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SOME OBSERVATIONS ON THE ANTIBACTERIAL ACTION OF SODIUM SALICYLATE.

WITH 2 FIGURES IN TEXT.

By G. IVÁNOVICS, I. CSÁBI and E. DICZFALUSY

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(RECEIVED FOR PUBLICATION 15. 2. 1948.)

According to earlier observations (e. g. *Christian*,) (2) sodium salicylate has only a weak antiseptic effect, and to prevent bacterial deterioration of food, e. g. meat, fruit-pulp, concentrations of 1 per cent or more should be used. The antiseptic effect of sodium salicylate in such concentration is easily explained by its protein denaturing action observed by *Anson* and *Mirsky* (1) at concentrations of 0,1—0,25 M. In addition to this, as found recently, sodium salicylate exhibits another mode of antibacterial action under certain experimental conditions (*Iványovics*,) (3). In synthetic or semisynthetic media sodium salicylate prevents the growth of Bac. coli, Staphylococcus pyogenes, Bac. typhi, Bac. proteus vulgaris and some other species (*Iványovics*,) (5) at varying, but considerable lower concentrations (0,01—0,0002 M). This particular effect of sodium salicylate is specifically antagonized by minute amounts of pantothenic acid, and is absent in the case of bacteria which are exacting exogenous pantothenic acid with their growth (*Iványovics*,) (4).

From these observations it was concluded that the specific bacteriostatic effect of sodium salicylate is due to inhibition of pantothenic acid synthesis. As pantolactone, although in considerably higher concentration also antagonizes the bacteriostatic action of sodium salicylate, and as beta-alanin has no such effect, it is obvous that pantolactone synthesis is inhibited or interfered by sodium salicylate.

Altough many details of the antibacterial action of sodium salicylate have been studied recently, there are still some questions of primary importance which can not be answered by experimental facts at present. First of all, there is no evidence whether the cessation of bacterial growth caused by highly dilute sodium salicylate is a *real* bacteriostatic effect or only an *apparent* one due to gradual destruction of the bacteria. Another probleme waiting for an answer is whether inhibition of bacte-

rial growth by sodium salicylate does finally culminate in a lethal effect or whether after a considerable lapse of time growth still can be resumed.

MATERIAL AND METHODS.

Medium. — All experimets have been carried out in a synthetic medium recommanded by *Sahyun* et al. (7). In this medium the only source of carbon is glucose, and ammonium sulfate is serving as the sole source of nitrogen. Nine ml of the medium were distributed into test-tubes and sterilized at 105⁰ C for 20 minutes.

Inoculation of the Medium. — Test-tubes containing the medium were inoculated with a few thousands of Bac. coli grown on slant agar for 16 hours. This preliminary culture still actively growing at the beginning of the experiment was used for preparing inocula.

Technique. — Different dilutions of sodium salicylate were added to a set of tubes containing synthetic medium, heated to 37⁰ C, and inoculated with a certain number of bacteria obtained from the preliminary culture. Dilutions of inocula were made also with warm synthetic medium, and the volume of the tubes was filled up to 10 ml. The bacterium counts were established by plating in melted agar. Dilutions for plating were also made with warmed synthetic medium. Each bacterium count was established in duplicate, or triplicate test. Bac. coli, strain No. 8945 which proved to be highly sensitive to the action of sodium salicylate was used throughout these investigations.

EXPERIMENTAL.

Rate of growth of Bac. coli in the presence of sodium salicylate.

Different amounts of sodium salicylate were added into a set of test-tubes containing salt-glucose medium, and each was inoculated with the same number of bacteria. One of these experiments is represented by Fig. 1.

The numbers of living bacteria are decreasing rapidly in the presence of 0,1 M sodium salicylate, and no viable organisms were found by the third hour. Concentrations of 0,01 M, or less have no effect on the survival of inoculated bacteria, and growth occured in every case, however, the rate of mutiplication is significantly retarted. In the absence of sodium salicylate (control) there is a linear relationship between the log of the viable count and time, proving the rate of mutiplication to be

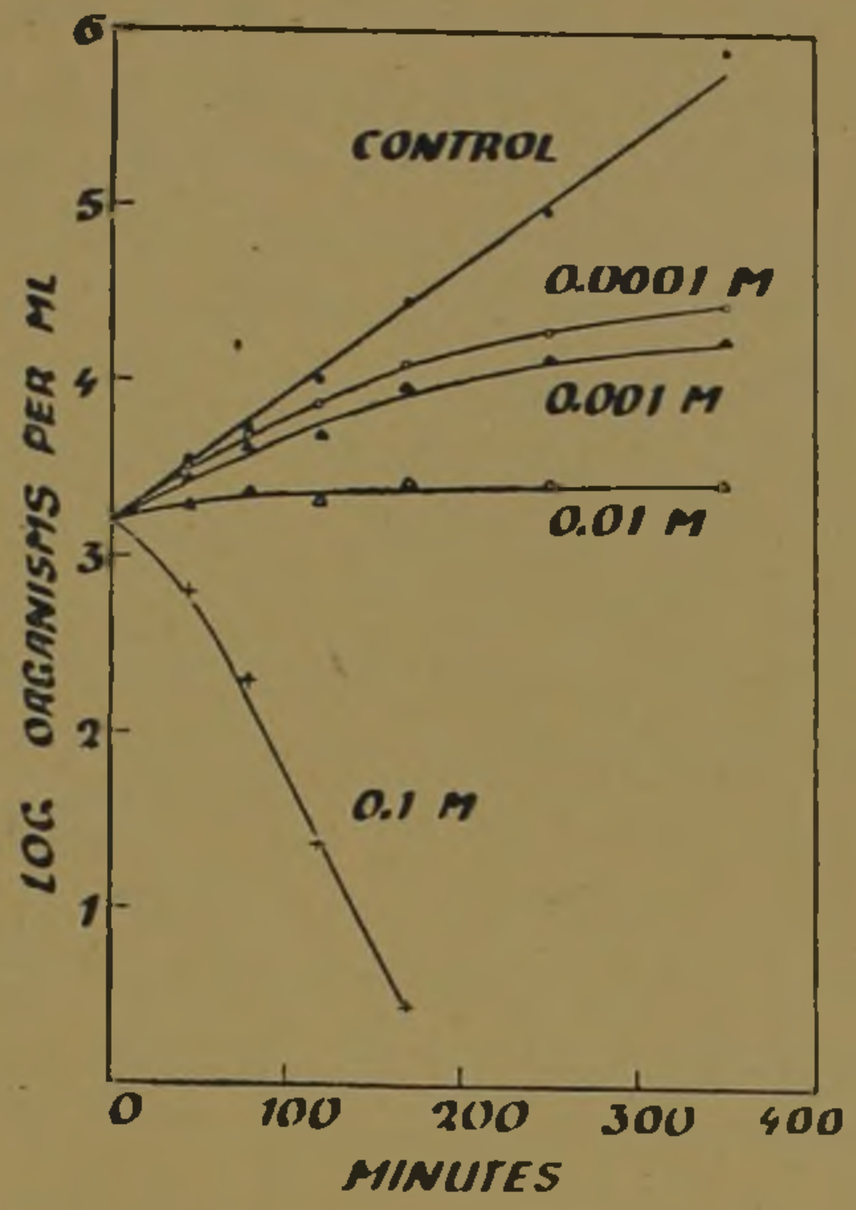

Fig. 1. Growth curves at different sodium salicylate concentrations.

constant. In the presence of the drug a curvilinear relationship is observed in which the slope of the curve gradually decreases until a certain point from which the trend of it is almost linear. This change of the growth-rate is very clearly demonstrated in those experiments in which the observation was extended for a considerable period of time (1500 minutes). See Fig. 2.

The change of growth-rate expressed numerically in terms of velocity constans as recommanded by *Kohn* and *Harris* (6) in their experiments with sulfanilamides, by the equation:

$$K = \frac{2.3}{T_2 - T_1} \log \frac{N_2}{N_1}$$

TABLE I.

The velocity constants of growth.

The period of the experiment	Velocity constants in diff. conc. of sod. salicylate			
	0,01 *M*	0,001 *M*	0,0001 *M*	no drug
0—45 minutes	0,0073	0,0153	0,0163	0,0170
46—80 „	0,0071	0,0132	0,0146	ditto
81—120 „	0,0003	0,0103	0,0103	ditto
121—170 „	0,0003	0,0064	0,0087	ditto
171—250 „	0,0002	0,0039	0,0050	ditto

The velocity constant of growth in the absence of sodium salicylate is constant, and its value is round 0,02, which is the same as found by *Kohn* and *Harris* in similar experimental conditions. The rate of growth is decreasing very markedly in the presence of 0,01 M sodium salicylate, and the value of the velocity constant after 80 minutes is practically constant. In this latter stage of the experiment the multiplication rate of the bacteria is roughly one hundredth of that observed in the absence of the antiseptic. The slowing of multiplication is less marked in higher dilutions of sodium salicylate, and at the end of these experiments the velocity of growth is only 1/5—1/8 of the control. As can be seen on the graph, and expressed numerically by velocity constants, a marked retardation of growth sets in only after 80—120 minutes.

The size of the inoculum has no effect on the results, and the retardation of growth by 0,002 M sodium salicylate was the same in every case, although the number of bacteria inoculated varied considerably.

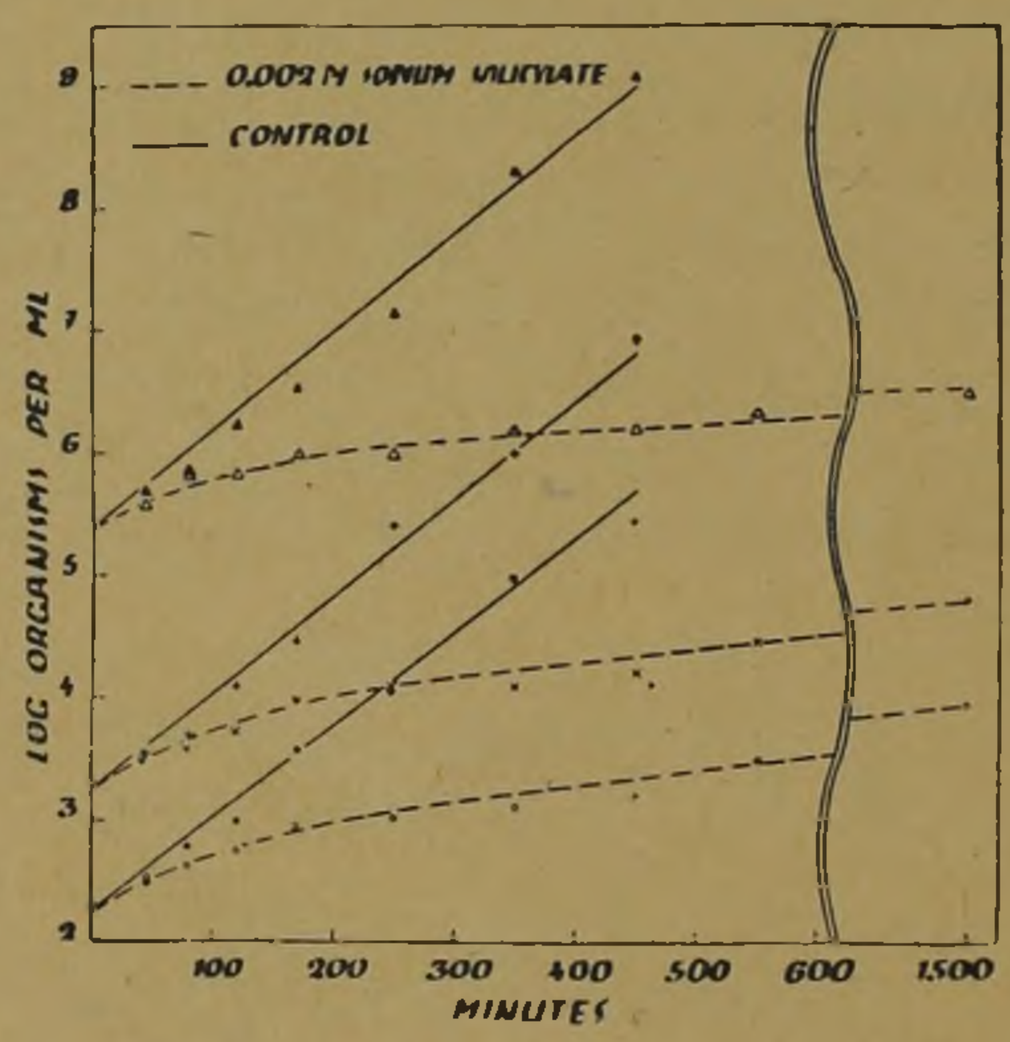

Fig. 2. The growth curves of Bac. coli at 0.002 M sodim salicylate concentration using different size of inocula.

As shown in foregoing experiments, variation of the concentration of sodium salicylate effected its action on Bac. coli. The organisms are destroyed by the drug if applied in high concentration as observed in the experiment in which 0,1 M was used. On the other hand, the bacteria multiply, though at a reduced rate, in 0,01 M of sodium salicylate. The change in the number of viable bacteria within these limits of concentration was studied and the results are tabulated in Table 2.

TABLE II.

The bacteriostatic effect of sodium salicylate of different concentrations in glucose-salt medium.

Minutes	The number of viable bacteria per ml indifferent sodium salicylate concentrations			
	0,2 *M*	0,1 *M*	0,05 *M*	0,033 *M*
5	1400	2725	2800	2820
20	0	1675	2480	2385
40	—	1100	2400	2325
60	—	545	2175	1870
90	—	485	1755	1510
120	—	98	1338	1418
170	—	2	902	1102
220	—	0	616	972
300	—	—	484	882

As can be seen, sodium salicylate concentrations of or above 0,033 M kill Bac. coli in glucose-salt medium, and the rate of destruction is proportionate with the concentration.

Reversibility of the antibacterial action of sodium salicylate.

Different concentrations of sodium salicylate in a series of tubes containing 4 ml glucose-salt medium were inoculated with appr. 6000 organisms of Bac. coli. The tubes were incubated, and after various intervals 1 ml of 0,001 M calcium pantothenate was added, and incubation allowed to proceed for another 96 hours. The growth of the bacteria was observed every 24 hours by recording turbidity. See Table 3.

TABLE III.

The growth of the cultures exposed to sodium salicylate for variable time and antagonized by calcium pantothenate.

Period of exposure	Contentration of sodium salicylate				
	0,1 *M*	0,05 *M*	0,02 *M*	0,01 *M*	0,002 *M*
5 minutes	—	—	+	+ +	+ + +
7 hours	—	—	+	+ +	+ + +
24 ,,	—	—	—	+ +	+ + +
72 ,,	—	—	—	+ +	+ + +

Remarks: 1. Same results were observed when 60,000 Bac. coli were used as an inoculum. 2. The first sign of growth was observed in 0,02 *M* of the drug only after 72 hours. 3. The growth was delayed but after 48 hours appeared in 0,01 *M*. 4. Full growth was already observed after 24 hours in 0,002 *M*. 5. Results obtained at different intervals and which did not differ from those included in the Table III. are ommited to save space.

This experiment shows that calcium pantothenate antagonizes the action of the sodium salicylate in concentrations of 0,01—0,002 M, and restores the growth of the cultures even after 72 hours of exposure to the drug. The same effect is observed in the case of 0,02 M sodium salicylate if the bacteria have not been exposed to its action longer than 7 hours. The two highest concentrations examined were lethal for the bacteria even in the presence of the vitamin.

As it was observed previously (*Ivánovics*,) (4), the bacteriostatic effect of sodium salicylate on Bac. coli in glucose-salt medium is interfered with amino acids. Lysine and valine were the most active and their antisalicylic-effect was even more pronounced in the presence of methionine. In order to ascertain whether lysine and valine are able to revert the bacteriostatic action of sodium salicylate if the Bac. coli was already in contact with the drug for a while, glucose-salt medium containing 0,002 M sodium salicylate was inoculated and incubated for 2 hours. After this time elapsed a bacterial count was made, and different amino acids were added, and the tubes further incubated. Multiplication of the bacteria was followed by plating in melted ager after different intervals. See Table 4.

TABLE IV.

The antagonistic effect of some amino acids on the action of sodium salicylate (0,002 M).

Minutes	Substance added to the medium in γ per *ml*						
	0 (control)	Ca. pant. 1	Valine 200	Methionine 200	Val. + Meth. 100—100	Glycine 200	Lysine 200
90	1,5	2,5	2,2	2,5	2,4	2,3	2,7
150	1,6	3,6	3,9	3,4	5,4	3,2	5,9
210	3,5	11,5	12,1	5,1	12,5	7,2	20,4
330	8,5	36,8	28,8	8,4	50,6	15,2	57,7
400	10,8	174,8	83,4	14,4	231,2	35,4	201,6

Remarks: 1. The numerical values are representing in thousands the number of viable bacteria per *ml*. 2. Inoculum: 620 per *ml*. 3. The bacterium count at the addition of the substances was 860 per *ml*.

This table shows that lysine and valine, particulary the latter in combination with methionin are enhancing growth, whereas glycine and methionine alone only slightly increased multiplication. It can be seen that some of the amino acids are restoring the growth of Bac. coli even after exposure to sodium salicylate. Similar experiments showed that the amount of amino acids effectivly countering the bacteriostatic action of sodium salicylate is a multiple that of pantothenic acid.

DISCUSSION.

Present investigations deal with the bacteriostatic action of sodium salicylate on Bac. coli in glucose-salt medium, in conditions therefore in which a maximal synthetic effort is required of the organisms. The rate of multiplication of the bacteria is gradually slowed by sodium salicylate in concentrations of 0,0001—0,01 M, and finally the rate of growth is only 1/5—1/100 of the original respectively. Since the slowing down of growth is proportional to concentrations, multiplication is almost zero at a sodium salicylate concentration of 0,01—0,02 M, and the cells developing are just replacing those which succumbed.

A sodium salicylate concentration of or higher than 0,02 M is not bacteriostatic in the true sense anymore, for it effects destruction of the cells. This destroying effect of the salt is gradually increasing until a concentration of 0,2 M is reached which already kills the bacteria almost instantaneously.

The bacteriostatic action of sodium salicylate is very similar to that of sulfanilamide. There is a latent period in both cases before marked retardation of growth occures. The latent period of the sulfanilamides observed by most workers is explained by the p-aminobenzoic acid hypothesis by extending this to include exhaustion of reserves. The same explanation might be of value to elucidate the latent period observed in the sodium salicylate experiments. Inhibition of the synthesis of pantothenic acid sets in probable at the moment of interaction of the drug and the bacteria, but it manifests itself fully only after the exhaustion of the reserves of the vitamin present in the cells.

The bacteriostatic effect of sodium salicylate can be eliminated by pantothenic acid, and this effect of the vitamin asserts itself even after a prolonged exposure of the cells to the drug. The antagonsitic effect of the vitamin manifests itself as long as living cells are present in the culture. Pantothenic acid, however, does not restore growth if the sodium salicylate concentration is 0,05 M or more.

It is supposed *(Iványovics,)* (3) that the bacteriostatic action of sodium salicylate is due to the inhibition of the synthesis of pantothenic acid in bacteria. The growth of Bac. coli requires a maximal synthetic effort of the cells in glucose-salt madium, and it is obvious that addition of essential metabolites of the bacteria alleviates the anabolism of the cells. As it was found previously, the velocity of the growth of Bac. coli as well as the biosynthesis of pantothenic acid by these bacteria is in the presence of a mixture of amino acids, e. g. casein hydrolysate higher, than in glucose-salt madium. These observations are offering an

explanation of the partial antagonistic action of amino acids on the effect of sodium salicylate.

Present experiments clearly show that some of the amino acids are effective even if the bacterium cells have been exposed for a while to the action of sodium salicylate and the rate of plasmasynthesis had been altered by the drug. As the amino acids are acting very differently in this respect, this line of investigation might prove of value in the study of the mechanism of pantothenic acid synthesis in Bac. coli.

SUMMARY.

The antibacterial action of sodium salicylate on Bac. coli was studied in glucose-salt medium. It was observed that the drug in concentration of 0,01—0,0001 M is considerably decreasing the multiplication of bacteria. This effect could be antagonized by pantothenic acid and some of the amino acids even if the cells have already been exposed to the drug for a considerable lenght of time. Sodium sylicalate in concentrations of or above 0,05 M gradually destroys the bacteria, and this effect is naturally irreversible. There is no sharp line between these two modes of action of sodium salicylate, and they overlap in a certain range of concentration.

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THE EFFECT OF BILE SALTS ON FIBRINOGEN AND ITS CLOTTING.

WITH 5 FIGURES IN TEXT.

BY E. MIHÁLYI

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(RECEIVED FOR PUBLICATION 21. 2. 1948.)

M. L. Anson (1) found that bile salts have a strong protein denaturing effect. In concentrations of 2,5 mg per ml solution, they cause a rapid denaturation of hemoglobin and egg albumin at the isoelectric point. Other hydrotropic substances have a similar effect, but require higher concentration and they act much more slowly. The protein denaturated by the hydrotropic is kept in a solution and precipitated only when the later is removed by dialysis. The proteins denaturated and precipitated by trichloracetic acid, wolframic acid, heat etc., can be dissolved by the hydrotropic substances, and when the hydrotropics are added before the precipitating agent, they prevent the precipitation of proteins. Anson was performing his studies also with a series of synthetic detergents, wich have the effect as the bile salts.

The first extensive work about the action of a hydrotropic on fibrinogen and its clotting was carried out by *I. Meisner* and *E. Wöhlisch* (2). They showed that urea, guanidine and hippurate do not denaturate fibrinogen In a solution of 30% urea the fibrinogen can be stored for months. The urea even prevents the spontaneous denaturation of fibrinogen. Removing by dialysis, the fibrinogen solution has all the properties of a fresh, untreated preparation. It was shown also, that 30% urea inhibits completely the clotting. More recently *W. F. H. M. Mommaerts* (3) investigated the effect of these substances on clotting and demonstrated that urea, guanidine etc. inhibit the first step of clotting i. e. the action of thrombin on the fibrinogen molecules. He found, that probably the the second step of clotting, the gelification of fibrinogen which has already undergone the action of thrombin, is also inhibited by urea.

It seems necessary to extend our knowledges about the action of hydrotropic substances on fibrinogen and clotting, by investigating the effect of bile salts too. The results concerning the interactions between proteins and bile salts are restricted only to a few proteins. In this respect too the elucidation of the problem presents some interest.

EXPERIMENTAL.

REAGENTS.

Fibrinogen: The experiments were carried out with fibrinogen prepared from fresh oxalated cattle plasma. The fibrinogen was precipitated by adding, under vigourous stirring one third volume of saturated ammonium sulphate. The precipitate, separated by centrifugation, was dissolved in one fifth of the original plasma volume of 0,9% *NaCl* solution containing 0,2% sodium oxalate. The insoluble part and eventualy present cytological elements were centrifuged out, and the fibrinogen precipitated by adding an aqual volume of saturated *NaCl* solution. The precipitate was washed with cold distilled water and dissolved in 0,9% *NaCl*, containing 0,2% sodium oxalate, of a volume equal to 1/10 of the original plasma volume. The solutions contain 20—40 mg fibrinogen per ml and are slightly opalescent.

The fibrinogen content of the preparations was estimated by K. *Laki*'s method (4), clotting under suitable conditions the fibrinogen with thrombin. The clot is washed several times, dried at 105 C^0 and weighed.

The total protein content was estimated by means of trichloracetic acid precipitation, washing and drying the precipitate and weighing till the weight was constant. The fibrinogen preparations used were of 80% purity in the average.

Bile salts: 0,1 M solutions of bile salts were prepared and then diluted to the desired concentration. Sodium salts of *Th. Schuchardt* were used, except the sodium choleinate which was prepared from choleinic acid neutralised with 0,2 *N NaOH*. The sodium taurocholate is to some extent hygroscopic, therefore it was dried for two hours at 105 ^{0}C before weighing. The choleinic acid is a molecular complex of 8 molecules of desoxycholic acid and one molecule of oleic acid. The prepared solution was 0,1 molar in respect to the desoxycholic acid, taking $^1/_8$ of the complex molecular weight as the basis of calculation.

Thrombin: Thrombin was prepared after *K. Laki* and *L. Lóránd* (5), and the strength was determined in the way described by these authors.

METHODS.

The clotting time of fibrinogen solutions was determined in small tubes noting the moment when the tube could be inclined without displacing the meniscus. The tubes with plane bottom have the following dimensions: 14 mm diameter, 50 mm length.

In order to test the influence of different bile salts in different concentrations and at different *pH*, the following reaction mixture was prepared:

0,5 ml fibrinogen solution,
0,4 ml 0,2 M phosphate buffer,
1,0 ml bile salt solution,
0,1 ml thrombin solution, about 10 units per ml.

In my experiences both fibrinogen and bile salt concentrations and the *pH* of buffer solutions were modified, in a manner which will be described in the text. The content of tubes was mixed thoroughly by closing the tubes with the finger and turning them over several times.

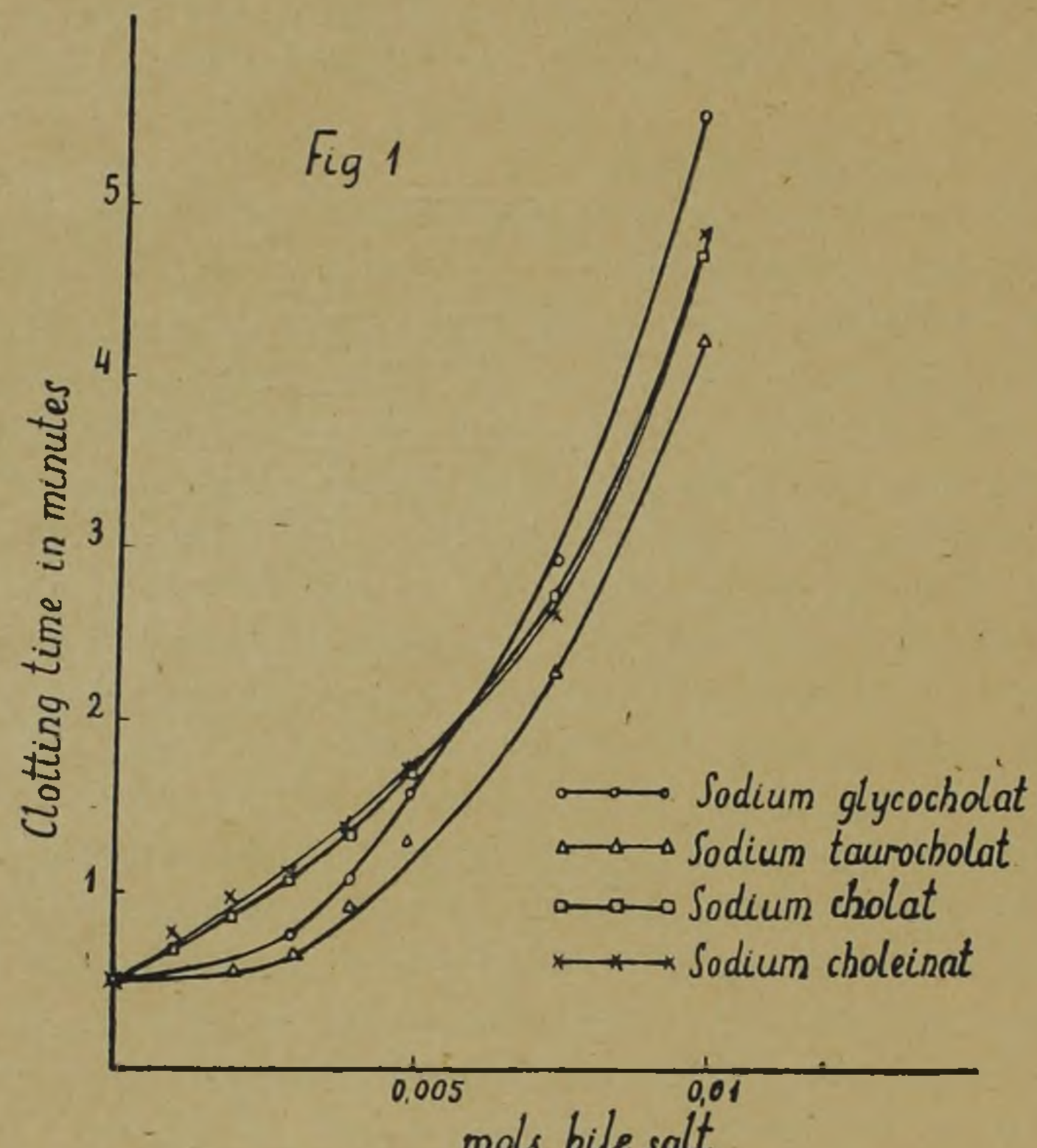
A Zeiss nephelometer combined with Stufenphotometer served for the determination of turbidities. The L_3 (green) filter and the No 4 standard turbid glass were used. The turbidity is expressed as the percent intensity of the light beam passing the standard turbid glass. The solutions were placed in the apparatus in the small tubes described above, in a proper celluloid holder. The fibrinogen was clotted in the tubes, pipetting in 0,75 ml fibrinogen solution, 0,6 ml 0,2 M phosphate buffer of *pH* 7,35, 1,5 ml bile salt solution and 0,15 ml thrombin of 10 units per ml. After 15 minutes the turbidity of the resulting clot was determined.

The bile salts are able also to prevent the turbidity caused by heating. The effect was investigated in a mixture similar to the above described, replacing the thrombin solution with distilled water. The tubes containing the mixtures were placed for 2 minutes in a boiling water bath and after cooling their turbidity measured.

RESULTS.

The effect of bile salts upon the clotting of fibrinogen with thrombin. The bile salts have a marked inhibiting property on clotting in relatively small concentrations. In fig. 1 is shown the action of different bile salts at *pH* 6,8 Ordinate: clotting time in minutes, abscissa: molar endconcentrations of the bile salts. As can be seen, the different bile salts have a very similar effect. There is a little difference in the form of curve of the simple bile salt (cholate) and the conjugated salts (glyco- and taurocholate). The former is rising nearly linear up to 0,005 M and is above the conjugated bile salts; the curves of the later after an approximately horizontal part rise steeper. We meet the greatest inhibitory effect by sodium glycocholate, although not much greater than by the others, which in 0,01 M endconcentration prolongs the clotting time more than ten times. 0,01 M endconcentration corresponds to a solution of 0,45%. The curves of sodium

Fig 1

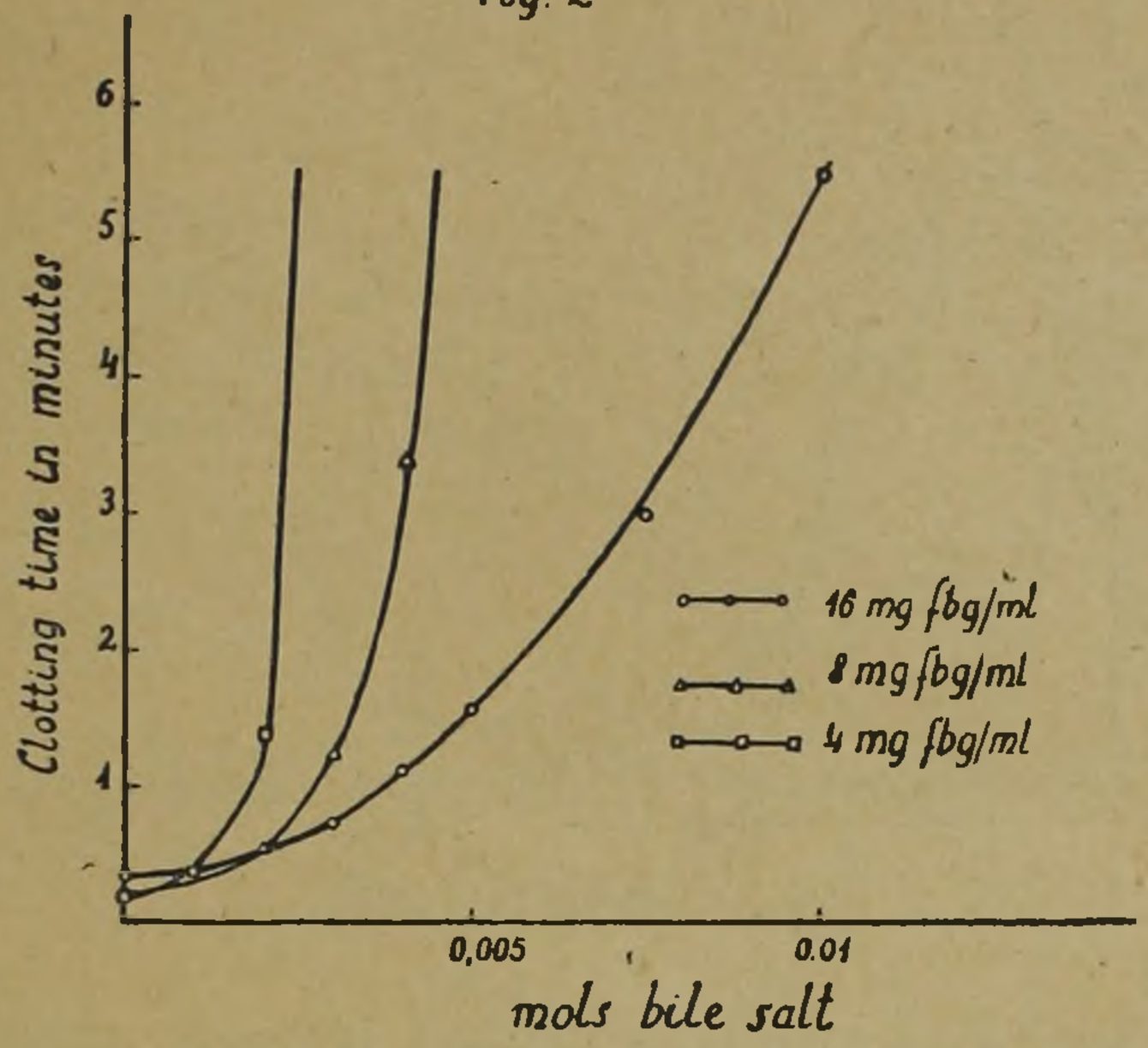


cholate and the complex sodium choleinate are completely superimposed. It seems that the complex formation has not any influence on the inhibiting power. At high bile salt concentration the clots are at first fragile, but in a few hours, even at 0,05 M endconcetration, they become very firm. At 0,05 M the clotting time is $1^1/_2$—2 hours. The clot is completely transparent.

The inhibiting power of bile salts depends on the fibrinogen concentration too. There is a close relation between the fibrinogen content and the bile salt concentration. The higher is the former, the higher must be the later in order to obtain a given inhibition. Fig. 2 shows this effect. The curves were taken with 4, 8, 16 mg per ml concentration of fibrinogen solutions at *pH* 6,8 and *Na*- glycocholate. The distances between the straight part of curves correspond approximatly to the relation of fibrinogen concentrations, e. g. the abscissae of $3^1/_2$ minutes clotting time are 0,0025, 0,004 and 0,007 for the three different fibrinogen concentrations.

Fig. 3 shows the effect of *pH* on the inhibiting activity. As can be seen the rise of *pH* causes an augmentation of inhibition. At *pH* 8,04 the inhibiting activity is two times higher than at 6,8. 0,2 M phosphate

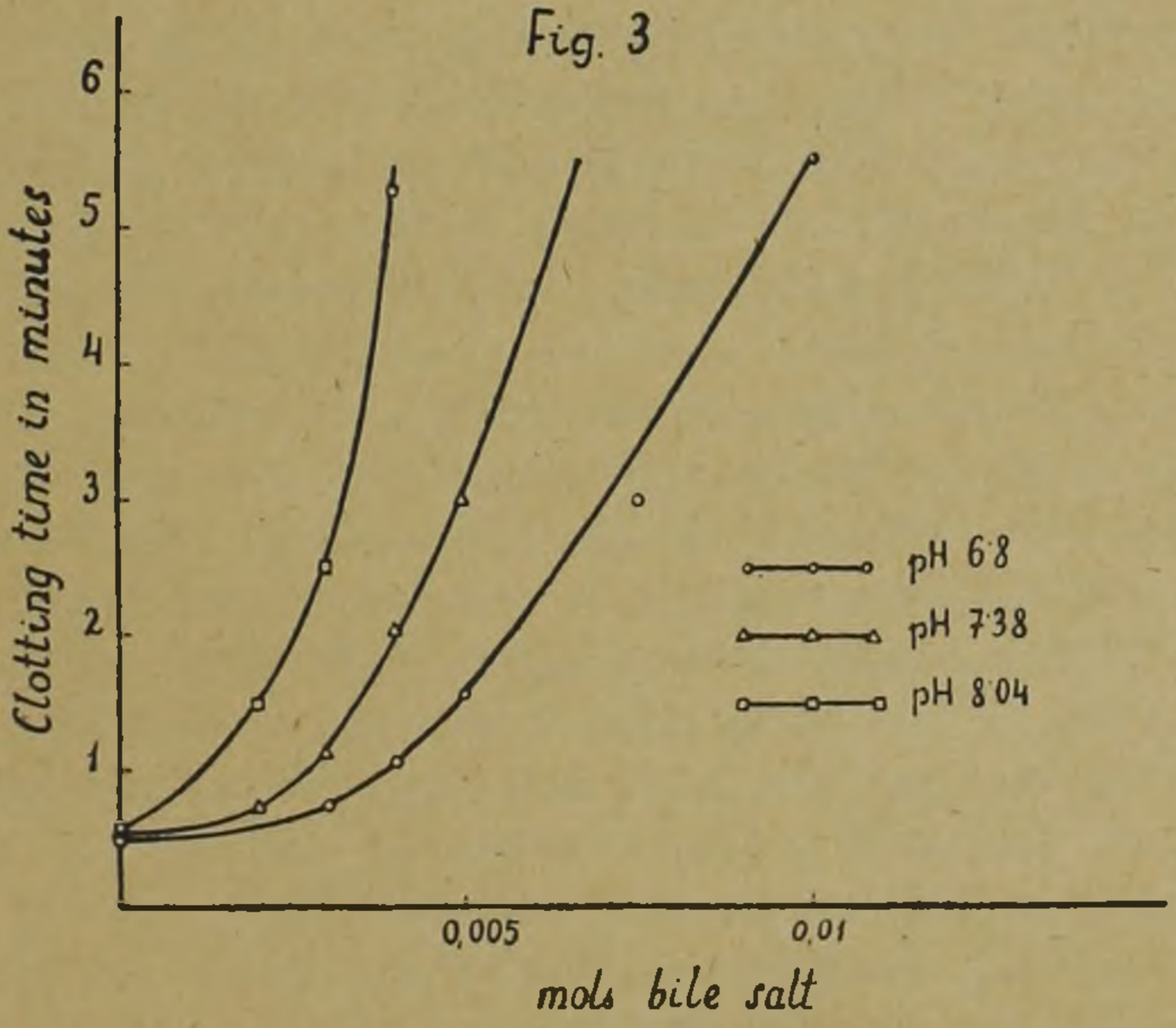
Fig. 2

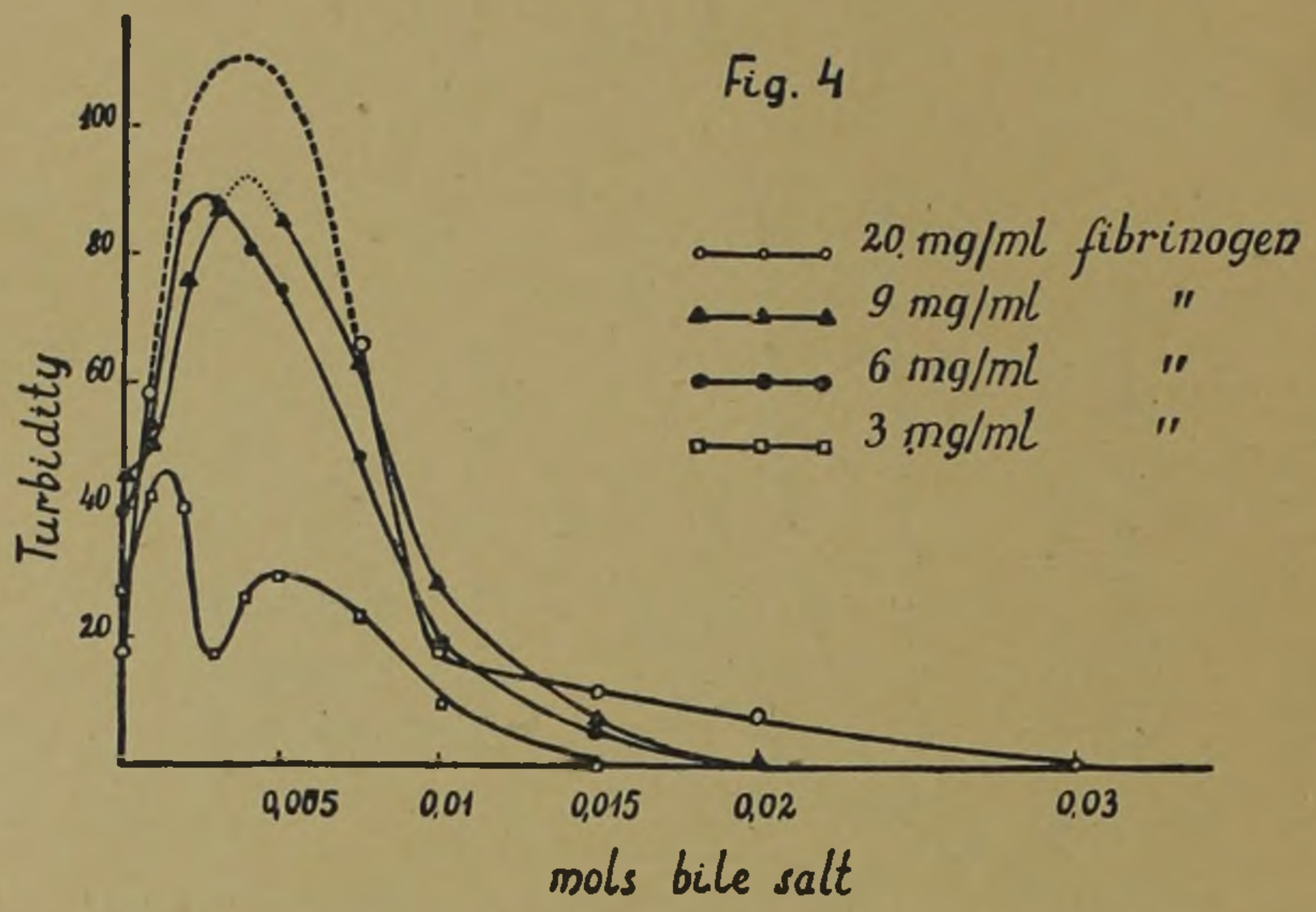


buffer of *pH* 6,8, 7,38 and 8,04 and *Na*- glycocholate were used. The fibrinogen concentration was 16 mg per ml.

The clots resulting by thrombin clotting are turbid, if the *pH* is not too alkaline. In the presence of bile salts, one can obtain a completely

Fig. 3

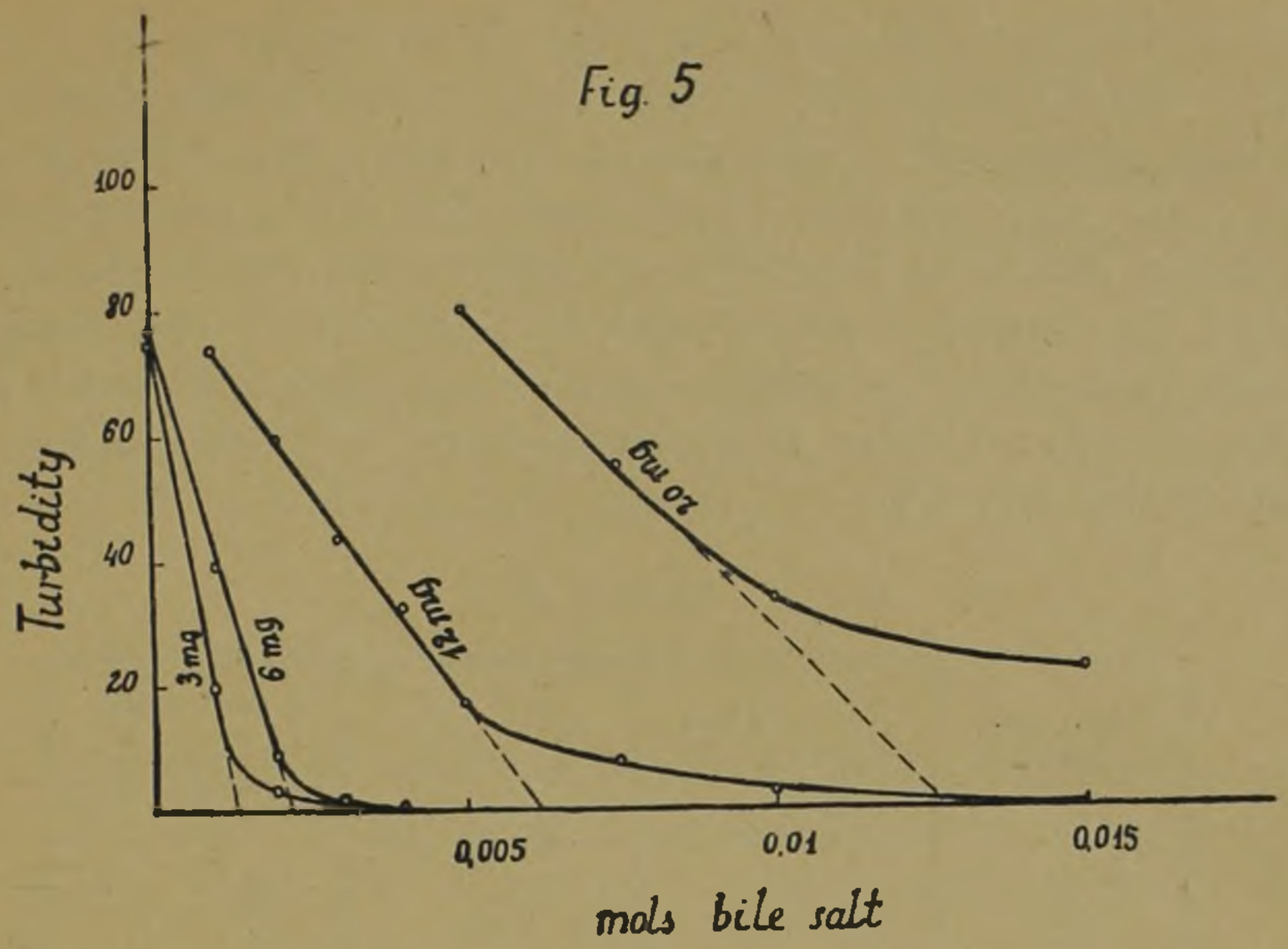




transparent clot even when the *pH* is such as to give otherwise a turbid clot. I investigated the manner in which this effect is connected with the fibrinogen and bile salt concentrations (Fig. 4). Solutions of 3, 6, 9, and 20 mg fibrinogen per ml were used. The clotting was performed at *pH* 7,38 in the presence of different amounts of *Na*- glycocholate. The broken part of curve corresponding to 20 mg per ml concentration of fibrinogen solution is arbitrary, since the turbidity of clots was so high that they scarcely transmit any light. Therefore the intensity of laterally scattered light is also greatly diminished and does not give the degree of turbidity. The curves are maximum curves and are shifted gradually with the increase of fibrinogen concentration towards higher bile salt concentrations. The shift is however not very important, and it would better to say, that the turbidity of clots falls to an insignificant value at 0,01 M bile salt concentration, independent of fibrinogen concentration. The curve of the lowest fibrinogen concentration has two maxima. Repeating the experiment with different fibrinogen preparations of the same concentration, this shape appears regularly. The clot corresponding to the minimum between the two maxima is very fragile, while all the others, even that corresponding to 0,05 M bile salt concentration, were firm.

The effect of bile salts on the heat coagulation of fibrinogen. The bile salts prevent the turbidity caused by heating the fibrinogen solutions too (Fig. 5). The curves were taken up with 3, 6, 12, and 20 mg per ml concentration of fibrinogen solutions. Phosphate buffer of *pH* 7,38 and 0,2 M concentration, and *Na*- glycocholate were used. It can be seen very nicely

Fig. 5



on the curves the connection between the fibrinogen and bile salt concentration. The curves start with a linear part. If we extend this part and consider the points of intersection with the abscissa, these are nearly proportional to the fibrinogen concentrations. But at high fibrinogen concentrations this proportionality does not hold good. The bile salt concentration will be much greater, in order to prevent the turbidity, than that calculated from the data of lower concentrations. But with rising concentration the tendency of aggregation, which is insignificant at low concentrations becomes more and more important. To prevent this new factor the bile salt concentration must be greater than without aggregation.

The effect of bile salts on the isoelectric precipitation and the salting out of fibrinogen. The bile salts do not affect the isoelectric precipitation or the salting out of fibrinogen.

Mineral acids precipitate the sparingly soluble bile acid from bile salt solutions. The acetic acid is weaker than the glycocholic acid. *K* for acetic acid is 0,0018, while that for glycocholic acid is 0,0132 and therefore it does not precipitate glycocholate. Acidulating a fibrinogen solution which contains sodium glycocholate by acetic acid, it is possible to precipitate fibrinogen without precipitating the bile acid. The precipitation occurs at about *pH* 5,3 as if the bile salt is absent and it is independent of bile salt concentration (0,05 to 0,2 M endconcentrations). The fibrinogen can be precipitated in similar conditions with 0,1 M acetate buffer of *pH* 5,3 too.

In the process of salting out a difficulty appears, namely the bile salt is also salted out. Fortunately this occurs at a higher salt concentration,

when fibrinogen is already salted out. The salting out of fibrinogen without bile salt, of bile salt alone and of a mixture of fibrinogen and bile salt were investigated. The fibrinogen was dissolved in 0,1 M phosphate of *pH* 6,8 (2 mg per ml) and a neutralised solution of ammonium sulphate at different degree of saturation was added. The results are shown in Table 1. The degree of precipitation is noted with +. The precipitating ammonium sulphate concentration is the same wether bile salt is present or not. The precipitation of fibrinogen is rather intensified than diminished by the bile salt.

TABLE I.

$(NH_4)_2SO_4$ saturation	1 *ml* fibrinogen 1 *ml* dist. water 2 *ml* ammon. sulph.	1 *ml* 0,2 *M* *Na* glycochol. 1 *ml* 0,1 *M* phosphate 6,8 2 *ml* ammon. sulph.	1 *ml* fibrinogen 1 *ml* 0,2 *M* *Na* glycochol. 2 *ml* ammon. sulph.
0,20	0	0	0
0,25	+	0	+ +
0,30	+	0	+ +
0,35	+	+ + +	+ + +
0,40	+	+ + + +	+ + + +
0,45	+	+ + + +	+ + + +
0,50	+	+ + + +	+ + + +

The denaturation of fibrinogen by bile salts. The bile salts denature fibrinogen, but the process of denaruration is very slow and appears only at high concentrations with the data as regards other proteins. Fibrinogen was allowed to stay at room temperature for half an hour in 0,05 M glycocholat solution. After this time it was put in cellophan bag and dialysed against distilled water. The dialysis is fairly slow. The solution contains after 24 hours 0,01 M bile salt as shown by surface tension determination with Traube's stalagmometer. The bile salt is therefore sufficient active during the dialysis too. The fibrinogen treated in such a way give with thrombin a very loose clot, which comes out to be only one tenth of the fibrinogen present originally. Repeating the experiment under similar conditions in 0,025 M *Na*- glycocholate, the clot formed corresponds to the total fibrinogen. — 5 ml fibrinogen (16 mg per ml), were mixed with 5 ml 0,2 M phosphate buffer of *pH* 6,8 and 10 ml 0,01 M *Na*- glycocholate. The mixture stood at room temperature and from time to time the clotting time of samples was determined. There was not any change in three hours, showing that any appreciable denaturation does not occur during this time.

Solubility of fibrin and denaturated fibrinogen in bile salt solutions. The bile salts are not able to dissolve fibrin. 5 ml fibrinogen (10 mg per ml),

2 ml 0,2 M phosphate of *pH* 6,24 and 0,5 ml thrombin were mixed. The resulting clot, after a few hours, was broken with a glass rod and 20 ml of a 20% solution of *Na*- glycocholate were added. The clot fragments had sharpe edges even after three days, showing that no dissolution occured.

The fibrinogen coagulated by heat or precipitated with trichloracetic acid does not dissolve in 20% solution of *Na*-glycocholate. The precipitates were allowed to stay with the bile salt solution for a day, after that they were centrifuged and the supernatant liquor acidulated with acetic acid. There was no precipitation at all, showing the absence of protein in the solution.

DISCUSSION.

The bile salts have a marked clot-inhibiting property. It is interesting that this effect scarcely differs from one bile salt to the other, in spite of the fact that simple and conjugated bile salts, and bile salt-fatty acid complex were investigated. This is an indication that rather the physical properties of the molecule are involved than their chemical structures.

The recent investigations have shown the importance of SO_3H groups in the action of heparin and synthetic anticoagulants. *Ch. Wunderly* (6) demonstrated that small molecules containing SO_3H groups also inhibited blood-clotting in vitro. He showed that by simple aromatic substances the anticoagulant activity run parallel to the number of SO_3H groups in the molecule. It was therefore expected that *Na*-taurocholate would have a much greater activity than *Na*-glycocholate. It was found in the contrary, thus demonstrating that the specific groups are not involved, only the physico-chemical properties are important.

The bile salts are characterised by a type of hydrophobic-hydrophilic structure. The hydrophilic, strong polar group of bile salts, is either the COO^- or the SO_3^- group and the hydrophobic, non polar group, is the sterol skeleton. The same type of structure can be found in numerous hydrotropics. Thus the soap and the great variety of synthetic detergents used in industry (Igepon, Duponol etc.) have a long fatty-acid chain and a polar group such as $COOH$, SO_3Na and sulphuric acid ester group (see *M. L. Anson* l. c.).

The bile salts are extremely surface active. Their hydrophobic part can combine with hydrophobic particles, when the hydrophilic groups are directed towards the solution, giving these particles a coating of hydrophilic groups. It is well known that the bile salts can emulsify the fats in above way. Anson in the quoted paper collected the available data about the solvent action of bile salts and detergents on proteins:

Bile salts have been used to extract the photosensitive pigment of the eye (7) and a chlorophyl compound from the chloroplast of spinach (8). It has been found also that synthetic detergents have a slow splitting action on the tobacco mosaic virus protein (9).

The clot-inhibiting activity and the prevention of the turbidity caused by heat, as would be shown, are related to the relative concentrations of fibrinogen and bile salt. This shows that a sort of compound must be formed between the protein and the bile salt. *M. L. Anson* arrived also at the same conclusion, when he found that the denaturing amount of detergents and bile salts rise parallel with the amount of hemoglobin to be denatured.

It may be suggested, that in the inhibition of clotting a certain number of bile salt molecules should be bound, giving the fibrinogen molecules such a degree of hydration, that they cannot approach each others sufficiently to bind together.

The light scattering power, i. e. the turbidity of protein solution was also investigated. It is difficult to interpret the results. The known equation of Rayleigh gives the intensity (I) of scattered light at right angles to the incident beam (monochromatic):

$$I = K\, \nu\, v^2 \left(\frac{N_1^2 - N_2^2}{N_1^2 + 2\, N_2^2} \right)^2$$

where K is a proportionality constant, ν the number, v the volume of particles, N_1 the refractive index of particles, N_2 that of the solvent.

In the performed experiments all these factors may change. The number of particles might have been diminished by aggregation and at the same time their volumes might have been increased. But the hydration also brings about a volume increase. The hydration may cause not only the increase the solvent coat, water may enter the interior of the particles too. In this case, naturally, the refractive index of particles is greatly altered. By a considerable hydration, which occurs frequently in biocolloids, the differences between the refractive indices of solvent and solute are so small, that their turbidity is only insignificant.

The theory of Lecomte du Noüy (10) concerning the heat denaturation of proteins may be used to explain the prevention of turbidity on heating. According to him, in serum the heating causes no aggregation, but increases the volume of single particles only. The protein molecules are surrounded by a field of forces which prevent the water molecules to enter into the molecule. On heating, the water molecules acquire a sufficient kinetic energy to overcome these forces. They enter the protein molecule

and stretch the polypeptide chains, increasing the volume of the whole particle. Therefore the turbidity on heating dilute protein solution is caused by the increase in volume of single particles and not by their aggregation.

It may be supposed, that the bile salts form a thin hydrophilic coat (the polar groups) and a much thicker hydrophobic one (the sterol skeletons) round the particles. The latter tends to prevent, or prevent completely the water molecules to enter. Thus the bile salt layer prevents the entry of water molecules, but does not hamper the chemical processes that may occur in the polypeptide chains. In spite of the fact that the heated solution remains clear, the protein is denatured. Precipitated from the bile salt solution with acetic acid, it does not dissolve again.

This effect is not limited to the bile salts alone. Lecithin can prevent also the turbidity of heated egg albumin solutions (unpublished result) and *P. D. Boyer, F. G. Lum, G. A. Ballou, J. M. Luck* and *R. G. Rice* (11) have shown that fatty acids have the same effect on serum proteins.

Recent investigations have shown that detergents form complexes of a definite structure with proteins. *H. B. Bull* (12) has demonstrated that one molecule of egg albumin can bind either 32 or 17 molecules of sodium lauryl sulphate. This figure corresponds exactly to the basic groups of egg albumin or to their half. *V. Desreux* and *C. Fabry* (13) found also that the complex formed between egg albumin and Aerosol *C* 6 contained 34 molecules of detergent to one molecule of egg albumin.

The fibrinogen-bile salt complex does not give any turbidity on heating. The decrease of turbidity is therefore proportional to the formation of the complex. At low bile salt concentrations all the bile salt molecules are bound to the protein. Therefore the turbidity decreases linear with the increase of bile salt concentration. It is possible to calculate the number of the bile salt molecules bound to the fibrinogen. 116 molecules of bile salt were found for 10^5 gramm fibrinogen. This figure is very close to 130.5, the number of basic groups of cattle fibrin calculated from the analytical data of *D. D. Van Slyke* (14).

The complexity of factors mentioned above, renders an exact analysis of the turbidity curves of fibrin clots impossible (fig. 4). It is apparent that the processes which cause the turbidity in clotting are quite different from those which occur in the turbidity by heating. The process of clotting brings about the aggregation of fibrinogen molecules in small bundles, but heat increases the volume of single particles. The processes involved are different and it is expected that the action of bile salts would be different too.

The bile salts have great influence on processes which depend on the hydration of particles but they do not affect the colloidal processes caused by the changes in the electrical charge of the particles. The isoelectric precipitation and the salting out are caused by the diminution of the electrokinetic potential. It seems that the bile salts cannot prevent the aggregation of particles when their charge falls below a certain limit.

Fibrin is dissolved by hydrotropics such as urea, guanidine, but the bile salts do not dissolve it. Perhaps in the dissolving action of urea the hydrogen bonds are broken. The bile salts have not this property and it may be said therefore, that they cannot dissolve fibrin and denaturated fibrinogen due to that reason.

The bile salts have a denaturing action on fibrinogen. But, the action in comparison with the findings in the case of egg albumin, hemoglobin etc. is very small. The process is much slower and occurs at much higher concentrations. Tobacco mosaic virus protein is also very slowly denatured by sodium duodecyl sulphate, a synthetic detergent (15) and the photosensitive pigment of eyes is reported also not denatured by extraction with bile salts (16). Perhaps, this is a general behaviour of fibrilar proteins, in opposition with the rapid denaturation of globular proteins by bile salts and detergents. The fibrinogen is meanwhile resistant to the denaturing action of urea and other hydrotropics too.

In conclusion I like to say that regarding pathological cases, the concentration of bile salts in blood is much smaller to cause any change in the clotting time. Rosenthal and Wislicky (17) found that in obstructive jaundice, the bile salt concentration of serum is 5—12 mg %. This value is about ten times lower, than the minimal bile salt concentration which causes a noticeable prolongation of the clotting time.

SUMMARY.

The bile salts have a marked clot-inhibiting property. The different bile salts have approximately the same effect.

They prevent the heat coagulation of fibrinogen solutions.

The bile salt concentration, which prevents clotting and heat coagulation, rises parallel with the fibrinogen concentration. This suggests that a sort of compound is formed between the bile salt and fibrinogen. The number of bile salt molecules bound is equal to the basic groups of fibrinogen.

The bile salts neither affect the isoelectric precipitation nor the salting out of fibrinogen.

They are not able to dissolve fibrin or denaturated fibrinogen.

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A STUDY ON THE SOLUBILITY OF FIBRIN CLOTS IN UREA.

WITH 1 FIGURE IN TEXT.

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According to *I. Meissner* and *E. Wöhlisch* (1) fibrin is insoluble in urea, while communications in the former literatures testify for the solubility of fibrin. *Ph. Limbourg* (2) has found that urea dissolves fibrin, though he attributed the same solvent effect to the neutral salt solutions, what is quite inadmissible. Since his experiments lasted sometimes for many days, it could not be stated, whether fibrinolysin, or the activity of decomposing bacteria etc. had no part in this procedure. *K. Spiro* (3) also emphasized the solubility of fibrin in urea, but conditions are only slightly mentioned in his descriptions, without giving any details.

The problem of the solubility of fibrin turned up in the course of our laboratory work, where fibrin solution was required. It has been found that the pure fibrinogen clotted by thrombin can be easily dissolved in 30% urea. The same phenomenon can be observed, when oxalated beef plasma is clotted by thrombin. However the fibrin clot obtained by recalcifying the oxalated plasma, is no more soluble in urea.

It seemed worth while to investigate closer the solubility of differently prepared fibrin clots. It was of a great help that crystallized fibrinogen and a thrombin of high purity could be used for these experiments. So it was possible to approach the problem in isolated systems.

PROPERTIES OF FIBRIN DISSOLVED IN UREA.

The pure fibrinogen clotted by thrombin can easily be dissolved in 30% urea. Neither the fibrinogen, nor the fibrin in urea solution show a double refraction of flow, unless in the case of fibrin, if registered in a very early state of the dissolution, when the double refraction of flow is due to the presence of some not yet depolymerized particles. — Dialysing out the urea from a fibrin solution, a firm fibrin clot is found again.

The solubility of the fibrin in urea allowed to compare the viscosity of such a fibrin solution with that of a fibrinogen solution. These viscosi-

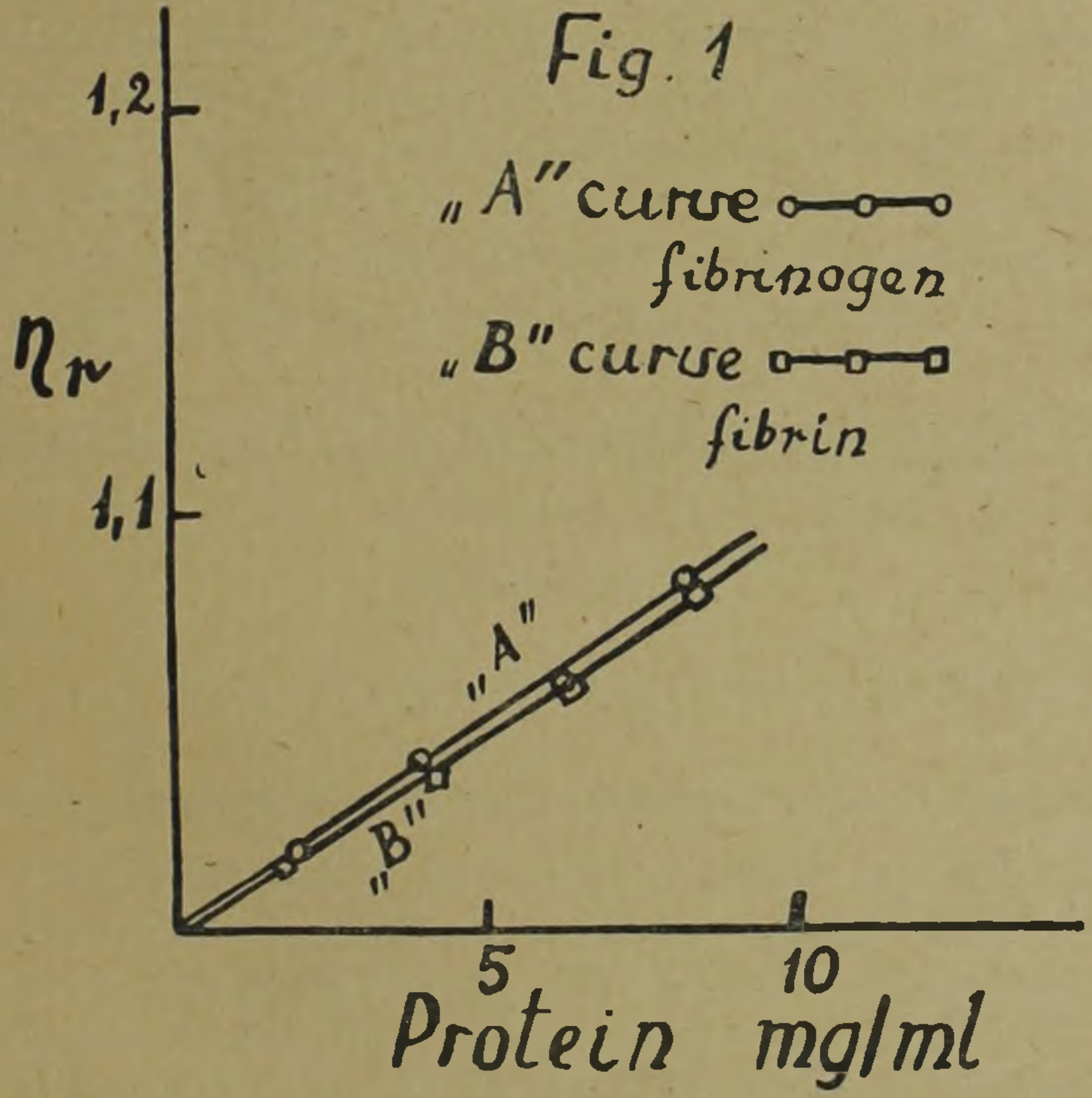
metric experiments show, that the viscosity of fibrin dissolved in urea is the same as that of the fibrinogen solution from which it has been prepared. A quite different observation was made by *L. Nanninga* (4), who found that the fibrin dissolved in a borate-*NaOH* buffer above *pH* 10, has a very high viscosity in comparison with the fibrinogen solution. It may be assumed that by the dissolution of urea, fibrin is splitted into the same particles as those which are present in the fibrinogen. But the alkali brings about the swelling of fibrin and the dissolution of the long fibrin threads without breaking them down into smaller particles.

The fig. (1) shows the results of an experiment of viscosity. It can be seen that the same concentrations of fibrinogen and fibrin solutions have the same relative viscosity. In the fig. 1 relative viscosities of fibrinogen (*A* curve) and fibrin (*B* curve) are plotted against their concentrations.

EXPERIMENTAL.

The experiment was carried out at room temperature of 20 °C, the viscosities were measured on 0 °C in Ostwald's viscosimeter.

Fig. 1



1. Fibrin solution: 3 ml fibrinogen (6,25 mg/ml of 1% *NaCl* solution, prepared after *D. Bagdy* and *E. Mihályi*), 1 ml M/5 aa phosphat buffer, 1 ml thrombin solution in dist. water. Clotting time 20 sec. Half an hour later 5 ml of 60% urea solution was added to the clot and after one hour standing, the viscosity was measured.

2. Control of arrangement 1.: 3 ml 1% *NaCl*, 1 ml buffer, 1 ml thrombin, 5 ml 60% urea. — Different dilutions of the fibrin solution were made with that.

3. Fibrinogen solution: 3 ml fibrinogen, 1 ml buffer, 1 ml dist. water. Half an hour later 5 ml 60% urea solution was added to the mixture and after one hour standing the viscosity was measured.

4. Control of arrangement 3.: 3 ml 1% *NaCl*, 1 ml buffer, 1 ml dist. water, 5 ml 60% urea solution. — Different dilutions of the fibrinogen solution were made with that.

THE ROLE OF CA-IONS AND OF A THERMOLABILE SERUM-FACTOR IN THE FORMATION OF FIBRIN CLOTS.

Working in isolated clotting systems with pure fibrinogen (5) and thrombin (6), an explanation was found, why certain clots are soluble in urea, while fibrins prepared in a different manner remain insoluble. It was shown, that only the fibrin clots formed in the presence of Ca-ions and serum, are insoluble in urea. To obtain a clot, resistent to the solvent effect of urea, none of these two factors can be disregarded, separately one of them is not sufficient. It has been found further, that a serum, inactivated by heating, could not be used as an effective serum in this respect.

These relationships are summarized in the following experimental part.

EXPERIMENTAL.

The experiments were carried out at the same time at room temp. of 24 °C.

1. *A clotting system containing Ca-ions and the serum-factor.*

2 ml fibrinogen solution (10 mg/ml) in the experiments always buffered with M/10 veronal-acetat, 2 ml serum (prepared in the following way: a beef plasma, containing 0,2% *Na*-oxalate is recalcified with aa volume of M/40 $CaCl_2$, clot removed, and after filtering serum is obtained), 1 ml M/40 $CaCl_2$, 2 ml thrombin solution. Clotting followed in 18 sec.

One hour after clotting 7 ml of 60% urea solution was added to the clot, but it remained insoluble for more than 24 hours.

2. *A clotting system containing only Ca-ions without the serumfactor.*

2 ml fibrinogen, 2 ml of a filtered solution of the aa volume mixture of phys. *NaCl* containing 0,2% *Na*-oxalate and M/40 $CaCl_2$, 1 ml M/40 $CaCl_2$, 2 ml thrombin. Clotting time 20 sec. One hour later 7 ml of 60% urea solution was given to the clot, which has been dissolved very easily in 20 min.

3. *A clotting system containing neither Ca-ions nor the serumfactor.*

2 ml fibrinogen, 2 ml phys. *NaCl*, instead of serum, 1 ml phys. *NaCl*, instead of *Ca*-ions, 2 ml thrombin. Clotting time 20 sec. One hour after clotting 7 ml of 60% urea was added to the clot, an easy dissolution followed in 20 min.

4. *A clotting system containing oxalated serum, no Ca-ions.*

2 ml fibrinogen, 2 ml serum containing 0,2% *Na*-oxalate, 1 ml 0,2% *Na*-oxalate, 2 ml thrombin. Clotting time: 24 sec. One hour later 7 ml of urea was added, the clot was dissolved in 20 min.

5. *A clotting system containing Ca-ions and serum inactivated by heating.*

2 ml fibrinogen, 2 ml serum inactivated by heating on 65 °C for 10 min., 1 ml M/40 $CaCl_2$, 2 ml thrombin. Clotting time: 22 sec. One hour after clotting 7 ml of 60% urea was added to the clot, and in 30 min. a homogenous solution was obtained.

The results of the experiments described above in detail, are summarized schematically in the table 1.

TABLE I.

1.	2.	3.	4.	5.
Fibrinogen Serum Ca-ions Thrombin	fibrinogen — Ca-ions thrombin	fibrinogen — — thrombin	fibrinogen oxalated serum — thrombin	fibrinogen serum inactivated by heating Ca-ions thrombin
insoluble	soluble	soluble	soluble	soluble in urea

The results seem to be in congruence with the findings of *K. C. Robbins* (7), who investigated the solubility of fibrins in dilute acids and alkalis. From the solubility-studies of fibrin in urea described above, we also share the view that *Ca*-ions and a thermolabile serum-factor are taking part in the formation of the normal fibrin clot. So the role

of *Ca* and of a new serum component became evident in the transition of fibrinogen into fibrin at the normal blood clotting. So far — except the thermolability — no details could be detected on the nature of this serum-factor, but we presume it might be a protein or several proteins of the serum, which are essential partners of fibrinogen in the formation of a normal fibrin clot. If this serum-factor is not present, the obtained fibrin clot is only by out-look similar to a normal one. But it differs from the normal beeing not resistent, for example to the solvent effect of urea.

SUMMARY.

1. A physiologically formed normal fibrin clot can not be dissolved in 30% urea. The same phenomenon may be observed, when a clotting system containing fibrinogen, *Ca*-ions, serum and thrombin is composed.

2. A clot obtained by the effect of thrombin on fibrinogen can easily be dissolved in 30% urea.

3. To have a clot resistent to the solvent effect of urea, both *Ca*-ions and serum are necessary, none of them can be disregarded. A serum inactivated by heating looses his activity in this respect.

4. Neither the fibrinogen nor the fibrin dissolved in urea show a double refraction of flow.

5. The viscosity of fibrin dissolved in urea is the same as that of the fibrinogen solution from which it has been prepared.

6. Dialysing out the urea from a fibrin solution a firm fibrin clot is found again.

I am deeply grateful to prof. *K. Laki* for his continuous help and advices in carrying out this work.

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THE REDUCING PROPERTIES OF FIBRINOGEN. THE ROLE OF SH GROUPS IN THE CLOTTING OF FIBRINOGEN.

WITH 5 FIGURES IN TEXT.

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Some authors have suggested, that the conversion of fibrinogen into fibrin gel is the result of oxydation of *SH* groups, which unite to form -*S*-*S*- bridges between adjacent molecules. *R. N. Lyons* (1), (2) supported this view, demonstrating by polarographic determinations, that the clotting by thrombin occurs in two steps. The first step is characterised by liberation of *SH* groups, the second one by the disappearance of them. He also has come to the conclusion on the role of *SH* groups from the inhibition of clotting by an excess of thiol compounds and by substances which react with thiol groups: mercury, arsenic compounds and cyanides. But this very nice theory became doubtful when *L. B. Jaques* (3) demonstrated that pure fibrinogen preparations did not give the nitroprussid test. *E. Chargaff* et al. (4) showed also that fibrinogen reduced a very small amount of porphyrindin. The amount of *SH* groups in fibrinogen is therefore very small, if they are at all.

For the binding of fibrinogen molecules, there are necessary only two *SH* groups for each molecule. Thus there is a possibility of a continuous polymerisation and the fibrinogen molecules can bind together to form long threads. In a protein particle with a molecular weight near 500.000 two *SH* groups means only 0,004 millimols per gm, which is a quantity almost impossible to determine chemically. For this reason we cannot exclude the possibility of a role of *SH* groups in blood clotting, even when they are not detectable chemically in fibrinogen.

These contradictory findings led us to investigate the problem of the *SH* groups in fibrinogen.

The problem of the protein *SH* groups by no means is definitely solved. Due to the merit of *M. L. Anson* especially, the elucidation of the problem of *SH* groups has been obtained to a great extent. He has done careful investigations about the *SH* groups of egg albumin and has described a great number of methods to determine them quantitatively. It was

apparent that this difficult problem can be solved only by using many methods, if it is possible of different chemical nature (oxidations, heavy metal compound binding, organic halogen compound binding etc.), and one can decide only when all these methods show concordance.

The difficulty was recognised early, that the findings of one protein cannot be transferred to other proteins generally. For example the *SH* groups of egg albumin are quantitatively oxidized by Iodine at 0^0 in presence of 1 *M KI*. In these conditions the *SH* groups of the tobacco mosaic virus protein are not oxidized at all (5).

Some reagents react with the *SH* groups of native proteins, the others react only with the denatured ones. But the mode of denaturation is also important. The egg albumin denatured by heat shows different properties in this respect to that denatured in presence of urea or guanidine. The reduction of ferricyanide is less when the protein is coagulated by heat or trichloracetic acid, as if it is denatured by heat in presence of urea or detergents, which keep the denatured protein in solution.

It is clear from the above statements, that to solve the problem of *SH* groups in fibrinogen, as many as possible methods should be employed on native and differently denatured fibrinogen.

We have performed clotting experiments (in order) to exclude the possibilities mentioned above, whether SH groups are involved in the clotting process, even in that case their amount being so small they cannot be determined surely. The fibrinogen was treated with different reagents, which in the experiments with other proteins destroyed all the *SH* groups. With thrombin, the clotting property of such preparations was checked. If the thrombin brings about clotting by a preparation where it is sure that the *SH* groups present have vanished, we can state that the *SH* groups are not involved in the clotting process.

EXPERIMENTAL.

The experiments were performed with a fibrinogen preparation of about 100% purity. The fibrinogen precipitated with ammonium sulphate was treated with an alkaline urea solution and after this was reprecipitated several times with ammonium sulphate. The details of the methods will appear shortly in this review. The preparation is converted quantitatively to fibrin by the action of thrombin. In the liquid expressed from the clot one cannot detect any nitrogen.

The thrombin preparation used was prepared after *K. Laki* and *L. Lóránd* (6).

A) *Nitroprusside test.*

The nitroprusside test was carried out after the descriptions *M. L. Anson* (7). To 0,5 ml of 1 or 5% fibrinogen solutions were added 2 drops of m/10 *KCN*, 2 drops of m/1 phosphate buffer of *pH* 6,8 and 0,7 gm of urea. The test tube was placed for 3 minutes in a water bath of 37 C⁰ and afterwards cooled in ice water. 1 drop of freshly prepared 5% sodium nitroprusside and 1 drop of 27% ammonium hydroxide were added. We have made also several experiments by placing the tubes in boiling water bath for 3 minutes, instead of at 37 C⁰. The test was always negative.

With impure preparations we have got positive reaction, but the intensity was always parallel to the degree of impurity and we have never seen positive reaction by pure preparations.

B) *Reactions with iodine.*

a) *Oxidation with iodine at pH* 3,2. *M. L. Anson* (8) has demonstrated that the *SH* groups of native egg albumin can be oxidized by iodine selectively at *pH* 3,2. The amount of consumed iodine corresponds exactly to the *SH* content of egg albumin. We have performed the reaction by mixing 5 ml of 2% fibrinogen solution with 35 ml of distilled water and adjusted the *pH* with *N*/1 *HCl*. The *pH* was determined with the glass electrode. By acidifying the fibrinogen precipitates first, than redissolves and gives a water-clear solution. To the acidifyed solution was added 5 ml of *N*/100 iodine and after keeping at room temperature for an hour the excess iodine was titrated with *N*/100 Sodium Thiosulphate. At the same time a blank test was done, which was acidifyed also to *pH* 3,2 and after keeping an hour was titrated. The iodine consumption was calculated from the difference of the two titrations. It was found that 100 mg fibrinogen consumed 0,11 ml of *N*/100 iodine, i. e. 0,011 millimols per gm. This figure is much less than that calculated from Jacques curve.

b) *Oxidation with iodine at* 0 *C⁰ in the presence of M*/1 *KI*. The *SH* groups of egg albumin can be titrated also according to *M. L. Anson* (9) at neutral *pH* at 0 C⁰ in the presence of *M*/1 *KI*. We have investigated the iodine consumption of fibrinogen in presence of *KI* at 0⁰ C.

The reaction mixture was as follows:

2 ml fibrinogen solution of (10 mg per ml)
1 ml *M*/1 Borate buffer of (*pH* 8,6)
0,5—5,0 ml *KI* 2 *M*
5,0—0,0 ml distilled water.

The mixture was placed in ice water, and after 10 minutes 2 ml of *M*/200 iodine was added. After 30 minutes the excess iodine was titrated

with *M*/200 Thiosulphate. It was found that the iodine consumption rises quickly on lowering the *KI* concentration. Beyond 0,6 *M KI* there is no iodine consumption at all. The urea increases greatly the iodine consumption. By adding to the reaction mixture 3 gm urea, the iodine consumption continued as far as 1,0 *KI* concentration (Fig. 1). The course of reaction in the presence of 3 gm urea and at different *KI* concentrations is show in Fig. 2. It can be seen that in presence of 1,0 *M KI* there is no iodine consumption even after three hours. The reaction has a great temperature coefficient. In presence of *M*/1 *KI* and 3 gm urea there is no iodine consumption at 0 C⁰, but at 37 C⁰ the consumption is very important (Fig. 3). The effect of *pH* is shown in Fig. 4. Borate buffers of different *pH* were used at 0 C⁰ in presence of 0,2 ml *KI* and 3 gm of urea. The consumption was noted after a period of a half an hour. Below *pH* 7,5 there is no iodine consumption at all and from this point it rises quickly.

Similar results were found by *Jaques* (3). He worked at room temperature without any *KI* addition and found that the iodine consumption begins at *pH* 2,3. The addition of *KI* and the lowering of temperature caused very marked shift on our curve towards the alkaline region. He also found that 0,3 *M KI* is sufficient to inhibit completely the reduction of Iodine at *pH* 6,6. The increase of *pH* caused also a shift to the inhibiting *KI* concentration to 0,6 without urea and to 1,0 *M* with urea. From his experiments he concluded that the reductive groups of fibrinogen cannot be *SH* groups and he supported the view that Tyrosine and Tryptophan groups are oxidased.

M. L. Anson (10), *D. E. Bowman* (11) etc. demonstrated that the oxidation of tyrosine is exactly in the same way influenced by the *KI* concentration and the *pH* as it was found in Jacques and our own experiences are also in the same line. Bowman also demonstrated that the oxydation of tyrosine has a very great temperature coefficient of about 10 for a change of temperature of 10 C.⁰ This was found by us too in the reaction in presence of 1,0 *M KI*.

It can be assumed that iodine acts on the tyrosine groups and in a paper which is in preparation, we will deal in more details with this reaction. The *KI* in *M*/1 concentration does not influence the oxidation of cysteine even at *pH* 6,8. At this *KI* concentration we have not found any reduction of iodine even at *pH* 8,6. But from these results we cannot conclude definitely on the absence of *SH* groups. *W. M. Stanley* and *M. L. Anson* (5) have shown by using different methods that all the sulfur of the Tobacco mosaic virus protein is present in the form of *SH* groups. In spite of this fact the tobacco mosaic virus protein does not reduce any iodine in the described conditions.

Fig. 1

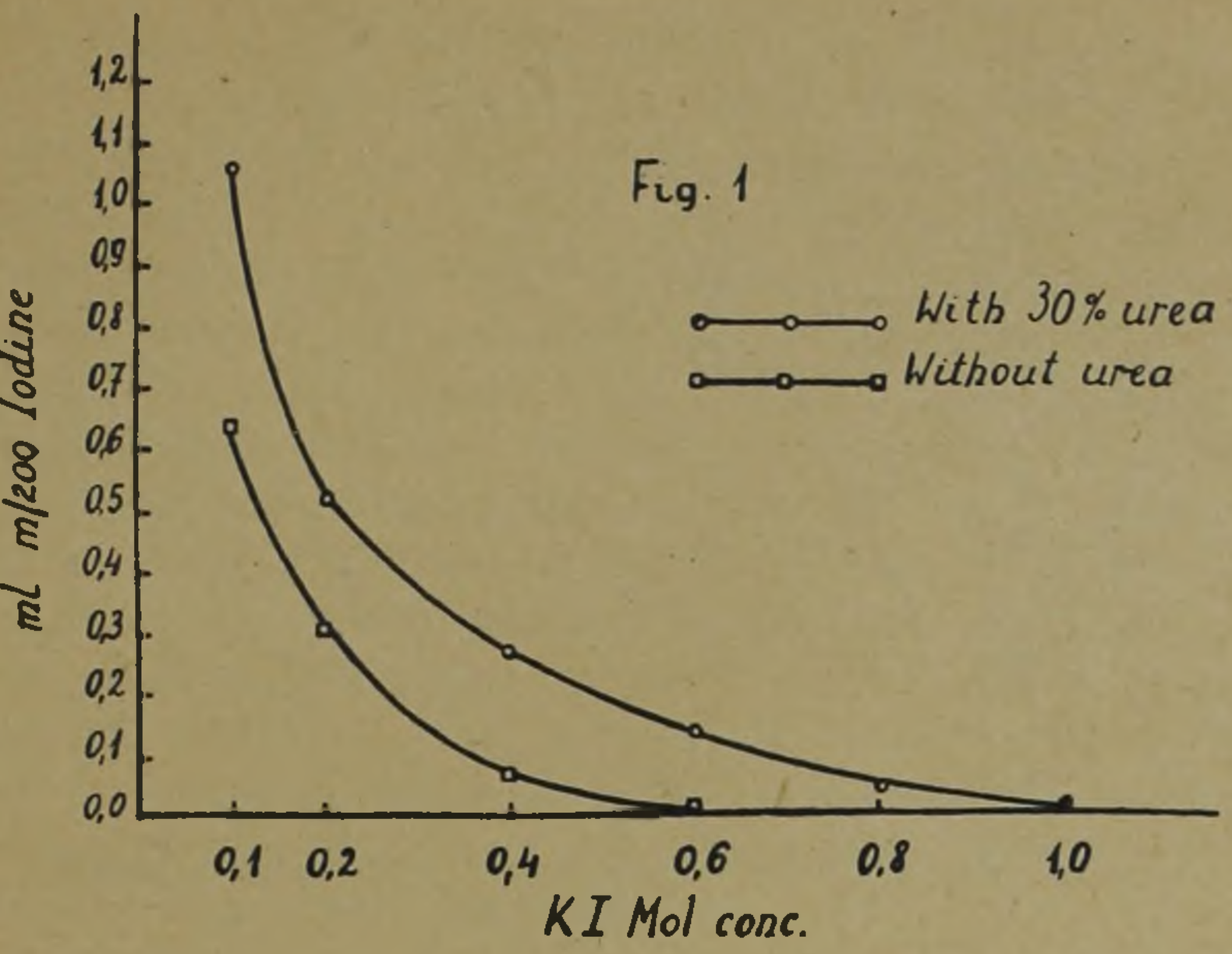

With 30% urea
Without urea
ml m/200 Iodine
1,2
1,1
1,0
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0,0
0,1
0,2
0,4
0,6
0,8
1,0
KI Mol conc.

Fig. 2

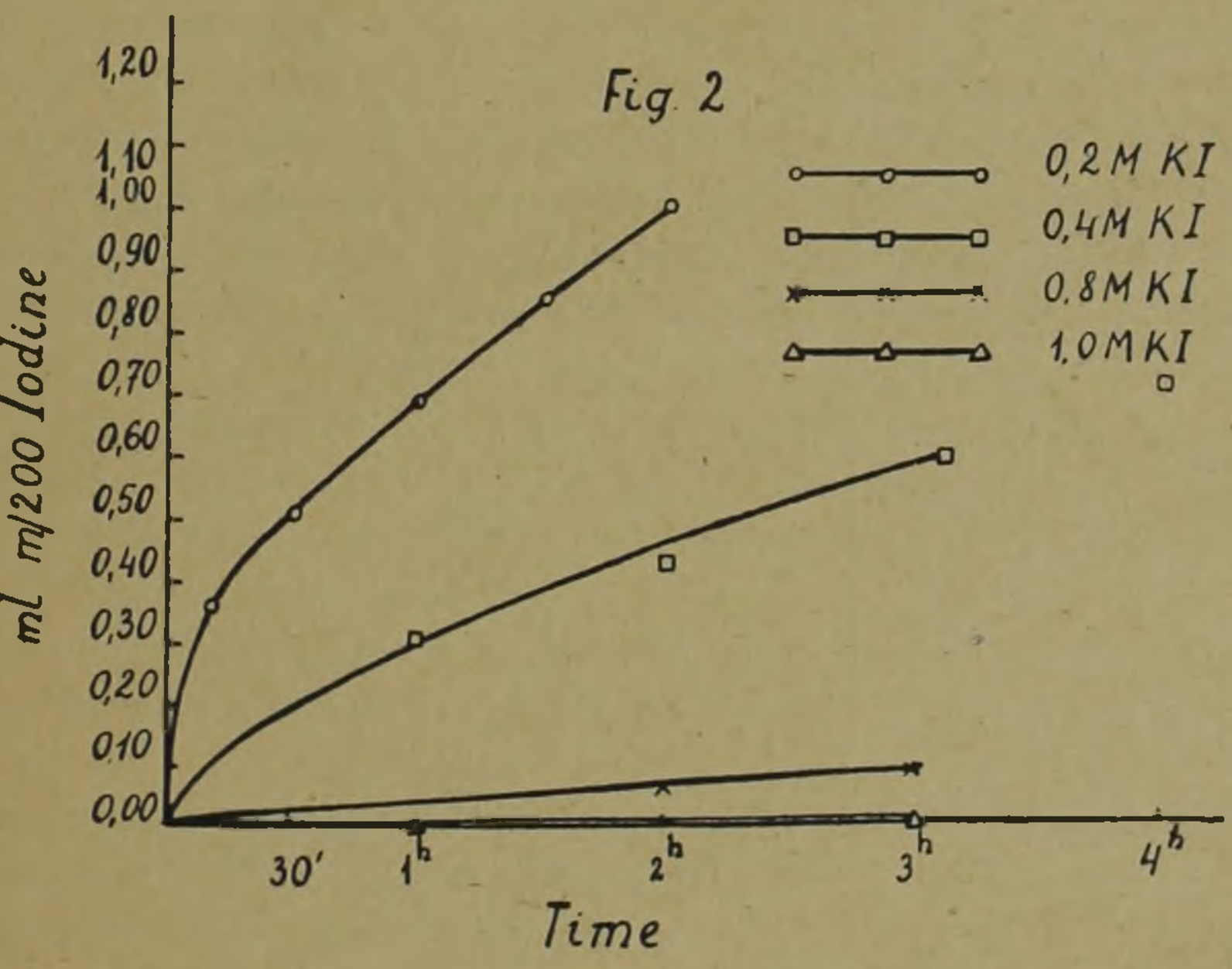

0,2 M KI
0,4M KI
0,8M KI
1,0M KI
ml m/200 Iodine
1,20
1,10
1,00
0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
30'
1h
2h
3h
4h
Time

Fig. 3

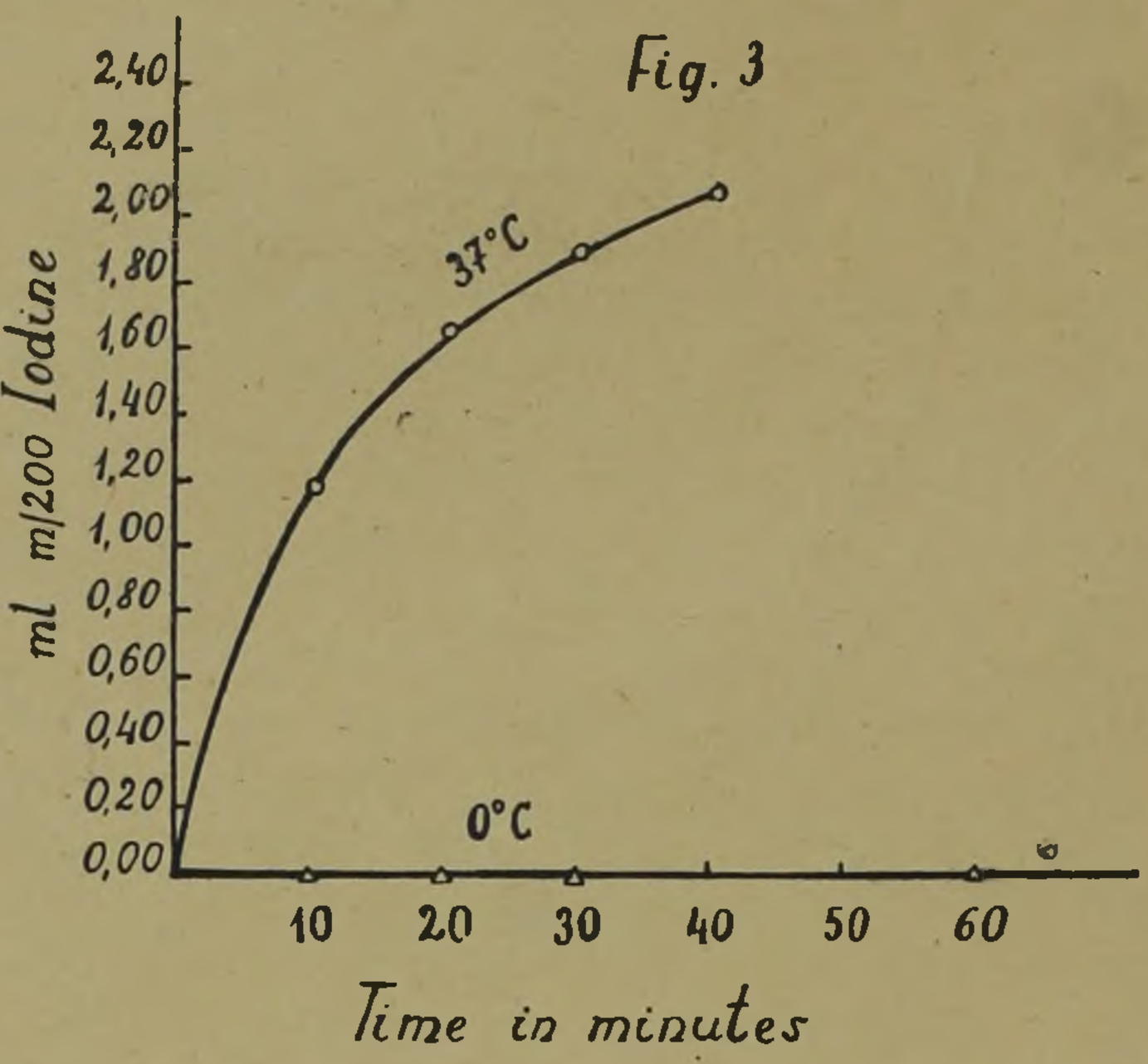
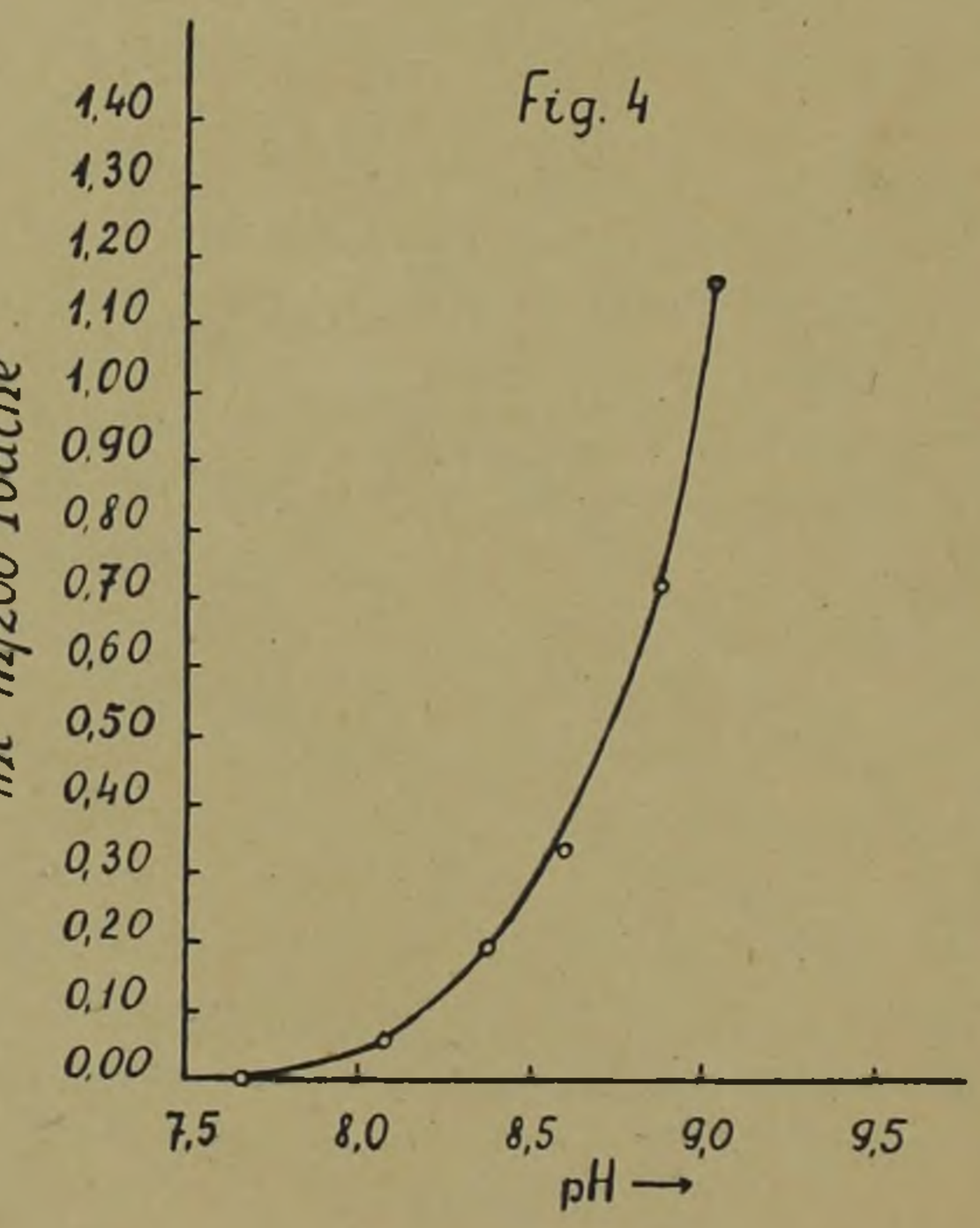

ml m/200 Iodine
2,40
2,20
2,00
1,80
1,60
1,40
1,20
1,00
0,80
0,60
0,40
0,20
0,00
37°C
0°C
10
20
30
40
50
60
Time in minutes

Fig. 4


ml m/200 Iodine
1,40
1,30
1,20
1,10
1,00
0,90
0,80
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
7,5
8,0
8,5
9,0
9,5
pH ⟶

The fibrin can be dissolved in urea solutions. The above experiments were performed in presence of urea to compare the reactions of dissolved fibrin with that of fibrinogen. 2 ml fibrinogen were clotted with 0,1 ml of thrombin. After an hour and a half 3 gm urea were added. The resulting solution was treated with iodine in the way described above for fibrinogen. No difference was found in the behaviour of fibrinogen and fibrin.

According to *M. L. Anson* (12) the *SH* groups of egg albumin combine with formaldehyde in alkaline solution and the protein treated in such a way does not reduce ferricyanide and does not give the nitroprusside test. 15 ml 2% fibrinogen, 7,5 ml *N*/10 *NaOH* and 4,5 ml 40% formaldehyde solution were mixed. After keeping 10 minutes at room temperature it was put in a cellophan bag and dialysed for two days. After dialysis it did not give the phenylhydrazine ferricyanide reaction of formaldehyde. The iodine consumption of this preparation at different *KI* concentrations and *pH* was exactly the same as that of an untreated fibrinogen.

C) *Reduction of ferricyanide.*

In this part of investigations the methods given by *M. L. Anson* (12, 7) were followed also.

a) *Reduction of ferricyanide by heat denaturated fibrinogen.* To 4 ml of 1% fibrinogen added 1 ml of *M*/1 *HCl* and the tube place in a water bath at 50 C⁰ for 10 minutes. After cooling 1 ml of *N*/1 *NaOH* and 2 ml of distilled water were added. If a 2% solution is used it becames gelatinous during heating. The 1% solution remained liquid and is slightly opalescent. In the neutral range of *pH* the denaturated fibrinogen is precipitated. It stays in solution only below *pH* 4 and beyond *pH* 10. The solution adjusted at *pH* 6 is very turbid, contains a very fine precipitate. To 2 ml of denaturated fibrinogen solution were added 0,1 ml of *M*/1 phosphate buffer of *pH* 6,8 and 1 ml of either *M*/10 or *M*/100 ferricyanide. The test tubes were placed for different times in a water bath of either 37 C⁰ or 100 C⁰. After the operation they were cooled and 2 ml of 20% trichloracetic acid added. The precipitation of the protein was complete in a few minutes and after the addition of 4,4 ml of water the precipitate was removed by centrifugation. The protein precipitation is intensified with the addition of the buffer and the tubes placed in the boiling water bath the denatured fibrinogen form a firm coagulum.

To avoid precipitation of denaturated protein, the reduction of ferricyanide by denatured fibrinogen in presence of urea was investigated also.

b) *Reduction of ferricyanide in urea solution.* To 0,5 ml of 2% fibrinogen solution 0,6 gm urea and 2 drops of *N*/1 *HCl* were added. After keeping the test tube at 37 C⁰ for 5 minutes 4 drops of *M*/1 6,8 phosphate

buffer, 2 drops of *N*/1 *NaOH*, 0,8 gm of urea and 1 ml of *M*/10 or *M*/100 ferricyanide solution were added. The tubes were placed for different times in a water bath of 37° or 100°. After the reaction time was over 2 ml of 20% trichloracetic acid were added and on complete precipitation, the volume was made up with water to 9,5 ml. The precipitate was centrifuged out.

The ferrocyanide formed was estimated in both cases by the colorimetric method. After addition of ferric sulphate reagent of O. *Folin* and *H. Malmros* (13), the blue color was determined with a Stufenphotometer using a 5 mm cuvette and *S* 75 filter. The standard extinction curve was taken up in the following way: to 1 ml of ferricyanide were added different amounts of a *M*/1000 ferrocyanide solution and 2 ml of trichloracetic acid. The volume was completed to 9,5 ml with water and 0,5 ml of ferric sulphate solution was added. The extinction of the solutions was noted after 5 minutes. The extinction rises lineally with the concentration of ferrocyanide.

The results are summarised in the table I. At 100° are some formation

TABLE I.

Amount of fibrinogen *mg*	How denatured	Ferricyanide	Reaction time min.	Temperature °C	*pH*	Ferrocyanide formed *mM.* per *gm.* fbg.
10	*HCl* + heat	m/100	30	37	6,8	0
10	*HCl* + heat	m/10	30	37	6,8	0
10	*HCl* + heat	m/100	10	100	6,8	0
15	urea	m/100	20	37	6,8	0
30	,,	m/100	20	37	6,8	0
15	,,	m/10	20	37	6,8	0,0016
30	,,	m/10	20	37	6,8	0,0013
10	,,	m/100	10	100	6,8	0
10	,,	m/10	2	100	6,8	0,040
10	,,	m/10	5	100	6,8	0,106
10	,,	m/10	10	100	6,8	0,118
0,001 *mM* (1,18 *mg*) tyrosin	—	Ferricyanide	Reaction time min.	Temp. °C	*pH*	*mM.* per 1,18 *mg* tyrosin
11	—	m/100	30′	37	6,8	0
11	—	m/10	30′	37	6,8	0
11	—	m/100	10′	100	6,8	0,00013
11	—	m/10	10′	100	6,8	0,00073

of ferrocyanide in a blank tube too. Therefore it was made in this case a control without any fibrinogen and its extinction substracted from that of the experiment.

It can be seen that neither the heat denaturated, nor the fibrinogen denatured with urea reduce any ferricyanide at 37 C⁰. The reduction of ferricyanide by the urea denatured fibrinogen only occurs at 100 C⁰ and in presence of *N*/10 ferricyanide. In this case the amount of ferricyanide reduced increases with the time. The non-reductivity of heat denaturated fibrinogen may be due to its precipitation. Similar thing was observed by *M. L. Anson* (14) and *A. E. Mirsky* (15) with egg albumin. The heat denatured egg albumin has reduced much less ferricyanide than that denatured by urea.

From the above data it is clear that the fibrinogen do not contain any *SH* groups. The *SH* groups are readily oxidized by ferricyanide at 37⁰ but it is found that fibrinogen reduces ferricyanide only at 100⁰ and with high ferricyanide concentration. *A. E. Mirsky* and *M. L. Anson* (16) have shown that different proteins, which do not contain *SH* groups or their *SH* groups have been oxidized with cystine, are able to reduce ferricyanide at *pH* 9,6. They have compared the reducing properties of proteins with that of tyrosine and tryptophan and have concluded that these groups are responsible for the non *SH* reducing power of proteins. We have also compared the oxidation of tyrosine with ferricyanide under the same condition as used by fibrinogen. 1 ml of *M*/100 or *M*/1000 tyrosine was mixed with 0,2 ml of 6,8 phosphate buffer and 1 ml of ferricyanide. The tube was placed in water bath of 37⁰ or 100⁰ and after different intervals of time cooled, 2 ml of 20% trichloracetic acid added and the volume completed to 9,5 ml with water. The color developed with 0,5 ml ferric sulphate reagent was noted. The findings as shown table I suggest that in this case also the tyrosine groups were oxidized by the ferricyanide. At 37 °C neither the *M*/100 nor *M*/10 ferricyanide oxidize tyrosine. The tyrosine is oxidized at this *pH* only at 100⁰. The oxidation is insignificant *M*/100 ferricyanide. It became greater only with *M*/10 ferricyanide. The formation of ferrocyanide, in spite of the fact that the amount of tyrosine present is about three times higher as that present in 10 mg of fibrinogen, is smaller than that by 10 mg fibrinogen. We must conclude that beside tyrosine, there are also other reducing groups in fibrinogen. The amount of ferricyanide reduced by tyrosine was the same in the presence and in the absence of 30% urea.

D) *Catalysis of the Iodine sodium azide reaction.*

According to *R. N. Lyons* (2) the reaction

$$2\,NaN_3 + I_2 = 2\,NaI + 3\,N_2$$

is catalysed specifically by the free *SH* groups. The rate of reaction was determined in the Warburg manometer. 0,12 *M* iodine and 0,12 *M* sodium

azide were used. The reaction mixture was the following: 1 ml sodium azide, 0,5 ml *M*/5 *pH* 7 phosphate buffer, 2 ml of solution to be investigated and 0,5 ml iodine in the side arm. For the control cysteine solution of different concentrations were taken and the rate of Nitrogen evolution estimated. It was found that there is indeed, a parallelity between the Nitrogen liberated and the cysteine concentration. The specificity of reaction was checked by investigating the effect of cysteine and cystine. It was found that they catalysed the reaction exactly in the same degree. The same was found for reduced and oxidized glutathione. It seems that the reaction is not specific for the *SH* groups, but the sulfur atom is involved, independent of the *SH* or -*S*-*S*-stage.

The method should be rejected, as a method specific for the *SH* groups and therefore the experiments carried out with fibrinogen and fibrin does not produce any interest as to our problem. We summarized them only to complete. 30% urea does not alter the rate of N_2 liberation, which gives a possibility to investigate both fibrinogen and fibrin dissolved in urea. As could be seen from Fig. 5 their activity is exactly the same.

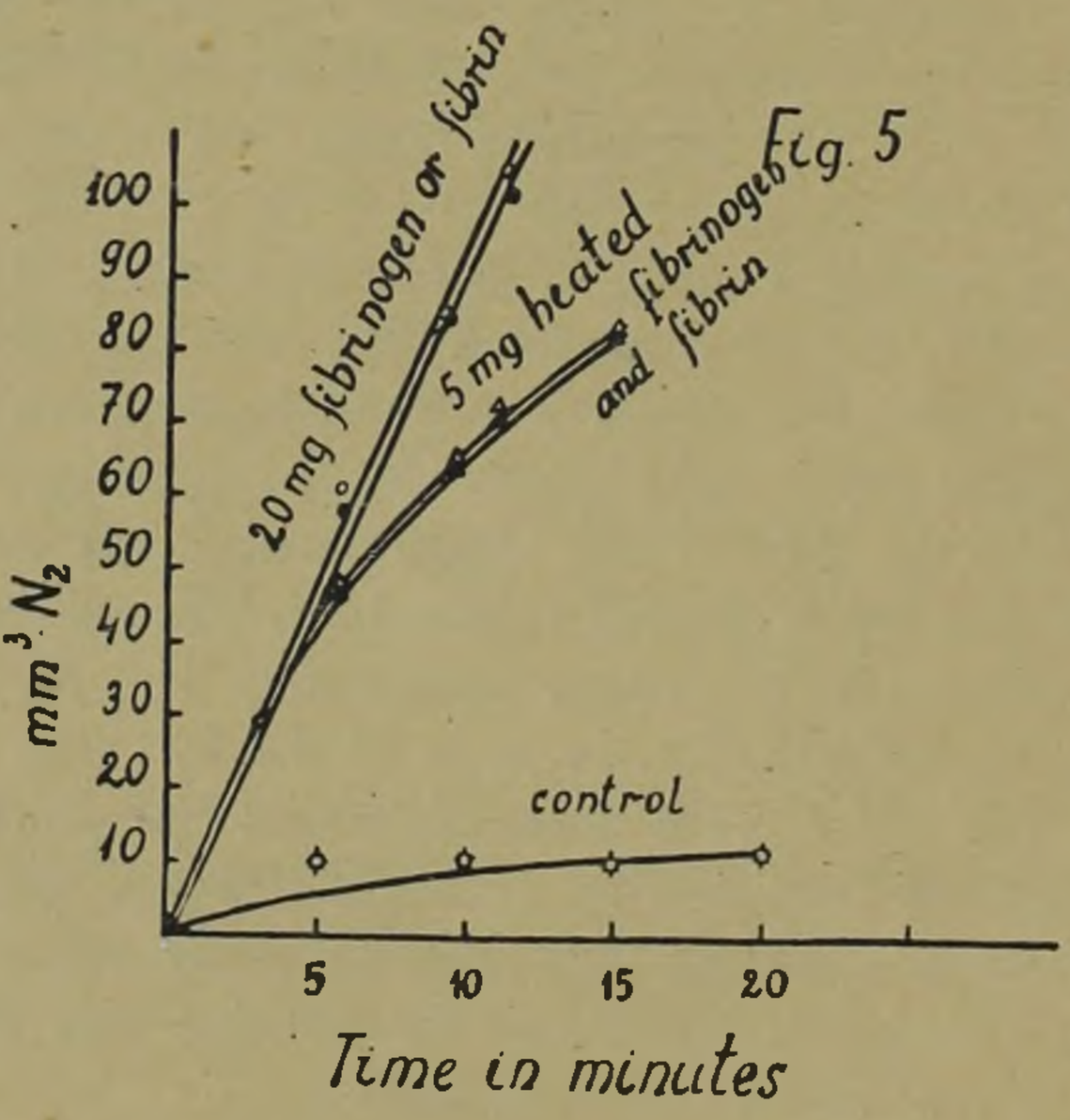


Fig. 5

It was found that 20 mg fibrinogen or fibrin catalyse the reaction to the same extent as do about 0,25 mg cysteine. This is in agreement with 1,2% half cystine content of fibrinogen. After boiling the fibrinogen in urea solution the activity is increased about 3—4 times. With 20 mg protein the N_2 development is so quick that it is scarcely measurable. In Fig 5.

is shown the effect of 5 mg boiled fibrinogen and fibrin. Both of them have the same activity, which approach that of 20 mg unboiled protein.

E) *Binding of Bromacetophenone.*

M. Dixon and *D. M. Needham* (17) have demonstrated that the *SH* groups of native protein react specifically with Chloracetophenone. *F. Guba* (18) has shown that bromacetophenone react quite in a similar manner. He elaborated a colorimetrical method for the estimation of the bromacetophenone binding. The method will appear in this journal and therefore we do not deal with its details. After the reaction the protein is precipitated with alcohol and centrifuged out. The excess of bromacetophenone is determined colorimetrically with meta-dinitrobenzene in alkaline solution.

The results are summarised in Table II. The binding capacity of native fibrinogen in *M*/10 veronal acetate buffer of *pH* 7 and that with heat denatured fibrinogen were investigated. The 1% fibrinogen solution

TABLE II.

Amount of fibrinogen *mg*	Native or denatured	Time min.	*pH*	Bromacetophenone added *mg*	Bromacetophenone found. *mg*	Bromacetophenone bound. *mg*
10	Native	10	7	0,2	0,2	0
10	,,	10	7	0,2	0,2	0
20	,,	10	7	0,2	0,195	0,005
0	,,	10	7	0,2	0,2	0
10	Heat denatured	10	7	0,2	0,2	0
20	,, ,,	10	7	0,2	0,2	0
20	,, ,,	10	7	0,2	0,2	0

in veronal buffer was placed in boiling water bath for 10 minutes. The protein was allowed to react with bromatecophenone for 10 minutes.

It is clear from the data that neither the native, nor the heat denatured fibrinogen bind any bromacetophenone.

F) *Clotting experiments.*

a) Clotting of fibrinogen treated with Iodine. L. B. Jaques (3) demonstrated that the fibrinogen treated with iodine at neutral *pH* is not clotted by thrombin. But the groups which are necessary for the action of thrombin are still present, while the Iodine treated fibrinogen is precipitated by thrombin. It may be said that the rigorous oxidation with Iodine changes only the colloidical properties fibrinogen in such a way that a gel could not be formed.

It was found by us that after a treatment of fibrinogen with Iodine at *pH* 8,6 in 0,2 *M KI* solution and at 0^0 the thrombin form a normal clot. 5 ml. of 1% fibrinogen, 1 ml *M*/1 Na_2HPO_4, 1 ml 2 *M KI* were mixed

and placed in ice water. After cooling 3 ml of *M*/200 Iodine were added and the mixture allowed to stay for 30 minutes. Then dialysed against distilled water. The dialysed preparation gave with thrombin a normal clot. Carrying out the reaction in presence of 3 gm Urea, still there is a normal clot. 10 ml of 1,2% fibrinogen was acidulated to *pH* 5 with *N*/10 *HCl*. 5 ml *N*/100 Iodine was added and the solution was allowed to stand at room temperature for 30 minutes. The excess Iodine was titrated with 10% sodium thiosulphate. The fibrinogen was precipitated by Iodine and the precipitate did not dissolve when Iodine was titrated, but they were readily dissolved on neutralising the solution. Adding phosphate buffer of *pH* 6,8 and thrombin a normal clot was obtained.

By the action of more concentrated solution of Iodine, there was no clotting, only a precipitate is formed by the action of thrombin and by more prolonged action of Iodine there was no change at all. We shall deal with these findings in another paper, which will appear shortly.

b) Clotting of fibrinogen treated with ferricyanide. 2,5 ml of 1% fibrinogen were mixed with 2,5 ml of *M*/5 and *M*/10 potassium ferricyanide. After an hour and a half the solution was dialysed for 2 days. The dialysed solution gave a normal clot with thrombin. The experiment was repeated in presence of 30% urea. Still both of the preparations clotted with thrombin, but the fibrinogen treated with *M*/5 ferricyanide gave a fragile clot. Perhaps some of the fibrinogen was denatured on the combined effect of such high ferricyanide and urea concentrations. The fibrinogen treated with *M*/10 ferricyanide gave a normal clot.

c) Clotting of fibrinogen treated with Chloracetophenone. The fibrinogen was treated with chloracetophenone in alkaline solution. 6 ml of 1% fibrinogen, 2 ml *M*/1 phosphate of *pH* 8 and 2 ml 0,2% Chloracetophenone in 96% alcohol were mixed. A control was prepared in the same way, adding instead of chloracetophenone solution, the same amount of 96% alcohol. The chloracetophenone did not alter the clotting of fibrinogen even after keeping for 3 days in a cold room. The experiment and the control gave always the same clotting time.

The same result was obtained, if the fibrinogen was incubated with chloracetophenone at 37 °C for 90 minutes.

Fibrinogen was incubated also with chloracetophenone in presence of 30% urea. After removal of urea by dialysis, the fibrinogen can be clotted by thrombin and the clotting time does not differ from that of a control, which is incubated with urea in the absence of chloracetophenone.

The effect of chloracetophenone on the formation of profibrin in acidic medium was investigated too. The fibrinogen was incubated

at room temperature with thrombin at *pH* 5 in presence of chloracetophenone. At this *pH* the addition of thrombin does not cause clotting, but after neutralisation the fibrinogen clots. (19, 20.) The clotting time is shorter when the incubation in acid medium was longer, and with an incubation of several hours the clotting occurs immediatly after neutralisation. It was found, that the chloracetophenone has no influence on this process. The clotting time after neutralisation is the same in presence or in absence of chloracetophenone.

L. Lóránd (21) found that the fibrin dissolved in urea clotted again on dialysing out the urea. The effect of above reagents on this process was investigated too. The fibrin dissolved in 30% urea was treated exactly in the manner described already; with Iodine, ferricyanide, and chloracetophenone. All these preparations, after the urea is removed by dialysis, give a normal clot.

From all these experiments it is clear that even in the case, when the number of the *SH* groups of fibrinogen is so small that it can not detected chemically, they have not any role in the process of clotting. The *SH* groups are very readily oxidized *(M. L. Anson)* before the other groups of the proteins. Iodine oxidizes *SH* groups of native as well as of denatured proteins. After Iodine treatment cannot persiste any unaffected *SH* group and the fibrinogen still clots. Ferricyanide does not oxidize the native proteins, but *R. N. Lyons* (2) has claimed that the *SH* groups of fibrinogen are liberated in the first step of clotting by thrombin and they are available for the oxidizing agents. But the clotting occurs even in the presence of *M*/10 ferricyanide and it is difficult to conceive that the liberated *SH* groups were not oxidized by the strong ferricyanide before the thrombine could act on them.

The experiments of *Jaques* (3) also showed that after a great consumption of H_2O_2 the fibrinogen is still, clotted by thrombin. It cannot belived that after the treatment with such strong oxidizing agents any *SH* groups can persiste in the fibrinogen molecule.

CONCLUSIONS.

In pure fibrinogen preparations one cannot detect any *SH* groups by different chemical methods.

The fibrinogen clots normally after treatment with such strong oxidizing agents, which surely have oxidized the *SH* groups and also after a treatment with chloracetophenon.

Even in the case when the number of *SH* groups is small and it cannot be detected chemically, they have not any role in the clotting process.

We are much indebted to Prof. *K. Laki* for his continuous guidance and kind help.

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INFLUENCE OF *KCl* AND *ATP* ON SUCCINO-DEHYDROGENASE AND CYTOCHROM-OXYDASE.

WITH 2 FIGURES IN TEXT.

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As it was shown in a previous paper the oxydation of succinic acid by washed muscle was strongly influenced by the ions present and by *ATP*. Oxydation reaches its maximum in 0,05 *M KCl* and by adding *ATP* is further increased with 100%, so the activity is maximum when the contractile matter of muscle is maximally precipitated or contracted. It is well known that oxydation of succinate involves at least three systems: cytochromoxydase, the cytochrom system and succinodehydrase. The first and the third, i. e. the oxygen-activator and the hydrogen-activator cannot be separated from the insoluble structure-proteins of the cell. It seemed interesting to investigate which part of the whole system was responsible for this behaviour. In the present paper we examined how far the succinodehydrase and cytochromoxydase activities of washed rabbit muscle depend on salt concentration and *ATP*.

EXPERIMENTAL.

As enzyme preparation minced and washed rabbit muscle was used as described in our previous paper (1).

Succinodehydrase activity was determined by two methods: *a)* by the classic Thunberg-method, measuring the time of decolorisation of a certain amount of methylene-blue anaerobically. *b)* The cytochrom system was poisoned with *KCN* and methylene-blue was added as oxygen transporter. Thus the oxygen uptake measured in Warburg respirometer was due directly to the succinodehydrase activity.

Cytochromoxydase activity was measured as oxygen uptake by the paraphenylenediamine-muscle system in Warburg respirometer. The base, neutralised with *HCl* to *pH* 6,9 close before beginning: was placed into the side tube by pouring it into the main part the reaction was started. Autooxydation of the paraphenylene-diamine was estimated in

separate blanks and corrected for. Exact composition of the samples is given with the results.

RESULTS.

Succinodehydrase activity as measured by the Thunberg method was not altered by *KCl* in concentrations 0,035—1,00 *M*, nor even if *ATP* was added (Table I).

TABLE I.

Time of methylene blue decolorisation varying the *KCl* concentration. The reaction-mixture contains: 300 mg muscle, 1·0 ml, 0·2 *M* borate-buffer: *pH* 8·7, 0·2 ml 0·2 *M K*-succinate, 0·1 mg methylene blue, varied *KCl*. Final volume 4 ml.

A) M KCl	T	*B)* M KCl	T No ATP	T with 5 mg ATP
0,035	36 min.	0,036	26 min.	26 min.
0,050	30 ,,	0,136	32 ,,	27 ,,
0,100	32 ,,	0,236	20 ,,	32 ,,
0,260	30 ,,	0,436	20 ,,	27 ,,
0,500	27 ,,	0,636	28 ,,	32 ,,
1,000	30 ,,	1,036	27 ,,	—

Measuring the activity by the oxygen uptake there was no decrease between 0,07 and 0,25 *M KCl* (Table II. column 1). Omitting the *KCN* and the dye a sharp decrease was observed as in the previous paper (column 2).

TABLE II.

Oxigen consumption in 20 minutes varying the *KCl* concentration.
I. Muscle-methyleneblue-*KCN*-succinate system. Reaction mixture: 600 mg muscle, 1 ml 0·2 *M* borate buffer: *pH* 8·7, 0·2 ml 0·2 *M K*-succinate, 0·5 mg methyleneblue. Final volume 4 ml.

II. Muscle-succinate system: Reaction mixture: 600 mg muscle, 1 ml 0,2 *M* borate buffer: *pH* 8·7, 0·5 ml, 0.2 *M K*-succinate. Final volume 4 ml.

M KCl	I.	IL
0,07	38	50
0,10	35 mm^3	41 mm^3
0,15	40 ,,	20 ,,
0,20	47 ,,	5 ,,
0,25	41 ,,	0 ,,

Results regarding the cytochromoxydase activity are given in Fig. 1. The oxygen uptake was plotted against the logarithm of the *KCl* concentration. There is a slight maximum at 0,05 *M*, then towards higher *KCl* values a slow decrease follows. The curve is essentially the same with *ATP* too.

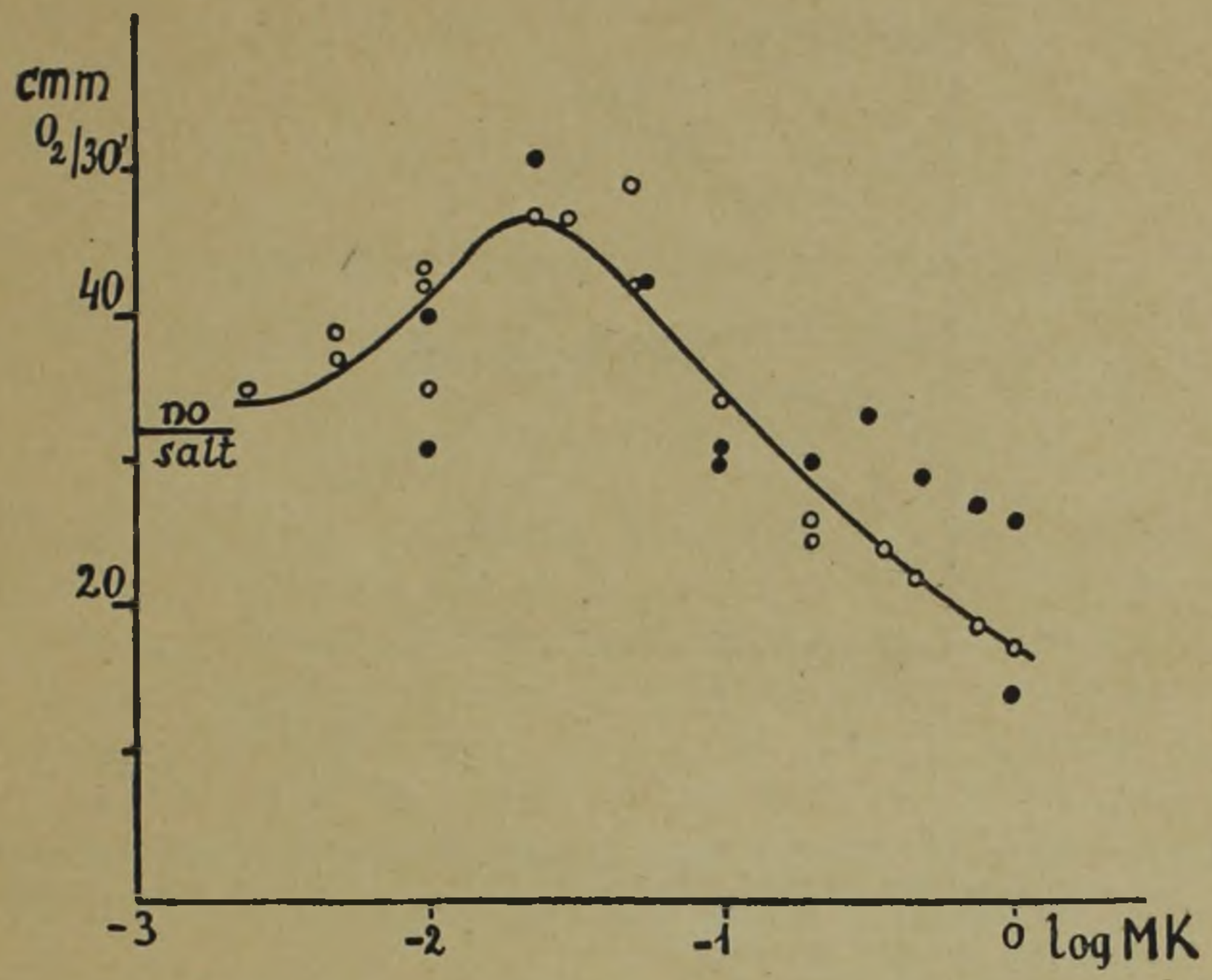

Fig. 1. — Oxygen uptake of 300 mg washed muscle in 30 minutes in 0,1 *M* p-phenylen-diamine. circles: without *ATP* dots: 5 mg *ATP* added.

Addition of *Ca* and *Mg* (besides *ATP*) in concentrations 0,001 *M* caused no change, if however their concentration was raised to 0,005 *M* — which in presence of *ATP* completely checks the succinoxydase — the slight maximum of *p*-phenylenediamine-oxydation at 0,05 *M KCl* disappeared and the oxygen consumption, independently from the *KCl* concentration, was the same as in salt free medium (Table III).

TABLE III.

Oxigen consumption in 30 minutes. Muscle-paraphenilenediamine system in the presence of $CaCl_2$, $MgCl_2$ and *ATP* varying the *KCl* concent ration.

Reaction mixture: 300 mg muscle, 1·0 ml 0·4 *M* paraphenylenediamine, 5 mg *ATP*, varied *KCl*. Final volume 4 ml.

M KCl	0·001 M $CaCl_2$ 0·001 M $MgCl_2$	0·005 M $CaCl_2$ 0·005 M $MgCl_2$
0,010	52 mm^3	37 mm^3
0,025	50 ,,	37 ,,
0,050	47 ,,	39 ,,
0,075	—	36 ,,
0,100	39 ,,	35 ,,
0,250	—	32 ,,
0,500	28 ,,	28 ,,

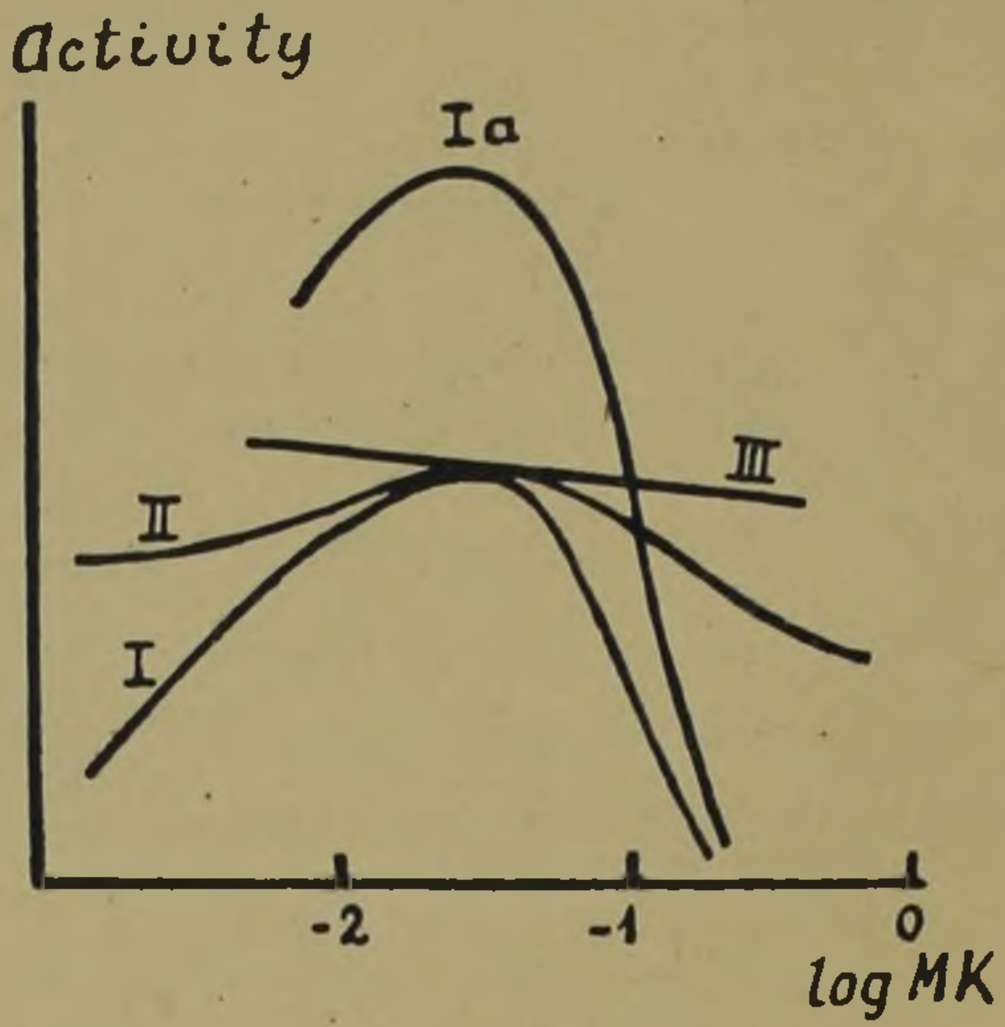

Fig. 2. — Activity of succinoxydase, succinodehydrase and cytochromoxydase at varied *KCl* concentrations, without and with *ATP*, in arbitrary units, taking the maximal activity without *ATP* equal in the three cases.

Abscissa log *M KCl* present.
Ordinate: Activity,
Curve I. Succinoxydase without *ATP*
„ I/a. „ 5 mg *ATP* added
„ II. Cytochromoxydase with and without *ATP*
„ III. Succinodehydrogenase with and without *ATP*.

SUMMARY.

Our results along with the main previous ones are summed up in Fig. 2. Succinoxydase activity has a sharp maximum in 0,05 *M KCl*, strongly increased by *ATP*: the activity of cytochromoxydase and succinodehydrase however, when determined separately, does not considerably depend on *KCl* concentration and on presence of *ATP*. So the behaviour of the whole system cannot exactly be explained by qualities of its separate components known so far.

LITERATURE.

1. Hungarica Acta Physiol. Vol. I. p. 9 (1946).

OBSERVATIONS ON WASHED MUSCLE.

BY N. A. BIRÓ AND A. SZENT-GYÖRGYI jun.

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(RECEIVED FOR PUBLICATION 2. 3. 1948.)

Washed muscle is generally considered as a well reproduceable preparation in connection with investigations on insoluble enzymes bound to the stroma. According to our experiences however, washed muscle may exhibit greatly differing physical qualities depending on the way of its preparation. We obtain either voluminous, strongly hydrated samples with little dry weight (minimum 4%) or hard, well filterable pasteous material with high dry weight (max. 25%) just according to the temperature, the *pH* and the time that elapsed since killing of the animal.

EXPERIMENTAL.

Minced rabbit muscle was used as experimental material. The animal was decapitated, rapidly skinned, eviscerated and dipped in ice-cold water for 20 minutes. The deep back muscles were cut out with cooled instruments and minced through a cooled mincer (holes 2 mm diameter). The whole procedure was carried out in the cold room at 0^0. The pulp was suspended in 20 vols of destilled water, stirred gently for 10 minutes, filtered and pressed sharply through a cloth with hands. Then the residue was resuspended and the whole procedure repeated.

Dry weight was estimated by having a sample dried to constant weight at 120^0.

RESULTS.

Experiment 1. Minced muscle was washed with distilled water of various temperature. The dry weights of the preparations are given in Table I. As may be seen the lower the temperature the lower are the figures of dry weights and the higher the grade of hydratation. With increasing temperature also the dry weights increase.

Effect of pH. By shifting the *pH* of the washing to the alkaline side the hydratation of muscle is enhanced.

Experiment 2. Part of muscle V. used in exp. 1. was washed with distilled water containing a few drops of $KHCO_3$ to adjust the *pH* to 7,8. The dry weight was only 6% whereas it was 10% after washing with plain distilled water of *pH* 6,5.

TABLE I.

Muscle	Temperature of the destilled water					
	0°	4°	13°	16°	20°	37°
	% dry weight					
I.	10	13	—	20	—	—
II.	10	12	12	18	—	—
III.	7	—	—	—	14	—
IV.	7	14	—	13	—	15
V.	10	—	17	19	—	23

Effect of ATP. The hydratation of the washed muscle depends also on how far its *ATP* content was split at the very moment of the first washing.

Experiment 3. Before washing it with distilled water of 0° the minced muscle was kept at 0° for various length of time. Dry weight increased but the amount of easily hydrolysable phosphorus decreased with the time. (Table II.)

TABLE II.

Muscle No.	The time of storage in minutes				
	0	30	90	180	210
	% dry weight				
I.	10	16	19	25	—
II.	12	14	20	—	23
	Hydrolysable phosphorus per 1000 mg muscle				
I.	0,15	0,16	0,05	—	0,00

The hydratation of the washed muscle decreased after resuspending it in presence of *ATP* at higher temperature and washing it again. By doing that in absence of *ATP* the dry weight was not changed (Exp. 4, 5).

Experiment 4. A sample that was previously washed at 0° and had a dry weight of 4% was resuspensed in 0,15 *M KCl* with and without ATP and washed again with distilled water of 0°. With *ATP* the dry weight was increased at higher temperatures. (Table III.)

TABLE III.

Temperature °	Dry weight %	
	without ATP	with ATP
0	5	7
1	6	12
5	4	8
12	5	10

Experiment 5. Samples of 10 resp. 12% dry weight were resuspended in 0,15 m *KCl* containing *ATP* at 16° and washed with distilled water at 16°. Dry weight was increased to 21, resp. 22%.

Reversibility. The experiments described above are not reversible. Samples of high dry weight (washed in „hot" water) were resuspended at 0° in the presence of *ATP* and washed again with water at 0°: in spite of this treatment the dry weight did not decrease but remained on high level (Exp. 4 and 6).

Expriment 6. A sample which was previously washed with water at 16° and had the high dry weight of 20% was suspended at 0° in 0,16 *M KCl* in the presence of *ATP* and washed again with distilled water at 0°: its dry weight did not change.

Part of muscle No. II. (Exp. 3. Table II) after having kept it 210 minutes was suspended in 0,15 m *KCl* containing *ATP* at 0° and after a few minutes washed again with distilled water at 0°. Its dry weight amounted to 20% and was so invariably high as compared with the previous 23%.

The *ATP*-ase activity of the various samples of different grade of hydratation was practically the same if calculated on the dry weight.

SUMMARY.

The hydratation of the washed muscle depends to a great extent on the temperature of the washing, on the presence of the *ATP* and on the *pH*. The higher the temperature the higher is the dry weight and vice versa, quite parallel with the physico-chemical behaviour of acto-myosin, the main constituent of the washed muscle fibre.

THE MECHANISM OF THE CLOTTING OF FIBRINOGEN WITH FORMALDEHYDE AND QUINONE.

INTERACTIONS BETWEEN FORMALDEHYDE AND THROMBIN.

WITH 9 FIG. IN TEXT.

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(RECEIVED FOR PUBLICATION 17. 3. 1948.)

Pure fibrinogen solutions can be clotted beside thrombin by a number of other substances too. These are in part of enzymatic nature as the papain, the snake venoms, and in part they are simple organic substances. *E. Chargaff* and *A. Bendich* (1) have found a number of such substances which may be divided into two classes: 1. naphthoquinone derivatives and 2. carbonyl derivatives, such as salicyl aldehyde, ninhydrin etc. According to *Chargaff* most of these substances are able to oxidize amino acids and peptides containing free amino groups. He believed that in the process of clotting by these substances the following mechanism are possible: "1. oxidation of aminoacyl groups present in the protein, 2. oxidation of other susceptible groups, such as sulfhydryl, 3. combination of the clotting agent with the protein." Although *Chargaff* seems to be inclined to the oxidative theory, the evidences supporting this, are not very convincing. It may be said that the exact mechanism of the action of artificial clotting agents is not elucidated still. There are some authors who even deny that fibrinogen can be clotted by these substances.

R. N. Lyons (2) would supply the opposite findings, by demonstrating the existence of two different forms of fibrinogen. Fibrinogen *A*, which is prepared from fresh plasma does not clot with 2 methyl- 1,4 naphthoquinone and has very few thiol groups and fibrinogen *B*, which can be prepared from aged plasma after a treatment with $Ca_3\ (PO_4)_2$, contains relatively large number of thiol groups and is readily clotted by synthetic clotting agents.

Lyons supposes that in the natural clotting too, the *SH* groups are involved. Thrombin component *A* reacts with fibrinogen liberating *SH* groups to form the intermediate fibrinogen *B*. Thrombin component *B*,

which seems to be a quinone compound, oxidizes the liberated *SH* groups to — *S*—*S* — linkages. Lyons'theory is not confirmed. With chemical methods it is impossible to determine any sulfhydryl groups in fibrinogen and it has been demonstrated that in the natural clotting the *SH* groups have no role (3). Therefore, the assumption, that the differences in clotting by synthetic clotting agents may be due to differences in the free *SH* content of different fibrinogen preparations, should also be rejected.

We have started our experiments to throw light on the clotting mechanism in the case of the synthetic clotting agents, to find the exact circumstances of this reaction and to elucidate in this way the cause of opposite findings too. On the other hand, by comparing the action of these substances with that of thrombin and by investigating the interactions between this and the synthetic clotting agents it could be possible to get some data on the mechanism of thrombin action too.

The experiments were performed with formaldehyde and p. quinone.

EXPERIMENTAL.

The fibrinogen was prepared from fresh oxalated cattle plasma according to *D. Bagdy* (4). The method is characterised by an ammonium sulphate precipitation followed by an isoelectric precipitation. The preparations were of 85—90% purity. The fibrinogen content was determined with the method due to *K. Laki* (5). The amount of total protein was determined by means of trichloracetic acid precipitation. The purity of fibrinogen could be brought to about 98% if the solutions were allowed to stay and the fibrinogen crystallised. In the experiments which will be described, generally the crystallization was omitted and the solutions of 85—90% purity were used.

Thrombin was prepared according to *K. Laki* and *L. Lóránd* (6).

The formaldehyde solutions were prepared by diluting a formaldehyde solution of 40% (Formaldehydum solutum Ph. Hg. IV). After dilution the solution was carefully neutralized. The neutralization of the concentrated solution was avoided, when a rapid polymerisation of formaldehyde occurs and a great amount of precipitate settles down. Solutions used were always freshly prepared.

To avoid erroneous interpretations it must be stated that in this paper always the formaldehyde final concentrations are given and not that of formalin.

Merck's quinone preparation was used. The quinone is somewhat difficultly soluble in cold water. It was dissolved by moderate heating,

than filtered. On standing the solution became dark, due to the polymerisation of quinone. Therefore, daily a fresh solution was prepared.

The clotting time was determined in a way already described (7). The solutions were mixed in a small glass tube and from time to time the tube inclined in order to see the moment of gelification.

In some experiments the technic described by W. F. H. M. Mommaerts (8) was used. The solutions were pipetted in one of the holes of a glass plate, as used in drop analysis and the time noted after which a fibrin thread could be drawn from it with a fine glass hook.

The experiments were carried out at room temperature (20 C⁰). It was found that the nature and concentration of ions had a great influence on the reactions which will be described. To keep the conditions as constant as possible in order to obtain comparable data, there were used always *M*/1 phosphate buffers. It was necessary to extend the experiments over a very wide *pH* range. The primary-secondary phosphate buffer range was not sufficient. It was completed with secondary-tertiary phosphate mixtures. We did not find in literature the *pH* values of such mixtures, therefore we have determined them with the hydrogen electrode. The mixtures were prepared from *M*/10 secondary and *M*/10 tertiary sodium phosphate. Merck's pro analysi preparations were used. The *pH* values are given in Table I.

TABLE I.

M/10 secundary phosphate	*M*/10 tertiary phosphate	*pH*
9,5	0,5	10,35
9	1	10,70
8	2	11,07
7	3	11,29
6	4	11,48
5	5	11,60
4	6	11,75
3	7	11,89
2	8	12,01
1	9	12,17
0,5	9,5	12,25

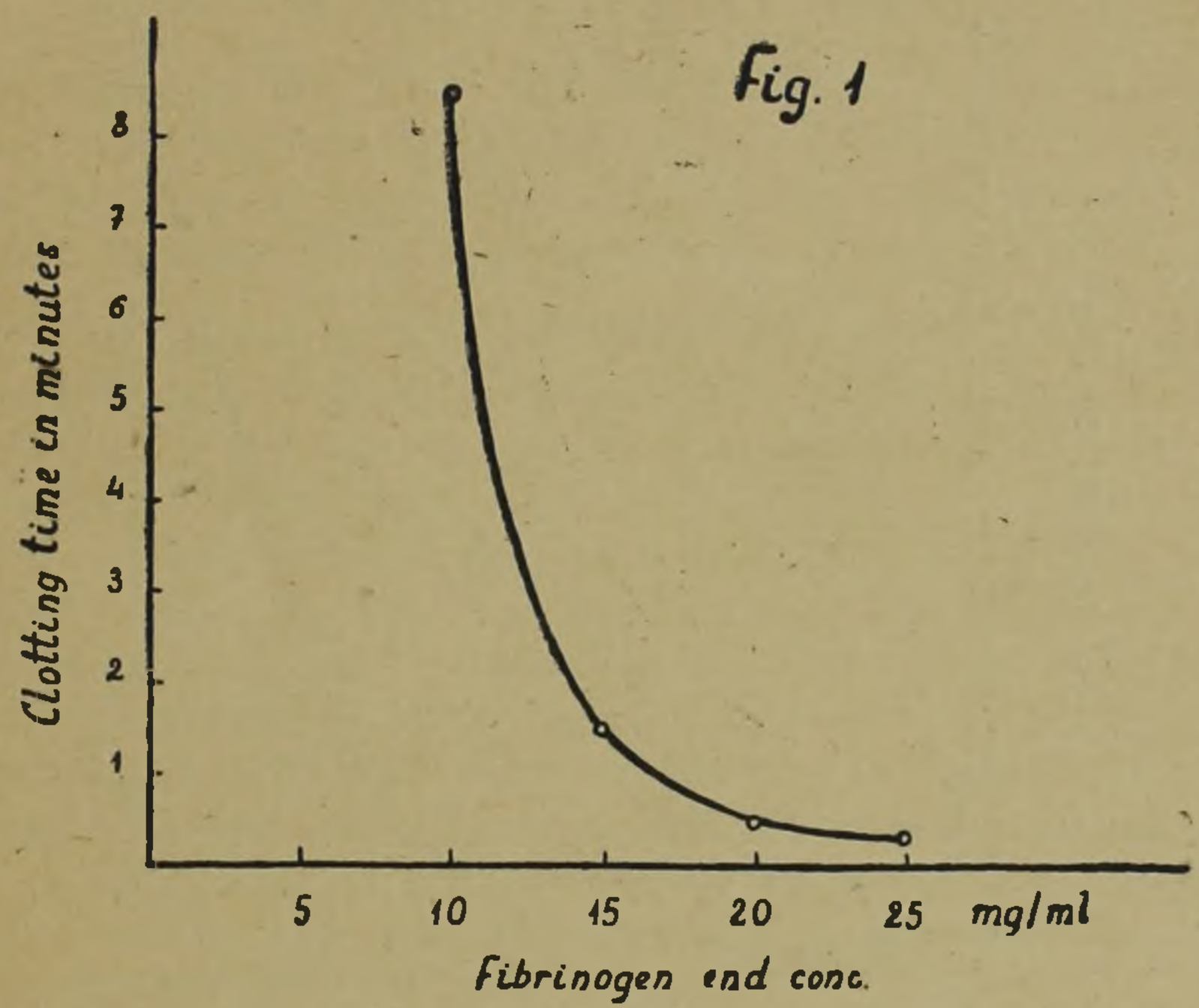
A) *Clotting activity of formaldehyde and p. quinone.*

It was found that the clotting time with formaldehyde and p. quinone is dependent on four factors: *a)* the concentration of fibrinogen, *b)* the concentration of clotting agent, *c)* the *pH* of the system, *d)* the

nature and concentration of ions present. These factors influenced not only the speed of clotting, but they determined whether the fibrinogen would clot or not.

a) Plotting the clotting time against the fibrinogen concentration when formaldehyde or quinone concentration are kept constant a curve like that shown in Fig. 1. is obtained. The reaction mixture was the following: 0,8 *ml* fibrinogen, 0,2 *ml* *M*/1 phosphate buffer of *pH* 8,0, 0,5 *ml* 4% formaldehyde or 1,0 *ml* 1% quinone. Fig. 1. shows the curve obtained with formaldehyde. The abscissae represent the fibrinogen final concentrations in *mg* per *ml*. It can be seen that below 10 *mg* per *ml* there was no clotting at all, the solution became only turbid on the action of formaldehyde. Rising the fibrinogen concentration the clotting time decreases quickly.

Fig. 1

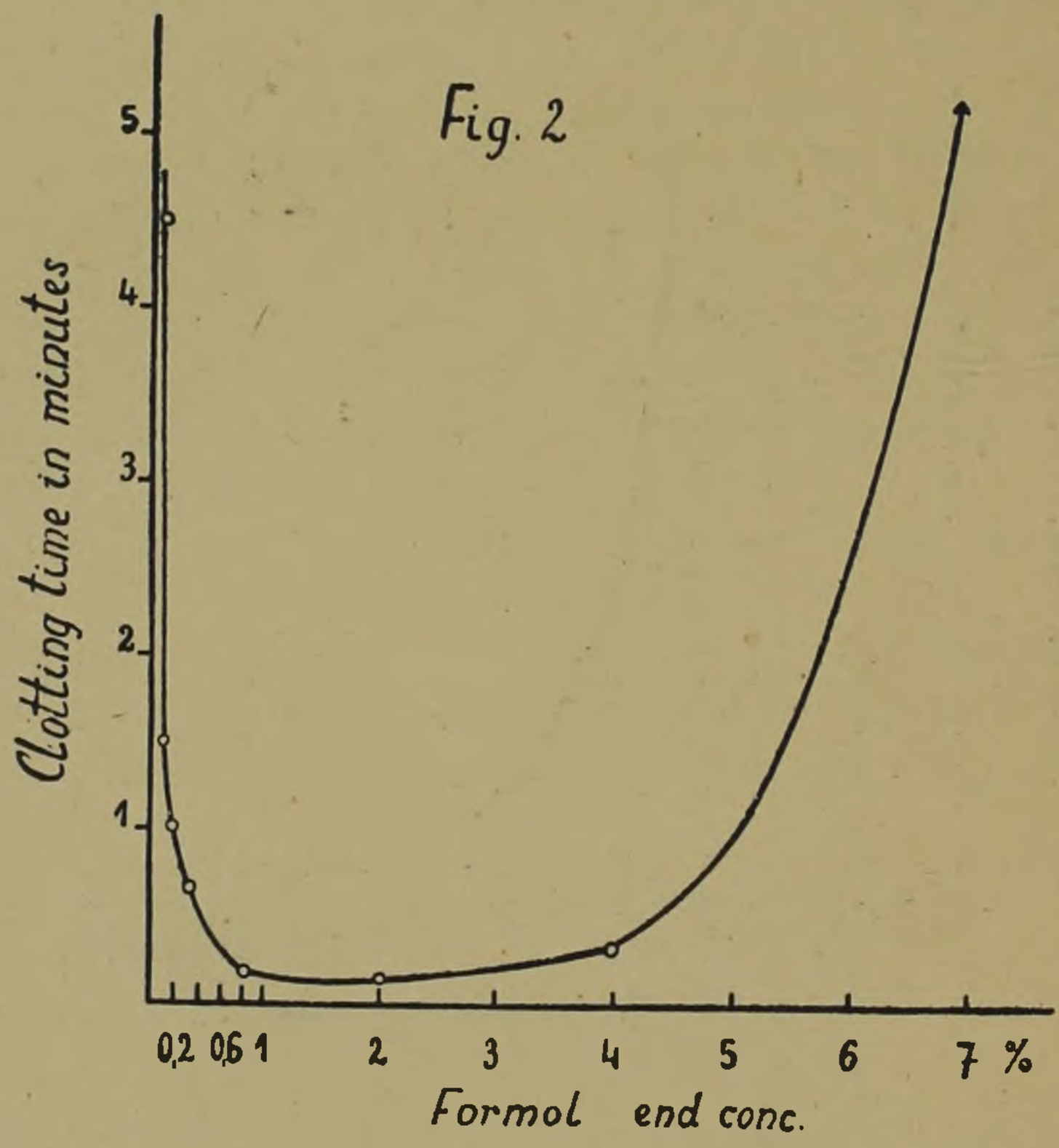


The curve obtained with quinone is of the same shape, only it is shifted towards smaller fibrinogen concentrations. The quinone is able to clot even a solution of 2 *mg* per *ml* fibrinogen. At this concentration the clotting time is 40 minutes and the clot is very loose. At 20 *mg* per *ml* endconcentration of fibrinogen the clotting time is only 1 min. 30 sec. and a very firm clot is formed.

b) The effect of different formaldehyde concentrations is shown in Fig. 2. The reaction mixture was the same as before, only instead

of the fibrinogen, the formaldehyde concentration was varied. Rising the formaldehyde concentration, the clotting time decreases rapidly. Between 0,5—4% formaldehyde concentrations the curve is flat and above this it begins to rise again. In 10% formaldehyde the clotting time is more than 4 hours and above this it is not clotted at all. In the experiment showed in Fig. 2. the fibrinogen endconcentration was 16 *mg* per *ml*. The clots brought about by formaldehyde were very turbid, but at higher formaldehyde concentrations they became more and more transparent. In the tube containing 16% formaldehyde a syrupy, transparent liquid resembling honey was formed after 12 hours. The clot was not so firm as by thrombin.

Fig. 2



The results obtained with p. quinone are very similar. At 0,02% quinone endconcentration the clotting time was 75 min. Below this concentration the fibrinogen was not clotted. Increasing the quinone concentration the clotting time decreased quickly and at 0,5% it reached 1 min. 30 sec. Higher quinone concentrations were not available for solubility difficulty of quinone.

The clotting with quinone is much slower than with formaldehyde. In the same conditions 1% formaldehyde clots the fibrinogen in about 10 seconds, but 1% quinone in more than one minute. The clots formed by quinone are brownish-red. The color is developed successively. After the addition of quinone, the solution becomes more and more dark. The colour development is quicker when the quinone concentration is higher. The solutions are at first clear and after the colour has reached already a considerable degree, they become turbid and shortly clot. The turbidity appears at about 2/3 of the clotting time. The clots are very resistant, even more than those obtained by thrombin.

The formaldehyde clots are dissolved readily by 30% urea. Removing the urea by dialysis, a water-clear solution is obtained, which does not clot either with formaldehyde, or with thrombin.

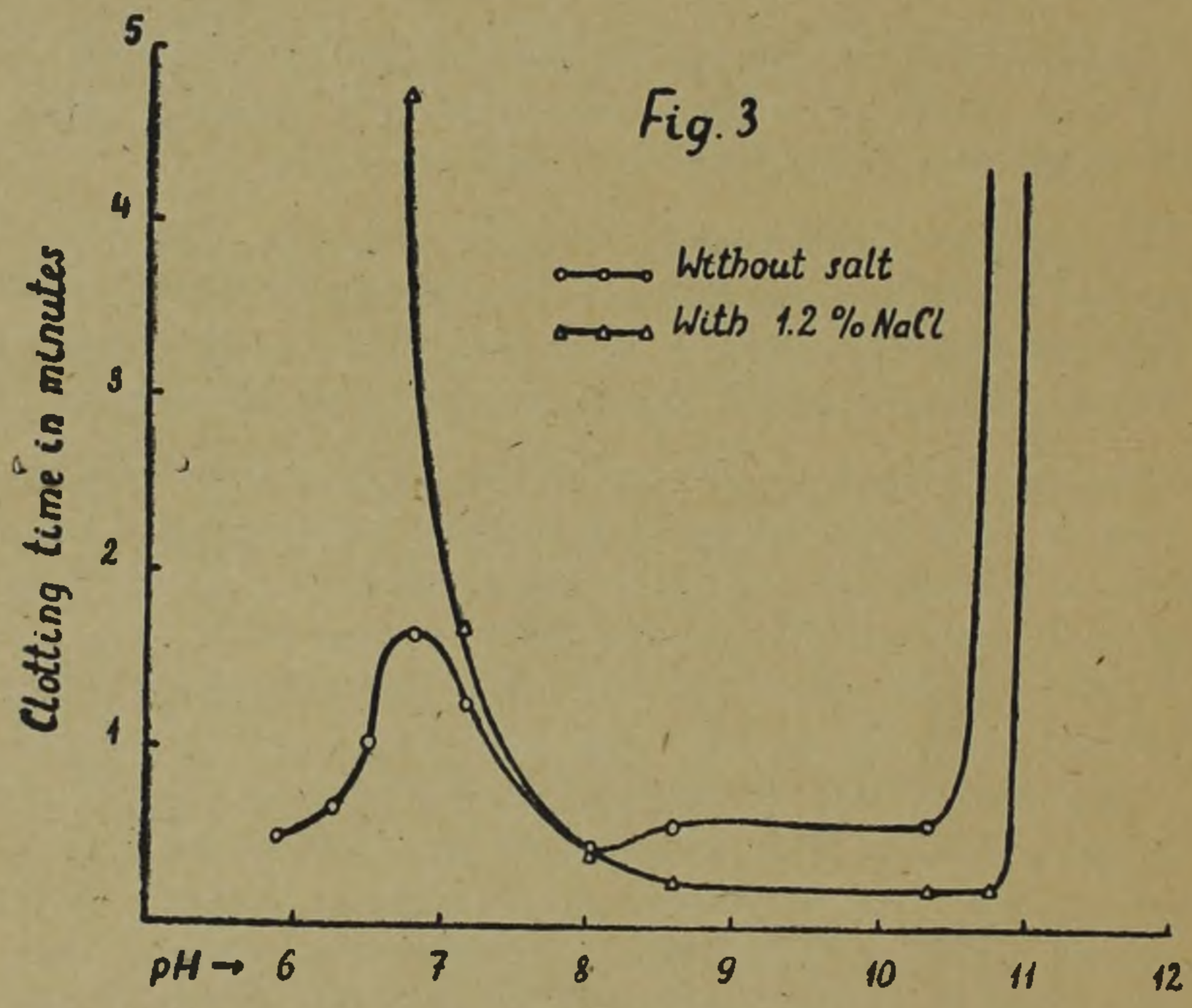
The quinone clot is insoluble even in 60% urea. The red colour is also very firmly bound to the clot. No coloured substance goes in solution if the clots are allowed to stand in water or in 60% urea for several days.

The reactions described above, are not a special property of the fibrinogen preparation used. The fibrinogen prepared from fresh oxalated cattle plasma by a single ammonium sulphate precipitation, clots in the same way at suitable *pH* and with suitable concentrations of fibrinogen, clotting agent and salt. Further, the fresh oxalated cattle plasma can be clotted with formaldehyde or quinone. 0,9 *ml* oxalated plasma, 0,1 *ml* *M*/1 phosphate of *pH* 8, 0,5 *ml* 8% formaldehyde or 1 *ml* 1% quinone were mixed. The clotting time by formaldehyde lies between 8 and 17 minutes with different plasmas. The quinone clots more slowly, the clotting time is about one hour. The fibrinogen concentration in plasma is only about 3 *mg* per *ml*, which comes below the minimal concentration when pure fibrinogen solution can be clotted with formaldehyde. It may be supposed therefore, that the other plasma proteins have also some role when plasma is clotted by formaldehyde or quinone.

c) The *pH* has a great influence on the clotting time and the quality of clots. The experiments were performed in the following way: 0,8 *ml* fibrinogen of 25 *mg* per *ml*, 0,2 *ml* *M*/1 phosphate buffer of different *pH*, 1,0 *ml* 2,5% formaldehyde or 1,0 *ml* 1% quinone were mixed and the clotting time determined. It was found that the course of the curves was different, if the salt concentration was different. Therefore, two sets of experiments were done: 1. with minimal salt concentration, in which case only the salt given with the buffer was present, 2. with 1,2% *NaCl* end-concentration, when to the fibrinogen solutions, which were practically

salt free, an amount of *NaCl* was added, to reach the mentioned concentration.

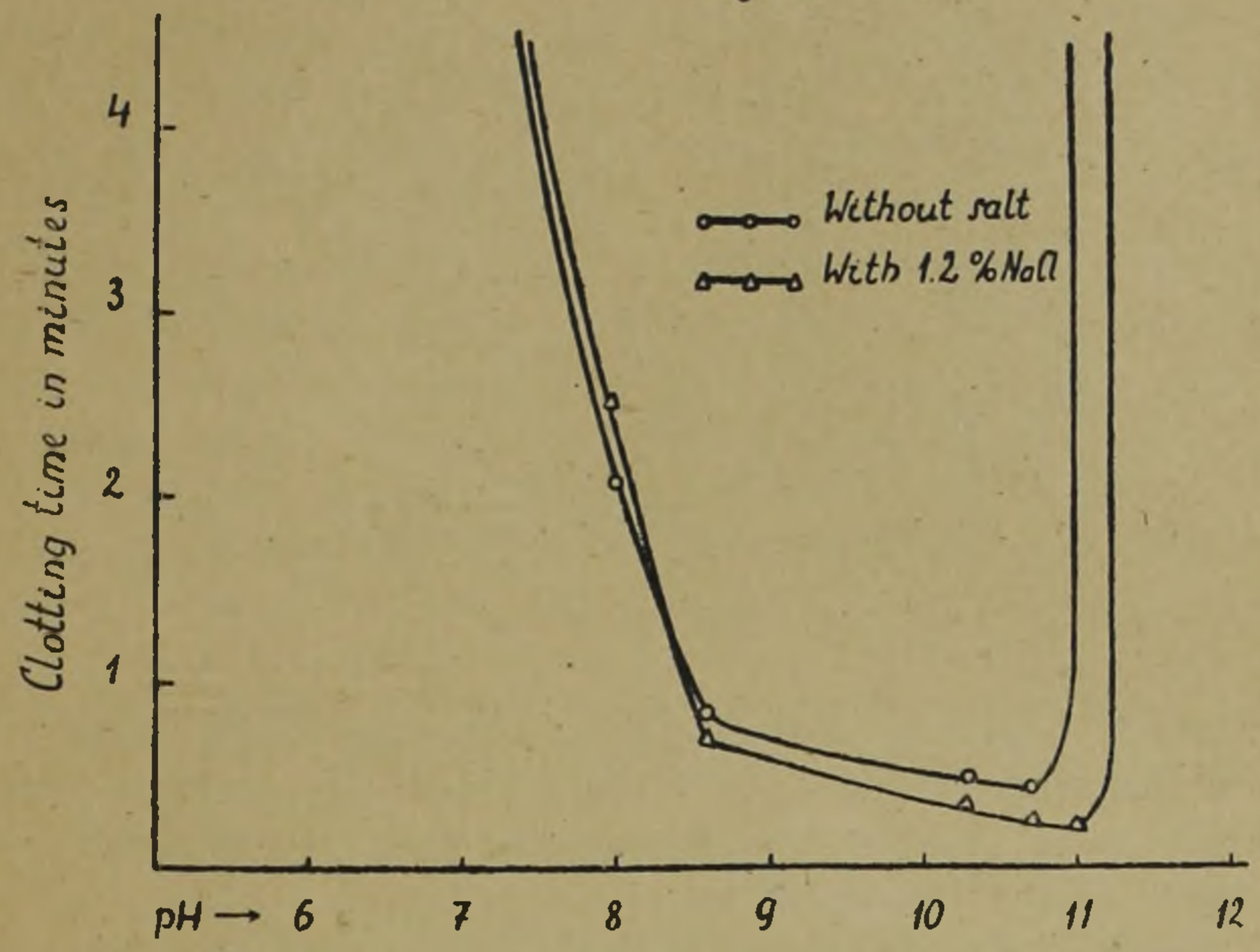
The effect of *pH* on the clotting with formaldehyde is shown in Fig. 3. Without salt, there is no clotting below *pH* 6,8. Increasing the *pH* the clotting time decreases quickly till 8,5 and from this point is nearly constant till *pH* 10,6, where it springs up suddenly. Above 11 the fibrinogen does not clot, it gives only a syrupy, transparent fluid. Between *pH* 7 and 10 the clots formed are solid and very turbid. Above 10 the resulting clots are of decreasing turbidity and firmness.



The experiments with salt addition give a quite different curve. The fibrinogen is clotted even at acidic *pH* where without salt it does not clot at all (below 6,8). The clotting time is increasing till *pH* 6,8 where it has a maximum, than decreases till *pH* 8 where it begins again to rise slowly till 10,3 and suddenly above this *pH*.

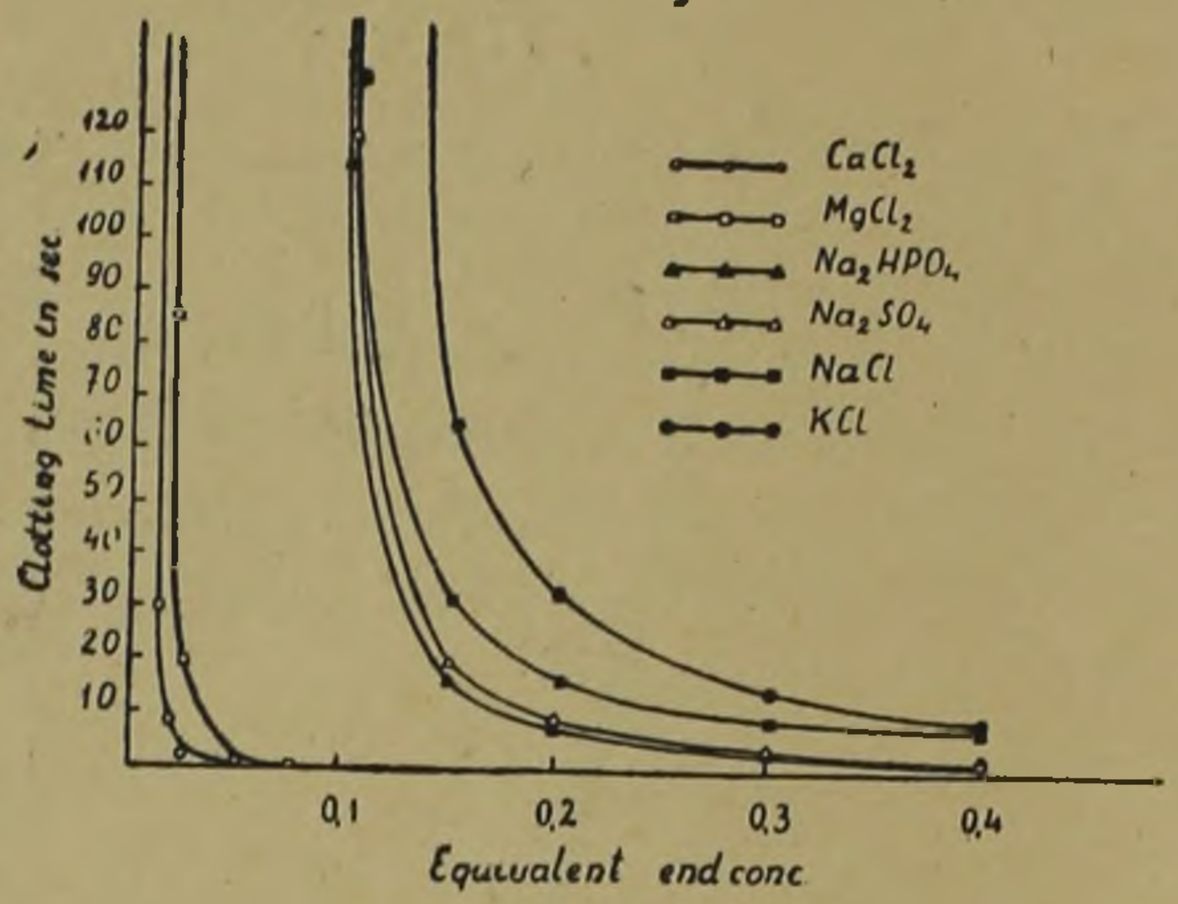
The clotting times with quinone, without and with added salt, are plotted in Fig. 4. as the function of the *pH*. The two curves are very similar only their position is somewhat different. The curve with added salt is a bit shifted in alkaline direction. The development of the red-brown colour is quicker when the *pH* is higher. The darkness of clots is also increasing with the increase of alkalinity.

Fig. 4



d) The clotting of fibrinogen by the two investigated synthetic clotting agents occurs only in the presence of small amounts of neutral salt. Varying the salt concentration it was found, that below a certain concentration there is no clotting. Increasing the salt concentration the clotting time decreases rapidly. The activity of different salts was investigated. It was found that in equivalent concentrations the salts with the same cation, but different anion have almost the same activity. The effect was investigated in the following mixtures: 0,8 *ml* fibrinogen of 35 *mg* per *ml*, 0,2 *ml*, *M*/1 borate buffer of *pH* 8,6, 0,5 *ml* salt solution, 0,5 *ml* 2,5% formaldehyde or 0,5 *ml* 1% quinone. If, instead of the salt solution, there was added distilled water, neither formaldehyde nor quinone were able to clot fibrinogen. The amount of salt introduced in the system with the buffer (0,025 equiv. endconc. of *Na*) is not sufficient to bring about clotting. The experiments are plotted on Fig. 5. for the clotting with formaldehyde and in Fig. 6. for the clotting with quinone. The abscissae are the equivalent endconcentrations of the added salts, the ordinatae the clotting times. By the calculation of the salt endconcentrations the amount of salt introduced with the buffer was not taken in account. The secondary phosphate at *pH* 8,6 dissociates totally into 2 *Na* and HPO_4 ions, therefore it was taken as a salt with a bivalent anion. It can be seen that the activity of monovalent cation salts is very similar.

Fig. 5



The curves of them forme a distinct group. The curves of bivalent cations forme a second group, at a 4—6 times lower concentration than the monovalent ones. The clots are of increasing firmness and turbidity with the increase of the amount of salt.

The effect of salts is the same in the case of quinone clotting, as in the case of formaldehyde clotting. There are only quantitative differences, the quinone is able to clot the fibrinogen in the presence of 0,015 equivalent concentration of *NaCl*, whereas formaldehyde only in presence of 0,1.

The concentration relations discussed above are not independent from each other, but they are in close interdependence. In presence of a

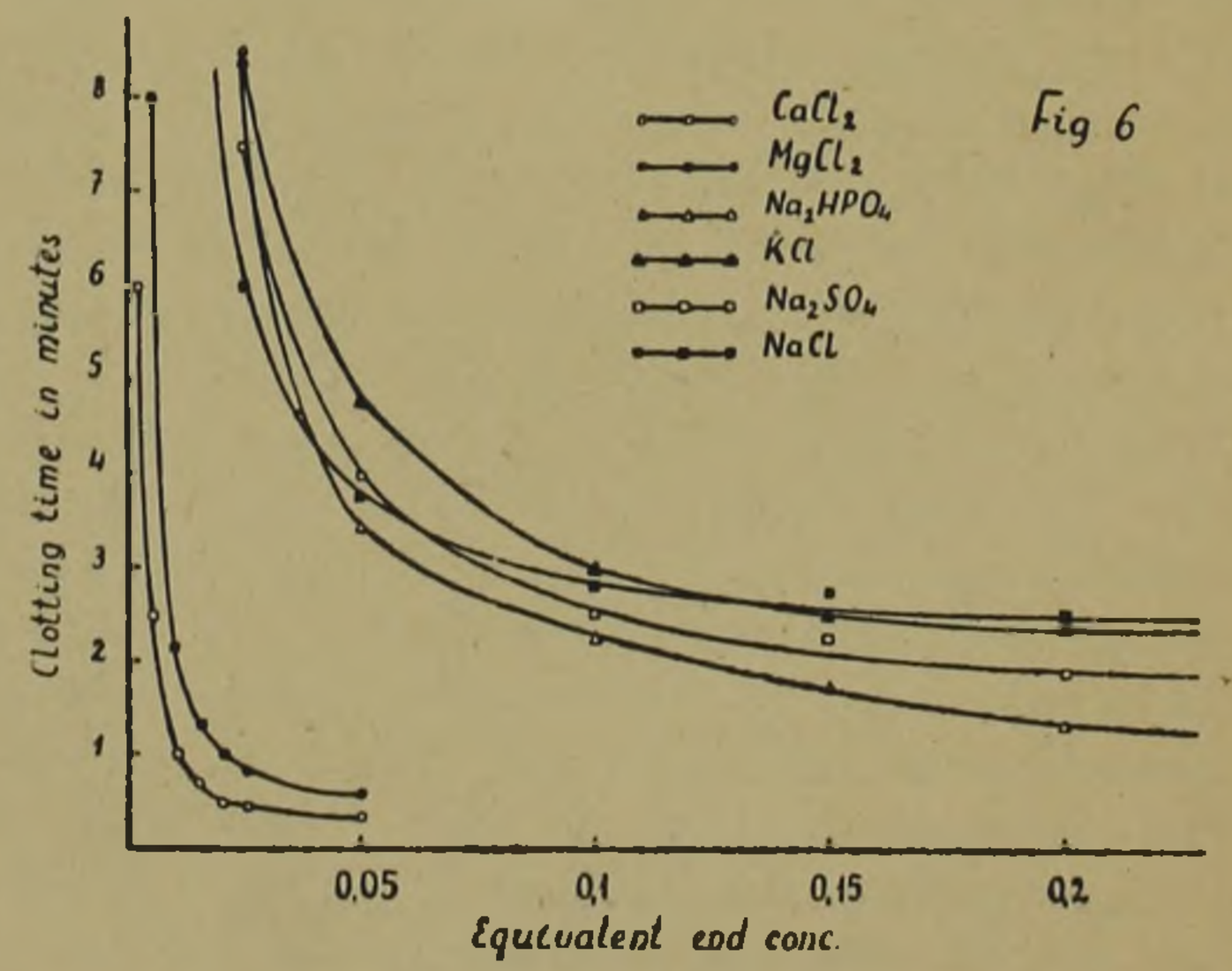


Fig 6

higher salt concentration for example, the minimal formaldehyde concentration which causes clotting is lower as in the case of a smaller salt concentration. Similarly the increase in the formaldehyde concentration brings about a widening of the clotting range in acidic direction. Generally one can say, that the increase of concentration of one factor causes a widening of the clotting territory of the other factors.

Omitting the salt in the above mixture, naturally the clotting does not occur. If we add to such a mixture the salt 10 minutes after the addition of clotting agent, again it does not happen anything, in spite of the fact that adding the clotting agent after the salt, the fibrinogen clots in about one minute. The experiment was carried out as follows: 0,8 *ml* fibrinogen of 25 *mg* per *ml*, 0,2 *ml* *M*/1 borate buffer of *pH* 8,6, 0,2 *ml* *M*/1 secondary sodium phosphate, and 0,8 *ml* 4% formaldehyde were mixed. The mixture clotted in one minute 30 sec. If the 0,2 *ml* of sodium phosphate was not pipetted in, the mixture did not clot, only a small turbidity appeared. Adding the salt solution after 10 minutes, there was no change even after hours.

B) *Interaction of formaldehyde with thrombin.*

The fibrinogen in special conditions is clotted by formaldehyde but if the conditions are of such a nature (too low fibrinogen concentration for example), that clotting by formaldehyde cannot occur, it inhibits the clotting activity of thrombin. The effect was investigated by determining the clotting time of fibrinogen with thrombin in the presence of different amounts of formaldehyde. The method of threads drawing was used in the following mixture: 0,1 *ml* fibrinogen of 10 *mg* per *ml*, 0,1 *ml* *M*/5 phosphate buffer, 0,1 *ml* formaldehyde of different concentration and 0,1 *ml* thrombin of 10 units per *ml*. The inhibiting effect has a great *pH* dependence, therefore buffers of different *pH* were used.

It was found, that at *pH* 6,8 and 7,17 the inhibiting activity of formaldehyde even in 0,5% final concentration is insignificant. At *pH* 7,75 the effect is very marked and even more at 8,04, where in presence of 0,5% formaldehyde there was no clotting at all. The inhibiting activity is very much increased with increase in *pH*. The experiment is shown in Fig. 7. In abscissae there are plotted the formaldehyde endconcentrations.

Even in the case when formaldehyde has not an immediate action on the clotting of fibrinogen with thrombin, it has a slow action on fibrinogen, modifying greatly its clotting ability. Such a concentration of formaldehyde and *pH* were chosen, where the formaldehyde had no immediate

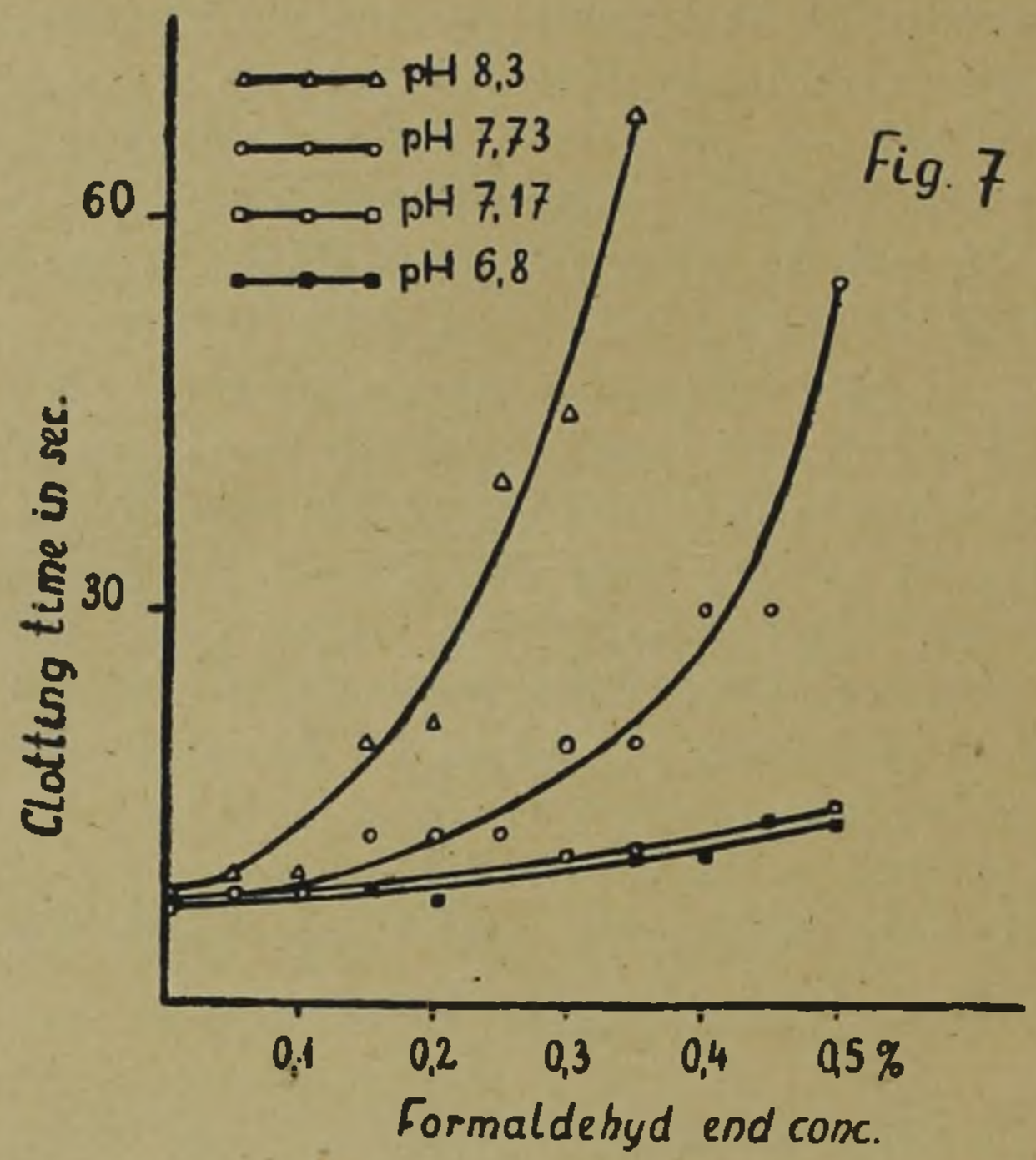
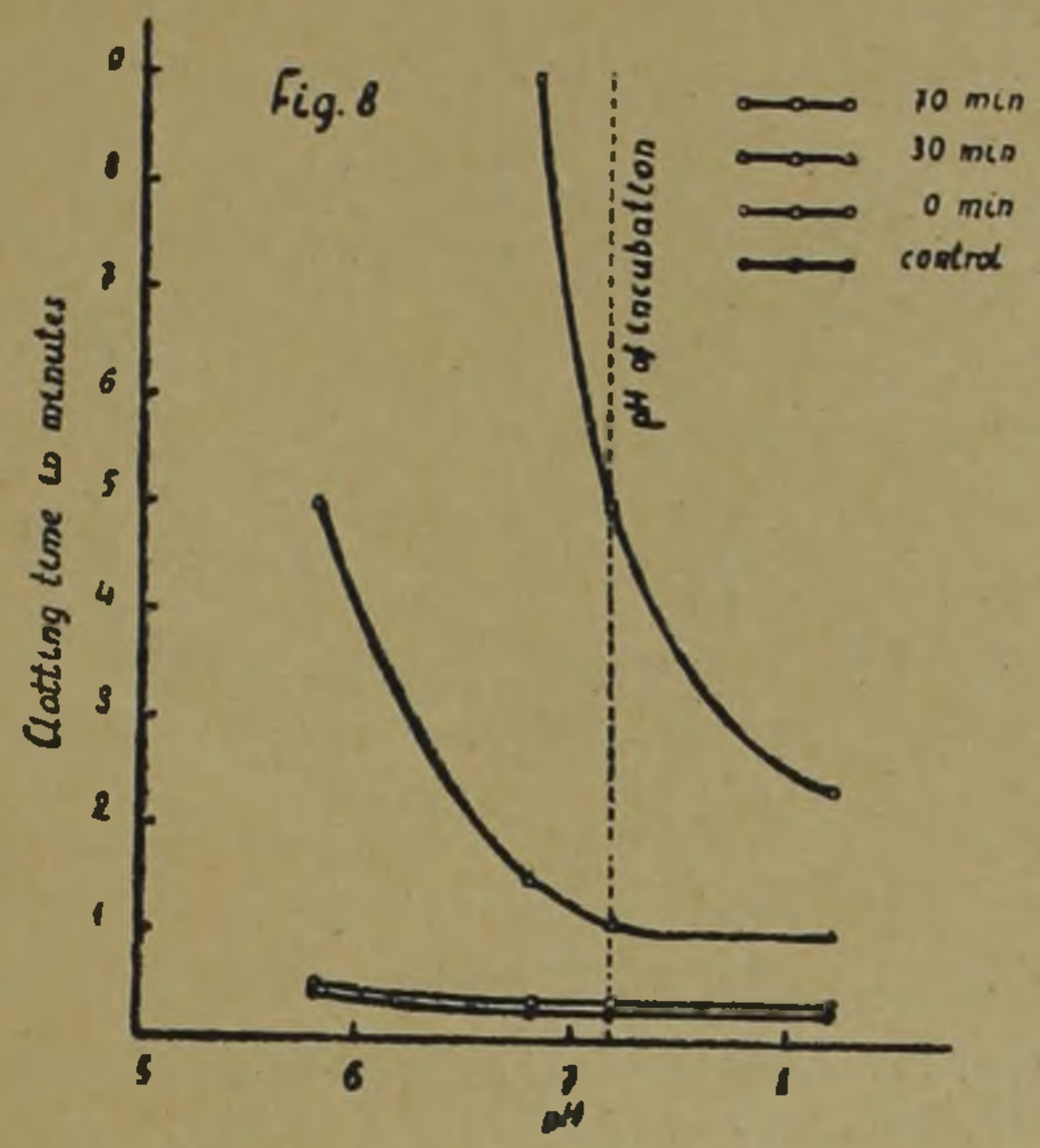


Fig. 7

inhibiting activity and the fibrinogen was incubated in these conditions at 37 °C. Checking the clotting time with thrombin of samples from the above mixture, it was found that the fibrinogen clotted more and more slowly as the incubation time was longer.

The effect is much pronounced at lower *pH*, therefore the clotting of formaldehyde treated fibrinogen samples was investigated at different *pH*. The experiment was carried out as follows: 10 *ml* fibrinogen of 10 *mg* per *ml*, 5 *ml* *M*/20 phosphate buffer of *pH* 7,17 and 5 *ml* of 1% formaldehyde were mixed and incubated at 37 °C. In the same time a control tube was arranged, which contained distilled water instead of formaldehyde. Samples were taken out immediately after the formaldehyde addition, after 30 and after 70 minutes. The formaldehyde treated fibrinogen and the control, were brought to different *pH* by means of phosphate buffers and the clotting time with thrombin determined in the following mixture: 0,2 *ml* fibrinogen, 0,1 *ml* *M*/2 phosphate buffer of different *pH* and 0,1 *ml* of thrombin of 10 units per *ml*.

The clotting times of control were the same during the whole experiment. The formaldehyde treated fibrinogen did not differ from the control at the begining of the experiment, but after 30 minutes of incubation there were considerable divergences. The experiment is plotted on Fig. 8.



It can be seen that the formaldehyde treatment has a much greater effect at lower *pH*. The clotting time at *pH* 8 is about three times that of the control, but below *pH* 7 the divergences are greatly increased. The phenomenon is more accentuated after 70 minutes of incubation. At *pH* 8 the clotting time is about seven times longer than that of the control and below 6,8 the fibringen did not clot at all.

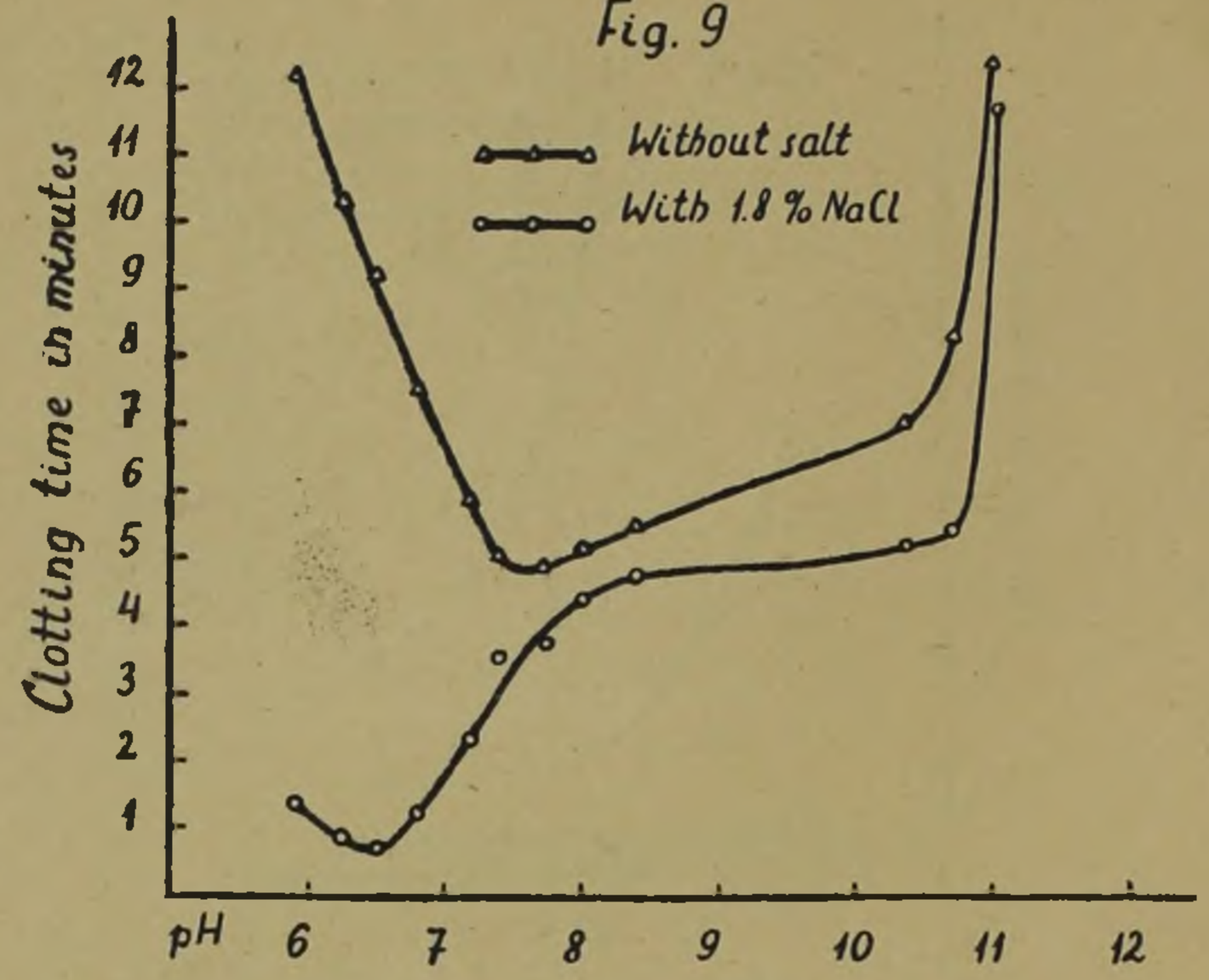
C) *The effect of pH on the clotting with thrombin.*

In order to compare the effect of *pH* on the clotting with formaldehyde and p. quinone with that of the clotting with thrombin, the *pH* dependence of the later was also investigated. As in the experiments already described, two series of determinations were made: one without any extra salt addition and the other in presence of 1,8% *NaCl* endconcentration. The salt was added to the fibrinogen solution. The clotting time was determined with the thread drawing method. 0,3 *ml* fibrinogen of 20 *mg* per *ml*, 0,1 *ml* *M*/5 phosphate buffer of different *pH* and 0,1 *ml* thrombin of 10 units per *ml* were mixed.

The curve obtained in presence of 1,8% *NaCl* is very similar to that obtained in the same conditions with formaldehyde and p. quinone. But whereas by the synthetic clotting agents the clotting time decreases continuously till *pH* 10,6, by thrombin it shows a minimum at *pH* 7,3 and from this point slowly increases till 10,6.

The results are shown in Fig. 9. The curve without salt is the mirror image of that of formaldehyde in similar conditions. It shows a

Fig. 9



marked minimum at *pH* 6,5 whereas the clotting with formaldehyde has shown a maximum at *pH* 6,8.

DISCUSSION.

The reactions of formaldehyde with amino acids and proteins have been studied extensively. Formaldehyde is able to react with a great number of functional groups. Most frequently occurs the reaction between formaldehyde and an uncharged nitrogen containing group, such as amino, imino, amide, guanidino groups of amino acids and proteins. Under conditions which do not denature the protein, the sulfhydryl group is still able to react with formaldehyde, while the hydroxyl group of serine presumably do not react with it.*

The amino acids which contain more than one reactive group, can react with formaldehyde in a complex manner with formation of cyclic structures. Naturally the possibilities for such complex reactions is much greater in proteins, where a great number of reactive groups are available. But even the simple amino acids, such as glycine, can react with formaldehyde in many different ways.

K. H. Gustavson (9) infers from his studies on the tanning of native collagen by formaldehyde, that the reaction is determined by the combina-

* See for details *D. French* and *J. T. Edsall,* The reactions of Formaldehyde with Amino Acidsand Proteins. Advances in Protein Chemistry. Vol. II. p. 277. Academic Press. New-York 1945.

tion of formaldehyde with the ε-amino groups of lysine. Reactions with the guanidino groups and peptide linkages occur only above *pH* 8 and do not contribute to the formation of the lattice structure. He assumes, that there are methylene bridge formation involving the amino groups of lysine. *H. Nitschmann* and *H. Hadorn* (10) also show that as in collagen, the lysine ε-amino groups have a decisive role in the tanning process of casein too. They give evidence, that the ratio of bound formaldehyde and of lysine is nearly 1 : 1, which excludes the cross linkage between two lysine groups, the ratio in this case would be 1 : 2. It is more probable that the methylene bridge is formed between a lysine ε-amino and another functional group, such as amide group or the peptide linkage.

Although we have not given direct evidences, it may be assumed that in the conditions described (*pH* 8, relatively low formaldehyde concentrations) the reaction between the lysine ε-amino group and the formaldehyde is the essential step in the formaldehyde clotting. The imidazol ring does not react with formaldehyde and the *pH* is lower as to have a reaction with the guanidino groups. Recent investigations have shown, that fibrinogen does not contain sulfhydryl groups, therefore the possibility of reaction with this group may also be excluded. The *S—S* groups of keratin are able to react with formaldehyde, but this is a very slow reaction and occurs only at very alkaline *pH*, which is not the case in our experiments.

The most frequently observed reactions between formaldehyde and an amino group are the following:

1. $R - NH_2 + CH_2O = R - NH - CH_2OH$
2. $R - NH_2 + 2\,CH_2O = R - N(CH_2OH)_2$
3. $2\,R - NH_2 + CH_2O = R - NH - CH_2 - NH - R$

By an excess of formaldehyde, reaction 1. or 2. occurs. Every amino group binds either one or two molecules of formaldehyde with formation of methoxy derivatives. It is very unprobable that such derivatives can bind together with formation of polyoxymethylen bridges. By the extensive studies of reactions between amino acids and formaldehyde, only by glycine was found a ring formation involving a polyoxymethylene structure. If the concentration of formaldehyde relative to the amino groups is small, reaction 3. occurs with formation of a methylene bridge.

According to reaction 3. formaldehyde is able to link adjacent molecules by methylene bridges, when the two amino groups belong to different molecules. The fibrinogen molecules have many accessible

amino groups, therefore a true lattice structure may be formed. It is very much probable, that this is the clotting mechanism with formaldehyde.

In an excess of formaldehyde every amino group reacts with one or two molecules of formaldehyde and naturally no linkage occurs between the molecules. This was actually observed by adding a great excess of formaldehyde. In this case no clot was formed and the solution did not become turbid, showing that there was no aggregation at all.

The clotting occurs only if the fibrinogen concentration is above a limit. If the concentration is too low, perhaps there are steric difficulties for the formation of a lattice. The fibrinogen molecules are placed at far greater distances from each other to form methylene bridges. The favorable condition for the bridge formation is rare and the methoxy compound undergoes a secondary reaction (cyclisation in the molecule itself etc.) before the bridge formation. Nevertheless in this case also, as shown by the development of turbidity, there is aggregation to some extent, but a clot cannot be formed.

Naturally the formaldehyde concentration also determines whether clotting occurs or not. If the concentration is too low, the few methoxy compounds formed undergoes secondary reactions, before the conditions were favorable to a bridge formation. The fibrinogen molecules have many amino groups which bind formaldehyde, but only a few are in a favorable situation for bridge formation. At low concentration the formaldehyde is bound in greater part in such ineffective linkages and the probability of bridge formation is very low.

The effect of *pH* can be explained very easily. At the isoelectric point almost all the amino groups are charged. The number of uncharged groups available for the formaldehyde is very small. With the increase of *pH* the dissociation of amino groups is diminished and the number of groups susceptible to react with formaldehyde becomes more and more great. The result of this is the shortening of the clotting time and the increasing compactness of the clots.

The clotting process occurs at the alkaline side of the isoelectric point of fibrinogen. The molecules are carrying thus a negative net charge which is increased when the *pH* is increased. The particles have a charge of the same sign, therefore they repel each other. The neutral salts are very effective in eliminating the net charge of protein particles. It is expected thus, that the neutral salts will favour greatly the approach of fibrinogen molecules and their aggregation. Cations decrease negative,

anions eliminate positive charges. Polyvalent ions are more efficient than monovalent ions. The fibrinogen molecule is charged negatively, therefore the cation of the added salt must be decisive in the above phenomenon and the kind of anion must have little or no influence. Actually this is found in the experiments. It was shown that the clot-promoting activity of salts depends only upon the cation and the valency of anion is immaterial. The valency rule of *Hardy—Schulze* is in force. The bivalent cations are about 5—6 times more active than the monovalent ones. The succession of cations corresponds to their polarisability ($K < Na$ and $Mg < Ca$).

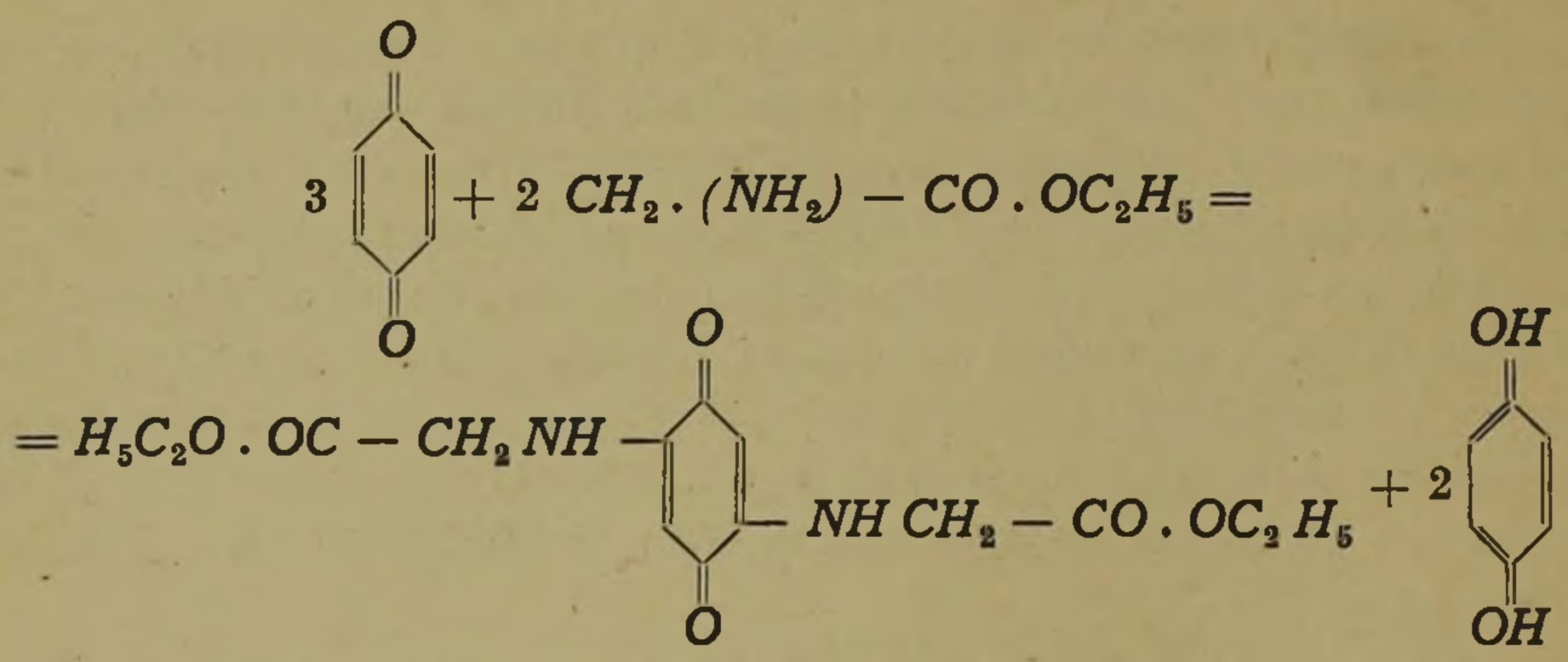
The negative effective charge thus hinders the clotting. The increase of *pH* increases the net charge of particles too. But in the same time also the number of uncharged amino groups is increased. The rise in *pH* causes in this way a double effect: 1. the increase of the negative net charge of the particles hinders the clotting 2. the increase in the number of uncharged amino groups available for the formaldehyde promotes it. It may be assumed that the maximum shape of the clotting curve is the resultant of these two contrary effects. At low *pH* the hindrance is in force, than the promotive effect and in more alkaline solutions the two effects nearly counterbalance each other and the clotting time is constant.

In the presence of salt the effect of charge is vanished. By the increase of *pH* there is apparent only the decrease of clotting time due to the increase of number of uncharged amino groups.

The formaldehyde can react with the fibrinogen in absence of salts too. But in this case the molecules cannot approach each other sufficiently to be bound by methylene bridges. All the amino groups are bound and secondary reactions occur which make the methoxy groups unable for bridge formation. If it is allowed a sufficient time for the accomplishement of the reaction between the formaldehyde and fibrinogen, the addition of salt does not bring about clotting or even turbidity in spite of the fact, that the repelling action of charge is now eliminated.

The reactions of p. quinone with amino acids and proteins are very similar to those of formaldehyde. p. Quinone reacts, as formaldehyde does, with the amino and sulfhydryl groups. The mechanism of reactions involved is far less elucidated as by formaldehyde.

E. Fischer and *H. Schrader* (11) obtained crystalline products from glycine aethylester and p. quinone in alcohol. The reaction is accompanied with hydroquinone formation and can be sketched as follows:



$$3 + 2\ CH_2 . (NH_2) - CO . OC_2H_5 =$$

$$= H_5C_2O . OC - CH_2 NH - NH\,CH_2 - CO . OC_2 H_5 + 2$$

S. Hilpert and *F. Brauns* (12) have also obtained well defined compounds between different quinones and glycine anilide. They investigated also the tanning effect of p. quinone on skin powder in different conditions. It is possible, by determining the amount of quinone consumed and the hydroquinone formed, to decide whether one or two amino groups react with one molecule of quinone. This can be seen from the following equations:

1. $2\,C_6H_4O_2 + NH_2 - R = C_6H_3O_2\,(NH - R) + C_6H_6O_2$.
2. $3\,C_6H_4O_2 + 2\,NH_2 - R = C_6H_2O_2\,(NH - R)_2 + 2\,C_6H_6O_2$.

In acid medium the reaction between the skin powder and the p. quinone was of type 1. In neutral or weak acid medium the amount of hydroquinone formed was much less. They assume, that in this conditions a great part of the hydroquinone is bound to the quinone amino compound formed in a way similar to the quinhydron formation.

In alkaline medium the process is complicated by the polymerisation of quinone. *Hilpert* and *Brauns* claimed, that the resulting polymer oxyquinone is bound in an adsorptiv way by the skin powder.

The sulfhydryl group is also able to react with p. quinone, as it was demonstrated by *R. Kuhn* and *H. Beinert* (13) who obtained heterocyclic compounds from cysteinester and p. quinone.

It is clear, that in the above experiments the quinone has combined with the amino groups of fibrinogen. The sulfhydryl group can be excluded for the already mentioned circumstances. The oxidative action of quinone is also very unprobable. Only the *SH* groups can be oxidized in the described conditions, but the fibrinogen does not contain *SH* groups.

The reaction between quinone and fibrinogen is accompanied with the same colour development as in the case of the reaction between

glycine and quinone. With the exact nature of the reaction involved we shall deal more extensively in an other paper. It can be stated only, that the reaction involves the free amino groups of fibrinogen. The amount of hydroquinone formed is very small, which is in agreement with the findings of *Hilpert* and *Brauns* by skin powder in neutral medium.

The quinone concentration which causes clotting of fibrinogen is much lower, than that of formaldehyde. Similarly the minimal fibrinogen and salt concentrations in presence of which clotting occurs is much lower by quinone than by formaldehyde. Perhaps, in this case the dimensions of the formaldehyde and quinone molecules are the causes of the quantitative differences. The quinone can bind molecules more distanced, than the small formaldehyde molecule.

The salt concentration required by the quinone clotting is about 1/5 of that with formaldehyde. This is the explanation why the *pH* curves of quinone clotting are the same, whether the clotting is performed in the presence or in the absence of added salt. Namely, the salt given with the buffer is sufficient to bring about the salt effect.

From the experiments performed with formaldehyde and thrombin the conclusion can be drawn, that the point of attack of both of them is the same, i. e. the amino groups of fibrinogen. At neutral reaction only the imidazol groups of hystidine are in uncharged form, but the formaldehyde does not react with these groups. The amino groups of lysine are dissociated, therefore the formaldehyde can react with them only very slowly. This is the reason why the formaldehyde has no immediate action upon the clotting with formaldehyde at neutrality. As the *pH* increases the number of undissociated amino groups is also increased. The formaldehyde can react with these instantaneously, substracting them from the action of thrombin. The formaldehyde inhibition is in this way more and more marked as the *pH* rises, when the available amino groups are more and more numerous.

This is the situation if the thrombin is added immediately after the formaldehyde addition. But even in the case when formaldehyde cannot react instantaneously a slow reaction takes place. The equilibrium between the charged and uncharged amino groups is disturbed by the removing of uncharged forms, which combine with formaldehyde. To reestablish the equilibrium new uncharged groups are forming, which may react now with formaldehyde. In this way slowly all the amino groups can bound formaldehyde even at a *pH* where the amount of uncharged amino groups is very low. With the course of reaction the number of free amino groups of fibrinogen is diminished. It may be supposed that the action of thrombin requires also uncharged amino groups and the

clotting is quicker if the number of these is greater. With this assumption it is easy to explain the behaviour of formol-treated fibrinogen against thrombin at different *pH*. The *pH* curve of clotting is more and more shifted towards alkaline *pH* with the duration of the formaldehyde action. The total number of free amino groups is diminished by the action of formaldehyde, therefore to have the same number of undissociated amino groups one must raise in the clotting test the *pH* gradually as the reaction with the formaldehyde proceeds.

We meet difficulties in the explanation of the *pH* curves of thrombin clotting. It is obvious from the differences between the curves obtained without and with salt, that here again electrostatic forces play also a role. In the *pH* range 5,9 to 10,5 the curves of thrombin and formaldehyde clotting without salt are as if the mirror images of each other. We cannot give the cause of this. The curves with added salt are very similar in all the three cases investigated. The range of clotting is nearly the same by thrombin and the synthetic clotting agents. The conclusion can be drawn, that clotting with formaldehyde, quinone and thrombin are related phenomenons. We do not suggest, that thrombin or a part of it is a compound similar to formaldehyde or quinone, but only that the principle of the mechanism of thrombin clotting may be similar to that of these clotting substances.

We are indebted to Prof. *K. Laki* for his kind advices and guidance.

SUMMARY.

1. The clotting of fibrinogen with formaldehyde and p. quinone depends on four factors:

a) the concentration of fibrinogen,

b) the concentration of formaldehyde or quinone,

c) the *pH*,

d) the concentration and nature of the ions present.

2. Formaldehyde has an inhibiting activity on the clotting of fibrinogen with thrombin. The effect shows a great *pH* dependence.

3. The incubation of fibrinogen with formaldehyde diminished the clotting ability of fibrinogen with thrombin.

4. The *pH* curve of thrombin clotting was investigated.

5. The results are discussed and concluded, that the clotting with formaldehyde and p. quinone is determined by their reaction with the amino groups of fibrinogen. The same groups are involved in the clotting of fibrinogen with thrombin too.

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STUDIES ON THE TITRATION CURVES OF FIBRINOGEN AND CLOTTED FIBRINOGEN.

WITH 5 FIG. IN TEXT.

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Till now with different theories, the problem of clotting of blood is not yet solved. Sufficient confirmation for a single theory disproving others is not there. *E. Chargaff* (1) is right when he says that the problem will not be solved completely before clotting of pure fibrinogen is fully understood. He further suggests that studies with very pure components e. g. fibrinogen, fibrin, thrombin etc. will help a great deal to elucidate the problem. Further, the physico-chemical data of fibrinogen and fibrin — the two essential proteins in the process of blood clotting — are in no way exhaustive. *E. Chargaff* (1) has mentioned "most analyses have been carried out with fibrin thus assuming what remains to be shown, namely, that the chemical changes involved in the conversion of fibrinogen to fibrin are too small to affect the chemical composition of the respective proteins noticeably".

The present work was initiated keeping above point of view in mind with twofold ideas firstly, to study the titration curves of fibrinogen to compare with the existing data of fibrin and fibrinogen and secondly, to study the curves when fibrinogen is clotted with thrombin in an isolated system.

Fibrinogen of very high purity could be obtained after the method of *D. Bagdy* (2) and thrombin could be obtained after the method of *K. Laki* and *L. Lóránd* (3). The difficulty in this type of measurement when the protein is precipitated and a heterogenous system is formed, is solved in the present work by carrying out the experiments in a 10% urea solution (4). It is known that only at the extreme *pH* range urea has any acid and base binding capacity (5). The urea solution shifts the isoelectric point and influences ionisation (6) and it can be assumed that the effect of urea on the curves of fibrinogen and clotted fibrinogen is the same. Thus the curves obtained in urea solution may be compared for our purposes.

From the titration curve of fibrinogen in urea solution the above effects are observed but no drastic effect on the molecule of the protein is observed.

EXPERIMENTAL.

The fibrinogen used in the experiments is prepared always after the method of *D. Bagdy* (2) and it contained a small amount of *NaCl* as the solution of protein was dialysed against 2% *NaCl*. The amounts of *NaCl* present in the experiments are given with each experiment.

To have a titration curve of fibrinogen and to calculate the acid and base binding capacities, the experiments are done with *N*/10 *HCl* and *N*/10 *NaOH* containing the same concentration of *NaCl* using a *U*-tube Hydrogen electrode in a Cambridge Portable *pH*-meter. The equivalent acid and base bound to the protein is calculated following the method which *M. Dubuisson* used for myosine (7).

To find the action of urea solution on the titration curve of fibrinogen, fibrinogen solution and fibrinogen in 10 and 20% urea solutions are titrated with *N*/10 *HCl* and *NaOH* using a Glass electrode system in the mentioned instrument.

To compare the curves of fibrinogen solution in 10% urea and those of thrombin clotted fibrinogen solution in the same urea solution, the experiments are done in the above way and the values obtained for equivalent base and acid bound are plotted against the *pH*.

The formol titration curve, which is obtained by titrating in the above way a solution of fibrinogen containing 2,5% formaldehyde, is compared with that of fibrinogen titration curve under the same conditions only addition of formaldehyde is ommitted in the later. From the titration values the equivalent acid and base bound is calculated as before and the curve is plotted against *pH*.

a) *Titration curve of fibrinogen and calculation of acid and base bound to the protein :*

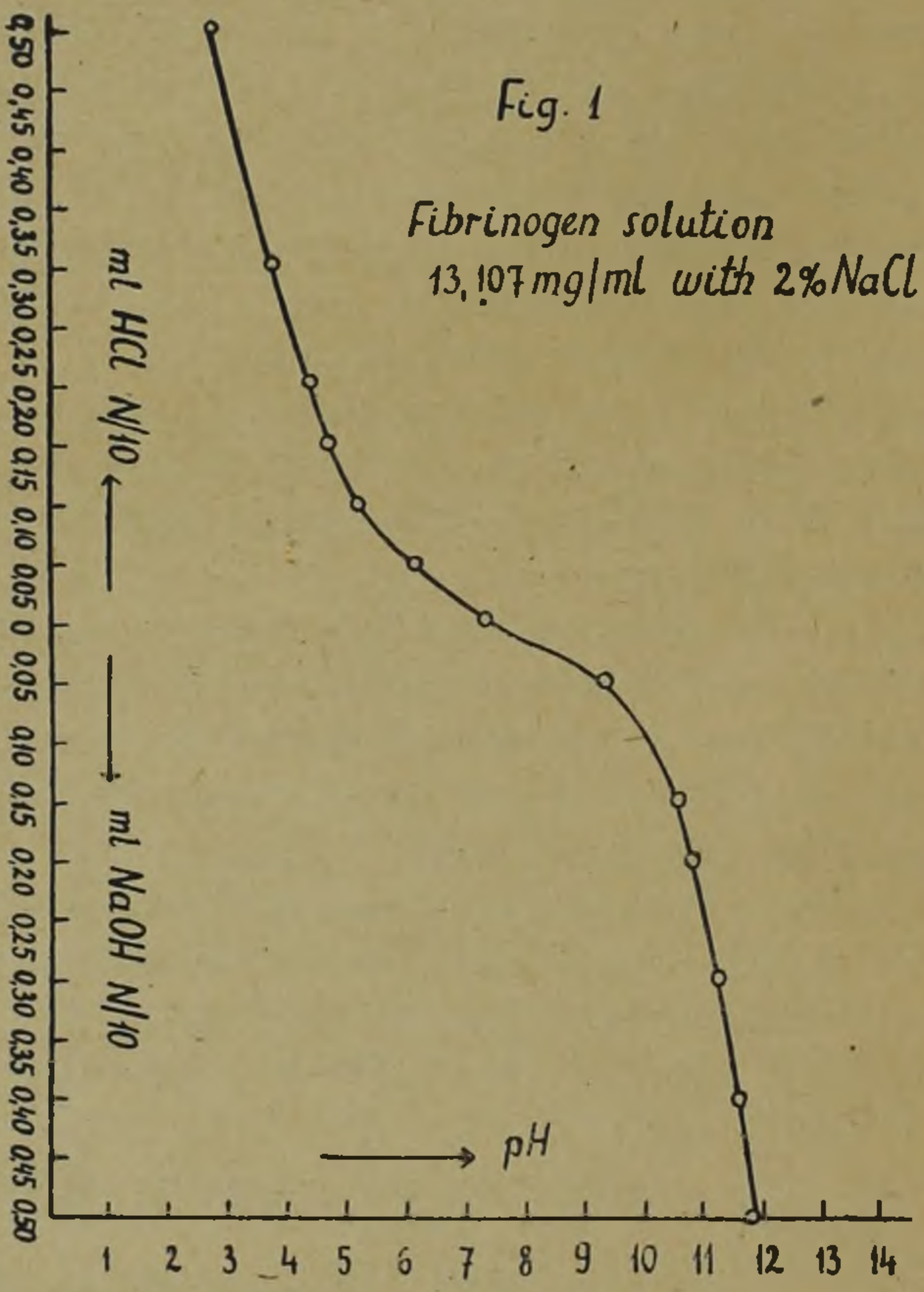
Three different solutions containing different amounts of fibrinogen are titrated separately. The amount of fibrinogen for each solution is estimated from Kjeldahl Nitrogen.

The procedure used is as follows:

To 1 *ml* of fibrinogen solution containing 2% *NaCl*, different amounts of *N*/10 *HCl* or *NaOH* are added from a microburrette separately just before each experiment. The acid and base also contained 2% NaCl. The volume of the mixture in every case was brought to 3 ml by adding the required amount of 2% *NaCl* solution. The solution without acid

or base is brought to 3 *ml* volume by adding 2 *ml* of 2% *NaCl* solution, and taken to be 0 *ml* acid or base.

The solution is taken into the *U*-tube Hydrogen electrode and the e. m. f. is measured as usual, e. m. f. measured in millivolts is converted to *pH* with necessary temperature corrections. In Fig. 1 a titration curve is shown. The end concentration of fibrinogen in this case is 13,107 *mgm/ml.*



In Fig. 2 the curve is drawn from three titration curves with end fibrinogen concentration of 10,152, 13,107 and 19,662 *mgm/ml.* The equivalent acid or base/*gm* $\times 10^{-5}$ of fibrinogen is plotted against the *pH*. The calculation is done as *M. Dubuisson* (7). The isoelectric point is chosen as 5,3. In calculation this point is chosen as the 0,0 point and the bound acid and base is calculated from the amount of added acid or base taking all the time consideration of the amount of free acid and base at different *pH*. The activity coefficient is considered to be unity

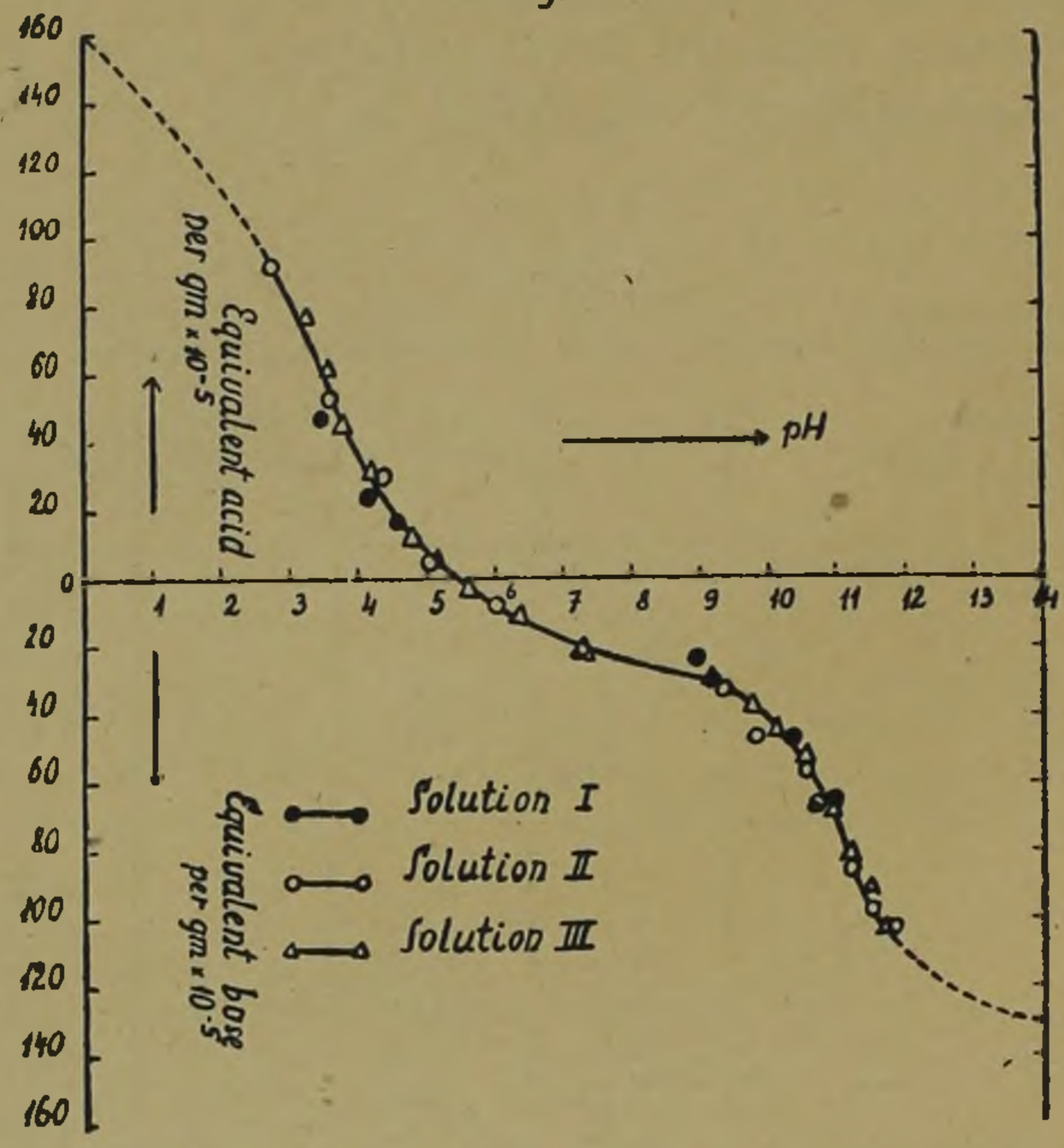
between the *pH* range of 3—11. Beyond this range it is calculated under the same conditions from the determinations of *pH* of *N*/10, *N*/100, and *N*/1000 solutions of *HCl* and *NaOH*. In Table 1 the results of cal-

TABLE I.

End concentration Of Fibrinogen Solution	*pH*	Acid or Base added $m \times 10^{-4}$	Free Acid or Base $m \times 10^{-4}$	Acid or Base Bound $m \times 10^{-4}$	Equivalents of Acid or Base per $gm \times 10^{-5}$
I. 1 *ml* solution contains 10,152 *mgm*	8,94	2,5	0,0075	2,49	24,5
	10,33	5,0	0,184	4,816	47,4
	10,90	7,5	0,684	6,816	67,1
	4,24	2,5	0,057	2,443	24,0
	3,45	5,0	0,357	4,643	45,8
II. 1 *ml* solution contains 13,107 *mgm*	7,27	0,00	0,000	2,66	20,3
	9,30	1,66	0,017	4,315	32,9
	9,81	3,33	0,055	5,944	45,3
	10,50	5,00	0,272	7,394	56,4
	10,76	6,66	0,495	8,837	67,4
	11,19	10,00	1,400	11,266	85,9
	11,54	13,33	3,142	12,857	98,0
	11,81	16,66	5,851	13,481	102,8
	6,06	1,66	0,0009	−1,0075	−7,6
	5,10	3,33	0,0079	0,659	5,02
	4,62	5,00	0,024	2,32	17,7
	4,32	6,66	0,047	3,953	30,1
	3,69	10,00	0,204	7,13	54,3
	2,77	16,66	1,716	12,284	93,7
III. 1 *ml* solution contains 19,662 *mgm*	7,27	0,00	0,000	4,00	20,3
	9,16	1,66	0,012	5,654	28,7
	9,71	3,33	0,044	7,289	37,0
	10,11	5,00	0,111	8,889	45,2
	10,50	6,66	0,272	10,394	52,8
	10,90	10,00	0,684	13,316	67,7
	11,11	13,33	1,11	16,222	82,5
	11,43	16,66	2,317	18,349	93,3
	11,67	20,00	4,027	19,973	100,5
	6,27	1,66	0,000	−2,34	−11,9
	5,68	3,33	0,002	−0,669	−3,4
	5,06	5,00	0,008	0,992	5,0
	4,75	6,66	0,0177	2,649	13,4
	4,24	10,00	0,0575	5,943	30,2
	3,83	13,33	0,148	9,185	46,7
	3,69	16,66	0,204	12,462	63,3
	3,29	20,00	0,513	15,487	78,7

culation are shown. The maximum acid and base binding capacity of the fibrinogen solution has been obtained by extrapolation as shown in Fig. 2 in the dotted lines, in the extremities.

Fig. 2



b) *Effect of urea on the titration curve of fibrinogen:*

30 *ml* of a fibrinogen solution containing 2,83 *mgm/ml* is taken. The solution contained 0,3% *NaCl*. To this, sufficient solid urea is added to bring the urea concentration to 10% and 20%.

The solution with fibrinogen and that with urea of above two concentrations are titrated with *N*/10 *HCl* and *NaOH* in a Glass electrode system. Curves are drawn with the values against the amounts of acid or base added. These curves are shown in the Fig. 3. The *pH* range of the experiment was between 3—11.

c) *The titration curve of fibrinogen and clotted fibrinogen in* 10% *urea solution:*

35 *ml* of fibrinogen solution containing 9,767 *mgm/ml* and 0,7% *NaCl* are taken. Sufficient solid urea is added to bring the concentration of urea to 10%. The solution is titrated with *N*/10 *HCl* and *NaOH* as before in the Glass electrode system.

For clotted fibrinogen, to 35 *ml* of above fibrinogen solution without urea, 1 *ml* of pure thrombin solution is added. The clot is allowed to stay for 30 minutes and dissolved by adding solid urea. The amount of urea added was sufficient to make the concentration of it in the solution 10%, calculated on the basis of 35 *ml*. The solution is titrated, when the protein has gone into solution, as in the previous case.

Fig. 4

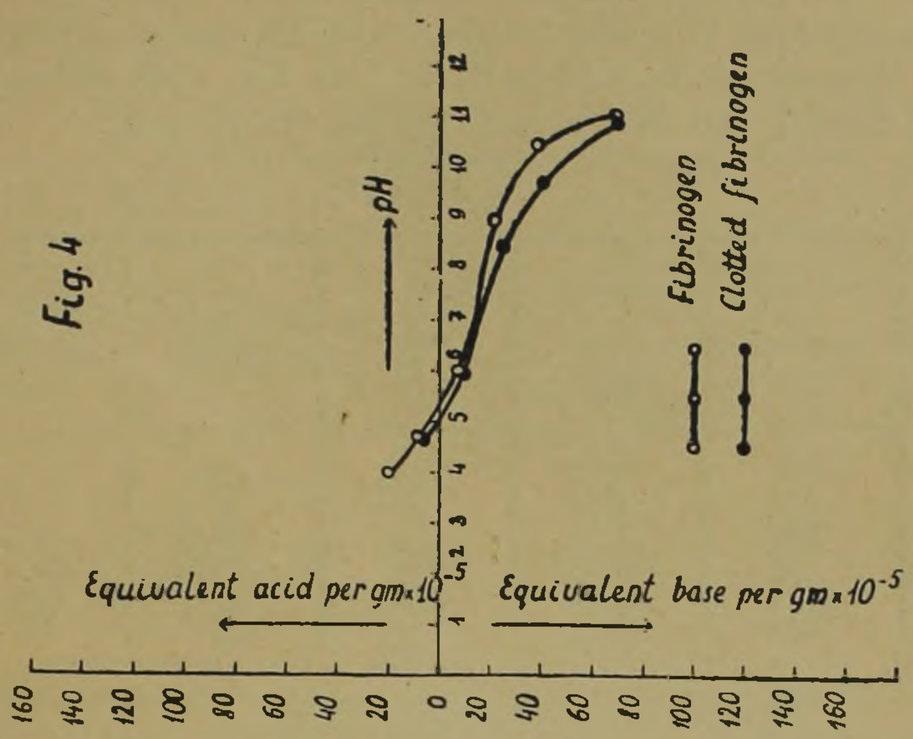
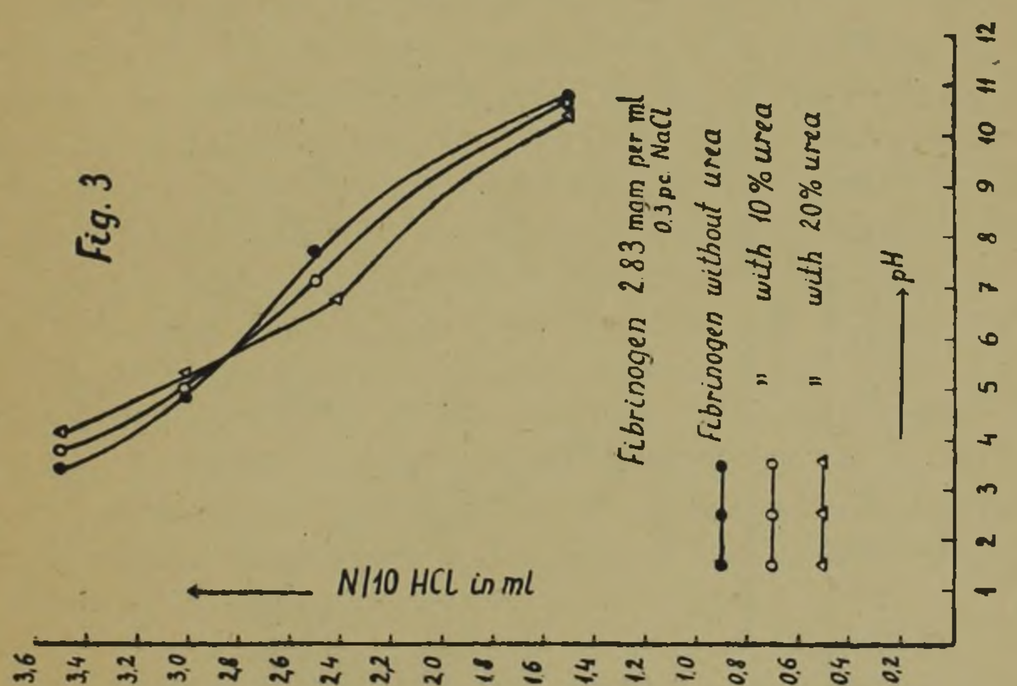

pH
Fibrinogen
Clotted fibrinogen
Equivalent acid per gm × 10
Equivalent base per gm × 10⁻⁵

Fig. 3


Fibrinogen 2.83 mgm per ml
0.3 pc. NaCl
Fibrinogen without urea
" with 10% urea
" with 20% urea
N/10 HCl in ml
pH

From the curves obtained the equivalent acid and base bound is calculated without taking into consideration the amount of free acid or base as within this range of *pH* 4—10 the amount is very small. Teh values obtained can be seen in Table 2. In Fig. 4 the curves drawn with equivalent acid and base against the *pH* can be seen.

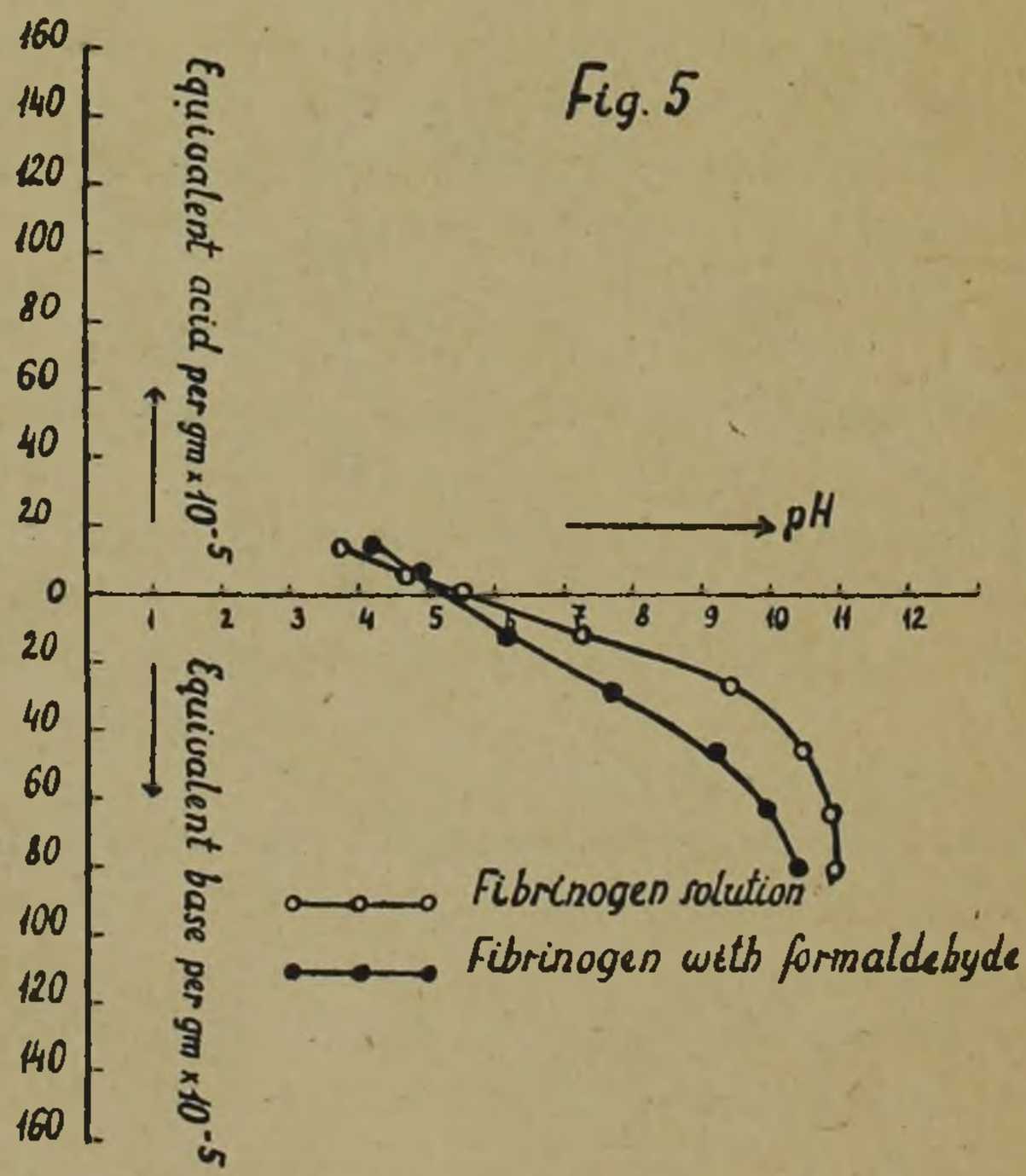
TABLE II.

Concentration of fibrinogen solution 9,767 *mgm/ml*		Acid or Base added $m \times 10^{-4}$	Acid or Base Bound $m \times 10^{-4}$	Equivalents of Acid or Base per *gm* $\times 10^{-5}$
fibrinogen *pH*	clotted fibrinogen *pH*			
5,95	6,10	0,00	−0,785	−8,5
4,72	4,62	1,42	0,642	6,5
3,90	3,80	2,82	2,043	20,9
8,96	8,50	1,42	2,212	22,6
10,40	9,55	2,82	3,613	36,9
10,94	10,90	5,71	6,499	66,5

d) *Titration curve of fibrinogen and that with* 2,5% *formaldehyde:* 35 *ml* of fibrinogen solution containing 14,652 *mgm/ml* and 1% *NaCl* are taken. It is titrated in the previous manner with *N*/10 *HCl* and *NaOH*.

To the same amount of previous solution sufficient neutralized 40% formaldehyde is added to make the final concentration 2,5%. A loose gel is formed. It is titrated as before.

Fig. 5



From the curves obtained the equivalent acid and base bound is calculated as in (c). The results are in Table 3. The curves drawn with these values against *pH* can be seen in Fig. 5.

TABLE III.

Concentration of fibrinogen solution 14,652 *mgm/ml*		Acid or Base added $m \times 10^{-4}$	Acid or Base bound $m \times 10^{-4}$	Equivalents of Acid or Base per *gm* $\times 10^{-5}$
fibrinogen *pH*	fibrinogen in 2,5% formaldehyde *pH*			
5,52	5,58	0,00	0,375	2,5
7,20	6,10	1,25	1,625	11,0
9,56	7,64	3,75	4,125	28,1
10,54	9,20	6,25	6,625	45,2
10,80	9,90	8,75	9,125	62,2
10,96	10,36	11,25	11,625	79,3
4,70	4,75	1,25	0,875	5,9
3,85	4,18	2,50	2,125	14,5

DISCUSSION.

The titration curve of fibrinogen (Fig. 1) is similar to any protein titration curve.

The curve obtained by plotting equivalent acid and base bound against *pH* (Fig. 2) is very similar to that of myosine obtained by *M. Dubuisson* (7). The maximum binding capacity of base by fibrinogen as obtained by extrapolating the curve in the alkaline region comes to 130. The value obtained by *D. Van Slyke* (8) for cattle fibrin from analytical data is 130,5. The calculated value on the basis of amino acid data supplied by *J. T. Edsall* (9) comes to very close to 130 for fibrinogen.

From the acid region the maximum acid binding capacity comes to 160. The value could not be calculated from the analytical data as the amount of amide could not be obtained from the literature available. The value obtained by *R. Nordbő* (10) with fibrinogen comes to 164.

Thus the values obtained by the experiments are in accordance with the available data. The values on the other hand confirm that pure fibrinogen which is used does not differ in free carboxyl and amino groups from fibrin as the analytical data of fibrin shows.

From Fig. 3 the curves for fibrinogen and fibrinogen in 10 and 20% urea solutions do not differ much. The change obtained with urea can be compared with the curve of carboxy-haemoglobin under different ionic strengths as obtained by *E. J. Cohn, A. A. Green* and *M. H. Blanchard* (11). Also the values of egg albumin under different ionic strengths

as obtained by the study of *R. K. Cannan, A. Kibrick* and *A. H. Palmer* (12) show a similar change. In these cases under the influence of different ionic strengths, the dissociation is affected.

Urea in the range of the experiment may be supposed to have a dipolar structure and thus it brings about similar effect as a polar ion does (5). The results from the curve show this change. In the urea solution the curve is rotated. The rotation again, increases with higher concentration of urea. In urea solution there is no increase in the titrable amino or carboxyl group and the acid-base binding capacities are not altered. Thus the study in the titration of fibrinogen and clotted fibrinogen in urea solution is possible and thus the advantage of study in an homogenous system is provided.

In Fig. 4 in the curve of clotted fibrinogen compared to that of fibrinogen there is a difference, which can be explained by assuming a shift in the *pK* value of basic group. The shift comes in the region of ε-amino group of lysine and shows that the basicity decreases.

The curve with formaldehyde (Fig. 5) on the other hand shows a marked shift almost double that we have with thrombin, and the shift occurs in the same region. With formaldehyde it is known that the basicity of ε-amino groups of lysine is decreased. The amino groups undergo a reaction with formaldehyde and change to secondary and tertiary groups. In the case of clotted fibrinogen it can be suggested that a similar change has occurred.

L. Nanninga (13) has demonstrated by formol titration that clotted fibrinogen contains about half compared to the amino group of fibrinogen. In the experiment here the diminishing of amino groups supports the finding of *L. Nanninga*.

Also, it has been found by *E. Mihályi* and *L. Lóránd* (14) in working with synthetic clotting agents on fibrinogen that the decisive step in clotting is a reaction with amino groups and with thrombin a similar reaction occurs.

The data as obtained in the above experiments show that it is impossible that there is an increase in amino groups as claimed by some workers (15). There is a shift in the *pK* value of amino group of lysine only. The experiment thus goes against the proteolytic theory of the clotting process. The shift in the *pK* value suggests that the amino groups of lysine take part in the process of clotting. Comparing the curves of formol titration with those obtained by titration of fibrinogen and clotted fibrinogen in urea solution, one is inclined to uggest that during the clotting process a similar change occurs. Thussit supports the theory of compound formation during the process

SUMMARY.

1. Maximum acid and base binding capacity of fibrinogen is studied and compared with the data of fibrin and fibrinogen. They are found to be very close.

2. Titration of fibrinogen in different strengths of urea solution is done and it is found that fibrinogen does not change in free amino or carboxyl group in the urea solution.

3. Acid and base equivalents of fibrinogen and thrombin-clotted fibrinogen are studied in isolated system and a shift in the *pK* value of amino groups of lysine is only observed.

4. The clotted fibrinogen curve when compared to the formol titration curve it is found that the changes occured are similar.

I am grateful to Professor *K. Laki,* who initiated me in the work and I am much indebted to *Dr. E. Mihályi,* who helped me in all possible way.

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INDUCED MUTATION ON BACILLUS ANTHRACIS AND BACILLUS MESENTERICUS.

WITH 8 FIG. IN TEXT.

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It has been established by a number of worker *(Stamatin, (5) Schaefer, (6) Sterne, (7) Buza (2)* and others) that virulent anthrax bacilli which on agar plates under usual atmospheric conditions form medusoid *(„R',)* colonies consisting of non capsulated bacilli, under morde suitable conditions, especially in a CO_2 containing atmosphere, develop *„S"* colonies built up by capsulated bacilli. Similarly it is known that from the peripherq of such mucous *"S"* colonies now and then projections may spread (fig. 1) consisting mainly of non capsulated bacilli mixed with more or less numerous capsulated specimens. By careful isolation of the non capsulated bacilli it is possible to obtain pure lines of bacilli absolutely devoid of the capsule forming property. The loss of te capsule forming property is the consequence of a mutation, the mutant being characterized in cultures on agar, even in presence of CO_2 by the formation of *„R"* colonies with the well known medusoid surface (fig.3). The lack of the capsule forming property is concomitant with the loss of virulence. This fact was observed in our Institute by *Buza* on more than 200 strains. It is to be emphasized that the disappearance of the capsule forming property in case of the dissociation from the *„S"* to the *„R"* type is, as mentioned, by all means the result of a mutation, for it has been evidenced by careful tests that anthrax bacilli which have lost their capsule forming capacity and therewith their virulence, never do regain in subcultures these properties.

Similar observations have been made in this Institute on a number of strains of the non pathogenic Bacillus mesentericus (vulgatus). Strains of this species immediately after their isolation from the soil showed a very extensive capsule forming ability and developed on agar plates

"*S*" colonies of a very definite sliminess, but, in contrast to Bacillus anthracis, also under normal atmospheric conditions. The "*S*" colonies of Bacillus mesentericus, more regularly than colonies of Bacillus anthracis spread also filaments into the surroundings, these projections consisting as in the case of Bacillus anthracis, mainly of uncapsulated bacilli mixed with a few capsulated individuals (fig. 2). The appearance of the non capsulated form, similarly as in the case of Bacillus anthracis, is the result of a mutation, and this mutant in pure cultures develops "*R*" colonies characterized by a granular surface and the lack of any kind of sliminess (fig. 4).

Thus by a careful isolation of both uncapsulated anthrax bacilli and uncapsulated mesentericus bacilli we obtained a number of pure mutants which even after a long series of subcultures (concerning some of our strains after more than hundred subcultures) never produced new variants and never regained their capsule forming property.

It is of importance to note that *Griffith* (3) and others were able to change non capsulated pneumococci of an "*R*" type into a capsulated "*S*" form by mixing them in the animal body or outside of the animal body with large amounts of killed capsulated pneumococci or extracts of pneumococci of an "*S*" type. Besides the new "*S*" form was correlated with the appearance of virulence and of new antigenic properties. *Avery, Mc Leod* and *Mc Carty*, (1) and *Mc Carty* and *Avery* (4) were even able to demonstrate that there is the question of an induced alteration caused by the transforming ability of a hypothetic substance, presumably protein, carried by a desoxyribonucleic acid. If it is allowed to speak in bacteriology of genes, we should like to say that in this case the mentioned desoxyribonucleic acid served as gene in producing a new combination in the hereditary material of the original pneumococci.

In view of these facts the question arose whether it might be possible to transform uncapsulated mutants of Bacillus anthracis and Bacillus mesentericus into capsulated variants by mutual interchange of their genes.

In an approach of this idea our first attempt was as follows. Non capsulated living anthrax bacilli were triturated in a mortar with a bacteria free extract of capsulated mesentericus bacilli. After the inoculation of agar plates with such mixtures, there appeared, even under usual atmospheric conditions, in some cases among typical "*R*" colonies of anthrax bacilli smooth colonies. These "*S*" colonies showed either the characteristics associated with the usual "*S*" type of capsulated mesentericus bacilli with dark umbonate centre (fig. 5), or were of a highly developed sliminess, but homogeneous without forming an elevation amidst (fig. 6),

both forms of colonies consisting of capsulated bacilli. The more, the bacilli receiving the capsule forming ability, were provided with a peritrichal flagellar apparatus and in consequence motile.

The same result has been obtained by inoculation of nutrient broth containing a bacteria free extract of mesentericus bacilli with living non capsulated anthrax bacilli and after a 24 hours incubation at 37 C⁰ by separation of the grown bacilli on agar plates.

By these procedures we obtained till now in 6 out of 24 experiments pure lines of anthrax bacilli which acquired a new capsule forming property and in addition to that a flagellar apparatus too. We feel entitled to conclude that the acquisition of these features has to be looked upon as the result of an induced mutation, with particular reference to the fact that the new variant maintained its new hereditary material in the same manner as do originally capsulated anthrax bacilli or mesentericus bacilli. A close examination of the sofar obtained 30 subcultures could not disclose the loss of either the capsule forming property or of the flagellar apparatus. The only change we observed, was the fact that some colonies of the new mutant, more frequently than do capsulated anthrax bacilli, but more seldom than do capsulated mesentericus bacilli, showed the appearance of projections built up mostly by uncapsulated bacilli. Thus in this respect the mutant behaved like originally capsulated anthrax and mesentericus bacilli which, as we pointed out, show also the tendency to develop by dissociation a non capsulated mutant. It is of interest to note that the non capsulated mutant which participates in the projections of the colonies of our described mutant, forms colonies of the type of non capsulated mesentericus bacilli.

As to the morphological character of the mutant we obtained by treating uncapsulated anthrax bacilli with extracts of capsulated mesentericus bacilli, it might be added that the bacilli on the whole show a resemblance to capsulated mesentericus bacilli rather than to anthrax bacilli. Apart from the fact that the bacilli are flagellated (fig. 7), their capsules are of a less compact structure than is the case in capsulated anthrax bacilli, but appearing exceedingly slimy they show the tendency to dissolve, so that parts of the capsule forming substance may be detected in the neighbourhood of the bacilli in the form of slimy stripes and crumbs (fig. 8). Likewise the bacilli are somewhat smaller than anthrax bacilli and resemble also in this respect mesentericus bacilli.

Judging from what appears to be a mere morphological viewpoint the suggestion might be impressive that the new mutant, as to its formal behaviour, derived its hereditary material mainly from the mesentericus bacilli, while the anthrax bacilli played part as the donor of its vitality

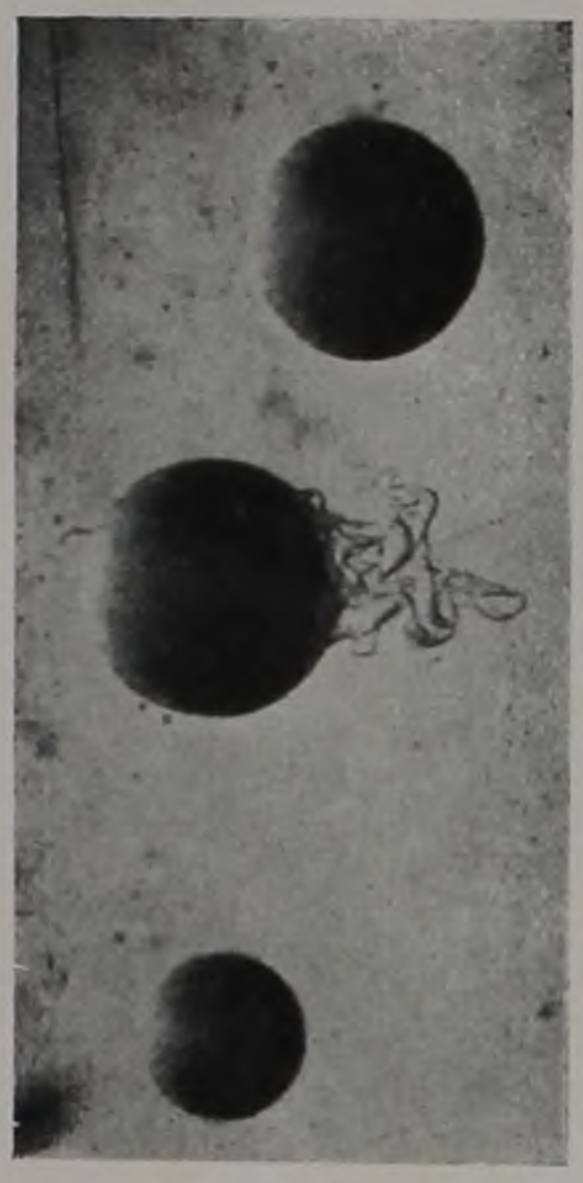

Fig. 1. — *Bacillus antracis*. „S" colonies grown on agar in CO_2 containig atmosphere, after 24 hours. One colony with projections. 1:10.

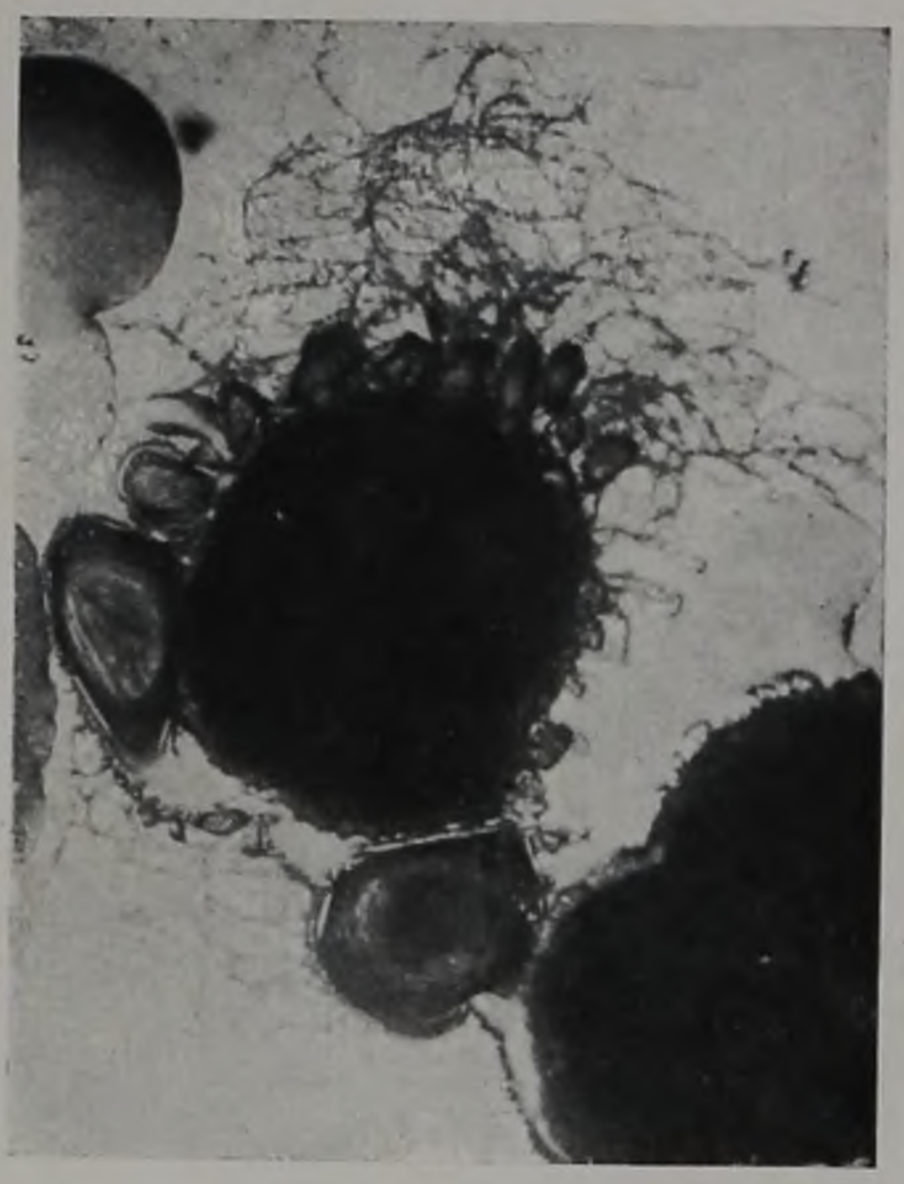

Fig. 2. — *Bacillus mesentericus*. „S" colonies grown on agar under usual atmospheric conditions, after 24 hours. One colony with projections. 1:10.

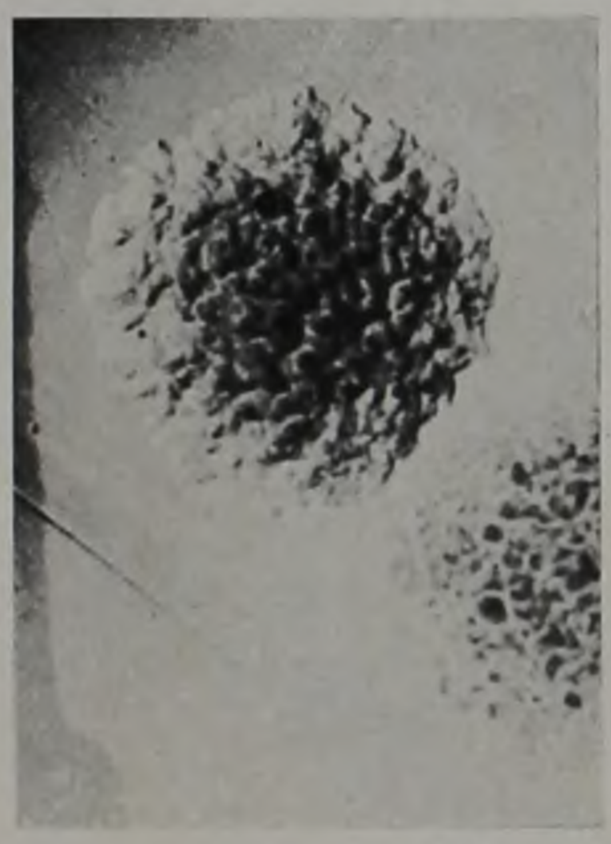

Fig. 3. — *Bacillus anthracis*. „R" colony grown on agar in a CO_2 containing atmosphere, after 16 hours. 1:20.

Fig. 4. — *Bacillus mesentericus*. „R" colonies on agar grown under usual atmospheric conditions, after 16 hours. 1:20.

Fig. 5 and 6. — *Bacillus anthracis after treatment with extract of Bacillus mesentericus.* „S" colonies grown on agar under usual atmospheric conditions, after 16 hours. 1:20. Fig. 5: confluent colonies with dark umbonate centre, fig. 6: a homogeneous slimy colony.

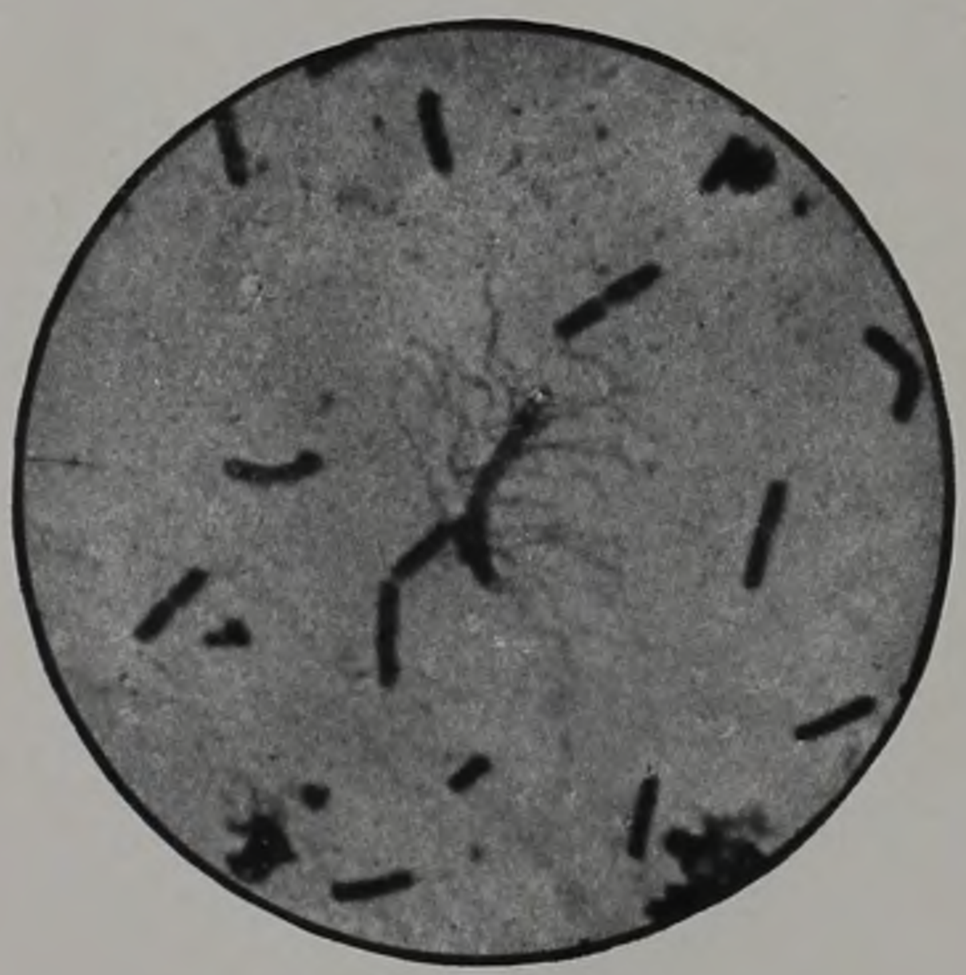

Fig. 7. — *Bacillus anthracis after treatment with extract of Bacillus mesentericus.* Flagellated bacilli. Stained after *Zettnow*'s method. 1:2,000.

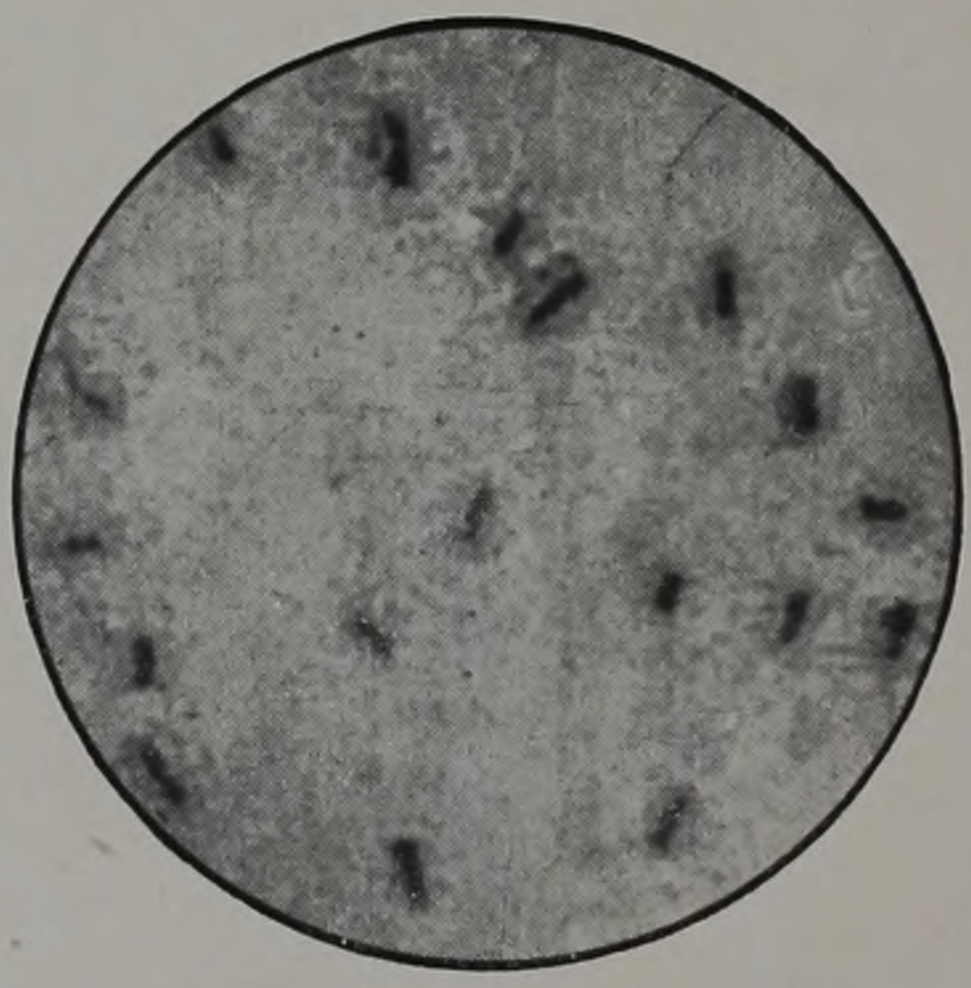

Fig. 8. — *Bacillus anthracis after treatment with extract of Bacillus mesentericus.* Capsulated bacilli. Stained with carbol thionin. 1:2,000.

only. There may be, however, no doubt that as far as the participation of both bacilli in constituting the mutant is concerned, a close chemical and serological investigation might be expected to throw light upon the details of inheritance.

Further experiments led to the observation that the uncapsulated mutant of Bacillus mesentericus after treatment with bacteria free extracts of capsulated anthrax bacilli may also undergo a mutation, as after such a procedure it is possible to isolate capsulated mesentericus bacilli. Thus we succeeded in obtaining such mutants in 3 out of 18 experiments. The mesentericus bacilli which were gaining in this way a capsule forming capacity, showed the same morphological features as originally capsulated mesentericus bacilli, and even their colony formation resembled that peculiar to this species. It must be noted, however, that the bacilli of the new mutant grow under other conditions in slimy ("*S*") colonies as either originally capsulated mesentericus bacilli or originally capsulated anthrax bacilli. On ordinary agar, in contrast to originally capsulated mesentericus bacilli, the bacilli of the mutant do not develop capsules and form, therefore, instead of slimy, as a rule "*R*" colonies, while for instance on Huddleson's liver agar they grow capsulated and form slimy colonies, but, in contrast to capsulated anthrax bacilli, also under normal atmospheric conditions, i. e. in absence of CO_2.

The variant obtained by mixing living non capsulated mesentericus bacilli with bacteria free extracts of capsulated anthrax bacilli seems to be, like the above described mutant of anthrax bacilli, the result of a true mutation, as until the 20th subculture we dispose sofar, we maintained it in its unchanged possession of the capsule forming property. However, it is to be noted that, similarly to the described anthrax mutant, several of the slimy colonies spread filaments containing non capsulated bacilli which as a result of a mutation have lost their capsule forming property and therewith the capacity of forming slimy colonies.

SUMMARY.

By mixing non capsulated living anthrax bacilli with bacteria free extracts of capsulated mesentericus bacilli, and similarly by mixing non capsulated living mesentericus bacilli with bacteria free extracts of capsulated anthrax bacilli there have been obtained constant strains of capsulated and flagellated bacilli which develop "*S*" colonies. It is pointed out that in both cases there is the question of the result of an induced mutation caused by the introduction of genes of the one species into the other.

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THE EFFECT OF METHYLTHIOURACIL ON HORMONAL THERMOREGULATION IN COLD ENVIRONMENT.

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(RECEIVED FOR PUBLICATION ON 1. 4. 1948.)

In homoiotherm animals oxydative processes increase in cold environment even during perfect rest. Increased heat production enables the animal to maintein a constant temperature. This mechanism was termed by *Rubner* (1) chemical thermoregulation.

In 1904 *A. Montuori's* (2) experiments have already shown, that humoral factors play a rôle in defence against cold. In 1909 *Mansfeld* and *Pap* (3) have found, that isolated hearts of rabbits consume in cold environment more glucose, than hearts in ordinary or warm environment. They considered this to indicate an increased metabolism. They showed also that the blood-serum of cooled animals contains a principle which can raise sugar consumption of the hearts of normal animals.

Mansfeld (4) followed up this problem further and showed that the blood-serum of animals exposed to cold increases O_2- consumption of sceletal muscle. No effect was observed after the removal of the thyroid, or of the hypophysis. Injection of thyroxine to thyroidectomized animals restored the capacity to produce the active principle in the course of cooling, while sera of hypophysectomized animals were found inactive even after administration of thyroxine. These experiments indicate that in a cold environment an increased thyroxine secretion sets in which ellicits in the hypophysis the secretion of a substance, which augments oxydation in resting sceletal muscle even if it is severed from the central nervous system. The "heating-hormone" of pituitary origin can be demonstrated also in sera of cooled animals after the elimination of proteins and lipids. The pituitary "heating-hormone" acts, contrasting with the action of thyroxine, without latency.

Many American and English workers observed [see e. g. *Astwood* (5)] in animals treated with derivates of thiourea or thiouracil the formation of goiter and hypothyreosis. According to biochemical investigations thyroxine secretion is inhibited. Recent experiments of *B. Berde* (6)

showed, that thermothyrin *A* production is also inhibited by methylthiouracil. It may be therefore concluded, that the so called antithyroid drugs lead to a general inhibition of the internal secretion of the thyroid. As a consequence of thiouracil treatment histological changes have been described in the basophil cells of the anterior lobe of the hypophysis, resembling those observed after thyroidectomy.

Considering that thiouracil derivates inhibit thyroxine production and cause histological changes in the pituitary it seemed of interest to examine their influence on those humoral reactions against cold in which, as we have seen, both glands play a rôle.

Rabbits (8 animals) were exposed for 4 hours to a temperature of 0 C^0, and consequently a sample of blood was taken from the jugular vein. Proteins were precipitated with four times the volume 96% alcohol. After evaporation of the alcohol in vacuum the lipids were shaken out with ether. Traces of ether were eliminated. The serum of each rabbit was tested on two rats. Three days before the experiment the rats received 0,3 *mg* thyroxine daily, for according to *Mansfeld* (7) the effect of sera of cooled animals is enhanced by treating the test animals with thyroxine. After removing (under sterile conditions) one gastrocnemius of the rats 2,5 *ccm* of the rabbit serum praeparation were injected subcutaneously. Two hours later the second gastrocnemius was removed. 0_2-consumption of the muscles was determined in pure 0_2-atmosphere by the method of *Warburg*, as modified by *Mansfeld* and *Scheff—Pfeiffer* (8) on the intact muscle, who described that corresponding muscles of untreated animals consume identical quantities of 0_2 even if an interval of 24—48 hours elapses between the removal of the two corresponding muscles. After administering to the rabbits for 4 weeks daily 0,10 *gr/kg* methylthiouracil per stomach-tube (Basethyrin Richter), the animals were exposed to cold and their sera were tested on rats in the manner above described.

Result: Sera of cooled normal rabbits augmented 0_2-consumption of muscle. The average increase for a period of two hours was + 21%. Practically no effect was observed after 4 weeks of methylthiouracil feeding, the differences were within the limits of error. The difference between the effect of sera obtained before and after the thiouracil treatment is highly significant (9): $P < 0{,}001$ (see Table I).

These experiments show that methylthiouracil treatment of 4 weeks inhibits the production of the "heating-hormone". Considering the inhibition of thyroxine production by thiouracil, there seemed little doubt that the secretion of the pituitary "heating-hormone" is inhibited by lack of thyroxine. Nevertheless we could not quite exclude the possib-

TABLE I.

The effect of serum praeparations of cooled rabbits on the O_2-consumption of the musc. gastrocnemius of rats.

(Differences in per cents.)

Nr. of rabbit	Before feeding with methylthiouracil				After feeding with methylthiouracil			
	Nr. of rat	first hour	second hour	two hours average	Nr. of rat	first hour	second hour	two hours average
I.	1.	+ 19	+ 26	+ 23	—	—	—	—
	2.	+ 38	+ 46	+ 42	—	—	—	—
II.	3.	+ 25	+ 27	+ 26	17.	— 1	+ 8	+ 3
	4.	+ 17	+ 10	+ 13	18.	+ 6	+ 22	+ 12
	—	—	—	—	19.	— 5	+ 3	— 2
III.	5.	+ 13	+ 41	+ 24	20.	— 7	+ 1	— 3
	6.	+ 22	+ 45	+ 32	21.	± 0	+ 2	+ 1
IV.	7.	— 1	+ 11	+ 4	22.	— 13	+ 7	— 4
	8.	+ 2	+ 21	+ 10	23.	— 6	+ 7	± 0
V.	9.	+ 25	+ 16	+ 21	24.	— 2	+ 8	+ 2
	10.	+ 11	+ 18	+ 14	25.	— 5	— 12	— 8
VI.	11.	+ 42	+ 26	+ 35	26.	— 1	+ 20	+ 7
	12.	+ 67	+ 44	+ 52	27.	+ 5	+ 16	+ 9
VII.	13.	+ 19	+ 46	+ 30	28.	+ 10	+ 5	+ 8
	14.	— 7	— 3	— 4	29.	— 12	— 12	— 12
VIII.	15.	+ 6	+ 26	+ 14	30.	+ 8	+ 1	+ 5
	16.	+ 3	+ 11	+ 7	31.	— 3	— 15	— 9
Mean		+ 19	+ 26	+ 21		— 2	+ 4	+ 1

$P < 0{,}001$

ility that thiouracil may have an effect on the hypophysis as well, and inhibit the production of the "heating-hormone" directly. To decide this problem we fed same rabbits (V—VIII) for another two weeks with methylthiouracil, and then injected 1 *mg* of thyroxine, and tested the sera obtained an hour later on rats in the same manner as in experiments described above. As *Mansfeld* demonstrated, sera obtained after injection of thyroxine ("thyroxine serum") have the same effect on the oxidation of muscles as sera of cooled animals, because thyroxine ellicits the secretion of the pituitary "heating-hormone". Sera of rabbits treated for six weeks with methylthiouracil, obtained 1 hour after injection of thyroxine increased 0_2-consumption of the gastrocnemius during the period of a two-hours experiment by an average of 32% (see Table II).

TABLE II.

The effect of ‚thyroxin-sera" of methylthiouracil fed rabbits on 0_2-consumption of musc. gastrocnemius of rats.

(Differences in per cents.)

Nr. of rabbit	Nr. of rat	First hour	Second hour	Two hours average
V.	32.	+ 70	+ 100	+ 86
	33.	+ 74	+ 113	+ 90
VI.	34.	+ 23	+ 21	+ 22
	35.	+ 5	+ 7	+ 6
VII.	36.	+ 7	+ 30	+ 16
	37.	+ 20	+ 18	+ 19
VIII.	38.	+ 16	+ 18	+ 17
	39.	+ 8	+ 4	+ 6
	Mean	+ 28	+ 39	+ 32

These experiments show that the hypophysis does not loose its ability to produce the "heating-hormone" in the course of methylthiouracil treatment, and that the defective hormone production of the treated animals is due to an inhibition of thyroxine-secretion.

SUMMARY.

1. Results of *G. Mansfeld* and his collaborators, that exposure to cold is followed by the appearance of a principle which augments 0_2-consumption of sceletal muscle are confirmed.

2. After methylthiouracil treatment no pituitary "heating-hormone" was detectable in the sera.

3. Methylthiouracil has no direct effect upon the hypophysis, because thyroxine ellicits the production of the pituitary "heating-hormone" equally in treated and untreated animals.

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