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THE INFLUENCE OF COLLOIDS UPON THE DIFFUSION OF HÆMOLYSINS.

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Plates XXVI, XXVII, XXVIII, XXIX, and XXX.

The study which forms the subject of this paper was suggested by the experiments made by one of us (Flexner[1]) upon the restraining influence of certain colloids upon the injurious action of bile salts upon the pancreas. The results of the present work not only confirm the view there expressed, namely, that colloidal substances reduce the degree and rapidity of diffusion of certain crystalloids, but they would seem to have even a wider biological bearing. It is for these reasons that they are given, in some detail, in this place. In order that the diffusion of very complex molecules (such as certain hæmolysins, etc.) might be studied it was necessary to devise a method of testing diffusion that would give approximately accurate results without having to resort to chemical analyses which are, indeed, useless with many of these bodies. We found that a suspension of blood corpuscles in certain colloids supplied a useful and accurate means of determining the rate of diffusion of simple and complex molecules which bring about hæmolysis. By reversing the experiment, namely by placing the chemical bodies in the colloids the rate of diffusion into water and saline could also be determined by estimating the quantity of the hæmolytic substance which appeared in these fluids after a given time.

The earlier studies on the influence of colloids upon the rate of diffusion showed, for the most part, that the rate remained unchanged. Graham[2] believed that no inhibition followed from the presence of colloids. He dissolved, among other things, 10 per cent. of sodium chloride in a 2 per cent. agar-agar jelly and overlaid this in a tube with another portion of the jelly

[1] *Jour. Exp. Med.*, 1906, viii, 167.

[2] *Phil. Trans. Roy. Soc.*, 1861, cli, Part I, 183; *Liebig's Annalen*, 1862, cxxi, 1.

minus the salt. At the end of eight days the amount of salt which diffused into the plain agar was found to be the same as that diffused into water. De Vries [3] failed to notice any marked difference in the diffusion of solutions of potassium chromate and copper sulphate into pure water and 4 per cent. silicic acid after a contact of four months. Voigtländer [4] investigated the rate of diffusion of certain simple acids and salts into agar-agar of from 1 to 4 per cent. While the rate of diffusion was at first in favor of the weaker concentration the actual quantities of salts and acids which diffused were about the same in all cases. Reformatsky [5] observed no inhibition of the catalytic reaction of acetate of methyl and hydrochloric acid by a 1.25 per cent. agar suspension; and Levi [6] ascertained that 1.57 per cent. silicic acid, 1 per cent. agar, and 0.6 per cent. gelatin did not alter electric conductivity, lower the freezing point, or inhibit the inversion of cane sugar by hydrochloric acid.

Not all experimenters have found that colloids act in a wholly indifferent manner upon the process of diffusion. Stefan [7] recalculated the values of Graham's experiments and ascertained that the diffusion constant was smaller with agar-agar jelly than with pure water. Chabry [8] found that gelatin and cartilage delayed the action of acids on orcein. While our own experiments were in progress a paper appeared by Kurt Mayer [9] in which the inhibiting action of gelatin, agar, and egg white upon diffusion is described. The bodies employed by him—sodium chloride and potassium chromate—were simple, and his results point to an unmistakable retardation of diffusion by colloids, the retardation being approximately proportional to the concentration of the colloids. With agar the differences are less marked than with gelatin, on account of the wider variations in concentration practicable with the latter substance. Similar results were obtained by Nell [10] who studied the effect of gelatin on the diffusion of a number of chemical bodies. Nell also observed that the electrical conductivity of solutions of copper sulphate are reduced by gelatin when present in amounts of from 1 to 20 per cent.

The number of substances the diffusion of which can be satisfactorily observed by the hæmolytic method is considerable. We studied acids, alkalies, sodium taurocholate, saponin, solanin, cobra venom, and tetanus toxin. In the case of the complex bodies, snake venom and tetanus toxin, the capacity for diffusion of the different constituents, namely, hæmolysins, neurotoxin, tetanospasmin, was also determined.

[3] *Recueil des travaux chimiques de Pays-Bas*, 1884, iii, 375.
[4] *Zeitschr. f. physik. Chemie*, 1889, iii, 316.
[5] *Zeitschr. f. physik. Chemie*, 1891, vii, 34.
[6] *Gazetta chimica italiana*, 1900, xxx, Parte II, 64.
[7] *Wiener Sitzungsberichte*, 1879, lxxix, II Abt., 161 and 215.
[8] *Journal de Physique*, 1888, vii, 115.
[9] *Beiträge z. chem. Physiologie u. Pathologie*, 1905, vii, 393.
[10] *Annalen d. Physik*, 1905, IV Folge, xviii, 323.

The particular points which were noted were: the influence of concentration of colloids upon the rate of diffusion; the time relations of the rate of diffusion; and the comparative degree of diffusibility of different substances.

The diffusible bodies were made to diffuse (1) from saline solution into colloids, (2) from colloids into colloids, and (3) from colloids into saline solution.

METHODS.

In preparing the various solutions and blood mixtures a fair degree of asepsis was observed. Where the experiments extended over any length of time greater precautions were taken. The blood was obtained aseptically and the colloids were sterile. The blood mixtures with agar and gelatin were well preserved at the end of a week or even longer. The experiments were made at room temperature which was about 20° C. The agar suspensions were made with agar-agar which had been thoroughly washed in running water. The agar was dissolved in distilled water, clarified, filtered, and 0.9 per cent. sodium chloride added. The gelatin was dissolved in distilled water, neutralized with NaOH, clarified, filtered, and salt in 0.9 per cent. added.

The highest concentrations of the colloids having been made, the weaker strengths were obtained by the addition of 0.9 per cent. salt solution. Acids, alkalies, and salts were made into normal solutions. These solutions were further diluted according to the hæmolytic strength of the different chemical bodies. In making the mixtures of chemical body and colloid a concentrated solution of the first was added to the second so as to avoid undue dilution of the colloid.

The colloids were pipetted into tubes of 8 mm. to 15mm. diameter, care being taken to prevent flowing over the wall of the tube above the layer. The quantities employed were, usually, 2 c.c. each of colloid and fluid. The colloid column measured from 32 to 33 mm. in tubes of 8 mm. diameter.

Dog and rabbit blood in 3 to 5 per cent. suspensions in 0.9 per cent. salt solution were used. The blood and colloid were mixed at low temperatures after which they were congealed. Ice was used to congeal the gelatin blood mixtures.

The readings were made with a scale one degree of which was 0.7 mm. All the figures were subsequently reduced to millimeters.

In determining the quantity of hæmolytic or toxic body which diffused from the colloid into the supernatant saline the latter fluid was removed at intervals and the hæmolytic or toxic strength determined. These values could be expressed in hæmolytic and in toxic units.

DIFFUSION OF HÆMOLYSINS FROM FLUID INTO COLLOID MEDIA.

There is no difficulty in observing the extent to which the hæmolytic body has penetrated into the blood-colloid suspension since

the originally opaque mixture becomes perfectly transparent as the result of the laking of the corpuscles. The line of penetration is usually sharp and straight, but in some cases it is irregular.

Series I.—Hydrochloric, nitric, oxalic acids, sodium hydroxide, and sodium carbonate in $\frac{1}{10}$ N. solutions were employed. The figures for hydrochloric and nitric acids are almost identical, although the degree of diffusion of hydrochloric is very slightly higher than of nitric acid. The values in millimeters for hydrochloric acid at the different periods (h. equalling hours) are as follows:

	9 h.	18 h.	42 h.	60 h.	84 h.
25% gelatin	4.5	7.7	13.0	16.5	18.2
10% "	5.4	9.1	16.2	20.	22.4

The values for oxalic acid are lower:

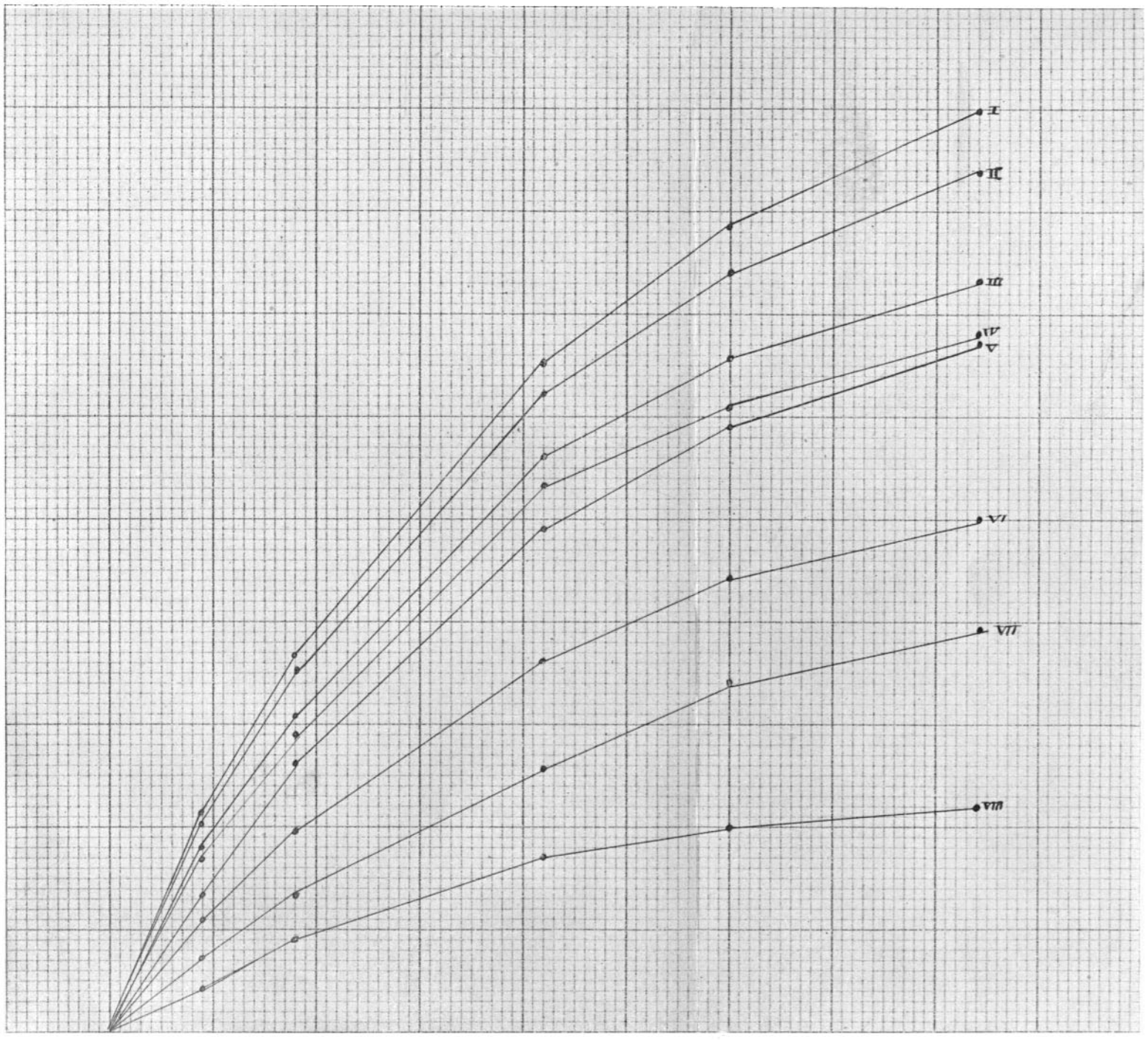
	9 h.	18 h.	42 h.	60 h.	84 h.
25 % gelatin	2.75	4.9	9.1	11.2	12.6
10 % "	3.4	6.3	12.3	14.0	16.8

And the values for sodium carbonate are still lower:

	9 h.	18 h.	42 h.	60 h.	84 h.
25 % gelatin	1.25	2.24	4.2	4.9	5.6
10 % "	1.75	3.3	6.5	8.4	9.8

The comparative rate of diffusion into gelatin of these simple bodies can be readily expressed graphically in the form of a curve (Plate XXVI). The influence of concentration of colloid on the rate and degree of diffusion is clearly seen in the curve. Lines I and II indicate the diffusion of hydrochloric acid into 10 per cent. and 25 per cent. gelatin respectively. Lines III and IV indicate nitric acid, V and VI oxalic acid, and VII and VIII sodium carbonate. The black dots show the reading periods and the marginal figures stand for millimeters.

On comparing the diffusion values for 10 and 25 per cent. gelatin the relation can be expressed by the following formula: *The amount of diffusion is approximately inversely proportional to the square root of the concentration of the gelatin.* The deviations from this formula can possibly be accounted for by considerations of experimental error, impurity in the gelatin, and the resistance introduced by the erythrocytes. The velocity of diffusion decreases steadily with increase in time.



1/10 N. HCl { I From saline into 10% gelatin. II From saline into 25% gelatin.

1/10 N. HNO_3 { III From saline into 10% gelatin. IV From saline into 25% gelatin.

1/10 N. $C_2H_2O_4$ { V From saline into 10% gelatin. VI From saline into 25% gelatin.

1/10 N. Na_2CO_3 { VII From saline into 10% gelatin. VIII From saline into 25% gelatin.

The concentration limits for agar are smaller than for gelatin and consequently the differences in degree and velocity of diffusion are also smaller with that colloid. The strengths employed in the next experiments were 0.5, 1, and 2 per cent. Two examples only, namely of nitric acid and sodium hydroxide, will be given.

HNO_3 $\frac{1}{10}$ N.

	15 min.	30 min.	1 h.	2 h.	3 h.	4 h.
2% agar	5.6	9.5	13.5	18.5	21.3	24.5
1% "	5.8	9.75	13.5	18.75	21.5	24.6
0.5% "	5.95	9.8	13.8	19.0	21.8	24.8

NaOH $\frac{1}{10}$ N.

	15 min.	30 min.	1 h.	2 h.	3 h.	4 h.
2% agar	5.6	9.5	13.5	18.5	21.3	24.5
1% "	5.6	9.5	13.5	18.5	21.5	24.5
0.5% "	5.95	9.6	13.75	18.75	21.5	28.

Series II.—In the next experiments sodium taurocholate, saponin, and solanin were employed. The hæmolytic values were first determined in the ordinary manner. They can be expressed for rabbit blood in the following figures: 0.0001 (saponin), 0.0007 (solanin), 0.007 (sodium taurocholate). The value of hydrochloric acid, under the same conditions was 0.02.

Sodium Taurocholate $\frac{1}{10}$ N. Rabbit Blood 5%

	3 h.	6 h.	12 h.	24 h.	42 h.	60 h.	84 h.	
25% gelatin	1.0	2.0	3.15	4.3	5.6	7.0		1st determination.
		1.2	2.2	3.9	5.6	7.0	8.4	2d "
10% "	1.4	2.6	4.3	6.6	7.8	9.0		1st "
		2.0	3.5	5.6	7.7	9.3	11.2	2d "

Saponin $\frac{1}{100}$ N. Rabbit Blood 5%

	3 h.	6 h.	24 h.	48 h.		
25% gelatin	1.0	2.0	4.2	6.3	1st	determination.
		2.4	5.0	7.0	2d	"
10% "	1.4	2.9	6.6	9.15	1st	"
		2.85	6.0	8.4	2d	"

Solanin $\frac{1}{100}$ N. Dog Blood 3%.

	6 h.	24 h.	48 h.
25% gelatin	3.6	7.5	10.5
10% "	4.6	9.0	13.5

The relation which exists in the case of gelatin, between the degree of diffusion and the concentration of the colloid is, as this

experiment shows, the same for certain chemicals of high and of low molecular weight. When agar-agar is substituted for gelatin the differences are less marked, although a difference can still be made out. Taking saponin as an example the following figures are obtained:

Saponin $\frac{1}{100}$ N.

	3 h.	6 h.	24 h.	48 h.	
1% agar	2.45	5.0	9.8	14.0	1st determination.
0.5% "	3.85	8.4	17.5	24.5	" "
1% "	1.7	3.5	8.9	10.5	2d "
0.5% "	1.8	3.8	9.3	11.2	" "

Series III.—The next experiments were made with the complex lysins of cobra venom and tetanus toxin. Two specimens of cobra venom were available: one a fresh solution of 0.4 per cent., one cubic centimeter of which contained for dog blood 2000 minimal hæmolytic doses; and a second solution of the same strength which had been on ice seven months and was ten times weaker. The samples of tetanolysin contained 33 minimal hæmolytic doses per cubic centimeter.

The experiments with dog blood and 0.1 per cent. cobra venom were not wholly satisfactory, since the line of demarkation was not always sharp. The influence of concentration of colloid is, however, shown even in this case.

	3 h.	9 h.	24 h.
25% gelatin	0.84	2.1	6.0
10% "	2.1	5.6	31.5

The next experiment was made with cobra venom, lecithin, and rabbit corpuscles, in which case the diffusion is regular.

Cobra Venom 0.2% 1 c.c., Lecithin $\frac{1}{100}$ N. 1 c.c.

	12 h.	24 h.	42 h.	60 h.	84 h.
25% gelatin	1.7	2.8	4.20	4.9	5.6
10% "	1.8	3.15	4.55	5.55	6.3

The employment of the old cobra venom gave, instead of a curve, an almost straight line.

Passing now to the results with cobra venom 0.1 per cent. and dog blood in agar, definite inhibition in the higher concentration is found to occur.

	6 h.	24 h.	48 h.
2% agar	1.4	5.25	9.1
0.5 "	2.1	7.0	12.6

Cobra venom diffuses into agar more readily than into gelatin. The rate of diffusion into agar is proportional not to the square root of the time but almost directly to the time.

Tetanolysin penetrates gelatin (strength below 10 per cent.) more quickly than it does agar of 0.5 per cent., which makes an exception to the rule.*

DIFFUSION OF HÆMOLYSINS FROM ONE COLLOID INTO ANOTHER.

The next experiments were designed to show whether any further inhibiting effect would be developed if the hæmolysin were enclosed in the colloid. Since the velocity of diffusion is smaller the higher the concentration of the colloid, it was presumable that the introduction of a second colloid medium would further reduce the velocity.

Sodium taurocholate, saponin, solanin, and pyrogallic acid were studied. In the first experiment to be given sodium taurocholate in $\frac{1}{10}$ N. solution was enclosed in gelatin of 10 and 25 per cent., and dog blood of 3 per cent. was suspended in agar of 0.5 and 2.0 per cent.

2% Agar and Dog Blood.

Sod. taurocholate in	6 h.	12 h.	24 h.	48 h.	66 h.
25% gelatin	1.55	2.4	3.15	4.0	5.6
10% "	1.7	2.6	3.6	4.9	7.0
Saline (control)	2.55	3.55	5.15	7.3	9.8

* Arrhenius and Madsen (*Contributions from the University Laboratory for Medical Bacteriology*, Copenhagen, 1902) have applied the method of diffusion into gelatin to a determination of the molecular weight of diphtheria toxin. They ascertained that diphtheria and tetanus toxin diffused into 10 per cent. gelatin; that the former underwent no change in the process and the latter diffused more rapidly than the former. The corresponding antitoxins passed into the gelatin more slowly than the toxins.

Craw (*Proc. of the Royal Soc.*, Series B., 1906, lxxvii, 311) determined that bacillus megatherium lysin passes more slowly through a porous filter impregnated with 15 per cent. than with 7.5 per cent. gelatin.

0.5% Agar and Dog Blood.

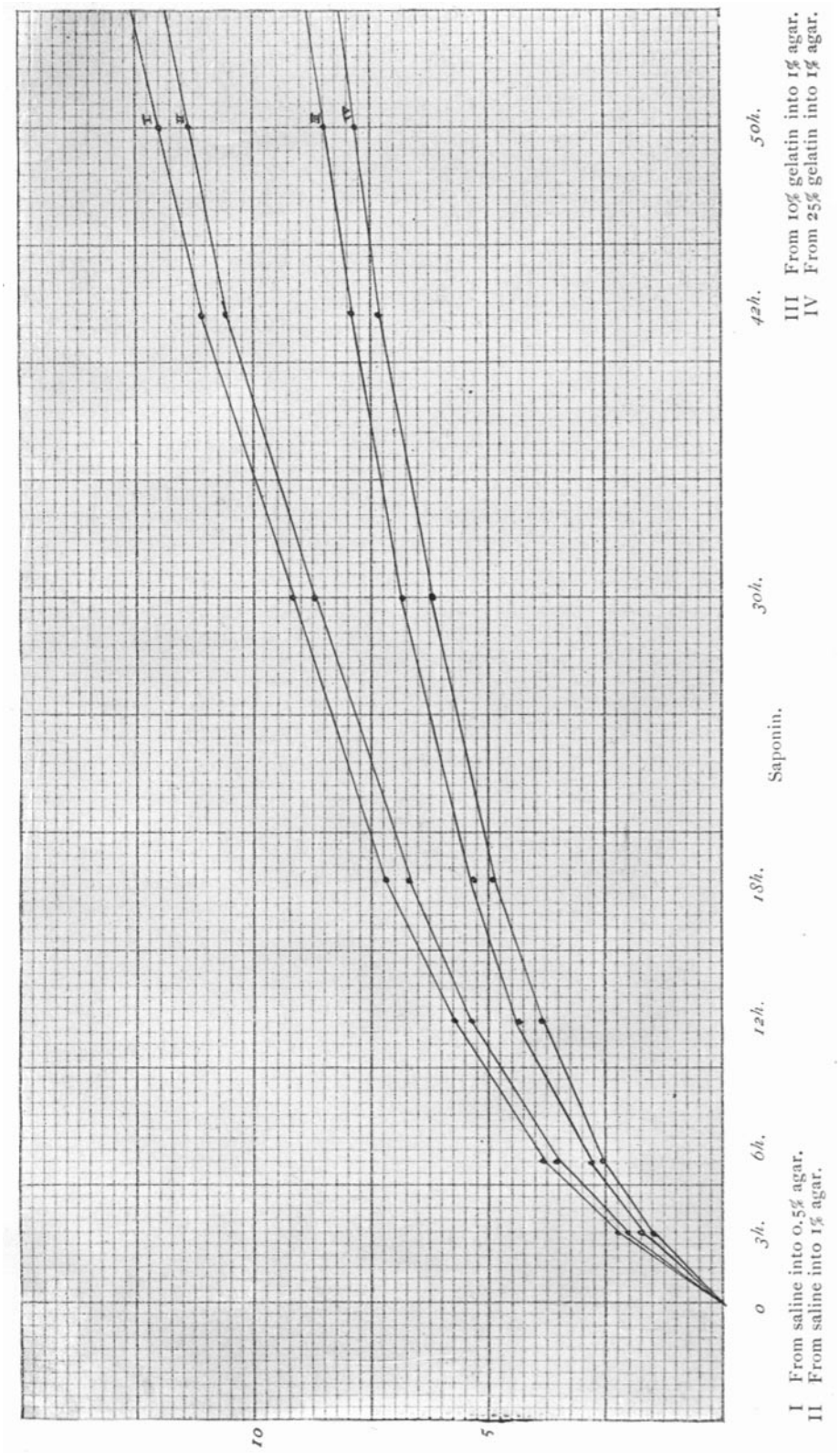
Sod. taurocholate in	6 h.	12 h.	24 h.	48 h.	66 h.
25% gelatin	1.5	2.45	3.15	4.55	5.6
10% "	1.8	2.8	3.8	5.0	7.0
Saline (control)	2.6	3.85	5.2	7.3	9.8

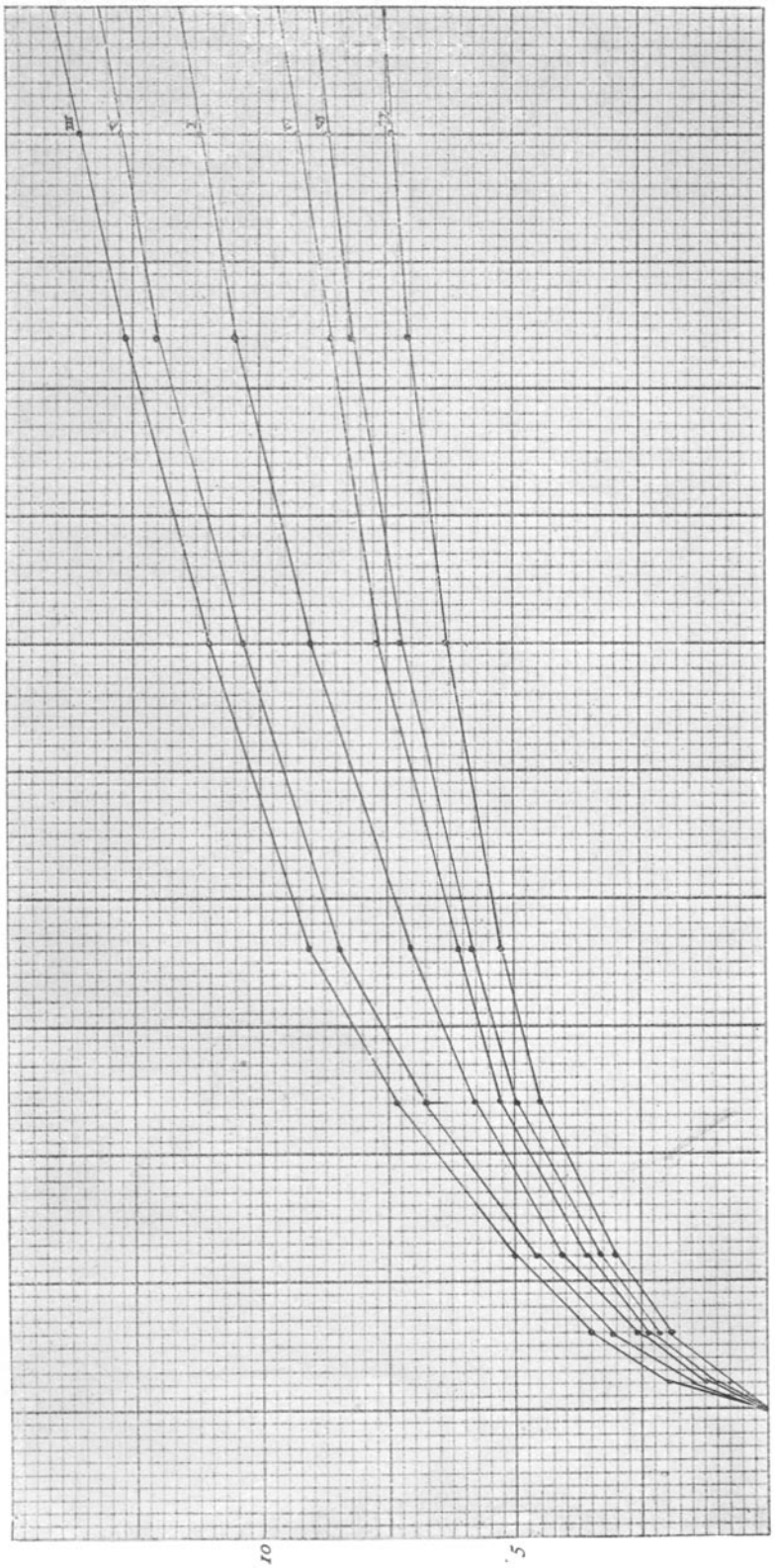
Hence taurocholate of sodium diffuses from 25 per cent. gelatin into agar more slowly than it does from 10 per cent. gelatin. The difference in concentration of the agar has only a slight influence on the rate of diffusion. In a second experiment the salt was dissolved in agar and the blood suspended in gelatin. The influence of concentration (0.5 and 2.0 per cent.) of the agar was very small, and the diffusion from agar was slightly greater than from saline solution (control).

The experiments with $\frac{1}{100}$ N. saponin dissolved in agar and dog blood suspended in gelatin showed diffusion to take place more rapidly into 10 than into 25 per cent. gelatin, and much more rapidly from agar than from the saline control. When, however, the colloids were reversed, namely, the blood suspended in agar and the solanin desolved in gelatin, the influence of the concentration of the gelatin came out, the diffusion from the saline being greater than that from the colloid. Plates XXVII and XXVIII express these effects in the form of curves.

The explanation of this discrepancy is to be found, possibly, in the sum of inhibitions exerted by the gelatin and the corpuscles, of which the first is the greater; while, as has frequently appeared in these experiments, the greatly weaker agar concentrations exercise slight inhibition only. A similar effect, although a smaller one, is produced in the case of pyrogallic acid.

In the study of the more complex hæmolysins of venom it was found, in spite of certain irregularities of diffusion, that (1) the velocity of diffusion from agar into the gelatin blood is slower in the case of 25 than in the case of 10 per cent. gelatin, (2) the hæmolysin of cobra venom is diffused from agar into gelatine more slowly than from these colloids into saline, (3) concentrations of agar between 0.5 and 2.0 per cent. act about the same, and (4) the relation of time to diffusion does not correspond to Fick's law.


I
II
III
IV
0
3h.
6h.
12h.
18h.
30h.
42h.
50h.
Saponin.
10
5
I From saline into 0.5% agar.
II From saline into 1% agar.
III From 10% gelatin into 1% agar.
IV From 25% gelatin into 1% agar.


10
5
0 1h. 3h. 6h. 12h. 18h. 30h. 42h.
Solanin.
I From saline into 10% gelatin.
II From saline into 25% gelatin.
III From 0.5%agar into 10% gelatin.
IV From 0.5% agar into 25% gelatin.
V From 1% agar into 10% gelatin.
VI From 1% agar into 25% gelatin.

DIFFUSION FROM COLLOID INTO SALINE SOLUTION.

Sodium taurocholate, saponin, and solanin were dissolved in 10 and 25 per cent. gelatin and poured into test-tubes of 20 mm. diameter. Five cubic centimeters of the colloids were put into each tube after the congealation of which an equal quantity of saline solution was poured in. The saline fluid, after contact, was removed at stated periods, and the quantity of hæmolysin present in it was estimated colorimetrically. Three

TABLE I.

SAPONIN $\frac{1}{100}$ N.

Period of Contact	Degree of Hæmolysis	25% gelatin		10% gelatin	
		Quantity in c.c. required to produce stated degree of hæmolysis	Corresponding number of hæmolytic units in 1 c.c. of saline	Quantity in c.c. required to produce stated degree of hæmolysis	Corresponding number of hæmolytic units in 1 c.c. of saline
1 hour	100%				
	30%	1.8	0.55	1.	1.
	5%	0.9	1.1	0.55	1.8
2 hours	100%				
	30%	1.4	0.7	0.6	1.6
	5%	0.7	1.4	0.5	2.
4 hours	100%			1.	1.
	30%	0.75	1.33	0.5	2.
	5%	0.5	2.	0.35	2.8
24 hours	100%	0.7	1.4	0.5	2.
	30%	0.3	3.3	0.2	5.
	5%	0.18	5.5	0.14	7.1

SOLANIN $\frac{1}{100}$ N.

1 hour	100%	0.55	1.8	0.33	3.
	30%	0.14	7.	0.09	11.
	5%	0.125	8.	0.085	12.
2 hours	100%	0.33	3.	0.25	4.
	30%	0.09	11.	0.075	13.3
	5%	0.085	12.	0.07	14.
4 hours	100%	0.25	4.	0.18	5.5
	30%	0.07	14.	0.055	18.
	5%	0.055	18.	0.04	25.
24 hours	100%	0.1	10.	0.07	14.2
	30%	0.03	33.	0.025	40.
	5%	0.025	40.	0.02	50.

grades were used: 100, 30, and 5 per cent. It was found that sodium taurocholate was largely held back for at least 48 hours by the gelatin, probably because of a chemical union between the salt and the proteid. Only a trace of hæmolysis was obtained at the end of this period. The results obtained with saponin and solanin are given in the accompanying table (Table I), and they show that the quantities of these bodies diffused from 25 and 10 per cent. gelatin bear the proportion of 1 : 1.25–1.40. *Hence the velocity and amount of diffusion from colloid into saline, like that from saline into colloid, is inversely proportional to the square root of the concentration of the colloid.*

INFLUENCE UPON THE RATE OF DIFFUSION OF DIAMETER OF SURFACE, DEPTH OF MEDIUM, AND CONCENTRATION OF HÆMOLYSIN.

TABLE II.

Diameter of Tube	Amount of Gelatin Blood	Amount of Saponin Solution	Period of Contact	$\frac{1}{100}$ N. Saponin 25% gelatin	$\frac{1}{100}$ N. Saponin 10% gelatin	$\frac{1}{1000}$ N. Saponin 25% gelatin	$\frac{1}{1000}$ N. Saponin 10% gelatin	$\frac{1}{10000}$ N. Saponin 25% gelatin	$\frac{1}{10000}$ N. Saponin 10% gelatin
8 mm.	4c.c	4c.c.	6 hours	2.4	2.85		1.9		
			24 "	5.	6.	4.2	4.55	1.75	1.75
			48 "	7.	8.4	6.45	7.35	3.85	3.85
	4 c.c.	2 c.c.	6 hours	2.4	2.85		1.9		
			24 "	5.	6.	4.2	4.55	1.75	1.75
			48 "	7.	8.4	6.45	7.35	3.85	3.85
	2 c.c.	4 c.c.	6 hours	2.4	2.85		1.9		
			24 "	5.	6.	4.2	4.55	1.75	1.75
			48 "	7.	8.4	6.45	7.35	3.85	3.85
15 mm.	2 c.c.	2 c.c.	6 hours	2.4	2.85	>			
			24 "	5.	6.	4.2	4.55	2.	2.1
			48 "	7.	8.75	7.	7.7	3.85	3.85
	2 c.c.	2 c.c.	6 hours	2.4	2.85	>			
			24 "	5.	6.	4.2	4.55	2.	2.1
			48 "	7.	8.75	7.	7.7	3.85	3.85
	1 c.c.	2 c.c.	6 hours	2.4	2.85	>			
			24 "	5.	6.	4.2	4.55	2.	2.1
			48 "	7.5	9.1	7.	7.7	3.85	3.85

The substances used in these experiments were solanin and saponin in saline solution and dog blood mixed with gelatin. The results are given in Tables II and III and they show that no marked influence is exercised on the diffusion by diameter of surface and depth of medium. But other facts may be noted.

TABLE III.

Diameter of Tube	Amount of Gelatin Blood	Amount of Solanin Solution	Period of Contact	$\frac{1}{100}$ N. Solanin 25% gelatin	$\frac{1}{100}$ N. Solanin 10% gelatin	$\frac{1}{1000}$ N. Solanin 25% gelatin	$\frac{1}{1000}$ N. Solanin 10% gelatin	$\frac{1}{10000}$ N. Solanin 25% gelatin	$\frac{1}{10000}$ N. Solanin 10% gelatin
15 mm.	4 c.c.	4 c.c.	6 hours	3.6	4.6		1.25	Slight trace in 48 hours.	ditto.
			24 "	7.5	9.5	1.4	3.85		
			48 "	10.5	13.5	2.1	5.95		
	4 c.c.	2 c.c.	6 hours	3.6	4.6		1.25		
			24 "	7.5	9.5	1.3	3.85		
			48 "	10.5	13.5	2.1	6.		
	2 c.c.	4 c.c.	6 hours	3.6	4.6		1.25		
			24 "	7.5	9.5	1.4	3.85		
			48 "	10.5	13.5	2.1	5.95		
8 mm.	2 c.c.	2 c.c.	6 hours	3.6	4.6		1.25	Slight trace in 48 hours.	ditto.
			24 "	7.5	9.8	1.4	3.85		
			48 "	10.5	13.5	2.1	6.		
	2 c.c.	1 c.c.	6 hours	3.6	4.6		1.25		
			24 "	7.5	9.8	1.4	3.85		
			48 "	10.5	13.5	2.1	5.9		
	1 c.c.	2 c.c.	6 hours	3.6	4.6		1.25		
			24 "	8.	9.8	1.4	3.85		
			48 "	10.5	13.5	2.1	6.		

The relative capacity for diffusion of saponin and solanin from saline solution into gelatin blood is expressed in hæmolytic units by the proportion of 1 : 1.5. These figures are to be compared with those in which the hæmolysin is enclosed in the colloid (Table I) and allowed to diffuse into saline, in which series the solanin is shown to diffuse from seven to eight times more hæmolytic units in a given period than the saponin. But

as solanin is nearly twice as strong an hæmolytic agent as saponin the proportion of diffused hæmolytic units would stand, as regards diffusion from colloid into saline, as 1:4 against 1:1.5 for saline into colloid. The diffusion velocity for solanin is, under the former conditions, four times as great as for saponin; and the inhibiting influence of the colloid is greater when the crystalloid seeks to enter it from than when it attempts to leave it for a fluid medium.

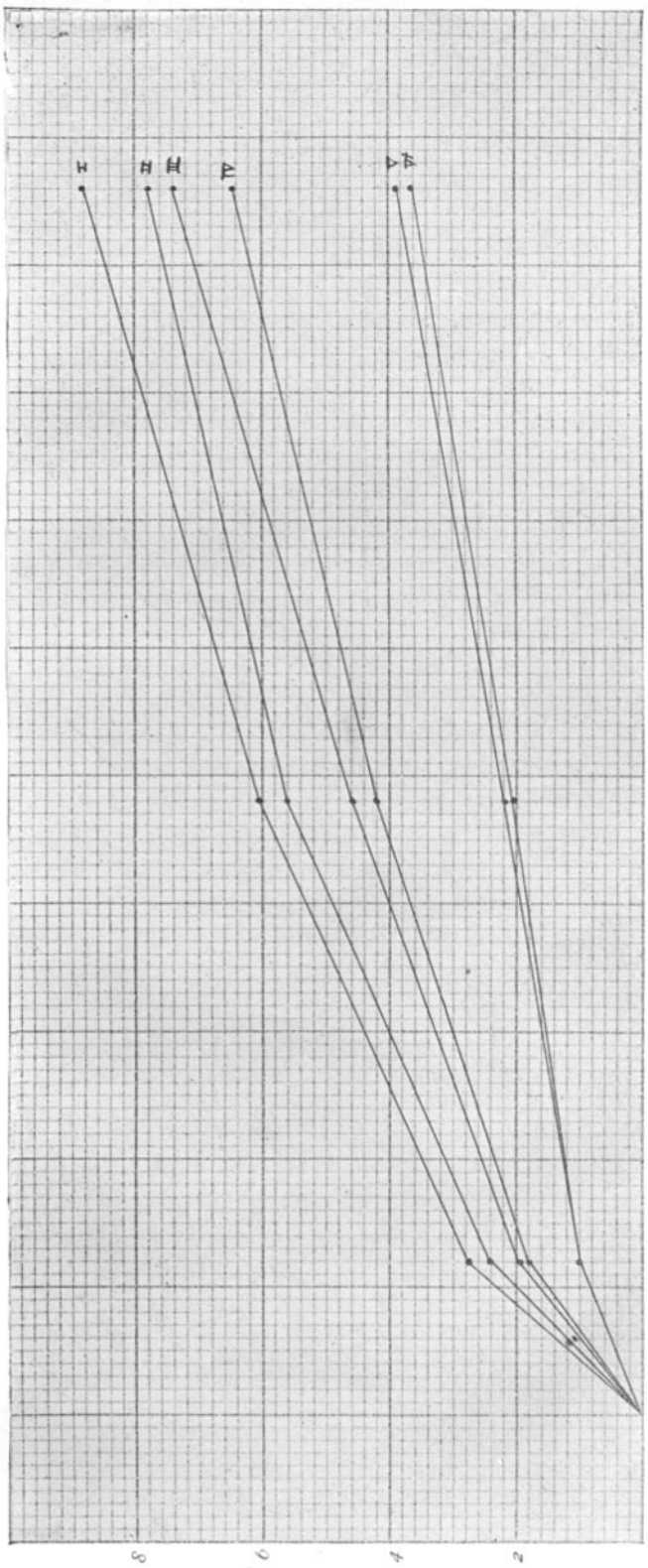
The concentration of the active substance plays a considerable part in relation to the rate of diffusion. The effects of this concentration are best seen by observing Plates XXIX and XXX. In the case of saponin while the difference in diffusion between $\frac{1}{100}$ N. and $\frac{1}{1000}$ N. is very slight, that between $\frac{1}{100}$ N. and $\frac{1}{10000}$ N. becomes as about 2:1. In the case of solanin the difference between $\frac{1}{100}$ N. and $\frac{1}{1000}$ N. is as 2:1, and between $\frac{1}{1000}$ N. and $\frac{1}{10000}$ N. more than 10:1.

In Plate XXIX, the following values are given: I and II represent the degree of diffusion of $\frac{1}{100}$ N. saponin from saline into 10 per cent. and 25 per cent. gelatin respectively; III and IV represent the degree of diffusion of $\frac{1}{1000}$ N., and V and VI, the degree of diffusion of $\frac{1}{10000}$ N. saponin under the same conditions. The values for Plate XXX are as follows: I and II represent the degree of diffusion of $\frac{1}{100}$ N. solanin from saline into 10 per cent. and 25 per cent. gelatin respectively; and III and IV the degree of diffusion of $\frac{1}{1000}$ N. solanin under the same conditions.

TABLE IV.

Scale of Hæmolysis*	Saponin				Solanin			
	25% gelatin		10% gelatin		25% gelatin		10% gelatin	
	$\frac{1}{100}$ N	$\frac{1}{1000}$ N	$\frac{1}{100}$ N	$\frac{1}{1000}$ N	$\frac{1}{100}$ N	$\frac{1}{1000}$ N	$\frac{1}{100}$ N	$\frac{1}{1000}$ N
a	1.		0.6		0.25	2.	0.16	1.5
b	0.3		0.25		0.06	0.7	0.05	0.5
c	0.2	2.	0.15	1.5	0.045	0.5	0.035	0.35

*The scale of hæmolysis was an arbitrary and descending one—a being highest and c lowest.


8
6
4
2
0
6h.
24h.
48h.
Saponin.
1/1000 N. Solution { III From saline into 10% gelatin. IV From saline into 25% gelatin.
1/100 N. Solution { I From saline into 10% gelatin. II From saline into 25% gelatin.
1/10000 N. Solution { V From saline into 10% gelatin. VI From saline into 25% gelatin.

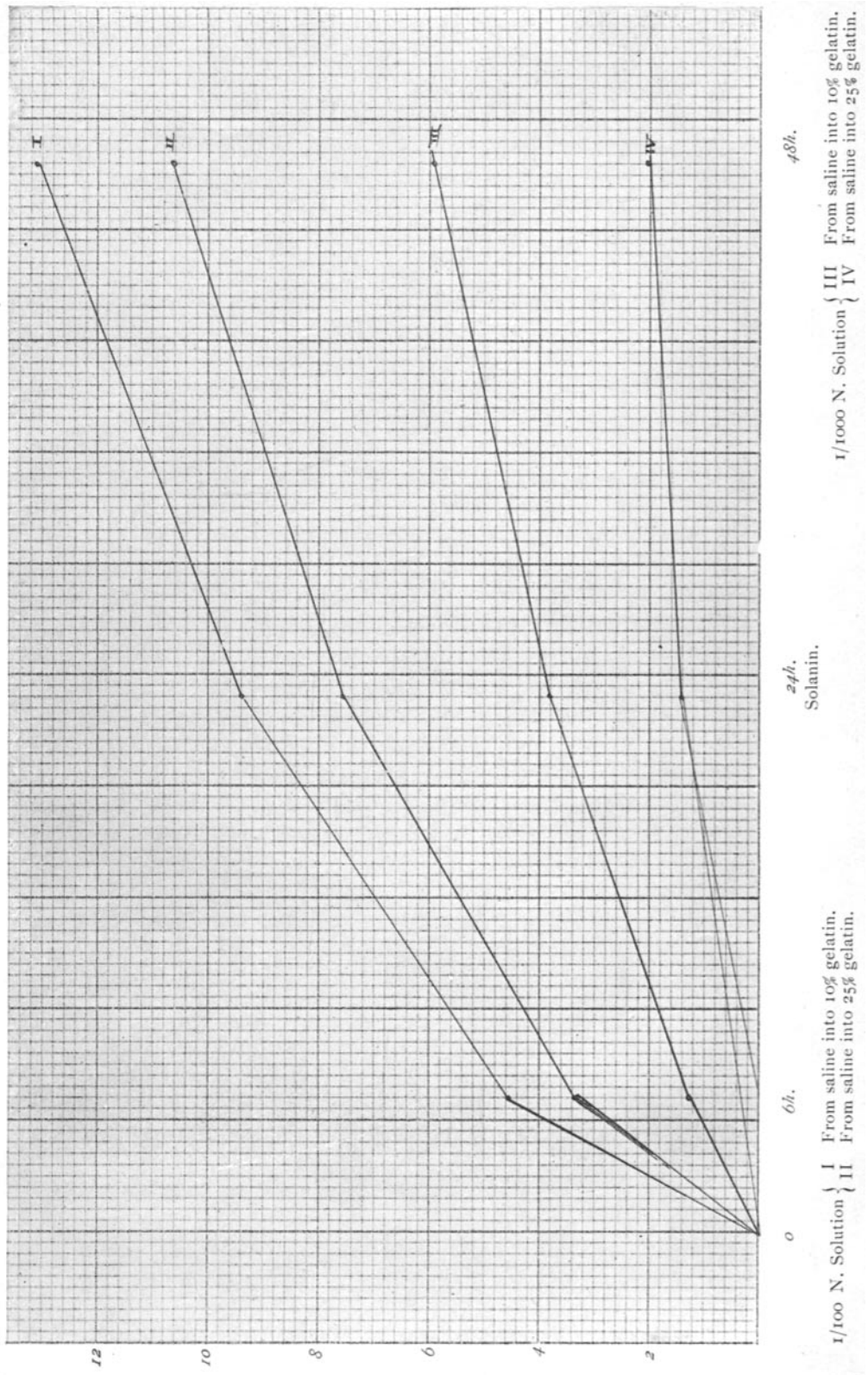

I
II
III
IV
12
10
8
6
4
2
0
6h.
24h.
48h.
Solanin.
1/100 N. Solution { I From saline into 10% gelatin. II From saline into 25% gelatin.
1/1000 N. Solution { III From saline into 10% gelatin. IV From saline into 25% gelatin.

TABLE V.

Period of Contact	Degree of Hæmolysis	$\frac{1}{100}$ N. Solanin		$\frac{1}{1000}$ N. Solanin	
		25% gelatin	10% gelatin	25% gelatin	10% gelatin
1 hour	30%	0.14*	0.09	1.5	1.
	5%	0.125	0.085	1.	0.65
2 hours	30%	0.09	0.075	1.	0.8
	5%	0.085	0.07	0.7	0.55
4 hours	30%	0.07	0.055	0.7	0.6
	5%	0.05	0.04	0.5	0.4
24 hours	30%	0.03	0.025	0.03	0.25
	5%	0.025	0.02	0.25	0.2

* These figures indicate the quantity of supernatant saline required to produce the degree of hæmolysis stated.

The differences noted are not entirely easy of explanation; and it is especially difficult to account for the behavior of solanin, in that while diffusing much more readily than saponin, it should, at the same time, be so greatly influenced by concentration. These considerations apply only to the special case in which the hæmolysins are contained in the saline; when they are enclosed in the colloid and allowed to diffuse into saline the amount of diffusion is directly proportional to the concentration of the hæmolysins.

DIFFUSION OF COBRA VENOM AND TETANUS TOXIN INTO COLLOIDS.

It has been stated that the hæmolysins of cobra venom and tetanus toxin pass from saline solution into gelatin and agar. A series of experiments was made to determine the effect of enclosing the toxins in one colloid (agar) and the blood in another (gelatin), and the reverse. Using a 5 per cent. suspension of dog blood in 10 per cent. gelatin and 0.1 per cent. cobra venom

in 2 per cent. agar, it was found that complete hæmolysis occurred in twenty-four hours, while when the blood was mixed with the agar and the venom with the gelatin, the hæmolysis proceeded much more slowly. It was also ascertained that the venom hæmolysin passes more quickly into gelatin from agar than from saline solution. In this respect it agrees with the more definite chemical hæmolysins.

The next question considered was whether the entire series of venom principles pass into the colloids, and if so whether they diffuse with equal velocity. To test this the hæmolysed portion of the gelatin- and agar-blood mixtures was carefully separated from the other in which the blood corpuscles were still intact, and each portion was injected into guinea-pigs to determine the toxicity. In the case of the gelatin-blood mixture the portion to be injected intraperitoneally was first melted, and in the case of agar it was emulsified in saline solution. The gelatin used was 10 per cent., and the agar 2 per cent. for enclosing the venom, and 0.5 per cent. for enclosing the blood.

Two cubic centimeters of the venom-colloid mixture were used in each experiment. The number of minimal lethal doses contained in this quantity, for guinea-pigs, of about 180 grams weight, was sixteen. It was ascertained that three m.l.d. of the venom passed in the hæmolysed portion from the agar into the gelatin blood, and one m.l.d. from the gelatin into the agar blood. It was further ascertained that the non-hæmolysed portion of the colloid-blood mixture does not contain the cobra neurotoxin in appreciable quantities. In other words, cobra lysin and cobra neurotoxin diffuse together.

Since the two principles diffuse together it was desirable to ascertain, if possible, whether they diffuse accurately in the proportion in which they exist in venom; that is, whether the whole venom passes unchanged into the colloid, or whether a separation of the components occurs. It has been stated that, using two cubic centimeters of a 0.1 per cent. cobra venom suspension in agar, one fifth of its toxic constituents passed into the gelatin-blood mixture in twenty-four hours. It was ascertained that two cubic centimeters of the venom mixture contained 600

complete hæmolytic doses of cobra lysin (for dog blood). Since the rate of diffusion of cobra lysin is very little affected by time, as it proceeds almost in a straight line, all that is required to determine the quantity of diffused lysin is to multiply the amount of diffusion by the time. It was found by experiment that an amount of cobra lysin necessary to hæmolyse two cubic centimeters of a 5 per cent. suspension of dog corpuscles will require from three to four hours to pass from 10 per cent. gelatin containing 0.1 per cent. venom to saline solution; and that in twenty-four hours about twenty-four complete hæmolytic doses will have diffused into the saline. Hence the rate of diffusion of cobra lysin is as 24: 600 or 1:25. In the same time, as we have seen, the quantity of cobra neurotoxin which diffused from agar into gelatin was as 1:5. In other words, cobra neurotoxin diffused five times faster than cobra lysin from agar into a gelatin-blood mixture. This fact interesting in itself is rendered more suggestive in view of Faust's [11] recent statement of a relationship between cobra neurotoxin and the saponin substances.

A series of experiments similar to the above was made with a tetanus toxin one cubic centimeter of which contained 1000 minimal lethal doses for guinea-pigs of 250 grams and rats of 60 grams weight, and 33 minimal hæmolytic doses. It was found that both tetanolysin and tetanospasmin pass together easily into gelatin and agar, since the non-hæmolysed portions of the blood-colloid cylinder do not contain an appreciable quantity (less than 0.0001 c.c.) of the toxin. It would, however, appear from our experiments that tetanolysin diffuses more rapidly into colloids than tetanospasmin, since the great disproportion in quantity of spasmin and lysin—1000:33—which exists in the tetanus toxin does not bring about a corresponding preponderance of the spasmin in the colloid.

SUMMARY.

Acids, alkalies, salts, glucosides, and certain toxins diffuse

[11] Die tierische Gifte, Braunschweig, 1906, p. 54.

more quickly into 0.9 per cent. salt solution than into agar-agar and gelatin suspensions.

The inhibitory effect of the colloids grows with increase in concentration, which increase affects both the velocity and extent of the diffusion.

In the case of gelatin the degree of diffusion is approximately in inverse proportion to the square root of the concentration. Agar-agar in strengths up to 2 per cent. inhibits far less than gelatin in 10 per cent. suspensions; and the difference in degree of inhibition exercised by 0.5 per cent. and 2 per cent. agar-agar is a small one.

Hæmolytic substances diffuse from gelatin into agar-agar more slowly than from saline into agar-agar. But the velocity of diffusion from agar-agar into gelatin is greater than from saline into gelatin.

The effects of differences in concentration of the hæmolytic agent vary according to the agent and the manner of its solution. When the hæmolyser is dissolved in salt solution the diffusion of $\frac{1}{100}$ N. and $\frac{1}{1000}$ N. solutions (saponin) is almost identical; while with solanin the stronger solutions diffuse faster. When the hæmolyser is dissolved in the colloid diffusion into fluid media is nearly proportional to the concentrations of the hæmolytic agent.

The velocity of diffusion into and from colloids is in general proportional to the square root of the time. Acids, alkalies, salts, and glucosides act in a manner which is in agreement with this rule. Cobra lysin and tetanolysin do not act in conformity with the rule.

Cobra lysin appears to diffuse into colloids more slowly, proportionally, than cobra neurotoxin, and tetanospasmin more slowly than tetanolysin.

The biological method described in this paper for studying diffusion in colloids is applicable to hæmolytic and some other toxic substances, and, with accuracy possibly only to such substances possessing relatively simple compositions.

Since all diffusion in the living body takes place within colloidal media of different concentrations, it would seem desirable to

perfect methods through which the interaction of toxic chemicals and the fluids and cells of the body may in a manner be imitated *in vitro*. Through this means our knowledge of toxicology may well be extended.