKES.

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I. STATEMENT OF PROBLEMS.

IN Part I. (Yerkes, :02) of this experimental study of the func-
tions of the sense organs and nervous system of *Gonionema* the
motor reactions of the organism to chemical, mechanical, and photic
stimuli were considered without special attention to the part played
by the central nervous system in such reactions. The present paper
is primarily concerned with the relation of these motor reactions to

¹ Since the name of the genus is derived from *γωνία* (angle) and *νήμα* (thread,
i.e., tentacle), the proper spelling is *Gonionema*, not *Gonionemus*, which, in accord-
ance with the prevailing custom, was used in Part I. of this paper.

the functioning of the central nervous system, and with the bearing of certain experiments upon problems of the functional importance of this system.

The chief problems of the paper may be formulated thus:

1. Do the special reactions of the tentacles, manubrium, and bell depend upon the activity of the central nervous system?
2. What is meant by irritability and spontaneity, and in what relation do they stand to the central nervous system?
3. Does coördination depend upon the functional activity of the central nervous system?
4. Is there any evidence of the existence of special nerve centres of spontaneity and coördination?
5. Finally, what are the functions of the central nervous system?

II. ANATOMICAL CONDITIONS.

Since the histological conditions of the nervous system of *Gonionema* have never been investigated, we are forced to make use of what is known about the structure of the nervous system in other *Hydromedusæ* which closely resemble *Gonionema*. In the following discussion we shall have cause to refer to gross anatomical conditions only, so the lack of definite and detailed knowledge concerning the nervous system of *Gonionema* will not seriously affect the interpretation of the experiments under consideration; moreover, there is every reason to suppose that the following description of the nervous system of *Hydromedusæ* holds good for *Gonionema*.

According to the studies of the brothers Hertwig (O. und R. Hertwig, '78) *Hydromedusæ* possess a double nerve ring in the margin of the swimming bell, and a plexus of nerve fibres and cells in the subumbrellar tissue of the bell. The dorsal portion of the nerve ring, lying on the exumbrellar side of the mesogloea, is known as the upper nerve ring, and the ventral portion, lying on the subumbrellar side of that layer of tissue, as the lower nerve ring. These rings, though united by fibres which traverse the mesogloea, differ in constitution, the lower containing larger fibres and more cells than the upper. This double nerve ring constitutes the so-called central nervous system of the *Hydromedusæ*. The fibres and cells found in the subumbrella constitute the peripheral nervous system.

III. THE REACTIONS OF CERTAIN ORGANS AND PARTS OF
GONIONEMA CONSIDERED IN THEIR RELATIONSHIP
TO THE CENTRAL NERVOUS SYSTEM.

A. The reactions of the tentacles.—The tentacles of normal *Gonionemata* react to nearly all stimuli by a contraction which simply shortens the organs, but to some foods and to motile touch stimuli they frequently react by twisting into the form of a corkscrew in contracting. Nagel has described this as the “corkscrew reaction.” It may now be asked, Are these reactions of the tentacles dependent upon the central nervous system?

This question finds its answer in the results of experiments upon isolated tentacles. Tentacles were cut from the bell about a millimetre from their attachment and placed in Stender dishes containing sea-water. For a few minutes after excision they usually remained in a contracted condition; then expanded and became very active and sensitive to stimuli. Gelatine or meat applied to them called forth the “corkscrew reaction.” To other stimuli they responded with the usual straight contraction. It is therefore evident that the tentacle contains within itself the mechanism necessary for these reactions, and is not dependent upon the functional activity of the entire organism, nor upon the central nervous system for its ability to execute them.

Berger (:00, p. 13) states that the excised tentacles of the *Charybdæ* exhibit both “squirming” and straight simple contractions. Parker ('96, p. 112) finds that the tentacles of *Metridium*, after being cut off continue to make movements appropriate for carrying food to the mouth. The tentacles of *Carmarina hastata*, according to Nagel ('94, p. 525), exhibit both twisting and straight contractions. He, however, states that the general contraction and the twisting reaction are not given by excised tentacles.¹

The one point of agreement in the results of my study of the excised tentacles of *Gonionema* with those of Nagel for *Carmarina* is

¹ “Bezüglich der Reizbarkeit der Tentakel habe ich noch zu bemerken, dass abgeschnittene Tentakeln oder Theile von solchen wohl noch die lokale Contraction (in freilich undeutlicher Weise), nie aber das rasche Zusammensucken und die spiralige Krümmung zeigen. Zu dem Zustandekommen der letzteren bedarf es offenbar eines durch eine sensible Erregung ausgelösten rückläufigen Impulses, dessen funktionelles Centrum im Nerveringe zu suchen ist” (Nagel, '94, p. 525).

that the excised tentacle does not respond to motile touch with the corkscrew reaction, whereas the normal tentacle frequently does. But I do not feel justified by these experiments in drawing the conclusion that this reaction is dependent upon the central nervous system; for the stimulus is difficult of application to the isolated tentacle, and it is not improbable that the failure of the organ to react was due to the weakness of the stimulus.

B. The reactions of the manubrium.—The manubrium of normal animals reacts to food by bending toward it, moving the lips, and making what appear to be attempts to reach the substance. In case of a strong stimulation of any part of the bell the manubrium points toward the irritated region. Local stimulation of the organ usually causes it to bend toward the side irritated, unless a contraction of the bell occurs. Are these activities controlled by the central nervous system?

If the manubrium be cut away from its attachment to the umbrella, and the isolated organ pinned at its base to the wax-covered bottom of a dish, it will after a few minutes respond to stimuli in apparently the same way as when in connection with the functioning nervous system. There is some evidence that the excised organ responds more slowly than the normal, but my data do not suffice for a definite statement concerning this point.

So far as I have been able to determine, all the reactions of the manubrium—contraction and expansion of the mouth, lip movements, and the various phases of the food-taking reaction—can be executed by the organ when it is separated from the central nervous system.

My experiments on *Gonionema* have led me to conclusions *similar* to those of Romanes ('85, p. 34). He states that the excision of the margin of the bell does not affect, *in the smallest degree*, the activities of the manubrium, in either the naked- or covered-eyed medusæ, and that even after complete severance of the organ from the body it continues its actions. Nagel ('94, p. 520) obtained practically the same results with *Carmarina hastata*.

Whereas Romanes emphasizes the fact that the reactions of the mutilated animal are the *same* as those of the normal animal, I can only say that they are *similar*, for there is abundant evidence of important differences in reaction-times, and in the sensitiveness of normal and mutilated animals. All that we are justified in concluding from these experiments, therefore, is that the central nervous

system is not essential for the execution of the actions of *Gonionema*. We cannot say that the actions are executed as rapidly and as accurately when the nervous system is not concerned; we cannot say they are the *same*, we can say they are *similar*.¹

C. The reactions of the bell.—The most important reactions of the bell are the general contraction, which causes locomotion, and local contractions in response to strong stimuli. In response to stimuli of considerable intensity the normal animal ordinarily gives a rhythmic series of bell contractions, but the bell from which the sense organs and nerve ring have been cut away reacts to the same kind of stimulus by a single contraction, or in rare cases by two or three contractions; it never contracts rhythmically. Such a marginless bell is able to execute local contractions much as does the normal *Gonionema*.

In the rhythmic action we have found, it would seem, a reaction to stimuli which is dependent upon the central nervous system, or at least upon the structures contained in the margin and tentacles. It is of course conceivable that the reaction should depend upon the sense organs of the margin and tentacles rather than upon the nerve ring, but I have been unable thus far to make any experiments to settle the matter. In the following sections an attempt will be made to show the probable relation of rhythmic action to the nervous system.

IV. IRRITABILITY AND SPONTANEITY IN RELATION TO THE CENTRAL NERVOUS SYSTEM.

A. Irritability.—By the irritability, or sensitiveness, of a tissue we mean its susceptibility to the influence of changes occurring about it which we know as stimuli.

Now, the various parts of *Gonionema* differ widely in irritability. The tentacles, for example, are extremely sensitive; the exumbrellar surface and the jelly of the bell are insensitive. It is significant that in those tissues which give no evidence of sensitiveness neither nerve

¹ As a continuation of this study of the physiology of the nervous system of *Gonionema*, I propose to make quantitative determinations of the sensitiveness of normal and mutilated animals, and of the reaction-times of organs when in connection with the central nervous system and when separated from it. For, everything now indicates that it is from this kind of physiological work we may hope for important facts concerning the functions of the nervous system of simple organisms.

elements nor muscle cells have been discovered. Comparative physiology, however, does not permit us to conclude from this that irritability is a property peculiar to nervous and muscular tissues, but only that it is the chief or special characteristic of the former and an important, though secondary, characteristic of the latter, while it may be possessed in a less degree by other tissues. We may therefore suppose that in case of the exumbrella of *Gonionema* either the sensitiveness is so slight that no motor reaction can be produced by stimuli, or, the tissues being non-contractile, there can be no visible expression of irritability. As a matter of fact the exumbrella does not transmit impulses, neither is it contractile: under these conditions no ordinary tests would disclose irritability.

Furthermore, different groups of cells, or perhaps individual cells of the same tissue and cell group, possess at any instant different degrees of sensitiveness, and this presumably because of slight differences in their approach to a state of perfect equilibration. Certain cells, for example, because of more abundant nutriment, may be much more easily disturbed than are others which possess less perfect nutrition.

The general sensitiveness of *Gonionema* varies with the state of the animal and with external conditions. Hungry animals are much more sensitive to foods and react more quickly than do well-fed individuals. The continued action of strong stimuli, such as acids, certain salts, electricity, and heat or cold soon decreases sensitiveness. Experiments proved that acidulation of the water in which *Gonionemata* were swimming caused a gradual loss of sensitiveness to chemicals, but even after an animal had become entirely insensitive to chemicals it would sometimes react to strong mechanical stimuli.

In order to ascertain the effect of solutions of KCl upon the sensitiveness and the rate of the rhythmic contractions of intact *Gonionemata* and upon fractions of the animal, a series of experiments was made. A solution of KCl ($\frac{1}{4}$) acts as a stimulus for *Gonionema* and causes contractions of the bell. The experiments were planned to determine (1) whether the rate of the rhythm of the bell decreases or increases during the first four minutes of the action of the KCl solution; (2) whether the rate is the same for pieces of the animal as for the whole, and (3) whether the rate changes more rapidly for pieces than for the whole.

Four animals were taken, Nos. 1 and 2 (see table) being about 8 mm. in diameter, Nos. 3 and 4 about 14 mm. These animals were

placed, one at a time, in a $\frac{2}{4}$ KCl solution in sea-water, and the contractions of the bell counted in case of each animal, for the first, second, third, and fourth minutes after immersion. At the end of the four minutes the animals were returned to pure sea-water and left for half an hour that they might recover from the effects of their prolonged activity. The individuals were then, in turn, divided into halves by a vertical cut through the middle of the bell; one of the halves was left in the sea-water, the other was placed in the $\frac{2}{4}$ KCl solution, and its rate of contraction determined, as had been done for the whole animal. This having been accomplished, the half remaining in the water was again halved and the rate of the rhythm for a quarter of the animal determined as before. And, finally, the remaining quarter was halved in order that the same observations might be made with an eighth of the bell. Each of the pieces used contained at least one radial canal; the halves contained two.

The results as given in the table show: (1) that the whole animal has a more rapid rhythm than any fraction of it; (2) that the smaller the piece the slower is the rhythm; (3) that the rate of contraction for the whole animal and for the pieces gradually decreases during the time of observation; (4) that this decrease is more rapid for a part than for the whole, and more rapid for the smaller part than for the larger.

Mutilation of *Gonionema* causes no obvious decrease in sensitiveness, but the results just presented seem to show that fractions of an animal are not able to react as rapidly as can the whole. At times excised tentacles appear to be rendered more sensitive by being cut off; this irritability, however, is soon lost.

From the above considerations it is clearly impossible to regard irritability as a function of the central nervous system.

B. Spontaneity.—Normal *Gonionemata* make rhythmic movements of the bell when there is no evident stimulus present. It is to such actions that the term spontaneous is applied. All reactions are the result of changes which operate as stimuli, but in certain cases these changes are exclusively internal and cannot be detected by the experimenter, or they may be external changes of such a nature as to be unobservable by ordinary methods. Whenever we can discover no environmental cause for a reaction, we say it is spontaneous. Unmutilated *Gonionemata* exhibit spontaneity: What of individuals which lack the central nervous system? Experiments have shown that removal of the entire margin of the bell completely destroys

TABLE SHOWING NUMBER OF CONTRACTIONS PER MINUTE OF WHOLE BELLS AND OF FRACTIONS OF BELLS WHEN PLACED IN $\frac{1}{4}$ KCl SOLUTION.

Animal.	Minute.	Whole Animal.	Half.	Quarter.	Eighth.
Number 1	1st	87	67	95	65
	2d	74	55	89	65
	3d	69	48	54	47
	4th	58	60	40	38
Number 2	1st	96	102	79	0
	2d	75	81	50	5
	3d	75	63	57	11
	4th	70	48	49	3
Number 3	1st	91	98	78	95
	2d	72	67	62	84
	3d	80	59	45	77
	4th	66	50	43	58
Number 4	1st	77	44	50	39
	2d	70	47	47	42
	3d	70	41	41	27
	4th	55	38	47	18
Averages for the four animals. .	1st	88-	78-	75+	50-
	2d	73-	63+	62	49
	3d	73+	53	49+	40-
	4th	62+	49	45-	29+
General averages	74	60+	58-	42

spontaneous movements. The margin itself after the operation continues to move rhythmically at intervals for hours; it shows both spontaneity and sensitiveness to stimuli, but the marginless bell usually contracts only in response to some strong stimulus, and then

only irregularly and a few times, never rhythmically. It thus appears that the spontaneous activity of *Gonionema* is dependent either upon the nerve ring or upon the sense organs of the margin of the bell.

Romanes ('85, p. 27) is authority for the statement with respect to naked-eyed medusæ that, "Excision of the extreme margin of a nectocalyx causes immediate, total, and permanent paralysis of the entire organ." He adds (p. 28): "From this experiment, therefore, I conclude that in the margin of all the species of the naked-eyed Medusæ which I have as yet had the opportunity of examining, there is situated an intensely localized system of centres of spontaneity, having at least for one of its functions the origination of impulses, to which the contractions of the nectocalyx, under ordinary circumstances, are exclusively due."

These statements of Romanes are not true for *Gonionema*. In it paralysis is neither total nor permanent. A few times I have seen marginless bells contract when no stimulus could be detected, and repeatedly in these experiments mechanical or chemical stimulation of such bells has resulted in one or even a series of as many as five contractions.

These facts indicate that the margin (including the tentacles) is the most sensitive portion of the animal. Its removal simply renders the organism unresponsive to intensities of stimuli which uniformly call forth reactions of the normal animal. The marginless bell is sensitive to, and capable of responding to, strong stimuli; it is not sensitive to those unobservable stimuli to which spontaneous action is due; hence, no margin, no spontaneity of action. From this standpoint the so-called "centres of spontaneity" are only regions of greater irritability than other portions of the body. Spontaneity is a function of irritability. If the bell were as sensitive as the margin, that is if it contained cells capable of responding to such slight changes as do those of the margin, it would, after separation from the central nervous system, react in approximately normal fashion, as do the excised tentacles or manubrium.

Since nerve cells are the most sensitive cells of the organism, spontaneity is undoubtedly a function of the nervous system. But until it is proved that the sensory cells of the tentacles or margin, which are in direct connection with the nerve ring but are not a part of the central nervous system, do not mediate the spontaneous movements of *Gonionema*, spontaneity cannot be called a function of the *central* nervous system.

Loeb (:00, p. 383) has discovered that the marginless bell will beat rhythmically in pure $\frac{5}{8}n$ NaCl or NaBr solutions. "Irritability," he writes, "depends upon the various ions (Na, Ca, K, Mg) existing in definite proportions in the tissues." This tells us that the possibility of stimulating a tissue depends upon its chemical constitution, as well as upon the nature of the stimulus. Thus a further step is taken toward the explanation of activity in physical (chemical) terms. It is obvious, then, that irritability depends upon chemical transformations, whilst spontaneity is dependent upon irritability. Simply because nerve cells are extremely susceptible to chemical or electro-chemical changes occurring about them they are all important in the mediation of spontaneous actions.

C. Centres of spontaneity.—There is one phenomenon in my experiments which may be taken as evidence of the presence of centres of spontaneity in the margin of the bell. When the margin is excised and its continuity interrupted by a cut at one point, it has a tendency to assume a spiral form, and examination of such cases usually shows that the spiral consists of three or four more or less perfect volutions, all of which beat in the same rhythm when the margin is in pure sea-water. If acid is added to the sea-water in sufficient quantity to destroy the regularity of the beats, it is noteworthy that the volutions begin to beat in independent rhythms. From this we might argue that there are as many centres of spontaneity in the margin as there are volutions with independent rhythms in the excised margin. Conditions point to four as the number of such centres.

In explanation of the spiral form of the margin it may be said that the radial-canal regions are the most highly contractile portions of the bell, as is proved by the fact that when the contractions of the bell for any reason become irregular the form assumed by the contracted organism is always such as would result from stronger contraction at the radial canals than elsewhere, hence, when the margin is excised its contraction results in the formation of as many ring-like portions as there are regions of especially high contractility. Difference in contractility accounts for the rings; how are the individual rhythms assumed by these rings to be explained? The only suggestion I can make is that the rhythms are referable to centres of special sensitiveness. Each of the four radial-canal regions may be supposed to contain such a region, whose function would be that of a centre of spontaneity. It might also be said that the

inequality of contraction is in itself sufficient to break up the coördination of movement and establish as many rhythms as there are regions of strong contraction. I do not wish to lay much stress on this explanation, however, nor on the phenomenon of ring formation as evidence in favor of centres of spontaneity.

So far the reactions of *Gonionema* appear to depend entirely upon the irritability and contractility of muscular or neuro-muscular tissue. In one region a $\frac{1}{1000}$ HCl solution suffices to call out a contraction; in another a $\frac{1}{100}$ causes no motor reaction, simply because the cells either are not sensitive to it or are non-contractile. All the facts indicate, therefore, that the margin of *Gonionema* contains cells which, because of extreme sensitiveness, control the spontaneous activities of the bell.

V. COÖRDINATION AND THE TRANSMISSION OF IMPULSES.

A. Normal coördinated movements.—*Gonionema* ordinarily moves by reason of perfectly coördinated, rhythmic contractions of the bell. There is no visible irregularity in the movements, no sign of a contraction wave sweeping around the bell; it appears as if all portions of the organ entered the contraction phase of reaction at the same instant.

For the purpose of discovering, if possible, on what structures and what conditions coördination depends, experiments were made in which animals were cut in various ways or exposed to the influence of certain chemicals. We may consider first the effects of operations on the bell.

B. The effects of operations. *Experiment 1.*—The margin was cut at four equi-distant points, midway between each pair of radial canals. The quadrants continued to beat in perfect coördination, just as before the operation.

Experiment 2.—The incisions, instead of severing the margin only, were carried into the bell half way to the manubrium, thus dividing the animal into four parts, which were connected at the apex of the bell. As a result no change in coördination could be detected, but there was little progressive movement. The animals seemed even more sensitive than before the operation, but fewer contractions were given in series than usual, ordinarily not more than five. Stimulation of any part of the margin of one of the quadrants caused a general contraction in which all the quadrants participated, to all appearances

simultaneously. Since in this case the impulse must be carried from one quadrant to another through the substance of the bell, it is clear that transmission must be quite rapid in the bell. Touching the margin, tentacles, or subumbrella caused immediate reaction; touching the manubrium and exumbrella rarely, and in case of the latter only after several seconds' delay. This operation sometimes destroys coördination for a few seconds.

Experiment 3.—The same incisions were extended to the base of the manubrium, so that the quadrants remained in connection by a region at the apex of the bell which was not over four millimetres in diameter. The quadrants now bent in toward the mouth independently. In response to stimulation at any point they contracted, to all appearances simultaneously. Rarely there seemed to be slight irregularities, one part responding before the others. The animals fed imperfectly. Evidently the stimulation of one quadrant still produces an approximately simultaneous reaction of all because of rapid transmission. Since the tissue which connects the quadrants consists of three parts,—the outer, or exumbrellar layer, the inner, or subumbrellar layer, and the jelly substance of the bell between them,—we may now inquire which, if not all, of these layers transmits the nervous or muscular impulse. The following experiments furnish an answer to the question.

Experiment 4.—In an animal operated upon as in Experiment 3 the radial canal of one quadrant was severed and a small section of it removed so that no impulses could pass along that region. This was done because it seemed not unlikely that the radial-canal region was the chiefly important transmitting portion of the bell. After the operation coördination persisted, although at times the quadrant with mutilated canal region reacted somewhat slowly. It may well be, however, that this was due to a lessening of the contractile power of the quadrant, rather than to interference with transmission. The quadrants almost always contracted unequally. The reactions were slightly slower than in case of the other experiments, and there was considerable evidence that the radial-canal region transmitted impulses more quickly, perhaps because more directly, than other portions of the bell.

Experiment 5.—Incisions were made so that a segment of the bell between two radial canals was left attached to the remainder of the organism by a narrow strip of tissue at the apex of the bell. Stimulation of the larger portion of the organism caused it to react at

once, and after a delay of one half to one second the smaller segment contracted. Likewise stimulation of the smaller segment caused it to react immediately, whereas the larger segment contracted a fraction of a second later. This proves conclusively that *the radial-canal region transmits impulses more rapidly than the remainder of the bell substance.*

Since in this experiment coördination was destroyed, it is clear that coördinated movements depend upon the continuity and transmitting power of the tissues, in all probability of the muscular tissue as well as the nervous, and not upon the functioning of any special nerve centres.

Experiment 6.—After a segment had been cut as described in the preceding experiment, the *subumbrellar layer* of the strip of tissue which formed the connecting link between the small and the large segments was cut, so that the *exumbrellar layer* and the jelly substance alone connected the segments. Now the large portion, when stimulated, reacted, but the small one did not. Stimulation of the small segment caused it alone to contract. This experiment was supplemented by observation of reactions when the *exumbrellar tissue* and when both the *exumbrellar layer* and jelly were severed instead of the *subumbrellar layer*. In such experiments the two segments reacted to the same stimulus. This proves that *of the three layers of the bell the subumbrellar alone transmits impulses.*

Experiment 7.—From the smaller segment of an animal operated on as in Experiment 4 the margin was cut away, and the animal then placed in $\frac{2}{4}$ KCl solution. The main part of the bell beat rhythmically in response to the chemical stimulus, but the small segment deprived of its margin contracted only a few times, and then very irregularly.

From the results of the foregoing operations we may conclude: (1) That coördination is dependent upon the ability of the tissues to transmit impulses rapidly, not upon the continuity of the nerve ring; (2) that transmission in the bell is most rapid in the radial-canal regions; (3) that of the three layers of tissue composing the bell the *subumbrellar layer* alone transmits impulses; (4) that spontaneity is not interfered with by operations upon the margin and bell, but (5) that removal of the margin destroys spontaneous action because (6), as Experiment 7 indicates, the margin contains cells which are sensitive to stimuli that do not act on the remainder of the bell substance.

In his chapter on coördination Romanes ('85, p. 130) remarks that since in covered-eyed medusæ the stimulus wave does not move faster than the contraction wave, it is impossible to hold that the ganglionic centres control one another independently of the contraction wave. Romanes found that in the medusæ mentioned one or more of the lithocysts are either temporarily or permanently pre-potent over the others (*i. e.*, they are more sensitive), and that from these pre-potent regions start contraction waves, which pass rapidly to other regions; thus a general contraction of the bell is brought about by the sweep of a contraction wave from a pre-potent region to all other parts of the bell.

My study of the reactions of *Gonionema* furnishes evidence that Romanes' statements are true of this form also. There are many reasons for believing that the contraction of the *Gonionema* bell, even in what appears to us as perfectly coördinated movement, is really due to a contraction wave which is initiated at one, or possibly a few, very sensitive points, and passes so rapidly to adjacent regions that the eye can detect only the general contraction, except when fatigue or the action of some chemical lowers the rate of the wave's movement. In this connection the effects of chemical stimuli on coördination and the rate of movement of the contractile or nervous impulse are of interest.

C. The effects of chemicals.—*Gonionemata* put into $\frac{1}{150}$ solutions of acetic, hydrochloric, sulphuric, or nitric acid in sea-water, at first show a marked increase in the rhythm of the bell. But after a few seconds the beats become irregular, now slow, now fast, and finally pass into what is comparable with muscular fibrillation. In this condition the whole bell trembles, and there is no general contraction. It looks as if a multitude of regions in the margin were contracting independently of one another. At times, instead of this tremor, contraction waves appear. Unless the animal is removed to pure sea-water within a few moments after coördination is lost, it becomes completely and permanently paralyzed. But if, after the acid has acted for from ten to twenty seconds, it be transferred to sea-water, irregular, uncoördinated contractions continue for a time, then give place to coördinated action. In these irregular contractions first one region then another is seen to contract. Occasionally a contraction started at one point may be seen to pass around the margin as a contraction wave. This makes it not improbable that in the normal animal there is a contraction wave which is too rapid

to be perceived as such. The acid interferes with either the rate of the contraction or the nerve impulse wave and thus makes it visible.

KOH, so far as my observations go, never destroys coördination as do acids. KCl, on the other hand, does. Severe shocks, jars, and exposure to air frequently cause, for a time, irregular beats.

D. A theory of coördination.—The sensory regions of a normal *Gonionema* differ in sensitiveness among themselves at any instant, and from moment to moment. The changes, neural and muscular, or muscular alone, which result in reactions of the organism may be due (1) to a local stimulus which initiates a contraction wave at some point, or (2) to a general stimulus which acts upon one or more of the most sensitive regions (the pre-potent regions). When an animal is partially paralyzed,—as, for example, by certain chemicals,—transmission is comparatively slow and the progress of the contraction wave can be directly observed. (See Romanes, '85, p. 93, *et seq.*)

The coördinated actions here considered are such as involve the apparently simultaneous activity of a number of parts, which under certain circumstances act successively or in an irregular manner. In connection with the actions of the bell of *Gonionema* coördination has been used to refer to the simultaneous occurrence of systole or diastole in all parts of the bell; whereas, under certain conditions, as will be shown later, some regions may be in systole while others are in diastole.

Now, if every beat of the bell involves a contraction wave, it is evident that there is, strictly speaking, no such thing as the simultaneous contraction of all the contractile units of the organ, but instead a rapid succession of contractions. Assuming that the contraction of the bell results from the successive instead of simultaneous action of the units of contraction, how can the loss of coördination be explained?

It may be said that violent stimuli either temporarily increase the number of sensitive regions which are sending out nervous or muscular impulses at any instant, or decrease the contractility of the tissues. If the first occurs, instead of having one impulse (which has arisen in a pre-potent region) control the contraction by originating a wave, we have the phenomena of a number of regions responding at about the same instant to the stimulus. This results in a series of sharp, violent contractions at various points in the bell, which block one another and give the phenomenon of "tremor" instead of the usual contraction wave. If, on the other hand, the

contractility is decreased, certain regions, such as the radial-canal regions, are able to respond quickly, whereas others do not; hence there arises irregularity.¹

The theory of coördination which I have suggested is based on Romanes' pre-potency idea and on the experimental evidence of my study. It receives support from the work of Loeb, Lingle, and Porter. Loeb (:00^a, p. 25) writes, "Thus we see that the whole heart beats in the rhythm of the part that has the maximum number of contractions per minute. From this we must assume that the coördination of the heart's activity is due to the fact that the part which contracts most frequently forces the other parts to contract in the same rhythm." In the frog's heart it has been observed that the sinus venosus beats faster than the other parts, and therefore controls the rhythm. Also, in the ascidian heart the region sending out the first contraction controls the direction of the wave of contraction. Porter ('99, pp. 130 and 133) discovered that the synchronism of the mammalian ventricles is not dependent on nerve cells, but is in all probability due to muscular conduction. From what has already been said, it will be seen that this is probably true of *Gonionema* also.

As a result of the consideration of coördination in *Gonionema* the following are provisional conclusions: (1) Coördination depends on specific irritability and the transmission of impulses (it is uncertain whether the impulses are nervous or muscular), not on the action of special nerve centres; (2) there are no special centres of coördination; (3) all normal bell contractions consist of waves of such rapidity as to be invisible as such; (4) normally the most sensitive region of the animal, or a region which is affected by a local stimulus, initiates a contraction wave; (5) when the sensitiveness of the animal is so increased that many regions are constantly being brought into contraction at the same instant, or when the power of transmission is decreased so that the wave passes slowly, coördination disappears, and there results either "tremor" or a visible wave, and (6) strong

¹ In a consideration of the phenomenon of tonus and fibrillation in the mammalian heart, PORTER ('99, p. 129 foot-note) suggests an explanation of the breaking up of contraction waves in the ventricle of the heart which seems applicable in case of *Gonionema*. He argues that strong contraction at certain points (our regions of pre-potency) presumably blocks the wave and thus leads to a series of partial contractions ("tremor" of *Gonionema*), instead of the normal synchronous action.

local contractions tend to block one another, thus breaking up the general bell contraction into a series of small and partial contractions, as in muscular fibrillation.

VI. SUMMARY.

1. The reactions of special organs or parts of *Gonionema* are not dependent for their execution upon the functional activity of the central nervous system.

2. Irritability, or sensitiveness, is a property of all parts of the animal except the jelly of the bell and the exumbrellar surface; it differs widely in degree for different regions.

3. Spontaneity is not dependent upon the central nervous system, but upon the high degree of irritability of certain parts of the margin of the bell.

4. The marginless bell of *Gonionema* fails to show spontaneous movements, except in rare cases, simply because it is insensitive to other than strong stimuli.

5. The rate of the rhythm of the bell when subjected to a continued stimulation by $\frac{2}{4}$ KCl is higher for the whole bell than for any fraction of it. The smaller the part of the bell, up to an eighth, the slower the rate.

6. Coördination is not dependent on the functioning of the nerve ring or of any special nerve centres, but upon the rapid transmission of an impulse, which is either nervous or muscular, probably the latter.

7. All tissues except the jelly and exumbrella are capable of transmitting impulses.

8. Certain chemical stimuli destroy coördination by increasing the irritability or decreasing the transmitting power of tissues.

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THE EFFECTS OF POTASSIUM AND CALCIUM IONS ON STRIATED MUSCLE.

By W. D. ZOETHOUT.

(From the Hull Physiological Laboratory of the University of Chicago, Ill.)

INVESTIGATORS working on the effects of potassium and calcium ions on the heart have generally found that calcium produces contraction and potassium relaxation of the heart muscle.¹ To see whether this held true for all muscle tissue, Dr. Loeb suggested that I try the effects of Na-, K-, and Ca-ions on the striated muscle.

A series of experiments were made on the gastrocnemius of the frog, and to our surprise we found that K-ions cause a prolonged contraction of the muscle and that Ca-ions antagonize this action of potassium. Since these experiments were made, I discovered that Ringer,² in 1886, had also found that calcium counteracts this peculiar effect of potassium.

The property of potassium to cause an immediate and prolonged contraction of striated muscle was also demonstrated by Grützner³ and by Zenneck.⁴ Grützner found that a 4 per cent KCl solution, applied to the motor nerve, does not cause a contraction, while if it be applied to a curarized gastrocnemius, it will cause a prolonged contraction. This fact I fully corroborated.

In these experiments the femur of the muscle preparation was fastened to a suitable support, and the tendon of Achilles was connected with a muscle-lever writing on a drum. The muscle was then submerged in the solution whose action we wished to test.

When the gastrocnemius is placed in a bath of $\frac{m}{8}$ KCl, a powerful and prolonged contraction results. The latent period is very short,

¹ GREEN: This journal, 1899, ii, p. 82; HOWELL: This journal, 1898, ii, p. 47; HOWELL: This journal, 1901, v, p. 181.

² RINGER: Journal of physiology, 1886, vii, p. 291.

³ GRÜTZNER: Archiv für die gesammte Physiologie, 1893, liii, p. 85.

⁴ ZENNECK: *Ibid.*, 1899, lxxvi, p. 21.

for the muscle contracts almost immediately on touching the solution. The contraction reaches its maximum in two or three seconds, and may remain in this contracted condition for from twenty to one

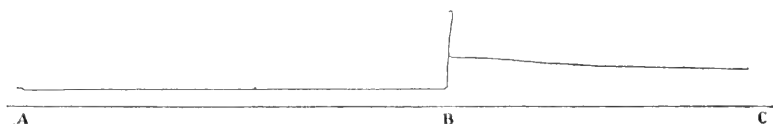


FIGURE 1.— At (A) the muscle is placed in a $\frac{m}{8}$ NaCl solution. At (B) the NaCl is replaced by a $\frac{m}{8}$ KCl solution.

hundred minutes. As a rule, the curve produced (see Fig. 1, 2, 3, and 4) is a smooth line, not showing any individual twitches.

The same results were obtained with the following salts of potassium:—

KClO₃, KNO₃, K₂SO₄, KI, KBr, K₂CrO₄, K₂Cr₂O₇, K-ferro-cyanide,
K-oxalate, K-cyanide, and K-tartrate.

If, instead of $\frac{m}{8}$ KCl solution, we use a less concentrated solution, the latent period is increased, the height and duration of the contraction are decreased, and the maximum contraction is not reached so speedily. The minimum strength of KCl capable of producing a contraction was found to be a $\frac{m}{80}$ solution. The contraction produced by a $\frac{m}{80}$ solution is not due to the greatly reduced osmotic pressure,



FIGURE 2.— At (A) the muscle is placed in a $\frac{m}{8}$ CaCl₂ solution. At (B) the CaCl₂ is replaced by a $\frac{m}{8}$ KCl solution.

for the same results were obtained by using a mixture consisting of 9 c.c. glycerine (isotonic with 0.7 per cent NaCl) + 1 c.c. $\frac{m}{8}$ KCl. Potassium solutions of greater concentration than $\frac{m}{8}$ produced more powerful contractions. When the $\frac{m}{8}$ KCl was diluted with $\frac{m}{8}$ NaCl, we found the same results as before, except that the minimum concentration was raised from a $\frac{m}{80}$ to a $\frac{m}{40}$ KCl solution.

If the KCl is diluted with CaCl₂, the power of the KCl to produce contractions is very much reduced. The calcium neutralizes the action of the potassium, as the following experiments demonstrate.

1. In a mixture of $\frac{m}{8}$ KCl and $\frac{m}{8}$ CaCl₂, the weakest solution capable of calling forth a contraction was found to be 4 $\frac{1}{2}$ c.c. of

$\frac{m}{8}$ KCl + $5\frac{1}{2}$ c.c. of $\frac{m}{8}$ CaCl₂. Compared with its dilution with water, the minimum concentration of the KCl solution increased from $\frac{m}{80}$ to $\frac{m}{18}$. This antagonistic action of Ca increases as the Ca molecules and ions increase. If, for example, the $\frac{m}{8}$ KCl solution is diluted with a $\frac{m}{2}$ CaCl₂ solution, the minimum concentration of the KCl is increased to $7\frac{m}{80}$ KCl.

2. The following still more clearly proves the neutralizing effect of calcium: At (A), in Fig. 1, a muscle was placed in a bath of $\frac{m}{8}$ NaCl. After fifteen minutes at (B), this was replaced by a $\frac{m}{8}$ KCl solution. The contraction which followed was the same as that produced by a perfectly fresh muscle.

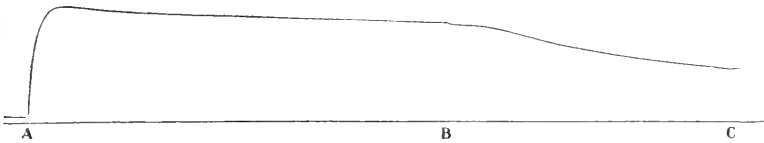


FIGURE 3.—At (A) the muscle is placed in a $\frac{m}{8}$ KCl solution. At (B) the KCl is replaced by a $\frac{m}{8}$ NaCl solution.

In Fig. 2 the muscle was treated with $\frac{m}{8}$ CaCl₂ for fifteen minutes, and then, at (B), subjected to the action of $\frac{m}{8}$ KCl solution. It will be noticed that the contraction is very gradual, and that the latent period is much longer than in the fresh muscle.

3. This antagonistic action is also very strikingly brought out as follows: At (A), Fig. 3, the muscle was placed in a $\frac{m}{8}$ KCl solution. After fifteen minutes at (B), the KCl was replaced by a $\frac{m}{8}$ NaCl solution. The relaxation did not set in till about one minute after the application of the NaCl, and was then very gradual, so that at the



FIGURE 4.—At (A) the muscle is placed in a $\frac{m}{8}$ KCl solution. At (B) the KCl is replaced by a $\frac{m}{8}$ CaCl₂ solution.

expiration of ten minutes the muscle had not yet regained its original length. In Fig. 4 a muscle was treated in the same manner, except that at (B) a $\frac{m}{8}$ CaCl₂ solution was used. In this case the relaxation began immediately and was very fast, the muscle regaining its original length in ten minutes.

4. From the experiments related above, we thought it might be possible to cause the muscle to contract and relax a great number of times by the alternate application of KCl and NaCl or KCl and CaCl_2 . This was, indeed, found to take place, and it is especially here that the antagonistic action of calcium appears. The KCl and CaCl_2



FIGURE 5.—The alternate application of KCl and CaCl_2 to the gastrocnemius muscle.

solutions were placed in two burettes, and so fixed that a little of the solutions could be squirted against the muscle placed in a muscle-holder. In this manner the curve in Fig. 5 was produced. In all, the muscle gave seventy-six contractions in one hour. At the expiration of that time the contractions became small and slow, although the irritability of the muscle towards repeated induction shocks was very great (Sec. coil at 12 cm.; one dry cell).

That this action of the CaCl_2 solution is not due to the washing away of the KCl was proven by alternately applying KCl and NaCl.



FIGURE 6.—The alternate application of KCl and NaCl to the gastrocnemius muscle.

In this manner the curve of Fig. 6 was produced. The muscle, in ten minutes, gave nine contractions, and then no longer responded to the KCl. Already at the expiration of five minutes the contraction produced by the KCl are extremely feeble.

CONCLUSIONS.

1. K-ions produce a prolonged contraction of the gastrocnemius muscle of the frog.
2. Ca-ions (and to a lesser extent Na-ions) antagonize this action of the K-ions.

WITTE'S PEPTONE: ITS DISSOCIATION, AND ITS COMBINATION WITH ACID AND ALKALI.

By TORALD SOLLMANN.

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THE combining power of albumose with acids and alkalies, particularly with the former, has been the subject of extensive experimentation. The albumoses used in these investigations have for the most part been such ill-defined chemical substances that the results, even when obtained by faultless methods, have not given us any clear insight into the constitution of the resulting compounds. Valuable data have, however, been brought to light. It seemed interesting to determine how far these would apply to more impure mixtures. The determination of certain physical and chemical data have, moreover, a peculiar practical interest in the case of Witte's peptonum siccum, since this substance is so extensively employed in experimentation on account of the ease with which it may be obtained and its fairly constant composition. It consists of a mixture of albumoses, containing from 60 to 70 per cent of "Propepton" (1),¹ a minimal quantity of true peptone, and considerable ash.

1. Reaction to various indicators.—1 gm. of Witte's peptone, in 5 per cent solution, required the following amounts of decinormal solutions to render it indifferent to the indicators named:

Methyl orange	13.0 c.c.	$\frac{N}{10}$ H ₂ SO ₄
Litmus	3.0 c.c.	$\frac{N}{10}$ H ₂ SO ₄
Phenylphtalein	4.0 c.c.	$\frac{N}{10}$ NaOH

Sjöqvist (2) (p. 352) finds that 3.12 gm. of his dialyzed "Peptonum siccum ex albumine," Schuchardt, render 100 c.c. $\frac{N}{20}$ HCl neutral to

¹ The numbers refer to the bibliography at the end of the paper.

tropæolin 00, so that 1 gm. of albumose requires 16 c.c. of $\frac{N}{10}$ acid. Fawitzky (3) finds that Witte's peptone binds $\frac{1}{9}$ of its weight of HCl with methyl-violet or phloroglucin-vanillin as indicators. This would equal 31.75 c.c. $\frac{N}{10}$ acid per gm. ! Many investigators neglect to state the reaction of their albumose toward indicators.

It is interesting to compare with these figures those given by Benedicenti (4) for other proteids.

TABLE I.
REACTION OF PROTEIDS.

	Methyl- orange.	Phenyl- phtaleïn.	Litmus.
1 gm. of peptone Witte			
SOLLMANN requires	13.00 c.c. $\frac{N}{10}$ H ₂ SO ₄	4.0 c.c. $\frac{N}{10}$ NaOH	3.3 c.c. $\frac{N}{10}$ H ₂ SO ₄
1 gm. of gelatine			
BENEDICENTI requires	9.60 c.c. $\frac{N}{10}$ HCl	1.8 c.c. $\frac{N}{10}$ NaOH	1.4 c.c. $\frac{N}{10}$ NaOH
1 gm. of egg albumin, natural, moist			
BENEDICENTI requires	1.75 c.c. $\frac{N}{10}$ HCl		
1 gm. of egg albumin, dry, Merck			
BENEDICENTI requires	13.00 c.c. $\frac{N}{10}$ HCl		
1 gm. of cattle serum			
BENEDICENTI requires	1.75 c.c. $\frac{N}{10}$ HCl		
1 gm. of fibrin			
BENEDICENTI requires	3.40 c.c. $\frac{N}{10}$ HCl		
1 gm. of casein			
BENEDICENTI requires	7.20 c.c. $\frac{N}{10}$ HCl		

It follows that the alkalinity of peptone Witte is just that of dried egg albumin.

Throughout this article, the reaction is always referred to methyl-orange.

If a solution of the albumose is heated, its reaction changes so that it requires a further 5.4 c.c. of $\frac{N}{10}$ acid per gm. to render it indifferent to the orange; on cooling it recovers its original reaction, so that now 5.4 c.c. of $\frac{N}{10}$ alkali must be added. This change can be repeated indefinitely. The same change occurs in solutions which are acid or alkaline: boiling solutions always require 5.4 c.c. more of $\frac{N}{10}$ acid to reduce them to the neutral point, than they do in the cold.

2. The ash.—The determination of the ash gives very variable results, pointing to fairly volatile constituents. Parallel determinations made on samples of 0.5 to 0.75 gm., with various degrees and time of heating, gave 2.4; 3.0; 3.3; 3.6; 4.0 per cent.

The mean of these—3.3 per cent—if dissolved in water, gives nearly the same depression of freezing point as the albumose from which it has been prepared, so that it may probably be presumed to be correct:

Δ of 0.75 gm. peptone dissolved in 25 c.c. of water = 0.104.

Δ of ash of 0.75 gm. peptone dissolved in 25 c.c. of water = 0.092.

Of this 3.3 per cent of ash, 1.27 are chlorides, calculated as NaCl.

The peptonum siccum ex albumine, Merck, with which Bugarszky and Liebermann (5) started, contained 16 per cent of ash; while their dialyzed product contained 1.27 per cent.

Δ of 4 gm. of peptone dissolved in 100 c.c. water = 0.033.

Δ of ash of 4 gm. of peptone dissolved in 100 c.c. water = 0.002.

Sjöqvist (2) (p. 348) finds in his purified albumose 0.63 per cent of ash, consisting mainly of Ca, SO_4 and PO_4 , with very little chloride.

The reaction of the ash, dissolved in water, equals 4 c.c. $\frac{1}{10}$ alkali per gm. of albumose (very near the alkalinity of the original albumose to litmus). This leaves 9.0 c.c. of the original alkalinity unaccounted for. The easy volatility of the ash suggests ammonium. However, the fact that the depression of freezing point of the ash is practically that of the proteids proves that the alkalinity is inherent in the proteid molecule. So does Sjöqvist's observation; his albumose contained but $\frac{1}{5}$ the ash of mine, yet it had the same alkalinity. However, ammonia, or some volatile base, is present in a loosely combined state. On boiling peptone with NaOH, the vapors blue litmus and paper moistened with dilute CuSO_4 . A determination by Schloesing's method yielded 1.8 c.c. $\frac{1}{10}$ volatile alkali per gm. of albumose.

3. Dissociability of the pure albumose solution.—(A) *Freezing points*: My first studies were made by determination of the freezing point, using Beckmann's apparatus.

The results are given in Table II, and Curve I, 1.

TABLE II.
FREEZING POINTS OF WATERY SOLUTIONS OF ALBUMOSE.

WITTE'S PEPTONE.					Results of BUGARSZKY and LIEBERMANN on their dialyzed albumose.	
I.	II.	III.	IV.	V.		
Gm. of albumose in 100 c.c. of water.	Δ	Δ p. gm. in 100 c.c.	Molecular concentration per gm. of albumose in 100 c.c.	Mean molecular weight.		
0.25	0.004	0.016
0.5	0.059	0.118	0.06243	160.2	0.008	0.016
1.0	0.072	0.072	0.03807	262.5	0.013	0.013
2.0	0.087	0.0435	0.02302	434.5	0.020	0.010
3.0	0.104	0.035	0.01852	540.0
4.0	0.133	0.033	0.01746	572.3	0.033	0.00825
6.0	0.193	0.032	0.01693	590.6
8.0	0.242	0.030	0.01587	630.0	0.060	0.0075

Explanation of Table II:—

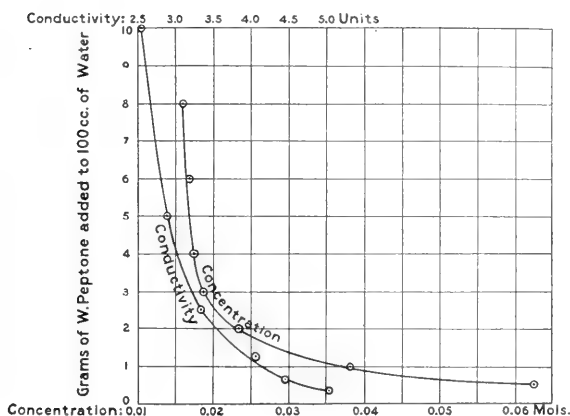
Column II: Δ = Depression of freezing point.

Column III: $= \frac{\Delta}{\text{gm. in 100 c.c.}}$

Column IV: $= \frac{\Delta}{1.89}$

Column V: $= \frac{\text{gm. p. Litre} \times 1.89}{\Delta}$

The columns II and III of Bugarszky and Liebermann correspond to these numbers in the above, and are calculated from the figures given on page 71 of their article (5).



CURVE I.—WITTE'S *peptone dissolved in water*. The molecular concentration and conductivity are reduced to 1 gm. in 100 c.c., and correspond to the Columns IV, Table II, and III, Table III.

(B) *Electrical Conductivity*: This was taken by a modification of the Kohlrausch method, using the apparatus employed by G. N. Stewart in his work on the permeability of blood corpuscles.

The result is given in Table III and Curve I.

TABLE III.

WITTE'S peptone.			SJÖQVIST.	
I.	II.	III.	IV.	V.
Gm. of albumose in 100 c.c.	Conductivity at 5° C.	Conductivity per gm. in 100 c.c.	Gm. of albumose in 100 c.c.	Specific conductivity.
$\frac{5}{16}$	1.568	5.018	1.0	0.0897
$\frac{5}{8}$	2.781	4.450	2.0	0.1552
$1\frac{1}{4}$	5.055	4.045	4.0	0.2505
$2\frac{1}{2}$	8.345	3.338	6.25	0.3385
5	14.58	2.916	8.33	0.3952
10	25.34	2.534	9.99	0.4249
....	12.49	0.4481

Explanation:

Column II. Reciprocal ohms $\times 10^8$ reduced to 5°C .

Column III. $\frac{\text{Conductivity (= Column II)}}{\text{gms. in 100 c.c.}}$.

Columns IV and V are taken from p. 348 (2); Column V varies as Column II.

Both the freezing point and conductivity indicate a dissociation of the albumose. Such has, indeed, been the opinion of all previous observers. The number of molecules which arises varies, however, with the different samples. From the curves it will be seen that an 8 to 10 per cent solution is practically undissociated, while the dissociation is practically complete with a content of $\frac{1}{4}$ to $\frac{1}{2}$ per cent. With the latter concentration, each gram of Witte's peptone causes nearly four times $\left(\frac{0.06243}{0.01587}\right)$ as much depression of freezing point as in an 8 per cent solution. Each "average" molecule is therefore capable of dissociation into four. With the albumose used by Bugarszky and Liebermann the dissociation is only into two $\left(\frac{0.0160}{0.0075}\right)$. The form of the curve plotted from their data coincides closely with that drawn from Witte's peptone.

It seems beyond doubt that the ash is responsible for the greater dissociation observed in Witte's peptone. The average molecular weight $\left(\frac{\text{gm. p. L.} \times 1.89}{\Delta}\right)$ of the latter, inclusive of ash, is in the undissociated condition 630, when dissociated 160. Subtracting the concentration due to the ash, the average molecular weight of the ash-free Witte's peptone lies about 4600. The albumose employed by Bugarszky and Liebermann had the molecular weight, when non-dissociated, of 2520 (*i. e.*, about four times that of the Witte's inclusive of ash); when dissociated its molecular weight was 1180. Their albumose was, however, a mixture of unknown composition. This is still more true of the Witte's peptone, and the problem of what molecules exist in the non-dissociated and in the dissociated solution, is hedged about by so many sources of confusion that speculation about it appears valueless. However, that Witte's peptone is dissociated in the manner described, and that the number of dissociated molecules bears so simple a ratio to the non-dissociated, are interesting phenomena.

If the albumose is added to 1 per cent NaCl solution, dissociation

does not seem to occur. The Δ of the NaCl solution was 0.653. Adding 3 per cent of albumose it became 0.740. Assuming that the dissociation were as in water, it would have been 0.757; if no dissociation had occurred, it would be 0.743 — within 0.003 of what was actually found. Even this small difference may be explained by the condensation of the NaCl.

The electrolytic dissociation gives us another aspect of the phenomenon. In Witte's peptone the conductivity is increased by dissociation to $\frac{5.018}{2.534}$ per gm., *i. e.*, doubled. Sjöqvist's albumose increases from 10 per cent to 1 per cent by $\frac{0.0897}{0.0425}$ per gm.; from 12 per cent to 1 per cent by $\frac{0.0897}{0.036}$ per gm. As the dissociation at 1 per cent is not quite complete, we may assume that the conductivity of his albumose is rendered three times as great by dissociation. The changes in conductivity do not admit of as clear insight or as simple interpretation as do those of the freezing point. Not all the fragments of the dissociated molecules need be conducting; the non-conductors will lessen the conductivity of the others; further, the conductivity of the different ions is far from uniform. However, the close correspondence in the changes of freezing point and of conductivity in the case of Witte's peptone seems to show that the two are parallel processes; and since the conductivity is merely doubled, whereas Δ is quadrupled, the conclusion appears to be justified that not all of the molecules arising in the dissociation are conducting. The albumose part of the original molecule is probably non-conducting. Ammonia (NH_3) is also a non-conductor (2, p. 331), and since the alkalinity increases when dissociation is brought about by heat, it appears a not unlikely hypothesis that this may also be split off from the albumose.

A very interesting difference appears when the *dissociation* of the albumose is compared with its *decomposition*, as by bacteria. G. N. Stewart (6) has investigated the molecular changes occurring in Witte's peptone solution through the growth of *B. subtilis*, and *B. proteus* Zenkeri. In Experiment III, p. 241, he used both the freezing point and conductivity methods. The sample of Witte's peptone with which he worked, evidently corresponded very closely with mine, since a 4.3 per cent solution gave Δ 0.124 and conductivity (using the same apparatus) of 13.68×10^{-8} at 5° . The growth of the bacteria was associated with a splitting up of the molecules. When this de-

composition had been carried to the maximum possible under the conditions of the experiment (No. 1), Δ was increased fifteen times, the conductivity sixteen times.

TABLE IV.
FREEZING POINTS. ACID AND ALBUMOSE.

WITTE's peptone.						BUGARSKY and LIEBERMANN.	
I.	II.	III.	IV.	V.	VI.	II a.	VI a.
Gm. of albumose added to 100 c.c. $\frac{1}{10}$ H ₂ SO ₄ SOLLMANN (or $\frac{2}{10}$ HCl BUGARSKY).	Δ	Molecular concentration.	Difference between observed concentration and the concentration of the acid (0.1291).	Sum of the concentration of acid plus undissociated albumose.	Difference between V and III; <i>i. e.</i> , concentration diminished by :	Δ	Difference between sum of freezing point of acid plus that of undissociated albumose and observed freezing point.
0.0	0.244	0.1291	0.186
0.25	0.184	0.004
0.5	0.178	0.012
1.0	0.167	0.0265
2.0	0.148	0.053
2.67	0.212	0.1122	-0.0169	0.1712	0.0590
4.0	0.197	0.1042	-0.0249	0.1923	0.0881	0.116	0.100
5.33	0.219	0.1159	-0.0132	0.2134	0.0975
6.0	0.234	0.1238	-0.0053	0.2239	0.1001
6.4	0.249	0.1317	+0.0026	0.2302	0.0985
8.0	0.156	0.090
8.1	0.277	0.1466	+0.0175	0.2571	0.1105
9.0	0.299	0.1582	+0.0291	0.2713	0.1131
10.0	0.332	0.1757	+0.0466	0.2871	0.1114

The conductivity in bacterial decomposition is therefore increased by even more than the amount corresponding to the liberated molecules, whereas in dissociation the conductivity is considerably less.

When the decomposition is only slight, the case approaches more nearly to dissociation; thus in No. 6 Δ is increased 2.2 times, conductivity 1.7 times. Kühne¹ investigated the action of *B. subtilis* and *B. prodigiosus* on solutions of protalbumose, from the chemical standpoint, and found that the phenomena resemble closely those of tryptic digestion. The conversion to tyrosin, leucin, and tryptophan was often almost complete.

4. **Combination of Witte's peptone with sulphuric acid.** — I investigated this subject by the freezing point and conductivity method. The results are given in Tables IV and V and in Curve II. I shall again place parallel with them the results of Bugarszky and Liebermann (5, p. 73).

Explanation of Table IV:—

Column II : Δ = observed depression of freezing point.

Column III: = $\frac{\Delta}{1.89}$

Column VI a: should be parallel to Column VI, both indicating the loss in molecules.

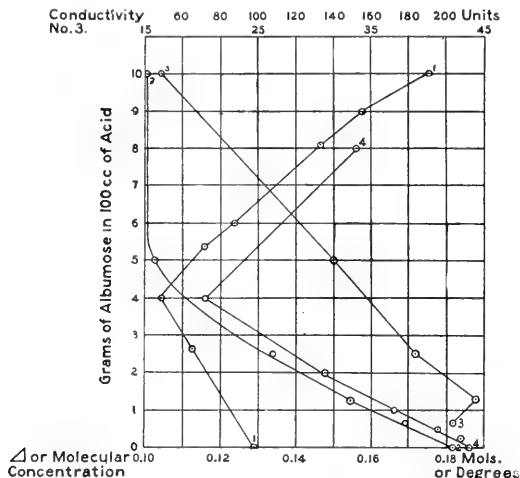
TABLE V.
CONDUCTIVITY OF MIXTURES OF $\frac{1}{10}$ H₂SO₄ AND ALBUMOSE.

I.	II.	III.	IV.
Gm. of albumose in 100 c.c. acid.	Conductivity at 5° C. $\times 10^{-8}$.	Absolute loss of conductivity.	Each per cent of albumose lessens conductivity by
0	203.9
$\frac{5}{8}$	177.9	26.0	41.6
$1\frac{1}{4}$	148.7	55.2	44.2
$2\frac{1}{2}$	107.4	96.5	38.6
5	45.15	158.75	31.75
10	41.5	162.4	16.24

The numbers and curves from the freezing points show that both Bugarszky and Liebermann's and Witte's peptone cause, in small amounts, a condensation of the molecules, so that the addition of albumose molecules to the acid, instead of increasing, actually

¹ Zeitschrift für Biologie, 1892, xxix, p. 37.

diminishes the number of molecules present. An increase in the size of the molecule — in other words, an actual combination — must therefore have taken place. The conductivity curves force to the same conclusion.



CURVE II. — Addition of WITTE'S peptone to $\frac{1}{10}$ H_2SO_4 . No. 1. Molecular concentration = Column III, Table IV. No. 2. Conductivity = Column II, Table V. No. 3. Reduction per gram of WITTE'S peptone = Column IV, Table V. No. 4. Δ of BUGARSZKY and LIEBERMANN'S albumose in $\frac{1}{20}$ HCl = Column II a, Table IV.

Further deductions as to the amount of acid combined with the albumose, and as to the structure of the resulting molecule, are rather hazardous. Even the freezing-point method does not give an unequivocal answer to these questions.

As has been said, the addition of albumose molecules produces even a decrease in the total number of molecules present. It causes similarly a decrease in the electrical conductivity, much greater than if it were an absolute non-conductor — for each gram the decrease is from 16 to 70 per cent of the conductivity, while other non-conductors cause only a decrease of from 1 to at most 4 per cent (albumin 1.52 per cent — Sjöqvist; hæmoglobin 1 per cent — G. N. Stewart). The lessened conductivity is explained at least in part by the lesser molecular conductivity of the newly formed ions. The lessening of the freezing point can only be explained by lessened dissociation, or actual entrance of the entire acid molecule into the albumose molecule. Counteracting the condensation resulting from this, we have the fact that the acid-albumose compound dissociates hydrolytically partly into its components in dilute solutions (Sjöqvist (2) p. 335). Attempts to calculate the freezing point by allowing for these factors must prove fruitless, until we can assign a numerical value to them. Even the point at which the acid is saturated by the albumose is open to doubt. Yet the tables and curves show some striking phenomena which demand discussion.

The freezing points show that the minimum concentration exists with Bugarszky and Liebermann's albumose at 4 gm. p. 100 c.c. $\frac{''}{20}$ HCl; with Witte's peptone at 4 gm. p. 100 c.c. $\frac{''}{10}$ H₂SO₄. At this point there is a sharp bend (Curve II, Nos. 1 and 4.) The concentration now increases regularly, and with Bugarszky and Liebermann's albumose, is almost exactly the molecular concentration of the added albumose. The combination of acid and albumose may therefore be supposed to be complete at the point where the angle occurs. Accordingly 1 gm. of Bugarszky and Liebermann's albumose binds $\frac{1.00}{4} \times \frac{1}{2} = 1.25$ *n* HCl; and since 4 gm. of their albumose in 100 c.c. = $\left(\frac{0.030}{1.89} =\right)$ 0.016 undissociated molecule, and 100 c.c. of $\frac{''}{20}$ acid = .050 molecule, 1 molecule of albumose combines with 4 molecules of acid. If the combination of Witte's albumose were also complete at this point, then 1 gm. of Witte's peptone binds $\frac{1.00}{4} \times \frac{1}{10} = 2.5$ *n* H₂SO₄ or 0.0632 molecule of albumose bind 1 molecule of $\frac{\text{acid}}{2}$; or 1 molecule of albumose binds $1\frac{1}{2}$ molecule of $\frac{\text{H}_2\text{SO}_4}{2}$.

However, Column VI of Table IV shows that the maximum condensation equals 0.0985 to 0.1114 molecule. The persistence of this figure very near to 0.1 seems to argue that this amount of albumose is bound by the acid. If this is its significance, then 1 molecule of Witte's peptone binds 1 molecule of $\frac{\text{H}_2\text{SO}_4}{2}$. This would correspond to $\left(\frac{0.1}{0.01587} =\right)$ 6 gm. of albumose per 100 c.c. $\frac{''}{10}$ acid, or 15.5 c.c. of $\frac{''}{10}$ acid per gm. of albumose — a figure very near to the alkalinity toward methyl orange; 6 gm. of albumose per 100 c.c. is also the point where the conductivity has reached its maximum.

These phenomena appear therefore to point to the existence of two maxima of combination, the first containing $1\frac{1}{2}$, the second 1 equivalent of H₂SO₄ per average molecule of the peptone.

The fact that the freezing point of the acid returns to the original when 6 per cent of albumose have been added, whereas it has not reached this point even when 16 gms. of Bugarszky and Liebermann's albumose have been added to 100 c.c. $\frac{''}{10}$ acid, is easily explained by the ash of the former. If this ash does not partake in the combination, it should raise the concentration proportional to the amount added (allowing for lessened dissociation). 10 gms. of undissociated ash in 100 c.c. would raise the concentration about 0.144 molecule.

So that a 10 per cent solution really has theoretically a concentration of 0.2731. The observed concentration being 0.1757, it is plain that Witte's peptone also does not really reach the original concentration when 10 per cent have been added.

The electrical conductivity curve corresponds in kind very closely to that given by Sjöqvist. From his curves combination with $\frac{2}{10}$ HCl was complete when 7 per cent of albumose had been added, as also for $\frac{2}{10}$ H₂SO₄. With Witte's peptone, the minimum conductivity was reached, when 6 gm. were added to 100 c.c. of $\frac{2}{10}$ H₂SO₄. With this it is $\frac{4}{20}$ of the original; with 4 per cent it is $\frac{6}{20}$.

The binding power of albumoses for acids has been very extensively studied, and the widely different methods which have been employed have given results which agree quite closely in certain particulars.

Sjöqvist's admirable paper (2) gives an extensive account of the earlier literature, particularly as concerns the combination of hydrochloric acid with albumin. As regards albumose, the first quantitative work quoted by him is that of Herth (1884). The latter precipitated hemialbumose from acid solution by NaCl and found that the precipitate washed with NaCl contained 5.3 per cent HCl (which would equal 14.6 c.c. $\frac{2}{10}$ acid per gm.) which by dialysis could be reduced to a minimum of 1.87 per cent = 5.1 c.c. $\frac{2}{10}$ acid per gram. The former figure agrees fairly closely with that obtained by me with Witte's peptone. The latter could perhaps be explained by hydrolytic dissociation. The results of Sjöqvist have already received sufficient mention, as also those of Bugarszky and Liebermann. The latter also measured the combination by the electromotive force, and obtained results which agree with the freezing method.

O. Cohnheim (7) investigated the subject by Hofmann's method, adding an excess of HCl and determining the uncombined acid by inverting cane sugar. He finds the interesting fact that with albumoses the amount of acid bound per gm. of albumose varies inversely as the dissociation, whether the latter is brought about by great dilution or by heating. This is the more remarkable in view of the fact that the alkalinity of Witte's peptone towards methyl orange increases by heating. Cohnheim, however, showed that antipeptone does not change its binding power by dilution. These differences may be explainable by differences in the constitution of the various albumoses. The lesser combining power in Cohnheim's dissociated albumoses

may be due to hydrolytic dissociation. The greater combining power of the dissociated Witte's peptone to the liberation of a basic (amin?) ion.

In 5 per cent solution the protoalbumose binds, at 40° C., 4.7 per cent of its weight of HCl, which would correspond to 12.9 c.c. of $\frac{N}{10}$ acid per gm. of peptone; deutoalbumose binds 5.48 per cent; heteroalbumose, 8.16 per cent; antipeptone, 15.78 per cent—the last three in 2.5 per cent solution. Cohnheim also used the salt-precipitation method for proto- and heteroalbumose, as also Gunzburg's reagent. These all gave practically identical results and showed that concentration above 2.5 per cent does not increase the combining power materially.

Table VI gives a brief survey of the results obtained by different investigators.

TABLE VI.

Kinds of albumose.	Investigator.	c.c. $\frac{N}{10}$ acid bound by 1 gm. of albumose.	Method used.
"Peptonum siccum ex albumine" Schuchard, dialyzed	SJÖQVIST (2)	16 c.c. HCl 7.4 HCl ¹ or H ₂ SO ₄	Titration against tropæolin 00 Conductivity
"Peptonum siccum ex albumine" Merck, dialyzed	BUGARSKY and LIEBERMANN (5)	12.5 HCl	Electromotive force and freezing point
Hemialbumose After prolonged dialysis	HERTH (2)	14.6 HCl 5.1 HCl	Precipitation with NaCl Precipitation with NaCl
Protoalbumose Deutoalbumose Heteroalbumose Antipeptone	COHNHEIM (7)	12.9 HCl 15.0 HCl 22.3 HCl 43.5 HCl	By inversion of cane sugar; precipitation by NaCl, and Günzburg's reagent.
WITTE'S dry peptone	SOLLMANN	13.0 H ₂ SO ₄ 18.4 25.0 15.5	Titration against methyl orange, ordinary temperature Titration against methyl orange at 100° C. Freezing point. Freezing point and conductivity.

¹ This is the point at which the conductivity is depressed no further by further addition of albumose. Sjöqvist himself does not state the conclusion that the acid is just saturated at this point.

5. **Combination of albumose with sodium hydrate.**—This subject appears to have received much less attention. Sjöqvist states that the behavior of albumoses to alkalies is similar to their reaction with acid, and promises further work on the subject. The only important work actually published, however, is found in the paper of Bugarszky and Liebermann already quoted. They investigated the problems by the electromotive force and by freezing points, the results of the two methods being in agreement.

I shall again place the results of these authors parallel with my own, which are set forth in Tables VII and VIII and Curve III.

The explanation of the columns is the same as in Table IV.

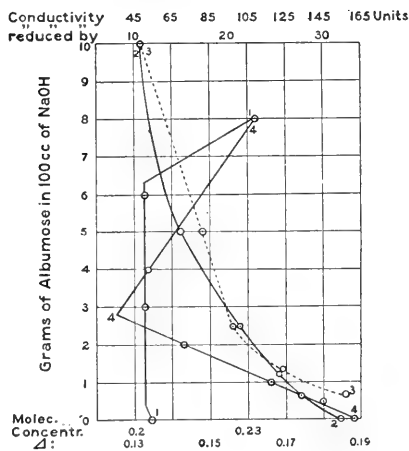
TABLE VII.
FREEZING POINTS. SODIUM HYDRATE AND ALBUMOSE.

WITTE'S PEPTONE.					BUGARSKY and LIEBERMANN.	
I.	II.	III.	IV.	VI.	II a.	VI a.
Gm. of albumose added to 100 c.c. $\frac{1}{16}$ NaOH (SOLLMANN) or $\frac{1}{20}$ NaOH (BUGARSKY and LIEBERMANN).	Δ	Concentration.	Difference between observed concentration and concentration of alkali.	Difference between observed concentration and theoretical; <i>i. e.</i> , sum of concentration of the NaOH plus undissociated albumose.	Δ	Difference between sum of freezing point of alkali plus that of undissociated albumose; and observed freezing point.
0.0	0.386	0.2042	0.188
0.5	0.180	0.0117
1.0	0.166	0.0295
2.0	0.143	0.0600
3.0	0.383	0.2026	-0.0016	-0.0490
4.0	0.133	0.0700
6.0	0.383	0.2026	-0.0016	-0.964
7.0	0.438	0.2317	+0.0275	-0.989	0.161	0.0870

TABLE VIII.
CONDUCTIVITY, ALKALI, AND ALBUMOSE.

I.	II.	III.	IV.
Per cent of albumose.	Conductivity at $5^\circ \times 10^8$.	Absolute loss of conductivity.	Each per cent of albumose lessens conductivity by
0	155.0
$\frac{5}{8}$	134.84	20.16	32.26
$1\frac{1}{4}$	122.60	32.40	25.92
$2\frac{1}{2}$	102.97	52.03	20.81
5	69.20	85.80	17.16
10	47.44	107.56	10.76

CURVE III. — Addition of WITTE'S peptone to $\frac{n}{10}$ NaOH. 1. Changes in the molecular concentration (= Column III, Table VII). 2. Changes in the conductivity (= Column II, Table VIII). 3. Reduction of conductivity per gm. of albumose (= Column IV, Table VIII). 4. Changes in freezing point, BUGARSKY and LIEBERMANN.



A remarkable difference is at once seen between the albumose used by Bugarszky and Liebermann, and Witte's. The former lessens the depression of the freezing point, the minimum being reached (by interpolation) at 3 per cent, and it has not returned to the original even at 8 per cent, in $\frac{n}{20}$ NaOH. Accepting the minimum as indicating complete saturation, 1 gm. albumose would bind $16.7 \frac{n}{10}$ NaOH, or 1 molecule of albumose = $\left(\frac{0.0225}{1.89} = \frac{0.050}{0.012}\right) = 4\frac{1}{6}$ molecules of NaOH.

However, Bugarszky and Liebermann did not determine the freezing point with 3 per cent of albumose. The minimum actual determination was at 4 per cent, which corresponds to 1 gm. binding 12.5 c.c. of $\frac{n}{10}$ of NaOH; or 1 molecule albumose binding just 4 molecules of NaOH. The results by the electromotive method agree with these figures.

Witte's peptone produced no alteration of freezing point until between 6 and 8 gms. had been added. The increase which then occurred corresponds to 1.7 gm. of albumose, so that the 6.3 gm. would saturate 100 c.c. $\frac{n}{10}$ NaOH; or 1 gm. 15.9 $\frac{n}{10}$ NaOH; or 0.09954 molecule albumose bind 0.1 molecule NaOH — *i. e.*, 1 molecule binds 1 molecule. This corresponds, practically, to the maximum binding proven for decinormal acid, found by Bugarszky and Liebermann and by Witte. We may, therefore, conclude that Bugarszky and Liebermann's albumose is tetravalent; whilst the smaller molecule of Witte's peptone is monovalent.

But another most significant phenomenon is, that while the addition of Bugarszky and Liebermann's albumose below four per cent causes the molecular concentration to fall, it is unaltered by Witte's. This is presumably attributable to the ash of the latter. It is certainly a remarkable coincidence that the ash should raise the freezing point by just the same amount by which the albumose depresses it. Yet it seems not improbable that this is the case. The addition of the ash of 6 gm. of undissociated albumose would increase the molecular concentration something like 0.0864 ($= 6 \times 0.0144$) so that the number of NaOH total molecules would be decreased to $\frac{2026}{2890}$; Bugarszky and Liebermann find them decreased at most by $\frac{13}{2}$ — not a very great difference.

The conductivity curve shows also a marked peculiarity from that seen with acids.

It might also be argued that the albumose inclusive of its ash combines with NaOH in such a way as not to change its ions; Na—OH giving, perhaps, Alb. Na—OH. However, that this could not take place without changing the dissociation appears unlikely, particularly in view of the fact that the conductivity is reduced by something like 20 per cent to 25 per cent for each gm. of albumose added.

CONCLUSIONS.

1. The research gives data concerning the reaction of Witte's peptone toward different indicators, the content of the ash, the dissociability of the pure albumose solution, the combination of Witte's peptone with sulphuric acid, and the combination of albumose with sodium hydrate.

2. Witte's peptone has an average molecular weight of 630, but is dissociable into four molecules by dilution. By heat it splits off an alkaline molecule.

3. Witte's peptone appears to be monovalent towards acids and bases.

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THE COMBINATION OF FORMALDEHYDE WITH WITTE'S PEPTONE.

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THE peculiar modifications induced by formaldehyde in solutions of various proteids have been the subject of considerable investigation, the literature of which is contained in a paper by Benedicenti (1).¹ Proteoses and peptone, however, have been but little investigated in this respect.

Charles Lepierre (4) presents in two short notes some observations on the action of formaldehyde on albumoses. His results, in so far as they treat of the same problems, differ considerably from my own. On account of the very scant details which he furnishes, it is impossible to account satisfactorily for these differences, and unprofitable to treat of them at length. I therefore take this occasion to point out the principal disagreements: — Lepierre states that the reaction does not occur, or at most very slowly, in the cold, but takes place quickly on boiling. Sometimes the substance is rendered insoluble, but more commonly (with commercial peptones) no conspicuous phenomenon reveals the reaction. The precipitate is insoluble in Na_2CO_3 .

I have found that "Witte's peptone" is precipitated by formaldehyde under certain conditions, whereas no precipitate occurs under other conditions. I have studied the factors influencing this precipitation so far mainly upon Witte's peptone, which I shall often designate briefly as "albumose."

¹ The numbers refer to the bibliography at the end of the article.

A: PHENOMENA OBSERVED WITH THE ORIGINAL WITTE'S PEPTONE.

1. **Appearance of the precipitate.**—The addition of formaldehyde to aqueous solutions of the Witte's peptone causes an immediate turbidity which passes in a few minutes into a white curdy precipitate or gelatinous clot, according to the concentration in proteid and formaldehyde. The precipitation occurs only when the solution has a slightly alkaline reaction, such as is possessed by the peptone. The precipitation is incomplete, for the filtrate still gives proteose reactions.

2. **Solubility of the precipitate.**—The precipitate is quite insoluble in water. When it is boiled with water for two hours the filtrate gives only the very faintest proteid reaction. It is, on the other hand, soluble in acids and alkalies, but the solubility is greatly diminished by the precipitate remaining in prolonged contact with the formaldehyde.

Immediately after its appearance, the precipitate dissolves readily and completely in 0.2 per cent, or stronger, hydrochloric acid, in acetic acid, and in 1 per cent, or stronger, sodium hydrate or carbonate. It remains in solution when the liquid is made neutral to methyl orange, but is reprecipitated when the reaction is made *slightly* alkaline.

If the precipitate has been left in contact with the formaldehyde even for only five minutes, the addition of the above solvents only causes the precipitate to become more transparent in the cold, little if any dissolving. On boiling, however, it slowly goes into solution. After standing half an hour with CH_2O , the solution occurs still more slowly. After sixteen hours' standing, the precipitate requires for solution nearly an hour's boiling on the water bath with 0.4 per cent hydrochloric acid, or about two hours' boiling with 10 per cent sodium hydrate; whilst two hours' boiling with 1 per cent sodium hydrate scarcely attacks it.

The rapidity of solution was always greatest with 0.2 or 0.4 per cent hydrochloric acid, next in 10 per cent sodium hydrate, least in 1 per cent sodium hydrate.

Drying at 110°C . only increases the insolubility of the precipitate. After being kept at this temperature, the precipitate is much more readily soluble in 10 per cent NaOH than in 0.5 per cent H_2SO_4 .

The precipitate is also soluble in fairly strong solutions of *neutral salts* of alkalies, magnesium, and zinc, particularly in ammonium salts, least in zinc salts. This will be discussed with more detail below.

3. **Reactions of the redissolved precipitate.** — The solutions of the precipitate, whether obtained at once and in the cold or after standing through prolonged boiling, in either acid or alkali, show the characteristic reactions of albumoses. The albumose can therefore be regenerated by bringing the formaldehyde precipitate into solution.

They can be reprecipitated by the formaldehyde in the solution, if the reaction is rendered faintly alkaline. They give a distinct biuret reaction; a yellow color and precipitate with nitric acid; the precipitate dissolving on heating; a considerable turbidity with acetic acid and potassium ferrocyanide.

After neutralization they give a copious precipitate with Almen's Tannin Reagent (Almen's does not precipitate with formaldehyde); a large precipitate on saturating or half saturating with ammonium sulphate; a good precipitate on saturating with sodium chloride.

Benedicenti (1) found that by driving off the formaldehyde from the proteid by steam, the proteids recovered their original properties. It appears from my results that albumoses may resume their characters even in the presence of formaldehyde, if the conditions for the actions of the latter are removed.

Lepierre (4) also obtained a re-solution of his precipitates by heating for two hours at 110° C. in the autoclave. This solution has also the characters of the proteid from which it was formed.

Although it follows from these experiments that albumose can be regenerated from the precipitate, the experiments do not show whether all the albumose can be recovered. The following experiment proved that this is the case.

A sample of an albumose solution was treated with an excess of formaldehyde, and left to stand for half an hour. An equal volume of 10 per cent sodium hydrate was added, and the mixture boiled to solution. Another sample was carried through precisely the same process, except that water was added in place of the formaldehyde. The same proportions were used, and the two samples were treated simultaneously. Both solutions, *i.e.*, with and without formaldehyde, gave the same intensity of precipitation with the tannin and with the ferrocyanide test.

I shall, further on, have occasion to show that the alkaline solution of the formaldehyde-albumose precipitate contains, not the original albumose, but its formaldehyde compound. The acid solution, on the other hand, contains the free albumose.

4. **Binding of the formaldehyde.** — Benedicenti (1) has already shown by a titration method (see below) that formaldehyde is very firmly bound to proteid precipitates. He believes this to disprove the contrary opinion of Elsner (1895) and Classen (1896). Using Benedicenti's method, I could corroborate his results also on albumose. I also succeeded in showing by other methods that the albumose enters into chemical combination with the CH_2O , and that the latter is retained by the precipitate, partly mechanically, partly in chemical combination.

(a) The presence of formaldehyde is very readily shown by the brown color which it develops when it is boiled with alkali. The color is bleached by acid, but returns on making the solution again alkaline. The precipitate gives the reaction even after very *prolonged washing*. Thus, it was washed in a steady stream of hot water for two hours, then left to stand under water for sixteen hours. This hot water gave no color on boiling with sodium hydrate, but the precipitate gave a deep amber.

The combination is also quite *stable at 110° C.* When the dried precipitate is heated at this temperature for as long as seven days or more, it remains quite insoluble in boiling water, but it dissolves slowly in boiling 10 per cent sodium hydrate, imparting to this a rather light amber color, yet four times deeper than is obtained by boiling the same amount of the original albumose with soda under the same conditions.

(b) When a formaldehyde solution is added to an alkaline solution of albumose, the depression of the freezing point of the latter is diminished by as much as 0.086, instead of being increased by 0.123 as happens when the formaldehyde is added to an equivalent amount of alkali alone.

(c) By Benedicenti's method it can be shown that the binding of the CH_2O occurs independently of the precipitation. Combination may take place without any precipitate, and the latter when formed usually does not contain all of the compound. Yet after the most thorough washing, with or without drying at 110° for a week, the precipitate yields, after decomposition with acid, one and one-half to three times as much CH_2O as the entire amount bound.

The phenomenon that a proteid precipitate retains so firmly, yet merely mechanically, a very volatile substance like CH_2O , and protects it against solution and against volatilization, does not stand without parallel. It is known that iodoform may also be retained in this manner (5).

B: REACTION WITH THE INDIVIDUAL ALBUMOSES.

It has already been mentioned that the filtrate from the CH_2O precipitate still gives proteose reactions. Even after standing thirty

days under the most favorable conditions the weight of the precipitate amounts to only 40 per cent, its nitrogen to 48.6 per cent of the albumose originally present. Under similar conditions the binding of the formaldehyde has reached its maximum in a much shorter time, so that the reaction must be considered as completed. There are also good grounds for the opinion that the precipitation is then complete. The non-precipitation of about 60 per cent Witte's peptone can, therefore, only be accounted for by supposing that this part of it is incapable of reacting with the CH_2O .

Half saturation of the filtrate in the above experiment with ammonium sulphate gave only a small turbidity; somewhat more occurred on saturating with NaCl . It appears, therefore, that the primary albumoses are practically completely precipitated. This was, indeed, shown to be the case on solutions of pure proto- and hetero-albumoses, isolated from the Witte's peptone and subjected to prolonged dialysis. However, the primary albumoses are not the only ones concerned in the reaction; for while the nitrogen of the precipitate amounts to 48.6 per cent of that of the entire peptone, the nitrogen of the primary albumoses amounts to only 18 per cent if they are precipitated by saturation with NaCl , or to only 10 per cent if they are precipitated by the zinc sulphate method of Zunz (6).

Some preliminary experiments, made to show whether deutero-albumose and peptone were precipitated by CH_2O , proved unsuccessful, since the preparations were not ash-free. I hope to be able to extend the present research to isolated albumoses, and perhaps to other proteids.

C: CONDITIONS WHICH MODIFY THE REACTION.

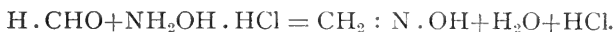
1. **Methods.** — I investigated the influence of various factors upon the reaction between the formaldehyde and Witte's peptone, mainly by the following quantitative methods:

- a **Gravimetric estimation of the precipitate.** — The mixed solutions are set aside for a specified time. Two small filters are exactly counter-balanced by cutting away the tip of the heavier filter, the entire filter being placed inside the mutilated one. The mixture and the gelatinous precipitate are decanted upon these filters. They are then washed free of salts with boiling water, then with 95 per cent alcohol, then with petroleum ether. They are then dried at 110°C , counterpoised and weighed.
- b **Titration of the bound alkali.** — To a certain quantity of the albumose (usually 10 c.c. of 5 per cent solution) of known reaction there was

added the solution of the substance to be investigated, and the formaldehyde (usually 4 c.c. of 2 per cent. CH_2O). The mixtures were allowed to stand undisturbed in bottles well fitted with greased glass stoppers for a specified length of time. At the end of this, methyl orange was added, and decinormal acid or alkali added to neutral reaction. The difference between this and the original reaction represents the quantity of the alkali bound.

c Titration of the free formaldehyde. — The method is essentially Benedict's modification of Brochet and Gambier's process (2). To the neutral solution remaining after the titration of the alkalinity (b), there are added 10 c.c. of 2 per cent solution of hydroxylamin hydrochlorid (Merck) for each 4 c.c. of 2 per cent CH_2O . This mixture is well shaken and set aside for an hour. Decinormal sodium hydrate is then run in drop by drop until the solution is neutral. As a control, another cubic centimetre of the alkali is added, and titrated back with decinormal sulphuric acid. The color change is by no means very sharp, unless compared with a standard sample. I would not place any significance on differences of 0.3 c.c. of $\frac{N}{10}$ acid, equal to 0.009 gm. formaldehyde. This difficulty was greater when the hydroxylamin was added shortly after mixing the albumose solution and the formalin. The amount of alkali required is subtracted from that required by the formaldehyde solution alone. The difference represents the formaldehyde bound by the proteid.

The reaction occurs according to the equation



Therefore, 1 c.c. of $\frac{N}{10}$ NaOH = 0.0030 gm. of formaldehyde.

I first tried whether standing effected any *changes in the pure formaldehyde solution*. I found that this lost a small and fairly constant amount of formaldehyde (0.003 gm.) on the first day; little, if any, later. I am inclined to attribute this loss to the distribution of the formaldehyde vapor into the air of the bottle.

Experiment: Into glass stoppered bottles there was placed 10 c.c. of water, and 4 c.c. of a 2 per cent formaldehyde (Schering). The formaldehyde itself has toward phenylphtalein an acidity of less than 0.08 c.c. $\frac{N}{10}$ for the quantity used; toward methyl orange it is absolutely neutral.

After adding the hydroxylamin, there were required for neutralization: —

At once	26.6 c.c. $\frac{N}{10}$ H_2SO_4 ¹
After having stood one hour	26.6 c.c. $\frac{N}{10}$ H_2SO_4
After having stood one to ten days	25.6 to 25.9 c.c.

¹ This corresponds to 0.0798 gm. of formaldehyde, instead of the theoretical 0.008 gm. The formalin (Schering) therefore contained 39.9 per cent of formaldehyde.

In all the following work, 26.6 c.c. $\frac{1}{10}^n$ was accepted as the standard for the 4 c.c. 2 per cent formaldehyde, if the titration was made within one hour; 25.6 c.c. if made in one to ten days.

Before my attention was directed to the binding of alkali by the compound, I reduced the reaction to the theoretical, not the actual, neutral, before adding the hydroxylamin. Results obtained in this manner allow no conclusion as to the actual amount of alkali, or CH_2O bound. They are, however, comparable amongst themselves, and I shall quote them as "combined alkali and CH_2O ."

d Direct estimation of the alkali and CH_2O in the precipitate.— For this purpose the washed or dried precipitate is placed in a flask with a specified amount of 0.1 *n* H_2SO_4 . The flask is closed with a perforated rubber stopper and connected with Liebig's absorption bulbs charged with hydroxylamin solution. The flask is heated on a water bath until the contents dissolve. The direct titration of the contents of the flask shows the alkali which has been liberated. The hydroxylamin of the bulbs is now added to the acid; the titration, after standing, shows the quantity of formaldehyde liberated.

e Freezing point.— The freezing points were estimated with the usual Beckmann's apparatus. Instead of the customary platinum wire, I employed a spiral of rolled gold wire, 5 cm. long, the turns of the spiral being 5 mm. apart. The thermometer is standardized before use by a known NaCl solution. Control readings agree to 0.002° C.

C. 2. The influence of alkalinity or acidity.— All the phenomena under consideration — the precipitation and the binding of the formaldehyde and the alkali — are greatly influenced by the reaction of the liquid.

The reaction in the following experiments is referred to methyl orange, towards which Witte's peptone has an alkalinity of 13 c.c. $\frac{1}{10}^n$ per gm., *i.e.* each gram of the peptone requires 13 c.c. $\frac{1}{10}^n$ H_2SO_4 to render it indifferent to the indicator. By a "neutral solution" I understand one to which this quantity has been added. A solution of an alkalinity of 2.5 c.c. *n* per gm. is one to which 25 c.c. — 13 c.c. = 12 c.c. $\frac{1}{10}^n$ alkali has been added for each gram of albumose, etc.

Before taking up the influence of changes in reaction upon the formaldehyde precipitation, it will not be out of place to record the

2 a. Effect of the reaction upon the clearness of peptone solutions.— Neutral solutions containing $3\frac{1}{3}$ per cent of the peptone are distinctly turbid. The turbidity is greatest with a reaction of between 0.77 normal acid and 2.3 c.c. normal alkali per 100 c.c.; actual observation, 0.103 c.c. normal alkali. It lessens as the reaction is increased in either direction.

It disappears completely with the alkalinity between 2.3 and 3.65 c.c. All acid solutions show some slight turbidity, which does not increase on adding hydrochloric acid until the solution contains 20 per cent of HCl.

2 b. Influence of the reaction on precipitation by formaldehyde.— This very conspicuous factor attracted my attention very early in this research, particularly as I have not seen it mentioned in detail in connection with the other proteids so far investigated. In this connection Benedicenti quotes the following:

“ Beckmann (3) shows that the hardening of gelatin is only partial when a small quantity of acid is present, and is altogether wanting if the acidity is strong.”

Benedicenti himself (2) (p. 238) found that egg albumin which has stood some time in contact with formaldehyde, is precipitated by small quantities of either acid or alkali, but redissolves in an excess of either reagent.

I made a rather lengthy series of experiments to determine the *limits of reaction* between which turbidity and precipitation occurs, and obtained the following results:

Mixtures possessing a greater alkalinity than 1.6 c.c. normal per gram of albumose are not visibly changed by formaldehyde. 1.6 c.c. just shows a slight turbidity on standing three to four days. When the alkalinity is reduced to 1.52 c.c. per gram, a slight turbidity occurs at once, which is a plainly marked precipitate between the limits of 1.45 and 0.9. If the alkalinity is less than 0.9, there is only turbidity within a few hours, growing more faint as the alkalinity is further reduced. With 0.4, the turbidity is still plainly discernible. Below this, it is doubtful. Absolutely none can be distinguished (in several hours) in neutral or acid solutions. The turbidity in weakly alkaline solutions grows more dense with time, and consolidates. A reaction of 0.8 alkali shows a clot in twenty-four hours; 0.4 in forty-eight hours. In three or four days a small, loose, flaky precipitate occurs even in neutral solutions. On boiling, the precipitate from neutral solutions is more abundant, yielding after twenty-four hours' standing a precipitate of 0.0305 gm., instead of 0.116 gm. given with an alkalinity of 1.3, under like conditions. Even on plain standing with a large excess of formaldehyde, an abundant precipitate occurs in neutral reaction, and apparently also in those containing 0.2 c.c. normal acid per gram of peptone. There is absolutely none with 0.5 c.c. and upward of acid even after standing four days.

The optimum reaction — *i. e.*, that which gives the largest precipitate in the shortest time — lies between 1.05 and 1.1 c.c. of normal alkali per gram. The natural alkalinity of the peptone (1.3 c.c.) is therefore somewhat too great. This is supported by a gravimetric estimation:

20 c.c. of 2½ per cent peptone solution with 1 c.c. of 40 per cent CH₂O gave on twenty-four hours' standing 0.124 gm. of dry precipitate. The addition of a trace of hydrochloric acid brought down a further 0.0115 gm. in twenty-four hours.

I desire to lay particular stress upon the fact that the limit beyond which further alkali prevents precipitation, is *not determined by the absolute amount of alkali present, but by the ratio of alkali to peptone.*

Thus, in a 1¼ per cent peptone solution, the presence of 2.125 c.c. of normal alkali in 100 c.c. will prevent precipitation (alkalinity = 1.7 c.c. per gram of peptone); whilst the same amount of alkali in a 2½ per cent peptone solution (= 0.85 c.c. per gram of peptone) will allow a very large turbidity. To prevent precipitation in the latter requires 4 c.c. of normal alkali (= 1.6 c.c. per gram). It follows from this that the reduction of the alkalinity by dilution with water does not cause precipitation. To secure the maximum of precipitation in a 1¼ per cent solution requires a total alkalinity of 1.3 c.c. normal alkali per 100 c.c.; in a 2½ per cent solution, 2.6 c.c.

Solutions which have stood four days with an excess of acid do not precipitate on neutralization. Those in which precipitation has been prevented by excessive alkalinity become turbid on being neutralized, the degree of turbidity being apparently proportional to the amount of alkali which had been added, up to about 2 c.c. *n* per gram. From here it again diminishes. With an alkalinity of 5 c.c. *n*, there is but a very small turbidity on neutralization; and none at all with 7.5 c.c. and above. Where a precipitate occurs with lower alkalinity, this remains after neutralization when the CH₂O has been removed by hydroxylamin. Solutions with an alkalinity above 7.5 c.c. fail to give precipitation with this treatment.

2 c. Effect of reaction on the binding of alkali and formaldehyde. — On adding various amounts of acid or alkali to a solution of formaldehyde, the reaction as well as the amount of formaldehyde are found unchanged, even when the mixture has stood for several days. Nor does the reaction change when peptone solution and acid or alkali are left in contact.

If, however, mixtures of peptone, formaldehyde, and alkali are left together, a definite amount of alkali is bound so firmly that it is not liberated in the cold by the addition of acid. At the same time, a certain amount of CH_2O is bound, the two being very nearly proportional. If the quantity of CH_2O and the absolute quantity of alkali are sufficient, a certain maximum — 6.4 c.c. $\frac{n}{10}$ alkali and 0.0447 gm. CH_2O per gram of albumose — will eventually be bound, but the time required is determined by the excess of alkali or of formaldehyde present.

As regards the influence of reaction upon the combination: In an *acid medium* no combination occurs; and the compound when already formed in alkaline medium, is decomposed by boiling with an acid, the original amount of alkali and CH_2O being liberated. The following experiment may serve as an instance:

Experiment 110. To two flasks containing 10 c.c. of 5 per cent albumose solution was added 4 c.c. of 2 per cent CH_2O and the same amount of CH_2O to two flasks containing 10 c.c. of water, and each pair treated just alike at the same operation. One pair was left one hour, then 25 c.c. $\frac{n}{10}$ acid added, left over night, neutralized, and hydroxylamin added. The peptone liberated 26.0 c.c. $\frac{n}{10}$ acid, the control 25.9 c.c. The other pair was left twenty-four hours, 25 c.c. $\frac{n}{10}$ acid added, and digested for $\frac{1}{2}$ at 100° , yielding a perfectly clear solution of the peptone. On cooling they were neutralized, and hydroxylamin added. The peptone liberated 20.5 c.c., the control 20.6.

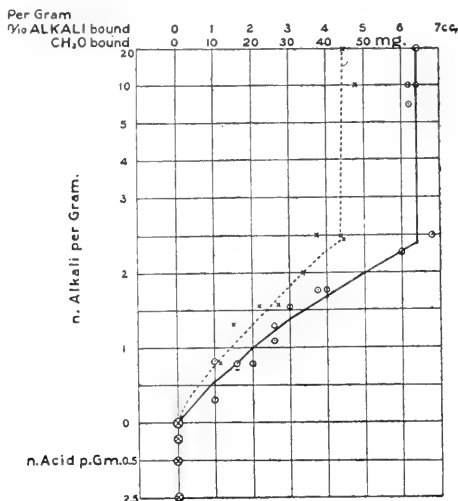
In alkaline solution, the extent of the reaction is exactly proportional to the amount of alkali present, as illustrated by Curve I.

The experiments from which the curve was drawn were made by mixing 10 c.c. of a 5 per cent albumose solution with enough of $\frac{n}{10}$ acid or alkali to give it the desired reaction. The mixtures were set aside for four days and titrated.

The maximum was reached under the conditions of the experiment when the alkalinity was 2.4 c.c. n per gram of albumose.

A somewhat peculiar phenomenon is noted when fairly strongly alkaline solutions — say 2 to 3 c.c. n per gram — are neutralized, for the amount of alkali which is bound appears to be larger at first. The first change of color would perhaps indicate a binding of 5.0 c.c. $\frac{n}{10}$; then the solution becomes yellow again, and more and more acid can be added, until the bound alkali corresponds to 3.2 or even 3.7 c.c. It appears as if a combination containing more alkali existed, from

which the alkali was slowly liberated—the whole change, however, occupying but a few minutes.



CURVE I.—The effect of reaction upon the binding of formaldehyde and alkali. The solid line and 0 represent the combined alkali, the dotted line and \times the combined formaldehyde.

The results of these observations show very conclusively that the reasons why precipitation does not occur in acid nor in alkaline media are very different. In the former, the combination does not take place; in the latter, the compound is soluble.

The *freezing-point method* also shows that a combination occurs in the presence of an alkali, but not in the presence of acid.

$$\Delta \text{ of } \frac{n}{10} \text{ NaOH} = 0.429^\circ \text{ C.}$$

$$\Delta \text{ of } 25 \text{ c.c. } \frac{n}{10} \text{ NaOH} + 4 \text{ c.c. } 2 \text{ per cent } \text{CH}_2\text{O} = 0.552^\circ \text{ C.} \quad \text{Difference} = +0.123^\circ \text{ C.}$$

$$\Delta \text{ of } 25 \text{ c.c. } \frac{n}{10} \text{ NaOH} + 0.5 \text{ gm. albumose} = 0.425^\circ \text{ C.} \quad \text{Difference} = -0.004^\circ \text{ C.}$$

$$\Delta \text{ of } 25 \text{ c.c. } \frac{n}{10} \text{ NaOH} + 0.5 \text{ gm. albumose} + 4 \text{ c.c. } 2 \text{ per cent } \text{CH}_2\text{O} = 0.524^\circ \text{ C.} \quad \text{Difference} = +0.095^\circ \text{ C.}$$

$$(\text{Alkalinity} = 6.3 n \text{ per gram.}) \quad \text{Condensation} = 0.028^\circ \text{ C.}$$

$$\Delta \text{ of } \frac{n}{10} \text{ H}_2\text{SO}_4 = 0.254^\circ \text{ C.}$$

$$\Delta \text{ of } 25 \text{ c.c. } \frac{n}{10} \text{ H}_2\text{SO}_4 + 4 \text{ c.c. } 2 \text{ per cent } \text{CH}_2\text{O} = 0.430^\circ \text{ C.} \quad \text{Difference} = +0.176^\circ \text{ C.}$$

$$\Delta \text{ of } 25 \text{ c.c. } \frac{n}{10} \text{ H}_2\text{SO}_4 + 0.75 \text{ gm. albumose} = 0.225^\circ \text{ C.} \quad \text{Difference} = -0.029^\circ \text{ C.}$$

Δ of 25 c.c. $\frac{N}{10}$ H₂SO₄ + 0.75 gm.

albumose + 4 c.c. 2 per cent CH₂O = 0.428° C. Difference = +0.174° C.

(Acidity = 3.3 *n* per gram.) Condensation = 0.002° C.

Examples of this could be multiplied.

C 3. Influence of the concentration in formaldehyde on the reaction.

— The effect of increasing the quantity of formaldehyde is like that of increasing the alkalinity, a more rapid combination and precipitation. The slower reaction with the lesser amount of formaldehyde is by no means due to a want of a sufficient quantity of CH₂O to complete the reaction, for I used in every case more than is actually bound (*viz.*, more than 0.0447 gm. CH₂O per gm. of albumose). The following figures support these statements: —

CH ₂ O (gm.) added per gm. of albumose.	Strength of the solution in albumose (natural re- action).	Time of standing.	Precipitate.	c c. of $\frac{N}{10}$ alkali bound.	CH ₂ O bound.
			Per gram of albumose.		
0.16	3.5	4 days	3.8	0.0156
0.4	2.0	4 days	5.6	0.0282
0.8	1.5	4 days	6.2
1.0	1.25	4 days	6.0
0.16	3.5	1 day	0.095
4.0	2.1	1 day	0.232
1.00	1.5	4 days	0.232
0.16	3.5	4 days	0.119	3.8	0.0150
0.08	7.0	4 days	0.109	5.9	0.0282

The last two experiments show how the retarding influence of a lesser concentration in CH₂O may be counterbalanced by other favoring factors, as in this case by greater concentration in albumose.

C 4. Influence of the concentration in albumose on the reaction. — Greater concentration of the solution in albumose also hastens the reaction, as is shown by the following results:

Strength of the solution in albumose (%), natural reaction.	CH ₂ O (gm.) added per gram of albumose.	Time of standing.	Precipitate.	c.c. of $\frac{N}{10}$ alkali bound.		CH ₂ O bound.
				Per gram of albumose.		
2.1	4.0	1 day	0.116			
3.4	4.0	1 day	0.1465			
5.0	4.0	3 days	0.2015	= (Maximum precipitation)		
3.5	0.16	4 days	...	2.7		(Combined)
5.5	0.16	4 days	...	3.21		(Combined)
3.5	0.16	4 days	0.119	3.8		0.0150
7.0	0.08	4 days	0.109	5.9		0.0282

C 5 Influence of standing upon the reaction. — I have already had occasion to mention that the reaction is a leisurely process. Even with the optimum conditions, scarcely any combination occurs at once, and the reaction is only a fifth completed in ten minutes, and is still incomplete in an hour. The precipitate also does not occur at once, under the most favorable conditions, and it consolidates and gelatinizes only in the course of time. The length of time required to complete the reaction varies inversely as the other favoring conditions. With the usual disposition of the experiment it is complete in less than eighteen hours, when the alkalinity is 2.5, whilst with an alkalinity of 1.3 it is not quite complete even in twenty-three days.

The following protocols and curve will bear out these statements:

a Precipitation :

0.5 gm. of albumose, 20 c.c. of water 5 c.c. 40 per cent CH₂O.

Precipitate after standing 1 day weighs 0.232 per gm. of albumose.

Precipitate after standing 21 days weighs 0.361 per gm. of albumose.

Precipitate after standing 30 days weighs 0.401 per gm. of albumose.

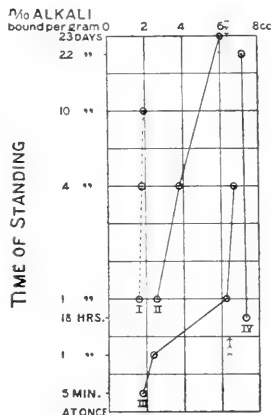
0.5 gm. albumose, 10 c.c. of water and 4 c.c. 2 per cent CH₂O.

Precipitate after standing 1 day weighs 0.085 per gm. of albumose.

Precipitate after standing 4 days weighs 0.107 per gm. of albumose.

Precipitate after standing 23 days weighs 0.1035 per gm. of albumose.

b Binding of alkali and CH₂O. — This is shown by Curve II, which represents only some of the experiments made, and which all illustrate the same facts. Since the binding of alkali and CH₂O are strictly proportional, the former is alone depicted.



CURVE II.—Effect of standing upon the reaction. No. 1: Reaction 0.8 alkali. The curve represents the “combined” figures. No. 2: Reaction 1.3 alkali. No. 3: Reaction 2.5 alkali. No. 4: Reaction 5.0 alkali. Nos. 1, 2 and 3 = c.c. $\frac{1}{10}$ alkali bound per gram. The arrow indicates the accepted maximum of bound alkali (6.4 c.c. $\frac{1}{10}$ per gram of albumose).

c Freezing points. — The results are presented in the form of a table:

Number of experiment.	Quantity of albumose. Grams.	Per cent of albumose.	Reaction. (c.c. $\frac{1}{10}$ per gram.)	CH ₂ O per gram.	Quantity of solvent.	Time of standing.	Calculated Δ of mixture lessened by*.	Precipitation.
195	0.5	2.5	2.5 alkali	0.16	20 c.c.	1 hour 1 day 4 days	x x + 0.048 x + 0.046	None “ “
197	0.5	2.4	2.7 alkali	0.16	21 c.c.	At once	0.040	“
197	1.0	4.9	2.1 alkali	0.08	21 c.c.	At once 1 day 4 days	0.020 0.071 0.086	“ “ “
198	3.0	11.0	0.47 alkali	0.037	29 c.c.	At once 1 day	0. 0.041	“ Considerable
199	0.75	2.6	3.3 acid	0.107	29 c.c.	At once 3 days	0. 0.	None “
205	2.5	8.6	0.26 alkali	0.032	29 c.c.	15 min.	0.	“
207	0.5	1.7	6.3 alkali	0.16	29 c.c.	At once 16 days	0.024 0.109	“ Few flakes

* This number refers to the difference between the Δ observed, and that which would have existed if no condensation had occurred. Thus, in Experiment 198: 25 c.c. $\frac{1}{10}$ H₂SO₄ gives Δ 0.254. The addition of 4 c.c. of 2 per cent CH₂O gives Δ 0.430. The Δ has increased by 0.176.

25 c.c. $\frac{1}{10}$ H₂SO₄ + 3 grams albumose, gives Δ 0.360. The addition of 4 c.c. of 2 per cent CH₂O should give $0.360 \pm 0.176 = 0.536$. The actual reading, immediately after mixing, is 0.540; no condensation has taken place. After four days, the reading is 0.495; difference $0.536 - 0.495 = 0.041$.

C 6 Influence of boiling on the combination. — The reaction is greatly hastened by boiling, and this is especially noticeable when the alkalinity is low. Boiling, as I have shown in the preceding paper, increases the alkalinity of peptone by 5.4 c.c. $\frac{n}{10}$ per gram. This is undoubtedly an important agent in the favorable influence of boiling, which acts in this way like the addition of alkali. That this is the case is shown by the fact that the boiling causes precipitation even in neutral solutions, and the latter acquire an acid reaction. However, this increase is not sufficiently large to account for the great rapidity of the reaction, so that this must be favored directly by the heat.

A neutralized solution of albumose is mixed with formaldehyde and methyl orange, boiled for an hour, and the precipitate is estimated after twenty-four hours. The solution turns pink. The precipitate is a third of that given with an acidity of 1.3 on standing twenty-four hours (0.0305, in place of 0.116). With alkalinity 1.3, boiling gives approximately the same quantity of precipitate in an hour as is found after standing at ordinary temperature for twenty-four hours (0.110 instead of 1.116). Boiling a solution of alkalinity 1.3 with 0.16 CH_2O per gram for an hour also gives the maximum binding of alkali, such as could only be obtained in the same solution by standing at ordinary temperature for over twenty-three days; or in several hours by increasing the alkalinity to 2.5.

Boiling causes no precipitate in solutions which contain an excess of alkali, nor in the presence of 0.8 *n* acid per gram.

C 7 Effect of salts upon the combination. — The precipitation of the albumose and the binding of the formaldehyde are both very conspicuously influenced by the presence of salts in the solution.

Small amounts of all the neutral salts — of alkalies, magnesium, and zinc — lessen the precipitation, and in greater amounts prevent it entirely. The most efficient are ammonium salts.¹ These also dissolve the precipitate immediately after it has been formed. On older precipitates they have little if any action, even on very prolonged boiling. As in the prevention of precipitation by alkalies, the *absolute quantity*, or concentration of the salts and of the albumose, are immaterial, the main factor being a certain minimum *ratio*

¹ It is well known that ammonium sulphate decomposes formaldehyde when heated with it. This is not, however, the main factor in my results: the solution still possessed a strong formalin odor, and precipitated when an excess of albumose was added.

of salts to peptone. A much less influence appears to be exerted by the amount of CH_2O in increasing the precipitation.

The presence of sufficient salts also prevents the binding of the formaldehyde completely.

PROTOCOLS.

a Salts preventing the precipitation. — 2 c.c. of 5 per cent albumose are mixed with 1 c.c. of the following solutions, and 1 c.c. of 40 per cent CH_2O . The solutions were : Water, NH_4Cl , 25 per cent ; Na_2SO_4 , KBr , MgSO_4 , ZnSO_4 , $(\text{NH}_4)_2\text{SO}_4$ — all saturated solutions.

In five minutes the water showed a large coagulum ; the $(\text{NH}_4)_2\text{SO}_4$ and ZnSO_4 showed a slight turbidity (precipitation of the albumose by the salt?). The others are quite clear. In twenty minutes the Na_2SO_4 is also somewhat turbid. 4, 8, and 16 c.c. of water were now added to each tube. The result was that the turbidity in the $(\text{NH}_4)_2\text{SO}_4$ cleared, whilst that in the ZnSO_4 became more pronounced. The other tubes did not undergo any change.

The ratio of 1 c.c. of saturated solution of any of these salts to 0.1 gm. of albumose prevented, therefore, the precipitation. There was a slight precipitate in the case of ZnSO_4 ; less with the Na_2SO_4 .

b Salts on already formed precipitates. — An albumose-formaldehyde precipitate, having stood with the precipitant for a day, is put in mixtures of two volumes of water and one volume of the solutions mentioned in the previous experiments. No perceptible solution occurred in any on boiling for two hours.

Portions of 2 c.c. of albumose are mixed with 1 c.c. of 40 per cent CH_2O . In an hour are added 1 c.c. of the above solutions. The mixtures all become clearer, the NH_4Cl most, then the $(\text{NH}_4)_2\text{SO}_4$. None dissolves completely. The tubes are set in the boiling water bath. The NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$ are both completely dissolved. They still have a strong CH_2O odor, and precipitate albumose. All the other tubes show only partial solution in twenty minutes.

10 c.c. of 5 per cent albumose are mixed with 5 c.c. of water and 4 c.c. of 2 per cent CH_2O . A precipitate occurs at once. In five minutes are added 5 c.c. of saturated NaCl . The mixture is a turbid solution in one hour.

As the last experiment, but the NaCl is added only at the end of an hour.

As a result the jelly-like precipitate becomes grumous. Boiling clears

¹ The zinc sulphate solution was acid to methyl orange. A small quantity of NaOH was added, so as to make it slightly alkaline to litmus, whilst still acid to the orange.

the mixture somewhat, but any real solution is doubtful, even after boiling for an hour.

An old precipitate dissolves very incompletely on repeated boiling and standing (a total of twenty-six hours of boiling and four days of standing) with fourth-saturated ammonium sulphate.

c **Ratio of salt required to prevent precipitation.** — I have shown above that a ratio of 10 c.c. of saturated solution to 1 gm. of albumose, prevented precipitation entirely with all the salts except zinc sulphate.

I tried to demonstrate the exact ratio required in the case of *sodium chloride*.

The difficulty of distinguishing a turbidity caused by the formaldehyde from that naturally present in the mixture is very great. This I attempted to obviate, with fair success, by keeping a control portion of the albumose-salt mixture before adding the CH_2O .

The results are succinctly set forth in the table.

EFFECT OF NaCl ON THE PRECIPITATION OF PEPTONE.

Unless otherwise noted, the amount of CH_2O was 0.5 to 10 % ; the time of standing, to two hours.

c.c. of saturated NaCl per gm. of peptone.	Results.	c.c. of saturated NaCl present in 100 c.c. of the mixture.	Gm. of peptone present in 100 c.c. of the mixture.	Remarks.
40.0	No precipitate in 4 days.	60.0	1.67	15% CH_2O
20.0	No precipitate in 1 day.	40.0	2.0	
20.0	No precipitate in 1 day.	13.0	0.67	
20.0	Large precipitate.	33.0	1.7	
10.0	No precipitate in 20 min.	25.0	2.5	
10.0	No precipitate in 20 min.	5.0	0.5	
5.0	No precipitate in 45 min.	9.9	1.8	
3.2	Slight turbid.	8.9	2.78	
2.9	No precipitate at once.	12.5	4.37	
2.8	Doubtful.	3.3	1.2	
2.5	No precipitate in 45 min.	9.9	3.6	
2.5	Slight precipitate.	11.1	4.44	
2.0	Good precipitate.	5.0	2.5	
2.0	Precipitate.	6.7	4.8	

It will be seen that precipitation is completely prevented when the ratio of saturated salt solution to albumose is between 2.5 and 3.2: 1. As I shall show later, very much smaller amounts materially *lessen* the precipitation.

I determined also the necessary proportion of *magnesium sulphate*. With this, a ratio of 7 c.c. of saturated solution to 1 gm. of albumose allows only a trifling turbidity on adding the CH_2O .

d Gravimetric estimation of the precipitates under the influence of sodium chloride. — I have mentioned elsewhere that the precipitate on mixing 10 c.c. of 5 per cent albumose solution, 10 c.c. of water, and 5 c.c. of 40 per cent CH_2O , and allowing to stand for a day, weighed 0.116 gm. On substitution of 1 c.c. of saturated NaCl for 1 c.c. of the water, the precipitate weighed 0.083 gm. The substitution of 10 c.c. of saturated NaCl for the 10 c.c. of water prevented precipitation entirely.

e Titration. — After allowing the following mixtures to stand for four days: —

(a) 10 c.c. 5 per cent albumose, 1 c.c. $\frac{N}{10}$ acid, 4 c.c. 2 per cent CH_2O .

(b) Above, plus 1 c.c. saturated solution of sodium chloride.

(c) Above, plus 20 c.c. saturated solution of sodium chloride.

5 c.c. of $\frac{N}{10}$ H_2SO_4 are added, then 10 c.c. of 2 per cent hydroxylamin hydrochloride, and after standing an hour, $\frac{N}{10}$ NaOH to neutralization.

(a) requires 23.4 c.c.

(b) requires 22.9 c.c.

(c) requires 26.0 c.c.

CH_2O alone requires 26.0 c.c.

No binding has therefore occurred in (c).

The effect of salts upon the precipitation of albumoses by formaldehyde is very different from their influence on precipitation by alcohol, for they favor the latter (Kühne 7). That they prevent the reaction of the albumose with CH_2O appears capable of scarcely any explanation, except that they enter into a compound with the albumose. This is also favored by the fact that a certain quantity, rather than a certain concentration, of salt is required to prevent the precipitation. Yet this is against the evidence of St. Bugarszky and Liebermann, and against the result obtained by myself (see previous paper) on mixtures of albumose and NaCl. I have not yet inquired further into the phenomenon.

D. DISCUSSION OF THE RESULTS.

1. **What becomes of the bound alkali?** — In the reaction of the alkali with the formaldehyde, a definite amount of the alkali is bound so that it is neutral to methyl orange. This amount is perfectly proportional to the quantity of CH_2O bound. The maximum binding, which is quite constant, however varying the conditions under which it has been produced, is 6.4 c.c. of the $\frac{N}{10}$ alkali for each gram of the albumose added. If the combination is broken up as by boiling with acid, the entire amount of the alkali is again liberated. Since the same amount of alkali disappears when no precipitation occurs (*i.e.*, in strongly alkaline medium) it is plain that its disappearance is not due to its being mechanically retained by the precipitate. Yet when a precipitate occurs, this contains part of the bound alkali, the quantity being proportional to the completeness of the precipitation. The alkali therefore forms an integral part of the compound, which latter behaves toward it as an acid. This acid apparently cannot exist in free form.

PROTOCOLS.

No. 150. 20 c.c. of $2\frac{1}{2}$ per cent albumose and 5 c.c. of 40 per cent CH_2O are set aside twenty-four hours. The precipitate (which by control (146) weighed 0.1095 dry) was washed in running water for three days. 25 c.c. of $\frac{N}{10}$ acid were added, and the flask was set to boil on a water bath. In two hours the solution was not quite complete. It was now titrated back, and required only 23.7 $\frac{N}{10}$ alkali — *i.e.*, 1.3 $\frac{N}{10}$ alkali had been bound by the 0.5 gm. of original peptone. A control shows that the alkali bound in the entire mixture under like conditions = 3.2 c.c. $\frac{N}{10}$. The precipitate therefore contains $\frac{1.3}{3.2}$ of the bound alkali.

No. 152. 10 c.c. of the 5 per cent peptone and 4 c.c. of 2 per cent formaldehyde were allowed to stand for three days. It was found that the mixture had bound 1.6 c.c. $\frac{N}{10}$ alkali. Half of the liquid was decanted; the other half was left on the precipitate over night. The latter required 0.2 c.c. $\frac{N}{10}$ acid to reduce it to neutral. No solution was apparent, and this probably corresponds to the mechanically retained alkali. 20 c.c. of $\frac{N}{10}$ acid were now added, and the mixture boiled. Complete solution occurred in half an hour. On titration it was found that 1 c.c. $\frac{N}{10}$ alkali had been given up by the precipitate.

D 2 Summary of relations. — The quantity of alkali and CH_2O entering into the combination bear a simple relation to each other and to the peptone.

The maximum bound under different conditions is per gm. of albumose: —

Of alkali, 6.0 to 7.4 c.c. $\frac{N}{10}$; mean, 6.4.

Of CH_2O , 0.042 to 0.048 $\frac{N}{10}$; mean, 0.0447.

This corresponds to 0.429 c.c. $\frac{N}{10}$ NaOH for 3 mg. CH_2O (= 1 c.c. $\frac{N}{10}$ CH_2O). The amounts bound under less favorable conditions vary in the different experiments.

For alkali, from 1.6 to 4.0; mean, 2.5 c.c. $\frac{N}{10}$.

For CH_2O , from 0.0108 to 0.0270; mean, 0.0147,

which corresponds to 0.51 c.c. $\frac{N}{10}$ NaOH for 3 mg. CH_2O .

It appears, therefore, that *the alkali and CH_2O are bound in the ratio of one molecule of the former to two of the latter.* I have already explained that there is reason to suppose that only part of the Witte's peptone participates in the reaction. This part is of course alone important in the present connection.

Just how much, however, enters into the reaction is difficult to determine. The maximum precipitation which has occurred under the most favorable conditions, was in one case 0.401; in another, 0.403 gm. per gram of the original albumose. Since these closely related results were obtained under very different conditions, — the former by very long standing, the latter by greater concentration in albumose with comparatively short standing, — it appears not unlikely that the entire quantity of the compound was precipitated. If this were the case, the formaldehyde albumose compound would contain per gram 0.1109 gm. of CH_2O , and have an alkalinity equal to 15.9 c.c. $\frac{N}{10}$ NaOH. This I attempted to control by decomposing precipitates through boiling with acid. The details of these experiments I have already described. The CH_2O content of the precipitate was invariably much greater than what would correspond to the above ratio. This is readily explained by the mechanical fixation of the CH_2O . The alkali liberated corresponded to 11.9, and 21.2 c.c. $\frac{N}{10}$ per gram of the precipitate. These figures are necessarily very faulty. In the first case the mixture was turbid, so that the decomposition was not quite complete; in the second we may assume a mechanical retention of the alkali. But they approach sufficiently to the theoretical number to lend it support. The compound, therefore, contains somewhat more alkali than the original albumose (1.6 : 1.3). It comes so near, however, that it suggests the view that the alkali which is fixed is mainly the "natural" alkali of the peptone, and if

this is true, it follows that one equivalent of albumose unites with two equivalents of CH_2O .

It is a most interesting fact that if the exact proportion of the ingredients are employed the combination occurs with extreme slowness, but is hastened very greatly when a greater concentration of any of the ingredients is present. I was at first tempted to explain this favoring influence of the increased alkalinity on the theory that the alkali-albumose compound mentioned in the preceding paper entered more readily into the reaction. However, since the same favorable influence is exerted by a greater concentration of any of the other constituents, I refer the effect of increased alkalinity to the same cause. I believe that we have here merely an illustration of the old phenomenon that concentrated solutions react more energetically than dilute. The great sensitiveness of the present reaction to concentration is interesting, but may probably be explained by the fact that the slowness of the reaction permits us to follow it more accurately.

Before concluding this paper, it may not be amiss to compare the formaldehyde reaction of the albumose with that of other proteids as determined by Benedicenti (1).

Proteid.	Alkalinity per gm. in c.c. of normal solution (methyl orange).	CH_2O bound per gm.	c.c. of $\frac{N}{10}$ alkali bound per gm.
		Maxima.	
Gelatin, liquefied by boiling	0.85	0.0135	1.0
Gelatin, liquefied by boiling pyocyaneus	0.85	0.0390	0.5
Egg, white, fresh	0.175	0.00375	0.2
Egg, white, dry	1.3	0.0180	1.75
Blood serum	0.175	0.00315	0.25
Fibrin	0.35	0.0115	3.1
Casein	0.72	0.0059	3.8
Albumose (SOLLMANN) .	1.3	0.0447	6.4

The albumose binds the greatest amount, both of the formaldehyde and of the alkali. In the former respect it is most nearly approached by the products formed from gelatin through bacterial action. It is

interesting to note that the combination of proteid with CH_2O is always accompanied by a binding of alkali. However, the ratio of the two substances entering into the combination varies with the different proteids. It does not appear to depend upon the alkalinity of the original proteid. The binding of the two substances proceeds parallel with most proteids; but with gelatin and fresh egg-albumin the combination of the alkali is completed considerably before that of the formaldehyde.

While on this subject, I deemed it interesting to investigate whether *acacia* also enters in combination with formaldehyde. This was found not to be the case. I found that *acacia* gives no precipitate with formaldehyde either naturally or on altering its reaction. The sample of *acacia* required for neutralization to methyl orange 1.9 c.c. $\frac{N}{10}$ acid. With the usual disposition, neutralizing before adding the hydroxylamin, 10 c.c. of 5 per cent solution of this *acacia* requires, after standing with 4 c.c. of 2 per cent formaldehyde for

One hour, 26.7 c.c. of $\frac{N}{10}$ alkali.

Four days, 26.2 c.c. of $\frac{N}{10}$ alkali.

Therefore none of the formaldehyde is bound.

CONCLUSIONS.

A. The addition of formaldehyde to a weakly alkaline solution of Witte's peptone causes the formation of a precipitate which develops somewhat slowly. The precipitate is dissolved by boiling with dilute acid or alkali, the solubility diminishing on prolonged contact with the precipitant. The solutions give the general reactions of the original proteose, and may be reprecipitated by reducing the reaction to faint alkalinity. The precipitate contains chemically combined formaldehyde. A certain amount of the latter is also retained mechanically, but so that it cannot be removed by prolonged washing and heating.

B. Only about 40 per cent of the proteids of Witte's peptone participate in the reaction. The primary albumoses are completely precipitated.

C. The formation of the precipitate occurs only within comparatively narrow limits of reaction, — 0.2 c.c. N acid to 1.6 c.c. N alkali per gram of peptone. The optimum reaction for precipitation is 1.1 c.c. N alkali per gram. Precipitation is also favored by greater concentration in albumose or in CH_2O , as also by standing or boiling.

The reason for the non-precipitation of the albumose with an acid reaction of the liquid lies in the fact that no combination occurs. Increased alkalinity, on the other hand, favors the combination, but precipitation does not occur, since the compound is soluble in alkaline liquids.

The binding of the CH_2O is associated with a binding of the alkali, the two entering into combination in a constant ratio. Both are liberated in the original quantity if the compound is decomposed by the action of acid.

The combination is favored by greater concentration in alkali, formaldehyde, or albumose, and by standing or boiling. A certain maximum is reached which is constant under whatever conditions it has been produced. The combination is prevented by the presence of neutral salts.

D. One gram of Witte's peptone is capable of binding 0.0447 gm. of CH_2O and 6.4 c.c. $\frac{N}{10}$ alkali. It appears probable that the compound weighs 0.402 gm., so that a gram of the compound contains 0.1109 gm. CH_2O and 15.9 $\frac{N}{10}$ alkali. The ratio of CH_2O to alkali is two equivalents of the former to one of the latter. Since the bound alkali is probably the natural alkalinity of the albumose, it follows that one equivalent of albumose binds two equivalents of CH_2O .

A binding of alkali is also seen with other proteids, according to Benedicenti's figures.

Acacia does not bind formaldehyde.

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A COMPARATIVE STUDY OF THE VISCOSITY OF THE BLOOD.

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IN the present investigation upon the determination of the viscosity of the blood in cold-blooded animals the method used is that given by Hürthle¹ for measuring the viscosity of the "living" blood in mammals. In brief, the method consists in determining the time which a measured quantity of blood, under a certain pressure, requires to pass through a capillary tube of known size. Cannulas are placed in both carotid arteries. One cannula communicates with the capillary, the other with a mercury manometer. When the blood escapes from the capillary it is caught on one of the two triangular glass plates which are attached to a vertical lever and which move in front of the opening at right angles to the direction of its lumen. The glass plates diverge below, each one leading to a receptacle. After one or two drops of the blood have dripped into one of the small glasses, the lever is rapidly moved to the other side. The blood now passes into the second receptacle, which is weighed afterward, together with the glass plate leading to it. After about twenty-five or thirty seconds the blood is again directed into the receptacle used before. The movements of the lever are recorded upon smoked paper beneath the record of a Jaquet chronometer, beating fifths of seconds. The viscosity coefficient K is obtained by calculation from the quantity of the blood, its specific gravity, the blood-pressure, the duration of flow and the length and internal diameter of the capillary.

In the present series of experiments two capillaries were employed. The diameter of each was measured with the aid of the microscope and was determined again by weighing columns of mercury contained in the lumen of the tube (Table I).

For capillary A the following values were taken : length, 249.0 mm.,

¹ HÜRTHLE, K.: *Archiv für die gesammte Physiologie*, 1900, lxxxii, pp. 415-442.

diameter, 0.525 mm. ; and for capillary B: length, 254.0 mm., diameter, 0.4955 mm.

TABLE I.
THE DIMENSIONS OF THE CAPILLARIES.

Capillary.	Length. (mm.)	Diameter. (mm.)	
		Microscope.	Mercury column.
A	249.0	0.5250	0.5250
		0.5254	
		0.5260	
		0.5252	
B	254.0	0.4950	0.4955
		0.4962	
		0.4951	

It was now necessary to determine whether with these capillaries values for the viscosity of distilled water could be obtained which would agree with the standard determinations given by Poiseuille. The results are shown in Table II. It will be observed that the

TABLE II.
EXPERIMENTS WITH DISTILLED WATER.

Exp.	Capillary.	Temperature C.	Time. in secs.	Pressure. mm. Hg.	Quantity. mg.	I. Coefficient f. dist. water.	II. Coefficient. Poiseuille.	Difference between I. and II.
1	A	21.0°	32.51	1025.0	3375.55	3282.4	3316.0	-34
2	A	21.0°	34.60	817.0	2873.1	3293.5	3316.0	-23
3	A	20.0°	38.40	770.0	2956.35	3240.0	3236.0	+4
4	B	22.0°	29.79	765.0	2415.95	3429.0	3406.0	+23
5	B	22.0°	26.59	703.0	1974.85	3423.0	3406.0	+17
6	B	22.2°	34.40	684.0	2519.6	3470.0	3406.0	+64

coefficients, obtained with the above-mentioned capillaries, differ only little from those of Poiseuille; indeed, the differences are too slight to be of importance.

THE VISCOSITY OF THE "LIVING" BLOOD OF THE FROG.

I had intended at first to determine the viscosity of the frog's blood both in the warm and the cold season of the year, but, as frogs in captivity rarely show a true winter sleep and as this sleep, were it present, would be more or less disturbed during the experiments, I have abandoned this idea for the present. Under these adverse circumstances which were increased by anatomical difficulties, I was forced to seek a more uniform basis for the experiments.

The frogs were caught late in the autumn. The experiments were made in the winter months, from November to February inclusive. The frogs remained in the laboratory for two days before the experiment, at a uniform temperature of from 20° to 22° C.

The specific gravity of the blood was determined by means of small, curved glass tubes, having very thin walls and measuring about 1 mm. in diameter. The relative values were obtained by weighing the tube when empty and when filled first with water and then with

TABLE III.
BULLFROG. *Rana catesbiana*.

Arteriæ ischiadicæ. Curare, 5 min. Weight, 290 gms.						
Capillary.	Temp. C.	Spec. grav.	Time in secs.	Pressure. mm. Hg.	Quantity. mg.	Coefficient.
A	20.0°	1036.5	29.24	260.0	322.46	1321.6
Arteriæ ischiadicæ. Curare, 5 min. Weight, 270 gms.						
A	20.0°	1042.0	23.69	250.0	233.68	1227.0

blood. This was necessary because the quantities of blood available did not permit the use of the pyknometer. Even the withdrawal of the cubic centimetre of blood necessary to fill a very small pyknometer would very likely have brought about changes in the viscosity.

When it became necessary in some of the later experiments to know the specific gravity before the determination of the viscosity was begun, the chloroform-benzol determination, described by Hammerschlag,¹ was employed. This method reveals decided changes in the specific gravity very clearly.

I first endeavored to use two corresponding peripheral arteries of small size, one of which could be connected with the mercury manometer and the other with the capillary, but even in the largest frogs these vessels are so small and delicate that it is impossible to introduce sufficiently large cannulas. With larger arteries, the ischiadicæ, satisfactory results were obtained in two bullfrogs. (See Table III.)

Having found the peripheral vessels so unsuitable for these experiments it seemed advisable to use the aortæ. These vessels are sufficiently large even in small frogs. Generally, the left aorta was connected with the capillary, while the right furnished the pres-

TABLE IV.
BULLFROG. *Rana catesbiana*.

Aorta. Ether-narcosis. Weight, 140 gms.						
Capillary.	Spec. grav.	Temp. C.	Time in secs.	Pressure. mm. Hg.	Quantity. mg.	Coefficient.
A	1.0335	21.0°	19.86	222.0	163.84	1515.2
Aorta. Curare, 3 min. Weight, 130 gms.						
A	1.038	21.0°	25.60	231.0	244.05	1290.2
Aorta. Ether-narcosis. Weight, 180 gms.						
A	1.048	20.5°	24.71	235.0	265.15	1147.6

sure curve. To make this method less open to objection the cannulas were introduced as near as possible to the dividing point of the truncus arteriosus. In this way the arteriæ pulmonales were on the proximal side of the cannulas and the lesser circulation was left unobstructed. Furthermore, the peripheral circulation of the right

¹ A. HAMMERSCHLAG: Zeitschrift für klinische Medicin, 1892, xx, p. 444.

side was not cut off until the experiment actually began, and the blood was permitted to flow through the capillary only from twenty to twenty-five seconds. In this way the grave errors which might result in consequence of the obstructions to the circulation were excluded as is shown by the close correspondence between the result of these experiments and those with defibrinated blood (Tables XII-XV).

Table IV shows the results obtained in three experiments with "living" blood by the procedure just described.

The five foregoing experiments, therefore, have given the following coefficients: —

TABLE V.

Experiments.	Temperature C.	Specific gravity.	Coefficient.
1	20.5°	1.048	1147.6
2	20.0°	1.042	1227.0
3	21.0°	1.038	1290.2
4	20.0°	1.0365	1321.6
5	21.0°	1.0335	1515.2

It, considering these results, it seems remarkable that, in spite of the apparent "thinness" of the blood of the frog, the average viscosity coefficient (1300) at 20° C. is almost the same as that in the rabbit (1350)¹. The explanation of the fact that the viscosity of the frog's blood is not less than that of the rabbit must undoubtedly be sought in the temperature. While the warmth of the blood of the former animal corresponds with the temperature of the surrounding media, the blood of the latter is constantly kept close to 37° C. *The great internal friction of the frog's blood is dependent upon the low temperature.*

This statement is supported by the following observation: With every increase in the temperature the viscosity decreases in a corresponding manner — *i. e.*, the coefficients become larger. When the blood of the frog was heated to 37° C. in the experiments with defibrinated and oxalated blood, to be described on pages 253 to 257,

¹ For the viscosity-coefficients of the rabbit's blood, see BURTON-OPITZ, R.: *Archiv für die gesammte Physiologie*, 1900, lxxxii, pp. 460-463.

the viscosity fell decidedly below that of the rabbit's blood, the coefficients then being — for the frog 1700 and for the rabbit 1350. Thus, the viscosity of the frog's blood determined at 37° C. showed the expected decrease, as compared with the internal friction of the blood of the rabbit. Under normal conditions the frog is never exposed to so high a temperature, and therefore the viscosity never falls to such a degree. The viscosity of distilled water at 20° C. is 3236 (Poiseuille), the blood of the frog is therefore 2.4 times more viscous. At 37° C. the viscosity of distilled water is 4696, that of the frog's blood (1700) is therefore 2.7 times greater.

Table V shows further that the changes in the specific gravity and the viscosity of the blood pursue a parallel course—namely, the greater the specific gravity, the greater the internal friction and *vice versa*. This relationship may become indefinite, or even contrary, if the difference between two samples of blood is very slight; but on the whole decided changes in the viscosity are always betrayed by equally conspicuous changes in the specific gravity.

THE INFLUENCE OF CURARE AND ETHER ON THE VISCOSITY OF THE "LIVING" BLOOD.

It is of course essential that the frog remain motionless while the experiment is in progress. To attain this end ether or curare was employed. The question arose therefore whether these agents change the viscosity of the blood. As it is impossible to determine the viscosity in the unnarcotized animal by the method used in this investigation, it is necessary in seeking an answer to the above problem to rely upon a comparison of the specific gravity of the blood of the unanæsthetized and anæsthetized animals. I hold myself justified in making this comparison, because the experiments with the blood of warm-blooded animals,¹ as well as those in the present series upon the frog, show conclusively that a very distinct parallelism exists between the specific gravity and the viscosity. However, in drawing these conclusions it is essential that the differences be large.

In the experiments we are now considering the specific gravity was determined by means of the chloroform-benzol mixture. The blood was withdrawn from the arteriæ ischiadicæ. These vessels were isolated from the surrounding tissues, and raised so that the blood

¹ BURTON-OPITZ, R.: Archiv für die gesammte Physiologie, 1900, lxxxii, pp. 447-463 and pp. 464-473.

could be conveniently caught in the vessel containing the chloroform-benzol mixture. A few drops of blood were first allowed to escape before the drop to be used was allowed to fall into the liquid. Two or three determinations were made. The wound was then closed, because in the second withdrawal of blood the other artery was used, two or three determinations again being made.

The details of the experiments with curare are given in Table VI.

TABLE VI.
THE EFFECT OF CURARE.

Exp.	Weight of frogs. gms.	Sp. gr. before injection.	Quantity injected. min.	Time elapsed. minutes.	Sp. gr. after injection.
1	125	1.035	3	15	1.0415
2	25	1.041	1-2	20	1.043
3	110	1.035	3	15	1.040
4	130	1.038	3	15	1.045
5	170	1.037	4	20	1.041
6	120	1.032	4	20	1.0395
7	90	1.044	3	20	1.048

The same method was employed in the experiments with ether. The frogs were allowed to inhale the vapor until dyspnoea resulted, because at earlier stages of etherization no marked change could be obtained. The first two frogs were subjected to a second narcosis some time after the first with the result as given in Table VII.

Both curare and ether, administered in the specified manner, materially increase the specific gravity of the blood of the frog. As it has been shown above, and in my earlier papers, that the specific gravity and the viscosity usually run a parallel course, it is probable that as the specific gravity is increased by curare and deep etherization, the viscosity is also increased. This result cannot destroy the value of the experiments with "living" blood, because, even if this increase takes place when ether and curare are employed, the coefficients are still correct. They betray the viscosity when the specific gravity possesses the indicated values.

TABLE VII.
THE EFFECT OF ETHER.

Exp.	Weight of frogs. gms.	Sp. gr. Normal.	Sp. gr. Ether-narcosis. I.	Interval. min.	Sp. gr. after I.	Sp. gr. Ether-narcosis. II.	Interval. min.	Sp. gr. after II.
1	120	1.036	1.0415	40	1.0375	1.041	50	1.038
2	160	1.033	1.037	50	1.0345	1.0375	60	1.0345
3	80	1.040	1.043					
4	25	1.037	1.0425					
5	120	1.0375	1.0415					

I determined the specific gravity in about thirty frogs, varying in size from twenty-five to two hundred and ninety grams. The values ranged from 1.033 to 1.050, the average being 1.037-1.038. This property of the blood bears no relationship to the size of the animal.

THE VISCOSITY OF THE DEFIBRINATED AND OXALATED BLOOD.

In attempting a comparative study of the viscosity of the "living" blood and blood which had been kept fluid artificially either by defibrination, or by the addition of some oxalic salt, I was again embarrassed by various difficulties. All the experiments with the unnarcotized animal failed more or less completely, and even if I had succeeded, the quantity of blood remaining would not have been sufficiently large for an adequate number of determinations of the latter type. Under these circumstances the specific gravity had to be taken as the only indication for the viscosity of the "living" blood.

The blood was withdrawn from the arteria ischiadica, to the first 3 c.c. being added from two to three drops of a 2 per cent solution of potassium oxalate. Then the remaining quantity was quickly withdrawn, but in order to prevent dilution by lymph no more than 5 c.c. of blood were collected and defibrinated.

The apparatus was arranged in the following manner: A pressure-bottle was connected by means of a Y-tube with a mercury manometer and a short wide glass tube which contained the blood, stopcocks being interposed in each case. The small reservoir for the blood could easily be joined with the Y-tube on the one side, and

with the capillary on the other. Only the ends of the glass tube projected, the remaining portion being surrounded by a mantle through which water of a certain temperature could be passed. When the experiment began it was only necessary to open the stopcocks, the blood then being driven through the capillary, where by means of the vertical lever it was collected in the weighing-glass.

A special arrangement to prevent sedimentation was unnecessary, because the blood was well mixed before being drawn into the glass tube. Moreover, the entire quantity of the blood contained in the receptacle was always forced through the capillary in each experiment. Finally, the duration of the experiment was shortened as much as possible, the different determinations usually not lasting longer than from twenty-five to thirty seconds. As the viscosity is materially increased when the blood is left standing for some time, these experiments were always finished in as short a time as possible after the withdrawal, generally in from three to four hours.

While the first two tables (VIII and IX) give the coefficients of both the oxalated and the defibrinated blood in each case, the last table (X) gives only the viscosity of the oxalated blood of two frogs.

The results of this group of experiments are essentially the same as those obtained with the blood of the dog. The viscosity of the "living" blood is not at all like that of the defibrinated or the oxalated blood. While by the addition of potassium oxalate the inner friction is perceptibly increased, the blood shows a decided decrease after defibrination.

TABLE VIII.

Rana catesbiana.

Specific gravity of "living" blood, 1.0442. Weight, 145 gms.									
Experiments.	Capillary.	Spec. gravity.	Temp. C.	Time in secs.	Pressure. mm. Hg.	Quantity mg.	Coefficients.	Difference.	Mean value.
1. Oxalated blood	A	1.0447	20.5°	21.92	800.0	688.40	1218.0	} 43.0	1196.5
	A	1.0447	20.5°	16.34	800.0	495.23	1175.0		
2. Defibrinated blood	A	1.0335	20.5°	25.16	762.0	903.0	1476.0	..	1476.0

TABLE IX.

Rana catesbiana.

Specific gravity of "living" blood, 1.0451. Weight, 155 gms.									
Experiments.	Capillary.	Spec. gravity.	Temp. C.	Time in secs.	Pressure. mm. Hg.	Quantity mg.	Coefficients.	Difference.	Mean value.
1. Oxalated blood	A	1.0455	20.0°	23.74	848.0	741.48	1141.5	} 16.5	1149.0
	A	1.0455	20.0°	31.21	820.0	956.16	1158.0		
2. Defibrinated blood	A	1.037	20.0°	28.17	756.0	854.64	1254.0	} 31.0	1269.5
	A	1.037	20.0°	49.61	320.0	652.80	1285.0		

TABLE X.

Rana catesbiana.

Weight about 90 gms. each.							
Capillary.	Sp. gr. of normal blood.	Sp. gr. of oxalated blood.	Temp. C.	Time in secs.	Pressure. mm. Hg.	Quantity. mg.	Coefficients.
A	1.034	1.0355	21.0°	31.83	703.0	944.9	1321.4
A	1.0365	1.0372	21.0°	32.43	686.0	913.05	1284.3

By adding slightly more of the potassium oxalate solution than is absolutely necessary to keep the blood fluid, the specific gravity can be reduced to normal again. This explains the insignificant increase in the specific gravity of the second frog, Table IX.

THE VISCOSITY OF THE SERUM.

As the quantity of serum collected from about 10 c.c. of blood is small, only two or three determinations could be made, the temperature being 20° C. Three frogs were used, weighing about one hundred and twenty grams each.

TABLE XI.
THE VISCOSITY OF THE BLOOD-SERUM.

Exp.	Capillary.	Temp. C.	Specific gravity		Time. in secs.	Pressure. mm. Hg.	Quantity. mg.	Coefficients.	Difference.	Mean value.
			of "living" blood.	of blood-serum.						
I	A	20.0°	1.048	1.025	15.94	703.0	664.1	1877.4	} 23.4	1866.0
	A	20.0°	1.048	1.025	7.65	703.0	314.58	1854.0		
II	A	20.0°	1.049	1.0255	29.92	756.0	1272.3	1777.0	} 57.0	1748.0
	A	20.0°	1.049	1.0255	17.55	761.0	726.85	1720.0		
	A	2.00°	1.049	1.0255	38.55	749.0	1607.35	1759.0		
III	A	20.0°	1.0405	1.0245	7.8	870.0	377.9	1761.5	} 16.5	1769.0
	A	20.0°	1.0405	1.0245	13.43	870.0	656.76	1778.0		

It can be seen very readily that after the removal of the solid parts of the blood the viscosity undergoes only slight variations, as compared with the inner friction of the "living" blood. This proves again that the somewhat far-reaching differences in the coefficients of the normal blood are largely dependent upon its usual solid constituents, and in a lesser degree only upon the circulating nutritive elements. If it be taken into consideration that the serums used in these experiments are slightly heavier than those with which the previous determinations were obtained,¹ the correspondence in the coefficients will be very evident. Thus, at 20° C. the blood serum of the frog shows the same viscosity as the serum of the dog. It is more than likely therefore that, when the temperature is changed, this agreement will be maintained.

THE EFFECT OF CHANGES IN TEMPERATURE.

The experiments in this group were undertaken for the purpose of testing the question whether variations in the temperature of the frog's blood produce the changes in the viscosity observed in dog's blood under similar conditions.

¹ BURTON-OPITZ, R.: *Archiv für die gesammte Physiologie*, 1900, lxxxii, p. 471.

The specific gravity was determined at 5°, 20°, and 37° C. The intervening values were obtained by calculation. The assumption was made therefore that with every increase or decrease in the temperature the specific gravity suffers a constant and entirely proportional change in its value.

The details of the experiments as well as their results are compiled in Tables XII and XV. Tables XIV and XV represent two experiments with "normal" blood. In the latter cases the specific gravity of the "living" blood was determined first. The remaining quantity was then defibrinated. To this sample of blood the sediment of the defibrinated blood of a second frog was added until repeated determinations showed that the specific gravity had again been raised to its normal value. The same result, as I have mentioned previously, can be obtained by adding to freshly withdrawn blood slightly more of the potassium oxalate solution than is necessary to keep it fluid. The specific gravity was determined at 20° C.

TABLE XII.
DEFIBRINATED BLOOD. EFFECT OF CHANGES IN TEMPERATURE.

Capillary.	Sp. gr. of "living" blood, 20° C.	Temperature C.	Sp. gr. defibrinated blood.	Time in secs.	Pressure. mm. Hg.	Quantity. mg.	Coefficients.	Difference.	Mean value.	Differences for neighboring values.
A	1.042	5°	1.0385	28.09	350.0	161.1	511.3	..	511.3	} 192.7
A	1.042	10°	1.0375	24.83	453.0	253.7	704.0	..	704.0	
A	1.042	15°	1.0365	35.20	352.0	378.2	954.2	} 20.6	964.5	} 260.5
				16.90	953.0	502.2	974.8			
A	1.042	20°	1.036	16.71	887.0	565.3	1193.0	} 14.0	1200.0	} 235.5
				13.79	887.0	472.1	1207.0			
A	1.042	30°	1.033	21.61	602.0	645.85	1593.6	..	1593.6	} 393.6

These results prove a close correspondence between the blood of the frog and that of the dog. When the temperature is altered, the viscosity is also changed, *i. e.*, the higher the former the less the latter, and *vice versa*. Besides, the differences in the values of the coefficients produced by variations in the temperature appear to be constant and proportional for every degree Celsius.

TABLE XIII.
DEFIBRINATED BLOOD. EFFECT OF CHANGES IN TEMPERATURE.

Capillary.	Sp. gr. "living" blood, 20° C.	Temperature C.	Sp. gr. defibrinated blood.	Time in secs.	Pressure, mm. Hg.	Quantity, mg.	Coefficients.	Difference.	Mean value.	Differences for neighboring values.
A	1.0445	5°	1.0415	43.30	395.0	255.9	465.5	..	465.5	} 270.7
A	1.0445	10°	1.040	39.40 40.08	636.0 636.0	610.1 584.0	758.6 713.8	} 44.8	736.2	
A	1.0445	15°	1.039	21.00 53.80	405.0 381.0	630.3 623.6	626.1 948.9	} 22.8	937.5	} 201.3
B	1.0445	20°	1.0385	34.28 29.76	429.0 425.0	421.56 373.1	1163.1 1197.0	} 33.9	1179.9	} 242.4
B	1.0445	30°	1.036	33.55	408.0	521.54	1549.7	..	1549.7	} 369.8
B	1.0445	37°	1.0335	27.08	579.0	650.5	1691.4	..	1691.4	} 141.7

TABLE XIV.
"NORMAL" BLOOD. EFFECT OF CHANGES IN TEMPERATURE.

Capillary.	Sp. gr. of "living" blood at 20° C.	Temperature C.	Sp. gr. of "normal" blood.	Time in secs.	Pressure, mm. Hg.	Quantity, mg.	Coefficients.	Difference.	Mean value.	Difference between neighboring values.
A	1.0391	10.0°	1.041	38.56	487.0	405.0	671.3	671.3	} 167.1
A	1.0391	15.0°	1.0402	35.96 39.25	480.0 475.0	453.95 514.3	817.4 859.4	} 42.0	838.4	
A	1.0391	20.0°	1.0394	32.50 24.26	593.0 582.0	645.8 481.0	1046.5 1062.1	} 15.6	1053.8	} 215.4
A	1.0391	30.0°	1.037	27.35 31.20	493.0 492.0	620.5 711.3	1438.0 1447.9	} 9.9	1443.0	} 389.2
A	1.0391	37.0°	1.0355	21.72	388.0	436.6	1621.1	1621.1	} 178.1

TABLE XV.
 "NORMAL" BLOOD. EFFECT OF CHANGES IN TEMPERATURE.

Capillary.	Sp. gr. of "living" blood at 20° C.	Temperature C.	Sp. gr. of "normal" blood.	Time in secs.	Pressure. mm. Hg.	Quantity. mg.	Coefficients.	Difference.	Mean value.	Difference between neighboring values.	
B	1.0375	20.0°	1.0372	33.62	737.5	744.0	1219.1	} 8.6	1223.4	} 372.1	
				30.08	737.5	670.4	1227.7				
B	1.0375	30.0°	1.034	24.31	727.0	685.04	1579.5	} 31.9	1595.5		} 171.5
				26.94	726.5	773.94	1611.4				
B	1.0375	37.0°	1.033	22.76	705.0	695.1	1767.0	1767.0		

The less the viscosity, the greater are the differences for every degree Celsius. Thus, while in this case a rise or fall of 5° C. produces a corresponding change in the viscosity of about 200, this difference was only half as large in the dog. The reason seems to lie in the fact that the coefficients of the frog's blood must travel over a larger numerical field to reach the zero-value.

At 20° C. the coefficients of the frog's blood range through about the same numerical field as those of the blood of the rabbit. If, however, the temperature is raised, the correspondence is rapidly destroyed; the viscosity of the frog's blood suffers a decided decrease. Although the coefficients of these experiments were obtained with defibrinated blood and blood raised to its normal specific gravity, the viscosity-values obtained may nevertheless be assumed to be the same as if "living" blood had been exposed to a temperature of 37° C. Even if the specific gravity and the viscosity of the defibrinated blood show a great decrease as compared with the "living" blood, the coefficients still hold good for blood which under normal conditions possesses the specific gravity of the defibrinated blood used in these determinations. The average value of the viscosity of the blood of the dog, as derived from my previous experiments, was 925, and that of the rabbit was 1350. The preceding three determinations show that at 37° C. the blood of the frog possesses the value of about 1700.

The question now arises whether the viscosity suffers changes within the body when the frog is exposed to different temperatures.

Naturally, the specific gravity must be taken as the only indication of possible variations in the viscosity.

Having previously obtained the specific gravity in four frogs which had been kept in a temperature of 20° C., this value was again determined after they had remained for an hour in a room at 8-10° C. They were then kept at about 25° C. for an equally long time, after which the third determination was made.

The following changes were obtained:

TABLE XVI.

THE SPECIFIC GRAVITY WHEN EXTERNAL TEMPERATURE IS ALTERED.

Experiments.	Weight of frogs. gms.	Specific gravity. 20° C.	Specific gravity. 8-10° C.	Specific gravity. 25° C.
1	110	1.0405	1.0413	1.040
2	90	1.046	1.0468	1.0457
3	120	1.044	1.046	1.0425
4	25	1.041	1.0417	1.0405

THE VISCOSITY OF THE BLOOD OF THE TORTOISE.

Having failed more or less completely in determining the viscosity of the "living" blood, the present series embodies only three experiments with defibrinated blood. In the last experiment, the

TABLE XVII.

DEFIBRINATED BLOOD OF TORTOISE. EFFECT OF CHANGES IN TEMPERATURE.

Capillary.	Sp. gr. of "living" blood at 20° C.	Temperature. C.	Sp. gr. of defibrinated blood.	Time in secs.	Pressure. mm. Hg.	Quantity. mg.	Coefficients.	Difference.	Mean value.	Difference between neighboring values.		
A	1.0345	15.0°	1.0292	21.08	797.0	725.0	1359.0	24.5	1371.2	} 238.5		
				19.60	795.0	684.6	1383.5					
A	1.0345	20.0°	1.0287	15.02	715.0	548.37	1608.2	2.9	1609.7		} 351.4	
				16.36	714.0	597.5	1611.1					
A	1.0345	30.0°	1.0272	17.02	708.0	740.24	1937.9	46.4	1961.1			} 351.4
				17.30	708.0	770.4	1984.3					

defibrinated blood was raised to the specific gravity of the "living" blood by the addition of a certain amount of the corpuscular sediment in the manner described previously.

TABLE XVIII.

DEFIBRINATED BLOOD OF TORTOISE. EFFECT OF CHANGES IN TEMPERATURE.

Capillary.	Sp. gr. of "living" blood at 20° C.	Temperature C.	Sp. gr. of defibrinated blood.	Time in secs.	Pressure. mm. Hg.	Quantity. mg.	Coefficients.	Difference.	Mean value.	Difference between neighboring values.	
A	1.0445	15.0°	1.0392	16.78°	792.0	425.6	998.6	35.2	1016.2	} 203.4	
				13.66°	790.0	357.77	1033.8				
A	1.0445	20.0°	1.0385	24.38°	785.0	742.1	1209.9	19.4	1219.6		} 199.4
				20.95°	780.0	643.8	1229.3				
A	1.0445	25.0°	1.038	17.23°	785.0	602.5	1390.6	56.9	1419.0		} 370.8
				19.80°	785.0	720.7	1447.5				
A	1.0445	37.0°	1.0345	18.50°	755.0	796.5	1789.8	..	1789.8		

TABLE XIX.

"NORMAL" BLOOD OF TORTOISE. EFFECT OF CHANGES IN TEMPERATURE.

Capillary.	Sp. gr. of "normal" blood at 20° C.	Temperature C.	Sp. gr. of defibrinated blood.	Time in secs.	Pressure. mm. Hg.	Quantity. mg.	Coefficients.	Difference.	Mean value.	Difference between neighboring values.	
A	1.0358	15.0°	1.0361	32.90	540.0	619.95	1091.4	28.9	1076.9	} 275.5	
				37.40	540.0	686.0	1062.5				
A	1.0358	20.0°	1.0355	25.20	829.0	897.0	1342.5	19.8	1352.4		} 186.7
				28.20	799.0	980.9	1362.3				
A	1.0358	25.0°	1.034	26.06	577.0	727.3	1515.7	46.8	1539.1		} 345.4
				22.80	577.0	655.9	1562.5				
A	1.0358	37.0°	1.0325	14.75	722.0	634.2	1869.0	31.3	1884.5		
				20.60	722.0	900.6	1900.3				

I used *Clemmys insculpta*, so common in the rivers of New England. The blood was withdrawn from the art. carotidea and the viscosity-determinations were made shortly after the defibrination.

Having found such a low specific gravity and a correspondingly slight viscosity in the first experiment, the other two tortoises were given some food two days before the determinations. The assumption that in this instance the somewhat unusual result was largely dependent upon a low state of nutrition is suggested by the viscosity-coefficients of the blood-serum.

TABLE XX.
 VISCOSITY OF BLOOD-SERUM IN TORTOISE I.

Capillary.	Temperature C.	Spec. gravity.		Time in secs.	Pressure. mm. Hg.	Quantity. mg.	Coefficients.	Difference.	Mean value.
		"Living" blood.	Serum.						
A	20.0°	1.0345	1.017	28.05	395.0	866.9	2493.0	} 59.6	2523.0
A	20.0°	1.0345	1.017	35.18	400.0	1127.3	2552.6		

The results of the last two experiments coincide very closely with those obtained with the defibrinated blood of the frog. Although the viscosity of the blood of the tortoise, when determined at 20° C., shows about the same value as that of the rabbit at 37° C., this relationship is destroyed by exposing the former to higher temperatures. Thus, at 37° C. the coefficients of the blood of the tortoise are very much larger than those of the rabbit's blood, the relationship being about 1800 to 1350.

SUMMARY.

1. The average viscosity-coefficient of frog's blood at a normal temperature of 20° C. is 1300; that of tortoise blood at 20° C. is 1285. The coefficient of rabbit's blood at a normal temperature of 37° C. is 1350.
2. The viscosity of frog and tortoise blood varies inversely as the temperature. The coefficient changes about 200 for every change of 5° in the temperature. The coefficient of frog's blood at 37° C. is 1700; that of tortoise blood at 37° C. is 1800.
3. The average viscosity of blood-serum at 20° C. is 1800.
4. The viscosity-coefficients of normal living blood range through a large numerical field, while the variation in the viscosity of serum

is small. The changes in viscosity are therefore largely dependent upon the normal solid elements of the blood and only in a less degree upon the constituents of the plasma.

5. The specific gravity of frog's blood is altered by changes in the temperature in which the frog is placed.

6. The specific gravity of frog's blood is increased by potassium oxalate, curare, and deep etherization, and diminished by defibrination. As similar changes are observed in the viscosity, considerable variations in the specific gravity may be taken as an index of variations in the viscosity.

THE OSMOTIC PROPERTIES OF COLLOIDAL SOLUTIONS.

BY BENJAMIN MOORE AND WILLIAM H. PARKER.

THE physical properties of colloidal solutions have recently furnished the object of much investigation, for the subject is one which presents problems of peculiar interest to both the physical chemist and the physiologist. To the physical chemist, because colloids yield a type of solution differing in many respects from both dissociated and non-dissociated solutions of crystalloids, the properties and nature of which are still unknown or at most may be said to be in the region of speculation. To the physiologist this subject is also full of interest and importance because most of the solutions occurring in the body are colloidal in character, and until our knowledge of the properties of these has been increased we can have little hope of progress in the study of the chemistry and physics of the cell.

The chief characteristic of colloidal solutions is the large size of the molecule *in solution* as compared with that of the crystalloid in solution. The large molecule in solution may be due either to the complexity of the dissolved molecule itself as has always been *assumed* to be the case for proteid molecules, or to the aggregation of several simpler molecules, with dissolved soap molecules, as we shall see to be the case from the experiments which we record in this paper, and as is the case with all colloidal solutions of inorganic substances such as silicic acid or ferric hydrate.

The weight of such an aggregate need bear no simple relationship to the chemical molecular weight of the dissolved substance since the aggregate may contain many chemical molecules combined in some physical fashion, and since we have as yet no experimental evidence that all the aggregates in a solution are of equal size, probable as such an hypothesis may appear. It is hence convenient to have some name for such a colloidal aggregation in solution, and since it may consist of as many as twenty to thirty or even more chemical molecules united together, it has seemed to us better not to term it a molecule at all and hence in what follows we shall usually refer to it

as the *solution aggregate*. It is upon the size and weight of this solution aggregate that the physical properties of a colloidal solution depend and not directly upon those of the molecular weight of the dissolved substance in the usual chemical sense.

It is obvious that the degree of aggregation of the same substance may vary with many physical circumstances, such as the temperature, chemical reaction, presence or absence of crystalloids in varying quantity, and mode of formation of the colloidal solution, so that although the size of the solution aggregate is always large it may vary within wide limits, and hence there are many grades of colloidal solution.

There is, in fact, no hard and fast line separating colloidal solutions from mono-molecular non-dissociated solutions. Even the original distinction, giving rise to the terms crystalloid and colloid is obscured by many exceptions, for in the solid state many typical colloids such as hæmoglobin, several vegetable, and some animal proteids, occur in distinctly crystalline form, and there are numberless examples of amorphous and gum-like bodies in the solid form which yet yield solutions of typically crystalloid character.

Nor does the complexity of the *chemical* molecule of a given substance attended by a high molecular weight, necessarily give rise to typical colloidal properties, for many substances of high molecular weight yield solutions, more especially with organic solvents, which possess typical crystalloidal characters, and on the other hand many substances of low molecular weight such as the soaps and certain inorganic substances yield typical colloidal solutions, by forming by physical union large solution aggregates.

It is hence possible, as we shall see, that many substances, *such as the proteids*, which we have been in the habit of considering as hopelessly complex in chemical nature, mainly from physical evidence of a high molecular weight, may owe their complexity chiefly to physical aggregation in solution.

It is well to bear clearly in mind therefore that a large solution aggregate and its accompanying physical attributes need not necessarily indicate a corresponding molecular complexity in the chemical sense.

It is also exceedingly difficult to draw in any practical way a distinction between crystalloidal and colloidal solutions which shall not be absolutely arbitrary.

The best one is probably that of Graham, that crystalloids diffuse through membranes while colloids do not so diffuse, but this is

obviously a purely arbitrary line of division and merely involves the question of the comparative size of the molecules or *aggregates* in solution and the pores of the membrane used. For, as Traube¹ has shown, membranes can be constructed which refuse passage even to the ions of dissociated solutions. Further, even in the case of typical colloids, passage is given by certain membranes and refused by others, although there are not in either case openings which can be seen under the highest microscopic powers. Thus, the facts of nutrition prove that the capillaries in all regions of the body must be freely permeable to the proteids of the plasma, but parchment paper or a gelatine membrane is impermeable to those same proteids. Again, if the same membrane, say parchment paper, be chosen as the standard it will be found to be permeable to various forms of albumose, dextrans, and certain gums which it is obviously unscientific to exclude from the colloid class, since they resemble the other members of that class in so many physical attributes, notably in giving on concentration viscid solutions which froth upon agitation.

Thus it is obvious that there is no demarcation between crystalloidal and colloidal solutions, but rather that the typical properties of the colloidal solution gradually develop *pari passu* with the growth of the aggregate in solution.

When the growth of the solution aggregate is traced towards the other extreme toward which it passes, viz., the emulsion, it becomes apparent that here also there is no abrupt transition, but that by imperceptible degrees the solution aggregates at first interfere with the shorter wave-lengths of light giving bluish opalescent solutions as is seen typically in dilute solutions of glycogen or caseinogen, then become yellowish as in more concentrated solutions where the aggregation is greater, later dull white or opaque, and finally yield an emulsion with fine granules visible under the microscope.²

Accompanying the gradual growth of the solution aggregate there are certain changes in the physical properties of the solution which as they develop clearly mark off the typical colloidal from the typical crystalloidal solutions. New characteristic phenomena appear, while certain of the most important properties of crystalloidal solutions fall within the limits of experimental error or entirely disappear.

¹ TRAUBE: Archiv für Anatomie und Physiologie, 1867, S. 87.

² PICTON and LINDER (Journal of the chemical society, London, 1892, lxi, p. 148; 1895, lxxvii, p. 63) have shown that several of these stages can be obtained with the same chemical substance, viz., arsenious sulphide.

Thus colloidal solutions are viscid, the viscosity increasing with the concentration until gums are obtained; hence they form tenacious films, froth on agitation, and conserve emulsions. Further, they separate on supersaturation, by alteration of the physical conditions of their solution, into two phases the properties of which have recently been investigated by Hardy,¹ who sees in such separation into two systems, one rich and one poor in colloids, a probable physical explanation of the mode of production of the histological structure of protoplasm, one phase forming the reticulum and the other the hyaline spaces. Under certain conditions, which will be referred to later, the separation occurs in granular form, while in other cases gelatinization takes place. These are some of the new properties which colloidal solutions acquire by the growth of the solution aggregate, but at the same time other properties, namely those connected with the osmotic pressure, such as lowering of freezing point and vapour pressure, become so depressed as to fall within the limits of experimental error. It is, indeed, obvious, since these vary inversely in value as the molecular weight in solution, that they must be depreciated proportionately to the degree of aggregation; but it becomes an important question whether they are merely so depressed or entirely disappear when the solution aggregate attains a certain size, and it is to this question which is one of the most important concerned in our experiments that we must now turn.

The question at issue is whether a "solution aggregate" consisting of many chemical molecules, in physical combination, behaves like a molecule of a crystalloid solution, and exerts osmotic pressure or is perfectly inert, as it were merely in suspension, with none of the properties of matter in solution. It is to be expected that even if the "solution aggregate" does behave like a dissolved molecule, the observed osmotic pressure will be small, for the pressure is proportional merely to the number of molecules in unit volume, or to what is termed the "molecular concentration," and is independent of the weight of the molecule, and in the case of a colloidal solution hence would probably be independent of the size of the solution aggregate. Accordingly the indirect methods of observing osmotic pressure which depend upon small variations, such as lowering of freezing point and raising of boiling point, have, as might have been expected, in the hands of most observers given results which they agree to state

¹ HARDY: *Journal of physical chemistry*, 1900, iv, p. 254; *Journal of physiology*, 1899, xxiv, p. 158.

as falling within the experimental errors of the method. On the other hand, *direct* estimations of osmotic pressure have given readings which have been interpreted by the observers as showing an osmotic pressure due to the dissolved colloid, but there has been in the minds of others, a great difficulty in accepting these results on account of the question of associated crystalloids. We have attempted to perform some work towards the solution of this problem, to which we shall revert after stating some of the results obtained by other workers. Sebanejew¹ found the lowering of freezing point by colloidal silicic acid within the limits of experimental error, and in the case of egg-albumin found with a percentage of 15.6 a depression of 0.02° C., and with a 30.35 per cent solution, a depression of 0.042° C., but the inorganic constituents present were more than sufficient to account for these depressions.

Tamman² estimated the difference in lowering of the freezing point of the serum of the horse before and after coagulating the proteids by heat and removing them, and found that the difference amounted to only 0.006° C., a figure which again lies within the limit of experimental error of the method.

Ludeking³ found that even 40 per cent of gelatine in solution did not perceptibly alter the boiling point from 100° C.

Picton and Linder,⁴ in a measurement by a copper ferrocyanide cell of the osmotic pressure of a 4 per cent solution of colloidal arsenious sulphide, obtained a pressure of only 17 mm. of water, which would be given by an unweighable trace of crystalloid in solution.

Dreser⁵ and Koeppe⁶ both found that removal of the proteid from albuminous fluids had no effect upon the osmotic pressure.

Krafft and Wiglow⁷ found that three parts of gelatine dissolved in thirty parts of water yielded a solution which had exactly the same boiling point as the water used.

¹ SEBANEJEW: *Berichte der deutschen chemischen Gesellschaft*, 1890, xxiii, p. 87; 1891, xxiv, p. 558.

² TAMMAN: *Zeitschrift für physikalische Chemie*, 1896, xx, p. 180.

³ LUDEKING: *Annalen der Physik und Chemie*, 1888, xxxv, p. 552.

⁴ PICTON and LINDER: *Journal of the chemical society*, London, 1895, lxvii, p. 63.

⁵ DRESER: *Archiv für experimentelle Pathologie und Pharmakologie*, 1892, xxix, p. 314.

⁶ KOEPE: *Archiv für die gesammte Physiologie*, 1896, lxii, p. 571.

⁷ KRAFFT F. und H. WIGLOW: *Berichte der deutschen chemischen Gesellschaft*, 1895, ii, p. 2566.

In the case of sodium and potassium soaps, Krafft¹ and his co-workers found different results, according to the concentration of the soap solutions employed. In the case of dilute solutions containing one per cent and under, very variable results were obtained in the boiling-point readings under apparently identical conditions, and the authors were unable to assign any reasons for the contradictory results. Thus, in some cases, with these dilute solutions a result was obtained indicating an apparent molecular weight in solution less than the chemically deduced mono-molecular weight, and hence pointing to dissociation, and in other cases to a solution molecular weight much higher than that of the mono-molecule and pointing to association. For example, in one experiment a solution of neutral sodium stearate in water containing 1.24 per cent gave a rise in boiling point (0.04° C.) which led to a molecular weight of 162, the chemical molecular weight of sodium stearate being 306. While another similar experiment with a solution containing 0.64 per cent, which ought, one would expect, to be more dissociated, led to a molecular weight of 372.² Similarly in the case of sodium oleate (mol. wt. = 304) in two solutions of almost equal strength it was found that while one solution containing 0.99 per cent led to a molecular weight of 177, another solution containing 0.91 per cent led to a molecular weight of 366, both solutions being quite clear from any cloudiness. Obviously no reliance can be placed upon such variable results as indicating either dissociation or aggregation.

On the other hand, Krafft and his fellow-workers obtained concordant results in the case of strong solutions of these soaps, which showed that the apparent molecular weights enormously increased with the strength of solution, pointing here to the fact that at such concentrations the soaps pass into the colloidal condition.

Thus a solution of sodium palmitate (mol. wt. = 278) containing 3.14 per cent gave an apparent molecular weight in solution of 466; a solution containing 7.66 per cent, a solution molecular weight of 885. As the amount of soap in "solution" was increased up to values lying between 20 and 30 per cent, it was found that the boiling point actually fell back to that of pure water, showing, according to Krafft,

¹ F. KRAFFT und A. STERN: *Berichte der deutschen chemischen Gesellschaft*, 1894, ii, p. 1747; F. KRAFFT und H. WIGLOW, *ibid.*, 1895, ii, pp. 2566, 2573; F. KRAFFT, *ibid.*, 1896, ii, p. 1334; 1899, ii, pp. 1584, 1596, 1608.

² In a *later* paper (*Berichte*, 1898, ii, 1592) KRAFFT obtained with a 0.925 per cent solution of potassium stearate a rise of only 0.004, a result which gives a much greater apparent molecular weight than any given here.

that then the soap was present in typical colloidal solution and so did not affect the boiling point *at all*, this physical property disappearing when the solution became a typically colloidal one. That the soaps are really present as colloids in these concentrated solutions, is further evident, according to Krafft, from their increased viscosity and other accessions of the usual typical properties of colloids.

For ease of contrast, we may state that our results on direct measurement of osmotic pressures in soap solutions lead us to agree with Krafft that soaps can behave as true colloids, but we disagree as to this being only true for *concentrated* solutions, for, even with half per cent solutions, *at temperatures above 50 C.*, we have observed that all the typical properties of colloidal solutions, such as viscosity, frothing, and power of retaining emulsions in suspension, still persist; further, the soaps in such strengths do not pass through parchment paper, as they undoubtedly would do if they were present, either dissociated or in mono-molecular solution; and finally the osmotic pressures recorded are only from $\frac{1}{20}$ to $\frac{1}{60}$ of those required for mono-molecular solutions. Also, although the pressures so recorded are small, *yet they are perfectly definite*, and hence from our experiments we do not conclude that this group of allied physical properties really disappears, but merely that these properties become depressed so that all those giving small constants for measurement, such as lowering of freezing point and raising of boiling point, fall within the limit of experimental error, while the direct measurement of osmotic pressure, giving rise as it does to large readings, can still be made.

This point is, of course, of fundamental importance as to the nature of colloidal solution, in deciding whether colloidal solution consists of matter in a condition entirely different from that of a crystalloidal solution or whether it can satisfactorily be explained by increased molecular aggregation.

In regard to Krafft's experiments on dilute solutions, we regard the results as in great extent due to the smallness of the variations in temperature being measured, which also probably explains their great variability from one experiment to another. The objections to taking the results of our experiments on dilute solutions of soaps as indicative of molecular aggregation, will be discussed at a later stage.

The osmotic pressures of proteids in blood serum has been directly measured by Starling,¹ who used for this purpose a gelatine mem-

¹ Science progress, April, 1896: Journal of physiology, 1896, xix, p. 312; *ibid.*, 1899, xxiv, p. 317; SCHÄFER'S textbook of physiology i, p. 307, *et seq.*

brane, and for outer fluid, a proteid-free fluid obtained by pressure filtration through a gelatinized Chamberland filter.¹

In his earlier paper this author found that the osmotic pressure of the serum proteids amounted to 30-40 mm. of mercury, and in his later one with a serum containing 7.5 per cent of proteid he obtained a pressure of 28 mm. of mercury or somewhat less than 4 mm. of mercury for each per cent of proteid.

Starling also experimented with serum in which the percentage of proteid had been increased either by pressure filtration through a gelatine membrane or by immersion of dry gelatine in the serum, and found that the osmotic pressure obtained was directly proportional to the amount of proteid present.

The question of whether the osmotic pressures observed are due to the proteids or to associated traces of crystalloids has been left undetermined by previous observers in the case of proteids.

The determination of the osmotic properties of colloidal solution is surrounded by great difficulties, and unlike the similar determinations in the case of crystalloidal solutions, it is only by the direct method of measurement that reliable results can be expected. In the case of the crystalloids the experimental difficulties in the preparation of membranes which shall be completely impermeable to the dissolved substance are enormous and hence the direct method can rarely be employed, but the indirect methods, viz., lowering of freezing point, and raising of boiling point, yield results from which the osmotic pressure can readily be calculated with a fair approach to accuracy. On the other hand the differences in freezing and boiling point, even with concentrated solutions of colloids are so infinitesimally small that no reliance can be placed upon these indirect methods for calculating the osmotic pressures of such solutions. Further, it is impossible to prepare colloidal solutions uncontaminated by at least traces of associated crystalloids which, by virtue of their much lower molecular weights in solution, affect the readings correspondingly more than the colloids, and it is impossible, because of the presence of the colloid, to determine the amount of such extraneous crystalloid material with sufficient accuracy to deduct the effect due to its presence from the result.

Since the direct method of determining the osmotic pressures of colloidal solutions appears then to be the only one at present available, we may next turn to the discussion of that method and examine

¹ MARTIN: *Journal of physiology*, 1896, xx, p. 317.

the difficulties in the way of its application and the criteria which can be furnished as to whether the readings obtainable are really due to the dissolved colloid or to associated crystalloid.

The desiderata in any membrane employed for direct measurement of osmotic pressure of colloids, are that it shall be perfectly permeable for the solvent, usually water, and also *for any crystalloid which may be present in common solution with the colloid*, while at the same time perfectly impermeable for the colloid. Under such conditions, no osmotic pressure is manifested by the crystalloid and the total osmotic pressure manifested is due to the colloid, and hence from the reading and a determination of the percentage of colloid present in the solution the weight of the solution aggregate of the colloid can readily be calculated.

The question of the impermeability of the membrane for the dissolved colloid is one which is easily settled by allowing osmosis to go on for a prolonged period, and then testing for traces of the colloid in the outer fluid. Membranes are easily obtainable which satisfy this condition, for as a rule colloids in solution do not penetrate membranes consisting of insoluble colloid. Such a colloid is ordinary parchment paper which we have chiefly used as a membrane, and after days of osmosis we have never found a trace of proteid in the outer fluid by the most delicate known tests even after concentration of the outer fluid to a small bulk. Further, after allowing for dilution due to osmosis, the same amount of colloid has been reobtained within the osmometer as at the commencement of the experiment.

It is much more difficult to obtain satisfactory evidence that the condition of perfect permeability for crystalloids simultaneously present in solution is being satisfied in any given experiment, and it is this fact which has caused many observers to look with distrust upon figures obtained by the direct method for the osmotic pressure of colloids.

Different crystalloids possess very dissimilar velocities of diffusion, and hence while some diffuse with such readiness as to assure the observer that they have no effect upon the result obtained, others diffuse so exceedingly slowly, if indeed they pass through the colloidal wall at all, that it is impossible to get rid of them by the most prolonged dialysis which is practicable, and hence other means must be sought to determine whether the osmotic pressure observed is due to their presence acting alone *qua* crystalloids, or whether they are integral parts of the colloidal aggregate and hence that the pressure is due to that aggregate.

The first method which suggests itself of getting rid of the disturbing effects of crystalloids is that of obtaining an outer fluid as solvent which shall contain exactly the same crystalloids in identical concentration, and thus exactly balance the pressure of the crystalloids upon the two sides of the membrane so that only the unbalanced pressure of the colloids upon the inner side becomes apparent in the manometer.

Unfortunately this method fails when an attempt is made to apply it. Two procedures suggest themselves as to the preparation of such solutions, isotonic as regards their dissolved crystalloids. One is to destroy the colloid in solution, either by coagulation and filtration, or by incineration and dilution of the ash to the original volume by distilled water, and to use this as an outer fluid. But such processes are rendered futile, first, by the fact that in coagulation those very crystalloids which diffuse slowly, such as the calcium salts, are thrown out of solution with the coagulum of colloid either mechanically or in loose chemical combination, so that there remains behind merely a hypotonic solution containing those readily diffusible salts which only temporarily interfere with the pressure even when distilled water is used as the outer fluid. Secondly, incineration alters the character of the salts, and hence the total osmotic pressure, and renders some constituents insoluble, while volatile salts and organic crystalloids are removed, so that the solution obtained is by no means isotonic in crystalloids with the original solution.

The second method which has been followed in the attempt to obtain a fluid which is isotonic as regards crystalloids appears at first sight more feasible. It consists in filtering the colloidal solution under pressure through a colloidal membrane, such as gelatine supported by a porous earthenware wall.¹

It has been shown by experiment, however, that this method likewise fails to yield a solution which is isotonic in crystalloids.²

The water and easily diffusible salts, such as chlorides and carbonates, filter through with equal ease to the solvent, so that the concentration of these salts in solution remains unaltered in the filtrate, but a number of the other crystalloids, which are just those that constitute the difficulty of the case, pass through much more slowly than the water, so that these are more dilute in the filtrate, which is accordingly hypotonic in total crystalloids.

¹ MARTIN: *Journal of physiology*, 1896, xx, p. 364; STARLING, *ibid.*, 1899, xxiv, p. 317.

² WEYMOUTH REID: *Journal of physiology*, 1901, xxvii, p. 161.

Such procedures do not accordingly help us in settling the question of whether colloids really possess an osmotic pressure in solution, or whether the pressures observed are due to the crystalloids.

The importance of this question is further emphasized when it is found by experiment that even after the crystalloids have been removed as completely as possible by weeks of dialysis against distilled water, as in our experiments recorded below upon egg-albumin, there still remains enough ash in the proteid thrown out of such solutions by heat coagulation to account, when its theoretical osmotic pressure has been calculated, for the total pressure observed. This inorganic matter remaining after prolonged dialysis is very closely attached to the proteid, all the more soluble salts such as chlorides, carbonates, and phosphates having been removed, as shown by chemical analysis of the fluid both inside and outside, and it is thrown out of solution whenever the proteid is precipitated. The oft-discussed question is here again involved, Is this inorganic matter an integral part of the proteid molecule?

We have attempted to obtain some positive evidence upon these points *by altering the size of the solution aggregate of the colloid* by the action of reagents which leave unaffected the amount of indiffusible or feebly diffusible salts in solution.

If, while the amount of colloid in solution remains unaltered, the size of the solution aggregate be broken down, the osmotic pressure recorded, if this be really due to colloid, ought to rise correspondingly, because the molecular concentration, *i. e.*, the number of molecules per unit volume, and hence the osmotic pressure increases. On the other hand, if the pressure observed were due to crystalloids, then, since the amount of such crystalloid is not increased in the experiment, the pressure ought not to increase on account of the production of more colloidal aggregates of smaller size per unit volume, but rather to decrease as the inorganic matter is rendered freely diffusible in the process of breaking down of the larger aggregates.

To test this point, the osmotic pressure of a sample of a sheep's serum was recorded in two experiments one after the other, and readings of 22 and 23 mm. of mercury respectively were obtained. The serum was then converted into alkali albumin by adding a sufficient quantity of ten per cent caustic soda solution to make a one per cent solution and boiling; the same percentage amount exactly of the caustic soda solution was next added to the outer fluid, and readings

of osmotic pressure were again recorded when values of 110 and 105 mm. of mercury were obtained. In a second similar experiment with a second quantity of serum, a reading of 98 mm. of mercury was recorded after alkalization. Such a result can, in our opinion, only arise from a splitting up of the solution aggregate of the coagulable proteid into smaller aggregates of approximately one-fifth the weight, for even had there been any error in the quantity of caustic soda added to inner or outer fluid, this salt is freely diffusible and would soon have equalized on the two sides. Also, since no other crystalloid was added in the experiment, the result appears to us to show that the pressure readings obtained are really due to the proteid in solution.

EXPERIMENTAL METHOD.

The osmometer.—The original form of Dutrochet osmometer presents certain advantages over more recent forms which have induced us to use it in our experiments. In the first place, a very thin membrane can be used which increases the rapidity of working. Secondly, the convection currents set up on the two surfaces of the membrane cause a constant natural removal of the liquid wetting those surfaces, which, by hastening the establishment of osmotic equilibrium, dispenses with the necessity of employing artificial rockers or shakers.

The chief disadvantage of the Dutrochet membrane in our preliminary experiments was the difficulty of insuring, for quantitative work, that there was no leakage around the attachments of the membrane to the rest of the instrument. It is very disappointing in using the usual methods of attachment to find at the end of an experiment which may have lasted for two or three days that some minute leakage, in spite of all initial care, has vitiated the result, as shown by the presence of colloid in the outer fluid.

After much experimentation upon methods of securing an efficient attachment, we determined to abandon the use of a glass receiver altogether, and constructed one of metal, in which the leakage difficulty was entirely overcome by screwing the membrane up against a rubber ring between two metal flanges.

This instrument we have found to work perfectly, and it possesses the great advantage that an experiment can be started in a few minutes, while there is absolute security against leakage, for we have never detected a trace of colloid in the outer fluid, even after days of standing. All chemical action of the solution upon the metal is excluded

by thickly plating the brass body with silver, and finally upon the inner surface with gold.

The design of the instrument is shown in section in the accompanying figure. It consists of a hemispherical receiver, which has a tubular continuation at its upper pole for connection with the manometer, and a broad flange around its circumference, which has a narrow circular groove, let into its under surface. In this groove lies a somewhat thicker rubber band, against the under surface of which the membrane is tightly pressed. The pressure is equally applied by means of four screws (two of which are shown in the section) passing through four equidistant holes in the outer part of the flange of the receiver, and engaging in four similar but threaded holes in a heavy ring of metal of similar dimensions to the flange. In fitting up the instrument for an experiment, the membrane of parchment paper is cut to the outer diameter of the flange and the position of the screw holes marked with a pencil; openings opposite to the holes are punched out, and the membrane is wetted and laid evenly over the rubber ring; then the screws are inserted and screwed home, and the receiver is filled with the solution. Connection is next made with the manometer by means of a piece of barometer tubing of thick wall and narrow bore.

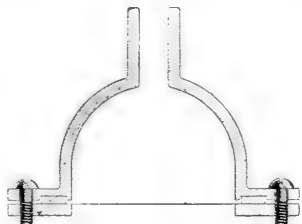


FIGURE 1.—Section of osmometer used for experiments; capacity 45 c.c.

The barometer tubing is covered at its end by a piece of rubber tubing, which is allowed to project about half a centimetre over the end of the glass tubing. The size of glass tube is so chosen that when covered by the rubber tubing the end can be pushed by exerting pressure into the opening of the brass tubulure of the receiver; in this manner a tight and efficient joint is secured. The remaining filling of the connecting tube is easily attained by the degree of movement which is possible in the stretched membrane. This can easily be pressed up a little when the fingers are applied underneath; a pipette filled with the solution is then applied to the open end of the tube, to which the solution within has been raised by the pressure of the fingers, and on this pressure being removed and the top of the pipette being simultaneously opened, the contents of the pipette run in as the membrane falls back beneath the pressure.

In the earlier experiments in which in addition to obtaining the final maximum pressure, we desired, to observe the initial velocity of

diffusion through the membrane, we employed as manometer a long straight piece of barometer tubing behind which was placed a centimetre scale. The pressures were recorded in centimetres of the solution, and these were afterwards reduced to mercury pressures. In this way the volume diffusing was increased, and hence a more accurate record of velocity of diffusion was obtained. But in later experiments we found it quicker and more advantageous to use an ordinary Ludwig's mercurial manometer of rather narrow bore, the style of which recorded upon a smoked drum revolving at the rate of about one centimetre in two hours. By this method a tracing was obtained of the rate of rise of pressure. The velocity of rise is at first practically constant, producing an inclined straight line tracing, but later this becomes asymptotic to the base line, showing that the maximum pressure has been reached.

The membrane.—The membrane we have used has been in most cases parchment paper. Such a membrane has the advantage of being preparable in a few moments; it is very constant in texture and thickness, so that the results of several experiments are strictly comparable; it is absolutely impermeable to the colloids we have tested, under the conditions in which we have employed it, and hence must give true records of the osmotic pressure, and finally it is rapid in action. We have further tested the readings obtained when such a membrane is used under the conditions of our experiments by performing otherwise identical experiments with different pieces of membrane, and have found very close readings of final pressures and almost parallel figures for velocities of diffusion.

A further advantage which such a membrane possesses over a gelatine membrane is that it can be employed for determinations at high temperatures. This is of great importance in the case of colloids such as the soaps, which are only appreciably soluble in water at temperatures of 50° C. to 70° C.; at these temperatures, gelatine membranes are of course useless on account of the high solubility of the gelatine.

EXPERIMENTAL RESULTS.

The following tables give a summary of the results obtained with the various colloidal solutions with which we have experimented:

TABLE I.
EXPERIMENTS ON COAGULABLE PROTEIDS.

Nature of inner and outer fluids.	Temp. in degrees C.	Maximum pressure in mm. of mercury.	Initial velocity of osmosis, in c.c. per hour per sq. c.m.	Percentage of proteid in osmometer.	Calculated pressure for each per cent of proteid.	Calculated velocity for each per cent of proteid.	Weight of "solution aggregate," calculated from osmotic pressure.
1. A one per cent solution of sodium chloride against distilled water	18°	4.0	0.0039	—	—	—	—
2. One half white of egg and one half distilled water against distilled water . . .	18°	62.2	0.0045	3.88	16.0	0.0012	9,944
3. One half white of egg and one half normal saline (0.7 per cent) against distilled water	38°	55.7	0.0122	3.99	13.9	0.0030	10,711
4. Same as No. 3	38°	59.0	0.0106	3.79	15.5	0.0028	9,541
5. Dialyzed white of egg against distilled water	38°	36.2	0.0045	4.75	7.6	0.0009	19,590
6. Same as No. 5	38°	43.4	0.0056	4.75	9.1	0.0012	16,360
7. Sheep's serum (sp. gr. 1019) against distilled water	38°	18.9	0.0044	6.02	3.1	0.0007	48,027
8. Same as No. 7	38°	18.5	0.0042	6.02	3.0	0.0007	49,628
9. Sheep's serum (sp. gr. 1025) against normal saline (0.7 per cent)	38°	18.0	0.0045	6.84	2.6	0.0007	57,263
10. Same as No. 9	38°	18.4	0.0043	6.84	2.7	0.0007	55,142
11. Same as No. 9	38°	18.7	—	6.84	2.7	—	55,142
12. Sheep's serum (sp. gr. 1030) against normal saline (0.7 per cent)	38°	18.0	0.0051	7.10	2.5	0.0007	59,553
13. Sheep's serum (sp. gr. 1027) against distilled water	21°	28.2	0.0050	7.20	3.9	0.0007	40,383
14. Same as No. 13	21°	28.2	0.0054	7.20	3.9	0.0007	40,383
15. Same serum as in No. 13 against normal saline (0.7 per cent)	21°	22.2	—	7.20	3.1	—	50,804
16. Same serum as in No. 13 against saline of 1 per cent strength	21°	19.3	—	7.2	2.7	—	58,327

Note. — In Experiment 1 the pressure given is the maximum noted; after this the pressure gradually dropped to zero. Hence, 4 mm. of mercury may be taken as the greatest error, due to a difference of 1 per cent in concentration of sodium chloride on the two sides. Since there was never anything greater than $\frac{1}{20}$ of this difference in the analysis of the inner and outer fluids for sodium chloride in the subsequent experiments, it may, we think, be taken that the disturbing influence of crystalloids in these later experiments was a negligible quantity. Thus, in Experiment 2 analysis gave: Inner fluid, 0.0625 per cent; outer fluid, 0.0104 per cent of sodium chloride. In Experiment 3: Inner fluid, 0.078 per cent; outer fluid, 0.028 per cent. In Experiment 9, 0.7 per cent in both outer and inner fluids. In Experiment 12, 0.705 in inner and 0.700 per cent in outer fluid.

The dialyzed white of egg used in Experiments 5 and 6 was obtained by dialyzing in a sausage tube of parchment paper, 300 c.c. of the undiluted egg-white for nine days against running tap water, and then in frequently-changed distilled water through which air was bubbled as a stirrer for four days more. The precipitated globulin and membranes were then filtered off and the filtrate used for the experiments. An analysis of the ash contained in this dialyzed solution, after coagulating, drying, and incinerating gave the following results:—

Total ash = 0.0490 per cent; CaO = 0.0178 per cent; MgO = 0.0047 per cent; SO₃ = 0.0227 per cent; P₂O₅ 0.0034 per cent; NaCl = 0.0026 per cent; KCl = 0.0002 per cent. The greater part of the ash was thrown out on coagulating the proteid. The osmotic pressure given even by these small traces of crystalloids amounts to from 60–70 mm. of mercury approximately without allowing for any dissociation. It will be observed that nearly all the ash consisted of sulphate of phosphate of calcium and magnesium.

The percentages of proteid given were obtained by causing heat coagulation in the presence of a trace of acetic acid, and weighing the coagulum dried to constant weight on a tarred filter paper. In all cases the determination of proteid was made in the filtered inner fluid after dialysis, but in no case was the precipitation of globulin in either serum or white of egg a considerable one.

TABLE II.
EXPERIMENTS ON ALKALIZATION OF SERUM.

Nature of inner and outer fluids.	Temperature C.	Maximum pressure in mm. of mercury.	Percentage of proteid in inner fluid.	Weight of solution aggregate calculated from osmotic pressure.
1. Inner fluid sheep's serum ; outer fluid 0.75 per cent saline	18°	22	6.65	54,680
2. Same as in No. 1	18°	23	6.65	52,303
3. Inner fluid the same serum as in Nos. 1 and 2, alkalized by 1 per cent caustic soda ; outer fluid 1 per cent caustic soda, and 0.75 per cent saline in distilled water	18°	110	5.98	9,834
4. Same as No. 3	18°	98	5.98	11,038

Note. — In these experiments no trace of either coagulable proteid or alkali albumin respectively were found at the end in the outer fluid. The smaller percentage of proteid in Experiments 3 and 4 is due to the necessary dilution with the caustic alkali. This change is of course discounted in the calculation of the solution aggregate in the last column.

TABLE III.
EXPERIMENTS ON SOAPS.

Nature of inner and outer fluids.	Temp. C.	Maximum pressure in mm. of mercury.	Percentage of soap dissolved.	Solution aggregate.	Approximate ratio of solution aggregate to molecular weight.
1. Half per cent sodium oleate against distilled water	55°	14.4	0.5	7,090	23 : 1
2. Three per cent sodium oleate against distilled water	40°	37.2	3.0	15,674	51 : 1
3. Half per cent sodium palmitate against distilled water	55°	6.2	0.5	16,420	59 : 1
4. Half per cent sodium stearate against distilled water	60°	6.5	0.5	15,861	52 : 1

Note. — The soap solutions did not contain any obvious matter in suspension at the temperatures of the experiments. There were no traces of soaps in the outer fluids evaporated down to dryness in the cases of the palmitates and stearates. But there was a slight trace of organic matter in the case of the oleates after about a week's continuous dialysis, not sufficient in amount to determine whether it was oleate or not. Practically the entire amount of each soap was recovered in the inner fluid on evaporating to dryness.

TABLE IV.
EXPERIMENTS WITH PERITONEAL MEMBRANE.

Experiment 1. Sheep's serum against distilled water, with fresh peritoneal membrane of the ox as dialyzer. The pressure fell in twenty hours from 9.3 cm. of the serum to 8.6 cm. At no period was there a rise, and the outer fluid at the end of the experiment gave the proteid reactions.

Experiment 2. Conditions similar: sheep's peritoneal membrane. Pressure fell from 2.9 cm. to zero. Proteid in outer fluid.

Experiment 3. Conditions similar: membrane from sheep. Pressure fell from 3.9 cm. to zero. Proteid in outer fluid.

Experiment 4. Sheep serum against distilled water, sheep's peritoneal membrane previously extracted with ether and washed to remove proteid. Pressure fell from 4.1 cm. to zero in forty-two hours, and there was proteid present in the outer fluid.

Note. The fall was so slow in these experiments as to indicate that there was no artificial leak in the membrane, so that the experiments show that peritoneal membrane is permeable to proteid.

DISCUSSION OF RESULTS.

The results given in these tables may be considered from two points of view; first, in regard to the physical properties of colloids in solution, and, secondly, as to their bearing upon the passage of substances in solution through living membranes. The experiments on alkalization of serum, which show such a marked rise in osmotic pressure, seem to us to indicate that the pressures observed are really due, in all cases, to the dissolved proteids.

Assuming this to be true, a striking result obtained is the variation in "solution aggregation" of the different proteids and the ease with which this physical property of aggregating changes with the conditions of solution. Thus the "solution aggregate" in the case of

sheep's serum is four to five times as great, under similar conditions, as that of egg albumin.

Further, the aggregation is practically doubled in the case of egg albumin by dialyzing off the salts, and this difference is not due to any osmotic pressure arising from differences in concentration of inorganic salts in the inside and outside fluids, for the percentages of sodium chloride in these was found by analysis to be practically identical at the conclusion of the experiment in each case. Nor is the result due to precipitation of globulin, for, in the first place, the percentage of globulin in egg white is many times too small to account for the difference, and, in the second place, the experiments were made in each case with filtered fluids, in which the percentage of proteid was afterwards determined, so that this source of error could not be introduced.

This variation in the solution aggregate with the nature of the solvent is somewhat important, since it indicates that the molecular weight, *in the chemical sense of the word*, cannot be obtained by such physical methods as determining the lowering of the freezing point or by osmotic pressure measurements.

It is, however, on the degree of aggregation that the osmotic pressure and other physical properties of colloidal solutions depend, and hence, so far as the behavior of proteids in the cell and in building up protoplasm is concerned, this constant is probably of more value than the weights of the various constituent molecules which go to build up the solution aggregate.

There is no experimental reason for believing that the chemical molecules constituting a "solution aggregate" need be identical in their chemical constitution, and differences in type in the various coagulable proteids might easily arise from the formation by means of different physical combinations of different "solution aggregates." The differences by which coagulable proteids are at present classified depend chiefly on differences in solubility, in water, and in salines of different strength and quality, or upon different temperatures of coagulation.

These are *physical* points of difference such as might arise from the formation into large, and somewhat differently arranged, aggregates of simpler chemical molecules. Thus the proteid molecule may be made to appear complex and large, more from physical aggregation than from chemical complexity. The small percentage of such elements as sulphur, iron, or calcium, present may also easily be ex-

plained on the supposition that these elements are present only in a small number of the chemical molecules which unite to form the solution aggregate.

This view that in colloidal solutions we are dealing with the physical properties of a "solution aggregate," that is to say, with an association of a considerable number of chemical molecules to form an "osmotic" molecule or unit, derives support not only from the variations in the *apparent* molecular weight of the same proteid in solution under varying conditions of the solvent, but also from the determinations of the osmotic pressures of soap solutions given above in Table III.

In the case of proteids, the chemical molecular weight is unknown, and hence it is possible that variations in osmotic pressure might be due to chemical dissociation, but in the case of the soaps the chemical constitution is well and definitely known, and hence when the readings of osmotic pressure lead to a "molecular" weight *in solution* lying between twenty and sixty times the value of the chemical molecular weight, some such explanation as that given above of a "solution aggregate" presents itself with much greater force.

There are of course other explanations of the low osmotic pressures obtained with soap solutions which may be worth putting forward for discussion. In the first place, the low values are not due to partial permeability of the membrane to soap molecules, for even after days of dialysis no soap is found in the outer fluid.

A second explanation which might be suggested is that the soap is not in solution but merely in suspension at the beginning of the experiment, or further it undergoes dissociation into acid soap or free fatty acid and alkali, and, the alkali freely diffusing through the parchment membrane, the amount of dissociation goes on increasing until finally nothing but insoluble free fatty acid is left inside.¹

It may be replied to this, in the first place, that the solutions at the temperature at which they were tested in the osmometer were quite clear from suspended particles and had merely the faint opalescence seen in most organic colloidal solutions, and that there were no particles visible under the microscope, so that the soaps must have been as truly in colloidal solution as the carbohydrate in a starch or glycogen solution or the proteid in a caseinogen solution. To say

¹ Any marked dissociation into free fatty acid and alkali only occurs at dilutions greater than those employed by us.

that such is a suspension is merely begging the question at issue, since a true and definite osmotic pressure is shown by such solutions. Further, the solutions remained clear at the end of the experiment at a temperature equal to that of the experiment, showing that no separation by dialysis had taken place; the amount of carbonate present after incineration of the soap at the end of the experiment in the inner fluid corresponded closely to that demanded for a neutral soap, and finally there was no free alkali or alkaline carbonate in the outer fluid.

If any separation by dissociation into insoluble free fatty acid or acid soap and free alkali or alkaline carbonate had occurred in our experiments the free alkali or carbonate would have diffused through the parchment membrane while the free fatty acid or insoluble acid soap would not have so passed through; but, as above stated, we were not able to demonstrate any alkali in the outer fluid even after evaporating down to a small bulk. This point was still further tested by adding one per cent of free caustic soda, in each case, to a half per cent solution of sodium stearate inside and to distilled water outside, so that any possible dissociation should be depressed by the presence of excess of free alkali; the result was as in the other cases, a small but definite osmotic pressure many times lower than that demanded by the chemical molecular weight.

We are hence inclined to think that no appreciable dissociation occurred in the solutions used for our experiments, and we believe that the small pressures observed arose from aggregated groups of soap molecules in colloidal solution.

Another view which might be put forward is that the soap was present in two phases and that the pressures observed are due to a small fraction of the soap which is dissolved in mono-molecular form in one phase, viz., that of soap dissolved in water, while the remainder of the soap is present in multi-molecular aggregates in which a solution of water in soap exists, and that these multi-molecules do not affect the osmotic pressure because they behave as a solvent and hence lie inert in the interspaces of the weak solution of soap. According to this theory only a small fraction of the soap would really be dissolved in the water of the inner fluid, and hence the observed osmotic pressure would only be a correspondingly small fraction of that theoretically calculated.

Against this view it may be argued that, in the first place, there is no obvious separation into two phases, and in the second place

it is extremely improbable that mono-molecules of the soaps, on account of their small molecular weights, would not pass through the pores of such a free membrane as parchment paper.

If such mono-molecules of soap did exist, it is more than probable that they would act as crystalloids, would pass freely through the membrane, and hence could give rise to no osmotic pressure. It is only by the aggregation of many molecules into such a solution aggregate as we have described above, that we are capable of conceiving the soaps acquiring the property of indiffusibility through such pores as exist in parchment paper and give passage to such large molecules as those of the albumoses and dextrines.

In fact, in our earlier experiments we did not expect to find evidence of such aggregations nor that soap solutions would prove to be such perfect colloids as to completely fail to pass through parchment paper. We began with the intention of working out the comparative diffusibility of soaps in pure aqueous solution, and in solutions of bile salts respectively, when we were met at the outset by the experimental result that *soaps in aqueous solution do not diffuse at all even through such a membrane as parchment paper*, and hence turned aside to investigate their osmotic properties.

The formation of such solution aggregates as we have been considering above is also interesting in connection with the physical changes which go on in the cell causing the formation of protoplasm, and the deposition or solution of granules in the cell, since it indicates that *purely physical aggregations may take as important a part in energy transformations in the cell as do chemical reactions in the narrower sense of the word*. Under the conditions existing in the cell, materials absorbed may be built into physical aggregations with those already present, and the nature of such aggregation will vary with the nature of those already present in the cell, so that in different cells different types may arise and so give rise to different reactions, for the *liability* of the aggregate must vary with the nature of the aggregation, and decomposition of different types of aggregates must yield different dissociation substances. Again, absorption by the cell must be favored by such a process of aggregation of absorbed products to those already existing in the cell, so that absorption may continue, on account of the absorbed substance being present no longer *as such* in the cell; hence the concentration of a given substance inside the cell is kept under that outside in the lymph, and absorption continues.

When the number of molecules present in a solution aggregate reaches a certain limit, the aggregate begins to be visible by interfering with the light rays, and at a later stage is visible as a granule, so passing by gradation from the dissolved colloidal condition to the granular form. The granules then go on increasing in size by deposition of fresh aggregates from solution. In a similar manner the fibrils and reticular network of the protoplasm may possibly be formed.

In strong solutions the continuous increase in size of the dissolved aggregations, when the conditions change, occurs more rapidly; and just as in crystallizing from a crystalloid solution, rapid crystallization gives rise to a magma of small crystals, while slow cooling gives rise to a smaller number of larger crystals, so here in colloidal solution more rapid formation in a strong solution gives rise to a large number of smaller aggregations, which finally unite continuously to one another to form an apparently homogeneous mass or hydrogel. Thus, slow formation results in granular deposition and rapid formation gives origin to jellies or gels. In this respect the various colloid solutions differ markedly, some possessing a tendency to form large aggregates, and so become precipitated out in particulate or granular form, while others have a tendency to form small aggregates in proportionately greater number, and so tend specially to form jellies or hydrogels. Analogous results are seen in solutions of crystalloids from some of which large crystals are obtained with ease, while from others only micro-crystals can be formed under any conditions.

When a solution from which small crystals have been rapidly formed is allowed to stand, as is well known, the larger crystals grow at the expense of the smaller ones, so that in the end very large crystals are obtained. An exactly parallel occurrence is seen in hydrogels, for these in many instances tend to become granular and particulate on standing, although subjected to the action of no reagent. This is exceedingly well seen in the case of the soaps with which we have worked; even a one per cent solution of sodium stearate made at boiling point will on cooling set into a jelly; a 2 per cent solution will form a stiff jelly which can be inverted and in which no granules are visible *at first* under the microscope, but on standing for twenty-four hours, a granular precipitate sinks to the bottom of the flask, leaving a thin, watery fluid which only contains a trace of stearate. Here the larger aggregations grow, as in the crystalloid, at the expense of the smaller, until granules visible to the eye are finally formed.

*Gelatinization is favored by strong solution and rapid cooling, while granulation is favored by weak solution and slow cooling.*¹ This is also seen when the degree of aggregation is increased by heat coagulation; when this occurs in strong solution the precipitate is more gelatinous, when in weak solution more granular.

The change in the opposite direction towards less aggregation is also interesting; as is shown by our experiments on alkalization of serum, the solution aggregates become reduced in size to about one-fifth. Similar changes are seen in the case of the complex carbohydrates, by the action of acids or alkalies, and probably the digestive enzymes cause a similar separation. As a result of these inverse changes, the dissolved substance finally approaches or passes into the mono-molecular or crystalloid type of solution, or may even become ionized.

BEARING OF RESULTS ON LYMPH FORMATION AND GLOMERULAR ACTIVITY.

In conclusion, we may briefly criticise the applications which have been made of the osmotic pressure of proteids to the processes of lymph absorption and glomerular activity.

Starling,² who has obtained in his measurements of osmotic pressure *slightly* higher results than those which we here record, viz., about 4 mm. of mercury for each per cent of proteid, or 25 to 30 mm. for average serum, claims that this pressure has great importance in influencing lymph absorption and glomerular filtration.

Starling's argument briefly is, that the walls of the capillary blood vessels act as a membrane which is more or less impermeable to proteids.

Thus the osmotic pressure of the proteid, though small compared to that of the crystalloids, becomes effective, and causes absorption of lymph by the capillaries. The degree of capillary permeability varies in different parts of the vascular system, according to Starling, being probably greatest in the liver and least in the limbs. This conclusion is drawn from the fact that in the resting limb no lymph can be ob-

¹ A similar phenomenon, probably also due to the formation of aggregates of varying size is seen in the solidification of colloidal solids, such as paraffin wax; here rapid cooling gives a translucent mass of small aggregates, homogeneous under the microscope, while slow cooling gives rise to a granular or sometimes crystalline mass of large aggregates.

² *Loc. cit.*

tained from the lymphatic trunks, and from the fact that liver lymph is rich in proteid, showing that little proteid has been restrained in passage through the capillary walls.

In opposition to this is the theory put forward by Heidenhain and recently supported by the work of Ascher and others, that lymph is a secretion formed in proportion to the wants and activity of the part, and governed by the condition of activity of the capillary cells.

The amount of osmotic pressure due to proteids, tending to cause absorption of lymph by the vascular capillaries,¹ obviously depends upon two factors, viz., the *difference* in concentration in proteid between the fluid inside (blood plasma) and that outside (the lymph), and secondly upon the degree of permeability of the wall to proteid. If there be equality of concentration on the two sides there will be no osmotic pressure manifested, or if, on the other hand, there be perfect permeability there will be no osmotic pressure no matter how great the difference in concentration on the two sides.

In considering whether the movements of lymph are influenced *at all* by the osmotic pressure due to proteid, we have hence to consider the difference in concentration in proteid of lymph and plasma, and the degree of permeability of the capillary wall, *premising* that the total available pressure, in case the walls were perfectly impermeable to proteids, and proteids were completely absent in the lymph before it reaches the cells and present in full concentration in the blood-vessels, can possibly only amount to from 18 to 28 mm. of mercury.

In considering the question as to whether there is any appreciable difference in concentration of proteid *on the two surfaces of the wall of the capillary blood-vessel*, we are at once met by the difficulty that it is impossible experimentally to catch the lymph and ascertain the concentration of proteid in it immediately after it has passed through the capillary wall, and before its percentage of proteid has become reduced by feeding the tissue cells. We cannot even obtain it in the lymphatic capillaries after it has fed the tissue cells, but can only obtain it from the main lymphatic trunks after it has passed through a lymphatic gland, to the cells of which it doubtless yields further quantities of proteid. Apart entirely from any *partial* impermeability of the capillary wall to proteids, it is hence not to be wondered at that lymph collected from a large lymphatic trunk contains a smaller percentage of proteid than the blood plasma. It is easily

¹ We are considering the capillary walls here as physical membranes, apart from their vital properties.

seen that the percentage loss in the proteid of lymph may be very considerable from actual consumption in the passage through the tissues, when it is remembered how *slow* the lymph stream is, compared with that of the blood. It is from the lymph and not directly from the plasma that the tissues are supplied with proteid, hence there must always be a diminution in proteid of the lymph, and the percentage diminution will vary directly as the time of passage of the lymph through the tissues; in other words, with a rapid flow of lymph from a part its percentage of proteid will approach that of the plasma, while with a slow flow of lymph the amount of proteid taken out will increase, and the percentage of proteid may fall to one-half of that of the plasma, or even lower. This seems to us a much simpler explanation of the difference of percentage in proteids of lymph from different parts, and under varying conditions, from the same part, than any hypothesis that the capillary wall in certain tissues has a greater impermeability to proteid than in other tissues, for *there is no experimental evidence that there exists even partial impermeability of the capillary walls to proteid in any part of the body.*

The question at issue may be perhaps made clearer by referring to the feeding of the tissues with a simpler substance, viz., oxygen; no one supposes that the capillary wall is partially impermeable to oxygen, because the percentage of oxygen in the tissues is lower than in the plasma, but rather that oxygen is being used up in the tissues. Similarly, proteid percentage in the tissue lymph is lower than in the plasma not because the capillary walls are impermeable to proteid, but because proteid is being continuously used up by the tissue cells.

Hence the view that there is any such difference in concentration of proteid *on the two sides of the thin capillary wall* as is indicated by the differences in proteid percentage shown by analyses of blood plasma on the one hand, and lymph taken from a main lymphatic trunk after it has fed the tissues on the other hand, must be erroneous; there can only be an *infinitesimally* small fraction of such a difference in concentration and accordingly not more than a small fraction of a millimetre of difference in osmotic pressure corresponding to the fraction which the thickness of the wall is of the whole distance in which the fall takes place.

The view that a greater impermeability for proteids is shown in the case of the limbs by the experimental fact that there is no lymph flow from a limb at rest is also in our opinion quite a fallacious one. In the first place, the limb capillaries must be fairly permeable to

proteid, or else the tissue cells would suffer from proteid starvation, and certainly in *luxus* proteid consumption the percentage of proteid oxidized even in the resting muscles of the limbs must be a fairly high one.

An easy explanation of the absence of a lymph flow in a resting limb and of the presence of a flow in an active limb, can, it seems to us, be readily given without any invocation of impermeability or partial impermeability of the capillary vessel to proteids.

There are two physical factors in operation in the transference of *any dissolved substance* from the blood plasma into the lymph of the tissue spaces or *vice versa*, viz.: First, a translation of the fluid as a whole due to pressure filtration carrying the dissolved substance along with the solvent and at the same rate; and secondly, a process of diffusion, due to differences of concentration in different regions giving rise to different solution pressures, and hence a movement of dissolved matter tending towards equalization.

The first of these two factors can only cause transference when there is a lymph flow, but the second can operate when there is a difference of concentration of any given substance within and without, *provided the wall of the vessel is permeable to the substance*, causing in this way a transference of dissolved substance without any accompanying transference of solvent, or even in opposition to any current of solvent, as for example in the carriage of carbon dioxide against the lymph stream from lymph to blood plasma.

In the normal limb at rest the diffusion factor alone supplies sufficient lymph to feed the tissues with nutrient material and to carry away all waste products, but in the case of the active limb the first factor must be called in to aid in these processes, the *modus operandi* being probably an increased hydrostatic pressure in the capillaries causing increased filtration into the tissue spaces and hence an increased lymph flow. In an active limb this is further aided by a pumping action on the valved lymphatic vessels due to the contracting muscles causing variation in pressure, and so pumping on the lymph.

There is thus no experimental evidence either that there exists any impermeability of the vascular capillaries to proteids or any sudden drop in concentration of proteid on the outer side of the wall, such as would produce an osmotic pressure tending towards lymph absorption. We have in short no evidence that the capillary wall acts towards proteids otherwise than towards other dissolved substances,

or that the proteids of the plasma play any special part in either lymph production or absorption.

It may also be pointed out that the manifestation of any such impermeability of the capillary wall to proteid with its attendant osmotic pressure would not only interfere with the normal nutrition of the part by proteid, but would hinder the flow of lymph into the tissue spaces, and onward through the lymphatic vessels into the great veins. For any osmotic pressure which appeared would diminish by its amount the driving power due to the capillary blood-pressure which as well as causing the filtration of the lymph through the capillary wall gives it that head of hydrostatic pressure which sends it on to the veins through the lymphatic system.

To illustrate this statement let us make the hypothesis that the concentration of the lymph immediately outside the capillary wall in proteid is only half that of the plasma within, and further that the coefficient of permeability of the capillary wall for proteid is one half, so that one half of the theoretically possible osmotic pressure for a perfectly impermeable wall becomes manifested. Taking the average figure of 24 mm. of mercury for the total osmotic pressure of the plasma against an otherwise isotonic non-proteid solution, this figure would have to be reduced by one half, *i. e.* to 12 mm. of mercury, on account of one half the amount of proteid being present outside, and again this would have to be reduced by one half or to 6 mm. of mercury on account of the partial permeability of the capillary wall. Therefore, we would have as a result a pressure of 6 mm. of mercury opposing itself to the flow of lymph from the capillary into the tissue spaces. A balance would then be maintained with a slower flow of lymph, on account of the head of pressure causing filtration from the capillaries under capillary pressure being lessened by this amount; *but there would be no current of lymph absorption from the tissue spaces due to this osmotic pressure*, for the osmotic pressure never could exceed the pressure in the capillary vessels even in the case of the lowest percentage of proteid in lymph ever observed experimentally. In fact, even granting that the lymph contained no proteid, and that the capillary walls possessed *perfect* impermeability, the total osmotic pressure does not exceed that of the capillary pressure, and hence even under such widely impossible conditions, there could be no absorption of lymph by the capillaries due to osmotic pressure brought about by proteid. Therefore all that such a manifestation of osmotic pressure could accomplish, did it exist, would be a

slightly variable slowing of the rate of lymph flow, but never an absorption of anything from the lymph spaces.

As absorption of substances in solution from the lymph spaces undoubtedly does take place, and that with great rapidity, as shown by Starling in the case of methylene blue injected into the peritoneal cavity, such absorption must accordingly be due either to the second physical factor indicated previously in this paper, viz., diffusion in solution, or to secretory activity of the endothelial cells of the blood-vessels.

In the foregoing discussion of the subject we have treated it upon entirely physical lines, and on such a basis have, we believe, given a demonstration that osmotic pressure phenomena due to proteids have no effect, or at most an inappreciable one, upon lymph production, or lymph absorption by the blood capillaries; but at the same time we would not have it thought from this that it is our view that physical processes, apart from vital activity, are *alone* concerned in the government of the nutrition of the tissues, by the rate of lymph flow and character of lymph supplied.

The endothelial cell lining the capillary vessel is a living cell, and in our opinion possesses the properties of a living cell, and a power of modifying both in quantity and quality the substances passing through it, just as does a secreting cell.

The vital properties of the endothelial cell are clearly shown by Lister's¹ early researches on the changes in reaction of the vessel wall, and corresponding change in the blood flow, on stimulation by irritants which excite but do not destroy the lining cells; by Heidenhain's² experiments on lymphagogues, especially those of the colloidal class, such as albumoses, which in small doses cause such a disproportionately large increase in lymph formation, and by Ascher's³ recent researches on the relationship between physiological activity and lymph flow.

These phenomena appear to us to find their most natural solution in the fact that although the lining cells of the capillary take on the form of a membrane, they still act as living cells, and possess a secreting action quantitatively, and to a lesser extent also qualita-

¹ See The HUXLEY Lecture to the Charing Cross Medical School, British medical journal, 1900, ii, p. 971; Lancet, 1900, ii, p. 987.

² HEIDENHAIN: Archiv für die gesammte Physiologie, 1891, xli, p. 239.

³ ASCHER: Zeitschrift für Biologie, 1897, xxxvi, p. 155; 1898, xxxvii, p. 261; 1900, xl, 180-333.

tively, upon the solutions passing through them. A similar view must in our opinion be taken of the functions of the cells lining the serous cavities and governing in amount and kind the secretions which they contain, as also of the action of the epitheloid cells of the pulmonary alveoli and of the kidney glomeruli. For the *glomerular* cell the case for secretion appears to us to be conclusively proven by the facts of urinary secretion under pressure, although Starling cites such experiments as proof that glomerular activity is due to filtration under pressure, because urinary secretion, according to this observer, stops when the pressure in the ureters is less than that in the blood-vessels of the glomerulus by an amount equal to the osmotic pressure of the proteids, which are present in the plasma, but not in the glomerular filtrate.

It appears to us, however, that the figures given by Starling in his experiments upon this point appeal strongly against the interpretation which he places upon them. Starling found that when the secretion of urine ceased the pressure in the ureters was less than that in the *carotid arteries* by from 38 to 43 mm. of mercury. Now the pressures to be compared are not those in the carotid artery and ureter, but those on the two sides of the glomerular membrane, which is a point of considerable importance; for, although the urinary flow is stopped and hence there would be a statical equality of pressure in ureter, pelvis, and urinary tubules back to the glomerulus, this is not so with the vascular circulation, and hence there must be a considerable fall of pressure between the carotid and the fine vessels which constitute the glomerulus. Now, even supposing proteids alone are kept back by filtration at the glomerulus (which is supposing a great deal, since dextrose and certain other crystalloids of the plasma probably do not pass through this structure), there would have to be made a deduction of at least 24 mm. of mercury from the differences in pressure, on the assumption that the proteids are separated solely by pressure filtration.

This would leave only 16 mm. of mercury for the fall in pressure between carotid artery and glomerular capillary, an amount which is in our opinion absurdly small, and which would not be sufficient to account for the fall in the arterioles leading to the vessel, not to mention forcing the blood along the fine vessels of the glomerulus itself. Even admitting that there can be considerable play in the distribution of the fall in pressure between renal artery and renal vein, by the relative degree of constriction of afferent and efferent

glomerular vessels, and in the *vasa recta*, it seems to us highly improbable that any appreciable blood-flow through the glomeruli could occur when there was a difference of pressure between carotid artery and glomerular capillaries of only 16 mm. of mercury. The more probable explanation of the result appears to us to be that the difference in pressure between ureter and carotid represented the fall in pressure between carotid arterial pressure and that in kidney capillaries, and that secretion of urine ceased because of occlusion of the glomerular vessels, when the pressure became equal on the two sides.

Further, the urine contains practically no dextrose and the plasma contains about 0.15 per cent of that substance, which represents an osmotic pressure amounting approximately to 200 mm. of mercury. Hence this substance cannot conceivably be separated by pressure filtration at the glomerulus, and we are driven either to the improbable conclusion that it is present in the glomerular filtrate and is reabsorbed by the cells of the urinary tubule and restored again to the blood, or to what seems to us the more probable view, that the glomerular epithelium secretes the fluid which passes through it, and hence is capable of modifying its constitution as do secreting cells in other parts of the body, in apparent opposition to the laws of osmosis as they are known for inert non-vitalized membrane, but in a fashion which we will be able to understand when the laws of vital energy as manifested by living cells cease to be an enigma.

Useful and hopeful as is the application of physical laws to vital phenomena, it is well to remember that the cell carries on energy transformations in a fashion, and with external results, peculiar to itself, which indeed necessitate the use of the words *vital* and *living*, to designate the fact that matter in such condition is capable of giving rise to energy transformations not seen in non-vitalized matter.

By the application of recent advances in our knowledge of the physical chemistry of solutions, and particularly of colloidal solutions, to the study of biological problems connected with the vitality of the cell, much will doubtless be learnt of the energy transformations which run their course in the living cell, but we venture to predict that although such studies will clear up many vital phenomena, yet *by the very act of doing so* they will thereby expose to our knowledge the existence in living matter of the power of developing a type of energy peculiar to itself and not found anywhere in non-vitalized matter. This "vital energy" of the future will bear much the same relationship to that of the past as our present ideas

regarding other forms of energy do to the old idea of the existence of "phlogiston." It will be robbed of much of its old mysticism, but still there will remain for it, as for the other forms of energy, the old, eternal, insoluble mystery of the nature of energy and the relationship of its various interchangeable forms to one another.

Our view of the problem, as it stands before us at present, is that in the living cell there exists a type of energy which is never found outside the living cell, which is capable of being produced from the non-vital forms of energy supplied by the "food" taken in by the cell, and which by acting on the matter present can call forth those transformations which we speak of as cellular activity and secretion. In all such transformations and transferences of matter, the law of the conservation of energy is followed by the vital energy, which in this respect behaves exactly as any non-vital form.

The study of the phenomena due to vital energy is the province of the biologist, and the recognition of this fact cannot, it seems to us have the effect of acting as a drag upon research, but rather as an increased stimulus to renewed effort.

CONCLUSIONS.

1. A definite osmotic pressure is exerted by colloids in solution, and it is shown that this pressure is not due to contamination by crystalloids.
2. There must be corresponding variations in freezing point and boiling point; but these would be so small as to fall well within the limit of experimental error; hence the direct method is the only one available for determining the osmotic pressure of colloids.
3. The osmotic pressure cannot be used for determining the molecular weight of colloids, since it gives in the case of substances of known molecular weight, such as the soaps, *apparent* molecular weights which are twenty to sixty times too large.
4. The important physical constant so given determines the osmotic properties of the colloidal solution, and we have termed it the "solution aggregate." The solution aggregate arises from the physical union or association of a variable number of molecules to form a single osmotic unit.
5. The weight of the solution aggregate varies with the conditions of solution. It is from four to five times as great for serum albumin

as for egg albumin. In the case of serum albumin it is reduced to approximately one-fifth its value by alkalization.

6. Even dilute solutions of sodium soaps in distilled water form colloidal solutions, at temperatures of from 50° C. to 70° C. Such solutions do not diffuse through a membrane of parchment paper.

7. Such dilute solutions of the sodium soaps yield hydrogels on cooling, which again yield solutions on heating. Such hydrogels become precipitated in granular form on standing.

8. Rapid cooling and increased strength of solution tend to the formation of hydrogels, while slow cooling and weaker solution tend to granule formation. These phenomena have their analogues in the passage of crystalloids from solution.

9. The differences in properties of the coagulable proteids may be due to different physical arrangements of chemical molecules to form different aggregates, and the complexity of the proteid molecule may be much more a physical than a chemical phenomenon.

10. Protoplasm may be built up by a continuation of such a process of aggregation; absorption of materials by the cell may be governed by the formation of varying aggregations with the protoplasm already built up in the cell, and similarly granule formation in the cell may be produced.

11. The osmotic pressure of the proteids does not in all probability take any share in lymph production or absorption; for there is no evidence that the capillary walls are impermeable to proteid, or that there is any appreciable difference in concentration on the two sides of the capillary wall.

12. The cells of the membranes in the glomerulus of the kidney probably act as secretory structures; for the differences in pressure are probably not sufficient to cause pressure filtration of a proteid-free filtrate, and are certainly many times too small to separate carbohydrate in this manner.

A NOTE ON THE INFLUENCE OF HEAT ON ENZYMES.

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THE influence of variations in temperature on the reactions brought about by enzymes has long been recognized. Recently Pozerski and Henri¹ have attempted to demonstrate that the inverting power of the invertin of beer yeast may be distinctly augmented by heating a solution of the enzyme to about 40°. It is necessary to raise the temperature of the solution for a short time only, in order to observe this modification; and the authors assume a change in the physical state of the solution. The method of experimentation was as follows:—

Invertin was prepared in the ordinary manner, without being subjected at any time to a temperature above 25°. A filtered solution of this product was employed in a series of portions, two per cent of sodium fluoride being present as a bactericide. One portion was kept at laboratory temperature (16°), while the others were heated separately at temperatures of 35°, 42°, 50°, 56°, for one half hour. They were all allowed to cool to 25°, then each was mixed with 50 c.c. of a five per cent saccharose solution and kept at 25°. The quantity of sugar inverted was estimated in each at various intervals. Thus at the end of one hour equal portions from the several trials showed the following:—

Temperature to which enzyme was subjected	25°	35°	42°	50°	56°
Sugar inverted, grams	0.409	0.540	0.675	0.500	0.257

From experiments of this type Pozerski and Henri conclude that when an enzyme solution is heated above 25° it does not resume its original condition after being cooled again to the same temperature. The maximum temperature for this favorable change is about 40°, and an extremely short time—less than ten minutes—suffices to produce the desired effects, which further heating for one half hour does not noticeably change.

¹ POZERSKI: Comptes rendus de la société de biologie, 1901, liii, p. 27; HENRI and POZERSKI: *ibid.* p. 28.

Observations like those described may, if verified, lead to important theoretical conclusions regarding the colloidal solutions in which enzymes occur.¹ In his study of the lipase of blood serum, Hanriot² has failed to observe similar variations resulting from changed temperature conditions. In view of the importance of the subject the writer has attempted, at Professor Mendel's suggestion, to repeat the experiments with invertin. The results have been practically negative in every case, the differences obtained with the previously heated enzyme solutions being minimal and variable, as the protocols below indicate. A few experiments with amylolytic enzymes resulted similarly.

Experiments with invertin. The enzyme solution was prepared by treating commercial compressed yeast with absolute alcohol containing one third volume of ether, as recommended by Albert.³ The precipitate was quickly filtered off by suction, washed repeatedly with ether, and dried in vacuo. A very active solution was obtained by extracting the resulting powder with water at room temperature. This solution was divided into two portions, one of which was kept at room temperature while the other was heated in a bath at 43° C. for twenty minutes, at the end of which time it was cooled to the same temperature as the unheated portion. For testing the rate of inversion a five per cent saccharose solution containing sodium fluoride (two per cent) was employed. The same volume of each portion of the enzyme solution was then added to equal parts of the saccharose solution. At the end of given periods the inversion was stopped by plunging the flask used into boiling water. The extent of inversion was measured by estimation of the power of reducing standard alkaline copper solution.

I. In this experiment the invertin solution was heated at 43° C. for twenty minutes and then cooled as described above. Ten c.c. of invertin solution and 50 c.c. of sugar solution were used in each trial. The inversion was allowed to proceed for two hours at 25° C. The estimations were made by the Allihn gravimetric method, the precipitate being collected on a Gooch filter, ignited and weighed as cupric oxide. The results are given in duplicate.

¹ HENRI and POZERSKI: *loc. cit.*, p. 28.

² HANRIOT: *Comptes rendus de la société de biologie*, 1901, liii, p. 58.

³ ALBERT: *Berichte der deutschen chemischen Gesellschaft*, 1900, xxxiii, p.

Preparation of invertin solution.	CuO found (grams).
Heated	{ 0.1831 } 0.1836
Not heated	{ 0.1813 } 0.1821

II. The conditions were the same as in Experiment I. The inversion was allowed to proceed for thirty-eight minutes at 25° C.

Preparation of invertin solution.	CuO found (grams).
Heated	{ 0.0632 } 0.0636
Not heated	{ 0.0641 } 0.0649

III. Four grams of the dried yeast powder were extracted for one half hour with 75 c.c. of water. The filtrate was divided into two portions, one of which was heated at 43° C. for one half hour and then cooled. Thirty c.c. of the enzyme solution were added in each case to 500 c.c. of the sugar solution. At intervals of one half hour portions of 100 c.c. were withdrawn, the inversion stopped by boiling, and the extent of this process determined by titration, Gerrard's cyano-cupric solution being used.¹ The results are expressed in the number of cubic centimetres of inverted solution required to reduce completely 10 c.c. of the standard cupric solution.

Preparation of invertin solution.	Volume of the solution required for complete reduction of the standard copper solution.			
	After 30 min.	After 60 min.	After 90 min.	After 120 min.
Heated	{ 38.7 } 38.7	28.3 28.7	22.3 22.6	17.5 17.6
Not heated	{ 37.5 } 37.8	28.5 28.8	24.0(?) 23.1	18.0 18.2

IV. In this experiment two grams of the yeast powder were extracted. The other details are like those in Experiment III.

¹ SUTTON: Volumetric analysis, 1896, p. 317.

Preparation of invertin solution.	Volume of the solution required for complete reduction of the standard copper solution.		
	After 1½ hours.	After 2 hours.	After 2½ hours.
Heated	{ 17.5 17.6	13.4 13.6	12.0 12.0
Not heated	{ 18.0 17.8	13.5 13.4	12.0 12.2

Experiments with saliva.—Mixed human saliva was filtered and diluted to ten times its original volume. One portion was heated at 43° C. and cooled to room temperature. Ten c.c. of the enzyme solution were added in each case to 100 c.c. of one per cent starch paste. At the expiration of varying periods the digestive mixtures were used in titrations with Gerrard's solution (10 c.c.) as above.

I. Diluted saliva heated for twenty-five minutes.

Preparation of the saliva.	Volume of the solution required for complete reduction of the standard copper solution.	
	After 20 min.	After 40 min.
Heated	{ 18.2 18.2	14.2 14.0
Not heated	{ 15.1 15.2	12.8 12.5

In this case the enzyme seems to have been weakened by the heating.

II. Diluted saliva heated for ten minutes.

Preparation of the saliva.	Volume of the solution required for complete reduction of the standard copper solution.		
	After 10 min.	After 20 min.	After 40 min.
Heated	{ 15.9 } 15.5	8.9 9.0	7.2 7.3
Not heated	{ 16.4 } 16.1	9.8 10.0	7.8 8.0

In criticism of the experiments with animal enzymes it may be said, in favor of Pozerski's theory, that they have already been subjected to body temperature and that therefore a further augmentation of enzyme activity is not to be expected. The following experiments were accordingly carried out with vegetable amyolytic enzymes.

Experiments with diastase. — In the first experiment an active extract of malted barley was prepared, and a portion of it heated at 43° C. for fifteen minutes. The further procedure was the same as with the saliva. In the second experiment the commercial preparation "Taka-diastase" — from *Aspergillus oryzae* — was employed.

Preparation of enzyme solution.	Volume of solution required for complete reduction of the standard copper solution.	
	After 20 min.	After 90 min.
I.		
Malt diastase heated	{ 19.1 } 19.3	10.9 10.8
Malt diastase not heated	{ 19.7 } 19.5	11.1 11.2
II.		
Taka-diastase heated	{ 9.8 } 9.8	6.8 6.9
Taka-diastase not heated	{ 9.9 } 9.8	6.8 6.7

Electrical conductivity.¹—Since it has been suggested that the heating of enzyme solutions produces changes in their physical character, conductivity experiments were carried out with two portions of the same invertin solution, one of which had been previously heated and cooled as in the inversion experiments. The specific conductivity of the solutions at 25° C. referred to reciprocal ohms was:

$$2.146 \times 10^{-3}, \text{ for the heated solution,}$$

$$2.138 \times 10^{-3}, \text{ for the unheated solution.}$$

The capacity of the vessel was standardized by means of a $\frac{1}{50}$ KCl solution, the specific conductivity of which was 2.765×10^{-3} .

Conclusion.—Under the conditions of experiment recorded no evidence was obtained of marked changes in the physical character of the enzyme solutions which have been subjected to a temperature of about 40° C.

¹ These experiments were carried out in the laboratory for physical chemistry. I desire to thank Dr. H. W. FOOTE for kind assistance.

FURTHER EXPERIMENTS ON ARTIFICIAL PARTHENOGENESIS IN ANNELIDS.

BY MARTIN H. FISCHER.

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I. INTRODUCTION.

THE following experiments were undertaken at the suggestion and with the coöperation of Dr. Loeb, and the results would have appeared in a joint paper had not pressure of work compelled Dr. Loeb to leave its publication in my hands.

The previous papers of Loeb upon artificial parthenogenesis had yielded the results that the unfertilized eggs of *Arbacia* or *Chætop-terus* can be brought to the swimming stage through changes in the sea-water. Unfertilized *Arbacia* eggs can be raised to the pluteus stage if they are permitted to remain for varying lengths of time in sea-water the concentration of which has been increased, and are then returned to ordinary sea-water. In *Chætopterus*, Loeb found that by slightly increasing the amount of potassium in the sea-water, without increasing its osmotic pressure, the unfertilized eggs can be caused to develop to the trochophore stage.

Our aim this year was to extend these observations to new forms, if possible, and to find out if there was a difference in the methods by which artificial parthenogenesis can be brought about in these various forms. We succeeded in raising the unfertilized eggs of *Amphitrite* to the trochophore stage by either adding a small amount of calcium to the sea-water, without increasing its osmotic pressure, or by mechanically agitating the sea-water in which the animals were. In *Nereis* we have been able to make only one series of experiments, which, however, seems to show that a certain increase in the osmotic pressure of the sea-water is able to bring about the development of the unfertilized eggs.

To guard against infection of the eggs with sperm, the precautions taken by Loeb¹ were followed. When first brought into the labora-

¹ LOEB, J. : This journal, 1901, iv. p. 424.

tory the animals were carefully and repeatedly washed in sea-water, and then put into separate dishes of running water. When ready for an experiment, the experimenter carefully washed his hands and instruments in fresh water. The animals were then changed to fresh sterile dishes, washed in fresh water, and opened in sea-water sterilized by heating to 50° C.

The greatest care was used throughout in changing the eggs from one dish to another. Unless specially indicated, a large-nozzled pipette was used in transferring the eggs, which were slowly sucked into the pipette, and then equally slowly permitted to flow out into the proper dish, while the mouth of the pipette was held under the surface of the water. The dishes were not moved about, nor the eggs shaken together, and every outside force that might jar the eggs was eliminated. It will be seen later why these extreme measures were necessary.

When eggs were removed from the various solutions for microscopic examination, it was done with as little mechanical disturbance as possible. The eggs removed were kept in covered watch crystals, and in no case were they returned to the dishes from which they had been taken. It is unnecessary to add that separate pipettes, properly sterilized in fresh water, were used in handling the eggs from each of the dishes.

II. THE SPECIFIC EFFECT OF CALCIUM IONS IN THE PRODUCTION OF SWIMMING LARVÆ FROM THE UNFERTILIZED EGGS OF AMPHITRITE.

We began our experiments upon Amphitrite by distributing a mass of eggs into a series of salt solution-sea-water mixtures and after varying intervals of time returning them to fresh sea-water. This first experiment yielded swimming larvæ only in the dish containing a mixture of sea-water and $2\frac{1}{2}$ *n* calcium chloride. We knew that the increase in osmotic pressure had not been the responsible agent, for in the other dishes in which the concentration of the sea-water had been increased to a like degree through potassium chloride or sodium chloride, no trochophores developed. This led us to the idea that the calcium ions had brought about the artificial parthenogenesis. When, however, we tried to determine the least amount of calcium that was necessary to cause the unfertilized eggs of Amphitrite to reach the swimming stage, we got uncertain results. This indicated that the addition of calcium was not the only variable

upon which the parthenogenetic development of the eggs depended. It did not take long to discover this second variable. A. P. Mathews¹ had just found that mechanical agitation was capable of producing artificial parthenogenesis in starfish, and it was no difficult task to show that mechanical agitation could bring about the same result in Amphitrite. A few experiments showed us that the unfertilized eggs of Amphitrite develop to the trochophore stage if, after a residence in sea-water of from one half to one hour, they be squirted from a small-nozzled pipette into another dish of sea-water. But the method is an uncertain one, and by no means can the eggs of every Amphitrite be caused to develop in this way. In order that mechanical agitation may be able to bring about parthenogenesis, the eggs must be in a certain state of "ripeness." Sometimes eggs are found that are so sensitive to mechanical manipulation that so slight a disturbance as the gentlest transference of the eggs from one dish to another may cause a few of them to develop. Previous residence in sea-water or in one of the sea-water-salt solution mixtures is, however, essential in order to make development possible. Only very occasionally does development result from mechanical manipulation immediately after the eggs are cut out of the animal.

These facts explain why such extreme care was exercised in the transference of the eggs from one dish to another. The control material in the following experiments was treated *exactly* like the eggs in the various solutions, so that every source of error that might have arisen from a development of the eggs through mechanical agitation has been eliminated.

Experiment I. August 17, 1901. — We wished first of all to decide if an increase in the amount of calcium in the sea-water could cause the eggs of Amphitrite to develop parthenogenetically. At 12.10 P. M. we distributed a lot of eggs, as gently as possible, into the two following dishes :

1. 98 c.c. sea-water + 2 c.c. $\text{Ca}(\text{NO}_3)_2$, $2\frac{1}{2}$ n.
2. 100 c.c. sea-water (control).

After half an hour (12.40) these eggs were transferred to sea-water. Eggs from both solutions were treated absolutely alike.

At 2.30 P. M. almost every egg which had been in the calcium solution was homogeneously black, contracted, and without a nucleus. The capsule was often swollen and the normal crenations obliterated. One

¹ MATHEWS : This journal, 1901, vi, p. 142.

egg was found in the two-cell stage, and an occasional one as an abortive morula (Fig. 1). The eggs from solution 2 contrasted markedly with those which had been in the calcium solution. Nearly all the eggs were nucleated and intact. Some were in the morula stage, but no typical black eggs were found.

At 3.30 every one of the eggs from solution 1 presented the appearance of the black eggs already described. Some were in the two- to four-cell stage, and occasional ones had reached the eight-cell stage (Fig. 1). Some morulae were found. These morulae corresponded in appearance to those found in solution 2. They were light colored, and not compact and contracted like the other eggs from the calcium solution. The eggs of solution 2 (ordinary sea-water) presented the same appearance as an hour previously, except that the number of morulae had increased.

At 4.30 cell division in the eggs from the calcium solution had progressed to the sixty-four-cell stage. These eggs were much darker and more compact than the morulae already described. The vast majority of eggs, however, showed no external signs of cleavage. One half of the eggs from solution 2 were in the morula stage, the other half were nucleated and intact.

At 5.30 the appearance of the eggs was practically the same as an hour previously. The segmented calcium eggs were still further advanced toward the morula stage. The eggs in the sea-water control were almost without exception in the morula stage.

7.00 P. M. showed the round, black eggs from the calcium solution lightening along the edge. They gave the impression of beginning trochophores. The morulae seemed to have reached a stationary point. Among the eggs from solution 2 no black eggs and no beginning trochophores could be found. All were in the morula stage.

At 9.00 P. M. the dish containing the eggs from the calcium solution was full of swimming larvæ (Fig. 1). An occasional giant was found. The control eggs which had been kept in solution 2 were all in the morula stage, but not a single swimming larvæ was found (Fig. 1).

Examination of the eggs left in the original dishes, 1 and 2, gave the following: In the calcium solution all the eggs were black and developed to the blastula stage, but none were swimming. In solution 2 (sea-water) the eggs were in the morula stage.

Examination of all the dishes the following morning (9.30 A. M.) showed the same condition of affairs as upon the night previous. None of the morulae had developed beyond this stage, and they were fast going to pieces. Many of the trochophores from the calcium solution were still swimming.

The experiment showed that if left undisturbed in sea-water the eggs of *Amphitrite* do not develop to the swimming stage, though they may show a beginning segmentation. If, however, the eggs

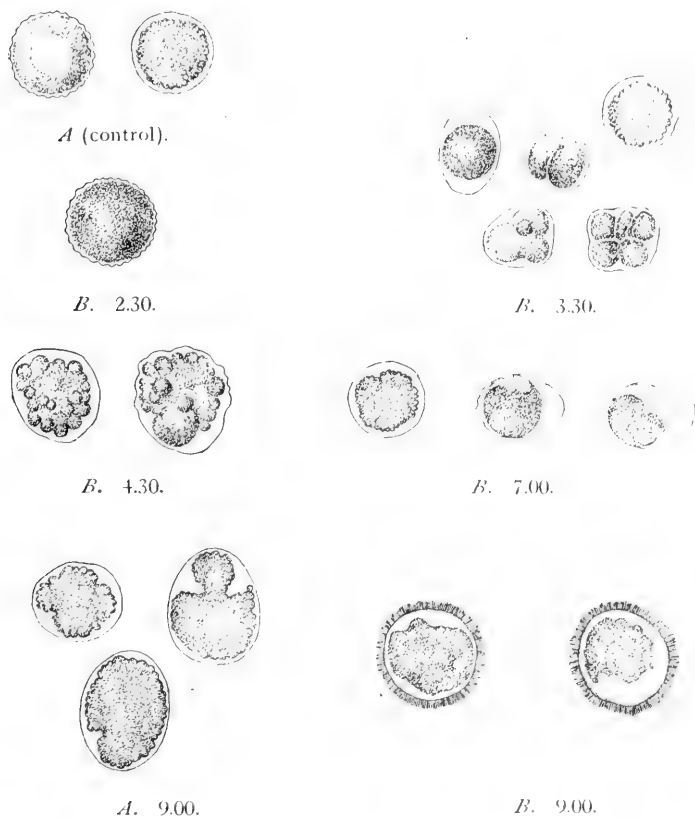


FIGURE 1.—*A.* Control eggs of *Amphitrite* left undisturbed in sea-water. *B.* Parthenogenesis in the same egg brought about through residence for thirty minutes in a mixture of 2 c.c. $\text{Ca}(\text{NO}_3)_2$, $2\frac{1}{2} n$ + 98 c.c. sea-water. The appearance of the two sets of eggs at various hours is given in parallel columns.

are left for thirty minutes in a solution of 98 c.c. sea-water + 2 c.c. $\text{Ca}(\text{NO}_3)_2$, $2\frac{1}{2} n$, and are then transferred to ordinary sea-water they develop into swimming trochophores. Since the osmotic pressure of the sea-water had been scarcely altered, the conclusion seemed justified that the calcium ion is capable of producing parthenogenesis in *Amphitrite*.

Experiment II. August 22, 1901.—It was necessary now to determine the smallest amount of calcium that must be added to sea-water in order to cause the eggs of *Amphitrite* to develop parthenogenetically. The following solutions were prepared :

1. $99\frac{3}{4}$ c.c. sea-water + $\frac{1}{4}$ c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
2. $99\frac{1}{2}$ c.c. sea-water + $\frac{1}{2}$ c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
3. 99 c.c. sea-water + 1 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
4. 98 c.c. sea-water + 2 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
5. 96 c.c. sea-water + 4 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
6. 92 c.c. sea-water + 8 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
7. $99\frac{3}{4}$ c.c. sea-water + $\frac{1}{4}$ c.c. MgCl_2 , $\frac{8}{8}$ *n.*
8. $99\frac{1}{2}$ c.c. sea-water + $\frac{1}{2}$ c.c. MgCl_2 , $\frac{8}{8}$ *n.*
9. 99 c.c. sea-water + 1 c.c. MgCl_2 , $\frac{8}{8}$ *n.*
10. 98 c.c. sea-water + 2 c.c. MgCl_2 , $\frac{8}{8}$ *n.*
11. 96 c.c. sea-water + 4 c.c. MgCl_2 , $\frac{8}{8}$ *n.*
12. 92 c.c. sea-water + 8 c.c. MgCl_2 , $\frac{8}{8}$ *n.*
13. 95 c.c. sea-water + 5 c.c. KCl , $\frac{4}{8}$ *n.*
14. sea-water (control).

The eggs were put into the solutions at 12.15 P. M., and one lot was removed after 45 minutes. The control eggs were changed at the same time.

A few of the eggs that had been in solutions 5, 6, 11, 12, 13, and 14, were removed to separate watch crystals and kept for observation. These eggs were not returned to the dishes from which they were taken. All the dishes were left absolutely undisturbed until after midnight, when the eggs that had been removed to the watch crystals showed swimming larvæ.

At 3.30 P. M. the two watch crystals containing the eggs that had been in the calcium mixtures showed a few black eggs. Those from the mixture of 92 c.c. sea-water + 8 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*, contained many more than those from solution 5. Some were in the early stages of segmentation. Solution 11 of the magnesium series, showed one black egg; solution 12 showed several, and two segmented eggs. The eggs from the potassium solution were absolutely unchanged. The control eggs, which had been subjected to the same mechanical manipulations, contained an occasional black egg.

At midnight the first swimming larvæ were found in the watch crystal containing the eggs from the mixture of 92 c.c. sea-water + 8 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.* This was full of swimming larvæ. The watch crystal of eggs from the mixture of 96 c.c. sea-water plus 4 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*, contained no swimming larvæ, but many of the eggs were in the blastula stage. Almost every egg from solution 11 of the magnesium series was nucleated and intact. One black egg was found, and a few morulæ. Solution 12 gave the same appearance, only it contained two swimming larvæ. The con-

trol eggs showed an occasional morula, and a few black and segmented eggs, but none were swimming.

At 1.30 A. M. we turned our attention to the original dishes. Except for an occasional black egg, every egg removed from the solutions containing $\frac{1}{4}$, $\frac{1}{2}$, or 1 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{1}{8}$ *n*, were absolutely unchanged. 98 c.c. sea-water + 2 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{1}{8}$ *n*, yielded fourteen swimming larvæ and many blastulæ. A greater number than this reached the trochophore stage in the dish containing the eggs from solution 5. The eggs from solution 6 gave a dish full of swimming larvæ.

We examined now the eggs which had been left in the original mixtures of sea-water and calcium nitrate. These had been left absolutely undisturbed since 1.00 P. M., when some of the eggs had been removed to ordinary sea-water. The eggs left in solutions 1 and 2 were, with the exception of three black eggs, absolutely unchanged. All were nucleated and intact. In solution 3 (99 c.c. sea-water plus 1 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{1}{8}$ *n*) one swimming larva was found. One-half of the eggs left in the dish were black and amœboid, or segmented. All the others were intact. Solution 4 was full of swimming larvæ. Those not swimming were in the blastula stage, or black and segmented. Solution 5 showed the same condition of affairs. In solution 6 (92 c.c. sea-water + 8 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{1}{8}$ *n*) over 25 per cent of the eggs were ciliated trochophores. The rest were blastulæ, or simply black eggs showing the early signs of segmentation. Not a single nucleated egg was found.

The control eggs which had been subjected to the same amount of mechanical disturbance as the eggs from the calcium solutions showed not a single segmented egg. Every egg was nucleated and intact. One black egg was found in a state of degeneration.

Two swimming larvæ developed in the dish containing the eggs transferred from one of the magnesium solutions. One swimming larva developed in another. Except for an occasional black egg or a blastula every one of the remaining dishes, both those containing the transferred eggs, and those left in the original solutions, contained nothing but nucleated eggs.

The experiment seemed to leave no room for doubt that the calcium ion is capable of bringing about parthenogenesis in *Amphitrite*. The minimal amount of calcium necessary for this purpose is the addition of 2 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{1}{8}$ *n*, to 99 c.c. sea-water. Many more, however, will develop to the swimming stage if the eggs are left permanently in this mixture than if they are, after thirty minutes, removed to sea-water. In every case in this experiment many more swimming larvæ developed from those eggs which were left perma-

nently in the mixtures of calcium nitrate and sea-water, than in those which were, after half an hour, transferred to normal sea-water. Furthermore, every egg left permanently in the calcium solutions became black, and often segmented. Of the eggs which were transferred to sea-water no less than 50 per cent remained unsegmented.

The eggs used in this experiment were in the proper state of "ripeness" to make parthenogenesis through mechanical disturbance possible. This accounts for the development of the occasional trochophores in the eggs removed from the magnesium chloride-sea-water mixtures. The original dishes from which these eggs had been taken were absolutely free from segmented eggs.

Experiment III. August 23, 1901. — We wished once more to assure ourselves of the truth of the results obtained in the last experiment. The eggs of an *Amphitrite* were distributed into the following solutions at 3.00 P. M. :

1. 99 $\frac{3}{4}$ c.c. sea-water + $\frac{1}{4}$ c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
2. 98 c.c. sea-water + 2 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
3. 96 c.c. sea-water + 4 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
4. sea-water (control).

In order to eliminate all sources of error due to mechanical agitation, the dishes were left absolutely undisturbed until 9.00 A. M. of the next day, when we knew that swimming larvæ would have developed.

Examination at this time showed the following: In the dish containing $\frac{1}{4}$ c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.* none were swimming and only one egg was in the three-cell stage. All the other eggs were homogeneously black and often undergoing disintegration. Solution 2 showed an occasional swimming larva, many blastulæ, and some segmented eggs. Solution 3 was full of swimming larvæ.

The control eggs, which had been treated precisely like the eggs in the calcium solutions, showed not a single swimming larva. All the eggs were black, and many were segmented into two and four cells.

Experiment IV. August 24, 1901. — We wished once more to determine the least amount of calcium that must be added to sea-water in order to cause parthenogenesis in the eggs of *Amphitrite*. We wished also to know if the abstraction of water from the egg could bring about its development. A lot of eggs were distributed into the following solutions at 11.00 A. M. :

1. 99 $\frac{3}{4}$ c.c. sea-water + $\frac{1}{4}$ c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
2. 99 $\frac{1}{2}$ c.c. sea-water + $\frac{1}{2}$ c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
3. 99 c.c. sea-water + 1 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
4. 98 c.c. sea-water + 2 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
5. 96 c.c. sea-water + 4 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*
6. 92 c.c. sea-water + 8 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ *n.*

7. 70 c.c. sea-water + 30 c.c. sea-water \times 2¹.
8. 60 c.c. sea-water + 40 c.c. sea-water \times 2.
9. 50 c.c. sea-water + 50 c.c. sea-water \times 2.
10. 40 c.c. sea-water + 60 c.c. sea-water \times 2.
11. sea-water (control).

The eggs in the calcium solutions were left undisturbed except for the removal of a few eggs from solution 6. These were kept in a watch crystal for observation, and were not returned to the original dish. The eggs in the concentrated sea-water and in the control were changed to fresh sea-water after 35 minutes. After this the eggs were not again disturbed.

At 11.30 P.M. when the watch crystal containing the calcium eggs showed many swimming larvæ (the day had been very warm, which accounts for the rapid development of the larvæ) conditions were as follows. Solutions 1, 2, and 3 showed, with occasional exceptions, nothing but nucleated and intact eggs. A few eggs were black, and an occasional egg had segmented to the four-cell stage. In solution 4 (98 c.c. sea-water plus 2 c.c. $\text{Ca}(\text{NO}_3)_2$, § *n*) we found a dozen swimming larvæ, and an equal number of blastulæ. Two thirds of the eggs were black and many were segmented. The rest were still nucleated. In solution 5 fully 25 per cent of the eggs were swimming trochophores. A large number were in the blastula stage, and some eggs were black and segmented. The solution containing 8 c.c. $\text{Ca}(\text{NO}_3)_2$, § *n*, presented the same appearance, but the number of swimming larvæ was less.

Control dish 11, which had been subjected to the same physical disturbance as the above, was free from swimming larvæ. With the exception of an occasional black egg, every egg was nucleated and intact. An occasional swimming larva was found among the eggs which were transferred from the concentrated sea-water to ordinary sea-water, but an equal number of larvæ was found in the dish containing the control eggs which had been similarly transferred. This led us to attribute the development of the eggs which had been in the concentrated sea-water to the mechanical disturbance they suffered in the transference to ordinary sea-water.

The experiment corroborated our previous finding, that calcium nitrate is capable of causing the development of swimming trochophores from the unfertilized eggs of *Amphitrite*, provided it is present in more than 2 c.c. of a normal solution to 98 c.c. of sea-water.

Experiment V. August 24, 1901.—The specificity of the action of calcium in the production of artificial parthenogenesis in *Amphitrite* is shown in the

¹ Sea-water was slowly evaporated to one half its volume.

following experiment. The eggs of a female were distributed into the following dishes at 9.00 P. M.:

1. $99\frac{3}{4}$ c.c. sea-water + $\frac{1}{4}$ c.c. LiCl, $\frac{1}{8}$ n.
2. $99\frac{1}{2}$ c.c. sea-water + $\frac{1}{2}$ c.c. LiCl, $\frac{1}{4}$ n.
3. 99 c.c. sea-water + 1 c.c. LiCl, $\frac{1}{2}$ n.
4. 98 c.c. sea-water + 2 c.c. LiCl, $\frac{1}{2}$ n.
5. 96 c.c. sea-water + 4 c.c. LiCl, $\frac{1}{2}$ n.
6. 92 c.c. sea-water + 8 c.c. LiCl, $\frac{1}{2}$ n.
7. 84 c.c. sea-water + 16 c.c. LiCl, $\frac{1}{2}$ n.
8. 95 c.c. sea-water + 5 c.c. NaCl, $\frac{1}{8}$ n.
9. 95 c.c. sea-water + 5 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}$ n.
10. sea-water (control).

The eggs were left absolutely undisturbed until the following morning at 9.30. Their condition then was as follows: Control 10 contained no swimming or segmented eggs, but every egg was black. In solution 1 four swimming trochophores were found. These had, no doubt, developed through mechanical agitation. In the remaining lithium and sodium solutions only an occasional black or segmented egg was found. Nearly all the eggs were nucleated and intact. The calcium-sea-water mixture, however, was full of swimming larvæ, while the eggs which did not actually swim were in the blastula stage. A few eggs were still nucleated.

III. THE DIFFERENCES BETWEEN THE PARTHENOGENETIC, NORMALLY FERTILIZED AND UNFERTILIZED EGGS OF AMPHITRITE.

The fertilized eggs of Amphitrite begin to cleave about forty-five minutes after the addition of the sperm. Within an hour practically all the eggs have reached the two- to four-cell stage. In two hours the eggs reach the eight-cell stage; in three hours the sixty-four- to one hundred and twenty-four-cell stage. Four hours after the addition of the sperm one half the eggs may be found in the swimming trochophore stage. The remaining eggs are in the morula stage, often showing a lightening along the edge (gastrula stage).

The eggs which are brought to the swimming stage through the addition of calcium to the sea-water, or through mechanical disturbance present a totally different appearance. An hour after the eggs have been put into a mixture of sea-water and calcium nitrate, the nucleus becomes obscure, and the protoplasm contracts. The bulk of the egg diminishes greatly, and becomes homogeneously black and opaque. The outline of the egg is perfectly sharp and distinct. The normally crenated egg capsule becomes smooth in outline, and swollen in appearance, due to the contraction of the egg proto-

plasm (Fig. 2). After two hours many of the black eggs may show a beginning of segmentation (Fig. 3). They may become indented upon one side, or may actually cleave into two or four cells. Rarely

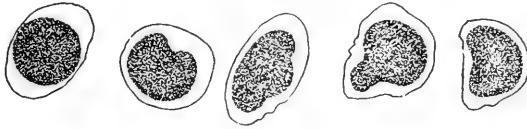


FIGURE 2.— Parthenogenesis in the eggs of *Amphitrite* brought about by the addition of 2 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{8}{8}n$ to 98 c.c. sea-water. The eggs have lost their nuclei and are in the amœboid state, changing their shapes while under observation.

the cleavage continues in a more or less regular order to the eight- or even twelve-cell stage. Sometimes the eggs go into the morula stage. The origin of the morulæ is somewhat obscure. The fact that they appear in solutions which have at no time contained any black eggs would suggest that the nucleated eggs can, in a very short time, give rise to morulæ without any external signs of cleavage. On the other hand, we have seen black eggs with numerous light lines running over their surfaces, as though they were suddenly to break up into a mass of smaller spherules (cells?). Sometimes an egg is found one half of which is homogeneously black and contracted, while the other half is broken up into a morula. What the correct answer to this question is must be left for further experimental study and histological examination. The majority of eggs, however, that cleave, never go beyond the two-cell stage. The eggs which show no segmentation become amœboid, and change their shape while under observation with the microscope (Fig. 2).



FIGURE 3.— Early stages of segmentation in parthenogenetic *Amphitrite* eggs.

In five to seven hours—varying with the temperature of the water, the ripeness of the eggs, etc.—the black eggs become somewhat less opaque at one pole. Later this light rim extends entirely around the egg. In eleven to thirteen hours the eggs swim. We cannot yet speak with perfect certainty upon this point, but from the fact that a much larger number of eggs become swimming larvæ than ever cleave, and that the number of eggs in the two- and four-cell stages seems not to diminish with an increase in the number of swimming

trochophores, it would seem that the eggs of *Amphitrite* can go into the blastula and trochophore stage without any external evidences of cleavage. This view is in harmony with the observations of Loeb¹ upon the eggs of *Chætopterus*. The appearance of the swimming trochophores (Figs. 4, 5), the ciliary activity, and the



FIGURE 4.—Swimming trochophore forty hours old. The parthenogenesis was brought about through residence for thirty minutes in a mixture of 95 c.c. sea-water + 5 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{1}{2}$ n.

FIGURE 5.—The same. This is the farthest stage to which it has been possible to raise the parthenogenetic eggs. The cilia are differentiated.

FIGURE 6.—A freak trochophore, from the same solution as that of Fig. 4.

general behavior of the parthenogenetic larvæ is exactly that of the normally fertilized larvæ. Often freaks of odd shapes are found (Fig. 6) and giant embryos made up of from two to ten eggs are not uncommon (Fig. 7).

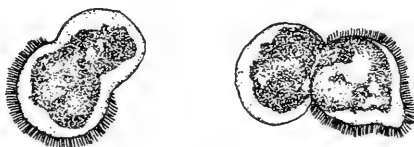


FIGURE 7.—Giant embryos from a mixture of 96 c.c. sea-water + 4 c.c. $\text{Ca}(\text{NO}_3)_2$, $\frac{1}{2}$ n. The eggs were left undisturbed in the solution.

The eggs of *Amphitrite* when left undisturbed in ordinary sea-water show, with rare exceptions, absolutely no change during the first five or ten hours. We have often seen a dish full of swimming parthenogenetic larvæ while the eggs of the control which had been left absolutely undisturbed in sea-water were every one of them nucleated and intact. After ten or fifteen hours, however, varying with the temperature and with the individual eggs, a large number became contracted and homogeneously black, like the eggs which have been described. If enough time is given, almost without exception, every egg will become black. A large number show the early

¹ LOEB, J.: This journal, 1901, iv, p. 423.

signs of cleavage, occasional ones going as far as the eight cell stage. None, however, reach the blastula or trochophore stage, and the eggs undergo a granular degeneration and die when the parthenogenetic larvæ are still swimming.

In Fig. 8 is shown in parallel columns the appearance of parthenogenetic calcium eggs, eggs left undisturbed in ordinary sea-water, and normally fertilized eggs.

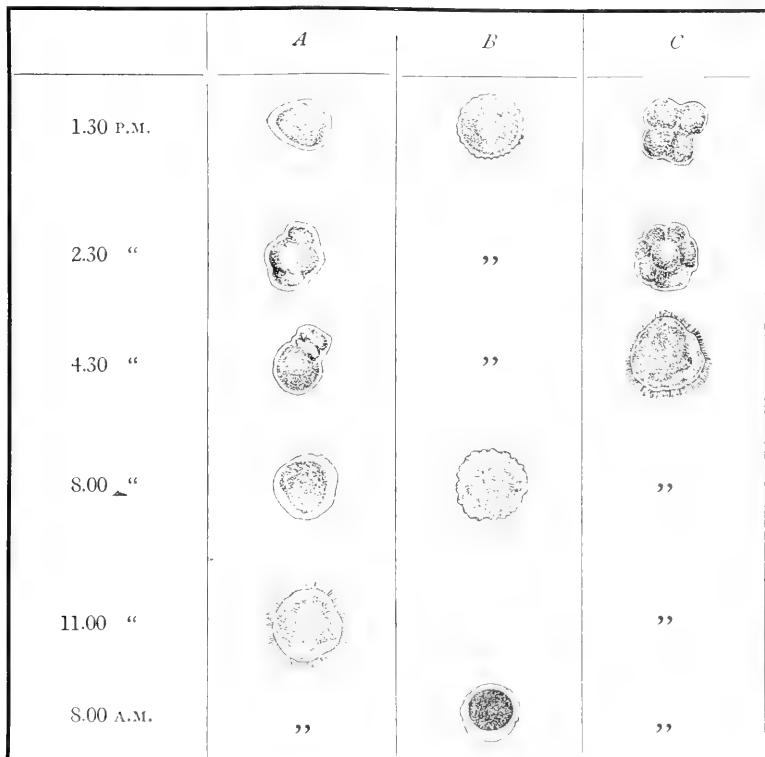


FIGURE 8.—*A*. Artificial parthenogenesis brought about by the addition of 5 c.c. $\text{Ca}(\text{NO}_3)_2, \frac{2}{3} \mu$ to 95 c.c. sea-water. The eggs remained in the solution thirty minutes. *B*. Control eggs left undisturbed in sea-water. *C*. Normally fertilized eggs. The eggs of *C* were fertilized at 12.30 P.M., when those in *A* were removed from the calcium nitrate-sea-water mixture. The appearance of the eggs at various times is given in parallel columns.

IV. ARTIFICIAL PARTHENOGENESIS IN NEREIS.

We wish to give here a brief report of a single experiment upon *Nereis limbata* in which we succeeded in bringing the unfertilized

eggs to the swimming stage. The impossibility of getting more material put an end to further work in this direction this year.

Our experiment consisted in distributing the eggs of two females into a series of dishes containing sea-water, the concentration of which had been raised to various degrees by the addition of $2\frac{1}{2}$ „ KCl. Our single experiment seems to justify the following conclusions: The eggs of *Nereis limbata* are not normally parthenogenetic, and will not, if left in ordinary sea-water, develop into swimming larvæ. A beginning cleavage, however, may take place after prolonged residence in ordinary sea-water. Swimming trochophores can be produced from the unfertilized eggs of *Nereis*, if they are immersed in a mixture of 80 c.c. sea-water + 20 c.c. KCl, $2\frac{1}{2}$ „, for thirty minutes, and are then returned to sea-water. The fact that parthenogenesis resulted only in mixtures of sea-water and potassium chloride in which the osmotic pressure had been considerably raised, would seem to indicate that it is brought about by an abstraction of water from the cell, and not through a specific effect of the potassium ion.

V. THEORETICAL CONSIDERATIONS.

The experiments upon *Amphitrite* and *Nereis* confirm the views of Loeb that the spermatozoon does not act as a "stimulus" to the egg that starts its development, but simply as a catalyzer that accelerates a process which starts of its own accord. The unfertilized eggs of either *Amphitrite* or *Nereis*, if left undisturbed in sea-water, show a dissolution of the nucleus, a deepening in the color and a contraction of the protoplasm, together with a beginning cellular division that correspond to the changes that occur in the fertilized egg, except that they begin at a much later time, and succeed each other much more slowly than in the fertilized eggs. The addition of calcium (in *Amphitrite*) or the abstraction of water (in *Nereis*) from the egg serves only as a method of accelerating a process that occurs anyway. But this accelerated karyokinesis is essential to the further development of the egg, for if left undisturbed in sea-water, the egg reaches only the first stages of segmentation and then dies.

It gives me pleasure to express my thanks to Dr. Hektoen, and to the faculty of Rush Medical College for the privileges of the Rush Table at Wood's Hole.

ON THE POWER OF Na_2SO_4 TO NEUTRALIZE THE ILL EFFECTS OF NaCl .

BY ANNE MOORE.

Introduction. — In an article¹ published last year I showed that the excised lymph hearts of the frog contract rhythmically in a pure $\frac{2}{8}$ NaCl solution. The contractions continue for a certain length of time and then cease. If CaCl_2 or some SO_4 compound, notably Na_2SO_4 , be then added to the solution in proper proportions, the hearts resume their contractions, thus proving that both CaCl_2 and Na_2SO_4 are effective in neutralizing the ill effects of NaCl .

Previously Loeb² showed that *Fundulus*, a marine fish, cannot live in a pure NaCl solution of the same concentration as sea-water, but can live in such a solution if a definite proportion of CaCl_2 be added. These experiments I repeated upon young trout and tadpoles, showing that for fresh-water animals as well as salt, CaCl_2 is efficacious in neutralizing the ill effects of NaCl .³ In view of my results on the lymph hearts which showed Na_2SO_4 to be effective in counteracting NaCl , it occurred to me to perform a series of experiments similar to those performed upon trout and tadpoles, to obtain, if possible, further evidence of this power of Na_2SO_4 .

Method. — As trout were not available, I used tadpoles and mosquito larvæ. The solutions were put into large covered dishes, which were kept at room temperature and were opened at intervals to renew the oxygen supply. Four to six specimens were placed in each dish and were examined from time to time. As described in my former paper, the activities were suspended in a definite order as death approached. The ordinary swimming motion first became somewhat abnormal, then motion ceased, later breathing ceased, and finally respiration. In order to have a uniform basis of comparison, loss of motion was considered a mark of death. The concentration of NaCl most favorable for testing the neutralizing effect of CaCl_2 ,

¹ MOORE, ANNE: This journal, 1901, v. p. 87.

² LOEB, J.: This journal, 1900, iii, p. 331.

³ MOORE, ANNE: This journal, 1900, iv, p. 265.

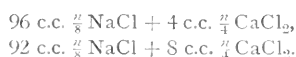
was found by my former experiments to be $\frac{1}{8}$. That concentration was therefore used in this series of experiments. Two parallel series of solutions were always made, each beginning with pure $\frac{1}{8}$ NaCl. In the one $\frac{1}{4}$ CaCl₂ was added in gradually increasing quantities, in the other equal amounts of $\frac{1}{4}$ Na₂SO₄ were added.

Results. — It was found that, when added to NaCl, Na₂SO₄ is as efficacious in prolonging the life of tadpoles and mosquito larvæ as CaCl₂. The following table gives evidence of the fact:

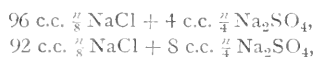
TABLE I. TADPOLES.

NaCl solutions + CaCl ₂	Average duration of life.	NaCl solutions + Na ₂ SO ₄	Average duration of life.
100 c.c. $\frac{1}{8}$ NaCl	4 $\frac{3}{4}$ days	100 c.c. $\frac{1}{8}$ NaCl	4 $\frac{3}{4}$ days
98 c.c. $\frac{1}{8}$ NaCl + 2 c.c. $\frac{1}{4}$ CaCl ₂	4 $\frac{1}{2}$ "	98 c.c. $\frac{1}{8}$ NaCl + 2 c.c. $\frac{1}{4}$ Na ₂ SO ₄	10 "
96 c.c. $\frac{1}{8}$ NaCl + 4 c.c. $\frac{1}{4}$ CaCl ₂	10 $\frac{1}{2}$ "	96 c.c. $\frac{1}{8}$ NaCl + 4 c.c. $\frac{1}{4}$ Na ₂ SO ₄	4 $\frac{1}{2}$ "
92 c.c. $\frac{1}{8}$ NaCl + 8 c.c. $\frac{1}{4}$ CaCl ₂	11 "	94 c.c. $\frac{1}{8}$ NaCl + 6 c.c. $\frac{1}{4}$ Na ₂ SO ₄	14 "
88 c.c. $\frac{1}{8}$ NaCl + 12 c.c. $\frac{1}{4}$ CaCl ₂	4 $\frac{1}{2}$ "	92 c.c. $\frac{1}{8}$ NaCl + 8 c.c. $\frac{1}{4}$ Na ₂ SO ₄	10 "
75 c.c. $\frac{1}{8}$ NaCl + 25 c.c. $\frac{1}{4}$ CaCl ₂	2 "	90 c.c. $\frac{1}{8}$ NaCl + 10 c.c. $\frac{1}{4}$ Na ₂ SO ₄	13 $\frac{1}{2}$ "
		88 c.c. $\frac{1}{8}$ NaCl + 12 c.c. $\frac{1}{4}$ Na ₂ SO ₄	7 "
		75 c.c. $\frac{1}{8}$ NaCl + 25 c.c. $\frac{1}{4}$ Na ₂ SO ₄	3 "

In the most striking experiment, the four tadpoles placed in $\frac{1}{8}$ NaCl were dead within three days. At the end of twenty-one days three specimens were still alive in the solutions



These died on the twenty-second day. In the solutions



two specimens died on the thirteenth day, the others on the twenty-third day.

Results of the same character were obtained with the mosquito larvæ. They were not quite so satisfactory for demonstrating the point, however, because there was much more individual variation in longevity among them than among tadpoles, and because the pupæ

moulted and left the solution before it had proved fatal. In $\frac{2}{8}$ NaCl they lived from less than twenty hours to nine days; in 88 c.c. $\frac{2}{8}$ NaCl + 12 c.c. $\frac{2}{4}$ Na₂SO₄ they lived from four days to fifteen days; in 98 c.c. $\frac{2}{8}$ NaCl + 2 c.c. $\frac{2}{4}$ CaCl₂ from twenty hours to fourteen days.

While engaged in other work last year I incidentally noticed a fact which adds further evidence of the power of Na₂SO₄ to neutralize the ill effects of NaCl. If frog muscles are placed in a solution of NaCl to which Na₂SO₄ has been added they retain their irritability longer than in the pure NaCl solution. In $\frac{2}{8}$ NaCl the muscles lost their irritability in from twenty-four to forty-eight hours. In the solution 92 c.c. $\frac{2}{8}$ NaCl + 8 c.c. $\frac{2}{4}$ Na₂SO₄ irritability lasted more than seventy-two hours. Further, I found that toad muscles, which contract rhythmically in pure NaCl, after exhaustion resume contractions if Na₂SO₄ is added to the solution. In one experiment the muscle contracted rhythmically in $\frac{2}{8}$ NaCl six hours. After it had become exhausted and had remained quiet one hour it was transferred to the solution 96 c.c. $\frac{2}{8}$ NaCl + 4 c.c. $\frac{2}{4}$ Na₂SO₄. Contractions were resumed and were continued until the next morning.

Loeb¹ has recently published an article on the effects of ions with special reference to their valency and their electrical charge. His experiments were performed upon freshly fertilized eggs of *Fundulus*. The eggs were placed in various solutions and the percentage of developing embryos determined. In the course of his work he tried a large number of experiments in which he attempted to annihilate the poisonous effects of a $\frac{5}{8}$ *m* NaCl solution by the addition of salts having a univalent, bi-, or trivalent anion. His results were negative throughout. In consequence he states: "It followed from these experiments that the toxic effects of salts with a monovalent cation and a monovalent anion can be annihilated only by bi- or trivalent cations but not by mono-, bi-, or trivalent anions." My results show very decidedly that in one case at least it is possible to annihilate the poisonous effects of a salt with a monovalent cation and a monovalent anion by a bivalent anion.

The question naturally arises: Is this effect of Na₂SO₄ a result of the bivalency of the anion? If so, other bivalent anions should have the same effect. With this in mind, I tried a similar series of experiments, using sodium oxalate instead of sodium sulphate. It was found that when used in the same proportions Na₂C₂O₄ was very

¹ LOEB, J.: This journal, 1902, vi, p. 411.

injurious, the specimens in NaCl to which $\text{Na}_2\text{C}_2\text{O}_4$ had been added dying much sooner than those in pure NaCl. The following solutions were used:

SERIES A.

1. 100 c.c. $\frac{1}{8}$ NaCl.
2. 98 c.c. $\frac{1}{8}$ NaCl + 2 c.c. $\frac{1}{4}$ $\text{Na}_2\text{C}_2\text{O}_4$.
3. 96 c.c. $\frac{1}{8}$ NaCl + 4 c.c. $\frac{1}{4}$ $\text{Na}_2\text{C}_2\text{O}_4$.
4. 94 c.c. $\frac{1}{8}$ NaCl + 6 c.c. $\frac{1}{4}$ $\text{Na}_2\text{C}_2\text{O}_4$.
5. 92 c.c. $\frac{1}{8}$ NaCl + 8 c.c. $\frac{1}{4}$ $\text{Na}_2\text{C}_2\text{O}_4$.
6. 90 c.c. $\frac{1}{8}$ NaCl + 10 c.c. $\frac{1}{4}$ $\text{Na}_2\text{C}_2\text{O}_4$.
7. 88 c.c. $\frac{1}{8}$ NaCl + 12 c.c. $\frac{1}{4}$ $\text{Na}_2\text{C}_2\text{O}_4$.
8. 75 c.c. $\frac{1}{8}$ NaCl + 25 c.c. $\frac{1}{4}$ $\text{Na}_2\text{C}_2\text{O}_4$.

In solutions 4, 5, 6, 7, 8, the six specimens placed in each dish were dead in less than seventeen hours. In solutions 2, 3, they were dead twenty-four hours later. Those in solution 1 lived five days.

An experiment in which the action of $\text{Na}_2\text{C}_2\text{O}_4$ was compared directly with that of Na_2SO_4 and CaCl_2 was very striking. The solutions were:

SERIES B.

1. 100 c.c. $\frac{1}{8}$ NaCl.
2. 98 c.c. $\frac{1}{8}$ NaCl + 2 c.c. $\frac{1}{4}$ $\text{Na}_2\text{C}_2\text{O}_4$.
3. 96 c.c. $\frac{1}{8}$ NaCl + 4 c.c. $\frac{1}{4}$ $\text{Na}_2\text{C}_2\text{O}_4$.
4. 92 c.c. $\frac{1}{8}$ NaCl + 8 c.c. $\frac{1}{4}$ $\text{Na}_2\text{C}_2\text{O}_4$.
5. 98 c.c. $\frac{1}{8}$ NaCl + 2 c.c. $\frac{1}{4}$ CaCl_2 .
6. 92 c.c. $\frac{1}{8}$ NaCl + 8 c.c. $\frac{1}{4}$ CaCl_2 .
7. 98 c.c. $\frac{1}{8}$ NaCl + 2 c.c. $\frac{1}{4}$ Na_2SO_4 .
8. 92 c.c. $\frac{1}{8}$ NaCl + 8 c.c. $\frac{1}{4}$ Na_2SO_4 .

The specimens in 3, 4, were dead in less than seventeen hours; those in 2 lived six days; those in 1 seven days, while in solutions 5, 6, 7, 8, some of the specimens were still alive on the fourteenth day.

Thinking that possibly the solutions of $\text{Na}_2\text{C}_2\text{O}_4$ were too strong and that it might be able to neutralize the effect of NaCl if used in weaker solutions, I tried a series of experiments in which the solutions were the same as those in Series A except that the concentration of the $\text{Na}_2\text{C}_2\text{O}_4$ was decreased from $\frac{1}{4}$ to $\frac{1}{6}$. As the $\text{Na}_2\text{C}_2\text{O}_4$ was weaker, it naturally proved less injurious. The average length of life in all the solutions was practically the same, not varying more than half a day, and without exception specimens were still living in the pure $\frac{1}{8}$ NaCl solution after those in the other solutions were dead.

As both Na_2SO_4 and $\text{Na}_2\text{C}_2\text{O}_4$ have bivalent anions and yet act so differently, these experiments, especially when taken in connection

with the results of Loeb's experiments with bivalent anions, would seem to indicate that the beneficial effect of Na₂SO₄ is not the immediate result of the bivalency of the anion. Apparently we must either regard Na₂C₂O₄ as specifically toxic or Na₂SO₄ as specifically beneficial. In view, however, of the work of Dr. Loeb, which suggests that some general law, either of valency or of the electric charge, governs the effects of ions, and in view of the fact that CaCl₂ as well as Na₂SO₄ is efficacious in neutralizing the ill effects of NaCl, it seems probable that the action of Na₂SO₄ is not a specific effect, but is a response to a general law, which is possibly an expression of valency or of electric charge. Further experiments must determine the point. Dr. Loeb found that although as a rule bivalent kations are able to annihilate the ill effects of NaCl, copper acetate and mercuric chloride gave negative results, "for these two ions (Cu and Hg) are so poisonous that the small amounts necessary to render inert the poisonous effects of a sodium chloride solution are sufficient to kill the egg." It is quite probable that sodium oxalate is poisonous in some such way.

Howell,¹ in a recent article on the automatic contractions of heart muscle, says concerning an experiment in which he used Na₂C₂O₄: "If one chooses to make the objection to this experiment that the oxalate prevents the contractions, not by precipitating the Ca, but by some direct action of the oxalic acid ion, one cannot prove the contrary directly since by the nature of the reaction the Ca cannot be removed without an excess of the oxalate and vice versa. The indirect evidence, however, shows conclusively that the effect of the oxalate is due to the precipitation of the Ca."

When one considers, however, that Na₂SO₄ also precipitates calcium and yet is able to cause the lymph hearts of frogs and toads' muscles previously exhausted in NaCl to resume contractions it would seem that the objection is not ill founded.

¹ HOWELL, W. D. : This journal, 1901, iv, p. 196.

ON THE CONTACT IRRITABILITY OF MUSCLES.

BY W. D. ZOETHOUT.

[From the Harvey Medical College, Chicago.]

IN a former paper, in which I discussed the effects of potassium and calcium ions on the tone of muscle,¹ it was proved that potassium increases the tone of the striated muscle, and that calcium, and to a lesser extent sodium, counteract this effect. I found that the minimum concentration of potassium capable of producing a slight increase in the tone of the muscle was represented by the following solutions:

- 1 c.c. $\frac{m}{8}$ KCl + 9 c.c. H₂O (or glycerine).
- 2 c.c. $\frac{m}{8}$ KCl + 8 c.c. $\frac{m}{8}$ NaCl.
- 4 $\frac{1}{2}$ c.c. $\frac{m}{8}$ KCl + 6 $\frac{1}{2}$ c.c. $\frac{m}{8}$ CaCl₂.

The fact that the effect of the potassium can be decreased by the previous or subsequent application of calcium clearly shows the antagonistic action of these two substances.

While repeating some of the experiments, it occurred to me that as the muscle always contains calcium salts, the presence of these salts raises the concentration of the KCl necessary to produce an increase in tone. If we could, therefore, decrease the calcium in the muscle previously to or simultaneously with the introduction of potassium into the muscle, the minimum concentration of the potassium necessary to increase the tone of the muscle would be decreased. And this we found to take place. If the muscle was treated with K-citrate, K-oxalate, or any potassium salt whose anion precipitates calcium, the minimum concentration was found to be:

- $\frac{1}{2}$ c.c. $\frac{m}{8}$ K-citrate + 9 $\frac{1}{2}$ c.c. H₂O, or
- 1 c.c. $\frac{m}{8}$ K-citrate + 9 c.c. $\frac{m}{8}$ NaCl.

Comparing this with the table of concentration of KCl it will be noticed that the concentrations are decreased by 50 per cent. Previous observers have pointed out that any salt whose anion pre-

¹ ZOETHOUT: This journal, 1902, vii, p. 199.

precipitates calcium may cause a slight shortening of the muscle;¹ the concentration of such salts, *e.g.*, Na-oxalate, necessary to produce a contraction is very much greater than that of the potassium salts.

The same point was also demonstrated in the following manner: A muscle was treated for one minute with a $\frac{m}{8}$ solution of a sodium salt which precipitates calcium and then subjected to the action of KCl or K_2SO_4 . In this case the minimum concentration was much reduced and the increase in tone was more marked.

The normal muscle is rich in potassium salts. According to Bunge,² and Katz,³ muscle contains from 1 to 1.5 per cent ash, and of this the K_2O constitutes from one quarter to one third. If the potassium increases the tone of the muscle, why is not the muscle always contracted? I think this is due to the calcium salts present in the muscle. The calcium in some way or other inhibits the effects of the potassium. If the potassium ions in the muscle are increased (by placing muscle in KCl solution) the normal equilibrium between the calcium and the potassium is destroyed and the excess of potassium produces an increase in tone. If we introduce potassium and calcium into the muscle simultaneously, for example, by placing a muscle in 3 c.c. $\frac{m}{8}$ KCl + 7 c.c. $\frac{m}{8}$ $CaCl_2$, the equilibrium is not disturbed and no increase of tone results. On the other hand, it is possible to destroy the ratio between the potassium and calcium by reducing the calcium; the potassium in the muscle will then cause the muscle to contract. This is, no doubt, the cause of the contraction produced by such salts as Na-oxalate, Na-fluoride, etc.

While working on the effects of the sodium salts whose anions produce insoluble calcium compounds, I had many occasions to observe the curious action of these salts in causing what Dr. Loeb⁴ has called "contact irritability." Loeb called attention to the fact that while a normal frog muscle is not stimulated by the contact of such bodies as air, water, oil, etc., a muscle which has been treated with a sodium salt whose anion precipitates calcium is thrown into activity when it comes in contact with the above-named bodies (contact reaction).

Now, it occurred to me that since potassium increases the tone of the muscle and calcium inhibits this action of the potassium, it might

¹ LOEB: This journal, 1901, v, p. 362.

² BUNGE: Zeitschrift für physiologische Chemie, ix, p. 60.

³ KATZ: Archiv für die gesammte Physiologie, 1896, lxxiii, p. 1.

⁴ LOEB: This journal, 1901, v, p. 362.

be possible that the precipitation of the calcium salts causes contact irritability because it destroys the normal equilibrium between these two salts in the muscle. Dr. Loeb himself suggested a disturbance in the equilibrium of the ions of the muscle when he said: "While all the facts thus seem to harmonize with the view that a decrease in the amount of Ca-ions in the tissues (and possibly an increase in the amount of Na-ions) is the essential condition for the production of the contact reaction, it is yet possible that the sodium salts whose anions form insoluble calcium compounds may have a specific effect upon other constituents of the protoplasm, *c. g.*, proteids."¹

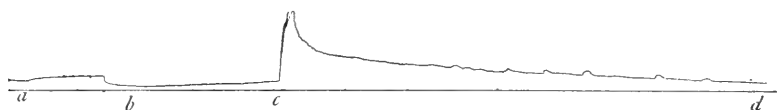


FIGURE 1.—At (a) the muscle is placed in 2 c.c. KCl + 8 c.c. NaCl. At (b) this is exchanged for 10 c.c. Na oxalate. At (c) the muscle is exposed to the air.

Upon testing this view we found it to be correct. The contact reaction produced by Na-citrate is increased if we previously or simultaneously introduce K-ions into the muscle. The experiments were made as follows: The left gastrocnemius muscle of a frog was prepared and placed in a bath of 2 c.c. $\frac{m}{8}$ KCl + 8 c.c. $\frac{m}{8}$ NaCl for six minutes. At the expiration of this time the muscle was treated with 10 c.c. of $\frac{m}{8}$ Na-oxalate for one minute. On exposure to the air the muscle was immediately thrown into contraction which developed into a more or less complete tetanus sustained for some time (see Fig. 1). The other gastrocnemius muscle was then prepared and placed for six minutes in 10 c.c. $\frac{m}{8}$ NaCl, after which it was treated



FIGURE 2.—At (a) the muscle is placed in 10 c.c. NaCl. At (b) this is exchanged for 10 c.c. Na oxalate. At (c) the muscle is exposed to the air.

in 10 c.c. Na-oxalate. On coming in contact with the air (Fig. 2, c) no contractions occurred, except after the lapse of about three minutes, when a slight contact reaction set in.

The experiments were repeated a great many times, and with different proportions of KCl. The best results were obtained by using

¹ LOEB: This journal, 1901, v, p. 362.

3 c.c. KCl + 7 c.c. NaCl. It is necessary for the KCl to act for at least three or four minutes in order to give the KCl sufficient time to penetrate the muscle tissue. If stronger solutions of KCl are used the subsequent use of Na-oxalate may throw the muscle into spasms and the contact reaction is not as good. As the irritability of different muscles varies greatly, and the irritability of the same muscle is greatly influenced by temperature and other conditions, it was found necessary always to use one of the gastrocnemius muscles of each frog as a control test. Sometimes the control was made first, sometimes last.

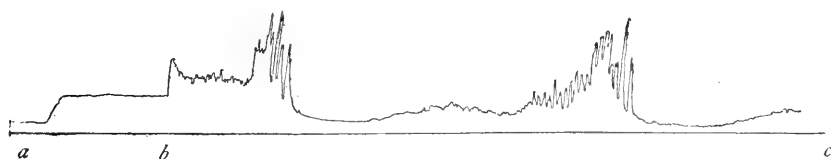


FIGURE 3.—At (*a*) the muscle is placed in 1 c.c. KCl + 9 c.c. Na oxalate. After 1 minute (at *b*) the muscle is exposed to the air.

These experiments were made with Na-oxalate, Na-citrate, Na-fluoride, Na-carbonate, and Na-tartrate, and demonstrate that the introduction of K-ions into the muscle renders the muscle more susceptible to contact reaction.

That the contact irritability depends upon a disturbance in the ratio of the ions in the muscle was also shown in the following manner. At (*a*), in Fig. 3; a muscle was placed in a solution composed of 1 c.c. $\frac{m}{8}$ KCl + 9 c.c. $\frac{m}{8}$ Na-oxalate. After one minute (at *b*) this solution was removed and the muscle was exposed to the air. By this the muscle was immediately thrown into a series of powerful contractions lasting for about three minutes. The control muscle was placed in 1 c.c. $\frac{m}{8}$ NaCl + 9 c.c. $\frac{m}{8}$ Na-oxalate. After one minute

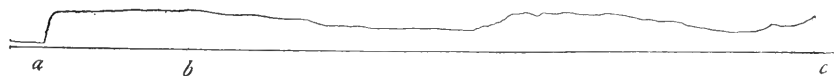


FIGURE 4.—At (*a*) the muscle is placed in 10 c.c. Na oxalate. After 1 minute (at *b*) the muscle is exposed to the air.

(Fig. 4, *b*) this solution was removed and the muscle exposed to the air. It will be noticed that extremely feeble contractions occurred which were somewhat intensified after two minutes. These experiments were also made with Na-fluoride, Na-carbonate, Na-citrate,

and Na-tartrate. Even as little as $\frac{1}{2}$ c.c. KCl added to $9\frac{1}{2}$ c.c. Na-oxalate greatly increased the contact reaction.

Experiments were also made in which the Na-oxalate was mixed with water, or with $\frac{m}{8}$ solutions of urca or cane sugar, but the mere dilution of the oxalate with these substances had practically no effect.

CONCLUSIONS.

1. If the calcium salts in the muscle are decreased the efficiency of the K-ions to increase the tone of the muscle is increased.
2. If the K-ions in the muscle are increased, the efficiency of such salts as Na-oxalate and Na-citrate to cause contact irritability is increased.
3. The contact irritability depends (as Loeb suggested) on the disturbance of the normal ratio of salts in the muscle. Perhaps it is the disturbed ratio between the potassium and the calcium salts which makes the contact reaction possible.

THE FORMATION OF FILM ON HEATED MILK.¹

BY LEO F. RETTGER.

(Research Scholar of the Rockefeller Institute.)

[From the Sheffield Laboratory of Physiological Chemistry, Yale University.]

IN the course of a series of experiments on the liberation of volatile sulphide from milk on heating,² I had occasion to make a number of observations upon the production of films on heated milk. Comparatively little attention has been directed to this subject. Hoppe-Seyler³ early assumed that the film consisted of lactalbumin and caseinogen. Halliburton⁴ states that it is probably in part due to the coagulation of lactalbumin, which carries a little caseinogen and fat to the surface. The same idea is presented in Schaefer's text-book of physiology.⁵ Harris,⁶ on the other hand, claims that the "skin" is precipitated caseinogen. "But by adopting Ringer's view," he continues, "we may say the heat has caused a certain amount of the phosphates (calcic phosphate) to be precipitated, and that has united with the caseinogen to form a solid pellicle." In Hammarsten's text-book of physiological chemistry the statement occurs that the film consists of coagulated caseinogen and lime salts.⁷

Recently Jamison and Hertz⁸ published the results of some experimental observations made on the film or "skin" of warmed milk, and of other proteid solutions. They hold that the formation of film on milk depends upon the presence of globules of fat or similar emulsifying substances. The nature of the suspended matter (milk-fat, cedar

¹ This research was carried on with the aid of an appropriation from the Rockefeller Institute for Medical Research.

² RETTGER: This journal, 1902, vi, p. 450.

³ HOPPE-SEYLER: Archiv für pathologische Anatomie und Physiologie (Virchow), 1859, xvii, p. 420.

⁴ HALLIBURTON: Text-book of chemical physiology and pathology, 1891, p. 583.

⁵ SCHAEFER: Text-book of physiology, 1898, i, p. 126.

⁶ HARRIS: Journal of anatomy and physiology, 1895, xxix, p. 192.

⁷ HAMMARSTEN: Text-book of physiological chemistry (translated by Mandel), 1900, p. 386.

⁸ JAMISON and HERTZ: Journal of physiology, 1902, xxvii, p. 26.

oil, paraffine, etc.) forming the emulsion is, according to them, immaterial. The kind of proteid in solution is likewise immaterial; and finally, exposure of the milk to the influence of unsaturated air is essential. In other words, drying is a necessary condition in the formation of a film.

Soon after the publication of the last named paper, there appeared a communication by W. A. Osborne¹ on caseinogen and its salts. This author, contrary to Jamison and Hertz, holds that a fat-free solution of calcium or magnesium caseinogenate develops a film on heating, and that therefore fat is not necessary in this process. He agrees, however, with the previous authors that the nature of the proteid matter is immaterial. "When solutions of magnesium or calcium caseinogenate are warmed, an emulsion of free caseinogen is produced by hydrolytic dissociation, which may be sufficient to cause the phenomenon in question."

It is the purpose of this paper to discuss some of the conditions of film-formation, upon the basis of further experimental observation.

The nature of the proteid matter which goes to make up the film is immaterial, as Jamison and Hertz have already shown. This statement, however, requires to be modified to a certain extent. For example, egg albumin solution was made to yield a film on heating, after the addition of a sufficient quantity of dilute lime-water to make the proteid solution (1 to 2 per cent) slightly alkaline, and lessen its tendency to coagulate when heated. On warming at about 85° C. a delicate film was formed. Below this temperature no "skin" was visible. Milk, on the other hand, begins to develop a film at a temperature as low as 40-45° C. The solutions of egg albumin were heated both with and without added portions of fat (cod-liver oil, etc.). A solution of calcium caseinogenate containing fat will also form a film much more readily than one of egg albumin. This difference is evidently due to the nature of the proteids, the egg albumin being more deficient in the process of film formation than caseinogen.

Not only will different proteids form a "skin" on heating, but the carbohydrate glycogen acts similarly.² On heating an almost saturated aqueous solution of glycogen, at 80° C. for 40-45 minutes, a film became quite apparent, but redissolved on cooling.

Numerous observations have indicated that fat facilitates film-forma-

¹ OSBORNE, W. A. : Journal of physiology, 1902, xxvii, p. 388.

² This fact was first observed by CHITTENDEN, in his study of the glycogen in *Pecten irradians*. American journal of science, 1875, x, p. 26.

tion. Further, different kinds of fat will do this, especially rancid cod-liver oil. This behavior, therefore, is not peculiar to milk fat. That fat is essential, however, to the production of film, as Jamison and Hertz claim,¹ is not borne out by my observations. Fresh milk was extracted in the Taylor extraction apparatus² with ether until free from fat (five or six days), as chemical and microscopic tests showed. The ether was removed by warming, and by a current of air. This fat-free milk developed a visible film on heating at 65° C. for thirty minutes. On the application of more heat, the film became easily apparent. It inclosed no fat, as was seen by staining with osmic acid, and microscopic examination. In water the film quickly sank to the bottom; it also sank in the milk when the latter was agitated.

A pure solution of calcium caseinogenate yielded a film on heating. The material was prepared by precipitating the caseinogen of fresh, skimmed milk with dilute acetic acid, filtering, redissolving the precipitate in a weak solution of sodium hydrate, and again precipitating with acetic acid. This purification was repeated eight or nine times, until the caseinogen was entirely free from fat. The final precipitate was dissolved in a small quantity of lime-water. On filtering, a solution of calcium caseinogenate which was practically neutral, and entirely fat-free, was obtained. It contained two per cent of the proteid, and was strongly opalescent. On warming, it became opaque. With acids it was precipitated; also with rennin. In short, it had the typical properties of a pure solution of calcium caseinogenate. When this solution was warmed at 75° C. film formation became quite apparent within thirty minutes. The same solution diluted with an equal volume of water also gave a distinct film, while in weaker solutions, film formation was scarcely noticeable.

My experience does not agree with the statement of Jamison and Hertz that exposure of the heated milk to the drying influence of air is necessary in the formation of a "skin." Film formation was quite apparent in vessels sealed air-tight and heated to 60° C. or above. These observations were made at different times, and under varying conditions. For example, self-sealing beer bottles of a capacity of 400 c.c. were employed. At the outset, 100 c.c. of fresh milk were introduced into the bottle, and the latter stoppered (air-tight) and immersed in a heated water bath. The temperature of the bath and

¹ JAMISON and HERTZ: *Journal of physiology*, 1902, xxvii, p. 26.

² This journal, 1899-1900, iii, p. 183.

the time within which a film became apparent, were recorded. In less than twenty minutes a distinct "skin" occurred at a temperature of about 98° C. This film was more or less wrinkled and tenacious, and in every way resembled ordinary milk film. To demonstrate whether the air space (300 c.c.) remaining in the sealed bottle might account for this "skin" production, varying quantities of milk were used. Similar results were obtained in every case. Even when the air space was less than 30 or 40 c.c. the film still developed. Furthermore, if the air in the bottle was replaced by hydrogen or carbon dioxide,¹ and the bottle was sealed, a film of the usual appearance and texture was obtained.

These facts make it quite evident that neither air nor drying is absolutely necessary for film formation. To substantiate these results further, different quantities of milk were heated in stout glass cylinders of 25 c.c. capacity, which were firmly closed with rubber stoppers. A film was formed during sixty minutes of heating, at 60° C. It was not very tenacious, although quite apparent. With increased temperature, the "skin" became more abundant. It enclosed considerable fat, and was characteristic in appearance, being very tenacious and slimy.

It must be noted that surface evaporation is conducive to film formation, but it is not essential. Fresh milk begins to form a "skin" at 40° C. when exposed to the atmosphere. At 50–60° C. the film is developed in considerable quantity. It is insoluble in pure water, but soluble in lime-water. Above 60° C. the films were so changed as to be insoluble in lime-water. The high temperature had evidently coagulated the proteid in the film.² During the heating the bottles or cylinders were moved as little as possible, since agitation of the milk greatly lessens the tendency to distinct film formation.

A third method employed was that of heating the milk in a flask

¹ HOPPE-SEYLER had observed that film-formation took place when the air was removed by passing a continuous current of carbon dioxide through the milk, during the heating, in an open vessel. *Archiv für pathologische Anatomie und Physiologie* (Virchow), 1859, xvii, p. 420.

² These observations are in accord with the view presented in a previous paper (RETTGER: *This journal*, 1902, vi, p. 450), that the formation of a film on milk is due to changes in the composition of the milk, rather than a purely physical change. The caseinogen being in combination with calcium in fresh milk, is not coagulated by heat until it has been liberated as free caseinogen (accepting the view that the proteid in the film is free caseinogen), in which state it is coagulable by heat, and therefore undergoes more or less change.

which was closed with a stopper possessing a small slit. On cooling, the slit was sealed with paraffine, so as to exclude the air. The same results were obtained as before. Milk which has been covered with paraffine fails to produce a film on heating. This seems to indicate that surface tension plays a very important part in the process.

In composition, the ordinary film on milk is almost wholly proteid and fat. That the proteid is not lactalbumin, as a number of writers have claimed, is shown by the fact that film continues to be formed almost indefinitely after each removal of the "skin" already formed; it yields a quantity, therefore, too large to consist of lactalbumin, as the percentage of the latter in milk is very small. That the proteid is probably caseinogen, is shown by several facts. First, when the film is formed at a temperature under 60° C. it is soluble in lime-water. This points to a formation of soluble calcium caseinogenate. From this solution, the caseinogen is again precipitated by rennin. Second, a pure solution of calcium caseinogenate develops a film, with or without fat; the film is likewise insoluble in water, but soluble in dilute lime solution, except when coagulated. Lastly, the amount of caseinogen in milk is comparatively large, and can readily account for the continued formation of new film after the removal of the old.

The suggestion of Osborne¹ that the proteid in the film is free caseinogen which has been separated from its combination with the calcium, by a process of hydrolytic dissociation, is very plausible. This view is especially supported by the fact that when a pure solution of calcium caseinogenate is warmed to 40° C. the solution becomes quite opaque. On cooling, the original opalescence is again acquired. After heating above the coagulating temperature, however, the increased turbidity becomes permanent. The turbidity is in all probability due to the separation of free caseinogen, which being insoluble in water, is suspended in the form of minute particles. In the production of the film, these particles are carried to the surface, and there coalesce to form the characteristic pellicle. The fat which is present naturally tends to carry these particles to the surface; but, as stated above, this process is not fundamentally dependent upon the agency of the fat.

In conclusion, the following brief summary seems appropriate:—

1. The formation of film on heated milk is dependent upon proteid in the milk.
2. This proteid is caseinogen.

¹ OSBORNE, W. A.: *Journal of physiology*, 1902, xxvii, p. 388.

3. The presence of fat facilitates film formation, but is not essential.

4. While surface evaporation facilitates film production, it is not necessary.

Again I am indebted to Prof. Lafayette B. Mendel for his assistance in the work upon which this paper is based.

EXPERIMENTS TO DETERMINE THE POSSIBLE ADMIXTURE OR COMBINATION OF FAT OR FATTY ACID WITH VARIOUS PROTEID PRODUCTS.¹

BY E. R. POSNER AND WILLIAM J. GIES.

[From the Laboratory of Physiological Chemistry of Columbia University, at the College of Physicians and Surgeons, New York.]

IN one of the recent papers from this laboratory on the qualities of connective tissue mucoïd, attention was drawn to the "lack of particular uniformity in percentage composition" of osseomucoïd preparations. Since the analyzed products had been made with the greatest care, we were led to the deduction "that the mucin substance of bone varies in composition just as does the glucoproteïd from other sources . . . a conclusion not only in accord with our analytic results, but in harmony, also, with the deductions drawn under similar conditions, for other tissues and products, by various observers."²

Later, and for the same reasons, we came to identical conclusions regarding tendomucoïd.³ In discussing the suggestion of Chittenden and Gies,⁴ that possibly "mucin obtainable from tendon is prone to carry with it a certain amount of some other form of proteïd matter which the ordinary methods of purification are not wholly adequate to remove," we indicated that there is "no longer any reason to believe that proteïd impurity is responsible for the observed variations." It was further stated, at that time, that "we know of no other substance in tendon which would resist the washing treatment and, by mechanical admixture or chemical combination, account for the orderly variations observed in the analytic series."

After the latter account of our experiments had gone to the printer,⁵ Nerking's paper on "fat-proteïd compounds" reached us. His results

¹ POSNER and GIES: Proceedings of the American Physiological Society, This journal, 1902, vi, p. xxix.

² HAWK and GIES: This journal, 1901, v, p. 415.

³ CUTTER and GIES: *Ibid.*, vi, pp. 167 and 169.

⁴ CHITTENDEN and GIES: Journal of experimental medicine, 1896, i, p. 194.

⁵ CUTTER and GIES: *Loc. cit.*, p. 169 (foot-note).

and views made it seem possible that the variations noted in analytic results for mucoids as well as for other proteids, may have been due to combinations or intimate admixtures of the proteid substance with fat or fatty acid.¹

Recalling Dormeyer's² physical explanation for the retention of that portion of fat which can be removed from tissues only after their digestion, Nerking suggested that there is quite as much reason for concluding that such fat is chemically united in the tissue as that it is held mechanically, and, therefore, that it is removable with difficulty merely because of such intimate combination. Bogdanow's³ observation, that the fat obtained in the later tissue extracts contains an increased proportion of free fatty acid, might seem to give strength to such a view, were it not for the probability that the increased amount of free fatty acid under such circumstances results by hydration of fat in the long-continued extraction process in boiling ether.

That fatty or fatty acid radicles are combinable with proteid is clearly evidenced in the example of the so-called lecithalbumins,⁴ which do not yield their fatty radicles to ordinary extraction with ether, but can be broken up into fatty and non-fatty matter by appropriate methods.

Obtaining results which seemed to point to the conclusion that blood serum contains combined fat, non-extractable with ether until after digestion in pepsin-hydrochloric acid, Nerking, at Pflüger's suggestion, looked for similar combinations in various proteid products as they are now commonly prepared.

His results indicated that several proteid substances, which had been prepared and purified by the usual methods, contained varying amounts of fat or fatty acid in close combination. Further, this fatty radicle could be broken off and determined quantitatively by Dormeyer's method. No such combination with ovomucoid was shown, but about three per cent of extractive matter was separated from sub-maxillary mucin, among other products. Most of the proteids examined gave negative results. Albuminoids were not studied.

The quantities of substance extracted, and the amounts of extract obtained in the process, were comparatively small in each of the posi-

¹ NERKING: *Archiv für die gesammte Physiologie*, 1901, lxxxv, p. 330.

² DORMEYER: *Ibid.*, 1895, lxi, p. 341; 1896, lxxv, p. 102.

³ BOGDANOW: *Ibid.*, 1896, lxxv, p. 81; 1897, lxxviii, p. 408.

⁴ A résumé of the literature concerning these bodies is given by COHNHEIM: *Chemie der Eiweisskörper*, 1900, p. 203.

tive cases. When the ordinary unavoidable sources of error in work of this kind are kept in mind it is difficult to lay very much stress upon extractive quantities as slight as those obtained in Nerking's experiments. It should be noted, further, that in no case was more than one sample of each particular proteid analyzed.

Nerking does not make it clear to the reader of his paper that his products were given the great care in preparation, particularly the extended extraction in hot alcohol-ether, which is necessary for their complete purification. He does not state that he was careful to use anhydrous ether, nor, indeed, that the samples of ether he employed had even been distilled by him previous to their use. Possibly he was not certain, therefore, that the extractive fluid itself would not sometimes yield residual matter on evaporation. He states nothing regarding the quantity of fatty material contained in the samples of enzyme used in his digestions. Preparations of pepsin such as he employed contain appreciable proportions of ether-soluble material.

Toward the end of his paper Nerking himself comments on the obvious weakness of his experimental evidence on the existence of "fat proteid compounds." He adds, also, that all his efforts to effect special combinations of proteid with fat have resulted negatively.

With such doubts in our minds as were raised by the omissions above referred to, and at the same time appreciating the suggestiveness of Nerking's results, particularly in connection with the mucoids, we set to work to ascertain the facts regarding the proteids referred to below.

PROTEID PRODUCTS INVESTIGATED.

Preparation. — All of the proteids worked with in these experiments, with the few exceptions to be noted, had been prepared and purified very carefully by improved or accepted methods for special research in other connections, some of the data of which have already been published. This fact is emphasized at this point to show that such results as were obtained in these experiments were not dependent on unusual care in this particular instance, in the separation of the proteids, but are typical for these substances as we are accustomed in this laboratory to prepare them.

Method of estimating extractive substance. — Care was taken to follow Nerking's general extractive procedure. The substance, dried at 100–105° C. to constant weight, was extracted for fifteen to twenty days continuously in a Soxhlet apparatus with anhydrous ether pre-

pared in bulk by us and freshly distilled in glass apparatus before use. On evaporation, large quantities of the ether completely disappeared without leaving a residue.

After preliminary extraction, the proteid was digested in a moderate excess of 0.2 per cent hydrochloric acid containing 0.8 gram of commercial pepsin scales per litre. Digestion in this fluid was rapid and complete. This quantity of the pepsin preparation (0.8 gm.) contained 2 to 4.8 milligrams of extractive material. After the digestion the extractions were conducted as in Dormeyer's method.

All ether extracts, those obtained before as well as after digestion, were filtered, the papers thoroughly washed with ether and the washings added to the main filtrate. Separation of the ethereal extract from the fluid digestive mixture was always made exactly, in a separatory funnel. There was no tendency to persistent emulsion at this point in any of our experiments. The amount of indigested matter was at most very slight, even with the mucoids.

The ether extracts, after filtration, were evaporated in vacuo in small beakers. Care was taken entirely to exclude dust particles after filtration. A very small amount of water was left behind on evaporating the ether which had been in contact with the acid fluid. The amount of solid matter dissolved in it must have been very slight in absolute quantity, although forming an appreciable proportion of the weight of the extract. See table, pages 338 and 339.

Mucoids. — It has been known for a long time that a certain amount of ether-soluble matter is admixed with connective tissue mucoid when the latter is first precipitated from its solution in alkali by acids.¹ The difficulties in the way of removing this admixture have been appreciated by various observers, but no one has determined the chemical nature of the extractive substance. These glucoproteid products therefore appeared to offer particularly interesting objects for study in this connection also.

Tendomucoid. — Our mucoids from tendon were prepared for the experiments recently described by Cutter and Gies.² We used samples of their analyzed preparations Nos. 1 to 5 inclusive. Our extractive results were practically negative for each of these.

A portion of preparation No. 1, which by accident had been left in the air-bath for a few days — a somewhat longer period than was necessary to carry it down to constant weight — had become slightly

¹ LOEBISCH: *Zeitschrift für physiologische Chemie*, 1886, x, p. 58.

² CUTTER and GIES: *Loc. cit.*

brownish (oxidized?) just as filter paper does, for example, under similar circumstances. On extracting this material the ether became yellowish at first, then reddish yellow in color.¹ The extracted substance was very slight in quantity, however, the high tinctorial effect having suggested a greater amount of solid matter in solution than was actually found.²

A sample of the same preparation when dried to constant weight in vacuo, instead of in the air bath, gave essentially the same negative results. Of course, no pigment was developed.

The results with purified tendomucoid having been negative, we determined next the amount of extractive matter in the crude material, which various observers, as we have already noted, have found it very difficult to remove in the purification process. For this purpose we used a sample of crude tendomucoid prepared originally for digestive experiments now in progress. This product was obtained in the usual way from the Achilles tendon of the ox. After its first precipitation from lime-water the substance was washed free of acid, then partly dehydrated in 50 per cent alcohol and dried in the air in thin layers on plates. 10.8 grams of this finely powdered product, in spite of the treatment with alcohol in its preparation, yielded 0.3 gram of extractive matter, a large part of which persisted in the substance even after two weeks' extraction.³

The extractive matter thus obtained was yellow in color and oily in consistency, but did not contain any fat crystals. Even when dissolved in ether and allowed to evaporate spontaneously at room temperature, crystals failed to develop. That it contained fat, however, was shown by the yield of fatty acid. It did not contain cholesterin or lecithin in sufficient quantity to respond to the familiar tests.

Our result in this connection emphasizes the need of thorough extraction in the purification of tendomucoid.

¹ The previous extracts were colorless. So were all subsequent ones except that from crude mucoid.

² That the usual treatment in an air-bath at 100-110° C. for the removal of water from proteids is an unsatisfactory method has long been recognized. Such results as the above, which indicate gradual decomposition, also emphasize the desirability of an improved method of drying proteid products for analysis.

³ This fact may have been due to the compactness of the powder particles, since the product had been dried before it was completely dehydrated. It was not light and fluffy, as is the dry, purified, dehydrated mucoid. All of this extractive substance, it will be remembered, can be eliminated from the freshly precipitated mucoid without the aid of the digestive process.

Osseomuroid and chondromuroid.—These preparations had been analyzed by Hawk and Gies.¹ The former was their preparation No. 6; the latter, preparation "b." Like the tendomuroid, these products were found to be free from fatty material.

Albuminoids.—Each of our albuminoid products was prepared by improved method. All were found to be practically free from extractive material.

Collagen.—One sample of collagen from the femur of the ox had been made by us from ossein shavings for other experiments not yet reported. Osseomuroid, etc., had been removed with lime-water and the albumoid² eliminated by digestion in alkaline trypsin solution.³ A sample of tendon collagen from the Achilles tendon of the ox had been made in the same way, for the same purpose, and was available for these experiments.

Gelatin.—Products prepared from bone, for other experiments in progress for some time, were used. They were made from ossein shavings obtained from the rib and the femur of the ox, after removal of the muroid and albumoid as above. The ligament gelatin used by us was analyzed by Richards and Gies.⁴ Through the kindness of the writer's former colleague, Dr. W. G. Van Name, we were able, also, to use samples of two of his preparations of tendon gelatin—C and D.⁵

Elastin.—Our samples of elastin were prepared and analyzed by Richards and Gies.⁶ Their preparations Nos. 7 and 8 were used.

Simple proteids.—These also gave practically negative results in the two experiments with purified products.

Globulin.—We used a sample of cocoa edestin obtained by Kirkwood and Gies⁷—their preparation No. 5. The endosperm of the cocoanut, from which this preparation of edestin was made, contains large proportions of fat and fatty acids, a condition particularly favorable to admixture or combination with proteid, if such had occurred.

Alkali albuminate.—This product had been made by Fried and Gies⁸ from a mixture of myosin and muscle "stroma substance." It

¹ HAWK and GIES: *Loc. cit.*

² HAWK and GIES: This journal, 1902, vii, p. 340.

³ EWALD and KÜHNE: Jahresbericht der Thier-Chemie, 1877, vii, p. 281.

⁴ RICHARDS and GIES: This journal, 1902, vii, p. 128.

⁵ VAN NAME: Journal of experimental medicine, 1897, ii, p. 124.

⁶ RICHARDS and GIES: *Loc. cit.*, p. 104.

⁷ KIRKWOOD and GIES: Bulletin of the Torrey Botanical Club, 1902, xxix, p.

had not been thoroughly extracted with ether in the purification process.

Commercial products. — These substances were dried egg albumen, Witte's peptone, somatose, and chloralbacid. An appreciable quantity of extractive matter was separable from the albumen, but the proportion of such substance obtained from it was not as great as that from crude mucoid.

DISCUSSION OF RESULTS.

The table on pages 338 and 339 summarizes the data obtained in these experiments. It will be observed that the figures for composition of the purified products agree with the accepted average data for each class of substances. Further, it is seen that the absolute amounts of extractive substance are very slight — so minute, in fact, as to be practically nothing except for the crude products with their usual extractive impurities. The influence of ordinary, unavoidable defects of manipulation on such small quantities of residual substance is obvious.

The perceptible decrease in the weight of many of the extracts during the drying process in the air-bath might be interpreted as indicating a loss of volatile fatty acid. This decrease, however, is seen to be very slight in absolute amount. It is much more probable that the loss was water only. The small beakers in which the ethereal extracts were evaporated were light in weight but of a capacity of 80 c.c. While even this size was somewhat disadvantageous as far as drying and weighing were concerned, smaller vessels could not have served very well in other respects. It is probable that, in their stay in the desiccators over sulphuric acid, not all of the moisture was removed from them. In the air-bath it was, of course, driven off and the total weight thereby reduced somewhat.

CONCLUSIONS.

We conclude from the data of these experiments that the above proteids of the simple, compound and albuminoid types, which were prepared by the best methods now in use, are not "fat-proteid compounds."

It is obvious, also, that these substances bear no resemblance to products of the lecithalbumin type.

Preliminary extraction in ether, 15-20 days.			Extraction for 7 days in ether after digestion in pepsin — HCl.			Total extract.			
Extract dried over H ₂ SO ₄ 24 hrs.	Extract dried in air-bath at 100° C.		Extract dried over H ₂ SO ₄ 24 hrs.	Extract dried in air-bath at 100° C.		Dried over H ₂ SO ₄ 24 hrs.	Dried in air-bath at 100° C. 24-48 hrs.		
	24 hrs.	48 hrs.		24 hrs.	48 hrs.		Mgms.	%	Mgms.
Milligrams.						Mgms.	%	Mgms.	%
....	1.0	0.8	1.8	0.037
1.2	0.7	0.8	0.9	0.7	0.6	2.1	0.09	1.4	0.062
....	0.7	0.7	1.7	1.1	1.3	2.0	0.041
1.8	0.8	0.5	1.5	1.0	1.4	3.3	0.12	1.9	0.070
1.1	0.2	0.3	0.7	0.4	0.6	1.8	0.08	0.9	0.044
5.9	2.5	2.3	3.6	2.0	1.8	9.5	0.23	4.1	0.100
2.3	1.1	1.3	0.5	0.0	2.8	0.10	1.3	0.046
114.2	108.9	107.5	202.6	195.7	316.8	2.93	303.2	2.800
0.7	0.6	0.7	0.7	0.1	0.0	1.4	0.03	0.7	0.016
2.3	1.5	1.7	0.8	0.2	0.0	3.1	0.05	1.7	0.030
2.7	0.7	1.7	0.7	4.4	0.10	1.4	0.033
1.2	0.8	0.5	0.3	1.7	0.04	1.1	0.029
1.7	1.3	0.8	1.8	1.0	3.5	0.08	1.8	0.041
1.6	1.2	0.4	2.2	1.3	3.8	0.08	1.7	0.034
0.4	0.1	0.0	1.2	0.5	1.6	0.05	0.5	0.017
1.5	1.1	1.2	0.4	0.0	1.9	0.06	1.2	0.034
1.5	1.0	0.9	1.5	0.6	3.0	0.05	1.5	0.026
1.5	0.7	0.1	1.7	0.8	3.2	0.06	0.9	0.016
1.3	0.7	0.0	1.3	0.5	2.6	0.03	0.5	0.006
1.2	0.8	0.8	2.7	1.8	3.9	0.09	2.6	0.061
1.1	0.5	0.7	6.8	5.4	7.9	0.16	6.1	0.127
24.1	12.7	0.7	17.6	17.2	41.7	0.51	17.9	0.218
9.3	6.5	4.6	15.6	14.7	24.9	0.30	4.6	0.056
2.4	1.6	0.0	1.0	0.0	3.4	0.26	0.0
8.0	3.2	3.0	6.5	4.1	3.9	14.5	0.12	6.9	0.056

ON THE COMPOSITION AND CHEMICAL PROPERTIES OF OSSEOALBUMOID, WITH A COMPARATIVE STUDY OF THE ALBUMOID OF CARTILAGE.¹

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AT the time of the first announcement of the writer's discovery of glucoproteid among the constituents of osseous tissue,² it was pointed out that the method of preparing osseomuroid furnishes residual material particularly well adapted to the study of other organic substances in bone. This method, it may be recalled, consisted, briefly, in preliminary softening of superficial layers of bone by removing inorganic matter with dilute acid (*e.g.*, 0.2 per cent HCl). The softer tissue was next transformed into thin shavings by scraping it with a scalpel, and finally, after hashing the material and washing it free of acid in water, was extracted with dilute alkali, such as half-saturated lime-water. The residual product thus obtained naturally contained collagen, also any other albuminoid constituent possibly present in the tissue; other soluble proteid substances, such as lymph proteids or nucleo-compounds, having been eliminated. The weakness of the acid and alkali used in the process of preparing the ossein

¹ A preliminary account is given in the Proceedings of the American Physiological Society: This journal, 1902, vi, p. xxvii.

² GIES: Proceedings, *Ibid.*, 1900, iii, p. vii.

makes it very probable, further, that any albuminoid constituents other than collagen are entirely unaffected chemically by such extraction process.

These observations induced us to study the elastin-like substance of bone. This constituent has been referred to by several investigators. They have given us anything but a clear idea of its qualities, however. In this connection it was found desirable, also, to make a comparative study of the albumoid of cartilage, which has been referred to by various observers quite as indefinitely.

OSSEOALBUMOID.

Historical.—Numerous investigators have made chemical studies of osseous tissue. In their researches, the organic, proteid residue left behind after solution of the salts in acid, the so-called ossein, has usually been regarded as consisting entirely of collagen. Lymph proteids and nucleo-compounds have been recognized, however, and elastic fibres are admittedly present in normal bone and in ossein, though in comparatively small number.¹

Broesike² some years ago reviewed the data of microchemical study of osseous tissue, and published, also, the results of several experiments by himself, which led him to believe that keratin is among the normal bone constituents. The substance he called keratin was evidently located, in part at least, in the lining of the lacunæ and canaliculi. His conclusion that this substance was keratin was dependent on its seeming indigestibility, and, further, on its lack of solubility in various reagents in which keratin, also, is unaffected chemically.

Smith³ soon after, under Kühne's supervision, made it very evident that Broesike had fallen into a number of experimental errors, and that, as a consequence, the latter observer's chief chemical deductions were fallacious. Instead of finding ossein indigestible in pepsin-hydrochloric acid, for example, Smith clearly demonstrated, as several others seem also to have done before him, that very little solid matter remains after treatment of the organic elements of bone with an *active* enzyme solution. He observed, further, that such residual substance as is resistant either completely disappears, when subjected

¹ HALLIBURTON: Schäfer's Text-book of physiology, 1898, i, p. 111.

² BROESIKE: Archiv für mikroskopische Anatomie, 1882, xxi, p. 695.

³ SMITH: Zeitschrift für Biologie, 1883, xix, p. 469.

to the influence of a new pepsin-acid solution, or is converted into a slight proportion of nuclein-like material entirely different from the keratins.¹ Although Smith did not establish the identity of the substance which Broesike called keratin, his work suggested that the material was elastin.²

With nothing very definite on the point of chemical identity we therefore proceeded with our attempts to isolate sufficient material for analysis.

General method of preparation. — All our preparations were made from the femur of the ox. We have already indicated that the preliminary part of the preparation process consisted first in transforming bone into ossein shavings, then putting the shavings through a hashing machine and extracting the mucoid, nucleoproteids, etc., from the finely divided tissue.³

After this treatment, the alkali remaining in the shavings was removed by repeated washing in water. When this process had been completed the hash was heated in water in a large, agate-ware kettle until gelatinization of the collagenous elements was complete. In the later preparations the kettle was kept covered so as to elevate the temperature of the mixture to the highest point possible under the circumstances. When it was desired to renew the hydrating fluid, the mixture was at first strained through fine cloth or a sieve. When it became more finely divided, toward the later stages of the disintegration, filtration on a hard filter sufficed for ready separation of the solid matter. Conclusion of the gelatinization process was determined not only by the almost complete disappearance of fibrous structure from the residual flocks, but also by the nearly negative reaction of the filtered fluid with picric acid. Such slight reaction with this reagent as persisted after a few days' boiling was due undoubtedly to proteoses formed from the residual matter.

¹ The results of Smith's experiments are obviously in harmony with the fact that large quantities of bone are ordinarily digested in the alimentary tract of carnivora. We ourselves have witnessed the complete digestion of small pieces of fresh bone in a large proportion of normal gastric juice taken from a fistula in a dog, only a small proportion of nuclein-like material remaining undissolved.

² This has since generally been taken for granted. See Text-books of physiological chemistry by HALLIBURTON (1891, p. 493), GAUTIER (1897, p. 107), NEUMEISTER (1897, p. 454), and HAMMARSTEN (1899, p. 326).

³ This method was given in detail in the second contribution from this laboratory on the subject of the preparation of osseomucoid. This journal, 1901, v, p. 393.

The resultant product contained the elastin-like substance, which was purified in boiling alcohol-ether in the customary manner.

Modifications of, and additions to this method are noted below under each preparation.

Preparation No. 1.—Our first product was made by the general method just outlined. In this case the ossein shavings were boiled in water for 12 hours and the residue heated continuously in a flask over the boiling water of a bath for 328 hours—as long as the substance appeared to diminish in bulk. The final product was dehydrated, and extraneous matter removed, by treatment in alcohol and ether in the usual process of proteid purification.

The material thus obtained was light and fluffy, and grayish brown in color. The moist substance was lightly flocculent, dark brown, granular for the most part, but consisting in small degree of fibrous fragments—probably elastic material.¹ To our great surprise the supposedly pure product contained 76.32 per cent of ash, mostly calcium phosphate.² The ash-free substance contained the following:³

C	H
49.81%	6.68%

In pepsin-hydrochloric acid, samples of this product digested very readily, proteoses forming in good proportion.

It was very evident from these results that the soft ossein shavings, obtained after treatment with dilute acid as above, still contained considerable inorganic matter, which remained in part in the organic residue even after its complete disintegration in hot water.

Preparation No. 2.—The remaining substance of preparation No. 1, about 4 grams, was washed in 0.025 per cent hydrochloric acid re-

¹ The reader need hardly be reminded of the great difficulty in the way of absolute purification of residual tissue constituents, particularly when such products form a comparatively small proportion of the original structure. Products of the kind before us here, which are never dissolved, filtered, and precipitated, are very apt to accumulate dust particles, fragments of various extraneous matters, etc. The greatest precaution is insufficient to entirely prevent such adventitious admixture. In all of these preparations the greatest care was constantly taken to diminish such accidental adulteration, and before analysis was begun, each product was very thoroughly looked over for particles of foreign material.

² The ash was brick-red in color. The same color characterized the ash from all of these products—both from bone and cartilage. A fairly large proportion of iron was detectable in these inorganic residues.

³ The methods of elementary analysis used throughout this work were those in general employment already described by us: *This journal*, 1901, v, p. 403.

peatedly for a week, until only slight quantities of phosphate could be detected in the washings. After dehydration, etc., this product still contained 46.25 per cent of ash.¹ The physical condition of the previously dried material was doubtless unfavorable to complete elimination of the saline matter in the very weak acid used.

This product was found to be entirely insoluble in cold dilute potassium hydroxide, even when as strong as 1 per cent. No biuret reaction could be obtained in the filtrate after the substance had been frequently stirred with the alkali for about a day.

In dilute hydrochloric acid — 0.2 per cent or less — the substance diminished in quantity by reason of the solvent action on the admixed phosphate, but no biuret reaction could be obtained with the acid extract even after it had been in contact with the substance for twenty-four hours.

The preparation itself gave the Millon's, xanthoproteic, and biuret reactions very distinctly. The composition of the ash-free substance was as follows:

C	H	N
49.71%	6.62%	16.11%

Preparation No. 3. — This was obtained from several pounds of shavings which had been made in 0.5 per cent hydrochloric acid² and preserved during their accumulation in 10 per cent alcohol. After the removal of the mucoid the shavings had been kept extracting in large excess of 0.25 per cent potassium hydroxide for four months, for complete elimination of traces of mucoid and nucleo-compounds.

When the alkali had been washed out, the ossein hash was kept in boiling water ten hours daily for thirteen days. At first the hot water became faintly alkaline each time it was renewed, because of

¹ The persistently high proportion of ash in these two preparations brought to mind the old question of possible chemical combination between some of the inorganic and organic substances of bone. (Consult the discussion of this matter by DRECHSEL in Hermann's *Handbuch der Physiologie*, 1883, v, (1), p. 609). Our later results, however, as will be seen, do not offer the same indications as those of the first two preparations. From our later data it appears that there are only mechanical obstacles to the ready removal of the inorganic matter, and that, when these are overcome by more thorough acid treatment, the amount of ash is not much above that found associated with the average proteid from other sources.

² Shavings for the preceding preparations were made from bones treated with 0.2 per cent HCl. The shavings had been kept in 25 per cent alcohol before extraction of the mucoid.

liberation of mechanically held alkali on disintegration of the tissue pieces. This alkali had persisted in spite of the previous thorough washing. Finally, however, the warmed mixture was entirely neutral. The boiling process was continued much longer than appeared to be necessary merely to make certain that all collagenous matter had been transformed into soluble material.

In order to remove more thoroughly inorganic matter from the substance remaining after the boiling process, the product was repeatedly washed for ten days in cold hydrochloric acid of a strength increasing at first from 0.05 per cent to 0.2 per cent, and later decreasing to 0.05 per cent. Much phosphate was taken out in this way. A slight biuret reaction was obtainable in the washings with the 0.2 per cent hydrochloric acid. This was not obtained with the 0.1 per cent acid at first, although as the phosphate content diminished the residual proteid became more susceptible to the action of the acid and slight solution in 0.1 per cent acid finally occurred.¹

After purification in alcohol-ether, etc., 1.36 gram of substance remained. This preparation, in spite of the long-continued washing in acid just before dehydration, contained 5.85 per cent of ash. Samples of this substance gave the usual proteid color reactions and digested easily in artificial gastric juice. The digestive product was mostly proteose, after twenty-four hours at 40° C.

The analytic results for this preparation were as follows :

Carbon and Hydrogen. 0.1021 gm. substance gave 0.0576 gm. H₂O = 6.31 per cent H; 0.1030 gm. substance gave 0.1764 gm. CO₂ = 46.71 per cent C, and 0.0580 gm. H₂O = 6.30 per cent H.

Nitrogen. 0.1599 gm. substance gave 0.02413 gm. N = 15.09 per cent N.

Total Sulphur. 0.6440 gm. substance gave 0.0490 gm. BaSO₄ = 1.05 per cent S.

Ash. 0.1213 gm. substance gave 0.0071 gm. Ash = 5.85 per cent Ash; 0.2580 gm. substance gave 0.0151 gm. Ash = 5.85 per cent Ash.

Sulphur of the Ash. 0.2580 gm. substance left 0.0151 gm. Ash, which gave 0.0046 gm. BaSO₄ = 0.16 per cent S.²

¹ Note remarks on solubility, etc., of ligament elastin by RICHARDS and GIES : This journal, 1902, vii, p. 104.

² This amount of sulphur is not deducted from the quantity calculated for ash-free substance. The large amount of sulphur in the substance makes it probable that the SO₄ of the ash was derived by oxidation of organic sulphur. This applies equally well to all of our preparations, both from bone and cartilage.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.¹

					Average.
C	49.61	49.61
H	6.70	6.69	6.70
N	16.03	16.03
S	1.11	1.11
O	26.55

Preparation No. 4. — This product was obtained from shavings made about six months previously from bones treated with 0.5 per cent hydrochloric acid. The shavings were washed once in 0.1 per cent hydrochloric acid and thereafter kept in acidified 25 per cent alcohol until several pounds of material had been obtained. During the six months after removal of the mucoid the ossein hash was repeatedly washed in 0.3 per cent potassium hydroxide. Finally, after the alkali had been removed as usual, hydration was effected in boiling water, repeatedly renewed and made faintly acid with acetic acid. From this point the process of treatment was identical with that for preparation No. 3.

A marked physical difference between this and the former products was observed. The residual material, although quite resistant to the action of the boiling water, was somewhat gelatinous in appearance. Though divided into minute flocks, these were somewhat adherent, and tended to collect at the top of the hot water in a semi-gelatinous layer. This was easily broken up into flocks on stirring. The product was finally much diminished in bulk and appeared more soluble in dilute acids than any of the preceding preparations. We did not obtain sufficient for quantitative analysis. The residual substance gave the proteid color reactions. It appeared to be a transformation product resulting from the action of the acid in the boiling fluid during the process of hydrating the collagen, although, aside from differences in physical form and solubility, it was identical with the other products. It contained loosely-bound sulphur, was digestible, and did not yield reducing substance on decomposition with acid.

Preparation No. 5. — Ossein shavings were freshly prepared after treatment of the bones with 0.2 per cent hydrochloric acid. Several kilos of the material were made. While they were accumulating, the

¹ The substance was found to be entirely free from phosphorus in organic combination. Phosphate was the chief constituent of the ash.

shavings were kept in 0.1 per cent hydrochloric acid. This was frequently renewed. After elimination of the mucoid with lime-water, the shavings were washed free of alkali with very dilute acetic acid. The rest of the process was essentially the same as that for preparation No. 3.

The fluid poured off at first, after the hydration had been begun, was very faintly alkaline, showing, as in previous instances, that, in spite of the acid treatment, some of the lime-water was held unaffected in the tissue. This product appeared to be somewhat more soluble in 0.2 per cent hydrochloric acid than preparation No. 3. About one gram of purified substance was obtained.¹

The ash of this preparation amounted to 5.88 per cent. The analytic data obtained for ash-free substance were:²

C	H	S
50.57	7.17	1.17

Preparation No. 6. — The results of the ash analysis of our previous preparations made it very evident that more attention was necessary to the removal of phosphates. Although treatment of the residual substance with dilute acid was effective in removing most of the phosphate held in it, it was impossible to use sufficiently strong acid for the purpose at that point because of the solvent and transforming action of the same on the remaining proteid. It seemed desirable, therefore, to give still more attention to the removal of inorganic matter from the shavings in the first place.

A large quantity of hashed ossein made with 0.5 per cent hydrochloric acid, from which the osseomucoid had been removed and which had been under 0.25 per cent potassium hydroxide for eight months, was washed free of alkali in water and then thoroughly stirred with 0.8 per cent hydrochloric acid at intervals for a day. Much phosphate was removed in this process. The hash was given similar treatment in 0.6 per cent hydrochloric acid, with the same result. A third washing was made in 0.4 per cent hydrochloric acid. Thereafter the hash was washed for several days in 0.2 per cent acid

¹ It should be kept in mind, of course, that the quantities of substance finally obtained do not represent fully the amounts of osseoalbumoid in the tissue. A considerable proportion is transformed into soluble products with the collagen in the hydration process, as well as lost mechanically in purifying.

² Our determinations of phosphorus of this and subsequent preparations showed that there is none present in organic combination.

until only traces of phosphate were being removed. At this point the washings did not yield a biuret reaction.

After the acid had been thoroughly washed out, leaving in a readily soluble form¹ such traces of phosphate as might still be in the tissue, the usual hydration process was carried out. The final residual substance had a somewhat gelatinous appearance, just as in the case of preparation No. 4. In this instance, also, the initial hydration was made in the presence of a trace of acid which had not been thoroughly washed out. Only about 0.6 gram of substance was obtained in the process.

The amount of ash in this preparation had been reduced by the improved method to 3.07 per cent. Analytic percentage data obtained for the ash-free substance were:

C	H
50.45	7.24

A microscopic study was made in this connection of the changes in the ossein during the heating process. Samples were taken each day during the ten days that the boiling was continued. Each sample was placed in 70 per cent alcohol after it had been washed in water.

At the end of the first day in the boiling water the fibrous structure of the material still remaining undissolved was but little modified, but much granular matter was present in the hydration fluid. The fibrous structure gradually disappeared, however, and long before the completion of the hydration process practically nothing but small collections of granular matter represented the original structures. An occasional fragment of what appeared to be an elastic fibre could be detected, however.²

Preparation No. 7. — Shavings, which had been made seven months previously from ossein obtained in 0.5 per cent hydrochloric acid, were kept in 0.25 per cent potassium hydroxide until ready for use in these experiments. After most of the alkali had been removed with water the hash was washed for several days in hydrochloric acid

¹ This method of concluding the preliminary extractive process with acid had the special advantage, over the previous methods, of transforming tri-basic earthy phosphate into acid modifications. The washing with alkali alone naturally had little or no extractive action on the earthy phosphates, but, on the contrary, tended to convert residual phosphates of calcium and magnesium into fixed forms.

² See foot-note, page 343.

increasing in strength to 0.2 per cent. When the acid appeared to be removed by subsequent washing in water the hydration process was begun. The fluid soon acquired an acid reaction, however. This reaction persisted in several of the first warm washings.

The product soon became quite gelatinous. It was very resistant to the further action of the boiling water. Eventually nearly all of the substance went into solution, although the renewed fluids remained neutral. At the end of a week's boiling, daily for about ten hours, too little remained for quantitative analysis.

Preparation No. 8.—The results obtained with preparations Nos. 4, 6, and 7 indicated that the presence of acid, however little it might be in the fluid during hydration, tended to effect transformation into somewhat gelatinous material. It was evident that this substance was not gelatin. At the same time it was clear that it was different from the residue obtained in the absence of acid or in the presence of alkali. That the difference was mainly physical was indicated by the fact that the analytic results for the semi-gelatinous form were essentially the same as for that obtained without the influence of acid in the hydration process. It seemed best to avoid this unnecessary complication, and in this preparation it was accomplished.

About 3 kilos of shavings were freshly prepared from bones treated with 0.5 per cent hydrochloric acid. After removal of the mucoid with lime-water, as usual, the shavings were kept in 0.3 per cent hydrochloric acid for three weeks to remove inorganic matter. The acid was frequently renewed. At the end of this time only a trace of phosphate reaction was obtainable in the acid washings. The acid was very thoroughly removed by repeated washing in cold and warm water. The boiling process in large volumes of frequently renewed water continued for 112 hours. The moist material was flocculent, granular, cream colored, and had no gelatinous qualities.

The usual treatment with acid before boiling in alcohol-ether was omitted. 16.6 grams of purified product were obtained. The ash amounted to only 2.08 per cent. It had the usual brick-red color.

The analytic results for this preparation were as follows :

Carbon and Hydrogen. 0.2032 gm. substance gave 0.3640 gm. $\text{CO}_2 = 48.86$ per cent C, and 0.1251 gm. $\text{H}_2\text{O} = 6.89$ per cent H; 0.2035 gm. substance gave 0.3683 gm. $\text{CO}_2 = 49.36$ per cent C, and 0.1254 gm. $\text{H}_2\text{O} = 6.90$ per cent H.

Nitrogen. 0.4184 gm. substance gave 0.06573 gm. N = 15.71 per cent N; 0.2420 gm. substance gave 0.03803 gm. N = 15.71 per cent N.

Total Sulphur. 0.5012 gm. substance gave 0.0406 gm. $\text{BaSO}_4 = 1.12$ per cent S; 0.5050 gm. substance gave 0.0421 gm. $\text{BaSO}_4 = 1.15$ per cent S.

Total Phosphorus. 0.4008 gm. substance gave 0.0078 gm. $\text{Mg}_2\text{P}_2\text{O}_7 = 0.54$ per cent P.

Phosphorus of the Ash. 0.0174 gm. Ash gave 0.0100 gm. $\text{Mg}_2\text{P}_2\text{O}_7 = 0.33$ per cent P.

Ash. 0.4850 gm. substance gave 0.0102 gm. Ash = 2.10 per cent Ash; 0.4838 gm. substance gave 0.0099 gm. Ash = 2.06 per cent Ash.

PERCENTAGE COMPOSITION OF THE ASH-FREE SUBSTANCE.

							Average.
C	49.90	50.41	50.16
H	7.04	7.04	7.04
N	16.04	16.04	16.04
S	1.14	1.17	1.16
O	25.60

This preparation, after purification and drying, was found to be entirely insoluble in water, 10 per cent sodium chloride, 0.2 per cent hydrochloric acid, and 0.5 per cent sodium carbonate; but slowly soluble in 10 per cent hydrochloric acid and 10 per cent potassium hydroxide. Solution was more rapid in the alkali than in the acid. In all of the reagents except water and sodium chloride, complete solution took place speedily on boiling. Albuminates were formed in this treatment and could be precipitated on neutralization. Part of the sulphur in the substance could be split off on heating with potassium hydroxide and detected as sulphide with lead acetate. The larger proportion of the sulphur was closely united, however.

The substance gave the typical proteid color reactions distinctly and digested in pepsin hydrochloric acid, with a formation of albuminate and proteoses. A small proportion of an albumid-like residue remained undissolved. This was soluble in dilute alkali and insoluble in dilute acid. Peptone could not be detected — probably only traces had been formed from the small quantity of substance used in the test.¹ On decomposition with 2 per cent hydrochloric acid the product failed to yield reducing substance.

Preparation No. 9. — This was made by essentially the same process

¹ Compare the similar results obtained with ligament elastin by RICHARDS and GIES: This journal, 1902, vii, p. III.

as that for preparation No. 8. The original shavings, about 2 kilos, were washed in acid for about three weeks longer than those of the previous preparation, even after practically no more phosphate could be detected in the extracts. The acid was very completely washed out in cold and warm water before hydration was begun. The boiling process was discontinued at the end of eighty-two hours.

The physical properties of the product were identical with those of preparation No. 8.¹ Between 5 and 6 grams of purified substance were obtained. The ash amounted to only 2.76 per cent. It had the usual brick-red color.

This product was found to be identical, in qualitative chemical characteristics, with preparation No. 8. The results of its quantitative analysis are appended :

Carbon and Hydrogen. 0.1510 gm. substance gave 0.2710 gm. CO₂ = 48.95 per cent C, and 0.0944 gm. H₂O = 6.99 per cent H; 0.1520 gm. substance gave 0.2709 gm. CO₂ = 48.61 per cent C, and 0.0900 gm. H₂O = 6.63 per cent H.

Nitrogen. 0.2435 gm. substance gave 0.03847 gm. N = 15.80 per cent N; 0.2715 gm. substance gave 0.04317 gm. N = 15.90 per cent N.

Total Sulphur. 0.5042 gm. substance gave 0.0418 gm. BaSO₄ = 1.14 per cent S; 0.5050 gm. substance gave 0.0437 gm. BaSO₄ = 1.19 per cent S.

Ash. 0.4007 gm. substance gave 0.0108 gm. Ash = 2.69 per cent Ash; 0.4014 gm. substance gave 0.0114 gm. Ash = 2.84 per cent Ash.

PERCENTAGE COMPOSITION OF ASH-FREE SUBSTANCE.

							Average.
C	50.34	50.00	50.17
H	7.19	6.82	7.01
N	16.25	16.35	16.30
S	1.17	1.22	1.19
O	25.33

Conclusions from analytic data.—The summary on the next page shows at a glance the average results of all our elementary analyses. It also brings into comparison the figures for composition of typical preparations of keratin, elastin, collagen, and albumoid.

¹ Preparations Nos. 8 and 9 at this stage very closely resembled the similar products from cartilage to be described farther on.

SUMMARY OF ANALYTICAL RESULTS FOR PERCENTAGE COMPOSITION OF OSSEOALBUMOID.

Preparation.	Ash-free Substance.					Ash.
	C	H	N	S	O	
1	49.81	6.68	76.31
2	49.71	6.62	16.11	46.25
3	49.61	6.70	16.03	1.11	26.55	5.85
4
5	50.57	7.17	1.17	5.88
6	50.45	7.24	3.07
7
8	50.16	7.04	16.04	1.16	25.60	2.08
9	50.17	7.01	16.30	1.19	25.33	2.76
Average.						
1-7	50.03	6.88	16.07	1.14	25.88	
8-9	50.16	7.03	16.17	1.18	25.46	
1-9	50.07	6.92	16.12	1.16	25.73	
Albumoid ¹	50.46	7.05	14.95	1.86	25.68	
Albumoid ²	53.12	6.80	16.62	0.79	22.67	
Collagen ³	50.75	6.47	17.86	24.92	
Keratin ⁴	49.45	6.52	16.81	4.02	23.20	
Elastin ⁵	54.14	7.33	16.87	0.14	21.52	

¹ From cartilage. See page 357 of this paper.

² From the crystalline lens. MÖRNER: *Zeitschrift für physiologische Chemie*, 1894, xviii, p. 78.

³ From gelatin. HOFMEISTER: *Ibid.*, 1879, ii, p. 322.

⁴ From white hair. KÜHNE and CHITTENDEN: *Zeitschrift für Biologie*, 1890, xxvi, p. 291.

⁵ From ligamentum nuchae. RICHARDS and GIES: *This journal*, 1902, vii, p. 104.

The chemical qualities of the albumoid product separated from bone in these experiments indicate that the substance is neither a collagen, a keratin, nor an elastin. This may also be seen from the analytic figures. Unlike the collagens, it does not yield gelatin. It is readily digestible, whereas the keratins are indigestible. It contains an abundance of loosely united sulphur; elastins contain only slight quantities of sulphur,—some of them, no loosely bound sulphur at all. The properties of our product, while somewhat different, as we have said, approach to a certain extent those of the elastins of ligamentum nuchæ¹ or the aorta.² They appear to be identical for the most part with those of the albumoid of cartilage.³

Since all the albumoids are residual tissue constituents of variable qualities and composition, though of typical resistance to the action of solvents,⁴ it seems proper to classify the product we have obtained from bone as an elastin-like albumoid and to refer to it, therefore, as osseoalbumoid. We freely admit that, while our chemical knowledge of the albuminoids remains as slight as at present, such classification has the virtue of only temporary convenience.

No attempt has been made in these experiments to ascertain the exact location of osseoalbumoid in the tissue. It appears probable, however, that the substance is the same as that regarded as keratin by Broesike and which was found by him in the lining of the lacunæ and canaliculi. We are inclined to believe, also, that the elastic fibres of the bone, perhaps also elastic portions of blood-vessels in the Haversian canals, have contributed substance to our preparations.⁵ It is possible, of course, that the residual matter prepared by the method we have employed is composed of more than one substance, although the harmony in our analyses, of preparations made by a changeable process, indicates that the products obtained are not admixed to any appreciable extent with variable constituents.

The proportionate amount of osseoalbumoid in bone is small. It

¹ RICHARDS and GIES: *Loc. cit.*

² SCHWARZ: *Zeitschrift für physiologische Chemie*, 1894, xviii, p. 487.

³ MÖRNER: *Skandinavisches Archiv für Physiologie*, 1889, i, p. 234. See also page 357 of this paper.

⁴ COHNHEIM: *Chemie der Eiweisskörper*, 1900, p. 299.

⁵ Recent staining methods show that bone contains very little elastic material. See Abstract of MELNIKOW-RASWEDENKOW'S paper, in *American Medicine*, 1901, ii, p. 466.

appeared somewhat greater, however, than the quantity of the corresponding constituent of cartilage.¹

CHONDROALBUMOID.

The qualities of the albumoid obtained from bone were found to be so nearly the same as those ascribed to the albumoid in cartilage that a comparative study of the latter body appeared to be particularly desirable in this connection.

Historical. — It will be recalled that in his classical researches on the constituents of hyaline cartilage, Mörner² separated a product which he considered an albumoid. This body was a residual substance obtained from the tracheal cartilages of the ox after complete hydration of the collagenous elements in boiling water in a Papin's digester at 110–120° C.

The substance obtained in this way was entirely insoluble in 1 per cent potassium hydroxide, but slightly soluble in 5 per cent solution of the same reagent. It was readily soluble in boiling 0.1 per cent alkali. It digested completely, with a formation of albuminate, proteose, and peptone. It contained considerable loosely united sulphur, but did not yield reducing substance on decomposition with acid.³ Its resemblance to keratin and elastin in some respects, and its difference from them in others, made it necessary for Mörner to consider it a proteid of the indefinite albumoid type.

The quantities of albumoid obtained in Mörner's experiments were too small to offer favorable opportunity for elementary analysis. He transformed into albuminate such material as was available, however, for the sake of removing insoluble extraneous matter, and then determined the nitrogen content of the derived products. In two determinations the alkali albuminate made with boiling 0.1 per cent potassium hydroxide contained 15.87 per cent nitrogen; that made with boiling 0.5 per cent potassium hydroxide had 16.02 per cent. Neither of these results was for ash-free substance, the ash not having been determined. The nitrogen content, also not ash-free, of one preparation, made in boiling 0.5 per cent hydrochloric acid, was 15.43 per cent. Mörner concluded that the albumoid itself has a content of nitrogen ranging between 15 and 16 per cent.

¹ Further reference to osseoalbumoid is made on page 357.

² MÖRNER: *Loc. cit.*

³ Compare with the results of our analysis of osseoalbumoid, page 353.

Nothing further has been done to determine the characters of chondroalbumoid. When we recall that albuminates are products in which the proportion of nitrogen is usually different from its proportion in the substance from which the albuminates are derived, particularly when obtained with *boiling* reagents, it is obvious that Mörner's analytic results tell us very little about the composition of the original body.

The substance identified by Mörner was absent from the tracheal cartilages (the only ones examined) of calves. Mörner concludes, from this fact, that immature cartilage is essentially different from the mature form of the tissue in its lack of the albumoid constituent. This conclusion is based on only a few observations. If, however, it is found later to be correct, the fact that osseoalbumoid appears to be present in bone in greater proportion than in cartilage from the same animal would suggest that, in the development of bone from cartilage, the proportion of the albumoid constituent increases.

Method of preparation. — In these experiments we used the cartilaginous portion of the nasal septum of the ox. Several pounds of these pieces of typical cartilage, about ten inches long and three inches wide, were used. The outer membranes were removed, the pure cartilage put through a hashing machine, the resultant hash thoroughly washed in running water; mucoid, nucleo-proteid, etc., thoroughly eliminated in several extractions with dilute alkali after preliminary treatment with 0.1–0.2 per cent hydrochloric acid; and the alkali-free residue thoroughly hydrated in boiling water for several days under conditions identical with those for the preparation of osseoalbumoid. The final product was also extracted with 0.1 per cent sodium carbonate and 0.5 per cent hydrochloric acid in which the substance seemed to be entirely insoluble.

The physical appearance of the final products was practically identical with that of preparations Nos. 8 and 9 of the albumoid from bone. It accorded also with the appearance of the material described by Mörner.

Records of analysis. — After purification in boiling alcohol-ether, as usual, the following analytic results were obtained for the two preparations made by us:

Preparation A.

Carbon and Hydrogen. 0.1998 gm. substance gave 0.3542 gm. $\text{CO}_2 = 48.35$ per cent C, and 0.1200 gm. $\text{H}_2\text{O} = 6.72$ per cent H; 0.2008 gm. sub-

These preparations possessed the same reactions as those summarized by us on page 354 from Mörner's paper, and also those referred to in some detail in connection with preparations Nos. 8 and 9 of our osseoalbumoid. The reactions for loosely bound sulphur were, however, very much stronger for the cartilage preparations than for those prepared from the femur. On the other hand, sulphur obtainable from chondroalbumoid, on boiling with 2 per cent hydrochloric acid, appeared to be less in comparative tests than for the bone products.

The following summary brings into contrast the analytic averages for the albumoid products from both sources :

PERCENTAGE COMPOSITION OF ALBUMOIDS FROM CARTILAGE AND BONE.

Elements.	Chondroalbumoid.			Osseoalbumoid.
	Preparation A.	Preparation B.	Average A-B.	Average. Preparations 8-9.
C	50.02	50.90	50.46	50.16
H	6.97	7.14	7.05	7.03
N	14.94	14.96	14.95	16.17
S	1.85	1.86	1.86	1.18
O	26.22	25.14	25.68	25.46

Conclusions from the data of analysis. — The properties of this substance are found to be those ascribed to it by Mörner. That it is not exactly the same as osseoalbumoid is indicated by its higher content of sulphur and its considerably lower content of nitrogen. The larger proportion of sulphur obtainable from it on cleavage with alkali has already been referred to.

These differences are not sufficient, however, to prevent the conclusion that the two substances are closely related members of the same class of proteids.

The relative amount of the substance in cartilage appears to be less, as we have already said, than the proportion of osseoalbumoid in bone.¹

¹ For facts regarding location of albumoid in cartilage see MÖRNER'S paper, *Loc. cit.*

SUMMARY OF CONCLUSIONS.

1. Osseous tissue contains a residual proteid substance, obtainable after hydration of the collagen, which is neither keratin nor typical elastin, although it resembles the latter body.

This substance is present in bone in only comparatively small proportion, though apparently in greater relative quantity than the corresponding constituent of cartilage.

The average percentage elementary composition of the purest products was found to be as follows, calculated for ash-free substance:¹

C	H	N	S	O
50.16	7.03	16.17	1.18	25.46

The analyzed products were free from organic phosphorus.

The substance appears to be very similar to some of the albumoids, particularly to that from cartilage. It has therefore been termed osseoalbumoid.

No attempts have been made to ascertain its location in the tissue, but it appears to be identical with the substance referred to erroneously by Broesike as keratin and found by him in the lining of the lacunæ and canaliculi. It is possible, also, that the elastic fibres of the bone have contributed substance to the preparations.

2. Further investigation of the qualities of chondroalbumoid confirmed most of Mörner's conclusions regarding it.

In addition, its elementary composition has been determined, with the following percentage results for ash-free substance:

C	H	N	S	O
50.46	7.05	14.95	1.86	25.68

This product is likewise devoid of phosphorus in organic combination.

¹ Average of preparations Nos. 8 and 9, our purest products. See page 349.

IS ADRENALIN THE ACTIVE PRINCIPLE OF THE SUPRARENAL GLAND?

By T. B. ALDRICH.

PROFESSOR ABEL has recently attempted to show¹ that adrenalin, since it reduces Fehling's solution, is not identical with the native principle as found in the gland, since the former reduces Fehling's solution, while the latter does not.

This lack of conformity of reaction, he claims is explained by the fact that adrenalin is a reduced form of the native principle, since in the method used by me for the isolation of adrenalin, hydrogen sulphide, a reducing agent, was used. To substantiate this contention that the native principle does not reduce Fehling's solution, Abel cites one of his previous papers,² in which especial attention was given to this point, also other papers by v. Furth,³ Fraenkel,⁴ Moore,⁵ and Metzger,⁶ all of whom were unable to reduce Fehling's solution with their questionable products supposed to contain more or less of the native principle. The failure of these products as obtained by these investigators to reduce Fehling's solution might possibly be attributed to some disturbing substance which interferes with the reaction. It is probable that this assumption may be true in part, but it seems to me there are other reasons why these workers have failed to obtain the reduction just alluded to:

(1) In all of these preparations there is a possibility and a strong probability that partial oxidation of the native principle had taken place. Abel admits as much where he says,⁷ in speaking of v. Furth's

¹ ABEL, J. J.: Johns Hopkins Hospital Bulletin, 1901, vii, p. 337.

² ABEL, J. J.: *Ibid*, 1897, viii, p. 151.

³ VON FURTH: Zeitschrift für physiologische Chemie, 1898, xxiv, p. 142.

⁴ FRAENKEL: Wiener medicinische Blätter, 1896, xxvi, p. 409.

⁵ MOORE, B.: Journal of physiology, 1895, xvii, p. xiv.

⁶ METZGER: Zur Kenntniss der wirksamen Substanzen der Nebennieren, Dissertation, Würzburg, 1897.

⁷ ABEL: *Loc. cit.*, p. 338.

iron salt: "The possibility of an oxidation in the preparation of this compound is not denied." The autoclave product as obtained by Abel may be, and no doubt is, a partially oxidized product or, at least, a product fundamentally changed from the native principle, but still having some properties in common with it.

(2) It is a well known fact that the quantity of adrenalin, the native principle, in the gland, is very small—estimated all the way from 0.05–0.1 per cent, possibly more; therefore it is possible that too little of the native principle was present in the extracts of the various authors to obtain a perceptible reduction. For even adrenalin, the pure principle, requires about 0.5 mgm. (under conditions given below) to give a noticeable reduction, while with possibly disturbing substances, as is the case in the various extracts, it would presumably require more.

I consider the failure of the various workers in this field to obtain a product capable of reducing Fehling's solution due to a changed form of the active principle, or to using too small an amount of the extract. All the extracts were more or less active in raising the blood-pressure, to be sure, but when one considers the small amount necessary to produce a marked rise in blood-pressure (0.01 mgm. produces a number of millimetres rise) as compared with 0.5 mgm. (50 times as much)—the amount necessary to produce a noticeable reduction—the facts I have advanced seem still more probable. Neglecting all these more or less probable suppositions, we have the following to show:

(1) That the active principle obtained in the form of an extract without the use of an oxidizing or reducing agent will reduce Fehling's solution about proportionately to the amount of adrenalin present.

(2) That the mother-liquor from adrenalin does not reduce Fehling's solution except in comparatively large amounts.

(3) That adrenalin prepared without the use of a reducing agent will reduce Fehling's solution.

(4) That adrenalin, the copper-sulphate-reducing body, and the blood-pressure-raising body are identical.

PREPARATION OF AN EXTRACT OF THE GLAND THAT REDUCES FEHLING'S SOLUTION.

The finely divided glands were extracted in the usual manner with water heated to about 50–80° C. The united extracts were then boiled to coagulate extracted proteids, a little acetic acid being added

to facilitate this coagulation. After being freed from proteid as far as possible, the filtrate was evaporated in vacuo to a small volume, and then precipitated with 94 per cent alcohol which removed for the most part some amorphous bodies. The alcoholic solution could readily be decanted, as this precipitate, being gummy, adhered to the bottom and sides of the vessel. This alcoholic liquid was evaporated in vacuo again to a still smaller volume than at first, and again precipitated, this time with strong alcohol. A body similar to the one previously obtained with alcohol was thrown down, and the alcohol removed by decanting in the same manner as above. The last alcoholic solution was evaporated in vacuo and the resulting more or less colored liquid was used for the reduction tests with Fehling's solution. The method employed in obtaining this extract, Abel claims, precludes the occurrence of either reduction or oxidation, except in so far as the latter might be induced by exposure to air.

All the reduction tests given below were carried out as follows:

Five c.c. of the freshly prepared mixed Fehling's solution diluted with 50 c.c. of water, was placed in a casserole and brought to boiling. The reducing solution was then added, and the mixture boiled two minutes. When two or more milligrams of adrenalin were present in a solution the reduction was readily noticeable by inclining the vessel to one side without cooling. When, however, there was very little reduction it was necessary to allow the solution to cool and then decant carefully the liquid, whereby the faint reddish tinge caused by the reduced copper could be readily seen against the white background of the casserole. If the reduction was very slight, the color became more noticeable by allowing a drop of dilute acid to flow across the bottom of the vessel. By this last method the faintest trace can be detected, and by its use I have been enabled to detect as little as 0.5 mgm. of adrenalin in solution. The rapidity of reduction will depend naturally on the amount of reducing substance contained in the extract. Many extracts from different lots of glands were prepared according to the method given above, and each one promptly reduced Fehling's solution on boiling. We can draw but one conclusion from these facts: The extract as prepared above contains a body originally in the gland which reduces Fehling's solution on boiling. Now this body which causes this reduction may or may not be identical with adrenalin, which also reduces Fehling's solution. If, however, after the removal of adrenalin from this extract, it can be shown that what is left does not appreciably reduce this solution, it is certain

that this native principle and adrenalin are identical. This has virtually been done.

Five c.c. of extract prepared as above was diluted with H₂O and made up to 50 c.c. — this was placed in a burette for convenience in measuring.

The mixed Fehling's solution was placed in a casserole and heated to boiling, then measured portions of the diluted extract were added and boiled two minutes, eight tests being made in all, as follows :

5.0 c.c. gave very marked reduction.	0.2 c.c. gave faintest trace.
1.0 c.c. gave very marked reduction.	0.2 c.c. gave faintest trace.
0.5 c.c. gave marked reduction.	0.1 c.c. gave no reduction.
0.3 c.c. gave noticeable reduction.	

The amount of reduction shown by any extract or the amount of adrenalin obtainable from an extract will depend, to a certain extent at least, upon the freshness of the glands from which the extract is prepared.

REMOVAL OF ADRENALIN, OR THE NATIVE PRINCIPLE, FROM THE SUPRARENAL EXTRACT.

As in the preceding, 5 c.c. of the original extract was taken, and to it 10 c.c. of a saturated solution of pure sodium carbonate was added. A few drops of ether were added to facilitate the precipitation and, after agitating a little, set aside. The adrenalin was precipitated in a very short time. After allowing it to stand for two hours to be sure of as complete a precipitation as possible, it was filtered under suction, and the adrenalin washed first with water, then with absolute alcohol and ether in succession. The alcohol-ether wash fluid was caught separately. After allowing to dry thoroughly the adrenalin weighed 0.1633 gm. (There was, no doubt, a little lost, as it is practically impossible to wash it thoroughly without dissolving some.) This adrenalin was dissolved in a little water to which a few drops of hydrochloric acid was added and the solution made up to 50 c.c.

The reduction power of this solution was tested, as in the previous experiment, with the original extract, and with the same amount of Fehling's solution, same dilution, and boiling the same length of time (1-2 minutes). The results were the following :

0.5 c.c. gave very marked reduction.	0.2 c.c. gave uncertain result.
0.3 c.c. gave very noticeable reduction.	0.1 c.c. gave no reduction.
0.2 c.c. gave uncertain result.	

I will add that the rapidity of reduction depends on the strength or amount of adrenalin present. 0.5 c.c. of this strength of solution shows reduction in less than one minute, while 0.3 c.c. required a minute or more before it was noticeable.

MOTHER-LIQUOR FROM SUPRARENAL EXTRACT AFTER REMOVAL OF ADRENALIN.

The filtrate from adrenalin plus wash water (but not alcohol-ether wash liquid) was diluted to exactly 50 c.c. and its reducing power towards Fehling's solution also tested. This was carried out as given above with the following results :

5 c.c. gave no reduction after boiling 2 min.¹

10 c.c. gave no reduction after boiling 2 min.

35 c.c. gave no reduction after boiling 2 min.

ON THE IDENTITY OF ADRENALIN, THE COPPER-SULPHATE-REDUCING BODY, THE BLOOD-PRESSURE-RAISING SUBSTANCE, AND THE NATIVE PRINCIPLE.

It has been shown in another part of this paper that the removal of adrenalin from an extract of the suprarenal gland causes a diminution in its reducing power as shown toward Fehling's solution; in fact it has been shown that the adrenalin can be removed so completely that the filtrate from the same, even in comparatively large amounts, is not capable of reducing Fehling's solution to any great extent. (I will add at this point that the reducing power of the filtrate also depends upon the precipitant used in throwing out the adrenalin. When ammonium hydrate is used more adrenalin is obtained than when a saturated solution of sodium carbonate is employed, and in consequence the filtrate where the former is used contains less adrenalin, and therefore requires more of it to show a marked reduction of Fehling's solution.) Since in the previous

¹ Usually 10-20 c.c. of the mother-liquor reduced Fehling's solution perceptibly. Since, however, the amount of reduction depends on the quantity of adrenalin present, and since this amount varies considerably, we should expect to find that the amount of mother-liquor required to cause reduction would vary also. In the case cited above, 35 c.c. did not cause a perceptible reduction, which shows that the precipitation of adrenalin was very complete; in other cases I found that 10 c.c. would often give a faint reduction showing that more adrenalin remained in solution.

experiments adrenalin is shown to be the copper-sulphate-reducing body either when isolated or in the original extract, it remains to be shown whether or not the reducing body is identical with the blood-pressure-raising body as well, and therefore the latter also identical with adrenalin.

The following experiments were carried out to demonstrate the identity of the three substances:

Two concentrated aqueous extracts (A) and (B), prepared from two different lots of bovine suprarenal glands, and in the manner previously described, were employed for this purpose. Five solutions of each were used, viz.: —

- I. Containing 5 c.c. respectively of the original concentrated extracts (A) and (B), diluted with water to 100 c.c.
- II. Containing 188.5 mgms. of adrenalin (A extract); 158.6 mgms. (B extract) dissolved in acidulated water and made up to 100 c.c. respectively. The adrenalin in each case was the total amount obtained from 5 c.c. of the original concentrated extracts, 3 c.c. of a saturated solution of sodium carbonate being employed in each case to effect the precipitation. After allowing to stand for one hour, the precipitated adrenalin was collected on a filter and washed in succession with water, strong alcohol, and ether, and finally weighed.
- III. Consisting of the mother-liquors from (II) made up with water to 100 c.c., none of the wash liquids being allowed to mix with these.
- IV. Containing respectively 200 mgms. of adrenalin (A extract); 193.4 mgms. (B extract) dissolved, etc., as by (II). This adrenalin was also obtained from 5 c.c. of the original concentrated aqueous extract, 3 c.c. of ammonium hydrate (10 per cent) being employed to effect the precipitation. The precipitated adrenalin was treated further as by (II) and finally weighed.
- V. Consisting of the mother liquors from (IV) made up with water to 100 c.c., etc., as by (III).

BLOOD-PRESSURE EXPERIMENTS.¹

In these experiments chloretone was used as the anæsthetic, and sufficient time was allowed after each injection for the blood-pressure to assume its normal height. The solutions used in each case were made up from the stronger solutions previously alluded to, and were diluted for convenience and accuracy in measuring. The amount of dilution will be given under the proper heads below.

¹ This work was carried out through the kindness of Dr. Mogk.

Extract (A)

- (1) 1 c.c. of (I) diluted to 100 c.c. Each c.c. of this solution contains 0.0005 c.c. (0.5 mgm.) of the original concentrated extract. This amount produced a rise of 19 mm. of mercury.
- (2) 1 c.c. of (II) diluted to 100 c.c. Each c.c. of this solution contains 0.01885 mgm.; 0.6 c.c. of this solution contains 0.01131 mgm. and caused a rise of 12 mm. of mercury.
- (3) 10 c.c. of (III) diluted to 100 c.c. Each c.c. of this solution contains approximately 0.005 c.c. (5 mgms.) of the original concentrated extract, the greater part of the adrenalin having been removed, and 0.5 c.c., containing 0.0025 c.c. (2.5 mgms.), causes a rise of 17 mm. of mercury.
- (4) 1 c.c. of (IV) diluted to 100 c.c. Each c.c. of this solution contains 0.02 mgm.; 0.5 c.c. of this solution contains 0.01 mgm. and produced a rise of 12 mm. of mercury.
- (5) 10 c.c. of (V) diluted to 100 c.c. Each c.c. of this solution contains approximately 0.005 c.c. (5 mgms.) of the original concentrated extract, the greater part of the adrenalin having been removed, and 0.5 c.c. containing 0.0025 c.c. (2.5 mgms.) causes a rise of 12 mm. of mercury.

Extract (B).

- (1) 1 c.c. of (I) diluted to 100 c.c. Each c.c. of this solution contains 0.005 c.c. (0.5 mgms.) of the original concentrated extract and produced a rise of 25 mm. of mercury.
- (2) 1 c.c. of (II) diluted to 100 c.c. Each c.c. of this solution contains 0.01586 mgms. of adrenalin, and 0.5 c.c. containing 0.00793 mgms. produces a rise of 11 mm. of mercury.
- (3) 10 c.c. of (III) diluted to 100 c.c. Each c.c. of this solution contains approximately 0.005 c.c. (5 mgms.) of the original concentrated extract, the greater part of the adrenalin having been removed, and 0.4 c.c. contains 0.002 c.c. (2 mgms.) and produces a rise of 14 mm. of mercury.
- (4) 1 c.c. of (IV) diluted to 100 c.c. Each c.c. of this solution contains 0.01934 mgms. of adrenalin, and 5 c.c. contains 0.00967 mgms. and produces a rise of 13 mm. of mercury.
- (5) 10 c.c. of (IV) diluted to 100 c.c. Each c.c. of this solution contains approximately 0.005 c.c. (5 mgms.) of the original concentrated extract, the greater part of the adrenalin having been removed, and 0.5 c.c. contains 0.0025 c.c. (2.5 mgms.) and produced a rise of 10 mm. of mercury.

REDUCTION TEST WITH FEHLING'S SOLUTION.

These tests were made in the manner previously described, and are comparative.

Extract (A).

- (1) 0.5 c.c. of (I) gave a very positive reduction.
0.3 c.c. of (I) gave a very slight trace.
0.2 c.c. of (I) gave the merest trace.
- (2) 0.5 c.c. of (II) gave a very positive reduction.
0.3 c.c. of (II) gave a very slight trace.
0.2 c.c. of (II) gave no reduction.
- (3) 5.0 c.c. of (III) gave no reduction.
10.0 c.c. of (III) gave a faint reduction.
15.0 c.c. of (III) gave a very positive reduction.
- (4) 0.5 c.c. of (IV) gave a very positive reduction.
0.3 c.c. of (IV) gave a very faint reduction.
0.2 c.c. of (IV) gave the faintest trace of reduction.
- (5) 5.0 c.c. of (V) gave no reduction.
10.0 c.c. of (V) gave no reduction.
15.0 c.c. of (V) gave no reduction.
25.0 c.c. of (V) gave a trace of reduction.

Extract (B).

- (1) 0.5 c.c. of (I) gave a very positive reduction.
0.3 c.c. of (I) gave a slight trace of reduction.
0.2 c.c. of (I) gave the slightest trace.
- (2) 0.5 c.c. of (II) gave a positive reduction.
0.3 c.c. of (II) gave the slightest trace.
- (3) 5.0 c.c. of (III) gave no reduction.
10.0 c.c. of (III) gave no reduction.
15.0 c.c. of (III) gave a questionable reduction.
- (4) 0.5 c.c. of (IV) gave a very positive reduction.
0.3 c.c. of (IV) gave the slightest trace.
- (5) 5.0 c.c. of (V) gave no reduction.
10.0 c.c. of (V) gave no reduction.
15.0 c.c. of (V) gave no reduction.
25.0 c.c. of (V) gave a questionable reduction.

For convenience in comparison the results obtained above are tabulated on page 367.

In each case the amount used in this table for injecting or for the reduction tests was calculated back to the first 100 c.c. solution, and each quantity is therefore this portion of that solution.

In column (1) we have the results obtained from the original extract with all the inert material present; in columns (2) and (4) those obtained from the adrenalin gotten out of the same amount of extract (5 c.c.) as used in (1); in columns (3) and (5) those obtained

from the mother liquor after removal of most of the active principle, but which contained naturally a small amount of adrenalin.

Comparing column (1) with columns (2) and (4) we notice that its reducing power is approximately the same as these and that its blood-pressure-raising property, when everything is taken into consideration, is also not very far removed from these. (2) and (4) should show less activity because they do not contain all of the adrenalin, as found in (1); this variation is readily accounted for by

	(1)	(2)	(3)	(4)	(5)
Extract (A). Mm. of mercury	19.0 mm.	12.0 mm.	17.0 mm.	12.0 mm.	12.0 mm.
Amount injected of first 100 c.c. solution	0.01 c.c.	0.006 c.c. (0.011 mgm. Ad.)	0.05 c.c.	0.005 c.c. (0.01 mgm. Ad.)	0.05 c.c.
Amount required of first 100 c.c. solution to show reduction	0.2 c.c.	0.3 c.c.	10.0 c.c.	0.2 c.c.	25.0 c.c.
Extract (B). Mm. of mercury	25.0 mm.	11.0 mm.	14.0 mm.	13.0 mm.	10.0 mm.
Amount injected of first 100 c.c. solution	0.01 c.c.	0.005 c.c. (0.008 mgm. Ad.)	0.04 c.c.	0.005 c.c. (0.0097 mgm. Ad.)	0.05 c.c.
Amount required of first 100 c.c. solution to show reduction	0.2 c.c.	0.3 c.c.	15.0 c.c.	0.3 c.c.	25.0 c.c.

columns (3) and (5) which show some activity. Now the results given in (2) and (4) were obtained from the adrenalin solutions, and since these results correspond so closely with those of (1), which contains the unaltered active principle, and whereas (3) and (5) have very little activity, it is evident that adrenalin is the blood-pressure-raising body, the copper-sulphate-reducing body, and therefore the active principle as found in the adrenal glands.

It is also shown by this table that the adrenalin obtained by using a saturated sodium carbonate solution as a precipitant (2) shows practically the same results as that obtained by using ammonium hydrate (4). I will also state in this connection that the crystalline form of the two precipitates was identical. It was observed, however, that ammonium hydrate usually precipitated the adrenalin more quickly than the sodium carbonate solution.

CONCLUSIONS.

(1) All concentrated aqueous extracts of the suprarenal gland reduce Fehling's solution on boiling.

(2) The adrenalin obtained from a certain amount of the aqueous extract reduces Fehling's solution approximately in the same proportion as the original extract from which it was obtained.

(3) The mother liquor after removal of the greater part of the adrenalin, except in comparatively large amounts, does not reduce Fehling's solution.

(4) Adrenalin is not a reduced form of the native principle, since it was obtained without the use of a reducing agent, such as hydrogen sulphide.

(5) Adrenalin is the same whether obtained by using sodium carbonate solution or ammonium hydrate as a precipitant.

(6) Epinephrin, and the other questionable products obtained from the gland, must be oxidized or at least a changed form of adrenalin, the active principle, since they do not reduce Fehling's solution.

(7) Adrenalin is identical with the copper-sulphate-reducing body, the blood-pressure-raising substance, as found in the gland, and is therefore the active principle of the same, and not a modified or changed form, as Abel contends.

A CASE OF VOLUNTARY ERECTION OF THE HUMAN HAIR AND PRODUCTION OF CUTIS ANSERINA.

By S. S. MAXWELL.

CUTIS ANSERINA, goose-flesh, or goose-skin, is usually thought of as a purely reflex phenomenon. The contraction of the muscle fibres, the *arrectores pilorum*, in connection with the slantingly placed hair follicle, pulls the axis of the follicle into a position more or less perfectly perpendicular to the surface of the skin, causing the erection of the hair, and at the same time draws the whole mass of tissues surrounding the follicle toward the surface and causes the papule to protrude. The reflex may be brought about in various well known ways; *e. g.*, by exposure of the skin to cold, by the play of the emotions under certain conditions or by mechanical stimulation of certain regions of the skin.

The kind and amount of response to mechanical stimulation (rubbing the skin with the wooden handle of a paper knife) has been studied especially by Féré.¹ Individual differences are very great. In some persons the goose-skin appears only in the region stimulated, in others it may extend over nearly the whole body. An example coming under my own observation will illustrate this. Three young men, college students, exercising in the gymnasium, had the upper part of the body naked. I stimulated each in turn by drawing the handle of a scalpel along a line about 5 cm. from the sternal midline and extending from the third to the seventh rib. In A., a slender and rather nervous individual, the reflex was strongly marked, the papules rising promptly on the back, arms, and legs. In J., of stronger motor temperament and vigorous health, no response could be obtained except along the line of stimulation. K., who is somewhat phlegmatic, health good, gave no response on the arms, back, or legs, but the papules arose on a considerable region of the chest.

It is also well known that great individual differences occur in the

¹ FÉRE : Comptes rendus de la Société de biologie, 1898, p. 342.

readiness with which exposure to cold brings on the reflex. The same is true of the effect of the emotions. In some, the slightest physical fear brings up the papules; others experience the "creepy feel" produced by the hair movements, on reading a weird tale. One case is known to me of a lady who cannot listen to pathetic music without a succession of waves of contraction of the arrectores pilorum.

Cases are known in which voluntary production of goose-skin has been stimulated by calling up mental images, either of the sensation of a chill, or of some emotion which had previously been associated with the production of the reflex. It is not improbable that in unusually sensitive subjects it would be possible by practice to acquire a considerable appearance of voluntary control. So far as I have been able to examine the literature, no case of actual, direct voluntary control has been put on record heretofore, and the case herein reported appears to be unique. It is very probable, however, that, like voluntary control of the heart-beat, the publication of one case will call attention to others.

For the past eight months there has been under my observation a clearly marked case of voluntary erection of the hairs and production of cutis anserina, and I desire now to make a partial report of the results of my observations. The condition can be presented at will, showing itself in from two to ten seconds from the instant of the volition and disappearing with almost equal promptness. It is seen upon those regions of the body upon which in normal individuals it appears on cooling or chilling the skin, being especially prominent upon the hips and thighs, the back and the arms. There is no œdema. There is not the slightest resemblance to factitious urticaria or to any other of the well known reflexes sometimes seen in highly neurotic individuals. There is no marked change of color in the skin, only perhaps a very slight increase of pallor probably due to vasomotor changes described below. The condition is cutis anserina pure and simple. (See Fig. 1.) Where the hairs are relatively large, as on the lower part of the forearm, they can be seen coming up into a partially erect position as promptly and almost as decidedly as those on the tail of an angry cat. Where the hairs are minute one can see with a lens that they make a similar change of position. No one actually watching the performance doubts that the erection of the hair is produced by the contraction of the muscles, the arrectores pilorum, in connection with the hair follicles. Each papule

is plainly seen to have a hair at its apex. The condition extends to the scalp, but in lesser degree, and the head hair is too heavy to be actually erected.

The subject of this unusual power is Mr. S. B. McQuown, a young man twenty-seven years of age, a student in Monmouth College. He became aware of the possession of this peculiarity when he was eleven or twelve years of age. He does not think that it has changed in character or intensity since that time. He is afflicted with curvature of the spine, first noticed after a protracted illness in his fifth year. His hearing was impaired several years ago by disease following an attack of measles. He is color-blind to red. Notwithstanding these facts, his general health is good, and he cannot be classed as a neurotic, if that term has any definite meaning.

The conditions just mentioned will probably be thought of as furnishing a basis for exaggerated reflexes or other nervous disturbances. A remarkable development of the dermal structures has probably much more to do with the phenomenon in question. Mr. McQuown has unusual control of the facial muscles. He moves the skin of the scalp freely in various directions; he moves the ears simultaneously, alternately, or singly at will. He contracts the platysma myoides on either side singly or on both at once. In the muscles of his fingers and toes also the voluntary control is much more highly differentiated than in the average individual. These movements are not wholly nor very largely the result of practice. He discovered them by learning that they were possessed by his father. Indeed, if there is any difference, the father has the larger degree of development of the skin muscles. In most members of the family on his father's side there is a very decided tendency to extreme hairiness of the body, although he himself does not show that characteristic very strongly. His father is not able to erect the hairs voluntarily, but the condition can be produced reflexly with unusual ease. It is certainly deserving of attention that he possesses side by side two characteristics which co-exist in a marked degree in so many mammals; namely, a high development of the vari-

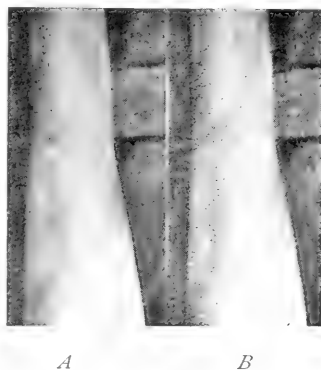


FIGURE 1.—Photographs of the forearm. *A* just before and *B* just after the signal for erection of the hairs.

ous elements of the panniculus muscular structures and the power of erection of the hairs.

The remarkable neuro-muscular development here presented reminds one at once of the description of those persons possessing the power of voluntary acceleration of the heart-beat. I may mention here that Mr. McQuown is absolutely unable to control or in any way directly to alter the heart-beat or to change the position of the heart. The close relation existing between this and the cases of voluntary control of the heart, however, will be seen in other particulars, especially in the close connection between the vaso-motor and the pilo-motor nerves.

Goose-flesh may be induced in some persons by the imagination of a chill or of some horrifying scene. This is certainly not the method used by Mr. McQuown. He is an intelligent student and takes a great interest in anything that tends to throw light on the explanation of the unusual power which he possesses. He has given every opportunity possible within the limits of his time here for the study of his case. He is absolutely sure that he does not call into play the imagination nor the emotions in bringing about the phenomenon. He does not "think a chill" nor picture some hair-raising scene. He affirms that he merely wills the erection of the papules, just as he wills the contraction of any skeletal muscle group, and the result follows the volition in the same way.

He does not wait for a favorable moment when his sensations might lead him to know that the erection is about to occur spontaneously; but contracts and relaxes the arrectores pilorum at any desired moment. In each of the accompanying kymograph tracings the lower line was made by an electrical signal operated by a spring key. A tap on the key depressed the lever and made a short slanting line. I had some portion of his body, usually the right forearm, under observation. The moment for the erection of the papules was chosen by myself not by Mr. McQuown. When the erection was desired I pronounced the syllable "On," tapping the key at the same instant. The signal thus made is marked *S* in all the tracings. The instant that I saw the papules rising I tapped the key again, and the mark resulting is indicated by *R* on the tracings. The reaction time is shown by the time curve in which each double vibration equals two seconds. The voluntary cessation is more striking than the inception. The signal for cessation was the word "Off" and the corresponding mark on the tracings is *S'*. The moment of complete depression of the

papules is marked *O*. It might be possible, but it is not probable, that at each word of command, repeated at frequent and variable intervals, mental images could be called up with such definiteness as invariably to incite the reflex. But it is scarcely possible that such images could be made to persist exactly so long as desired and to cease precisely at the desired instant. On the contrary, he is unable to produce the phenomenon by any amount of effort in calling up mental images or emotions of which the exciting cause is not present, or by imagining the sensation of chilliness. Concentration of the attention upon the appearance or the sensations of the skin does not produce the effect, although it does sometimes seem to make the erection of the papules easier. The phenomenon certainly comes as an act of will, just as in any other movement of the body.

Although no predisposing sensation is imagined, a sensation, "rather pleasurable than otherwise" and somewhat like a light chilliness, accompanies the erection of the hair. The sensation seems to pass in waves from an indefinite place, sometimes on the upper lumbar and sometimes on the lower cervical midline of the skin. This sensation gives some feeling of relief when the subject is suffering from headache and the frequent erection of the papules is resorted to on such occasions for that purpose.

That the phenomenon is caused by exposure of the body to a lower temperature than usual during the periods of observation is of course put out of the question by the facts already stated. The prompt appearance and disappearance of the papules at desire could not occur if change of temperature were the exciting cause. Besides, I have repeatedly observed the erection when only a small portion of the forearm above the wrist was exposed by pulling up the coat sleeve a very slight distance. Just as in other individuals, however, exposure of his skin to a low temperature does produce the goose-flesh reflexly. In this respect his skin is a little more sensitive than that of the average man, and he does not find shower baths or cold baths of any kind enjoyable.

The papules can also be raised reflexly by mechanical stimulation in the ordinary way, and the response is somewhat more extensive than in the average person. That the phenomenon can be produced reflexly, however, does not prove it to be reflex as opposed to voluntary in all cases, any more than that the muscles of a man's legs contract reflexly only because they can be made to do so by tickling his feet.

The erection can be continued for many minutes in succession, but as fatigue enters more effort becomes necessary. On watching the skin one sees that the papules frequently relax a little and then increase in size as if a new impulse had been sent to them. As the power becomes lessened by fatigue, these waves of motion become more obvious, and one can observe some relation between them and the respiration, although the fluctuating periods do not always coincide in number with the respirations, nor in time with any particular respiratory phase. The subject, so far as he can remember, has never through fatigue been unable to erect the papules, but when the power has been exercised for an unaccustomed length of time, the necessary effort becomes very great and a distinctly unpleasant feeling of interference with the respiration is experienced.

In attempting to discover the *modus operandi* of inciting the hair muscles to contraction, Mr. McQuown thought that a certain change

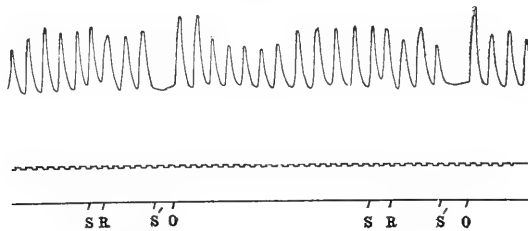


FIGURE 2.—Curve of respiration.

in the character of the respiratory movements was necessary. A sensation is usually felt as if the chest is held rather more than usual in the inspiratory phase, and the breathing deepened or momentarily suspended. To test this and to ascertain the actual conditions numerous pneumograph tracings were taken. In many of these a marked change in the respiration was shown and the change was not of the sort in his case found to be characteristic of mental attention. Fig. 2 is an average example; the lettering is as explained above. It soon became evident that these altered respiratory movements are not a necessary concomitant of the phenomenon, for tracings were obtained when the papules were as promptly and strongly produced as usual, but no apparently characteristic change occurred in the curve of respiration. Thus in Fig. 3 it would not be easy to show a connection between the respiration and the erection of the papules. An analysis of the conditions involved brought to light the fact that the

amount of respiratory disturbance depends largely upon the amount of fatigue incurred through repetition of the effort. As fatigue increases the erection requires markedly more effort and the changes in the respiratory movements become very great. This, it is well known, is not without parallel in other voluntary actions. Fig. 4 is a typical respiration curve during fatigue of the power. At the moment of relaxation there is not infrequently a momentary suspension of movement in the expiratory phase (Fig. 2), but this is by no means uniformly present. I am inclined to believe that the changed respiration is not a cause of the condition in the skin nor a medium through which a reflex is produced, but that the same nervous excitation may produce the effect in the skin alone, or may affect the skin and the respiration simultaneously.

In connection with the foregoing, it should be noticed that imitation of the changes in the breathing, holding the breath, deepening

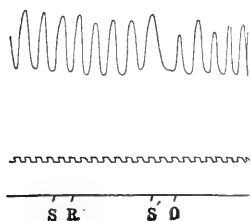


FIGURE 3.

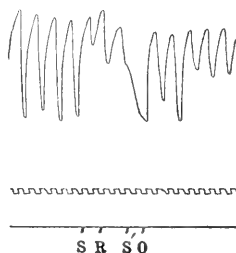


FIGURE 4.—Respiration when power of erection is fatigued.

the breathing, or increasing or decreasing the rate of the respiratory movements, did not in the slightest degree produce erection of the hair papules.

In view of the fact that in the lower mammals erection of the hairs is often associated with reactions comparable to certain emotional states, it was interesting to ascertain whether in this case anything similar occurs. It has been already mentioned that it was not possible by the imagination of causes for emotion to bring about the phenomenon. Nothing unusual could be discovered as to the effect of fear, sympathy, music, or other exciting cause of emotion, except in one particular: namely, that in witnessing an interesting football game the papules are erected most of the time, or can be brought up with unusual ease.

Langley¹ observed that in the regions in which erection of the hairs is obtained in the cat on stimulation of the sympathetic, the small arteries at the same time disappear from view and the skin becomes pale in consequence. This vaso-motor effect was seen to extend into lateral regions where no erection of the hairs occurred. Direct observation failed to show with certainty any change in the cutaneous vessels of Mr. McQuown during erection of the papules, although a slight appearance of increased pallor could usually be observed or imagined. On account of the distinctness with which they could be seen, especial attention was directed to the smaller vessels in the ears; but little could be detected with certainty until the following plan was used: The observations were made in an apartment lighted by a single north window, or else in direct sunlight. The observer was provided with a black pasteboard tube 245 mm. long and 20 mm. in diameter. The subject was seated between the

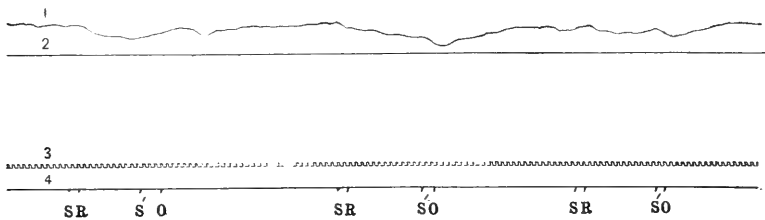


FIGURE 5. — Plethysmographic record of volume of the fingers during erection and relaxation.

observer and the window, and the tube was applied to the ear in such a way that the relative amount of blood in the ear was indicated by the depth of the pink glow. An assistant kept watch of the skin of the arm. The subject varied the condition of the skin at will and the observer and assistant kept watch of the ear and the skin respectively and later compared notes. It was possible in this way to prove that vaso-motor changes invariably accompany the erection of the hairs.

The vaso-motor changes were farther studied by the aid of a finger plethysmograph of the form devised by Professor Angell of the University of Chicago and with it absolutely definite results were obtained. The tracings show that invariably the erection of the papules is accompanied by a reduction in the volume of the fingers.

¹ LANGLEY: *Journal of physiology*, 1900, xxv, p. 468.

This is in perfect accord with Langley's observations that in the cat erection of the hairs is accompanied by contraction of the arterioles and that the vaso-motor action is more extensive in area than the pilo-motor. Fig. 5 is a sample tracing with the plethysmograph. Curve 1 was made by the tambour connected with the plethysmograph; 2 is a base line drawn for ease of comparison; 3 and 4 are identical in meaning with the corresponding curves in the respiration tracings. Without a single exception the curve falls in erection and rises at its close. During voluntary acceleration of the heart-beat there is a similar vaso-motor change; the blood-vessels of the skin contract and the volume of the extremities decreases.¹ In those cases, however, the constricted condition of the blood-vessels continues for some time after the heart-beat has returned to the normal. This is not true after erection of the hairs. The rise in volume of the fingers at the time of relaxation of the hair muscles is as prompt as the decrease at the moment of their contraction.

During erection of the papules, changes usually, or perhaps always, occur in the pupil of the eye. The observation of these is complicated and rendered difficult by the fact that the pupil dilates markedly during attention to anything requiring effort, the erection of the papules included. Thus when I asked Mr. McQuown to give the factors of $x^6 - y^6$, or to erect the hairs, the pupil dilated very similarly in the two cases. In numerous observations, however, I have never found the dilatation to be absent, at least at the moment of the beginning of the erection. The pupil does not always remain dilated during the whole period of the phenomenon. It usually undergoes considerable oscillation. These oscillations are probably connected with the wave-like changes in the prominence of the papules already referred to in connection with the respiration. It seems quite probable that the relation between the pupil dilator and the vaso-constrictor elements so well known by experiment on the lower animals obtains here also. The oscillations of the pupil would naturally be more marked and the dilated condition less constant than the corresponding constricted condition of the arteries, and hence a dancing of the pupil could occur during erection of the hairs while the calibre of the blood-vessels remained relatively constant. One remembers in this connection also the exaggerated dilatation of the pupil of the cat during erection of the hairs in anger or fright.

¹ TARCHANOFF: *Archiv für die gesammte Physiologie*, 1885, xxxv, p. 109.

Do the arrectores pilorum in this case contain striated muscle fibres? Nearly every competent observer of the phenomenon has asked this question. In order to secure an answer Mr. McQuown volunteered to submit to a biopsy. Doctors Patton and Linn, practising physicians of this city, kindly performed for me the operation of removing a small piece of skin from the anterior surface of the thigh. The portion selected contained two papules and included the entire depth of the skin down to the subcutaneous tissue. The piece was hardened, embedded in paraffine and sectioned perpendicularly to the surface in the plane of the muscle bundles. The sections do not show anything unusual or abnormal. The muscles were very distinctly seen to be made up of the ordinary plain fibres. No striated fibres were present. The muscle bundles seem, possibly, a little larger than is usual, but I cannot speak positively on this point because (1) I have not had sufficient opportunity to compare sections from the same region of the skin in other individuals and (2) only two papules were observed and no valid conclusion could be based on so limited an observation.

Recurring now to the question whether the erection of the hairs is to be considered a voluntary act or a reflex voluntarily initiated, the fact that plain and not striped muscle fibres are concerned makes no essential difference. It is well known that there are certain striated muscles which in the great majority of men and lower animals are wholly beyond the control of the will. The classification of plain muscles as "involuntary" and of striated as "voluntary" is clearly unscientific. A classification based on reaction time and the rate of movement during contraction is more rational, since striated muscles occur where prompt and rapid movement is necessary. The reaction time for the hair muscles was, in this case, three or four seconds, probably twenty-five times as long as for the skeletal muscles. This is the only essential difference in the response to the voluntary impulse, presented by the two kinds of muscular tissue. Striated muscles are usually voluntary, plain muscles involuntary; but there is no *a priori* reason why the reverse could not be true, and we now have exceptions on both sides. It seems to be not improbable that further observation will bring to light other instances of voluntary control of non-striated muscle.

I wish here to make acknowledgment of my indebtedness to Dr. Lingle of the University of Chicago and to Dr. Hyde of the Rush Medical College for valuable suggestions and assistance in the study of this case.

The results of my observations on this case may be briefly summarized as follows:

1. The erection of the papules can be brought about voluntarily.
2. The voluntary control of the arrectores pilorum seems to be correlated with an inherited, unusual neuro-muscular and dermal development.
3. The erection of the hairs is in this case accompanied by vaso-motor changes of such nature that the vaso-constrictors are stimulated while the arrectores pilorum are contracted.
4. Dilatation of the pupil occurs during the period of erection. The dilatation is most marked at the beginning of the erection and is very inconstant as to amount.
5. Modifications of the respiratory movements usually accompany the phenomenon, but are not necessarily connected with it.
6. The arrectores pilorum do not contain striated muscle.

ON THE LYMPHAGOGIC ACTION OF THE STRAWBERRY, AND ON POST-MORTEM LYMPH FLOW.

BY LAFAYETTE B. MENDEL AND DONALD R. HOOKER.

[*From the Sheffield Laboratory of Physiological Chemistry, Yale University.*]

IN his classic investigations on lymph formation, Heidenhain¹ assumed that the active constituents of his lymphagogues of the first class (peptone, extracts of leeches, crustacea, etc.) produce their particular lymphagogic influence by stimulating the endothelial cells of the blood capillaries, and that the latter thus exert a secretory function which results in the formation of lymph. He remarked that the ætiology of the urticaria which sometimes follows the ingestion of crustacea in the diet might be disclosed by such experiments as he carried out in this connection. In addition to this urticaria there arises not infrequently a diffuse œdema of the skin in certain regions. Similar symptoms are repeatedly observed in susceptible individuals as consequences of eating various forms of "shell-fish," such as oysters, crabs, lobsters, etc. They also occur occasionally after ingestion of berries, more particularly strawberries. Heidenhain has demonstrated the lymphagogic action of the former group; and we have examined the strawberry in order to learn whether comparable effects may be produced experimentally by this vegetable product. Since our attention was first directed to the subject, Clopatt² has published his observations on the lymph flow from the thoracic duct after intravenous injections of strawberry preparations. He observed that berry extracts introduced into the circulation of dogs exert an unquestionable lymphagogic action. Since such extracts contain sugar and inorganic salts, both of which have lymphagogic action when introduced in sufficient quantity into the bloodstream, it was necessary to take them into consideration. Clopatt showed that the quantities of these crystalloids present were (according to Heidenhain's experience) far too small to produce the

¹ HEIDENHAIN: *Archiv für die gesammte Physiologie*, 1891, xlix, p. 209.

² CLOPATT: *Skandinavisches Archiv für Physiologie*, 1900, x, p. 403.

results obtained with the strawberry extracts. Furthermore he injected solutions containing comparable quantities of sugar and salts, and failed to note any equally marked increase in lymph flow. No observations on other phenomena were reported; and the attempt to isolate the active constituent has not yet been successful.

In the present experiments, the details of which need not be repeated here, the observations of Clopatt were repeatedly verified. We determined, however, to investigate some other features of lymph production after injection of strawberry extracts, in order to obtain additional evidence that the action of this material is in fact comparable with that of the other lymphagogues of Heidenhain's first class — and not entirely due to the crystalloids present in the berry. In bringing about an acceleration of lymph flow the latter substances, represented by dextrose, sodium chloride, etc., give rise to a fluid which is, if anything, less rich in dissolved substance than the lymph normally flowing from the duct of the same animal. The other known lymphagogues, which include a number of miscellaneous substances, have in common their peculiar action in provoking lymph flow; the lymph to which they give rise, is, however, regularly found *richer* in solids than the corresponding normal lymph. Furthermore these substances exert no such diuretic action as do sugar and the inorganic salts referred to; and some of them (albumoses) produce a marked fall in arterial pressure. Attention was accordingly directed to differences of this sort; and a few observations on post-mortem lymph formation have also been made.

Experiments. — In the following illustrative experiment selected from our protocols, a dog of twenty kilos was anesthetized by inhalation of chloroform-ether after subcutaneous administration of morphine (15 cgm.) and atropin (15 mgm.). Lymph was collected from the thoracic duct; blood samples were removed from a femoral artery; the urinary flow was noted by means of cannulas in the ureters; and arterial blood-pressure was recorded with a mercury manometer. The details of the methods applied have been described elsewhere.¹ The dog had fasted for twenty-four hours previous to the experiment. Three injections with "strawberry extract" were made into the right facial vein. The extract was prepared by boiling

¹ See CHITTENDEN, MENDEL, and HENDERSON: This journal, 1898, ii, p. 142; also Studies in physiological chemistry, edited by R. H. CHITTENDEN, 1901, p. 279. Many observations on the action of lymphagogues are given in this paper, which includes a review of the literature.

forty grams of desiccated berries with two hundred and fifty c.c. of water and straining through cloth. The results are given in the table.

Time.	Lymph collected. c.c.	Lymph-flow in 10 min. c.c.	Total solids in lymph. Per cent.	Urine-flow in 10 min. c.c.	Blood-clotting time.
12.00-12.25	2.2	0.9	6.35	1.0	12.03 — 6 min. 12.08 — 10 min. 12.19 — 5 min.
12.25	Injection of 25 c.c. strawberry extract (30° C.).				
12.25-12.35	..	17.0	7.59	0.6	12.27 — 24 min. 12.28 — 20 hrs. 12.35 — 20 hrs.
12.35-12.45	..	11.8	7.34	none	12.40 — 20 hrs.
12.45-12.55	..	6.4	7.17	0.9	12.50 — 1 hr. 37 min.
12.55-1.05	..	3.7	6.80	1.5	12.56 — 13 min.
1.05-1.15	..	1.9	} 6.52	1.4	
1.15-1.25	..	1.3		1.0	1.18 — 7 min.
1.27	Injection of 43 c.c. strawberry extract (30° C.).				
1.27-1.37	..	3.2	6.55	4.0	1.30 — 8 min. 1.34 — 4 min.
1.37-1.47	..	1.7	} 6.75	3.7	1.44 — 5 min.
1.47-1.57	..	2.0		2.5	
1.57-2.07	..	1.9	} 6.53	0.7	
2.07-2.17	..	0.8		0.2	
2.20	Injection of 50 c.c. strawberry extract (30° C.).				
2.20-2.30	..	4.2	} 6.65	4.7	2.23 — 8 min.
2.30-2.40	..	2.7		2.0	2.29 — 10 min. 2.39 — 26 min.
2.40-2.50	..	2.1	} 6.47	0.3	2.48 — 17 min.
2.50-3.00	..	2.0		0.2	
3.00-3.10	..	3.2	} 6.57	none	
3.10-3.20	..	3.2		none	
3.20-3.27	4.2	6.0	6.61	2.8	
3.25	The blood-pressure falls suddenly and the dog is dead at 3.27.				

TABLE (continued).

Time.	Lymph collected. c.c.	Lymph-flow in 10 min. c.c.	Total solids in lymph. Per cent.	Blood-clotting time.
3.27-3.37	..	2.0	} 6.88	} post-mortem lymph.
3.37-3.47	..	2.0		
3.47-3.57	..	1.6		
3.57-4.27	2.0	0.7	} 7.48	
4.27-4.57	1.0	0.3		

After 5.00 the lymph ceased to flow.
The lymph collected 12.25-1.05 is reddish, and *does not clot*.

It will be observed that the acceleration of lymph flow was rather greater after the first injection than after subsequent ones. The effect of the extract in retarding the coagulability of the blood and lymph was likewise scarcely notable after the later injections. This fact accords with the observations frequently made on the "immunity" which is obtained towards repeated injections of other lymphagogues.¹ The effects on blood-pressure resembled those obtained after albumose injections, although the recovery from fall of pressure was rather rapid and each succeeding injection was less efficient than its predecessor. The tracings from this experiment are reproduced on p. 384.

Chemical examination of the strawberry extracts agreed with Clopatt's observation that the quantities of sugar and salts present in the volumes of extract used were too small to bring about the marked results on lymph flow noted. Analyses of twenty varieties of American strawberries have given the following averages:²

	Per cent.		Per cent.
Water	90.52	Ash	0.62
Dry matter	9.48	Crude fibre	1.55
Glucose and cane sugar	5.36	Ether extract	0.64
Free acid, as malic	1.37	Crude protein	0.99

¹ See CHITTENDEN, MENDEL, and HENDERSON: *Loc. cit.*; also SPIRO and ELLINGER: *Zeitschrift für physiologische Chemie*, 1897, xxiii, p. 121.

² STONE, W. E.: *Bulletin of the agricultural experiment station of the University of Tennessee*, 1889, ii, p. 77.



FIGURE 1.— One fourth the original size.

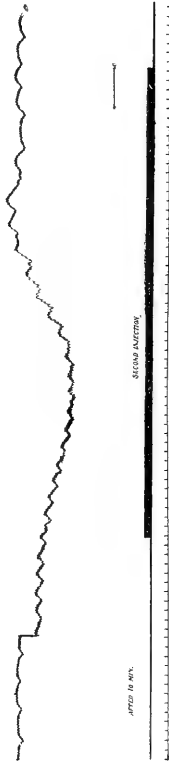


FIGURE 2.— One fifth the original size.

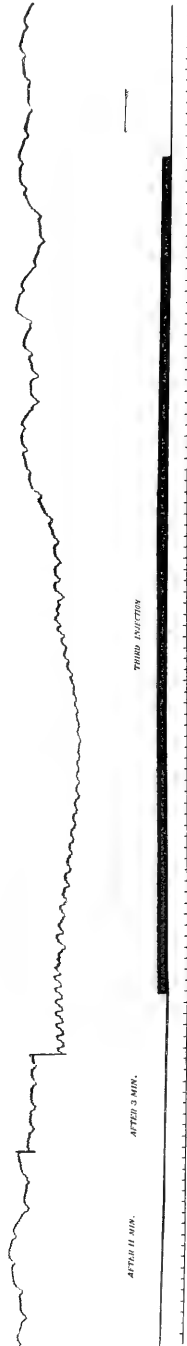


FIGURE 3.— One fourth the original size.

Assuming from these figures that one-half of the desiccated berries was made up of sugar, the maximum dose introduced in the largest injection would not exceed 0.2 gm. per kilo.¹ The accelerated flow of a lymph *richer* in solids, together with diminished coagulability of the blood, fall in pressure, etc., were similarly observed in other experiments. Thus, in various respects studied, *the action of strawberry extract resembles that produced by other typical lymphagogues of Heidenhain's first class.*

Post-mortem lymph flow. — The animal employed in the experiment just described died at 3.27 after a few respiratory efforts. The lymph however, continued to flow as indicated above, until 5 p. m. with an increasing content of dissolved solids. More striking, perhaps, were the observations made in another experiment. A dog of fourteen kilos had been prepared as in the preceding case. The animal had received two injections of strawberry preparations at intervals, viz., (1) an alcoholic extract (25 c.c.) of dried berries, and (2) 50 c.c. strawberry juice neutralized with NaOH. There was a transitory fall in blood-pressure in each case, but no noticeable effect on the clotting time of the blood, the latter being very short in every trial. Accordingly in order to ascertain whether this animal was naturally "immune," a test injection of five grams of Witte-pepton dissolved in fifty c.c. of water was made. The flow of lymph was greatly accelerated, but there was no delay in blood clotting. Eight minutes after the last injection the dog was killed by blowing air into the facial vein. *The lymph continued to flow for four hours, without any external mechanical assistance.* The table on p. 386 gives the data of interest in this connection.

These observations on the prolonged flow of concentrated lymph after somatic death are of interest in connection with the studies of Asher and Gies.² They noted an accelerated post-mortem flow of lymph following intravenous injections of sugar, and point to this fact to demonstrate that the lymph formation cannot be the consequence of capillary blood-pressure. The old filtration hypothesis has thus received its final blow. If the results are to be explained on a purely physical (in distinction from a "physiological") basis, they must be looked upon as the effect of the diffusion processes provoked by the crystalloid introduced. Fluid passes from the tissues into the

¹ Cf. CLOPATT: *Loc. cit.*, p. 407.

² ASHER and GIES: This journal, 1900, iii, p. xix; Zeitschrift für Biologie, 1900, xl, p. 207.

lymph channels, the osmotic pressure of the sugar playing the important rôle in calling forth the stream of lymph. Asher and Gies add, however, that other possibilities might be drawn upon in explanation; and in calling attention to the parallelism between post-mortem lymph formation and post-mortem salivary secretion they

Time and conditions.	Lymph-flow in 10 minutes. c.c.	Total solids in lymph. Per cent.
Before injection	4.4	4.57
After injection of the alcoholic strawberry extract	5.5	5.28
After injection of the neutralized straw- berry juice	13.3	6.33
After injection of 5 gms. of Witte-peptone	very large	7.78
Post-mortem lymph-flow in successive pe- riods of 10 minutes.		
First hour	10.1, 6.9, 4.0, 2.5, 2.3, 1.7	9.02
Second hour	2.0, 2.0, 1.5, 1.5, 1.0, 1.0	8.61
Third and fourth hours	0.8, 0.6, 0.6, 0.4, 0.3, 0.2, 0.1, 0.1, 0.1, 0.2, 0.05, 0.05	} 8.62
The lymph collected post mortem did not clot.		

suggest that distinctly "physiological" processes are involved in each case. Our own observations on post-mortem lymph flow produced by lymphagogues of a different class give support to this view and further emphasize the fact that we are not yet ready to deny the specific importance of the living (or surviving) cells for such physiological phenomena.¹

¹ Cf. MOORE and PARKER: This journal, 1902, vii, p. 291.

EXPERIMENTAL OBSERVATIONS ON PANCREATIC DIGESTION AND THE SPLEEN.

BY LAFAYETTE B. MENDEL AND LEO F. RETTGER.

[From the Sheffield Laboratory of Physiological Chemistry, Yale University.]

INTRODUCTORY.

IT has been known since the original classic experiments of Corvisart that the pancreas of animals does not show marked proteolytic power at all times. The amylolytic, and perhaps the lipolytic, properties can usually be demonstrated quite readily. But Corvisart was himself the first to note that during the actual progress of digestion the pancreas exhibits a variable solvent power for proteids. He observed the maximum digestive power at about the eighth hour after the ingestion of food, with a subsequent minimum after the thirteenth hour. Meissner noted that the pancreas of fasting animals exerts no proteolytic action. These observations were all verified by Schiff. He too found that the peptonizing power of pancreatic juice or pancreatic extracts was not constant and continuous, but rather variable and intermittent. The most pronounced effects were obtained soon after the culmination of digestion in the stomach. Schiff sought for some explanation of this periodic variation. The probability of a periodic central stimulation of the nerves was soon excluded; and Schiff turned his attention toward another organ which is likewise subject to periodic variations in its functional activity, viz., the spleen.

Schiff's chief paper embodying the results of his investigations on the possible interrelation of the spleen and pancreas was published in 1862.¹ It has, however, received little recognition or consideration at the hands of later investigators. The textbooks have usually avoided the subject. Thus in Schaefer's Textbook, which is as a rule rather exhaustive in its historical references, and in Bunge's new

¹ SCHIFF: Schweizerische Zeitschrift für Heilkunde, 1862; SCHIFF'S Gesammelte Beiträge zur Physiologie (herausgegeben von Herzen und Levier), 1898, iv, p. 167. A review of the earlier literature is given in this paper.

Lehrbuch, the question of an interrelation of the spleen and pancreas is not even referred to. In the American Textbook, the editor has written: "A theory has been supported by Schiff and Herzen, according to which the spleen produces something (an enzyme) which, when carried in the blood to the pancreas, acts upon the trypsinogen contained in this gland, converting it into trypsin." He then adds: "The experimental evidence upon which this view rests has not been confirmed by other observers."¹ Hammarsten, in his Physiological Chemistry, presents the evidence available and adds that "the statements on this question are still disputed."² It may be appropriate therefore, to recall briefly some of the facts ascertained.

Schiff carefully reviewed the recorded observations on the periodic swelling of the spleen and demonstrated a striking synchronism between the progress of splenic congestion after a meal and the distribution of trypsin in pancreatic juice or gland infusions. He then repeated his earlier pancreatic experiments on splenectomized animals or on animals in which the spleen function was eliminated by ligation of the blood vessels at the hilus. The methods used to ascertain the proteolytic power of the pancreas were various. In some cases gland extracts were employed; in others, the progress was studied by introducing albumin into a loop of duodenum or through a permanent duodenal fistula. In all of these many experiments, the observations were concordant in showing that when the spleen is extirpated or prevented from dilating normally, trypsin is absent from the pancreatic extract or secretion of the animals at those periods of the digestive cycle when it is found abundant in normal comparable animals. It thus appeared as if not only the presence of the spleen is necessary for the formation of trypsin, but also the congestion of that organ. The pancreas of an animal deprived of its spleen behaves (even in the height of the digestive period) like that of a normal animal during hunger. The explanation given by Schiff to the facts observed was adapted to conform with his well-known "peptogen" theory. Thus he concluded, in his own words, "that the spleen is an organ which so transforms a part of the peptogen bodies absorbed from the stomach that they are capable of forming the proteid-dissolving substance of the pancreas. After splenectomy the secretion or extracts of the pancreas lose the power of dissolving

¹ HOWELL: American Textbook of physiology, 1896, p. 273.

² HAMMARSTEN: Textbook of physiological chemistry (translated by MANDEL), 1900, p. 200.

proteids." With Heidenhain's demonstration of the formation of a zymogen in the pancreas itself, independently of the exterior influences, Schiff's *theory* was no longer tenable. But the facts remained unchanged; and Herzen modified the theory to conform with our present knowledge regarding the occurrence of trypsinogen. According to Herzen¹ the pancreatic zymogen is formed continually and therefore independently of the changes in the spleen. Trypsinogen accumulates in the pancreas during starvation in both normal and splenectomized animals. The rôle of the spleen consists in contributing something — an "internal secretion," as he calls it — which facilitates the transformation of the zymogen to trypsin. Furthermore Herzen has added, by entirely new methods, a further series of experiments bearing on the problem. He found that if an infusion of a congested spleen is mixed with an inactive extract of the pancreas obtained either from a fasting, or better from a splenectomized dog, the zymogen is readily transformed to trypsin; at any rate, whatever the explanation may be, a far more vigorous digestive power is manifested. The specific action of the splenic extracts is not due to the oxygenating power of the blood contained in them. For, according to Herzen, no other blood except that from the splenic vein gives results comparable with those produced by spleen infusions.

Herzen's observations have more recently been confirmed by Gachet and Pachon,² who have in turn brought another method to bear upon the problem. They have found that if a part of the pancreas is removed from a splenectomized dog and an extract at once prepared from it, and that if then the blood obtained from a congested spleen is injected intravenously and the second portion of the pancreas removed at the end of twenty minutes, and extracted as before, two unlike infusions are obtained. The first of these digests proteids only after a long time and very slowly; the second extract, obtained after the splenic injection, digests proteids at once and rapidly. The investigators further attempted to demonstrate the enzyme-like character of the specific splenic substance which facilitates the transformation of the zymogen to trypsin, by experiments

¹ HERZEN: *Revue général des Sciences*, 1895, 15 juin, p. 494; SCHIFF'S *Gesammelte Beiträge zur Physiologie*, 1898, iv, pp. 224, 235; *Archiv für die gesamte Physiologie*, 1901, lxxxiv, p. 115.

² GACHET: *Du rôle de la rate dans la digestion pancréatique de l'albumine*. Thèse de Bordeaux, 1897; GACHET and PACHON: *Archives de physiologie*, 1898, x, p. 363.

made *in vitro*. They found that a temperature of 100° C. destroys this peculiar power of the spleen infusions; and when active spleen infusions are precipitated with alcohol, the alcoholic extracts do not contain an active constituent. Since destruction by heat and precipitation by alcohol are well-known characteristics of enzymes in general, Gachet and Pachon conclude that the peculiar trypsinogenic properties of the spleen are probably due to something analogous to enzymes. Like Herzen, they also speak of an "internal secretion" of the spleen.

Since the completion of the present experiments Bellamy¹ has published further observations made in Professor Herzen's laboratory. The conclusions reached by him probably represent the latest standpoint of Herzen and may therefore be repeated here. He writes:

"(1) The pancreas of the dog presents two phases of activity, (a) a slow and continuous phase feebly evident after the decline of digestion and reaching its culmen during complete fast; during this period zymogen is accumulating in the gland cells, and at this time the contents of the latter, although consisting for the most part of pure zymogen, may, if the previous intake of food be not copious enough to occasion its entire removal, include a certain amount of true trypsin. (b) A rapid and intermittent phase coincident with the period of considerable gastric activity, in which, as advocated by Herzen, the inactive zymogen granules present in the cells receive from the spleen in the form of an "internal secretion" the agent with which to elaborate the active proteolytic ferment — trypsin, which passes forthwith into the glandular ducts.

"(2) The blood supplies the vehicle by which this product of the spleen is transported to the pancreas (Herzen). It appears to be carried by the solid elements of the blood, since serum does not contain it; if carried by the plasma it is destroyed by coagulation.

"(3) I find in agreement with Herzen that the pancreas of a dog deprived of its spleen exists in a condition of complete and permanent atrypsia; the elaboration of zymogen continues in the gland cells; this zymogen is excreted as such, and if it becomes subsequently changed so as to be of service to the organism this process is carried on outside of the pancreas and occurs as the result of some agency other than the spleen."

EXPERIMENTAL.

Experiments *in vitro*. — The present experiments were originally undertaken with the object of examining the validity of the Schiff-

¹ BELLAMY: *Journal of physiology*, 1901, xxvii, p. 334.

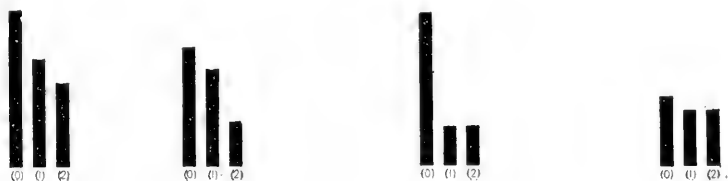
Herzen hypothesis.¹ Dogs were employed in almost every case. The first series, carried out *in vitro* entirely, was planned to indicate the specific effect of spleen infusions on inactive or feebly active pancreatic extracts. The relative proteolytic power was estimated by digesting weighed quantities of coagulated blood-fibrin. This proteid was selected because of its easy digestibility as compared with coagulated egg-white. Since the inactive extracts frequently contain zymogen which is spontaneously transformed into active trypsin on standing, the demonstration of original differences in the proteolytic power of the extract must be attempted as quickly as possible; hence the choice of blood fibrin. The latter was very thoroughly washed and selected, and then preserved in chloroform-water. Before use it was thoroughly boiled in water to destroy any enzymes of bacterial or other origin which might be adherent. For, as is well known, enzymes readily attach themselves to the fibrin; and experience in the laboratory has shown how readily they are formed when fibrin is allowed to stand in water for a time in a warm room. Subsequent washing and addition of antiseptics like chloroform may destroy all bacteria present without removing these foreign proteolytic enzymes. In our experiments all possibility of error in this way was excluded by the heating. The fibrin was pressed as dry as possible, comminuted, and weighed in moist condition. At the end of the digestive trials the undissolved residue was filtered off on a tarred paper, was washed very thoroughly, dried at 100° C., and weighed. The pancreatic extracts were prepared by treating a weighed portion of the glandular tissue for two hours with 50 c.c. of saturated boric acid solution, as recommended by Herzen. Putrefaction processes were thus checked without any very marked inhibition of proteolysis. The spleen was usually removed during chloroform-ether anæsthesia after subcutaneous administration of morphine, shortly before the digestion experiment and at a time after previous feeding when it was expected to be well distended with blood. This was usually about six hours. The operation was carried out with aseptic and antiseptic precautions and the splenectomized dogs were used after their recovery in subsequent experiments on the pancreas. Extracts of the spleen were

¹ A résumé of the history and general features of this theory is given by BELLAMY: *The Lancet*, 1900, October 27, p. 1185; and by HERZEN: XIII. Congrès international de médecine, Paris, 1900, *Comptes rendus*, Section de physiologie, physique et chimie biologiques, p. 108.

prepared with boric acid solution for the experiments in vitro. Control trials were made under comparable conditions with fibrin and boric acid solution alone. The protocols are given in the appendix to this paper. The following description will indicate the details of a typical experiment.

Equal portions (5 to 7 gms.) from the same part of the pancreas of a splenectomized dog were macerated for two hours at 39° C. with 25 c.c. saturated boric acid solution and filtered. To equal portions of the filtrate 25 c.c. of the same boric acid solution (1) and 25 c.c. of the boric acid extract of the spleen (2) respectively were added. The weighed quantity of fibrin was next introduced and the digestion allowed to proceed at 39° C. for varying periods of time, at the end of which the undissolved residue was filtered off and treated as described above.

The results are expressed graphically in the accompanying charts. The column marked (0) represents the weight of the undissolved fibrin (dry) recovered from the control trial, and thus corresponds with the quantity of proteid available for digestion; while the undissolved residue in the digestion trials is represented by (1) without splenic extract and (2) with splenic extract.



EXPERIMENT I. EXPERIMENT II.

Pancreas of splenectomized dog.
Extract of congested spleen.

EXPERIMENT III.

Pancreas of normal fasting dog.
Extract of contracted spleen
of same animal.

EXPERIMENT IV.

Pancreas of fasting cat.
Splenic extract of
a fasting cat.

Further investigation in this direction was discontinued after the publication of Hedin and Rowland's¹ observations on the occurrence of a proteolytic enzyme in the spleen. They ascertained that the expressed tissue juices from the spleen of the ox, horse, sheep, and pig undergo gradual autolysis in the presence of toluol. An acid reaction apparently favors the proteolysis, and the enzyme present is presumably not identical with trypsin. The splenic juices will

¹ HEDIN and ROWLAND: *Zeitschrift für physiologische Chemie*, 1901, xxxii, p. 341; also *Journal of physiology*, 1901, xxvii, p. xlvi.

also dissolve blood fibrin; but the proteolytic power does not appear to be very vigorous and it seems unlikely to us that the marked results obtained with pancreatic extracts and dilute splenic infusions such as we employed are entirely attributable to the activity of a proteolytic splenic enzyme; however, in the absence of special control trials, the results are fairly open to criticism. The experiments of the second series exclude this error.

Since, in the experiments to follow, it became necessary to use tissue from different parts of the pancreas in making the comparable digestion trials, it was desirable to see whether variations in proteolytic power might be associated with the different portions. Accordingly in Experiment V a portion (5 gms.) was removed from both (1) the "head" and (2) the "tail" of the pancreas of a splenectomized dog. Comparable extracts were prepared with boric acid solution and allowed to act upon 5 gms. of fibrin for twenty-two hours. As the chart shows, no noticeable differences were obtained.



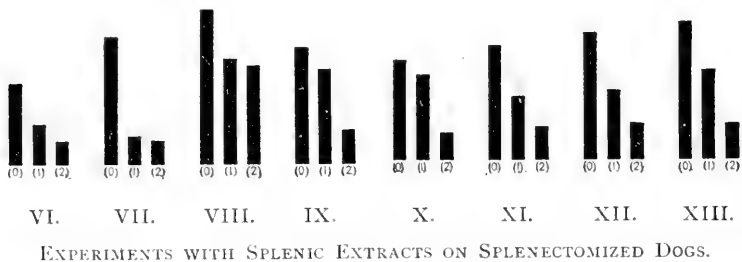
EXPERIMENT V.

Pancreas of splenectomized dog,
(1) from "head" of pancreas; (2) from "tail" of pancreas.

Experiments in vivo. — In the second series of experiments the method of Gachet and Pachon was followed quite closely. Splenectomized dogs which had completely recovered from the effects of the operation were employed. They were fed on dog biscuit and meat until two or three days before the experiment. The last meal consisted of chopped lean meat. After subcutaneous administration of morphine sulphate, chloroform-ether inhalation was begun and the duodenum and attached pancreas were withdrawn during anaesthesia through an opening in the linea alba. The free (lower) end of the pancreas which is detached from the gut was ligated and quickly removed. [Portion (1).] The organs were returned to the abdomen, which was then closed again. From 90 to 125 c.c. of a warm saline (0.7 per cent NaCl) extract of a congested spleen or other organ were now injected into the jugular vein. At the end of twenty minutes the animal was killed and the remainder of the

pancreas removed. [Portion (2).] Equal portions (4 to 7 gms.) of (1) and (2) were weighed out as speedily as possible, macerated and extracted for two hours at 39° C. with forty to fifty c.c. of saturated boric acid solution. The infusions were filtered through absorbent cotton, mixed with 5 to 7 gms. of blood fibrin and allowed to digest at 39° C. The other details were the same as in the first series. (See Appendix.)

The records from eight experiments on intravenous injection of extracts of congested spleens are presented.



It will be observed that our results agree in general with the experience of Gachet. It cannot be said that the pancreas of splenectomized animals was always in a condition of complete atrypsia. But in view of the readiness with which trypsinogen is converted to an active enzyme it can scarcely be expected that no proteolysis should occur in (1) even if no trypsin existed as such in the living tissue. The acid reaction and other conditions all favored the formation of the enzyme from its precursor. Unfortunately we have no satisfactory medium which will entirely check the gradual transformation of the zymogen. Glycerin¹ alone gives promise of usefulness, but its application introduces other difficulties. It is interesting to observe that the differences noted between the various trials were usually more marked early in the digestive period. Thus frequently the digestion was already under way in (2) before signs of solvent action were apparent in (1). Uniform results are scarcely to be expected where so many variables exist. The results are *concordant*, however, in showing a tendency toward increased content of preformed trypsin in the pancreas after introduction of splenic extracts into the circulation.

Are these effects specific for the extracts of the spleen, or do they

¹ Cf. VERNON: Journal of physiology, 1901, xxvii, p. 316.

merely represent the reaction of the gland to the injection itself, independently of the character of the material introduced? In attempting to answer this we have gone a step in advance of our predecessors. Gautier¹ has suggested that other organs doubtless possess a similar trypsinogenic power and Chepowalnikoff² has lately shown that the intestinal juice has the property of activating trypsinogen. Our experiments, which may be considered as a control series for the observations with splenic extracts included trials on splenectomized dogs as follows:

Experiments XIV, XV: injection of "physiological" NaCl solution; Experiment XVI: injection of a saline extract of fresh liver; Experiment XVII: injection of a saline extract of dog's pancreas; Experiment XVIII: injection of a *boiled* saline extract of congested spleen.

The results are given in the charts.

Injection of NaCl solution.

EXPERIMENT XIV.



EXPERIMENT XV.



Injection of organ extracts.



EXPERIMENT XVI.

Injection of liver extract.



EXPERIMENT XVII.

Injection of pancreas extract.



EXPERIMENT XVIII.

Injection of boiled spleen extract.

These experiments afford a striking contrast to those in which splenic extracts were employed. Differences in proteolytic power are not apparent in any case with the possible exception of the pancreas experiment. It has been suggested that the results noted with

¹ See XIII. Congrès international de médecine, Paris, 1900, Comptes rendus, Section de physiologie, physique et chimie biologiques, p. 114.

² CHEPOWALNIKOFF: La physiologie du suc intestinal. Thèse de St. Pétersbourg, 1899.

the splenic extracts are merely the expression of changes having a vaso-motor origin. If this explanation were correct might we not expect similar vaso-motor reactions in the pancreas after injection of boiled splenic extracts or of other tissues?

Herzen¹ has noted that the venous blood from a congested spleen alone is capable of increasing the proteolytic power of the pancreatic extract in vitro. Arterial blood from the systemic vessels does not act in this way. It seemed of interest, therefore, to try the effects of intravenous injection of splenic blood. The latter was collected directly from the vein of a congested spleen (in quantities of 40 to 50 c.c.), defibrinated, strained through cotton and injected into splenectomized dogs. Protocols from two experiments are given.

Injection of blood from the splenic vein.



EXPERIMENT XIX.



EXPERIMENT XX.

The nature of the evidence which Gachet and Pachon have brought forward in favor of the enzyme-like character of the trypsinogenic agent of the spleen has already been referred to. Such injection experiments as we have carried out are in accord with their observations in vitro. In Experiment XVIII the heated extract was used.

Injection of extract of alcoholic precipitate.



EXPERIMENT XXII.



EXPERIMENT XXIII.

To investigate the behavior of the "trypsinogenic agent" towards alcohol, active splenic infusions were precipitated with four to five

¹ HERZEN: *Revue général des Sciences*, 1895, 15 juin, p. 505; *Archiv für die gesammte Physiologie*, 1901, lxxxiv, p. 118.

volumes of alcohol. The precipitates were washed with alcohol and then extracted for two hours at 39° C. with physiological saline. Eighty to one hundred c.c. were used for injection. In Experiments XXII and XXIII this material was studied. In the latter, the trypsinogenic activity of the original extract used was first demonstrated by Experiment XII. It seems quite probable that the active principle is precipitated by alcohol. Comparable trials with the alcoholic filtrates were not made.

GENERAL CONSIDERATIONS.

The experimental data recorded in this paper verify and supplement those of previous investigators mentioned, in indicating that

1. Extracts of the spleen, prepared from the organ when congested during digestion, increase the proteolytic power of the pancreas. This was demonstrated *in vitro* and *in vivo*.

2. Injections of defibrinated blood from the splenic vein are likewise effective.

3. A boiled extract of the spleen is ineffective.

4. Extracts of other tissues (liver, pancreas) apparently have little action. The same is true of pure saline infusions.

5. The precipitate produced by addition of alcohol to active splenic extracts contains a trypsinogenic substance.

6. The extracts of the pancreas of splenectomized dogs are not always free from trypsin. Whether this is entirely attributable to an extra-pancreatic transformation of trypsinogen is not determined. Some investigators¹ have claimed that the pancreatic juice of splenectomized animals has normal proteolytic power, *i. e.*, contains preformed trypsin. This has been attributed by others² to the spontaneous changes described. That the secretion may contain the zymogen is unquestioned.

Thus far we have been dealing with observations alone. The Schiff-Herzen hypothesis goes further. "The spleen, by internal secretion, yields a substance of unknown character which has the property of transforming trypsinogen into trypsin."³ We are at

¹ HEIDENHAIN: *Hermann's Handbuch der Physiologie*, 1883, v, p. 206; POPIELSKI: *Jahresbericht für Tierchemie*, 1899, xxix, p. 353.

² HERZEN: *Archiv für die gesammte Physiologie*, 1901, lxxxiv, p. 124 note; BELLAMY: *Journal of physiology*, 1901, xxvii, p. 331.

³ HERZEN: *Archiv für die gesammte Physiologie*, 1901, lxxxiv, p. 126.

once led to inquire: What justification exists for the assumption of an "internal secretion"? Does this trypsinogenic rôle of the spleen have any physiological significance whatever? The criteria which are ordinarily applied to demonstrate the existence of a so-called "internal secretion" include the proof (a) that the organ involved contributes something specific to the blood which the latter does not bring to it, and (b) that functional suppression or extirpation of the organ is followed by the failure or omission of those reactions for which its "internal secretion" is assumed to be responsible. In the case of the adrenal glands, for example, both of these conditions are fulfilled.

For the spleen, likewise, the experimental evidence seems to indicate that it may contribute the peculiar trypsinogenic agent directly to the blood passing through it. (*Cf.* Experiments XIX, XX and Herzen's work.) Extirpation of the spleen, on the other hand, is not followed by any noticeable interference with digestive or other functions. Noël Paton¹ and his co-workers have shown that there may be no essential differences in the nitrogenous metabolism of dogs before and after splenectomy, and that the hæmopoietic functions are not seriously disturbed in splenectomized animals. Finally Frouin² has recently demonstrated that removal of the spleen from dogs with an isolated stomach does not apparently interfere with their nutrition even during a meat diet. The excellent nutritive condition of the splenectomized animals in which gastric digestion was excluded makes it improbable that pancreatic digestion failed; for there was no evidence of any deficiency in the digestive processes in the intestine. These facts do not, however, contradict the possibility of the formation of a trypsinogenic agent in the spleen. They merely indicate that the intestinal processes may proceed independently of the assumed rôle of the spleen in elaborating the trypsin.³ For the experiments of Chepowalnikoff⁴ and of Delezenne⁵ have indicated that the intestinal juice may also exercise an "activating" influence on pancreatic extracts. Here too the effective agent has the charac-

¹ NOËL PATON: *Journal of physiology*, 1900, xxv, p. 443; *ibid.* 1902, xxviii, p. 83. *Cf.* also MENDEL and JACKSON: *This journal*, 1900, iv, p. 163.

² FROUIN: *Comptes rendus de la Société de Biologie*, 1902, liv, p. 418.

³ *Cf.* GLEY: *Comptes rendus de la Société de Biologie*, 1902, liv, p. 419.

⁴ CHEPOWALNIKOFF: *Loc. cit.*; PAWLOW: *Le travail des glandes digestives*, Paris, 1901, p. 255; BELLAMY: *Journal of physiology*, 1901, xxvii, p. 332.

⁵ DELEZENNE: *Comptes rendus de la Société de Biologie*, 1901, liii, pp. 1161-1165; *ibid.*, 1902, liv, p. 281.

teristics of an enzyme and has been given the name of *enterokinase*. According to Delezenne it is found in various species of animals.

Thus it becomes clear, as Camus and Gley have lately pointed out, that the transformation of zymogen into trypsin *may* be brought about in at least two ways, viz., (1) by an intra-pancreatic process facilitated by the spleen (as suggested by Herzen) and (2) by an extra-pancreatic process facilitated by the intestinal secretion.¹ According to Wertheimer,² the pancreatic juice obtained from starving dogs reflexly after introduction of acid into the intestine does not contain preformed trypsin; whereas the enzyme is present in the secretion provoked by pilocarpine, even though the spleen may not be simultaneously congested. This fact also does not necessarily contradict the Schiff-Herzen hypothesis. For pilocarpine may have an action more or less analogous to that of the hypothetical splenic agent elaborated during normal digestion. Finally, even if the pancreas were at times found capable of transforming its tryptic zymogen into trypsin, the intervention of an agent such as the spleen has been assumed to elaborate might be an additional activating intra-pancreatic influence at those periods when a vigorous digestive secretion is required.

Conclusion. A review of the available experimental data fails to reveal evidence which absolutely controverts the Schiff-Herzen hypothesis that the spleen may yield an agent which can react directly within the living pancreas in elaborating trypsin. What physiological importance, if any, is attached under normal conditions to this reaction is at present uncertain. At any rate in the light of recent investigation it can no longer be accepted as the only factor in the conversion of the pancreatic zymogen. Favorable conditions for the activity of the pancreatic enzyme in the intestine are thus always assured.

¹ "Dès maintenant cependant il semble bien que l'on puisse dire qu'il existe deux processus de transformation du zymogène en trypsine, l'un intra-pancréatique, et l'autre extra-pancréatique, par l'action du suc intestinal (expériences de l'école de Pawlow); le second paraît d'ailleurs plus important que le premier. Reste qu'il faut se demander si dans la réalité ces deux processus peuvent se produire séparément ou s'ils ne sont pas toujours associés. Quoi qu'il en soit, par ce double mécanisme la digestion duodénale est toujours assurée." CAMUS and GLEY: *Comptes rendus de la Société de Biologie*, 1902, liv, p. 241; *cf.* also BELLAMY: *Loc. cit.*

² WERTHEIMER: *Comptes rendus de la Société de Biologie*, 1901, liii, p. 139; also CAMUS and GLEY: *ibid.*, p. 194.

APPENDIX.

Experiments in vitro. *a. Extracts of congested spleens.*

- I. Large dog, splenectomized one week before. Fed on lean beef for three days, then starved three days before the experiment. Two portions of pancreas (5 gms. each) were extracted at 39° C. for two hours with 25 c.c. saturated boric acid solution. Digestions at 39° C., as follows :

- (1) filtrate + 25 c.c. saturated boric acid solution,
 (2) filtrate + 25 c.c. boric acid extract of *congested* spleen.
 7 gms. of fibrin in each trial.

In ten hours digestion was apparent in (2), not in (1). At the end of twenty-four hours the residues were weighed :

- (0) 2.472 gms.,
 (1) 1.690 gms., or 68.3%,
 (2) 1.299 gms., or 52.5%.

- II. Large bitch, splenectomized one week before. Feeding, etc., as in Exp. I. Two portions of pancreas (4 gms. each) were extracted. Digestion trials as in Exp. I., 5 gms. of fibrin being used in each. Within twelve hours most of the fibrin was digested in (2), very little dissolved in (1). Residues filtered off after fourteen hours :

- (0) 1.850 gms.,
 (1) 1.513 gms., or 81.7%,
 (2) 0.619 gm., or 33.4%.

b. Extracts of contracted spleens.

- III. Normal dog. Starved three days. Pancreas extracted for two hours with 100 c.c. boric acid solution. Contracted spleen of the same dog extracted for three hours with 100 c.c. boric acid solution.

- (1) 25 c.c. boric acid solution, }
 (2) 25 c.c. spleen extract, }

and 30 c.c. pancreas extract and 8 gms. fibrin in each. Residue filtered at the end of sixteen hours.

- (0) 2.410 gms.,
 (1) 0.590 gm., or 24.5%,
 (2) 0.594 gm., or 24.6%.

- IV. Cat, splenectomized one week before. Fed on milk, then starved three days. The pancreas (8 gms.) was extracted for two hours with boric acid solution. Splenic extract was prepared by extracting the contracted spleen of another starving cat with 75 c.c. boric acid solution for three hours. Each digestion trial contained 25 c.c. pancreas extract, 5 gms. of fibrin and

- (1) 25 c.c. boric acid solution,
- (2) 25 c.c. spleen extract.

Residues filtered at the end of eighteen hours :

- (0) 1.588 gms.,
- (1) 0.876 gm., or 55.2%,
- (2) 0.866 gm., or 54.6%.

c. Activity of different parts of the same pancreas.

V. Small dog, splenectomized one week before. Fed three days, then starved three days. Five gms. of pancreas from (1) "head" and (2) "tail" of the organ were each extracted with 50 c.c. boric acid solution for two hours. To the filtrates 5 gms. of fibrin were added in each case. Control trial (0) with boric acid and fibrin alone. Residues filtered off after twenty-two hours :

- (0) 1.438 gms.,
- (1) 1.004 gms., or 69.8%,
- (2) 1.008 gms., or 70.1%.

Experiments in vivo. *a. Extracts of congested spleens.*

VI. Small dog, splenectomized two and one half months before. Starved three days before the experiment. Ninety c.c. of saline extract of congested spleen injected after removal of first portion of the pancreas. Five-gm. fractions of the pancreas portions were extracted with 50 c.c. boric acid solution at 39° C. for two hours. To equal portions of the filtrate were added 5 gms. of fibrin. Digestion at 39° C. In three hours beginning of digestion was evident in (2), not in (1). At the end of ten hours the residues were weighed :

- (0) 1.269 gms.,
- (1) 0.635 gm., or 51.0%,
- (2) 0.332 gm., or 26.1%.

VII. Large old dog, splenectomized one and one half weeks before. Fed on dog biscuit and starved two days before experiment. Injection fluid = 100 c.c. saline extract of a congested spleen. The animal died within eight minutes after the injection. Seven-gm. portions of pancreas used, as in VI. Six gms. of fibrin added and allowed to digest for four hours. Residues filtered :

- (0) 2.076 gms.,
- (1) 0.449 gm., or 21.6%,
- (2) 0.398 gm., or 19.2%.

VIII. Large dog, splenectomized one week before. Fed on lean meat three days, then starved three days. No morphine. Injection of 100 c.c.

saline extract of a congested spleen. Five-gm. portions of pancreas used, as in VI. Seven gms. of fibrin added to each trial. Visible signs of digestion in (2) in eleven hours; in (1) in sixteen hours. After twenty-four hours the residue was filtered off:

(0)	2.472 gms.,
(1)	1.690 gms., or 68.3%,
(2)	1.550 gms., or 62.7%.

IX. Large bitch, splenectomized one week before. Fed as in VIII. Injection of 125 c.c. saline extract of congested spleen. Four-gm. portions of pancreas used, as in VI. Five gms. of fibrin added. After twelve hours digestion was beginning in (1) while in (2) the greater part was dissolved. Residues filtered after fourteen hours:

(0)	1.850 gms.,
(1)	1.512 gms., or 81.7%,
(2)	0.477 gm., or 25.7%.

X. Small young bitch, splenectomized one week before. Fed as in VIII, then given a pound of lean meat six hours before the experiment. Injection fluid as in VIII. (Congested spleen.) Five-gm. portions of pancreas used as in VI. Five gms. of fibrin added to each. Residues filtered after 8 hours:

(0)	1.594 gms.,
(1)	1.199 gms., or 75.1%,
(2)	0.406 gm., or 25.5%.

XI. Dog, splenectomized twenty-five days before. Diet, etc., as in X. Seven gms. of fibrin used in each digestion. Within four hours (2) was about half dissolved. Residues filtered after five hours:

(0)	1.817 gms.,
(1)	0.975 gm., or 53.6%,
(2)	0.510 gm., or 28.0.

XII. Small young dog, splenectomized eighteen days before. Diet, etc., as in X, 80 c.c. of spleen extract being injected. Three-gm. portions of pancreas were used and 7 gms. of fibrin were added to each digestive trial. In three hours digestion was evident in (2). Residues filtered after six hours:

(0)	2.040 gms.,
(1)	1.072 gms., or 52.5%,
(2)	0.586 gm., or 28.7%.

XIII. Large bitch, splenectomized three and one half weeks before. Starved two days and fed dog biscuit six hours before the experiment. Injection of 150 c.c. saline extract of congested spleen. Five-gm. portions of pan-

creas used; 7 gms. of fibrin in the digestion trials. Residues filtered after ten hours:

- (0) 2.211 gms.,
- (1) 1.440 gms., or 65.1%,
- (2) 0.567 gm., or 25.4%.

b. Control experiments.

XIV. Small dog, splenectomized five days before. Starved three days before the experiment, which was the same as XIII, except that 0.7 per cent NaCl solution was injected in place of spleen extract. Residues filtered after four hours:

- (0) 1.533 gms.,
- (1) 0.760 gm., or 49.6%,
- (2) 0.765 gm., or 49.8%.

XV. Small dog, splenectomized two weeks before. Experiment like XIV, 100 c.c. salt solution being injected. Five-gm. portions of pancreas used; 5 gms. of fibrin in each trial. Residues were filtered off in four hours:

- (0) 1.621 gms.,
- (1) 0.452 gm., or 27.8%,
- (2) 0.450 gm., or 27.7%.

XVI. Dog, splenectomized one week before. Fed dog biscuit and meat, then starved two days. Injection of 100 c.c. saline (0.7 per cent NaCl) extract of *fresh liver*. Five-gm. portions of pancreas used; 5 gms. fibrin added. Residues filtered after twelve hours:

- (0) 1.510 gms.,
- (1) 1.135 gms., or 75.2%,
- (2) 1.137 gms., or 75.3%.

XVII. Dog, splenectomized two and one half weeks before. Diet, etc., as in XVI, meat being fed seven hours before the experiment. Injection of 100 c.c. saline extract of *dog's pancreas*. Five-gm. portions of pancreas used; 7 gms. of fibrin added in each trial. Residues filtered off after eight hours:

- (0) 2.211 gms.,
- (1) 0.619 gm., or 28.0%,
- (2) 0.549 gm., or 24.8%.

XVIII. Small bitch, splenectomized twenty-four days before. Starved two days and fed lean meat six hours before the experiment. Injection of 100 c.c. of extract of congested spleen which had been *boiled* for ten minutes and filtered. Five gm. portions of pancreas used; 7 gms. of fibrin added to each trial. Residues filtered off after four hours:

- (0) 2.162 gms.,
- (1) 0.623 gm., or 28.8%,
- (2) 0.661 gm., or 30.5%.

c. Injection of splenic blood.

XIX. Dog, splenectomized six days before. Starved two days before the experiment. Injection of 50 c.c. of defibrinated blood collected from the vein of a congested spleen. Digestion conditions as in XVIII. Residues filtered off after four hours :

(0)	2.249 gms.,
(1)	0.948 gm., or 42.1%,
(2)	0.727 gm., or 32.3%.

XX. Large bitch, splenectomized three weeks before. Starved two days before the experiment. Experimental conditions as in XIX, 40 c.c. of splenic vein blood being injected. Residues filtered off after eight hours :

(0)	2.225 gms.,
(1)	0.579 gm., or 26.0%,
(2)	0.505 gm., or 22.7%.

d. Influence of heat on splenic extracts.

XXI. Compare Experiment XVIII.

e. Influence of alcohol on splenic extracts.

XXII. Small dog, splenectomized two weeks before. Starved twenty hours and fed lean meat six hours before the experiment. Injection fluid prepared by precipitation of splenic extracts with alcohol, etc., as described in the text. 100 c.c. of saline extract of the alcohol precipitate were used. Digestions, etc., as in Experiment XVIII. Residues filtered off after four hours :

(0)	1.977 gms.,
(1)	0.761 gm., or 38.4%,
(2)	0.526 gm., or 26.6%.

XXIII. Small bitch, splenectomized fifteen days before. The injection extract was prepared as in Experiment XXII, except that the exposure to the action of alcohol was shorter. Eighty c.c. were used. Four-gm. portions of pancreas were employed; 7 gms. of fibrin added to each trial. Residues filtered off after ten hours :

(0)	2.040 gms.,
(1)	0.910 gm., or 44.6%,
(2)	0.606 gm., or 29.7%.

FURTHER EXPERIMENTS ON THE ANTITOXIC EFFECT OF IONS.

By HUGH NEILSON.

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PROFESSOR LOEB has shown in his work on the embryos of *Fundulus*, as well as in his work on muscular tissue,¹ that the toxic effect of a pure solution of NaCl, LiCl, NH₄Cl, or KCl can be annihilated by traces of certain salts with a bivalent or trivalent kation; also that large amounts of the salts with monovalent kation will have the same effect.

If small amounts of these salts with a bivalent or trivalent kation be added to a $\frac{5}{8}$ m NaCl, LiCl, etc., solution, the number of embryos developing is greatly increased. In these experiments the aim has been to apply the facts already worked out by Professor Loeb to the prolongation of the life of muscular tissue.

METHODS.

The gastrocnemius muscle of winter frogs was used. The muscles were always used in pairs, one muscle being placed in a Stender dish containing 100 c.c. of the solution whose toxic effect was to be determined. The other muscle was placed in 100 c.c. of the same solution, to which was added traces of the salts whose ions were to be tested for their antitoxic effect.

The dishes containing the muscles and the solutions were placed in a sink and running water was allowed to flow around them, in order to keep the temperature low and constant.

To determine the time of death electrical stimuli were applied. In testing the muscle it was first carefully dried by filter paper and then the electrodes of an induction coil were placed directly on the muscle tissue. A preliminary experiment was made in each case to find out when the muscles would commence to die in a given solution. This was taken as a basis to commence the testing of the muscle in the final experiment, so as to avoid unnecessary fatigue. The muscles

¹ LOEB: This journal, 1902, pp. 411-433.

were all treated exactly alike in handling, testing, etc. The increase in length of life of the muscle in the solution containing the antitoxic ion was calculated in per cent of the total life of the other muscle in the solution whose toxic effect was to be tested.

ANTITOXIC KATIONS.

I. Antitoxic effects of monovalent kations. — To 100 c.c. $\frac{m}{8}$ NaCl was added $\frac{1}{2}$, 1, 2, 4, 8 c.c. of $\frac{m}{16}$ or $\frac{m}{8}$ LiCl, NH_4Cl , or KCl. These salts in such small amounts had no antitoxic effect, as the life of the muscle was not prolonged. Even on the addition of LiCl, which is least poisonous, there is no effect. In the solution containing the KCl the life of the muscle was even lessened, due no doubt to the well-known poisonous effect of the K-ion on muscular tissue. The NH_4Cl is more poisonous than LiCl, but less so than KCl, yet it had no antitoxic effect. Larger amounts and stronger concentrations were next tried and positive results were obtained.

In 100 c.c. $\frac{m}{4}$ NaCl + 10 c.c. $\frac{m}{4}$ LiCl the muscle lived 15 per cent longer than in the pure NaCl solution. In 100 c.c. $\frac{m}{4}$ NaCl + 25 $\frac{m}{4}$ LiCl the muscle lived longer than in a pure NaCl solution, but died sooner than in the solution of NaCl plus only 10 c.c. $\frac{m}{4}$ LiCl. In 100 $\frac{m}{4}$ LiCl + 10 c.c. $\frac{m}{4}$ NaCl the muscle lived 20 per cent longer, and about 8 per cent longer in 100 c.c. $\frac{m}{4}$ LiCl + 25 $\frac{m}{4}$ NaCl than in the pure LiCl solution. This is in keeping with the fact found by Loeb that it requires stronger solutions of the salts with monovalent kation than of the salts with bivalent or trivalent kation to produce antitoxic effects.

II. Antitoxic effect of bivalent kations. — If to a pure solution of NaCl a trace of CaCl_2 be added, the life of the muscle is prolonged, as is shown by the following table:

Solution.	Percentage increase in length of life.
100 c.c. $\frac{m}{4}$ NaCl + $\frac{1}{10}$ c.c. $\frac{m}{32}$ CaCl_2	0
100 c.c. $\frac{m}{4}$ NaCl + $\frac{1}{8}$ c.c. $\frac{m}{32}$ CaCl_2	0
100 c.c. $\frac{m}{4}$ NaCl + $\frac{1}{4}$ c.c. $\frac{m}{32}$ CaCl_2	11
100 c.c. $\frac{m}{4}$ NaCl + $\frac{1}{2}$ c.c. $\frac{m}{32}$ CaCl_2	22
100 c.c. $\frac{m}{4}$ NaCl + 1 c.c. $\frac{m}{32}$ CaCl_2	23
100 c.c. $\frac{m}{4}$ NaCl + 2 c.c. $\frac{m}{32}$ CaCl_2	35
100 c.c. $\frac{m}{4}$ NaCl + 4 c.c. $\frac{m}{32}$ CaCl_2	22
100 c.c. $\frac{m}{4}$ NaCl + 8 c.c. $\frac{m}{32}$ CaCl_2	24

The antitoxic effect of SrCl_2 and MgCl_2 was approximately the same as that of CaCl_2 when added to NaCl solution, but BaCl_2 has very little effect.

If CaCl_2 , SrCl_2 , and MgCl_2 be added to KCl as the toxic solution instead of NaCl , the life of the muscle is prolonged from 20 to 50 per cent. BaCl_2 added to a KCl solution gives a much more decided result, the increase in life of the muscle being from 150 to 250 per cent, as the following table shows :

Solution.	Percentage increase in length of life.
100 c.c. $\frac{m}{8}$ KCl + 2 c.c. $\frac{m}{32}$ BaCl_2	250
100 c.c. $\frac{m}{8}$ KCl + 8 c.c. $\frac{m}{32}$ BaCl_2	150

CaCl_2 , BaCl_2 , and SrCl_2 , added to NH_4Cl , prolong the life from 15 to 20 per cent. With MgCl_2 , while it prolongs the life of the muscle, the per cent increase is not so high as is the case with CaCl_2 , SrCl_2 , and BaCl_2 .

MgCl_2 , SrCl_2 , CaCl_2 , added to LiCl , prolong the life from 10 to 60 per cent. BaCl_2 , on the other hand, has very little antitoxic effect on the poisonous effect of LiCl .

The salts NaCl , LiCl , NH_4Cl , and KCl , according to their toxic effect on muscle, fall into two classes: (1) NaCl and LiCl are the least poisonous; (2) NH_4Cl and KCl are much more so, KCl being especially poisonous to muscle tissue. The effect of BaCl_2 seems to bear some relation to this division. BaCl_2 added to pure solutions of NaCl or LiCl has no antitoxic effect, while on the other hand, if added to NH_4Cl , it prolongs the life of the muscle, and to a much higher degree when added to KCl , the most poisonous of these salts. This seems to be due to some specific ion effect and not to an antitoxic effect due to valency and electric charge of the ion.

$\frac{m}{16}$ CaCl_2 added to a pure NaCl solution prolonged the life of the muscle from 10 to 38 per cent. It also did the same when added to a LiCl -, NH_4Cl -, and KCl -solution.

ZnCl_2 , $\text{Pb}(\text{NO}_3)_2$, and $\text{Cd}(\text{NO}_3)_2$, added to pure solutions of NaCl , LiCl , NH_4Cl , or KCl , gave antitoxic effects, but the increase in the duration of life was not great. This may be due to the fact that these salts are very poisonous, and hence can only be used in weak concentrations. One to two c.c. of $\frac{m}{56}$ added to the pure solutions of the above salts give the best effect.

III. Antitoxic effect of trivalent kations. — If to 100 c.c. of $\frac{m}{4}$ NaCl is added a trace of $\frac{m}{84}$ AlCl_3 , the muscle lives from 33 to 60 per cent longer. $\frac{m}{84}$ $\text{Cr}(\text{NO}_3)_3$ prolongs the life from 5 to 28 per cent, although the results vary considerably in the different experiments. With $\text{Cr}(\text{NO}_3)_3$ the results were all positive, *i. e.*, it prolonged

the life of the muscle. FeCl_3 , on the other hand, while it gave some positive results, also gave results which were negative. The specific chemical action of the ferri ion seems stronger than the antitoxic effect due to its valency. The antitoxic effect of AlCl_3 , $\text{Cr}(\text{NO}_3)_3$, and FeCl_3 , when added to LiCl , is approximately the same as their effect on NaCl solution. When added to NH_4Cl or KCl solution, very little antitoxic effect is shown by the trivalent Al , Cr , or Fe ion, possibly due to the NH_4 and K ion being more poisonous than the Li and Na ion.

ANTITOXIC EFFECT OF ANIONS.

Na_2SO_4 was first tried. It gave no definite results on NaCl and LiCl , but on KCl the results were generally positive, although the percentage of increase in length of life of the muscle was not so high as in the other experiments, where kations were used, being only from 5 to 15 per cent. There were some positive results with NH_4Cl , but they still more varied, and the percentage was less than with KCl .

Na_3 citrate was next tried. It had no positive effect on NaCl and LiCl , but its effect on NH_4Cl and KCl was decidedly positive, although the percentage of increase was not high. These experiments again show that NaCl and LiCl fall in one class, and NH_2Cl and KCl in a second class, probably in some way connected with the more poisonous effects of the NH_4 and K ion.

CONCLUSIONS.

Small amounts of salts with mono-, bi-, or trivalent kations diminish the poisonous effects of pure NaCl , LiCl , NH_4Cl , or KCl solutions.

For salts with monovalent kation 10 c.c. $\frac{m}{8}$, for salts with bivalent kation 1 c.c. of $\frac{m}{32}$, and for salts with trivalent kation, 1 c.c. of $\frac{m}{84}$ added to 100 c.c. $\frac{m}{8}$ or $\frac{m}{4}$ of NaCl , LiCl , NH_4Cl , or KCl gave the best results.

Sodium salts with anions of higher valency when added to solutions of NaCl or LiCl have no antitoxic effect, but have a slight antitoxic effect when added to NH_4Cl or KCl .

My thanks are due Professor Loeb for suggesting this work and for his help in carrying out these experiments.

NOTE UPON THE EFFECT OF CALCIUM AND OF
FREE OXYGEN UPON RHYTHMIC CONTRACTION.

BY S. S. MAXWELL AND J. C. HILL.

THE fact that in the tissues substances occur the solubility of which is affected by the presence or the absence of oxygen has been proved by Loeb. He found that in *Ctenolabrus* eggs the cell wall dissolved in the absence of oxygen and reappeared when the oxygen was restored.¹ In certain infusoria liquefaction of the cell membrane follows deprivation of oxygen.² A muscle deprived of oxygen absorbs more water than a normal muscle of equal weight.³ Similar observations on increased solubility in the absence of free oxygen have been made by Kühne and others.

The existence of compounds of the metallic ions with the proteids has been argued by Loeb,⁴ and a considerable amount of experimental evidence has been presented by Lillie in his investigations on the effects of various salt solutions on ciliary and muscular action.⁵ Loeb has called attention to the similarity in the absorption of liquids by muscle and soaps. Potassium soaps are exceedingly soluble; sodium soaps are also quite soluble, but much less so than potassium soaps. Calcium soaps are quite insoluble. If the sodium in a sodium soap is replaced by calcium, water is given up. If calcium is replaced by sodium, water is absorbed. A perfect analogy exists between the absorption of water by muscle in isotonic solutions of KCl, NaCl, and CaCl₂ and the absorption by K, Na, and Ca soaps. Thus he found that a muscle remaining eighteen hours in a KCl solution increased in weight 45.7 per cent; in an equimolecular solution of NaCl there was an increase of 6 per cent; in CaCl₂ there was a decrease of 20 per cent in the same time.⁶

¹ LOEB: *Archiv für die gesammte Physiologie*, lxii, p. 249.

² BUDGETT: *This journal*, i, p. 210.

³ LOEB: *Archiv für die gesammte Physiologie*, lxxi, p. 471.

⁴ LOEB: *Festschrift für Fick*, Braunschweig, 1899.

⁵ LILLIE: *This journal*, vii, p. 25.

⁶ LOEB: *Archiv für die gesammte Physiologie*, lxxv, p. 303.

It was the good fortune of one of us to witness certain experiments about to be published by Dr. Lingle, in which, after the exhaustion of a strip of ventricular muscle in a pure NaCl solution, he succeeded in securing a recovery of the rhythmic action by the simple addition of a supply of free oxygen to the pure NaCl solution, and the recovery was as complete, as vigorous and as long lasting as the recovery in Ringer's fluid or in a NaCl and CaCl₂ mixture. It seemed possible that the similarity between the effect of free oxygen and of a trace of calcium in the solution might in each case be due in part to a decrease of solubility of some substance in the tissue; and it occurred to us to compare the effect of calcium and of free oxygen in another way; namely, to see if the toxic effect of a calcium solution could be increased or decreased by the presence or absence of free oxygen. Our experiments herein described will show that this is indeed the case.

For our purpose it was necessary to employ a tissue which would remain active for some time in a pure $\frac{1}{8} n$ CaCl₂ solution. In some preliminary work we found that the ciliated cells of the frog's œsophagus would continue to move in such a solution for a little less than one day. We then undertook to discover what effect the presence or absence of oxygen would have on the duration of ciliary movement.

The method of the experiments was as follows :

The solutions were made from the chemically pure CaCl₂ with water redistilled in glass. The solutions marked "Oxygen" were saturated before each experiment by allowing a stream of washed oxygen gas to bubble through the liquid for some minutes. The solutions marked "Boiled" were boiled for a period of not less than fifteen minutes to expel the dissolved oxygen; the loss from evaporation was made good by the addition of the proper amount of thoroughly boiled distilled water, and the vessels were then kept closely stoppered. This method, of course, did not secure a solution perfectly free from dissolved oxygen, but the oxygen content of the two solutions was markedly different.

The experiments were always made in pairs in the following way: A frog was pithed and the œsophagus was dissected out and split into right and left halves. One half was put into an "oxygen" solution and the other into a "boiled" solution. Small Stender dishes holding about twenty-five cubic centimetres were used. These dishes had very closely fitting ground covers. Microscopic examinations were made from time to time by teasing off upon a slide small

portions of the ciliated epithelium. If the first preparation showed no ciliary motion a second mount was made, and so on, until it was certain that ciliary motion was present or absent.

A sample set of experiments is given in the following table:

Exp.	Boiled.			Oxygen.		
	24 hrs.	30 hrs.	42 hrs.	24 hrs.	30 hrs.	42 hrs.
1	Moving	Moving	Stopped	Moving	Stopped	
2	Moving	Moving	Moving	Moving	Stopped	
3	Moving	Stopped	Moving	Stopped	
4	Moving	Stopped	Moving	Stopped	
5	Moving	Stopped	Moving	Stopped	
6	Moving	Moving	Stopped	Moving	Stopped	
7	Moving	Moving	Stopped	Stopped		

For the whole series of experiments the average duration of ciliary activity in the "boiled" solution was thirty-one hours; in the "oxygen" solution, twenty hours.

The facts above presented have an important bearing upon all experiments on the action of solutions on the living tissues. In order that a trustworthy comparison be made between the action of different salt solutions upon similar tissues, or of one solution upon different tissues, more especial attention must be given to the degree of oxygen saturation of the liquids employed. It is not improbable that we have here an explanation of the apparently discordant results of competent observers. If some of the solutions are made up from freshly prepared or thoroughly boiled distilled water, and others from water which has been standing for some time in contact with air in a partially filled vessel, or if the process of solution has been hastened by violent shaking, a difference in the oxygen content of the liquids could exist sufficient to cause wide variations in the results.

ON THE OXIDATIVE PROPERTIES OF THE CELL-NUCLEUS.

BY RALPH S. LILLIE.

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I. INTRODUCTION.

THE earlier researches of Pflüger and Hoppe-Seyler have proved that the greater part of the oxidations in living animals take place not in the body-fluids but within the living tissues. Schultzen and Naunyn¹ proved in 1867 that certain aromatic hydro-carbons (benzol, toluol) may undergo oxidation in the animal body; Bunge and Schmiedeberg followed in 1876 with the demonstration of the synthesis of hippuric acid in the kidney. Schmiedeberg² proved in 1881 that salicylic aldehyde may be oxidized to salicylic acid and benzyl alcohol to benzoic acid by the action of certain tissues (especially the kidney and lung). Jaquet³ in 1892, continuing Schmiedeberg's researches, made the important discovery that the oxidative action of the tissues in these transformations is dependent neither upon the continuance of vital processes, nor upon the preservation of anatomical structure, but that it is conditional (at least in part) upon the presence of a soluble ferment-like body (or oxidase) which can be extracted by salt solutions from both fresh and alcoholic tissues. More recent researches, especially those of Pohl,⁴ Abelous and Biarnès,⁵ Salkowski,⁶ and Spitzer⁷ have confirmed Jaquet's observations and have established the further important fact that the

¹ SCHULTZEN and NAUNYN: Du Bois Reymond's Archiv, 1867, p. 349.

² SCHMIEDEBERG: Archiv für experimentelle Pathologie und Pharmakologie, 14, 1881, p. 288.

³ JAQUET: *Ibid.*, 29, 1892, p. 386.

⁴ POHL: Archiv für experimentelle Pathologie und Pharmakologie, 31, 1893, p. 281.

⁵ ABELOUS et BIARNÈS: Archives de physiologie normale et pathologique (5), 7, 1895, p. 239, and 10, 1898, p. 664.

⁶ SALKOWSKI (mit JAMAGIWA): Virchow's Archiv, 147, 1897, p. 1.

⁷ SPITZER: Archiv für die gesammte Physiologie, 60, 1895, p. 303.

various tissues exhibit definite and constant differences among themselves in degree of oxidative power, this power being most marked in such organs as the spleen, liver, or kidney, which are characterized by unusual synthetic and metabolic activity, and least marked in nervous and muscular tissue. Finally Spitzer¹ in 1897 showed that certain nucleo-proteids from the liver, kidney, thymus, and blood-corpuscles possess the same oxidative properties as the original tissues; he accordingly referred oxidations to the nucleo-proteids of the cell. Shortly afterward Loeb² called attention to several facts³ which seemed to afford clear indication that the well-known absence of regenerative capacity in enucleated cells is due to a loss of the oxidative properties of the protoplasm; from these he drew the inference that the nucleus is the chief seat of oxidations in the living cell, and consequently also of the syntheses that depend upon these oxidations.⁴

II. METHODS.

The experiments about to be described have been made with the aim of testing the above hypothesis and of supplying, if possible, definite proof that the nucleus does actually play this rôle in the cell. To demonstrate the oxidative action of the tissues I have employed a variety of reactions by which colored oxidation-products may be formed within the tissues. Chief use has been made of a reaction first employed by Ehrlich⁵ in 1885 as an indicator of oxidations in the animal body, and more fully studied by Röhmann and Spitzer⁶ in 1895, viz.: the formation of indophenol, a deeply colored violet dye, by the oxidation of an alkaline aqueous solution containing small quantities of α -naphthol ($C_{10}H_7OH$) and para-diamido-benzene (or paraphenylene diamine: $C_6H_4(NH_2)_2$, 1, 4) in equimolecular proportions. This solution, at first colorless, becomes deeply tinted on oxidation. The reaction constitutes a true synthetic oxidation

¹ SPITZER: *Ibid.*, 67, 1897, p. 615.

² LOEB, J.: *Archiv für Entwicklungsmechanik*, 8, 1899, p. 689.

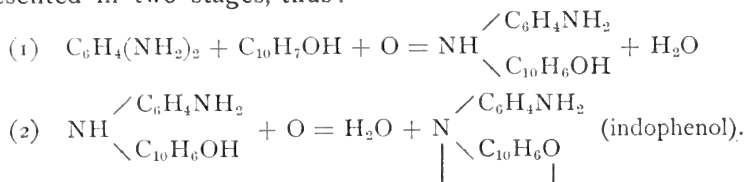
³ Especially certain physiological resemblances between enucleated cells and cells artificially deprived of oxygen.

⁴ For a more complete sketch of the history of research on the subject of oxidation in the animal body, see MEDWEDEW: *Archiv für die gesammte Physiologie*, 65, 1896, p. 249.

⁵ EHRLICH: "Das Sauerstoffbedürfniss des Organismus," Berlin, 1885.

⁶ RÖHMANN u. SPITZER: *Ber. d. deutsch. chem. Gesellschaft*, 1895, xxviii, p. 567.

in Schmiedeberg's sense,¹ condensation occurring with abstraction of water as a result of the entrance of the oxygen atom. It may be represented in two stages, thus:



Several other related color-reactions were also employed and are here briefly enumerated:

1. Oxidation of dimethyl p.-diamido-benzene with α -naphthol; result is an intense greenish blue dye.
2. Formation of quinone anilimide, a violet dye, by oxidation of phenol with p.-diamido-benzene.
3. Formation of the dimethyl derivative of the same compound, a deep blue dye, by oxidation of phenol with dimethyl p.-diamido-benzene.
4. Formation of phenylene blue by oxidation of aniline with p.-diamido-benzene.
5. Formation of the dimethyl compound of 4, a violet dye, by oxidation of dimethyl-aniline with p.-diamido-benzene.
6. Formation of "Bindschedler's green" by oxidation of dimethyl aniline with dimethyl p.-diamido-benzene.

For use with fresh tissues the above-named compounds were dissolved in physiological salt solution; in the case of alcoholic tissues the solvent was distilled water. The two constituents of each reagent were dissolved separately and the solutions mixed shortly before using.

Not all of the above oxidations are effected with equal readiness; the presence of the methyl groups appears to facilitate the reaction, and naphthol enters into such combinations more readily than phenol. The most sensitive reactions and those on which I have placed most reliance are thus the original indophenol reaction (α -naphthol and p.-diamido-benzene) and members 1 and 3 of the above group; the indamine reactions (members 4-6) have proved less serviceable although useful for purposes of control. The conditions of formation of all the above color-products are essentially alike; the following general statement while referring primarily to the first-named applies therefore with slight modifications to all.

¹ Cf. SCHMIEDEBERG: *Loc. cit.*

The formation of the indophenol dyes takes place slowly on exposure of the above solutions to the air, but is greatly accelerated by the addition of finely divided animal tissues. The tissue, if its oxidative activity is considerable (as is that of the spleen, kidney, liver, or lung) quickly becomes dyed an intense violet, and is soon surrounded by a cloud of the dissolved color-product which gradually imparts its tint to the rest of the solution. The formation of the dye is prevented by slight acidulation or by the presence of strongly reducing substances; the reaction is furthermore greatly retarded — although not entirely prevented — by the addition of certain poisons, especially hydrocyanic acid and its salts.

It has been shown¹ that the capacity of the tissues to form these colored synthetic products bears a direct relation to their capability of freeing oxygen from hydrogen peroxide, of bluing tincture of Guaiac, and of oxidizing salicylic aldehyde and benzyl alcohol to their respective acids. It thus seems probable that all of these oxidations are effected through the same agency, — namely, the freeing of active or atomic oxygen by ferment action.²

Further indications of the power of the tissues to liberate active oxygen are obtained by the use of alkaline solutions of pyrogallol or hydroquinone. The brown or orange-colored oxidation products appear most rapidly in those tissues which possess the most energetic oxidative activities. Similar indications may also be obtained by the use of solutions of Guaiac-tincture containing hydrogen peroxide. Such solutions immediately turn blue in tissues that free nascent oxygen.

The experiments about to be described have been made on the various organs and tissues of the frog, — liver, spleen, pancreas, lung, kidney, testis, ovary, the various regions of the intestinal tract, striated muscle, the various regions of the brain and spinal cord, and certain lymphatic structures, especially the thymus and the ventral “gill-vestiges.” The chief method has been to subject thin slices of alcoholic and fresh tissues (made freehand and with the freezing microtome respectively) to the action of the above solutions; control sections were dyed at the same time with methyl-green so as to afford an indication of the quantity and distribution of the nuclear matter in the respective tissues employed. The sections were left in the solutions for variable periods (fifteen minutes to several hours) until sufficiently

¹ Cf. SALKOWSKI, SPITZER, and ABELOUS and BIARNÈS, *loc. cit.*

² For a full discussion of this question see SPITZER, *loc. cit.*

impregnated with the colored oxidation products; they were then removed and examined; the distribution of the color could then be studied under any desired magnification.

In general it has been observed that the above oxidations take place most rapidly in those organs, and in those regions of organs, which contain the most numerous and most densely aggregated nuclei. In structures like the testis, intestine, kidney, etc., in which the nuclei show a very definite arrangement, the regions most deeply impregnated with the dye are always found to correspond closely with the regions in which the nuclei are chiefly situated, so that methyl-green sections and indophenol sections show in general a marked similarity with respect to the intensity and distribution of their respective stains. Indophenol, however, is not a differential nuclear stain, and its distribution in the sections is comparatively diffuse, — not sharply defined like that of the methyl-green. In sections of fresh tissues the localization of the dye is somewhat less precise than in alcoholic sections, a circumstance due probably to the readier diffusion of the oxidase into the solution from the uncoagulated tissues. Fresh tissues differ also from alcoholic tissues in possessing distinctly greater oxidative activity; in other respects the properties of the two differ but slightly under the above conditions of experiment.

III. OXIDATIVE PROPERTIES OF VARIOUS TISSUES.

The following is a summary account of the chief facts as yet ascertained by use of the above methods. The different regions of the alimentary canal, the spleen, kidney, liver, and blood-corpuscles will be considered in order.

The differences in the oxidative activities of the various elementary tissues are illustrated in a striking manner on placing cross-sections of the stomach-wall in the solution of *a*-naphthol and *p*-diamidobenzene. The mucous membrane soon assumes an intense violet coloration; this is most marked in the gastric glands which are thus sharply differentiated from the neighboring structures. The cells of the mucous epithelium also become deeply colored; the coloration is particularly intense at their inner ends, *i. e.*, the nuclear region, while the outer ends remain clear and only slightly stained. This peculiarity of the cells of columnar epithelium may also be observed in other regions, *e. g.* œsophagus and bile-duct. The muscular layers

take on a diffuse and relatively slight coloration, in accordance with the generally observed feeble oxidative activity of muscle; the connective tissue portions of the submucosa remain almost uncolored.

The above appearances are typical with the indophenol reactions. Immersion of thin sections of stomach in alkaline pyrogallol or hydroquinone solutions is similarly followed within a short period by the appearance of the characteristic brown or orange oxidation-products within the tissues. Their distribution is essentially as described above, coloration appearing first in the mucosa, especially in the gastric glands and the inner ends of the columnar epithelial cells.

Cross sections of other regions of the intestine (tongue, œsophagus, intestine, rectum) show a similar distribution of the indophenol coloration. In all these regions the mucosa colors soonest and most deeply; while muscular layers and submucosa assume a relatively slight stain. In the tongue the deep coloration of the lingual glands is noteworthy; the tall columnar cells of the ventral surface of this organ likewise color deeply, especially at their inner ends where the nuclei are situated. Similar conditions obtain in the œsophagus, oxidation being most active in the glands and in the mucosa; here also the position of the nuclei of the mucous epithelium is indicated by the deeper coloration of the inner ends of the cells. The same is very generally true of the mucosa in the small intestine and rectum, but neither here nor in the œsophagus is this appearance so well-defined as in the elongated columnar cells of the gastric epithelium. A similar appearance, however, is very definite in cross sections of the bile-ducts; the columnar epithelial cells stain deeply at their inner extremities and remain clear and almost unstained towards the lumen of the duct. In the cells of the intestinal mucosa the individual nuclei are usually indistinguishable; but in the gastric gland-cells the indophenol is frequently seen to be deposited largely in the form of granules surrounding a clear round central area which evidently corresponds to the nucleus. This peculiarity in the distribution of the oxidation-products is however more clearly exhibited in certain other tissues (see below).

It is noteworthy that in all regions of the intestinal tract the gland-cells exhibit an unusually powerful oxidative action; their intense stain always renders these cells highly conspicuous in indophenol sections. Apparently glandular activity and power of freeing active oxygen are closely related physiological characteristics; this is seen also in the case of the kidney and liver. The fact is of interest as

affording further indication of the close relation apparently existing between oxidations and syntheses in the animal body.

Indophenol sections of the small intestine and rectum invariably show numerous small intensely stained patches consisting of aggregations of indophenol granules situated chiefly in the submucosa immediately within the mucosa, and frequently between the epithelial cells. These evidently correspond to leucocytes which, as will be seen below, possess marked oxidative activity.

The spleen exhibits a remarkably intense oxidative activity, sections of this organ invariably becoming deeply colored after a few minutes' immersion in any of the above solutions. Microscopic examination shows that the entire pulp becomes deeply impregnated with the indophenol; the distribution of the dye is however not uniform; at intervals isolated areas are seen which are prominent from the unusual intensity of the stain. These evidently correspond to the aggregations of leucocytes or so-called Malpighian corpuscles.

Sections of lymphoid structures (such as the adult thymus and the ventral branchial vestiges) show an almost equally intense activity; in structure these organs (especially the latter) present considerable resemblance to the spleen; the nuclei are numerous, deeply staining, and densely aggregated.

Sections of the kidney are also extremely active, although somewhat less so than those of the spleen. In a mixture of α -naphthol and p.-diamido-benzene the tubules quickly assume a deep violet tinge; the Malpighian capsules, on the contrary, stain relatively slightly—a fact of some significance as affording additional indication of the respective physiological rôles of these two components of the tubule. Under high magnification it is seen that the nucleus of the tubule-cells remains comparatively clear and uncolored, and that the coloration of the cytoplasm is diffuse, but *typically deeper in the immediate neighborhood of the nucleus than elsewhere*—a clear indication that oxidations are especially active at the nuclear surface. With solutions 1, 2, 3, and 4 of the above series the stain is also seen very clearly to be deposited within the cytoplasm largely in the form of granules which are especially numerous on the surface of the nucleus; this structure itself remains clear and almost unstained.

Sections of the liver also give rise to very significant appearances. This organ is likewise very active in accelerating the above oxidations, although somewhat less so than the spleen. The following record is taken directly from my notebook with merely verbal modifications.

Thin slices of alcoholic liver were placed in Solutions 1, 2, 3, and 5 of the above series at 11.40 A. M., January 9. At 11.45 the sections in Solution 1 (*a*-naphthol and dimethyl *p*-diamido-benzene) are already distinctly greenish. Under high powers the color is seen to be aggregated chiefly in blotches *between* the liver-cells (*i. e.*, in the capillaries). At 12.05 P. M. examination of another section shows in addition that the liver-cells have themselves become stained, and that in the centre of many the green stain, although as yet faint, is aggregated as if surrounding the nucleus. In Solution 3 (phenol and dimethyl *p*-diamido-benzene) at 12.30 a similar aggregation of the stain in the centres of the cells is plainly observable under moderate magnifications.

At 2.42 P. M. the sections in Solution 1 are more deeply stained; the distribution of the stain remains the same as before, and its aggregation in and about the nucleus is unmistakable. In Solution 5 (dimethyl aniline and *p*-diamido-benzene) the distribution of the color-product shows similar relations to the nucleus, but the stain is too faint to be satisfactory. In Solution 3 (phenol and dimethyl *p*-diamido-benzene) the nucleus is sharply defined by the distribution of the blue stain; the stain is *in* the nucleus as well as aggregated in granules at its surface. In Solution 2 (phenol and *p*-diamido-benzene) the stain is not so deep as in 3; in other respects conditions are similar, the granules of color-product being aggregated chiefly in and about the nucleus.

The experiment was also made of running alkaline pyrogallol solution under the cover-glass of a mounted preparation; the browning of the liver-cells is gradual, but is clearly seen to begin at the nucleus, which in a few minutes becomes deep yellow in color. The cell-body also gradually assumes this tint, especially near the nucleus. With Solution 1 (*a*-naphthol and dimethyl *p*-diamido-benzene) similar appearances result; the color appears slowly in the cells, soonest in the nucleus which within half an hour is found to have assumed a distinctly greenish tinge.

The above experiments seem to leave little doubt that in the liver-cells at least the above oxidations are effected chiefly through the intermediation of the nucleus. An even more striking demonstration of the same fact is furnished by the use of leucocytes, which are readily obtainable in large numbers by fine subdivision of the spleen, thymus or ventral gill-vestiges. Many of these cells ("lymphocytes") consist essentially of free nuclei with merely a thin surface film of cytoplasm; their oxidative activity is remarkably energetic. When physiological salt solution containing such cells is added to the alkaline solution of *a*-naphthol and *p*-diamido-benzene the fluid in a few minutes assumes an intense violet coloration. If then the cells are examined

the dye is found to be largely distributed in granules on the surface of the nuclei. The most striking and unequivocal demonstrations are made by the use of Solution 1 above (*α*-naphthol and dimethyl p.-diamido-benzene). When this solution is introduced beneath the cover-glass of a fresh preparation of teased thymus or spleen, granules of the intense greenish blue oxidation-product shortly make their appearance within the leucocytes. Their first appearance is typically at the boundary between nucleus and cytoplasm; eventually

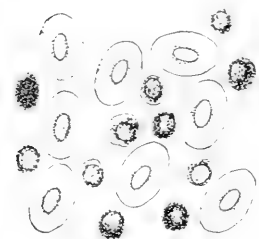


FIGURE 1. — Blood-corpuscles of frog showing distribution of granules of oxidation-product.

the latter may become so densely laden as completely to obscure the nucleus (Fig. 1). Frequently a single preparation presents all gradations between leucocytes on whose nuclei only a few granules have been deposited, and cells so densely laden as to present the appearance of mere aggregations of intensely colored granules similar to those seen in the intestinal submucosa.

Erythrocytes possess less energetic oxidative properties than leucocytes; granules of the stain appear more gradually and less abundantly in the cytoplasm; here also they are deposited especially in the immediate vicinity of the nucleus. This appearance is strikingly uniform; the preparation, after the lapse of thirty minutes, typically exhibits the appearance presented in the figure; in each erythrocyte the contour of the nucleus is distinctly marked by the deposition of minute intensely colored granules of the oxidation-product.

IV. GENERAL CONCLUSIONS.

The above facts furnish, it is believed, conclusive evidence that in many tissues the nucleus is the chief agency in the intracellular activation of oxygen; and, further, that the active or atomic oxygen is in general most abundantly freed at the surface of contact between nucleus and cytoplasm. If this relation of the nucleus to oxidation is a constant characteristic of the cellular organization — as seems probable — the inference is clear that the oxidative activities of organs must be largely a function of their extent of nuclear surface; the same conclusion applies also to synthetic processes in so far as these are dependent upon oxidations. The foregoing facts are plainly in harmony with this conclusion; a similar significance in all proba-

bility attaches to the large surface-extent presented by the branched nuclei of many invertebrate gland-cells.

The disposition or arrangement of the nuclei, quite apart from their number and united surface-areas, may also be of great importance in relation to the physiological activity of the organ. Thus it appears probable that the intense oxidative activity of the intestinal mucosa in both stomach and intestine, evidently a property of great physiological importance, is conditional in large part upon the prevailing columnar arrangement of the epithelial cells. The nuclei of these cells are situated near their inner ends; hence the nuclei of adjacent cells are almost in contact with one another. It is clear therefore that all substances absorbed by the intestine must of necessity traverse a surface in which they are exposed to the oxidative action of a layer of closely packed nuclei. It is thus inevitable that oxidations and their correlated syntheses should take place in the mucosa during absorption whenever the intestinal contents include substances capable of undergoing such changes. A columnar epithelium is very general in absorptive and secretory structures and it is possible that its physiological properties are closely dependent upon the peculiar distribution of nuclear matter resulting from such an arrangement of the cells.

SUMMARY.

1. A general similarity exists between the distribution of nuclear matter in the various tissues of the frog and the distribution of colored synthetic products formed within the same tissues by their oxidative action.

2. In a number of instances (especially liver- and kidney-cells and blood corpuscles) the colored oxidation products are seen to be deposited chiefly in and about the nucleus, especially at the surface of contact between nucleus and cytoplasm.

THE NUCLEOPROTEID OF THE SUPRARENAL GLAND.

BY WALTER JONES AND G. H. WHIPPLE.

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THE great variation in the relative quantities of xanthine bases which are produced by the hydrolysis of nucleic acids finds a rational explanation in Kossel's¹ assumption that there are four nucleic acids each of which yields only one base. If this view be accepted it follows directly that these four nucleic acids, unless by a curious coincidence, must differ in the relative ease with which they are formed or decomposed under a given set of physiological conditions so that any one may preponderate or be entirely wanting in a tissue and the xanthine base which is formed by its decomposition will be found in relatively large amount or fail to make its appearance among the hydrolytic products of the mixture. This theory of Kossel, if it may be so termed, is greatly strengthened by Bang's discovery of guanylic acid,² a nucleic acid obtained from the pancreas which, as its name indicates, yields only one xanthine base.

One might on first thought be inclined to the opinion that Bang was fortunate in examining a tissue in which three nucleic acids are incapable of existence; but a closer inspection of the method of preparation which he employed will suggest the possibility that guanylic is the only one of the four acids which could withstand the severity of the process, and that this interesting substance may in reality be a laboratory product.³

If this view of the matter be correct, guanylic acid should alone survive when any mixture of nucleic acids which yields guanine among its hydrolytic products is submitted to Bang's method.

The tissue which is best adapted to such an investigation is clearly

¹ KOSSEL und NEUMANN: *Berichte der deutschen chemischen Gesellschaft*, xxvi, p. 2754.

² BANG: *Zeitschrift für physiologische Chemie*, xxvi, p. 133.

³ BANG heated his material with 2 per cent caustic potash for half an hour at the temperature of boiling water.

suggested by a publication of Okerblom¹ which appeared under the title "The Xanthine Bodies of the Suprarenal Gland." Okerblom treated the finely divided glands with water for two days at the body temperature and after heating to coagulate the proteids, the aqueous extract was faintly acidified with acetic acid and evaporated to a small volume at 35°-40° under diminished pressure. There was formed a crystalline sediment which was found to consist mainly of xanthine, while 1-methylxanthine and hypoxanthine were found in smaller quantities, and epiguanine and adenine could be shown by qualitative tests, there being an insufficient amount of material for analysis. The complete absence of guanine is distinctly noted, a fact which is readily explained by what has already been stated, viz., that while other nucleic acids were decomposed even by such careful treatment, guanylic acid remained intact and might be found among the by-products.

On the other hand Schmiedeberg² has taken the ground that in the case of salmon nucleic acid residues of both guanine and adenine are contained in the one molecule. In view of these circumstances it seemed to us that an investigation of the phosphoproteid of the suprarenal gland and the products of its hydrolysis under various conditions could scarcely fail to yield interesting results.

PREPARATION OF THE NUCLEOPROTEID OF THE SUPRARENAL GLAND.

Sheep suprarenal glands were collected (about 200 at a time), trimmed of fat, ground in a machine, and preserved in 70 per cent alcohol. When a sufficient amount of material had been thus obtained the alcoholic fluid was pressed through linen and the residue treated in turn with 95 per cent alcohol, absolute alcohol, and ether, and after drying in the air, was ground to a fine powder. About one fourth of the total nucleoproteid of the gland remains in the first alcoholic fluid and can be recovered by a suitable process; but as the product obtained from this source is always rich in inorganic salts it had best be neglected.

The dried gland was made into a paste with 2 per cent ammonia and allowed to stand for an hour, when the fluid was pressed through linen and the residue extracted in the same manner with water. The

¹ OKERBLOM: *Zeitschrift für physiologische Chemie*, xxviii, p. 60.

² SCHMIEDEBERG: *Archiv für experimentelle Pathologie und Pharmakologie*, xliii, p. 57.

united extracts formed an emulsion impossible to filter and giving no sediment when submitted to centrifugation. The vessel containing the fluid was placed in cold water, and acetic acid was added a few drops at a time and with continual stirring until the fluid showed a marked acid reaction to litmus. A small quantity of dark slimy material was precipitated, which settled rapidly, leaving a perfectly transparent bright reddish brown fluid whose color becomes much darker on exposure to the air, — a phenomenon which is attributable to the oxidation of the physiologically active constituent of the gland. The liquid was filtered off and poured into four times its volume of 95 per cent alcohol, when the nucleoprotein was precipitated perfectly white and flocculent, leaving all traces of coloring matter in the solution. The precipitate was washed repeatedly by decantation with large volumes of 95 per cent alcohol, then with absolute alcohol, and finally with ether.

The substance thus obtained is a perfectly white amorphous powder which is soluble in water with the greatest ease. It gives off ammonia when treated with caustic potash in the cold, but was shown both by a check test and by special reactions to be free from ammonium acetate. The protein yields about $4\frac{1}{2}$ per cent of ash, which was found to consist mostly of silica and which contained only a trace of inorganic phosphates.

This substance, which is evidently an ammonium salt, was dissolved in water, and the solution was treated with extremely dilute acetic acid as long as the reagent caused an appreciable increase in the bulk of the precipitate formed. An equal quantity of dilute acetic acid was then added. The precipitate was filtered off, washed with water which had been acidified with acetic acid and rinsed into a tall cylinder where it was successively washed by decantation with large volumes of 95 per cent alcohol, absolute alcohol, and ether.

The nucleoprotein thus prepared is only slightly soluble in water, but dissolves instantly on the addition of a trace of caustic soda or ammonia, and is precipitated from a faintly alkaline solution by acidification with acetic acid. This precipitate requires a large excess of acetic acid to effect its solution. A solution of the protein in 2 per cent ammonia yields no precipitate upon acidification with acetic acid. Such a solution was poured into four volumes of 95 per cent alcohol. A flocculent precipitate was produced which, on drying with alcohol and ether, was found to be easily soluble in water.

The proteid responds well to the xanthoproteic and biuret tests, but gives Millon's reaction very poorly.

The yield of nucleoproteid is quite variable. In one experiment we were able to prepare 6 gms. of the substance from 100 gms. of the dried gland, but in order to obtain this maximum yield the glands must not be allowed to stand for any great length of time, especially in a warm place. The easy decomposition of the nucleoproteid while in the gland is strikingly shown by the fact that the residue from many pounds of suprarenals which had been exhausted with water acidified with acetic acid, contained so small a quantity of nucleoproteid as to be useless for our work.¹ Nevertheless the pure nucleoproteid may be allowed to stand dissolved in ammonium acetate and acetic acid without any appreciable decomposition.

The yield from the suprarenal gland of the beef is markedly less than from that of the sheep, being rarely above 2 per cent of the dried gland. The products from these two sources, however, have the same chemical composition, and in so far as we have been able to observe yield the same hydrolytic products.

ON THE CHEMICAL CHARACTER OF THE NUCLEOPROTEID.

Below are given the results obtained by the analysis of three specimens of nucleoproteid, obtained by the method described above, from the suprarenal gland of the sheep, the suprarenal gland of the ox, and the pancreas of the pig respectively. For the purpose of comparison we also quote Hammarsten's² analysis of the pancreas nucleoproteid which he obtained by a somewhat different method and from which Bang³ prepared guanlyic acid. We are well aware that analytical concordances are often simply due to similarity of method, but we propose to show that the agreement in this instance extends to the hydrolytic products.

Nucleoproteid from suprarenal gland of the sheep.

0.2649 gm. gave 0.0055 gm. of ash = 2.08 per cent.

0.2140 gm. gave 0.3550 gm. of CO₂ and 0.1150 gm. of H₂O.

0.3368 gm. gave 0.0567 gm. of Mg₂P₂O₇.

0.2167 gm. required 11.21 c.c. of H₂SO₄ (1 c.c. = 0.03392 gm. of N.)

¹ This material was kindly submitted to us by Messrs. Armour & Co., who obtained it as a by-product in the preparation of an extract of the gland for Dr. Abel. See Bulletin of the Johns Hopkins Hospital, March, 1902.

² HAMMARSTEN: Zeitschrift für physiologische Chemie, xix, p. 19.

³ BANG: *Loc. cit.*

Nucleoproteid from suprarenal gland of beef.

- 0.3168 gm. gave 0.0059 gm. of ash = 1.86 per cent.
- 0.2458 gm. gave 0.4140 gm. of CO₂ and 0.1385 gm. of H₂O.
- 0.3279 gm. gave 0.0555 gm. of Mg₂P₂O₇.
- 0.2341 gm. required 12.09 c.c. of H₂SO₄.

Nucleoproteid from pancreas of pig.

- 0.4004 gm. gave 0.0088 gm. of ash = 2.20 per cent.
- 0.2271 gm. gave 0.3683 gm. of CO₂ and 0.1359 gm. of H₂O.
- 0.4026 gm. gave 0.0729 gm. of Mg₂P₂O₇.
- 0.1897 gm. required 9.53 c.c. of H₂SO₄.

	Nucleoproteid of suprarenal gland of sheep.	Nucleoproteid of suprarenal gland of ox.	Nucleoproteid of pancreas of pig.	HAMMARSTEN'S preparation.
C	46.22	46.81	45.23	43.62
II	6.10	6.38	6.26	5.45
P	4.70	4.72	5.05	4.48
N	17.92	17.85	17.42	17.39

We have hitherto used the term "nucleoproteid" to include all compound proteids which yield the decomposition products of nucleic acids. The word is often used however in contradistinction to "nucleohistone." Lilienfeld¹ prepared from an aqueous extract of the thymus, two phosphoproteids, one of which he called nucleoproteid and the other, nucleohistone. Since Lilienfeld's work these two proteids have occupied the attention of several chemists whose differences of opinion upon many essential points have caused the matter to fall into a state of almost hopeless confusion. Bang² claims that nucleohistone has no existence and that the aqueous extract of the thymus contains a nucleoproteid, a histone, and a nucleic acid. Kossel³ flatly denies the logic of Bang's contention, and claims that analogous arguments might be employed to prove that sodium chloride does not exist. Malengreau separates the two proteids by fractional precipitation with ammonium sulphate and finds that both are nucleohistones, since both yield histone on hydrolysis.

¹ LILIENFELD: *Zeitschrift für physiologische Chemie*, xviii, p. 478.

² BANG: *Zeitschrift für physiologische Chemie*, xxx, p. 508.

³ KOSSEL: *Ibid.*, p. 520.

While these and other points are still subjects of contention, most or all writers on the subject agree that the two proteids differ markedly in chemical composition as shown in the following table.

	C	H	N	P
Nucleohistone	48.66	6.90	18.36	3.77
Nucleoproteid	51.90	7.37	16.48	0.94

If composition alone be considered, our nucleoproteid falls clearly within the group of nucleohistones.

On the other hand Huiskamp,¹ whose analyses we have quoted, states as a characteristic difference between the two proteids (and one on which he bases his method of separation) that the nucleohistone is precipitated from a solution in water by the addition of calcium chloride in such an amount that the solution contains 0.1 per cent of the salt.

A 5 per cent solution of suprarenal nucleoproteid was prepared with the use of so small an amount of ammonia that the fluid was scarcely alkaline to litmus. Ten c.c. of this solution were treated with a 0.3 per cent solution of calcium chloride a drop at a time until 10 c.c. of the reagent had been added, but at no time was a precipitate formed. This seems to show that the nucleoproteid is not a nucleohistone.

HYDROLYTIC PRODUCTS OF THE NUCLEOPROTEID.

In our earlier work the hydrolysis of the proteid was effected in an autoclave at 150° with 20 per cent sulphuric acid, but subsequent experiments showed that the decomposition can be brought about by boiling for twenty minutes with 5 per cent sulphuric acid. Just what are the limits in regard to strength of acid and time of boiling required for the hydrolysis we are unable to say. In one experiment a quantity of nucleoproteid from the beef gland was evaporated on the water-bath with 3 per cent acetic acid. On cooling, the concentrated fluid deposited crusts of guanine. The decomposition with nitric acid is extremely easy. A solution of the proteid in 5 per cent nitric acid was treated with an excess of silver nitrate and heated to the boiling point. On cooling, silver compounds of the xanthine bases were deposited in crystalline form.

¹ HUISKAMP: *Zeitschrift für physiologische Chemie*, xxxii, p. 145.

Thirty grams of the nucleoproteid from the sheep suprarenal gland were mixed with 200 c.c. of 5 per cent sulphuric acid and boiled for an hour under an inverted condenser. The dark-brown fluid was filtered from deposited pigment, and after diluting to $1\frac{1}{2}$ litres was treated with a hot concentrated solution of barium hydroxide until most of the sulphuric acid was neutralized. The fluid was then made almost exactly neutral with dilute barium hydroxide, treated with a small quantity of sodium acetate, filtered, and evaporated on the water-bath to about 25 c.c. As the evaporation proceeded guanine was continually deposited with coloring matter and was found in the form of dark-brown crusts adhering to the sides and bottom of the vessel. The fluid, which can be shown to contain no appreciable quantity of guanine, was filtered off and reserved for the other xanthine bases and thymine.

1. Guanine. — The dark-brown crusts were dissolved in very dilute sulphuric acid and the solution was decolorized with animal charcoal. The fluid was then treated with a 5 per cent solution of silver nitrate as long as the reagent produced a precipitate and ammonia was added to strong alkaline reaction. The silver precipitate was washed thoroughly with boiling water suspended in very dilute sulphuric acid and decomposed with sulphuretted hydrogen. After filtering off the silver sulphide the fluid was treated to marked alkalinity with ammonia and the bulky white precipitate of guanine was filtered off, washed with water, and dried with alcohol and ether.

The free base was dissolved in hot very dilute hydrochloric acid. On cooling, beautiful needles of the hydrochlorate were deposited. This salt seems to have been neglected by those who have worked with guanine, but we found it one of the most useful of all the derivatives of the base. The compound may be repeatedly recrystallized from dilute hydrochloric acid without serious loss, possesses a highly characteristic crystalline form and has no tendency to lose its water of crystallization unless placed in a desiccator. The compound can also be easily converted into the free base by repeatedly evaporating with water, thus rendering possible the preparation of the base absolutely free from ammonium salts, an end which we have been able to accomplish in no other way.

The hydrochlorate was weighed, recrystallized once from dilute hydrochloric acid, dried on filter paper and analyzed.

0.1789 gm. of the crystallized salt lost 0.0288 on heating at 105.

0.1501 gm. of the anhydrous salt required 16.47 c.c. of sulphuric acid
(1 c.c. = 0.003392 N).

	Theoretical for guanine.	Theoretical for xanthine.	Found.
2 H ₂ O	16.11	0.00	16.10
N	37.35	29.72	37.22

Part of the hydrochlorate was evaporated several times with water on the water-bath, then with alcohol, and finally with a trace of ammonia. The residue was washed on to a filter with warm water and dried with alcohol and ether. The substance dried at 110° gave the following analytical result:

0.1368 gm. required 18.56 c.c. of sulphuric acid (1 c.c. = 0.003392 N).

	Theoretical for guanine.	Theoretical for xanthine.	Found.
N	46.35	36.84	46.02

We have taken the trouble to establish the identity of this substance beyond question, because it far outweighs the sum of all the other bases formed by the hydrolysis of the nucleoproteid, while Okerblom failed to find a trace of guanine among the preformed bases of the gland.

From 30 gms. of the nucleoproteid of the suprarenal gland of the sheep were obtained 510 mgms. of guanine hydrochlorate. Similar experiments with the nucleoproteids of beef suprarenal gland (10 gms.) and pig pancreas gave on calculation to a basis of 30 gms. of proteid 501 and 517 mgms. respectively.

2. Xanthine bases other than guanine.—The original mother liquor from which guanine had separated during evaporation, the fluid from which guanine was precipitated with ammonia, and the mother liquor from the first crystallization of guanine hydrochlorate were united. The fluid was decolorized with animal charcoal, and after making alkaline with ammonia, was treated with a slight excess of a solution of silver nitrate in ammonia. The precipitated silver compounds were filtered off, washed with boiling water and submitted to an examination for xanthine bases by the method of Krüger and Solomon.¹ The

¹ KRÜGER and SOLOMON: Zeitschrift für physiologische Chemie, xxvi, p. 350.

filtrate was faintly acidified with nitric acid and reserved for an examination for thymine.

The gelatinous precipitate was washed into a flask and treated on the water-bath with a dilute solution of hydrochloric acid a drop at a time until the precipitate became granular and settled rapidly. An equal amount of hydrochloric acid was then added and the solution was boiled and filtered. The fluid was evaporated to dryness on the water-bath, water was added and evaporated, and the process repeated until the residue became coarsely granular. Alcohol was then substituted for water and the final residue was digested with water at 40° for several hours and filtered. The residue may contain xanthine, heteroxanthine, and 1-methyl xanthine, while in the aqueous fluid may be found hypoxanthine, epiguanine, adenine, and paraxanthine.

a. **The hypoxanthine fraction.**—The solution was treated with ammonia in slight excess. The bulky precipitate which was first formed redissolved except a trace of reddish brown flocculent material which was easily shown to be ferric hydroxide. Okerblom obtained a trace of what he supposed to be epiguanine at this point.

The filtrate from the ferric hydroxide was allowed to stand several days in a warm place in order to allow the excess of ammonia to escape, and then treated with a slight excess of a saturated aqueous solution of picric acid. A bulky bright yellow microcrystalline precipitate of adenine picrate was formed which was immediately filtered off, washed with water, and dried in a desiccator. The substance was found to melt sharply at 281° (adenine picrate melts at 282–283°). Krüger and Schittenhelm¹ state that the analysis of a substance melting at this point and obtained under the conditions stated is superfluous.

The amount of adenine picrate obtained from 30 gms. of the nucleoproteid was 212 mgms. Similar experiments with the nucleoproteids from the suprarenal gland of the beef (10 gms.) and the pancreas of the pig (22 gms.) gave on calculation to a basis of 30 gms. of proteid 210 and 215 mgms. respectively.²

The yellow filtrate from adenine picrate was acidified with sulphuric acid and the picric acid removed by shaking with ether. On treatment of the aqueous fluid with ammonia and silver nitrate an

¹ *Ibid.*, xxxv, p. 153.

² LEVENE has described a nucleic acid from the pancreas which yields both guanine and adenine. *Zeitschrift für physiologische Chemie*, xxxii, p. 541.

extremely small precipitate occurred. This precipitate, which is in all probability attributable to the slight solubility of adenine picrate, was too small for an exhaustive investigation. It was suspended in water, decomposed with sulphuretted hydrogen, and the aqueous fluid evaporated to dryness. Since the residue was scarcely weighable it was treated with a few drops of dilute nitric acid (10 c.c. of concentrated nitric acid + 90 c.c. of water), and allowed to stand twenty-four hours. No separation of hypoxanthine nitrate occurred.

Okerblom found hypoxanthine in comparatively large quantity among the preformed bases of the gland. We were unable to find the slightest trace of the substance among the hydrolytic products either of the nucleoprotein of the suprarenal gland of the beef or that of the pancreas of the pig.

b. The xanthine fraction.—The residue weighed only 62 mgms., and was somewhat contaminated with silver chloride. With so small a quantity it was found difficult to follow Krüger and Solomon's directions. It was treated with 3 c.c. of hot 3.3 per cent caustic soda and filtered. On standing no precipitate was formed. The fluid was warmed to 60° and treated with 2 c.c. of a mixture of 20 c.c. of concentrated nitric acid and 20 c.c. of water. No precipitation of xanthine nitrate occurred within 24 hours. Okerblom found xanthine in great preponderance among the preformed bases of the gland.

The question naturally arises, What is the material which forms our xanthine fraction? By exclusion we might conclude that it is 1-methyl xanthine, but this would simply throw the burden of proof upon the analytical method which we employed; so that in order to be able to give any satisfactory answer to the question, it will be necessary to work with much larger quantities of the nucleoprotein than we have hitherto been able to obtain.

It will thus be seen that guanine and adenine are the only xanthine bases which result in appreciable quantity from the hydrolysis of any of the three nucleoproteins with which we worked, and an examination of the quantitative results which have been given will show that the two bases are produced in these cases in nearly the same relative amounts. Thus:

- I. 30 gms. of nucleoprotein of sheep suprarenal gland gave 510 mgms. of guanine hydrochlorate and 212 mgms. of adenine picrate.
- II. 10 gms. of nucleoprotein of beef suprarenal gland gave 167 mgms. of guanine hydrochlorate and 70 mgms. of adenine picrate.

III. 22 gms. of nucleoprotein of pig suprarenal gave 374 mgms. of guanine hydrochlorate 155 mgms. of adenine picrate.

On calculation to a basis of 30 gms. of protein we have

	Guanine hydrochlorate.	Adenine picrate.	Molecular ratio.
I	510	212	4.11 : 1
II	501	210	4.07 : 1
III	510	220	3.89 : 1

3. Thymine. The solution was filtered from silver chloride, diluted with water and treated with silver nitrate and barium hydroxide according to the method already described.¹ There was no difficulty in obtaining a beautifully crystalline substance whose properties throughout agreed with those which have been ascribed to thymine.

0.1485 gm. of substance required 9.77 c.c. of H₂SO₄ (1 c.c. = 0.003392 N.)

	Theoretical for thymine.	Found.
N	22.22	22.32

From 30 gms. of nucleoprotein were obtained 131 mgms. of thymine, while from 30 gms. of material whose xanthine bases were treated by Neubauer's method, were obtained 138 mgms. of thymine. The substance results just as well from the suprarenal gland of the beef as from that of the sheep, but experiments with the pancreas gave unsatisfactory results. In the final liquid could be seen crystals which had every appearance of thymine, but which could not be separated from the viscous mother liquor.²

While one scarcely expects to obtain a quantitative yield of thymine, the numbers given show a very fair molecular concordance with those already given for guanine and adenine, thus:

30 gms. of nucleoprotein gave 510 mgms. of guanine hydrochlorate, 212 mgms. of adenine picrate, and 135 mgms. of thymine. Therefore the molecular ratio

$$\text{guanine : adenine : thymine} :: 4.11 : 1.00 : 1.93$$

SUMMARY.

1. The nucleoprotein of the suprarenal is a thymonucleoprotein, and probably not a nucleohistone.
2. The nucleoproteins of the suprarenal gland of the beef and the

¹ JONES: Zeitschrift für physiologische Chemie, xxix, p. 461.

² See also LEVENE: *Loc. cit.*

sheep are identical and scarcely differ in chemical composition from the nucleoproteid of the pancreas.

3. As closely as the analytical processes at our command can determine, the nucleoproteids of the pancreas and the suprarenal gland yield guanine and adenine in the same relative proportions.

4. In so far as these quantitative relations have value, they are in support of Schmiedeberg's contention, that one molecule of a nucleoproteid (or a nucleic acid) may yield two different xanthine bases.

5. We were unable to find xanthine, hypoxanthine, or epiguanine: and it is certain that these bases, if formed at all, can be demonstrated only by the use of extremely large quantities of the nucleoproteid.

6. It is possible that a trace of xanthine escaped our attention, owing to a defect in the method of Krüger and Solomon, which was pointed out by Krüger and Schittenhelm¹ after we had completed our work.

7. As Okerblom professed to find hypoxanthine, epiguanine, and an excessive amount of xanthine, while he failed to find any trace of guanine, we seem to be confronted by a most curious and interesting qualitative difference between the xanthine bases which are prepared in the gland and those which result from the hydrolysis of the nucleoproteid.

¹ KRÜGER and SCHITTENHELM: *Zeitschrift für physiologische Chemie*, xxxv, p. 153.

THE FLOW OF THE BLOOD IN THE EXTERNAL JUGULAR VEIN.

By R. BURTON-OPITZ.

[From the Physiological Laboratory of the Harvard Medical School.]

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METHOD.

THE blood-flow through the external jugular vein was measured in this investigation with the recording stromuhr devised by Hürthle and demonstrated by him to the members of the last physiological congress, held at Turin, September, 1901.

The stromuhr previously constructed by Hürthle contained two metal revolving cylinders. This new instrument consists of only one cylinder (*C*, Fig. 1) which is stationary and in which a piston (*P*) moves in the vertical direction. The blood reaches the cylinder through the peripheral cannula (*A*) and leaves it through the central cannula (*B*). In its course it passes through a disc (*D*) pierced with two openings. Through one of these the blood enters the cylinder at *E*, below the piston, while through the other it enters a curved tube which conveys it into the cylinder at *E'*, above the piston. At the beginning of the experiment the blood is admitted through *E* and forces the piston to the top of the cylinder. As the piston rises the contents of the cylinder (normal saline solution) are driven through *E'* into the outflow cannula *B*. When the cylinder is full, the perforated disc *D* is turned. The blood now flows from the inflow cannula, *A*, through *E'* into the top of the cylinder. The piston is driven downwards and the contents of the cylinder are forced out through *E* and the outflow cannula *B*.

The piston rod (*T*) is attached to a horizontal writing lever (*L*) by means of a long silk thread. When the piston is driven downward the writing-point of the lever moves upward, and when the piston moves upward, the writing-point moves downward. In Fig. 2 the lever has written two downward phases (*A* to *B* and *E* to *F*) and one upward phase (*C* to *D*). The reversal of the blood-stream by turning the disc has occurred between *B* and *C* and again between *D* and *E*.

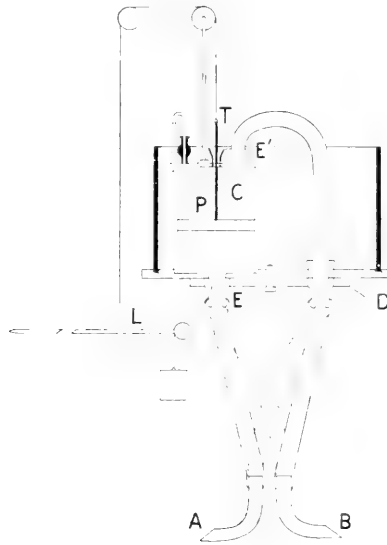


FIGURE 1.—Diagram of Hürthle's Recording Stromuhr.¹ *A* and *B*. Central and peripheral cannulas. *C*. Stationary cylinder. *D*. Revolving disc. *E* and *E'*. Openings in cylinder for entrance of blood. *L*. Writing lever with weight. *P*. Piston. *T*. Tip of piston, connected with lever by means of silk thread.

The cylinder being calibrated, every rise or fall of the lever indicates that a definite quantity of blood has entered or left the stromuhr. In the case illustrated by Fig. 2 a rise of 4 mm. shows that 1 c.c. of blood has entered the instrument. The time, absolutely essential in calculating the results, was recorded by a Jaquet chronometer, beating fifths of seconds. The time record served also as the abscissa for the curve of the blood-flow. The venous pulse and the respiratory movements were also recorded when the occasion demanded.

¹ ТШУЕВСКИЙ, F. A.: О Кровоснабжении Отдельных Органов (On the blood-supply of several organs), Charkow, 1902.

ABSCISSA OF
NEG. PRESSURE IN PLEURAL CAVITY

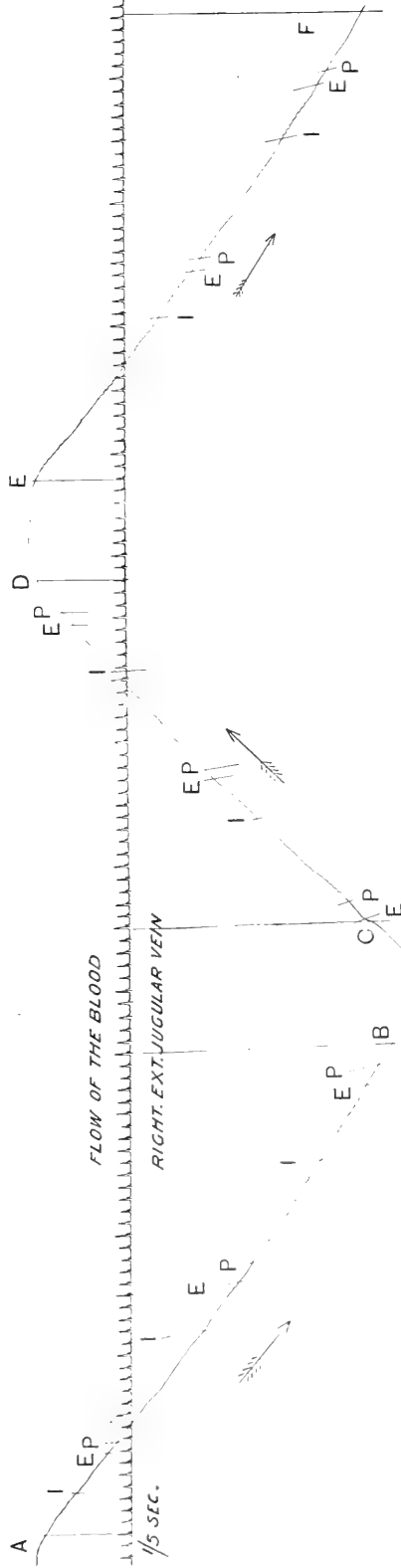


FIGURE 2. — The blood-flow in the external jugular vein.

THE VOLUME AND THE VELOCITY OF THE BLOOD-STREAM.

In making quantitative determinations of the blood-flow, especially in a vein, it is of course essential to reduce the resistance of the piston to a minimum. The external jugular vein being in an easily accessible position, the stromuhr could be placed horizontally. Thus, the blood was forced to make only a short lateral curve to enter the cylinder instead of being driven to a considerable height above the vein. The resistance was further decreased by counterpoising the writing-lever, indirectly therefore also the piston. A weight, just failing to move the piston by its own gravity, was placed upon the lever when moving downward and removed when the upward phase commenced.

In four of the experiments of this group the stromuhr, containing a warm 0.7 per cent sodium chloride solution, was inserted opposite the fifth to seventh rings of the trachea. In the fifth experiment its position was higher, the peripheral cannula being inserted close to the point of union of the internal and external maxillary veins. To prevent the possible entrance of air all parts of the stromuhr were made secure with beeswax and the opening for the piston was surrounded by a capsule filled with soft vaseline. It was also thought advisable to ligate the large communicating vein upon the larynx. The experiments were performed on medium-sized dogs, the right jugular vein being used exclusively. They were anæsthetized with morphine and chloroform.

In calculating the volume all variations in the blood-flow, dependent, as we shall see later, upon the respiratory changes in the intrathoracic pressure and the contraction of the right side of the heart, have been neglected. Only the relation of the height of the entire phase to its duration has been taken into account. Moreover, to avoid errors due to coagulation, only those phases were measured that were written during two minutes; the blood in the cylinder was still fluid from four to five minutes after the beginning of the experiment.

The internal diameter of the vein, necessary in calculating the velocity, was obtained by the method employed by Tschuowsky in the investigation referred to previously. A short portion of the vessel having been exposed, its outside diameter was determined by means of a spring caliper. The entire vessel was subsequently compressed between two narrow glass plates, the compression being just sufficient to let no blood pass. The thickness of the glass plates

was then subtracted from this measurement and the newly-derived value subtracted from the outside diameter.

The results of the experiments are given in Table I. The quantities obtained by measuring the various phases are reduced to a uniform value in each case, namely, the number of cubic centimetres per second.

TABLE I.
THE VOLUME AND THE VELOCITY OF THE BLOOD-STREAM.

Exp.	Weight of dog in kilos.	Volume. c.c. per sec.	Inside diameter. Mm.	Velocity. Mm. per sec.
1	10	1.8	0.4	143
2	12	2.1	0.42	146
3	12	2.4	0.43	163
4	13	1.9	0.4	151
5	21	3.7	0.6	131
Average	13	2.4	0.45	147

THE EFFECT OF STIMULATION AND SECTION OF THE VAGUS.

By stimulating either vagus with a sufficiently strong current the blood-flow can be made to cease, as can readily be seen by a glance at Fig. 3. In this experiment the left nerve was stimulated for about five seconds, the distance of the coils being 10 cm. (medium stimulus). The current was made at *A* and broken at *B*. A comparison with the abscissa will show that the blood-flow ceases a short time after the passage of the current and resumes its former value immediately after *B*.

The reverse relationship may be obtained by using a strong current of longer duration. In this case the blood-flow will stop almost the instant the current is made, but will not immediately become normal again. The curve after breaking the current will then show a staircase arrangement, indicating thereby that the strongly stimulated heart regains its normal frequency only gradually.

A staircase curve will also be written when the current applied to the vagus is weak, *i. e.*, not sufficiently strong to stop the heart entirely, but only to separate its beats more widely.

Such a curve is shown in Fig. 4. The current (distance of coils 15 cm., duration about three seconds) was made at *A* and broken at *B*. Three heart-beats, weaker than normal and of longer duration, occurred during this period. A less than normal onward flow is

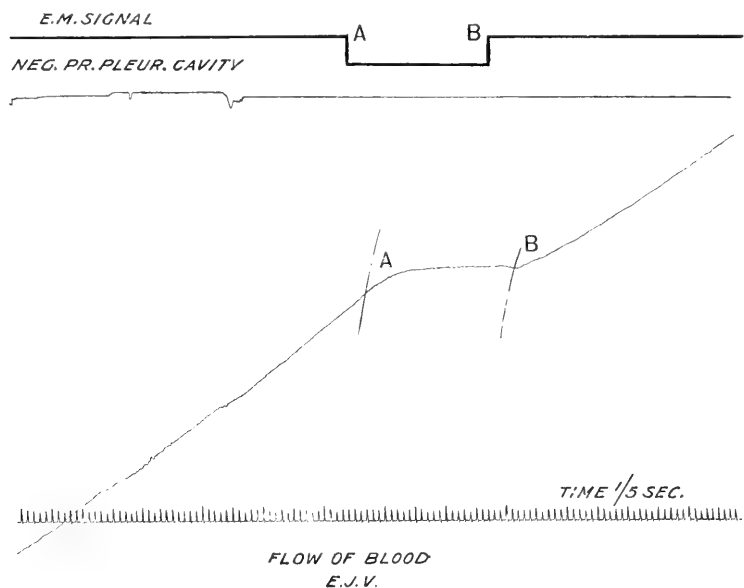


FIGURE 3. — Stimulation of left vagus (from *A* to *B*) with medium current.

observed to have taken place during the active period of the heart, from 1 to 2, while scarcely any flow was present during the pause, from 2 to 1. After discontinuing the stimulation the blood-flow regained its normal value again.

In two of the experiments both vagi were suddenly divided with scissors, while the curve of the blood-flow was being written. The

individual phases immediately became much steeper, showing thereby that a greater volume of blood was passing through the vein. A comparison was then made between the normal volume of blood and that propelled after the section of this nerve. The results of this calculation are contained in Table II.

TABLE II.
THE VOLUME OF BLOOD BEFORE AND AFTER SECTION OF THE VAGI.

Exp.	Weight of dog in kilos.	A. Normal flow. c.c. per sec.	B. Flow after section of vagi. c.c. per sec.	Increase. B times A.
1	10	1.8	5.9	3.2
2	8	1.2	2.8	2.4

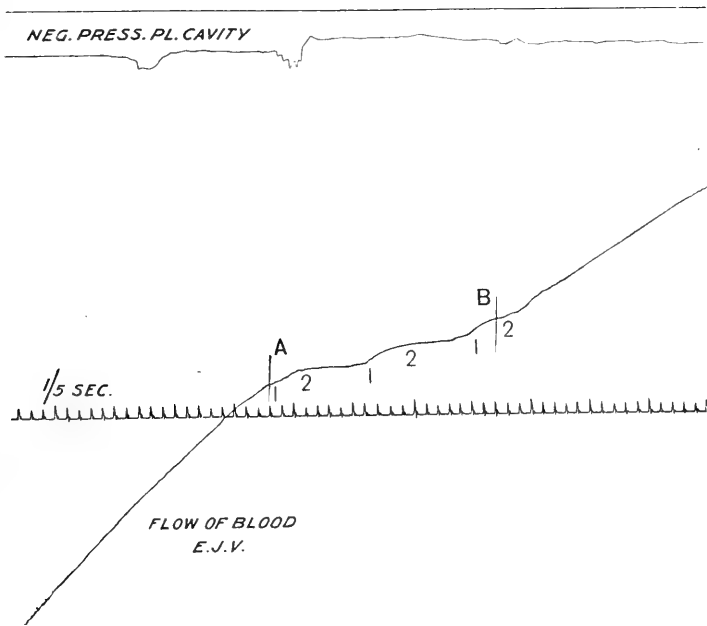


FIGURE 4 — Stimulation of left vagus (from A to B) with weak current.

THE CHANGE IN THE BLOOD-FLOW DURING THE COMPRESSION OF BOTH CAROTID ARTERIES.

Both carotid arteries were previously placed in ligatures opposite the third tracheal ring and were suddenly compressed during the ex-

periment by being tightly drawn against the flat ends of two probes. The great decrease in the blood-flow of the external jugular vein, produced by the compression, is clearly betrayed in Fig 5. The ligatures were tightened at *A* and released at *B*.

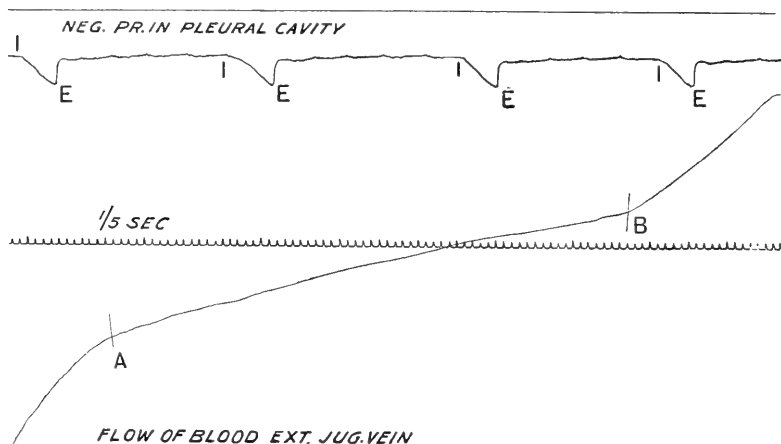


FIGURE 5. — Compression of both carotid arteries (from *A* to *B*).

This procedure was repeated several times in three different animals. In Table III. the normal volume of blood is compared with that passing through this vein during the compression.

TABLE III.
THE EFFECT OF COMPRESSION OF BOTH CAROTID ARTERIES.

Exp.	Weight of dog in kilos.	Normal volume. c.c. per sec.	Volume during compression. c.c. per sec.	Per cent of decrease.
1	8	1.5	0.7	53
2	10	1.7	0.68	60
3	10	1.8	0.87	52
Average	9	1.66	0.75	57

Among the conclusions arrived at so far, the following may be emphasized:

1. The normal volume of the blood-stream in the external jugular vein in a dog, weighing 13 kilos, is about 2.4 c.c. per second and the velocity 147 mm. per second.

2. By stimulating the vagus a total cessation of flow may be produced.
3. Section of both vagi increases the volume of the blood-stream 2.8 times.
4. By compressing both carotid arteries the quantity of blood in the external jugular vein is reduced 57 per cent.

THE RESPIRATORY VARIATIONS IN THE BLOOD-FLOW.

The curve of the blood-flow, as recorded by the lever of the stromuhr, is not a straight line, but shows regular periodic variations. The quantity of blood propelled is therefore not equally large at all times.

Two distinct types of variations will be considered in this paper, namely, those due to the changes in intrathoracic pressure during respiration, and secondly, those produced by the activity of the right auricle and ventricle. A third class, including all those changes in the venous flow that are dependent upon accidental causes, such as muscular contractions either near or more or less remote from the vein experimented on, will be reserved for future consideration.

In order to eliminate as much as possible the influence of the heart, the stromuhr in these experiments was inserted in the upper portion of the jugular, immediately below the union of the internal and external maxillary veins. Thus, a greater number of valves were interposed between the central cannula and the central venous trunks. This type of variations was most distinctly marked in those animals having a feeble heart-action normally or in those in which the anæsthetic had a depressing effect upon this organ.

The changes in intrathoracic pressure were recorded by a tambour, connected by means of a cannula with the left pleural cavity. The downward phase of the respiratory curve corresponds therefore to inspiration and the upward phase to expiration.

Fig. 6 is an example of this type of variations. We observe that the curve of the blood-flow shows regular and periodic deviations from its otherwise straight diagonal course, first turning further away from the abscissa and then further toward it. The changes occur at *I*, *E*, and *P*. We have therefore a period of increased flow, lasting from *I* to *E* and a period of slackened flow, extending between *E* and *P*. At the latter point the blood-flow assumes its normal value again.

When the ordinates of the negative pressure curve are compared with those of the blood-flow, it becomes evident that the variations

occur synchronously with the respiratory movements. During inspiration the flow is greater than normal and during expiration the flow is less than normal. Moreover, the increase takes place immediately

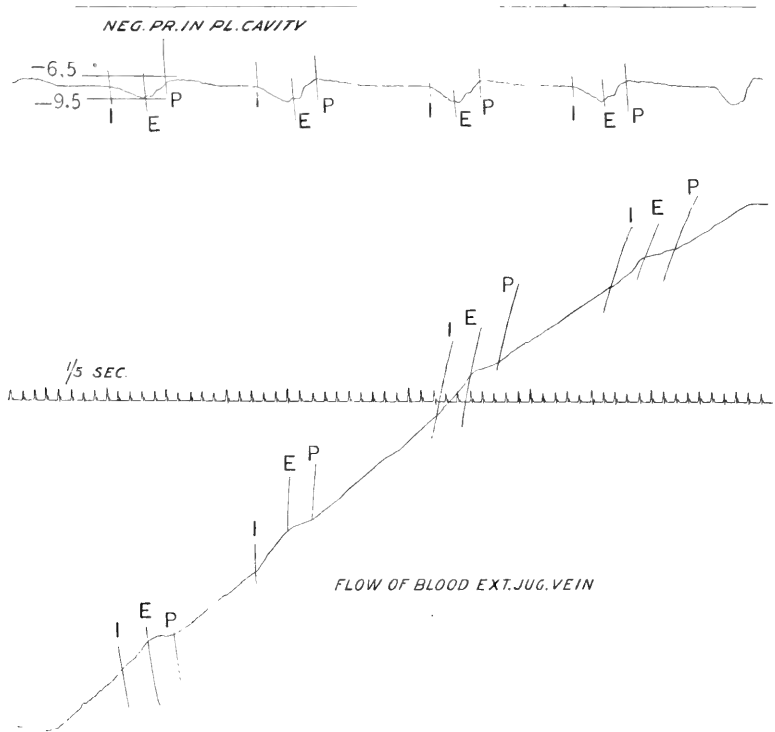


FIGURE 6. — The respiratory variations.

on inspiration, but becomes greater in the course of this phase, reaching its maximum value at the time of greatest negative pressure. The same parallelism is displayed during the expiratory phase. The shorter this phase, the shorter the duration of the period of decreased flow and the more decided the changes in the intrathoracic pressure, the more conspicuous are the changes in the blood-flow. Thus, forced expiration can produce a stoppage in the flow.

To show the dependence of the respiratory variations on the depth of the respiratory movements, the values for the following three experiments have been calculated. Table IV. shows the duration of the respiratory phases as recorded by the tambour connected with the pleural cavity.

TABLE IV.

THE RELATION OF THE DEPTH OF THE RESPIRATORY MOVEMENTS TO THE VARIATIONS IN THE BLOOD-FLOW.

Exp	Normal flow (during pause). c.c. per sec.	Inspiration.			Expiration.		
		Time (sec.).	Neg. pressure. Mm. Hg	Blood-flow. c.c. per sec.	Time (sec.).	Mg. pressure. Mm. Hg	Blood-flow. c.c. per sec.
1	17	0.95	-6.5 to -9.5	2.6	0.4	-9.7 to -6.5	1.2
2	1.2	1.0	-2.5 to -10.0	2.3	0.2	-10.0 to -2.5	0.0
3	2.4	1.5	-8.0 to -10.2	2.5	0.5	-10.2 to -8.0	2.1

The respiratory pause begins at *P*. The volume of the blood-stream during this period depends of course entirely upon the activity of the heart. Consequently we must come to the conclusion that under normal conditions the heart forms by far the most important factor in propelling the blood, while the respiratory movements play

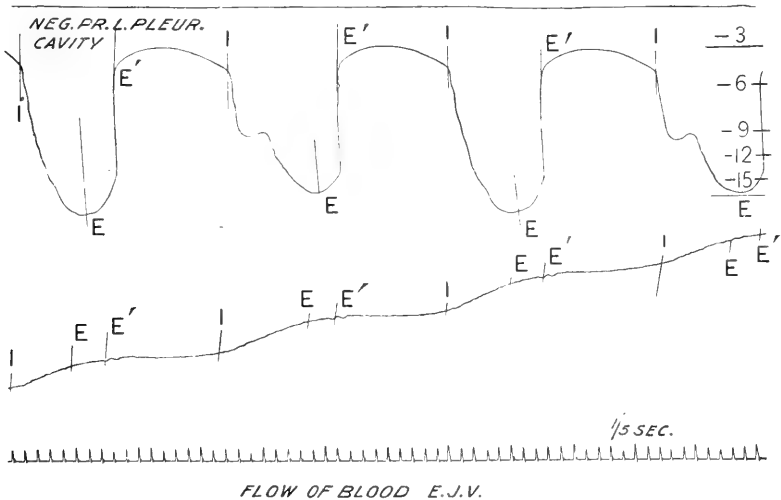


FIGURE 7. — The effect of forced respiratory movements.

only a secondary part. But, as their influence upon the blood-stream increases with their depth, it must necessarily also follow that they become the more important, the shorter the respiratory pause. How important they may become at times is clearly shown in Fig. 7.

This curve was written immediately after section of the left vagus in a lightly anæsthetized animal.

Normally the negative pressure in the pleural cavity varied from -4 to -8.25 mm. Hg and the curve of the blood-flow resembled very closely that given in Fig. 6. Immediately after the section the negative pressure showed the usual wider range toward as well as away from the abscissa (from -3 to -15 mm. Hg), but, instead of the usual decrease in the frequency of respiration, the section in this instance produced—at least for a short time—much quicker movements. The forced character of expiration is shown by the fact that the upper convex portion of the curve gradually approaches very near to the abscissa; a true pause is therefore absent.

The important influence of this type of respiration on the blood-flow in the jugular is apparent at the first glance. The curve has completely lost its normal character, represented in Fig. 6. To be sure the inspiratory movement is accompanied by a decided onward flow of the blood (*I* to *E*) which becomes most conspicuous during the time of greatest negative pressure. The period of slackened flow during expiration is not stopped at *E*, however, but continues as the negative pressure decreases still further. Finally, when the negative pressure reaches its lowest value, almost a complete stop in the blood-flow occurs. During the period of gradually increasing negative pressure, prior to the decided inspiratory movement at *I*, a correspondingly greater volume of blood is again moved onward. During the forced expiratory phase beginning at *E*, therefore, the part which the heart plays in propelling the blood was masked.

THE EFFECT OF STIMULATION OF THE PHRENIC NERVES.

Having found that the respiratory variations in the blood-flow are so closely related to the changes in the intrathoracic pressure, stimulation of the nervi phrenici was resorted to for the purpose of producing even more decisive changes in pressure. Both nerves were placed in covered electrodes opposite the outer border of the lower fourth of the sterno-cleido-mastoid muscle. A brief tetanic current was used, the strength of which was altered in such a way that either a slight or a great increase in intrathoracic pressure resulted. The current was made synchronous with the beginning of inspiration.

Fig. 8 may serve as an example of weak stimulation of these nerves. The current was in this case applied three times in succession (*A* to *B*)

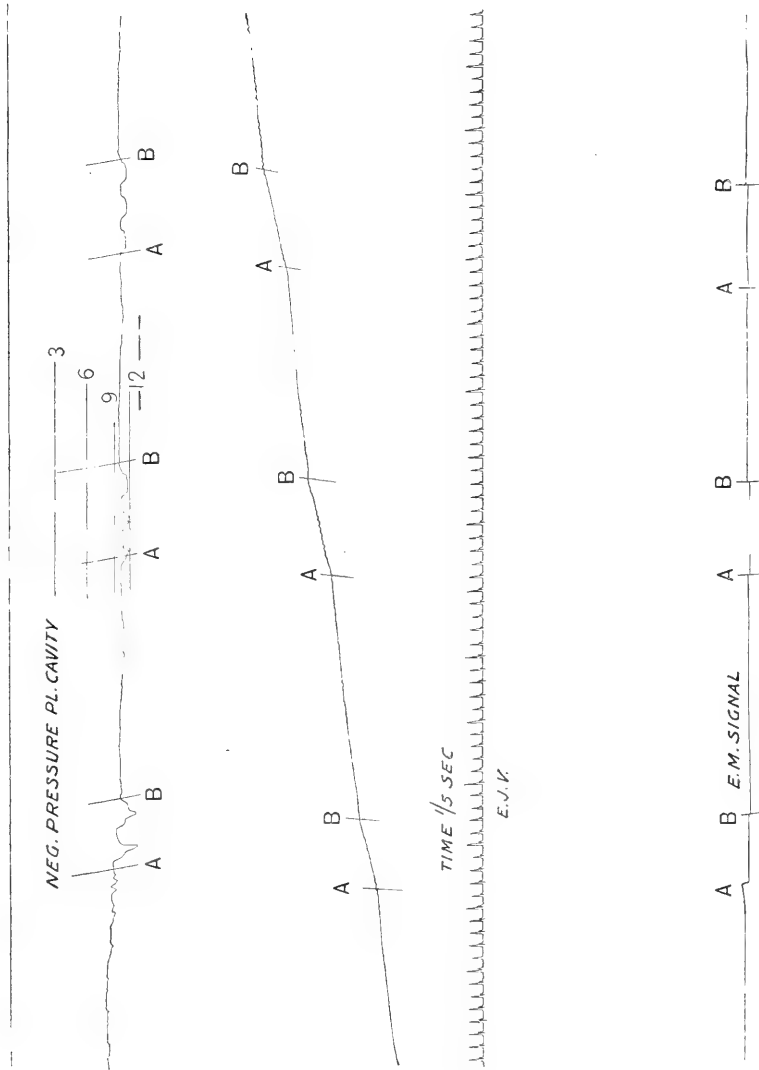


FIGURE 8. — Weak stimulation of the phrenic nerves.

and was sufficiently strong to increase the negative pressure to -12 mm. Hg, while normally it varied from -4.25 to -8 mm. Hg. The change in the blood-flow produced thereby was very decided. While normally only 1.2 c.c. per second were propelled, the average value during the periods of increased negative pressure (*A* to *B*) amounted to 1.9 c.c. per second.

In another case the intrapleural pressure was increased from -9.25 mm. Hg to -13 mm. Hg by stimulating the phrenic nerves. The blood-flow suffered at the same time an increase from 1.8 c.c. to 2.4 c.c. per second.

Increasing the negative pressure still further by stimulating these nerves with a strong current does not necessarily imply that a correspondingly larger quantity of blood is thereby moved onward. In fact, under these conditions the blood-flow usually decreases somewhat. In Fig. 9, for instance, the volume of blood was reduced from

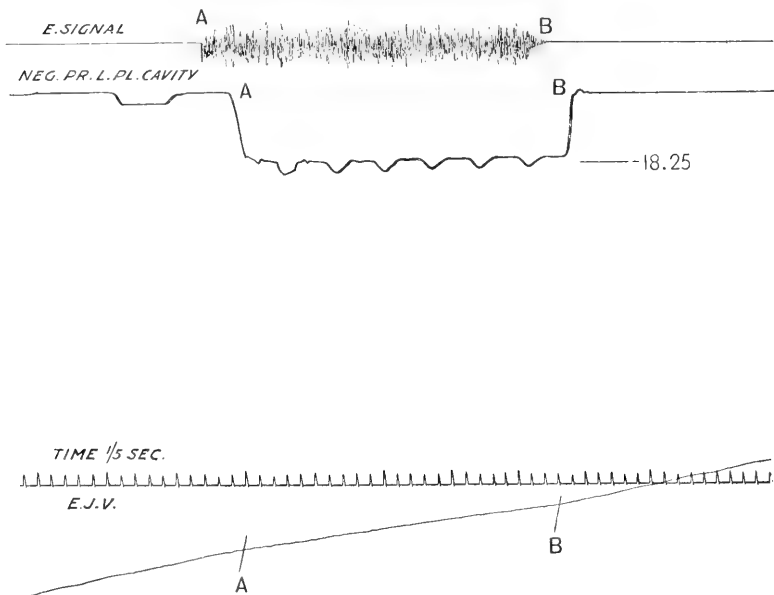


FIGURE 9. — Strong stimulation of the phrenic nerves.

1.8 c. c. to 1.6 c.c. per second during the period of stimulation from *A* to *B*. The negative pressure at this time was increased from its normal value, -9.25 , to -18.25 mm. Hg. It seems that in these instances the strongly contracted diaphragm presses upon the

abdominal organs, increasing in this way the flow in the inferior vena cava and lessening the influx of blood from the external jugular.

THE CARDIAC VARIATIONS IN THE BLOOD-FLOW.

The variations to be considered in this chapter are wholly dependent upon the changes in intra-auricular pressure occurring during each cardiac cycle. It is only natural to assume that the pressure-curve in the central venous trunks pursues a parallel course to that of the auricle, and that in the tributary veins these changes must be the more conspicuous, the closer we approach this venous reservoir.

Undoubtedly, under normal conditions the suction-action of the right auricle and ventricle on the blood in the great veins is the most important factor in the onward movement of the blood within them. The inspiratory movement by reason of its lesser frequency can be considered only as an "occasional" aid. The cardiac variations in the blood-flow of this vein will be recorded best when the respiratory movements are shallow and far apart and the heart beats forcibly.

In these experiments the stromuhr was inserted in the lower portion of the vein. Its central cannula, placed well into the groove between the neck and the shoulder, remained outside of the thoracic cavity and about 4 to 5 cm. from the subclavian vein. Three or four valves were thus left intact between the stromuhr and the inferior extremity of this vein. No valves were found between the latter point and the auricle.

The respiratory phases were recorded as described above. The venous pulse was recorded by a membrane manometer, connected by means of a T tube with the central cannula of the stromuhr. This record served me in obtaining the various phases of the cardiac cycle.

Venous pulsation may be due first to the contraction of the right auricle and ventricle, secondly to various accidental mechanical influences, and thirdly—at least in the smaller veins—to the arterial pulse. It is apparent that in these experiments only the first type has to be considered.

In interpreting the venous pulse curve obtained in these experiments I made use of the investigation of Fredericq¹ which, so far as

¹ FREDERICQ, L.: *Travaux du Laboratoire de Liège*, 1889-90, x, pp. 85-107.

I know, is the only recent paper on this subject. Fredericq recognizes two distinct rises in the venous pulse; the first he attributes to the systole of the right auricle and the second to the ventricular contraction. In general, the curves obtained by me correspond very closely with those in the paper referred to. Only one difference should be spoken of, and that is the fact that the auricular contraction always gave a strong rise, much higher than that of ventricular systole. In this respect the curve is identical with the curve of intra-auricular pressure shown in Fig. 10.

But it seemed essential that the curve of the blood-flow be compared directly with the curve of intra-auricular pressure. This, it seems to me, can be done without hesitation. In the paper cited above, Fredericq comes to the conclusion that the pressure-curve of the external jugular exhibits essentially the same characteristics as that of the right auricle.

Before proceeding further it might be well to consider briefly the different characteristic points of the intra-auricular pressure curve. According to Porter,¹ whose results substantiate and extend those of Fredericq, the auricular curve consists of:

A. The systolic rise, corresponding to the contraction of the auricle.

B. The first diastolic fall, corresponding to the relaxation of the auricle.

C. The first diastolic rise, from near the beginning of ventricular systole to the opening of the semilunar valves.

D. The second diastolic fall, from the opening of the semilunar valves to near the beginning of ventricular relaxation.

E. The second diastolic rise, from the end of the second diastolic fall to the beginning of ventricular relaxation.

F. The third diastolic fall, during some portion of ventricular relaxation.

G. The pause, from the third diastolic fall to the next systolic rise.

Upon this curve I shall largely rely in harmonizing the different features of the variations of the blood-flow with the changes in intra-auricular pressure.

Fig. 11 is an example of the type of variations to be dealt with in this chapter. The entire line recording the blood-flow exhibits numerous depressions similar in form and recurring at regular intervals. Between these indentations the curve pursues a wavy

¹ PORTER, W. T.: *Journal of physiology*, 1892, xiii. pp. 513-553.

course upward, first ascending very rapidly and then extremely slowly. We observe, therefore, that the flow is not equally great at

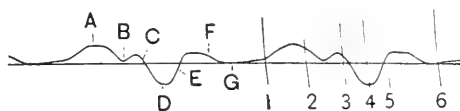


FIGURE 10. — Curve of intra-auricular pressure, taken with a large cannula in the auricular appendix (PORTER).

- | | |
|-------------------------------------|---|
| <i>A.</i> Systolic rise. | 1. Beginning of auricular systole. |
| <i>A.-B.</i> First diastolic fall. | 2. Beginning of ventricular systole. |
| <i>B.-C.</i> First diastolic rise. | 3. Opening of semilunar valves. |
| <i>C.-D.</i> Second diastolic fall. | 5. Beginning of ventricular downstroke. |
| <i>E.</i> Second diastolic rise. | 6. End of ventricular downstroke. |
| <i>F.</i> Third diastolic fall. | |
| <i>G.</i> Pause. | |

all times. There is a period during which a great quantity of blood is moved onward and a period during which very little flow takes place. Records taken at a greater speed (Fig. 12), in which the details of the curve are rendered visible, will show that in this second period there are times when the flow is completely arrested. These will be discussed below.

A comparison between the ordinates of the respiratory phases and those of the blood-flow in Fig. 11 will show immediately that the variations seen here are absolutely different from those described in the preceding chapter. But, if the ordinates of the blood-flow are compared with those of the venous pulse a complete correspondence is seen to exist.

The first elevation in the venous pulse curve, numbered 1, 2, etc. corresponds exactly with the bottom of the notch in the curve of the blood-flow, 1, 2, etc. The end of the period of slight flow is therefore synchronous with the end of auricular systole. As soon as the diastolic phase of this cavity begins, the blood-flow is greatly increased, from 1, 2, etc., to 1', 2', etc. The latter ordinates indicate the end of the period of greatest negative pressure during auricular diastole. Thus we see that the period of increased flow (1, 2, etc., to 1', 2', etc.) coincides with the entire diastolic phase of the auricle, from the end of auricular systole to the beginning of the second diastolic rise. From 1', 2', etc., to 1, 2, etc., a very slight rise in the curve of the blood-flow is noticeable. This period of slight flow commences with the second diastolic rise and ends with the next auricular systole.

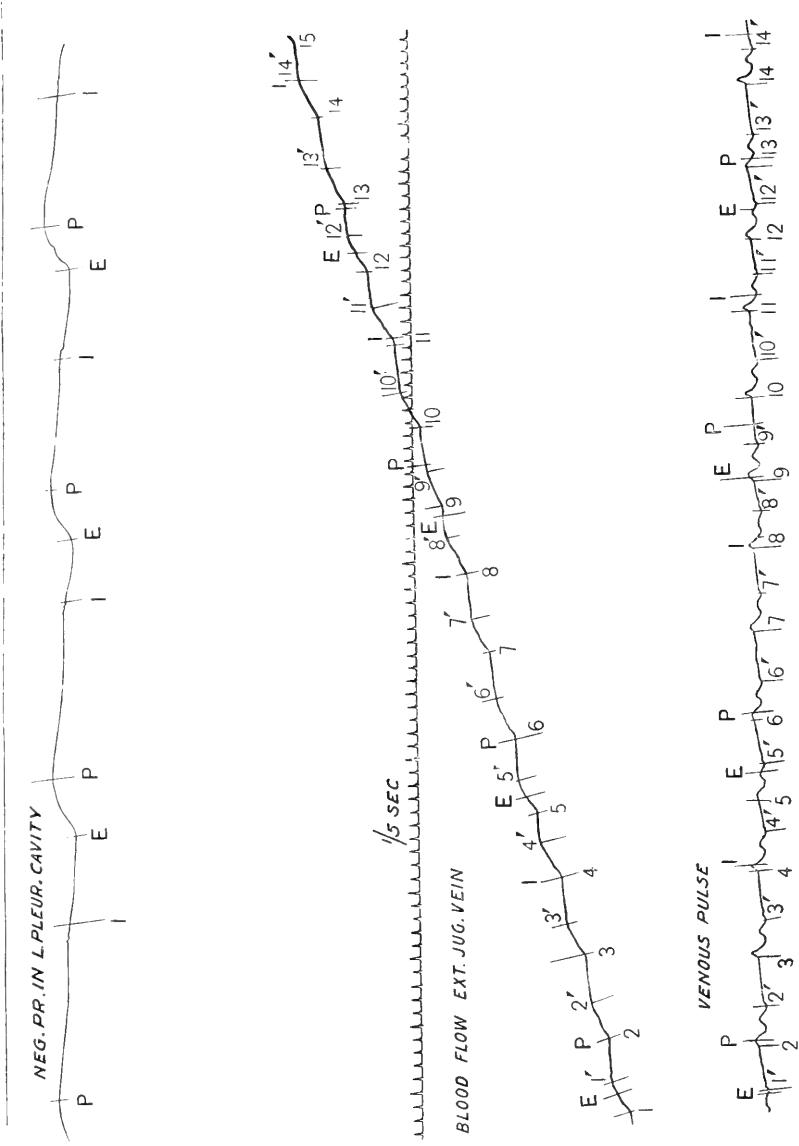


FIGURE 11. — The cardiac variations in the blood-flow.

A more careful inspection of the curve will also reveal the presence of a second slight notch about midway between the end of auricular systole (1, 2, etc.), and the end of the second diastolic fall (1', 2', etc.). This depression, shown clearly in only a few of the intersystolic phases of this curve, coincides with the beginning of ventricular systole. But, to show the full significance of this indentation and also to bring out more clearly the several features of the curve, it is necessary to study a curve written upon a rapidly revolving drum (Fig. 12).

When the different phases of the auricular cycle are in this way forced more widely apart, each intersystolic period shows the following details: The fall in pressure following the auricular systole is accompanied by an increase in the blood-flow (*A-B*) which continues during the entire first diastolic fall (*A-B*). During the first diastolic rise, *i.e.*, from the beginning of ventricular contraction to the opening of the semilunar valve, no onward movement of blood occurs, (*B-C*). The second diastolic fall (*C-D*), following the opening of these valves, produces a renewed increase in the blood-flow, which is again stopped during the second diastolic rise (*D-E*). There is another noticeable rise in the curve of the blood-flow at *E*, but it is much slighter than the two preceding and very likely takes place during *F-G*, the third diastolic fall. The quantity of blood propelled during the common pause (*F-G*) is extremely small. During the auricular systole (*G-A*) no onward movement of blood occurs.

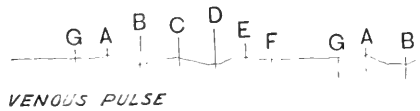
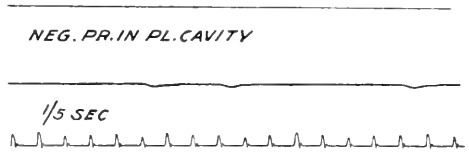


FIGURE 12.—The cardiac variations.

François-Frank¹ inserted a Chauveau's hæmodromograph in the external jugular vein and found that the systole of the auricle does not cause the column of blood to move backward. When he, however, destroyed the valves centrally to the instrument a backward movement of the blood took place. He therefore concluded that normally

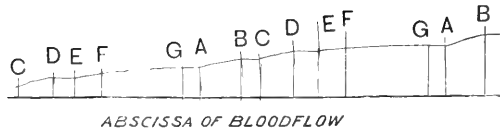
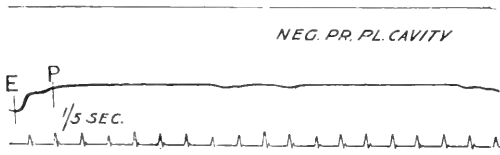


FIGURE 13.—The cardiac variations.

such an occurrence is made impossible by the interposition of the venous valves. He further states that a cessation of flow does not take place during this period.

While the experiments now under consideration prove the former statement to be correct, no evidence is contained in the curves to show that a stoppage in the blood-flow does not occur. When the abscissa is drawn and the ordinates of the curve of the blood-flow are continued downward, it can readily be seen that this curve pursues a parallel course to the abscissa in three places, namely, from *G-A*, from *B-C*, and from *D-E*. (Compare Fig. 13.)

When these periods are compared with the curve of auricular pressure it is found that no onward movement of blood in the external jugular vein takes place:

1. During the systole of the auricle,
2. During the first diastolic rise,
3. During the second diastolic rise.

¹ FRANÇOIS-FRANK, C. A.: Archives de physiologie, 1890, pp. 347-354 and pp. 395-410.

Thus, we must conclude that the volume of blood in this vein enters the central venous trunks and right auricle only during the periods of lesser pressure, namely:

1. During the first diastolic fall,
2. During the second diastolic fall,
3. During the third diastolic fall,
4. During the pause common to both auricle and ventricle.

These conclusions confirm and extend the results gained in Porter's study of the effect of changes in auricular and ventricular pressure upon the filling of the heart: He concluded from the origin of the systolic and the first diastolic rise, that the auricular pressure at these moments is higher than the pressure in the contiguous veins and that the venous inflow must at these times cease (page 525). With regard to the second diastolic rise his observations did not enable him to speak with confidence.

Regarding the third diastolic fall and the pause common to both auricle and ventricle, we have previously seen that the blood-flow during the third diastolic fall (*E* to *F*) is much less than during the first two periods of falling pressure. During the pause hardly any flow is noticeable.¹

The main factor in propelling the blood must therefore be the first and second diastolic falls. The second diastolic fall appears to exercise an even greater suction-action than the first. Although no exact determinations were made, this statement is well substantiated by the largest number of the curves. It harmonizes with the fact that according to Porter a greater fall in pressure occurs during this period. (Compare Fig. 10 and also page 533 of Porter's paper referred to previously.)

To show the relative importance of the various periods of the curve of auricular pressure I have calculated the value of the blood-flow (c.c. per second) for the first and the second half of each auricular cycle. The first half embraces the first diastolic fall, first diastolic rise, and second diastolic fall. The second half includes the values from the beginning of the second diastolic rise to the end of the next systolic rise. In the curves of Figs. 12 and 13 these periods are marked from *A* to *D* and from *D* to *A*. In the curve of Fig. 11 the first period extends from 1, 2, etc., to 1', 2', etc., and the latter period

¹ In two experiments there was hardly any increase in the blood-flow, even during the third diastolic fall. The force of the heart undoubtedly produces some slight alterations at times.

from 1', 2', etc., to 1, 2, etc. From thirty to forty intersystolic phases taken upon a rapid-moving drum, were measured in each case. This number seemed sufficient, because the individual deviations are very slight.

TABLE V.

THE RELATIVE VALUES OF THE BLOOD-FLOW FOR THE FIRST AND SECOND HALVES OF AN AURICULAR CYCLE.

Exp.	Weight of dogs in kilos.	Duration of first period from A to D (sec.).	Volume of blood. c.c. per sec.	Duration of second period from D to A.	Volume of blood. c.c. per sec.	Duration of auricular cycle in sec.	Volume of blood. c.c. per sec.
1	9	0.37	1.75	0.32	0.15	0.69	1.9
2	12	0.5	2.15	0.6	0.25	1.1	2.4
3	9	0.49	1.7	0.51	0.10	1.0	1.8

This table serves to prove again the observation made previously that by far the greatest volume of blood is moved onward during the first half of the auricular cycle, *i. e.*, during the period of greatest fall in pressure. During the second half, including the third diastolic fall and the common pause, the blood-flow is minimal. We observe that the former volume is about ten times as great as the latter.

This fact is of the highest importance to the frequent heart. For, when the heart beats quickly, the common pause is reduced in duration or entirely absent. It can readily be seen that only a very slight loss to the volume of inflowing blood is occasioned by the absence of the pause. Indeed, the auricular cycle might be reduced to about one half its normal duration and still there would not be an important decrease in the volume of the inflowing blood, so long as the duration of the first two diastolic falls remained unchanged.

Porter's measurements, on pages 531 to 533 of the paper already cited, show that the duration of the second diastolic fall remains practically unchanged even in the most frequent heart and that only the succeeding part of diastole, including the pause, suffers the shortening in time.

And even if the second diastolic fall should be slightly shortened when the heart beats very quickly and without a corresponding decrease in the length of the systolic periods, the loss in blood which

would in this case occur might be compensated by a correspondingly greater fall in pressure.

In order to show more clearly the relationship between the changes in intra-auricular pressure and the cardiac variations in the blood-flow, I have placed my curve, showing the volume of the blood-flow

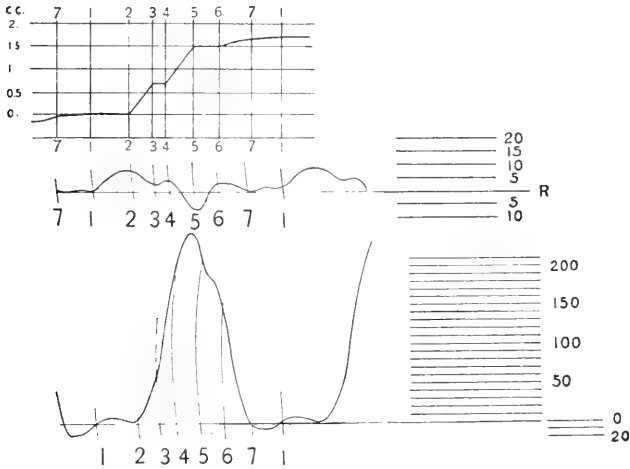


FIGURE 14.—The blood-flow in the external jugular vein in relation with the auricular and ventricular pressure curves. The uppermost curve shows the volume of blood flowing through the external jugular vein from the measurements in the present investigation. The middle and lowermost curves are respectively the intra-auricular and the intra-ventricular pressure curves, simultaneously recorded. The calibration of the auricular and ventricular membrane manometers are shown at the side. (PORTER, W. T., Figs. 1, 2, and 13, Plate XVIII.)

- | | |
|-----------------------------|------------------------------|
| 1-2. Systolic rise. | First cessation of flow. |
| 2-3. First diastolic fall. | First period of great flow. |
| 3-4. First diastolic rise. | Second cessation of flow. |
| 4-5. Second diastolic fall. | Second period of great flow. |
| 5-6. Second diastolic rise. | Third cessation of flow. |
| 6-7. Third diastolic fall. | Period of slight flow. |
| 7-1. Common pause. | Slight flow, or stoppage. |

through the external jugular vein, above the curves of pressure in the auricle and ventricle. (Fig. 14.) The time of the auricular cycle is one second and the quantity of blood propelled during this entire period is 1.7 c.c. The relative volumes for the different phases are given in 0.1 c.c.

Before concluding the discussion of the variations in the blood-flow, reference must be made to the changes which are produced by the simultaneous appearance of the respiratory and cardiac variations.

In Fig. 15 we observe that by far the largest number of the cardiac variations occur during the respiratory pause. Only two (3 and 7) coincide with respiratory phases. After the second and seventh systolic rises (auricular systoles) the blood-flow shows the usual increase (2 to *I* and 7 to *I*), but the succeeding period of slight flow during the last half of the auricular cycle is not present. Instead, there is an even greater onward flow (*I* to *E*), which entirely destroys the depression that would otherwise occur during this secondary rise.

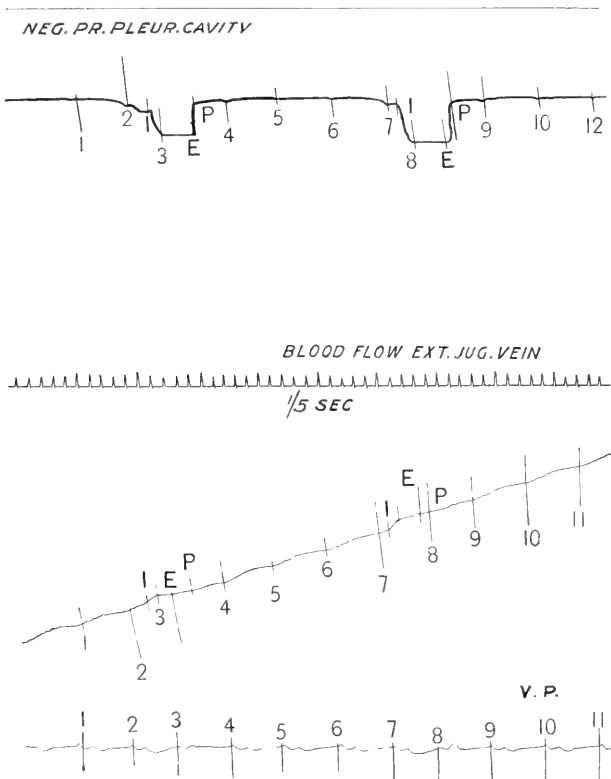


FIGURE 15. — The cardiac and respiratory variations.

The greater fall in intravenous pressure occasioned by the inspiratory movement (*I* to *E*) interferes therefore with the normal formation of the cardiac variations and gives rise to a blood-flow which is proportional to the sum of the intravenous pressures, caused by the force of the heart and by the depth of inspiration.

The same summation by interference occurs during the expiratory movement, but so soon as the respiratory pause commences, the cardiac variations again become prominent.

SUMMARY.

1. The normal volume of the blood-stream in the external jugular vein in a dog weighing 13 kilos amounts to about 2.4 c.c. per second. The velocity is 147 mm. per second.

2. By stimulating the vagus a total cessation of flow can be produced.

3. Section of both vagi increases the volume of the blood-stream 2.8 times.

4. By compressing both carotid arteries the quantity of blood is reduced 57 per cent.

5. The blood-flow in the external jugular vein is intermittent.

6. Two types of variations are present, namely those due to the respiratory movements and those caused by the changes in pressure during each auricular cycle.

7. Inspiration quickens the flow, expiration lessens it. The conspicuousness of the respiratory variations is dependent upon the frequency and the depth of the respiratory movements.

8. By increasing the intrapleural negative pressure slightly by stimulating the phrenic nerves the blood-flow is exaggerated. Strong stimulation of these nerves, on the other hand, generally produces a retardation.

9. During each auricular cycle the blood-flow ceases when the periods of rising pressure occur. The flow during the first and second diastolic falls is about ten times as great as during the other phases; therefore, in the frequent heart the duration of the entire cycle may be reduced to more than one-half before an appreciable loss in the quantity of inflowing blood would result. The second diastolic fall is more important than the first.

10. When the respiratory and cardiac pressure changes occur synchronously, the volume of the blood-stream is determined by the combined influence of both factors.

ON THE QUANTITATIVE DETERMINATION OF ACID-ALBUMIN IN DIGESTIVE MIXTURES.

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I. INTRODUCTORY

IN many of the experiments which have been carried out to determine quantitatively the proteolytic power of pepsin under various conditions, the chief deductions have been drawn directly from the amounts of undigested or residual matter rather than from the proportions of the digestive products themselves. In a majority of these cases the figures for undigested matter have doubtless suggested approximately correct conclusions in this regard, but it seems probable that, in some instances at least, quantitative studies of the albuminates, proteoses and peptones formed would have furnished more accurate and acceptable data.

The writer has recently been engaged in a study of the action of pepsin under varying degrees of acidity with a number of acids, and in the presence of different ions, the results of which will be reported later. In experiments of such character the increasing

or decreasing amounts of acid associated with the pepsin, to say nothing of its quality, variously affect the proteid indicator, irrespective of the influence on the latter of the enzyme. Different proportions of acidalbumin would be formed, also, with variations in the chemical character and physical condition of the proteid used to test relative zymolysis. If correct comparative deductions are to be drawn from the results of such experiments, it would seem that determining the amounts of albuminate present in each case would be almost if not quite as important as ascertaining the quantity of undissolved or undigested substance. It is conceivable that in comparative cases where, for example, the undigested matter might be decreased, the proportion of acidalbumin formed by the mere solvent action of the acid might be correspondingly larger. To assume from the fact of diminished quantity of original proteid, in such an instance, that zymolysis had been greater in the one case than in the other obviously would be unwarranted.

In the first of the writer's ion experiments, previously alluded to, purified fibrin was used as the indicator. At the end of the digestive interval the residue was filtered on a weighed paper and a given portion of the filtrate carefully neutralized for the precipitation and quantitative determination of the albuminate. After standing from twelve to twenty-four hours the precipitate was filtered on a weighed paper and, after washing and drying, estimated in the customary manner. Later, however, it was discovered that boiling the digestive fluid from which the neutral precipitate had been filtered, caused a further precipitate, presumably of albuminate, which was not separable by neutralization in the cold. The amount of this precipitate seemed comparatively small, but of course, for accuracy's sake, could not be ignored. The boiled fluid was either permanently turbid or minute flocks separated from it. The precipitate was obtained on boiling, in spite of the most careful neutralization of the digestive fluid. It likewise occurred independently of the character of the alkali used in neutralizing, the acid associated with the pepsin, the length of time between neutralization and filtration, and the volume of the digestive fluid.¹

These facts led us to make a special inquiry into the accuracy of the neutralization method for directly precipitating and determining the quantity of acidalbumin in digestive mixtures. This simple method is desirable and convenient not only for the special experi-

¹ See page 485 for further reference to the influence of volume.

ments in progress in this laboratory, but also for various other proteid studies. Further, such separation by neutralization alone is particularly advantageous in digestive experiments because it can be made without affecting the associated proteid products. Direct determinations of any substance, when they can be made accurately, always possess advantages over indirect determinations.

Very little attention has been given to the quantitative determination of acidalbumin. In those cases in which its approximate determination when present among other proteids has been desired, neutralization in the cold has been effected and then the precipitate has been filtered as in the writer's experiments just referred to. In most instances, however, acidalbumin has been determined as a part of albumin or globulin in the form of coagulated proteid; or, by reason of small amount or relative unimportance, has been ignored altogether.

As an example of direct determination quantitatively the process recently referred to by Effront¹ may be cited. In a general way this method has been in occasional use for years. In the experiments by Effront the acidalbumin ("syntonin") in a fluid mixture of proteoses, peptone, etc., was precipitated by careful neutralization. The neutral mixture was allowed to stand for two hours and the flocculent precipitate which had then separated was filtered on a weighed paper. Boiling was not a part of the process.

As we have already indicated, boiling the filtrate from the acidalbumin obtained in the cold fluid usually yields an additional flocculent proteid precipitate, an occurrence suggesting that mere neutralization is not sufficient for effecting separation if particular quantitative accuracy is desired.² Several theories to account for this fact suggest themselves.

It is usually stated that albuminates are insoluble in neutral salt solutions, although not all observers are agreed on this point.³ It

¹ EFFRONT: *Chemisches Centralblatt*, 1899, ii, p. 457.

² UMBER, among others, has noted, in cases where only a slight amount of acidalbumin, or none at all, could be precipitated on neutralizing, that the neutral filtrate remained clear on boiling, but additional "acidalbumin" separated on evaporation of the fluid to one-half its volume. See *Zeitschrift für physiologische Chemie*, 1898, xxv, p. 263.

³ HEYNSIUS: *Chemisches Centralblatt*, 1876, p. 807; MÖRNER: *Jahresbericht der Thier-Chemie*, 1877, p. 10; SAVIN: *Ibid.*, 1887, p. 2; NIKOLJUKIN: *Ibid.*, p. 5; HALLIBURTON: *Text-book of chemical physiology and pathology*, 1891, p. 128.

may be admitted that these derived proteid substances, and particularly the *dried* products, are for the most part insoluble in neutral saline media, but the moist, *freshly precipitated* digestive albuminate is clearly soluble in solutions of various salts, as we ourselves have definitely ascertained.¹ Consequently, on neutralization of its acid solutions, a portion of the acidalbumin remains in the salt solution formed in the process. On boiling the filtrate, however, some of this last residual portion is precipitated because albuminates are coagulable by heat in neutral saline fluids.²

It appeared probable, also, that in the experiments in mind the associated proteoses and peptones exerted solvent action on the albuminate, thus increasing the retaining power of the solution and thereby helping to prevent complete precipitation on simple neutralization.³

We were inclined to believe for a time that carbon dioxide in the fluid, which might have influenced the indicators (litmus and lacmoid papers), was driven out on boiling and its possible solvent action done away with, so that the rest of the albuminate was then thrown down.⁴

That the precipitate obtained on boiling was not due to earthy phosphate impurity in the reagents was definitely ascertained.

As the neutral point is approached in such experiments as these, it is possible that portions of the albuminate which have already been precipitated are redissolved and perhaps modified by the dilute alkali, added drop by drop to the nearly neutral fluid. These dissolved portions are not precipitated again in the cold, possibly,

¹ See page 487. Most of the statements regarding insolubility of acidalbumin refer to the dried product prepared from muscle tissue. By many writers these statements have been accepted as including the acidalbumin formed during pepsin proteolysis. Acidalbumin prepared from muscle tissue begins to diminish in solubility in dilute acid, even after standing under water for only a few minutes. Acidalbumin formed during peptic digestion is very different in this respect, for its solubility in dilute acid remains essentially the same, no matter how often it is washed, nor is its solubility altered by drying at 40° C. Even then it is soluble to a certain extent in dilute salt solution. Myosin albuminate under these conditions is quite insoluble. See pages 464, 472, and 486.

² We do not say above that *all* of the albuminate is precipitated on boiling, for the reasons given on page 474.

³ See page 479.

⁴ The alkaline fluids used in neutralizing were dilute KOH and NaOH. These naturally introduce some carbonate, no matter how carefully the pure solutions are handled in such experiments. See page 487.

because of the lack of acidity or on account of the solvent action of the increasing quantity of salts formed in the neutralization process.

We are not aware of any combinations of albuminate with proteose or peptone, nor of transformations of these substances under the conditions of these experiments, which would account for the precipitate thrown down when the neutral fluid is boiled.¹

It seemed desirable, then, to determine the influence of the various factors referred to and, particularly, to ascertain the proportions of albuminate lost on neutralizing, as well as the proportion thrown down on boiling the filtrate from which the neutralization precipitate had been removed. The facts we have ascertained, bearing on the sufficiency of the precipitation method of direct determination of acidalbumin, are indicated in the summaries of our experiments on pages 470-484.

II. EXPERIMENTAL.

Preparation of acidalbumin.—Two varieties of acidalbumin were used in these experiments. One was prepared from muscle with 0.2 per cent HCl at room temperature, the other from fibrin with pepsin—HCl (0.2 per cent) at 40°C.

Acidalbumin from muscle.—**A.** Several pounds of fresh, lean meat was finely minced in a meat chopper and the hash thoroughly washed in running water for thirty-six hours. After straining the last washings through cloth the hash was placed in an excess of 0.2 per cent HCl and kept there for twenty-four hours. At the end of that time the acid extract was filtered and the "syntonin" separated by neutralization with dilute KOH. The separated precipitate was redissolved in 0.2 per cent HCl and reprecipitated three times with dilute KOH for the complete removal of impurities. The final precipitate obtained from the filtered solution was frequently washed during twenty-four hours by decantation in seven to eight litres at a time, at first with ordinary water, at last with distilled water. All of the washings contained substance yielding the biuret reaction and causing very faint turbidity on boiling. A trace of this substance persisted in the washings, indicating a slight solubility of the freshly precipitated material

¹ The solutions were not sufficiently concentrated for the separation of heteroproteose, nor was there any acidity for the precipitation of acropoteose. The precipitate bore no resemblance to "coagulated" heteroproteose. There is no reason for believing that dysproteose separated under these conditions.

even in water. The precipitate was finally filtered off, spread on a glass plate in a thin layer and dried in a few hours in warm air at a temperature slightly under 40° C.¹ The dried material was eventually ground to a very fine powder before using. About 50 gms. were prepared.

- B.** A second preparation from washed meat was made by essentially the same method as that used for the separation of the first. This preparation was not dried, but the moist substance after thorough reprecipitation, washing, filtration, etc., was used in the sixth experiment, as indicated on page 478. In this preparation, also, the washings, in spite of their volume and frequency, contained, to the last, a trace of substance separable by boiling. Phosphates were absent from the later washings.

Acidalbumin from fibrin.—**C.** A considerable quantity of fibrin, which had been kept in 95 per cent alcohol for some time, was put through a meat chopper and the alcohol thoroughly washed out in running water. After the completion of the washing process the fibrin was placed in a moderate amount of HCl (0.2 per cent) containing only a very small proportion of pepsin and was kept at 40° C. for about an hour—until practically all of the fibrin had dissolved. The amount of pepsin selected was small, and the period of digestion short, so that the proportion of albuminate at this stage should be large. The digestive mixture was now brought quickly to the boiling-point, to destroy the pepsin; was kept at the boiling-point for a minute or two, and then immediately cooled to about 25–30° C. The cold filtrate was next neutralized with dilute KOH, and the heavy flocculent precipitate redissolved in 0.2 per cent HCl and reprecipitated once with dilute KOH, after which it was repeatedly and very thoroughly washed by decantation in large excess of ordinary water and, finally, in distilled water. The substance settled quickly and could be washed repeatedly in twenty-four hours. Even to the last, the washings gave biuret reactions and became turbid on boiling, just as with the product obtained from muscle. It seems necessary to conclude that in this case also the freshly precipitated material was slightly soluble in the water.

The freshly precipitated substance was used in the first experiment, as stated on page 470.

- D.** The second preparation of acidalbumin from fibrin by digestive process was made in essentially the same manner as the previous one. Fibrin boiled in water, and then extracted in alcohol and ether was used. The precipitate was washed in about fourteen litres of water frequently during twenty-four hours. Even to the last, the washings again became slightly turbid on boiling and on the addition of picric acid. On warming, the turbidity with the latter appeared to diminish somewhat and to increase

¹ The time required for the drying was too short for any perceptible bacterial changes to have occurred.

again on cooling, facts indicating the presence of proteose with acid-albumin.¹

The moist substance was finally dried in a thin layer² in warm air at a temperature below 40° C. The dried substance was finely powdered. It weighed 2.5 gms.

E. A third preparation of acidalbumin was made from fibrin by enzyme action. The fibrin had not been boiled, although it had been thoroughly washed in alcohol. This sample also was made by the general method just outlined. The neutralization precipitate was redissolved in 0.2 per cent HCl four times and as frequently reprecipitated with dilute KOH. Extreme care was taken to wash thoroughly and frequently. The precipitate was whipped up repeatedly in as much as fourteen litres of water at a time. The final washings in distilled water were almost entirely free from substance giving the biuret reaction and yielding turbidity on boiling or on treatment with picric acid. At this point the substance was divided roughly into two portions.

a. The first portion was dissolved in 0.2 per cent HCl, the solution diluted with an equal volume of water and filtered. The filtrate was precipitated with dilute KOH and the proteid, after thorough washing for a few hours, was spread on a glass plate and quickly dried below 40° C. as usual. Twelve grams were obtained.

b. The second portion was dissolved in 0.2 per cent HCl, allowed to stand several hours, without dilution, and then precipitated, washed and dried as was the first portion. It weighed about 30 gms.

The washings of both portions at first showed an increased content of acid-albumin. Merely a trace was present in the final washings in distilled water.

F. The fourth preparation of acidalbumin from fibrin was made from several hundred grams of the proteid which had not been boiled in water, but which had been very thoroughly extracted in alcohol and in ether. The method of preparation was the same as that for the previous products. The acidalbumin was thoroughly washed in fourteen litres of water eight times during forty-eight hours. At the end of the process only a mere trace of coagulable substance was detectable in the distilled water washings, and in the last two washings no satisfactory biuret reaction could

¹ The washing was done frequently during twenty-four hours, and there was hardly time enough for bacteria to develop and form proteose. It is possible that in the course of twenty-four hours slight bacterial changes did occur without our knowledge. The water used in washing all these preparations was kept free of antibacterial substances so as to prevent possible transformations of the desired products through such chemical agencies. See page 486.

² Higher temperature was avoided to prevent possible transformation into a less soluble product.

be obtained without concentration. The moist substance, after it had been allowed to drain, and after excess of moisture had been expressed from it through hard filter paper, was used in the experiment referred to on page 487.

G. A mixture of residues of **D** and **E** was used in the eleventh experiment, described on page 484.

Acids employed (salts formed). — In order to test the precipitation method as thoroughly as possible the following acids were used: hydrochloric, nitric, chloric, sulphuric, arsenic, phosphoric, acetic, lactic, oxalic, tartaric and citric. All these were carefully titrated with standard alkali and appropriate indicators, and made equivalent to $\frac{m}{10}$ NaOH.¹

In our experiments the acidalbumin was transferred to the acid and, after solution of the substance, most of the fluids were carefully made neutral to litmus. Mixtures in which acid salts were formed were tested with lacmoid paper. Alkali was added to these until all free acid was exactly transformed to *acid* salt.

In all cases permanent, bulky precipitates were formed even while some free acid still remained in the fluid, a fact in harmony with previous observations by various investigators.² The maximum effects were obtained at the neutral point, however, or when free acid was present only in inappreciable traces.³

¹ $\frac{m}{10}$ HCl contains 0.36 per cent HCl. $\frac{m}{20}$ H₂SO₄ contains 0.49 per cent H₂SO₄, $\frac{m}{30}$ H₃PO₄ contains 0.33 per cent H₃PO₄. These strengths of acid are approximately equivalent to those used in representative peptic digestive experiments.

² The salts formed on neutralization help precipitation. The more saline matter present in the fluid the greater the acidity may be without the exertion of solvent action on the part of the acid. This fact accounts for the heavy turbidity observed in some of the fluids, while free acid was still detectable in them. This precipitation occurred earlier in some than in others, doubtless because of the different influence of the anions. It appeared in the sulphate solution as quickly as in any, SO₄ seemingly being helpful to the precipitation of acidalbumin.

³ In reprecipitating several of our main products it was observed that when the solution was carefully carried from acid to *exact neutral reaction*, the supernatant fluid over the main bulk of the precipitate remained somewhat milky. On adding a little more dilute alkali the substance causing the turbidity became flocculent and settled out quickly under a perfectly clear fluid. The latter still remained neutral to litmus. In our quantitative experiments neutralization was carried to the point of flocculation in a perfectly clear fluid. This point corresponds very closely with the point of neutralization of acid and alkali. The filtrates from the neutral precipitates were "water-clear." See footnote, page 469.

Proteoses and peptones used. — Witte's peptone was used in all of the experiments in which we determined the influence of the digestive products on the precipitation of acidalbumin.

In such experiments weighed amounts of dry Witte's peptone and our acid-albumins were dissolved in given quantities of each of the acids above-mentioned. With most of the acids all of the substance comprising the "peptone" completely dissolved. In others, however, a permanent precipitate was formed, either immediately on admixture or later on neutralizing. In each case we determined accurately the amounts of the precipitates of such extraneous matter and made corresponding corrections in the data subsequently obtained.

The following summary gives our results in this connection, no precipitate as bulky or as heavy as that in HCl having been obtained with the acids not mentioned below.

Summary. — 100 c.c. of each acid was taken. All were equivalent to $\frac{m}{10}$ NaOH. Weighed peptone (Witte's) was dissolved in each.¹ The solutions were left standing several hours, and then neutralized with dilute KOH, litmus or lacmoid the indicator — in some cases before boiling, in others while the fluid was at the boiling point. After neutralization each mixture was allowed to stand over night and then was filtered. Subsequent boiling of the neutral filtrate failed to cause turbidity; neither did longer standing result in further separation of solid matter.

Acid.	Amount of pep- tone dissolved. Gram.	Time of neutralization.	Amount of neutralization pre- cipitate. Gram.
<i>A.</i> Oxalic	<i>a</i> 0.5	Before boiling	0.0214
	<i>b</i> 0.5	After "	0.0201
	<i>c</i> 1.0	Before "	0.0392
	<i>d</i> 1.0	After "	0.0376
<i>B.</i> Phosphoric	<i>a</i> 1.0	Before "	0.0146
	<i>b</i> 1.0	After "	0.0158
<i>C.</i> Citric	<i>a</i> 1.0	Before "	0.0091
	<i>b</i> 1.0	After "	0.0058
<i>D.</i> Tartaric	<i>a</i> 1.0	Before "	0.0118
	<i>b</i> 1.0	After "	0.0076
<i>E.</i> Hydrochloric	<i>a</i> 0.5	Before "	0.0032
	<i>b</i> 1.0	" "	0.0058

¹ Samples of the preparation used in the succeeding experiments. See page 469.

Compounds with calcium and with phosphoric acid doubtless account for the greater portion of the above precipitates. Witte's peptone contains both of these. The boiling process does not appear to have increased the quantity of the precipitated matter, but rather decreased it in most cases.

Quantities of solids and fluids taken.— Unless otherwise stated, 100 c.c. of acid was used for each experiment of a series. The quantities of acidalbumin were usually between 0.05 and 0.5 gm.; of peptone, between 0.5 and 1.0 gm.

We purposely used small amounts of both solids and fluids, believing that the method could be tested most effectively by so doing. With comparatively large quantities of the proteids and fluid, defects of manipulation are apt to cause appreciable errors. Small quantities are more easily and accurately handled. The amounts and proportions employed were such as have figured in the past in typical digestive experiments. In our main series of experiments we used portions of the same general supplies of the acids and Witte's peptone throughout. No variations were introduced, therefore, by reason of differences in the character of the materials used. In all cases where the dry acidalbumin was used we refer to the product dried below 40° C. The amount of water in the "air-dried" preparations was accurately determined by drying to constant weight at 100–105° C. in the usual manner and due correction made as indicated below.

Precipitation of acidalbumin.— The albuminate was first dissolved in the acids alone as already indicated, or in the acids with their content of Witte's peptone. The mixtures were usually allowed to stand in this condition for an hour or more, when careful neutralization was begun and completed as soon as possible.¹

The neutralized fluids with their precipitates were allowed to stand undisturbed until the following morning, when the solid matter was filtered on weighed papers, washed with water until free of soluble matter² and eventually dried in the air-bath in the customary manner. In the summaries farther on we give the corrected final

¹ The combining power of the "peptone" for the acid did not, as will be seen, appreciably influence the effect of neutralization. Acid combined by the peptone was doubtless too slight in amount to be of significance in this connection.

² In only a few instances were the filtrates turbid. By repeated filtration the solid matter was retained. The washings, also, on several occasions manifested initial turbidity, but the solid substance of these was likewise held after several filtrations. As these washings were always neutral it is difficult to account for the turbidity on any other than mechanical grounds.

weights in each case.¹ Other matters of method are indicated with the summaries of each experiment.

The completeness of precipitation of acidalbumin from its acid solutions by neutralization depends largely on the amount of saline matter present.² In our own experiments the acid was dilute and the final content of salt in the fluid on neutralizing was small, though sufficient for the precipitation of the amounts used. We were careful to carry the addition of acid to the point of exact neutrality or disappearance of free acid, which method, by cautious manipulation, was found to give the maximum amounts of precipitate.³ Various observers, among them recently Spiro and Pemsel,⁴ have noted the difficulty of completely precipitating acidalbumin on neutralizing and it is, perhaps, a fact not generally appreciated.

First experiment. — In this experiment we endeavored primarily to get an accurate idea of the relative proportion of substance thrown from the various neutral filtrates on boiling.

Summary. — Fibrin acidalbumin of preparation **C** was employed. Several grams of the latter was dissolved in a few c.c. of $\frac{m}{10}$ HCl and 3 c.c. of this solution added to each of the acids — 100 c.c. $\frac{m}{10}$ or equivalent thereto — and also to 100 c.c. H₂O. Neutralization was made with dilute KOH in this and the four succeeding experiments. The neutral filtrate was brought to the boiling-point and maintained there about a minute. The gravimetric results are shown in the table on page 471.

All the above filtrates that were obtained after boiling gave the biuret reaction and yielded slight amounts of proteid substance with alcohol.⁵ This precipitate dissolved in water and gave the proteose reaction with picric acid. This fact suggested that proteose was contained in the substance in spite of the thorough washing to which it had been subjected.⁶ Possibly, however, some of the proteose was derived from the albuminate on boiling.⁷

¹ See facts regarding precipitates of extraneous matter from the peptone, page 468. No allowance was made for the ash of the neutralization precipitates. The quantities of ash were entirely too slight to affect the figures given.

² See footnote 2 on page 467.

³ The formation of alkaline salts was avoided. See page 467.

⁴ SPIRO and PEMSEL: *Zeitschrift für physiologische Chemie*, 1898, xxvi, p. 236.

⁵ A large proportion of this precipitate was inorganic matter.

⁶ See page 486.

⁷ See page 489. The amount of saline matter present in the fluid was quite sufficient for complete precipitation of the acidalbumin.

In this experiment the precipitate obtained on boiling the neutral filtrate amounted, usually, to from 5 to 25 per cent of the quantity separated in the cold with dilute alkali. It is to be noted that no precipitate was obtained, on boiling, from the "control" nor from the solution of citrate. Further, it will be observed that the amount of albuminate recovered from the "water control" was greater than from any other solution. In this slightly acid mixture the amount of saline matter was comparatively small and solvent action on the acidalbumin greatly reduced therefore.¹

Acid.	Amount of neutralization precipitate. Gram.	Quantity of precipitate on boiling the neutral filtrate. Gram.
Sulphuric	0.031	0.003
Hydrochloric	0.031	0.004
Nitric	0.033	0.003
Lactic	0.033	0.008
Chloric	0.035	0.003
Oxalic	0.035	0.004
Phosphoric	0.036 ¹	0.003
Tartaric	0.036	0.004
Acetic	0.036	0.005
Arsenic	0.037	0.002
Citric	0.039	none
Water (control)	0.040	"
Average	(<i>a</i>) 0.035	(<i>b</i>) 0.003

Ratio. *a* : *b* = 12 : 1

Second experiment. — In the preceding series we did not know the exact amount of substance taken to begin with. Further, by dissolving the albuminate in HCl, and distributing it in such solution, we

¹ It will be remembered that 3 c.c. of $\frac{m}{10}$ HCl solution of acidalbumin was added to the water. The very slight proportionate acidity resulting thereby was sufficient to keep all of the substance dissolved.

introduced a small amount of the HCl into all of the acids and in the water, and thus, perhaps, tended to complicate matters. In this experiment we began with accurately weighed amounts of dry substance.

Summary.—0.2 gm. portions of fibrin albuminate of preparation **D** were weighed carefully and transferred to the acids. The weight of this amount of albuminate at 100–105° C. was found to be 0.181 gm.¹

Acid.	Amount of neutralization precipitate. Gram.	Quantity of precipitate on boiling the neutral filtrate. Gram.	Total amount of acidalbumin recovered. Gram.
Sulphuric	0.109	0.018	0.127
Oxalic	0.114	0.007	0.121
Chloric	0.114	0.017	0.131
Nitric	0.117	0.009	0.126
Tartaric	0.123	none	0.123
Hydrochloric	0.126	0.008	0.134
Lactic	0.133	0.001	0.134
Acetic	0.134	0.011	0.145
Citric	0.135	none	0.135
Phosphoric	0.139	0.003	0.142
Arsenic	0.143	none	0.143
Average	(a) 0.126	(b) 0.007	(c) 0.133

Average total quantity of acidalbumin lost, 0.048 gm. = 26.5 per cent.
 Average quantity of acidalbumin lost on neutralizing, 0.055 gm. = 30.4 per cent.
 Ratio. $a : b = 18 : 1$.
 $b = 3.87$ per cent of the original acidalbumin and 5.26 per cent of c .

Each final filtrate gave the biuret reaction and, when treated with 95 per cent alcohol, yielded a slight amount of substance, which appeared to be proteose in part. These and the above results seem to indicate that some of the original acidalbumin remains in solution. The proteose probably came from some of the dissolved albuminate on boiling. The average proportion of substance separable on boil-

¹ See footnote 2, page 466.

ing was somewhat less in this experiment than in that preceding. In several cases, however, it was unusually large, probably because of inaccurate neutralization in the first place. The variations in the total amounts recovered are slight, and within the limits of unavoidable experimental errors, which shows that there are only insignificant differences in the precipitative influences of the various salts formed from the acids under these conditions.¹

Third experiment. — The preceding experiment was repeated, but with myosin albuminate. Preparation **A** was used for the purpose.

Acid.	Amount of neutralization precipitate. Gram.	Quantity of precipitate on boiling the neutral filtrate. Gram.	Total amount of acidalbumin recovered. Gram.
Hydrochloric	0.216	0.006	0.222
Tartaric	0.222	0.008	0.230
Oxalic	0.228	0.009	0.237
Acetic	0.229	0.002	0.231
Nitric	0.229	0.004	0.233
Citric	0.232	0.005	0.237
Sulphuric	0.233	0.005	0.238
Chloric	0.233	0.007	0.240
Arsenic	0.237	0.002	0.239
Lactic	0.239	0.008	0.247
Phosphoric	0.243	0.003	0.246
Average	(a) 0.231	(b) 0.005	(c) 0.236

Average total quantity of acidalbumin lost, 0.039 gm. = 14.2 per cent.
 Average quantity of acidalbumin lost on neutralizing, 0.044 gm. = 16.0 per cent.
 Ratio. $a : b = 46 : 1$.
 $b = 1.82$ per cent of the original acidalbumin, and 2.12 per cent of c .

Summary. — 0.3 gm. portions of “syntonin” were carefully weighed and transferred to the acids. The weight of this quantity of substance after drying in the air-bath was 0.275 gm. Much of the material failed to dissolve in the acids, even after twenty-four hours with frequent stirring.

¹ This will be found the case in all our experiments. See footnote, page 467.

The drying had materially affected its solubility. The freshly precipitated, *moist* substance, however, is very easily dissolved in acids much more dilute than those used here. The sulphuric acid seemed to have the least solvent action. The *washings* from the precipitates obtained on neutralization of the hydrochloric and oxalic acid solutions gave respectively 3 and 6 mgms. of substance on boiling. These amounts were included in those for the neutralization precipitates, given in the table on page 473.

It will be observed that the average amount of precipitate obtained on boiling is very nearly the same as in the previous experiment, but that its proportion of the neutralization precipitate is less than before. The acidalbumin prepared from muscle is less soluble in neutral saline solution than that obtained from fibrin through the action of pepsin. The proportion of total substance recovered is large, 85.8 per cent, but, nevertheless, a slight loss resulted — a fact doubtless due, in great part at least, to transformation on heating.¹

The final filtrates gave typical biuret reactions and precipitates with alcohol which, when dissolved in water, responded faintly though distinctly to the proteose reactions with picric acid, potassium-mercuric iodide, etc. This fact emphasizes the conclusion stated above, and further convinces us that in the boiling process some of the albuminate held in solution is converted into proteose.

Fourth experiment. — The preceding experiments made it clear that a small though appreciable quantity of acidalbumin remains in solution when the acid holding it is neutralized; further that, on boiling the neutral filtrate, a part of this remaining albuminate is precipitable, whereas the larger portion appears to be converted into non-coagulable material. We next endeavored to ascertain the influence of proteoses and peptone on the precipitability of acidalbumin.

Summary. — 0.2 gm. samples of fibrin albuminate, preparation **E**, portion **a**, were used. This amount was equivalent to 0.180 gm. of substance dried at 100–105° C. to constant weight. The weights of Witte's peptone in this and subsequent experiments are for substance as it was received in the original package. The fluid in the first of each pair of experiments with oxalic and hydrochloric acids (**a**) was neutralized at the boiling point, after nearly all of the acid had been previously transformed to salt; that in the second (**b**) was neutralized as usual before boiling. Neutralization in all of the others was made as before in the cold.

¹ We do not overlook the fact that the usual errors of manipulation might account for the observed difference between the quantity taken and that recovered. Our weighings, filtrations, etc., were very carefully conducted, however, and such errors were reduced to an inappreciable minimum.

Acid.		Weight of peptone. Gram.	Amount of neutralization precipitate. ¹ Gram.	Quantity of precipitate on boiling the neutral filtrate. Gram.	Total amount of acidalbumin recovered. Gram.	
A. Hydrochloric	1	<i>a</i>	0.133	none	0.133
		<i>b</i>	0.154	"	0.154
	2	<i>a</i>	0.5	0.129	"	0.129
		<i>b</i>	0.5	0.127	0.006	0.133
	3	<i>a</i>	1.0	0.130	none	0.130
		<i>b</i>	1.0	0.127	0.007	0.134
B. Oxalic	1	<i>a</i>	0.146	none	0.146
		<i>b</i>	0.145	"	0.145
	2	<i>a</i>	0.5	0.141	"	0.141
		<i>b</i>	0.5	0.146	0.005	0.151
	3	<i>a</i>	1.0	0.129	none	0.129
		<i>b</i>	1.0	0.143	0.007	0.150
C. Tartaric	1	0.145	none	0.145	
	2	0.5	0.138	"	0.138	
	3	1.0	0.137	0.004	0.141	
D. Phosphoric	1	0.149	none	0.149	
	2	0.5	0.139	0.007	0.146	
	3	1.0	0.155	none	0.155	
E. Sulphuric	1	0.153	"	0.153	
	2	0.5	0.158	0.010	0.168	
	3	1.0	0.147	0.005	0.152	
F. Nitric	1	0.148	none	0.148	
	2	0.5	0.156	"	0.156	
	3	1.0	0.144	0.008	0.152	
G. Citric	1	0.161	none	0.161	
	2	0.5	0.159	0.006	0.165	
	3	1.0	0.175	none	0.175	
H. Lactic	1	0.152	"	0.152	
	2	0.5	0.151	0.001	0.152	
	3	1.0	0.140	0.001	0.141	
Average ²	1	(I) 0.151	(II) none	(III) 0.151	
	2	0.5	0.147	0.004	0.151	
	3	1.0	0.146	0.004	0.150	

Average total quantity of acidalbumin lost, 0.029 gm. = 16.1 per cent.

Average quantity of acidalbumin lost on neutralizing (cold), 0.032 gm. = 17.8 per cent.

Ratio. I : II (for 2 and 3) = 37 : 1.

II (for 2 and 3) = 2.22 per cent of the original acidalbumin and 2.65 per cent of III.

¹ Due correction has been made, as indicated on page 468.

² These averages do not include any of the figures for *a* in A and B. They represent, therefore, the average precipitation under uniform conditions throughout. See references in this connection on page 474.

The final filtrates from those fluids into which peptone had not been introduced gave the biuret reaction, faintly though distinctly. It was strongest in the chloride and oxalate fluids. The delicate precipitate obtained on treatment with alcohol was composed in part of proteose. The alcoholic turbidity also was greatest in the chloride and oxalate filtrates.

These results are in harmony with the preceding in showing slight losses of albuminate.¹ The peptone appears to be without any particular influence. The quantities of acidalbumin recovered seem to be below the average in the chloride solution and somewhat above it in the citrate. These data accord with the facts, however, that acidalbumin is fairly soluble in chlorides and less soluble in equivalent amounts of citrates. The quantity recovered from the citrate solution has been relatively high in the preceding experiments, also.

A singular occurrence in this experiment, one rather difficult to account for in the light of the results of succeeding series, was the fact that all of the cold neutral filtrates which were free from peptone, failed to yield a further precipitate on boiling. Most of the cold filtrates containing peptone, on the other hand, gave appreciable quantities of coagulum.

Fifth experiment. — In this experiment we repeated parts A and B of the fourth experiment. Fibrin albuminate from the second portion of preparation **E** was used.

Summary. — 0.2 gm. portions of the substance (**E**, **b**) were weighed into each beaker. This quantity corresponded to 0.179 gm. of substance dried to constant weight at 100–105° C.

The general results and conclusions of this experiment are the same as those of the fourth. It will be observed that second precipitates were obtained in only those fluids which had not been previously boiled. There is no particular difference in the action of the chlorides and oxalates. The proportion of unrecoverable substance in this experiment is practically the same as that of the preceding,

¹ The amount of saline matter contained in the original albuminate was small. Portion **b** of Preparation **E**, for example, contained only 0.86 per cent ash. It is hardly possible, therefore, that the loss of substance was due to removal of inorganic admixture on reprecipitation. All of our albuminate preparations, it will be recalled, were originally reprecipitated several times and frequently washed before drying, in which process inorganic matter was very thoroughly removed. See footnote 2, page 478.

although different preparations of acidalbumin were used. The final filtrates gave the usual proteose reactions.

Acid.		Weight of	Amount of	Quantity of	Total	
		peptone.	neutralization	precipitate on	amount of	
		Gram.	precipitate. ¹	boiling the	acidalbumin	
			Gram.	neutral filtrate.	recovered.	
				Gram.	Gram.	
A. Hydrochloric	1	$\left. \begin{matrix} a^2 \\ b \end{matrix} \right\}$	0.133	none	0.133
		$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	0.128	0.004	0.132
	2	$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	0.5	0.141	none	0.141
		$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	0.5	0.164	0.005	0.169
	3	$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	1.0	0.154	none	0.154
		$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	1.0	0.131	0.014	0.145
B. Oxalic	1	$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	0.146	none	0.146
		$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	0.145	0.002	0.147
	2	$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	0.5	0.149	none	0.149
		$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	0.5	0.148	0.002	0.150
	3	$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	1.0	0.152	none	0.152
		$\left. \begin{matrix} a \\ b \end{matrix} \right\}$	1.0	0.144	0.009	0.153
Average	1	<i>a</i>	(I) 0.140	(II) none	(III) 0.140
		<i>a</i>	0.5	0.145	"	0.145
		<i>a</i>	1.0	0.153	"	0.153
	2	<i>b</i>	0.136	0.003	0.140
		<i>b</i>	0.5	0.156	0.003	0.160
		<i>b</i>	1.0	0.137	0.012	0.149

Average total quantity of acidalbumin lost, 0.029 gm. = 16.2 per cent.
 Average quantity of acidalbumin lost on neutralizing (cold), 0.035 gm. = 19.5 per cent.
 Average ratio I : II (*b*) = 24 : 1.
 The average for II *b* (1-3) = 3.35 per cent of the original acidalbumin and 4.0 per cent of the average for III.
 Averages. A. I, 0.142; II, 0.004; III, 0.146 gm.
 B. I, 0.147; II, 0.002; III, 0.149 gm.

¹ See footnote, page 475.
² As in the previous experiment, *a* signifies *after* boiling; *b*, *before* boiling. See page 474.

Sixth experiment. — The preceding experiment was repeated. Muscle albuminate was used, instead of the product from fibrin. The freshly precipitated substance was taken because of the insolubility of the "air-dried" product.

Summary. — Preparation **B** was used. A little over 4 gms. of the moist substance was dissolved in 425 c.c. of each acid, giving about 1 gm. of the freshly precipitated material to each 100 c.c. Of this solution, 100 c.c. was taken, as usual, for each of the four tests of a series. The amount of solid substance in the moist syntonin was not determined directly. Dilute NaOH was used to neutralize the acids¹ in this and all subsequent experiments.

Acid.		Weight of peptone. Gram.	Amount of neutralization precipitate. ¹ Gram.	Quantity of precipitate on boiling the neutral filtrate. Gram.	Average total amount of acidalbumin recovered. Gram.
A. Hydrochloric	1 ²	0.054	0.002	0.059
	2	0.051	none	
	3	0.5	0.064	"	
	4	1.0	0.066	"	
B. Tartaric	1	0.066	0.001	0.073
	2	0.070	none	
	3	0.5	0.077	"	
	4	1.0	0.079	"	
C. Phosphoric	1	0.074	0.002	0.077
	2	0.074	none	
	3	0.5	0.081	"	
	4	1.0	0.079	"	
D. Oxalic	1	0.080	0.001	0.087
	2	0.084	none	
	3	0.5	0.089	"	
	4	1.0	0.095	"	

¹ See footnote, page 475.

² The first of each series was neutralized *before* boiling, the rest *after* the boiling point had been reached.

Each of the final filtrates from the fluids which had not received Witte's peptone gave delicate biuret reactions and slight precipitates in alcohol. These possessed proteose qualities.² The biuret reactions, as usual, were strongest in the chloride and oxalate solutions.

¹ No differences were observed in the effects of the alkalis used in the neutralization process. The anions of salts of the alkali metals vary somewhat in their effects. In these experiments, however, their influences have not been particularly appreciable. See page 467.

² We cannot believe that a trace of active pepsin adherent to the original acidalbumin caused the appearance of proteose at this point in all these experiments. The boiling of the digestive mixture before the first precipitation of the acidalbumin surely sufficed for the destruction of all of the enzyme. See methods of preparation, page 465.

The comparatively high results for acidalbumin precipitated from the phosphate and oxalate fluids are doubtless due in great measure to phosphate and calcium impurities in this particular preparation of the proteid. The amount of precipitate obtained in this experiment from the neutral filtrate on boiling is perceptibly less than in any heretofore. The failure to obtain such turbidity in the peptone mixtures may mean that the peptone has actually aided complete precipitation. On the other hand, there is just as much reason for assuming that the peptone holds the slight quantities referred to in solution. Only the first fluid of each series — neutralized before boiling — yielded a second precipitate.

It might be assumed that the peptone aids precipitation from the fact that the precipitates from the peptone mixtures are slightly greater here in each case than the precipitates not associated with peptone. We have just suggested a reason for this. Aside from the explanation already offered, the extreme difficulty of washing out last traces of peptone makes us still more doubtful that these slightly higher figures should be regarded as particularly significant.

We are justified, we think, in concluding from this and the foregoing experiments that the peptone has little if any constant, appreciable influence. Our further results harmonize with this deduction.

Experiments 7-10.—These experiments were carried out to ascertain the influence of increase and decrease in the quantities of digestive albuminate present in the fluids to begin with, the volumes of the latter remaining the same. The methods of the previous experiments were followed in detail.

Summary (7). — 0.1 gm. samples of fibrin albuminate of portion a, preparation **E** were used. This amount of substance at 100-105° C. = 0.090 gm. Results are tabulated on page 480.

Although the quantity of albuminate was reduced in this experiment, compared with the results of those in which 0.2 gm. was used, little proportionate difference is to be noted in the amount of precipitate obtained on boiling. Appreciable loss of acidalbumin was observed as usual. Preliminary boiling here did not seem to favor the highest quantitative precipitation. The process of first separating the neutralization precipitate and then boiling the filtrate appears to be best. In all probability preliminary boiling results in increased hydration.

Acid.	Weight of peptone. Gram.	Amount of neutralization precipitate. ¹ Gram.	Quantity of precipitate on boiling the neutral filtrate. Gram.	Total amount of acidalbumin recovered. Gram.		
<i>A.</i> Hydrochloric	1 $\left\{ \begin{array}{l} a^2 \\ b \end{array} \right.$	0.065 0.068	none 0.003	0.065 0.071	
	2 $\left\{ \begin{array}{l} a \\ b \end{array} \right.$	0.5 0.5	0.067 0.072	none 0.010	0.067 0.082	
	3 $\left\{ \begin{array}{l} a \\ b \end{array} \right.$	1.0 1.0	0.065 0.063	none 0.006	0.065 0.069	
	<i>B.</i> Oxalic	1 $\left\{ \begin{array}{l} a \\ b \end{array} \right.$	0.071 0.074	none "	0.071 0.074
		2 $\left\{ \begin{array}{l} a \\ b \end{array} \right.$	0.5 0.5	0.063 0.068	none 0.004	0.063 0.072
		3 $\left\{ \begin{array}{l} a \\ b \end{array} \right.$	1.0 1.0	0.067 0.059	none 0.004	0.067 0.063
Average. 1-3- <i>A</i> <i>B</i>	(I) 0.067 0.067	(II) 0.003 0.001	(III) 0.070 0.068		
Average total quantity of acidalbumin lost, 0.021 gm. = 23.3 per cent. Average quantity of acidalbumin lost on neutralizing (cold), 0.023 gm. = 25.5 per cent. Average ratio. I : II <i>b</i> = 34 : 1. The average for II <i>b</i> (1-3) = 2.22 per cent of the original substance and 2.90 per cent of the average for III <i>b</i> .						
¹ See footnote 1, on page 475.		² See footnote 2, on page 477.				

Summary (8). — 0.4 gm. samples of fibrin albuminate, portion **b**, preparation **E**, were used. Dried to constant weight, this amount contained 0.358 gm. substance.

Acid.	Weight of peptone.	Amount of neutralization precipitate. ¹	Quantity of precipitate on boiling the neutral filtrate.	Total amount of acidalbumin recovered.	
	Gram.	Gram.	Gram.	Gram.	
A. Hydrochloric	1 $\left\{ \begin{array}{l} a^2 \\ b \end{array} \right.$	0.281	none	0.281
		0.284	0.009	0.293
	2 $\left\{ \begin{array}{l} a \\ b \end{array} \right.$	0.5	0.288	none	0.288
		0.5	0.321	0.005	0.326
	3 $\left\{ \begin{array}{l} a \\ b \end{array} \right.$	1.0	0.296	none	0.296
		1.0	0.280	0.017	0.297
B. Oxalic	1 $\left\{ \begin{array}{l} a \\ b \end{array} \right.$	0.315	none	0.315
		0.305	0.008	0.313
	2 $\left\{ \begin{array}{l} a \\ b \end{array} \right.$	0.5	0.303	none	0.303
		0.5	0.312	0.009	0.321
	3 $\left\{ \begin{array}{l} a \\ b \end{array} \right.$	1.0	0.309	none	0.309
		1.0	0.291	0.014	0.305
Average 1-3-A B	(I) 0.292	(II) 0.005	(III) 0.297	
	0.306	0.005	0.311	
Average total quantity of acidalbumin lost, 0.054 gm. = 15.1 per cent.					
Average quantity of acidalbumin lost on neutralizing (cold), 0.059 gm. = 16.5 per cent.					
Average ratio, I : II $b = 60 : 1$.					
The average for II b (1-3) = 1.40 per cent of the original substance and 1.64 per cent of the average for III b .					
		¹ See footnote 1, on page 475.	² See footnote 2, on page 477.		

In this experiment four times as much acidalbumin was taken as in the previous one, yet the actual amount of precipitate obtained on boiling was only slightly increased; its proportion decreased. As in the preceding and some earlier experiments, the precipitate thrown down on boiling was obtained only from those fluids which had not been heated previous to their neutralization. In all the final filtrates, biuret reacting substance could be detected — doubtless proteose formed in the boiling process.

The amount of albuminate recovered from the chloride solutions was slightly less than from the oxalate, although in the previous experiment, and before that, little difference between the two was noted. Such differences as have been observed have not been at

all constant, probably for the reason that the variations are within the limits of unavoidable experimental error.

The conclusions drawn from experiments 7 and 8 will be found to hold for the results of the two following ones.

Summary (9). — 0.1 gm. samples of acidalbumin from fibrin were used, portion **a**, preparation **E**. This quantity of substance was equivalent to 0.090 gm., dried at 100–105° C.

Acid.		Weight of peptone. Gram.	Amount of neutralization precipitate. ¹ Gram.	Quantity of precipitate on boiling the neutral filtrate. Gram.	Total amount of acidalbumin recovered. Gram.
<i>A.</i> Oxalic	1	0.069	0.003	0.072
	2	0.069	none	0.069
	3	0.5	0.067	0.006	0.073
	4	1.0	0.057	none	0.057
<i>B.</i> Tartaric	1	0.064	0.004	0.068
	2	0.067	0.004	0.071
	3	0.5	0.068	none	0.068
	4	1.0	0.064	"	0.064
<i>C.</i> Phosphoric	1	0.072	0.003	0.075
	2	0.070	none	0.070
	3	0.5	0.058	0.007	0.065
	4	1.0	0.068	0.006	0.074
<i>D.</i> Hydrochloric	1	0.066	0.004	0.070
	2	0.070	0.003	0.073
	3	0.5	0.068	0.006	0.074
	4	1.0	0.068	none	0.068
Average (1–4)– <i>A</i>	(I) 0.065	(II) 0.002	(III) 0.067	
<i>B</i>	0.066	0.002	0.068	
<i>C</i>	0.067	0.004	0.071	
<i>D</i>	0.068	0.003	0.071	
Average total quantity of acidalbumin lost, 0.021 gm. = 23.3 per cent.					
Average quantity of acidalbumin lost on neutralizing (cold), 0.023 gm. = 25.5 per cent.					
General average ratio. I : II = 22 : 1.					
General average for II = 3.33 per cent of the original substance and 4.35 per cent of the general average for III.					
¹ See footnote, on page 475.					

Summary (10). — 0.340 gm. samples of fibrin albuminate, portion **b**, preparation **E**, were used. This quantity of substance corresponded with 0.340 gm. substance dried to constant weight at 100–105° C.

Acid.		Weight of	Amount of	Quantity of	Total
		peptone.	neutralization	precipitate	amount of
		Gram.	precipitate. ¹	on boiling the	acidalbumin
			Gram.	neutral filtrate.	recovered.
				Gram.	Gram.
<i>A.</i> Tartaric	1	0.275	0.002	0.277
	2	0.259	none	0.259
	3	0.5	0.257	0.007	0.264
	4	1.0	0.256	0.009	0.265
<i>B.</i> Oxalic	1	0.257	0.005	0.262
	2	0.266	none	0.266
	3	0.5	0.265	0.005	0.270
	4	1.0	0.264	0.008	0.272
<i>C.</i> Hydrochloric	1	0.274	0.002	0.276
	2	0.261	0.002	0.263
	3	0.5	0.267	none	0.267
	4	1.0	0.264	"	0.264
<i>D.</i> Phosphoric	1	0.284	0.004	0.288
	2	0.279	0.006	0.285
	3	0.5	0.272	0.006	0.278
	4	1.0	0.268	0.006	0.274
Average (1-4)- <i>A</i>		(I) 0.262	(II) 0.004	(III) 0.266
<i>B</i>		0.263	0.005	0.268
<i>C</i>		0.266	0.001	0.267
<i>D</i>		0.267	0.005	0.281
Average total quantity of acidalbumin lost, 0.033 gm. = 10.9 per cent.					
Average quantity of acidalbumin lost on neutralizing (cold), 0.037 gm. = 12.2 per cent.					
General average ratio. I : II = 67 : 1.					
General average for II = 1.31 per cent of the original substance and 1.85 per cent of the general average for III.					
¹ See footnote, page 475.					

The percentage of substance recovered on boiling was unusually low in this and in the eighth experiment, in which larger quantities of acidalbumin were taken, a fact suggesting that the loss is proportionately greatest with the least amounts of substance.¹

¹ See tables, pages 488 and 490.

Eleventh experiment. — The foregoing results show that in these determinations a small though appreciable amount of albuminate invariably was lost. The quantity of substance separated on boiling was slight and approximately the same throughout. Such differences as were perceptible appeared to depend mainly on the quantities of albuminate present to begin with. Thus, the proportion of this precipitate in the hot fluid to that on neutralization in the cold was usually greater the smaller the amount of albuminate originally taken.

This result would indicate that the method of neutralization in the cold is the more satisfactory the larger the quantity of albuminate involved. On the other hand, because the volumes and quantities of acid were uniform in all of these experiments, it might be assumed, that the solvent action of the salts formed was much the same, even though the amounts of substance used did vary somewhat. For this reason, also, the total loss of material noted may have been uniformly slight.

In order to test these points the following special experiment was carried out.

Summary. — 0.5 gm. samples of preparation **G** of fibrin albuminate (0.450 gm. substance dried at 105° C.) were dissolved in different amounts of $\frac{m}{10}$ HCl, varying from 50 c.c. to 800 c.c. The solutions were allowed to stand as usual for an hour or two, then were exactly neutralized with dilute NaOH, as before, and the bulky precipitate permitted to settle until the following morning. The weights of the acidalbumin recovered are given below. The filtrates were then brought to the boiling point and kept there a moment or two. Each became turbid. The turbidity was least in the smallest volume of fluid and most pronounced in the largest quantity. The precipitates soon settled under perfectly clear fluid and were easily filtered off, with the gravimetric results appended.

Volume of $\frac{m}{10}$ HCl. c.c.	Amount of neutralization precipitate. Gram.	Precipitate obtained on boiling the neutral filtrate.		Total amount of acidalbumin recovered. Gram.	Total quantity of substance lost. Gram.
		Gram.	Per cent.		
50	0.399	0.005	1.1	0.404	0.046
100	0.346	0.012	2.7	0.358	0.092
200	0.348	0.014	3.1	0.362	0.088
400	0.312	0.035	7.8	0.347	0.103
800	0.303	0.041	9.1	0.344	0.106

The results of this experiment show quite conclusively that, other conditions being equal, an increasing proportion of acidalbumin is lost as the volume of neutral fluid (NaCl here) becomes larger. We have no doubt it increases somewhat, also, with a rise in the proportion of saline matter and, vice versa, falls in amount with a decrease in the proportional content of neutral salt. Although the albuminate here was the same in amount throughout the series, an increasing quantity of coagulum was separable from this neutral filtrate, a result still further emphasizing the fact of solubility of acidalbumin in cold neutral saline solution.

Each of the filtrates gave the usual biuret and proteose reactions. The increasing loss of acidalbumin above was seemingly due to the greater hydration, inevitably induced by boiling, in the larger volumes.¹

In considering the value of this method, therefore, the volume of the digestive mixture as well as the percentage content of albuminate and neutral salts cannot be overlooked.

It appeared quite clear from this and each of the previous series of experiments that at least a small amount of acidalbumin was soluble in the cold neutral fluids containing it. Further, it was impossible to recover all of the albuminate used at the beginning of the experiment. It seemed desirable at this point, therefore, to ascertain definitely the solvent power of the various saline fluids made throughout these experiments in the process of neutralizing the acids.

Solubility of acidalbumin in saline solutions.— In the first of our special tests of this matter we ascertained merely the solubility of the

¹ The larger the volume the longer the time required, with a given flame of course, to raise the fluid to the boiling-point, and, therefore, the greater the exposure of the soluble substance to hydrating influence. Some hydration must occur before the solution reaches the boiling-point. In all probability the material which separates earliest and causes the initial turbidity is hydrated in part as the precipitate increases with the rise in temperature. Doubtless some of the material in solution is also hydrated before it can be precipitated. Perhaps heating to only 70–80° C. would have resulted in diminished loss of acidalbumin.

It would be natural to inquire in this connection why, on boiling, a small, fairly constant amount of substance usually remained as a coagulated precipitate, although hydration of the larger proportion, dissolved in the neutral filtrate, invariably occurred. The fact, however, that occasionally no such coagulation was observed, although loss of substance occurred, would indicate that *all* of the substance in the neutral filtrate was transformable into hydration products, and that, perhaps, the sameness of conditions attending the boiling process accounted for the similarity in the quantitative results. The very short boiling period was sufficient now and then to effect complete hydration of the dissolved residue.

dried fibrin albuminate in water and in 0.5 per cent NaCl, with the following results.

Summary (A).— Finely powdered samples of fibrin albuminate of portions **a** and **b**, preparation **E**, were used. 0.2 gm. was weighed for each test. This amount, dried to constant weight at 100–105° C., was equivalent to 0.180 gm. for portion **a**, 0.179 gm. for portion **b**. 100 c.c. of fluid was used as throughout all but the previous experiment. The mixtures were frequently stirred. They were allowed to stand over night, then filtered, etc., as in the previous experiments.

Solution used.		Weight of substance recovered. Gram.	
		Portion a .	Portion b .
<i>A.</i> Distilled water	<i>a</i>	0.1792	0.1738
	<i>b</i>	0.1779	0.1747
Average		0.1786	0.1743
<i>B.</i> 0.5% salt solution	<i>a</i>	0.1767	0.1663
	<i>b</i>	0.1701	0.1655
Average		0.1734	0.1659
Total substance taken in each of <i>A</i> and <i>B</i>		0.180	0.179
Average loss in <i>A</i>		0.001	0.005
Average loss in <i>B</i>		0.007	0.013

On boiling, the aqueous filtrates remained clear; but the saline fluids became opalescent. Practically nothing seems to have dissolved in water. In salt solution, however, a slight loss resulted in each test. The results with water show, if we grant that the dry acid-albumin is practically insoluble in water, that our preparations contained at most the merest traces of soluble salts or proteoses¹—obviously not in sufficient quantity to account to any extent for the loss of substance noted throughout all of our experiments. Consequently this experiment is particularly valuable in showing that such disappearance of substance as has been noted in all our previous tests has been due to loss of albuminate itself and not merely to removal of soluble impurity.

¹ See footnote, page 476.

We next tried the solvent action of the salts formed in the neutralization of the various acids previously employed; also the solubility in water alone and in water containing peptone.

Summary (B). — Preparation **F** of our fibrin albuminate (moist substance) was taken. 100 c.c. of various acids used in the preceding experiments were carefully neutralized to litmus and lacmoid with dilute NaOH, as already described. As indicated below, some of these neutral fluids were thoroughly boiled for a few minutes, without material loss by evaporation, for the removal of carbonic acid gas. Weighed amounts of our moist, freshly precipitated acidalbumin were transferred to the neutral fluids (the boiled ones had been cooled). The mixtures were repeatedly stirred and allowed to stand over night as in all of the experiments. At intervals samples from the main bulk of the moist precipitate were weighed into crucibles for the determination of dry solid matter, as indicated below.¹

It seems obvious, from the results on page 488, that acidalbumin is somewhat soluble in the salts formed on neutralizing acid fluids for its precipitation. Although practically insoluble in distilled water the acidalbumin appeared to be slightly soluble in water to which proteose and peptone had been added. We are not sure, however, that this result is not due to the solvent action of the saline matter present as impurity in Witte's peptone. The proportion of the amounts which dissolved in the cold neutral saline fluids to the total quantities originally taken is slight, however. With more decided acidity to begin with, and therefore more salts formed on neutralization than was the case in these experiments, doubtless the more decided would be the solution of substance, and the greater the quantity recoverable by coagulation.

The data just obtained also indicate that such slight amounts of carbonic acid gas as remained in the fluids on neutralization had little or no measurable influence on the results. The proportionate amounts of substance soluble in and recovered from the fluids which had been thoroughly boiled before the albuminate was put into them² and from which, therefore, the carbon dioxide had been removed,

¹ The moist substance was kept in a covered mortar. Before each sample was removed, the whole mass was thoroughly mixed. Errors caused by the slight evaporation of water under the circumstances were thus greatly minimized, and probably made inappreciable.

² The second of each series in the summary on page 488.

Solution.	Weight of substance taken.		Dry substance recovered.				Total average amount of substance lost (calculated). Gm.
	Fresh.	Dry (calculated). ¹	Insoluble.	Precipitate from the filtrate on boiling. Gm.	Total.	Ratio of <i>b</i> to <i>a</i> .	
1. Distilled water ²	3.163 3.415	1.232 1.330	(<i>a</i>) 1.2184 1.3397	(<i>b</i>) none none	1.2184 1.3397		0.004
2. Distilled water with 0.5 gm. peptone	4.250	1.656	1.6822	0.0076	1.6898		
Distilled water with 1 gm. peptone	4.456	1.736	1.7685	0.0121	1.7806		+ 0.078 ³
3. Chloride	2.491 3.240	0.971 1.262	0.7834 1.0552	0.0138 0.0066	0.7972 1.0618	1.76 0.63	0.187
4. Oxalate	4.574 3.178	1.782 1.238	1.5796 1.0634	0.0276 0.0212	1.6072 1.0846	1.75 1.99	0.164
5. Phosphate	2.701 2.482	1.052 0.967	0.9008 0.8618	0.0152 0.0148	0.9160 0.8766	1.69 1.71	0.113
6. Tartrate	2.480 2.682	0.966 1.045	0.8350 0.9275	0.0188 0.0216	0.8538 0.9491	2.25 2.33	0.104
7. Nitrate	2.911 3.678	1.134 1.434	1.0176 1.3136	0.0168 0.0286	1.0344 1.3422	1.65 2.18	0.095
8. Lactate	3.447 3.743	1.343 1.458	1.2398 1.3834	0.0124 0.0220	1.2522 1.4054	1.00 1.59	0.072
Average ⁴	1.221	(<i>a</i>) 1.080	(<i>b</i>) 0.0180	1.098	1.67	0.123

Average amount of substance lost = 10.07 per cent of that originally taken (average) and 11.39 per cent of the insoluble portion (average).

The average amount of precipitate obtained on boiling = 1.47 per cent of the original albuminate (average) and 1.67 per cent of the average quantity insoluble in the neutral fluids.

Ratio. *a* : *b* = 60 : 1.

¹ Portions of the moist substance were taken at the beginning of the experiment and after the third, fifth, and seventh series. The quantities of fresh material used for this purpose varied between 1.7334 and 3.9908 gms. The percentages of dry matter were found to be 38.38, 38.97, 39.20, and 39.29. See footnote, page 476.

² The second fluid of each pair throughout the series had been thoroughly boiled before receiving the albuminate.

³ This figure represents a gain of substance; peptone not completely washed out. See page 479.

⁴ The averages do not include the figures for the first two pairs of determinations.

were slightly greater than the others in some cases, but the same or less in others.

The results above likewise show an appreciable loss of substance even after the addition of the precipitate obtained from the boiled filtrate to that previously filtered off. This loss is doubtless due to formation of proteose in the process of boiling, as seems to have been the case in all of our previous experiments. It will be seen from the tabulated data, that this loss occurs in all of the tests, excepting the water alone and the water with peptone. The actual increase in amount recovered in the latter case is very probably due to adherent peptone which was very difficult to wash completely from the bulky precipitate. Since, also, the amount of solid matter in each quantity of the moist precipitate was calculated from special determinations of the dry substance contained in the fresh material, and not ascertained directly, we cannot lay too much stress upon it. All the results for "dry substance taken" may be a little high or low by reason of the unavoidable errors which usually accompany calculated data under such conditions, no matter how careful the experimenter may be to attend to every detail of manipulation in the comparative determinations. That appreciable loss occurred as usual, however, was clearly shown by the proteose content of the final filtrates.

The greatest losses appear, from the figures, to have been associated with the chlorides and oxalates. In our previous experiments, also, we noted that the biuret reactions in the final filtrates were usually strongest in the chloride and oxalate fluids.

III. GENERAL SUMMARY OF RESULTS.

The table on page 490 summarizes the more important average data of nearly all of our experiments.

IV. SUMMARY OF GENERAL CONCLUSIONS.

We conclude from these experiments that acidalbumin may be almost completely precipitated from acid digestive mixtures at ordinary temperatures by careful neutralization. The later stages in the neutralization process should be conducted with particularly dilute alkali.

The absolute quantity of acidalbumin remaining in such fluids after neutralization in ordinary experiments is small, its proportion to the

GENERAL SUMMARY OF RESULTS.

Experiment, ¹	Amount of acidalbumin taken.	Acidalbumin recovered.					Acidalbumin lost.		
		Average quantity of neutraliza- tion precipi- tate.	Average amount of precipitate obtained on boiling. ²	Ratio of neutralization precipitate to that obtained on boiling.	Precipitate obtained on boiling.		Total.	On neutralizing.	
					Proportion of the total substance taken.	Proportion of the total substance recovered.		Average amount. Gram.	Per cent.
		Gram.					Average amount. Gram.	Per cent.	Per cent.
Seventh	0.090	0.067	0.002	34 : 1	2.22	2.90	0.021	23.3	25.5
Ninth	0.090	0.067	0.003	22 : 1	3.33	4.35	0.021	23.3	25.5
Second	0.181	0.126	0.007	18 : 1	3.87	5.26	0.048	26.5	30.4
Fifth	0.179	0.145	0.006	24 : 1	3.35	4.00	0.029	16.2	19.5
Fourth	0.180	0.148	0.004	37 : 1	2.22	2.65	0.029	16.1	17.8
Third	0.275	0.231	0.005	46 : 1	1.82	2.12	0.039	14.2	16.0
Tenth	0.304	0.267	0.004	67 : 1	1.31	1.85	0.033	10.9	12.2
Eighth	0.358	0.299	0.005	60 : 1	1.40	1.64	0.054	15.1	16.5
B, p. 487 ³	1.221	1.080	0.018	60 : 1	1.47	1.67	0.123	10.1	11.4

¹ Only those are summarized for which data in each column were obtained. The first and sixth experiments gave incomplete results in this connection. The data of the eleventh experiment and under Summary A on page 486 are hardly comparable here. The results are so tabulated as to show at a glance the relations of amounts taken to those recovered.

² The averages in several cases do not include entirely negative results, as in the fourth experiment, for example.

³ Total average results for all of these experiments, with the original amounts of acidalbumin so different, would be at best only rough approximations, and also in some ways misleading.

main bulk of the albuminate depending largely on the amount of the latter, also on the volume of the fluid containing it and on the percentage of associated saline matter.

Some of this residual portion of acidalbumin may be obtained on boiling, although in this process the larger part, sometimes all, is retained permanently in solution, apparently because of its hydration into noncoagulable forms.

Proteoses and peptones, even when admixed in comparatively large proportion, do not materially affect the quantitative separation of the albuminate.

Neutralization at the boiling point does not insure the greatest quantitative precipitation of albuminate because of the increased hydration thereby resulting. The largest yield is obtained by neutralizing in the cold, heating the neutral filtrate and combining the two precipitates.

Acidalbumin, particularly that formed through the action of pepsin on fibrin and in the freshly precipitated condition, is somewhat soluble in various saline fluids.

The sodium and potassium salts formed on neutralizing various common acid solutions appear to exert approximately equal quantitative solvent action on the contained albuminate. Only insignificant differences in solvent power were noted.

Such quantities of carbon dioxide as are present in fluids neutralized with freshly prepared solutions of potassium or sodium hydroxides containing ordinary, minute amounts of carbonate, do not appear to materially affect the quantitative separation of acidalbumin by the neutralization process.

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