A Study of the Reducing Substances of the Blood with Additional Papers on (1) the Enzymatic Synthesis of Proteins with Special Reference to the Action of Pepsin and (2) a Method for the Determination of the Inorganic Sulphate Content of Blood Serum or Plasma.

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I N D E X.

A Study of the Reducing Substances of the Blood.

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A Method for the Determination of the Inorganic Sulphate Content of Blood Serum or Plasma.

A STUDY OF THE REDUCING SUBSTANCES OF BLOOD.

I N T R O D U C T I O N.

Within recent years considerable progress has been made in the determination of blood sugar. The values obtained by the older methods include varying amounts of non sugar reducing substances and since different procedures are affected in varying degrees by these compounds, considerable differences are observed when the same sample of blood is analysed by different methods.

De Wesselow (1919) found that the Lewis-Benedict picric acid method (1915) gave 30-50% higher results than the MacLean method (1916). Höst and Hatlehol (1920) comparing four methods over a wide range, came to the conclusion that Bang's method (1918) and the Hagedorn and Jensen method (1918) agreed fairly well, while that of Folin and Wu (1919) compared well with Bailey and Myers' modification (1916) of the Lewis-Benedict method, but that the two former gave lower results than the latter two. More recently Folin (1926) and Benedict (1927,1931) have evolved alkaline copper reagents with which they claim to obtain lower results than with those of Folin and Wu.

The more recent methods which have been evolved for the determination of sugar are based on the following principles.

(1) Total reducing and non-sugar reducing values are determined separately, true sugar values being obtained by difference.

(2)/

(2) Use of a protein precipitant which will precipitate all the non-sugar reducing substances.

(3) Preparation of reagents reduced by sugar but not by non-sugar reducing substances.

(1) Sugar is determined by difference by determining the reducing power of blood before and after removal of sugar. Sugar may be removed by yeast fermentation (Hiller, Linder and Van Slyke 1925,1926; Somogyi 1927), by glycolysis (Hiller, Linder and Van Slyke 1925), bacteria (Hubbard and Allison 1928,1930) or by copper sulphate and lime (Van Slyke 1917).

(2) For the removal of non sugar reducing substances prior to sugar determination, hydroxides of heavy metals have been used as protein precipitants. Bierry and Moquet (1924) and Harned (1925) have prepared blood filtrates free of non-sugar reducing substances by the use of mercuric nitrate and sodium hydroxide. Somogyi (1929) prepared filtrates with zinc sulphate and sodium hydroxide and found that they were almost free of non-fermentable reducing substances. Using these filtrates he obtained practically identical sugar values with the Shaffer - Hartmann (1921), Folin-Wu (1919) and Benedict (1927) methods. Benedict and Newton (1929) have stated that glutathione and thioneine, which are only present in the corpuscles, are the most important/

important non-sugar reducing substances present in the blood. Everett (1930) and Herbert Bourne, and Groen (1930) have shown that zinc filtrates prepared according to Somogyi's technique are free from glutathione. Folin (1930) and Herbert and Bourne (1930) by preventing haemolysis of the corpuscles previous to protein precipitation, prepared tungstic acid filtrates free of glutathione.

(3) Benedict (1927,1931) has evolved alkaline copper reagents which are so insensitive towards non-sugar reducing substances that true sugar values are obtained using tungstic acid filtrates even though they contain glutathione.

There have been many different types of reagents evolved for the determination of sugar. They may be summarised as follows:-

(1) Picric acid.

This method was evolved by Lewis and Benedict (1915) who utilised the power of glucose to convert alkaline sodium picrate solutions into red picramic acid when heated in a boiling water bath. This colorimetric method is undoubtedly rapid since the same reagent is employed for removing the proteins as for the determination of sugar, but it has been shown that substances other than sugar, produce the same reaction. Creatinine and the presence of phenols and phenol sulphates (Sweany and Johnson 1923) seriously/

seriously affect the results. Guy (1921) has shown that even 0.1 mg. of acetone leads to an appreciable alteration in the colour.

(2) Dinitrosalicylic Acid.

The use of this substance has been proposed by Sumner and Graham (1922) but it has not been subjected to extensive trials.

(3) 2:4 dinitro—1 naphthol—7 sulphonic acid.

Kingsbury (1927) has advocated the use of this substance. He states that this substance is unaffected by homogentisic acid but urea however has a marked influence. In view of the fairly high concentration of urea in the blood, this is a serious drawback to its application.

(4) Potassium Ferricyanide.

A method employing potassium ferricyanide was evolved by Hagedorn and Jensen (1918,1923). When glucose is heated with an alkaline carbonate solution of potassium ferricyanide in a boiling water bath, ferricyanide is reduced to ferrocyanide. After the reaction unchanged ferricyanide may be determined by the addition of potassium iodide and acetic acid when iodine is liberated according to the following reaction and may be titrated with a standard solution of sodium thiosulphate.

$$2\ H_3\ Fe\ (CN)_6 + 2\ HI \longrightarrow 2\ H_4\ Fe\ (CN)_6 + I_2$$

Since/

Since the above reaction is reversible, an excess of a zinc salt is added, when the reaction goes completely to the right, the ferrocyanide being precipitated out of the sphere of action as a zinc salt.

Such a method certainly offers advantages, especially since quantities of glucose less than 0.1 mg. may be determined. It has the disadvantage that the reagent is very sensitive to reduction by substances other than glucose. Herbert and Groen (1929) have shown that the non-sugar reducing substances of the blood reduce the Hagedorn and Jensen alkaline ferricyanide reagent to a much greater extent than they do the alkaline copper reagents. Hagedorn and Jensen have stated that the chemicals employed must be very pure since traces of impurities seriously affect the results.

(5) Mercury Salts.

Alkaline solutions of mercuric iodide have been employed by Fleury and Marque (1929) but they are unsuitable owing to their sensitivity to reduction by substances other than sugars.

(6) Alkaline Copper Reagents.

Many different types of these reagents have been evolved. They consist of copper sulphate, an alkali and a substance capable of keeping the copper in solution e.g. tartrate, citrate, bicarbonate etc.

One/

One of the earliest of these reagents was Fehling's solution which contained sodium hydroxide but owing to the destructive action of such a strong alkali upon sugars, carbonates are now much more generally used.

Upon heating such reagents with glucose, part of the copper is reduced to cuprous oxide which can be determined as follows:-

(A) Iodometric. (Bang 1918; Maclean 1916; Shaffer and Hartmann 1921.)

This method depends on the property of iodine of oxidising cuprous oxide in the presence of acid to the cupric condition. A known excess of iodine is added so that the unused iodine may be determined after the reaction by titration with a standard solution of sodium thiosulphate. It is usual for potassium iodide and iodate to be incorporated in the alkaline copper reagent so that upon acidification after the reduction process, iodine is liberated according to the following reaction.

$$KIO_3 + 5KI + 3H_2SO_4 \longrightarrow 3K_2SO_4 + 3H_2O + 3I_2$$

In the Maclean and Bang methods an excess of mineral acid is added after reduction. Shaffer and Hartmann (1921) state that under certain conditions in the presence of excess mineral acid the following reversible reaction tends to go to the right.

$$2\ Cu\ SO_4 + 4KI \rightleftharpoons Cu_2\ I_2 + 2K_2\ SO_4 + I_2$$

They found that when the concentration of copper is very low as in the Maclean and Bang methods, the tendency to go to the right is nil, but with higher concentrations of copper the reaction becomes apparent and increases with increase in concentration of copper. Shaffer and Hartmann in the preparation of their alkaline copper reagent to ensure complete liberation of iodine and yet to prevent the second reaction going to the right, incorporated sufficient potassium oxalate to neutralise the excess of mineral acid.

(B) Reduction of ferric sulphate by cuprous oxide to ferrous sulphate and subsequent determination of the reduced iron by titration with a standard solution of potassium permanganate.

This type of method has been used chiefly in macro determinations e.g. the Bertrand method (1906) for the determination of sugar in urine. Bierry and Voskressensky (1927,1928) have utilised this type of method in the determination of blood sugar. The end points however are not as delicate as in the iodometric methods.

(C) Reduction of the Phosphomolybdic acid reagent by cuprous oxide.

Cuprous oxide dissolves in the phosphomolybdic acid reagent of Folin and Wu (1919) to give an intense blue colour/

colour. Folin and Wu used this as a colorimetric method. Fontés and Thivolle (1921) state that the cuprous oxide may be determined after its solution in the phosphomolybdic acid reagent by titration with a standard solution of potassium permanganate, the end point being when all the blue colour has disappeared. The present writer has found that end points in iodometric titrations are much easier to define than in this latter method.

Since alkaline copper reagents are more specific to reduction by sugars, the present writer made a study of these only. It was decided to study the reduction of these reagents by glucose and other substances present in the blood, due allowance being made for the tenfold dilution of the constituents of blood which results from deproteinisation according to the technique of Folin and Wu.

Since the Shaffer-Hartmann method was to be used as a basis for this work, a preliminary examination of this method was made.

The Shaffer-Hartmann Method.

All the chemicals used in this work were of A.R. standard - British Drug Houses Ltd. The glucose was dried in an air oven at 100° for 1 hour before being weighed. It was found that a 1% solution of glucose made up in saturated benzoic acid kept a considerable time. The various strengths of sodium thiosulphate were freshly prepared each/

each day from a stock 0.IN solution which was tested from time to time against 0.IN potassium dichromate and 0.IN potassium iodate solutions. The keeping properties of the 0.IN thiosulphate were found to be increased considerably by the addition of 2 g. potassium bicarbonate per litre.

The method was examined from 3 standpoints:-

(1) using pure glucose. The reduction was carried out as described in the original paper:

(2) the reduction was carried out in an atmosphere of nitrogen:

(3) the reagent was made up in two separate solutions, (a) containing copper sulphate, tartaric acid and sodium carbonate; (b) containing potassium iodide, iodate and oxalate. The reduction was carried out with solution A, solution B being added afterwards. This reaction was studied both in an atmosphere of air and nitrogen.

The first line of investigation was carried out as follows:- Into an 8 x 1 inch test tube 10 cc. of glucose solution and 10 cc. of Shaffer-Hartmann reagent were mixed. This was placed in a boiling water bath for 10 minutes and then cooled. 10 cc. of N. H_2 SO_4 were added, the whole well shaken for about 2 minutes and then titrated with 0.0125N thiosulphate solution.

For the determination under (2) the test tube containing the mixture of glucose and alkaline copper reagent was/

was evacuated and then filled with a slow stream of nitrogen. This was repeated several times to remove air dissolved in the fluid. The determination was then carried out as under (1).

For the determination under (3) the following solutions are required:-

A.

Copper sulphate Cu $SO_4 \sim 5H_2O$	5 g.	per litre.
Tartaric Acid.	7.5 g.	
Sodium Carbonate (anhydrous)	40 g.	

B.

Sodium carbonate.	1 g.	per litre.
Potassium iodide.	20 g.	
Potassium iodate.	1.4 g.	
Potassium oxalate.	36.8 g.	

C. 2N. H_2SO_4

The technique in air was as follows. Into an 8 x 1 inch test tube 10 cc. of glucose solution and 10 cc. of solution A were mixed and put into a boiling water bath for 10 minutes. After cooling 5 cc. of solution B were added, followed by 5 cc. of 2N. H_2SO_4. After shaking the contents of the tube were titrated with 0.0125N thiosulphate. It will be noted that the final concentrations are the same as in the original method. The above procedure was repeated, the tube being filled with nitrogen as described under (2).

The results are shown in Table I, being expressed in/

in cc. 0.0125N thiosulphate and the difference in titration values of a blank using 10 cc. of distilled water and a determination using 10 cc. of a glucose solution.

TABLE I.

Concentration of Glucose (mg.per 100 cc.)	SHAFFER and HARTMANN Original Method.		Modified Solutions.	
	Air.	Nitrogen.	Air.	Nitrogen.
40	11.55	12.25	12.3	12.3
30	8. 55	9.25	9.3	9.3
20	5.5	6.0	6.05	6.05
10	2.4	2.85	2.9	2.9
8	1.85	2.3		
4	0.65	0.95		

It is well known that glucose when heated with a solution of an alkaline carbonate is easily oxidised by the oxygen dissolved in the solution. The higher results obtained in the Shaffer-Hartmann method when the reduction is carried out in an atmosphere of nitrogen is possibly due to the retarding action of the potassium salts upon the reduction so that part of the glucose is oxidised by the dissolved air before it reduces any copper.

This theory is confirmed by the fact that when the potassium salts are not incorporated in the alkaline copper reagent but added after reduction, the same results are obtained irrespective of whether the reduction is carried out in an atmosphere of air or of nitrogen. This demonstrates that the amount of glucose removed by atmospheric oxygen prior to reduction of copper is negligible under these conditions. These results agree with the results obtained with the original Shaffer-Hartmann reagent in an atmosphere of nitrogen.

In the next stage of this study the present writer investigated the effect upon the reducing values of glucose of varying the concentrations of the various items in the above alkaline copper reagent.

The Amount of Copper Reduced in Relation to the Concentration of Copper Sulphate, Tartaric Acid and Sodium Carbonate/

Carbonate.

Some substance must be incorporated in an alkaline copper solution which is capable of keeping the copper in solution. There are many substances possessing this property, the most important being, tartrates, citrates, bicarbonates and amino acids. Benedict (1926) originally recommended a high concentration of sodium citrate as he considered that such a reagent though not so sensitive to reduction as one containing a tartrate, is nevertheless more specific for sugars. Later (1927) he dispensed with citrate and used a low concentration of tartrate together with alanine and a high concentration of sodium nitrate. Folin and Wu (1919) and later Folin (1926) used a low concentration of tartrate - just sufficient to keep the copper in solution. These solutions containing low concentrations of tartrate are very sensitive to reduction by sugars and show very little autoreduction when heated with distilled water in a boiling water bath. Folin (1926) criticises Benedict's citrate reagent as being not sufficiently sensitive while on the other hand Benedict criticises Folin's reagents as being too sensitive in that they are reduced by such substances as formalin and chloroform. Folin (1929) criticises a modification of the Folin and Wu alkaline copper reagent made by Somogyi and Kramer (1928) in respect of the high concentration/

concentration of Rochelle salt used in place of the low concentration of tartaric acid. He states that reagents having high concentrations of citrates and tartrates have high blanks thereby being unsuitable for colorimetric methods. Somogyi (1926) modified the Shaffer-Hartmann reagent by replacing part of the sodium carbonate by bicarbonate. He states that a decrease in the alkalinity of the Shaffer-Hartmann reagent leads to an increase in reduction - this decrease in alkalinity being obtained by replacing part of the carbonate by bicarbonate. Somogyi appears to consider that alkalinity is the main factor since in two of his modifications of the Shaffer-Hartmann reagent which have been published, both contain the same concentrations of carbonate and bicarbonate but one contains 1.2% Rochelle salt, the other 2.5% (c.f. West, Scharles and Petersen 1929). Somogyi also uses the same concentrations of bicarbonate and carbonate in his modification of the alkaline ferricyanide reagent of Hagedorn and Jensen (1923). It is of interest to note that in MacLean's (1916) and in the Wood-Ost alkaline copper reagents no tartrate or citrate is used. Has the tartrate any effect on reduction other than simply holding the copper in solution? This was the first point to determine.

Reagents were prepared containing the same concentrations of copper sulphate and sodium carbonate but with/

with different concentrations of tartrate.

The following technique was employed in preparing the alkaline copper solutions. The requisite amounts of tartaric acid and sodium carbonate were weighed out and dissolved together in distilled water. The solution was then boiled to decompose any bicarbonate which might have been formed and then cooled. To this was added the requisite amount of copper sulphate dissolved in distilled water and the whole made up to the required volume.

The amount of copper reduced was determined as follows:-

Solutions.

1. Iodide - iodate - oxalate solution.

Sodium carbonate (anhydrous)	40 g.	per litre
Tartaric Acid.	7.5 g.	
Potassium iodide.	10 g.	
Potassium iodate.	0.8 g.	
Potassium oxalate.	18.4 g.	

2. N. H_2SO_4
3. 0.0125 N. Sodium Thiosulphate.

Into a 3 x 1 inch centrifuge tube 10 cc. glucose solution and 10 cc. of alkaline copper solution were mixed. The tube was then placed in a boiling water bath for 15 minutes/

minutes. It was then cooled and centrifuged. The supernatant fluid was decanted. 10 cc. of the iodide-iodate oxalate solution were then added, followed by 10 cc. N.H_2SO_4. The whole was stirred to dissolve the cuprous oxide and then titrated with 0.0125 N thiosulphate. A blank was performed under similar conditions using 10 cc. of distilled water. The difference between the blank determination and a determination using glucose was taken as a measure of the amount of copper reduced by the glucose.

It will be seen that the final concentrations of all the substances are the same as in the original Shaffer-Hartmann method with the exception of copper sulphate.

Tables 2 and 3 demonstrate the influence of concentration of tartaric acid, Table 4 the influence of concentration of sodium carbonate and Table 5 the influence of time of boiling.

TABLE 2.

Alkaline copper solutions containing:-

Copper sulphate $CuSO_4, 5H_2O$ 5g.
Tartaric Acid. 7.5-15g.
Sodium Carbonate (anhydrous) 40g.
} per litre.

Concentration of Tartaric Acid (g. per litre)	Amount of cuprous oxide formed by reduction by 10 cc. glucose solution in terms of cc. 0.0125N thiosulphate.				
	10 mg. glucose per 100 cc.	20 mg. glucose per 100 cc.	30 mg. glucose per 100 cc.	40 mg. glucose per 100 cc.	
7.5	3.0	6.05	-	12.3	
8.0	3.1	6.05	9.6	12.3	
9.0	3.3	6.6	9.6	12.7	
9.5	3.3	6.6	-	-	
10.0	3.3	6.6	9.9	12.8	
12.0	3.3	6.6	9.9	13.1	
15.0	3.3	6.6	9.9	13.1	

TABLE 3.

Alkaline copper solutions containing:-

Copper sulphate, $CuSO_4 . 5H_2O$ 10 g.
Tartaric Acid 7.5- 30 g.
Sodium Carbonate (anhydrous) 60 g.
per litre.

Concentration of Tartaric Acid. (g.per litre)	Amount of cuprous oxide formed by reduction by 10 cc. glucose solution in terms of cc. 0.0125N thiosulphate.				
	2.5mg. glucose per 100 cc.	5 mg. glucose per 100 cc.	10 mg. glucose per 100 cc.	20 mg. glucose per 100 cc.	40 mg. glucose. per 100 cc.
7.5	-	-	2.4	5.1	11.6
10.0	-	-	3.3	6.6	13.2
12.0	-	-	3.3	6.6	13.2
14.0	-	-	3.3	6.6	13.2
15.0	0.85	1.65	3.3	6.6	13.2
16.0	-	-	3.3	6.6	13.0
18.0	-	-	3.3	6.6	12.9
20.0	-	-	3.3	6.6	12.8
25.0	-	-	3.05	6.05	12.0
30.0	-	-	2.7	5.1	10.1

TABLE 4.

Alkaline Copper Solutions containing:-

Copper sulphate, $CuSO_4 \cdot 5H_2O$	10 g.	per litre.
Tartaric Acid	15 g.	
Sodium Carbonate (anhydrous)	30 - 80 g.	

Concentration of Sodium Carbonate (g. per litre)	Amount of cuprous oxide formed by reduction with 10 cc. glucose solution in terms of cc. 0.0125 N thiosulphate.					
	2.5 mg. glucose per 100cc	5 mg. glucose per 100cc	10 mg. glucose per 100cc	20 mg. glucose per 100cc	30 mg. glucose p.100cc.	40 mg. glucose per 100 cc
30	-	1.55	-	5.75	-	-
40	-	1.65	3.3	6.6	9.9	-
60	0.85	1.65	3.3	6.6	9.9	13.2
80	0.85	1.65	3.3	6.6	9.9	13.2

TABLE 5.

Alkaline Copper Solution containing:-

Copper sulphate, $CuSO_4 5H_2O$	10 g.	per litre.
Tartaric Acid	15 g.	
Sodium carbonate (anhydrous)	80 g.	

Time of Boiling (minutes)	Amount of cuprous oxide formed by reduction by 10 cc. glucose solution in terms of cc. 0.0125 N.thiosulphate	
	5 mg. glucose per 100 cc.	20 mg.glucose per 100 cc.
5	-	3.15
10	1.65	6.6
15	1.65	6.6
20	1.65	6.6
30	1.65	6.6

The results in Table 2 were checked using the following technique. Instead of centrifuging the mixture after reduction and separating the cuprous oxide from the supernatant fluid, 5 cc. of the following solution were added:-

Sodium carbonate	1 g.	per litre .
Potassium iodide	20 g.	
Potassium iodate	1.4 g	
Potassium oxalate	36.8 g.	

followed by 5 cc. of 2N. H_2SO_4. The whole was well shaken and titrated with 0.0125 N. thiosulphate.

The physical nature of the cuprous oxide appears to be related to the concentrations of the alkaline copper solutions. In the experiments reported in Table 2, the cuprous oxide was flocculent, light brown in colour and difficult to centrifuge whereas in those in Table 3 with concentrations of tartaric acid above 1.2%, the cuprous oxide was red, heavy and easily centrifuged.

Upon examining the results in Table 2 it will be observed that an increase in the concentration of tartaric acid from 0.75% to 0.9% resulted in increases in the amount of reduction. Further increases in concentration from 0.9 to 1.5% produced no further increase in reduction except in the case of the highest concentration of glucose examined.

In/

In Table 3 it will be seen that increase of concentration of tartaric acid leads to an increase in the amount of reduction but when the concentration becomes very high e.g. 2.5 and 3% the reduction is decreased. This inhibiting effect becomes apparent in lower concentrations than these, with the highest concentration of glucose used e.g. 40 mg. per 100 cc. The effect of increasing the concentration of sodium carbonate in the reagent containing 3% tartaric acid was studied. It will be seen from the results shown in Table 6 that an increase in concentration of sodium carbonate leads to an increase in the amount of the reduction to that of the maximum. It appears then that in high concentration tartaric acid exerts an inhibiting action which may be counterbalanced by increasing the concentration of sodium carbonate. It is interesting to note that increasing the concentration of sodium carbonate in the reagent containing 0.75% tartaric acid (c.f. Table 2) i.e. where the concentration of tartaric acid is insufficient to give the maximum reduction, had no influence upon the amount of reduction. The maximum reductions obtained in Tables 2 and 3 are the same even although the concentrations of copper sulphate are different i.e. o.5 and 1.0%. In all the reagents giving the maximum reduction, the amount of reduction is unaffected by increase in concentration of sodium carbonate (Table 4) or increase in time of heating (Table/

TABLE 6.

Alkaline Copper Solution containing:-

Copper sulphate, $CuSO_4 5H_2O$	10 g.	per litre.
Tartaric Acid	30 g.	
Sodium Carbonate (anhydrous)	60- 100 g.	

Concentration of Sodium Carbonate. (g. per litre)	Amount of cuprous oxide formed by reduction by 10 cc. glucose solution in terms of cc. 0.0125N thiosulphate.	
	10 mg. glucose per 100 cc.	20 mg. glucose per 100 cc.
60	2.7	5.05
80	3.3	6.6
100	3.3	6.6

(Table 5.) The amount of reduction of these reagents is directly proportional to the concentration of glucose as widely different as 2.5 to 40 mg. per 100 cc. The blank determination using distilled water increases with increase in concentration of tartaric acid, but this is not a disadvantage in a non-colorimetric method. It was found that carrying out the reduction in an atmosphere of nitrogen had no influence on the results.

Determination of Glucose by the Estimation of the Unreduced Copper.

Shaffer and Hartmann (1920-21) have determined the amount of glucose by estimating the amount of unreduced copper after the reduction of an alkaline copper solution. An excess of potassium iodide and acid was added when the following reaction takes place.

$$2\ Cu\ SO_4 + 4KI \longrightarrow Cu_2I_2 + 2K_2SO_4 + I_2$$

The liberated iodine was titrated with standard sodium thiosulphate solution. This method was investigated by the present writer and the results compared with those obtained by determining the amount of reduced copper directly. The following alkaline copper solution (c.f. Table 2) was employed.

Copper sulphate $Cu\ SO_4 . 5H_2O$	5	g.	per litre
Tartaric Acid	15	g.	
Sodium carbonate (anhydrous)	40	g.	

Into an 8 x 1 inch test tube 10 cc. of glucose solution and 10 cc. of alkaline copper solution were mixed and the whole placed in a boiling water bath for 15 minutes. It was then cooled and 10 cc. of saturated potassium iodide solution added followed by 5 cc. of 25% sulphuric acid. The whole was then well shaken and titrated with 0.0125N thiosulphate. A blank determination using 10 cc. of distilled water/

water was carried out at the same time. The difference between these two results being taken as equivalent to the amount of copper reduced. This will be termed the indirect method, the other the direct method. From the results shown in Table 7 it will be seen that the two methods give similar results.

TABLE 7.

Concentration of glucose (mg.per 100 cc.)	Amount of copper reduced by 10 cc. glucose solution in terms of cc. 0.0125 N. thiosulphate.	
	Direct Method.	Indirect Method.
30	9.9	9.9
20	6.6	6.65
10	3.3	3.3

THE EFFECT OF BICARBONATES.

Somogyi (1926) states that a decrease of alkalinity of the Shaffer and Hartmann alkaline copper reagent leads to an increased reduction. To obtain a decrease of alkalinity Somogyi substituted sodium bicarbonate for part of the sodium carbonate. He found that as the carbonate was decreased and the bicarbonate increased the reduction increased until the concentration of sodium carbonate was 2.0% and that of the sodium bicarbonate 2.5% but with a further increase of bicarbonate and decrease of carbonate, the reduction began to diminish. Somogyi asserts that with the above concentrations the optimum alkalinity for reduction is obtained. Somogyi evolved a modification of the Shaffer and Hartmann reagent from these experiments.

It was decided to investigate this problem along slightly different lines. The oxidising portion of the reagent had the following composition.

Copper sulphate $Cu.SO_4, 5H_2O$	6.5 g.	per litre.
Rochelle salt.	12 g.	
Sodium carbonate (anhydrous)	20 g.	
Sodium bicarbonate	25 g.	

In the first set of experiments the concentration of sodium carbonate was varied, keeping the concentration of/

of sodium bicarbonate constant and in the second set, the reverse was carried out. The potassium iodide, iodate and oxalate were not incorporated in the reagent but were added as a separate solution after reduction, otherwise the original technique of the author was carried out. The amount of acid added was varied in accordance with the variation in the concentration of ~~either~~ the carbonate or bicarbonate. The concentration of glucose used in all experiments was 20 mg. per 100 cc.

TABLE 8.

Concentration of Sodium Bicarbonate 2.5%

Concentration of Sodium Carbonate (anhydrous) (g. per 100 cc.)	Reduction by 10cc.glucose solution in terms of cc. 0.0125 N thiosulphate.
1.5	7.25
2.0	7.25
2.5	7.25
3.0	7.25
4.0	7.25

TABLE 9.

Concentration of Sodium Carbonate - 2%

Concentration of Sodium Bicarbonate (g. per 100 cc.)	Reduction by 10 cc. glucose solution in terms of cc. 0.0125 N thiosulphate.
0	4.3
0.5	5.35
1.0	5.95
1.5	6.5
2.0	6.9
2.5	7.25
3.0	7.2
4.0	6.85

In the first set of experiments it will be noted that an increase of concentration of carbonate has no effect on reduction. In the second set of experiments the reduction is diminished when the concentration of bicarbonate is either increased or decreased from that used by Somogyi in his reagent. The solution in Table 9 containing 4% of sodium bicarbonate was taken and the effect of increasing the concentration of sodium carbonate examined, Table 10.

TABLE 10.

Concentration of Sodium Carbonate (anhydrous) (g. per 100 cc.)	Reduction by 10 cc. glucose solution in terms of cc. 0.0125 N thiosulphate.
2.0	6.85
3.0	7.1
4.0	7.25

It will be observed that by increasing the concentration of sodium carbonate, the reduction is increased to the maximum value obtained with 2.5% sodium bicarbonate.

Bicarbonates can act in an alkaline copper reagent in the same manner as a tartrate or citrate c.f. MacLean's and the Wood-Ost reagent. In Somogyi's reagent the concentration of tartrate is insufficient to give the maximum reduction of tartrate reagents, so that by the addition of a substance of similar properties such as sodium bicarbonate, the reduction will be increased. It appears that a high concentration of sodium bicarbonate has an inhibiting action as well, this inhibiting action being neutralised by an increase in concentration of sodium carbonate. It will be noticed that similar results were obtained with solutions containing high concentrations of tartrates. (cf. Tables 3 and 6). These experiments hardly agree with Somogyi's views. It is interesting to note that the maximum reduction obtained with tartrate-bicarbonate solutions is a little higher than with tartrate solutions.

The Effect of Amino Acids upon the Reduction by Glucose.

Before a method can be accepted as suitable for the determination of blood sugar it must be shown that other substances present in protein free blood filtrates do not interfere. Holden (1926) states that under certain circumstances amino-acids are capable of influencing the results considerably. Normally the blood contains 40-70 mg. of amino acids per 100 cc. and in certain conditions these figures may be exceeded. Holden investigated the methods of Hagedorn and Jensen (1923) and the Wood-Ost method as modified by Cole (1920). The latter reagent contains copper sulphate, sodium carbonate and bicarbonate and the precipitated cuprous oxide is filtered off and washed prior to its determination. Holden examined the effects of glycine, aspartic acid, glutamic acid and cystine. With the alkaline ferricyanide, the amino acids were found to exert no influence upon its reduction by glucose. Cystine was the only one which reduced the reagent and the reduction was found to be the sum of the reducing values of the glucose and cystine determined separately. In the case of the Wood-Ost reagent, however, glucose in the presence of glycine, aspartic acid and glutamic acid caused a greater reduction than when alone. None of these substances were found to reduce the reagent. In the presence of cystine which itself reduced the reagent, the/

the reducing values were found to be greater than when glucose was alone even when the reducing value of cystine was allowed.

The present writer examined the influence of glycine, aspartic acid, glutamic acid and tyrosine. At this stage it was decided to limit the investigations to the following alkaline copper reagent.

Copper sulphate Cu. SO_4. $5H_2O$	10 g.	per litre
Tartaric acid.	15 g.	
Sodium carbonate (anhydrous)	80 g.	

The concentrations of glucose employed were 10 and 20 mg. per 100 cc. while the concentrations of amino acids used were as follows:-

1. glycine 10 mg. per 100 cc.
2. cystine 10 mg. per 100 cc.
3. aspartic acid 4 mg
   glutamic acid 4 mg.
   tyrosine 4 mg.
   } per 100 cc.

It was found that the reducing value of glucose was unchanged in the presence of these amino acids.

SULPHHYDRIL COMPOUNDS.

Cystine has already been considered but it may be mentioned that Hunter and Eagles (1927) failed to detect free cystine or cystein in blood using the Sullivan test (1926).

A/

A very important sulphur compound present in blood is the tripeptide glutathione. That it is present in the corpuscles and not in the plasma has been demonstrated by Holden (1925), Uyei (1926) Thomson and Voegtlin (1926), Hunter and Eagles (1927) and Hopkins (1929). Hunter and Eagles (1927) from the intensity of the nitroprusside reaction, calculated that whole blood contains about 50-100 mg. glutathione per 100 cc. Benedict (1931) states that the glutathione content of whole blood as determined by the Mason method (1930) is only of the order of 20 mg. per 100 cc. Herbert et alia (1930) stated that glutathione was responsible for the whole of the non-sugar reduction observed in certain methods. This however does not agree with the earlier results of Herbert and Groen (1929) who working with plasma obtained higher results for sugar using tungstic acid filtrates than with filtrates prepared by the use of heavy metals. If the theory of Herbert et alia is correct then Herbert and Groen should have obtained the same results for the sugar content of plasma irrespective of the deproteinising agent employed.

The present writer investigated the action of glutathione towards the alkaline copper reagent already described. Concentrations of glutathione of 5 and 10 mg. per 100 cc. were used and it was found that these concentrations did not exert any reducing action towards the alkaline copper reagent. These concentrations of glutathione were then mixed/

mixed with glucose in concentrations of 10 and 20 mg. per 100 cc. but no change in the reducing values of glucose was recorded.

The effect of another sulphydril compound was examined, namely dithioglycollic acid. It is doubtful whether this substance is present in the blood although Labbé and Nepveux (1931) claim to have detected it. This however requires confirmation. It was examined owing to its similarity in properties to glutathione and cystine. In concentrations of 20 mg. per 100 cc. it was found to exert no reducing action and did not affect the reduction of glucose.

It has been shown that neither glutathione, cystine nor dithioglycollic acid reduce the alkaline copper reagent nor affect its reduction by glucose.

Influence of Other Substances present in the Blood.

Creatinine Creatine and Uric acid:

Taylor (1924) has stated that creatinine delays the precipitation of cuprous oxide during the reduction of the Wood-Ost reagent by glucose. Hiller, Linder and van Slyke (1925) however found that uric acid, creatine and creatinine in the concentrations in which they are present in the blood have no appreciable effect upon the determination of sugar. The present writer has investigated the effect of these substances/

substances upon the reduction by glucose of the alkaline copper reagent he has adopted. Concentrations of uric acid creatine and creatinine up to 10 mg. per 100 cc. were used. Such concentrations greatly exceed the limits found in tungstic acid filtrates of blood. These substances were found to reduce the alkaline copper reagent slightly but as will be seen from the results shown in Table II, the reducing value of these substances as present in blood are inappreciable and may be neglected.

TABLE II.

Amount of Substance taken (mg. per 100 cc.)	Amount of glucose having same reducing value. (mg. per 100 cc.)
10 mg. creatinine.	3.3
10 mg. creatine.	0.3
10 mg. uric acid.	0.8

These results are in accordance with the views of Hiller, Linder and van Slyke. It was found that the reducing values of glucose in the presence of these substances in 10 mg. concentration, were exactly the sum of the separate reducing values so that uric acid, creatine and creatinine as present in blood do not interfere with the determination of/

of blood sugar.

Urea:

Holden (1926) has stated that urea retards the reduction of the Wood-Ost reagent by glucose. The present writer mixed urea in concentrations of 20 mg. per 100 cc. with glucose in concentrations of 10 and 20 mg. per 100 cc. It was found that urea exerted no reducing action and did not affect the reduction by glucose. Urea therefore in concentrations as present in blood does not interfere in the estimation of sugar.

Potassium Oxalate:

Since potassium oxalate was to be used for preventing coagulation of blood for sugar determination, it was decided to study the effect, if any, of this substance upon the determination of sugar. It was found that potassium oxalate could be added to blood up to 1% without any interfering action upon the determination of sugar.

Summary of the Preceding Investigations.

1. The amount of reduction of the Shaffer-Hartmann reagent by glucose is greater when carried out in an atmosphere of nitrogen than in air. When the potassium salts are not incorporated in the reagent but added as a separate solution after reduction the results are the same whether carried/

carried out in an atmosphere of nitrogen or air and are the same as those obtained with the original reagent reduced in an atmosphere of nitrogen.

2. The amount of reduction by glucose of alkaline copper reagents containing copper sulphate, tartaric acid and sodium carbonate depends upon the concentration of the latter two substances.

3. Bicarbonates may replace tartrates in alkaline copper reagents their concentration affecting the reduction by glucose.

4. Glycine, aspartic acid, glutamic acid, cystine and tyrosine in such concentrations as may be present in blood do not affect the reduction by glucose of the alkaline copper reagent examined.

5. Glutathione and dithioglycollic acid were found to exert no reducing action and no influence upon the reduction by glucose of the alkaline copper reagent examined.

6. Uric acid, creatinine and creatine in concentrations as present in blood do not affect the determination of sugar. Urea has no influence upon the determination of sugar.

7. Potassium oxalate, as an anti-coagulant may be added to blood up to a concentration of 1% without affecting a blood sugar result.

The Determination of Blood Sugar using the modified Alkaline Copper Reagent.

At this stage the modified alkaline copper reagent was applied to the determination of blood sugar. The method adopted was as follows:-

Solutions:

1. Alkaline copper solution.

Copper sulphate, $CuSO_4.5H_2O$	10g.	per litre.
Tartaric acid.	15 g.	
Sodium carbonate (anhydrous)	80 g.	

2. Iodide - Iodate - Oxalate Solution.

Sodium carbonate (anhydrous)	40 g.	per litre.
Tartaric acid	7.5g.	
Potassium Iodide	10. g	
Potassium Iodate	0.7g.	
Potassium Oxalate	18.4g.	

3. N. H_2SO_4.

Procedure.

Into a 3" x 1" centrifuge tube 10 cc. of a protein free blood filtrate and 10 cc. of the above alkaline copper solution were mixed. This was placed into a boiling water bath/

bath for 15 minutes. It is then cooled and centrifuged. The supernatant fluid was removed and 10 cc. of the iodide - iodate-oxalate solution were added to the cuprous oxide followed by 10 cc. of $N.H_2SO_4$. The whole was well stirred to dissolve the cuprous oxide and then titrated with 0.0125 N. thiosulphate. A blank determination using 10 cc. distilled water was carried out at the same time.

1 mg. glucose = 3.3 cc. 0·0125 N thiosulphate.

Smaller volumes of blood filtrate and smaller centrifuge tubes may be used, but for this work it was decided to use larger volumes for accuracy.

As a preliminary tungstic acid filtrates of various bloods were prepared according to the method of Folin and Wu (1919) and their reducing values as determined by the new technique and other methods then compared. The other methods included that of Shaffer-Hartmann (1921); Folin and Wu (1919); Folin (1926); and Hagedorn and Jensen (1923).

Zinc filtrates were not employed for the Hagedorn and Jensen (1923) method since this method was intended for unoxalated blood. Such figures would be unsatisfactory since all bloods used in this work were oxalated. Folin and Malmros (1929) and Kramer and Steiner (1931) have criticised the Hagedorn and Jensen method of deproteinisation since they state that/

that large quantities of reducing material are retained in the cotton plugs used for filtration. The results are shown in Tables 12 and 13.

TABLE 12.

	New Technique (mg.per 100cc. blood.)	Shaffer and Hartmann Method (1920-21). (mg.per 100 cc. blood.)	Folin and Wu Method (1919). (mg. per 100cc. blood.)
1	105	108	99
2	103	95	91
3	102	90	89
4	112	108	100
5	74	70	67
6	86	95	95
7	102	96	105
8	100	126	89
9	87	90	89
10	86	74	89
11	108	104	115
12	83	75	87

TABLE 13.

	New Technique. (mg. per 100cc. blood.)	Folin Method. (1926) (mg. per 100 cc. blood.)	Hagedorn and Jensen Method. (1923). (mg. per 100 cc. blood.)
13	99	84	127
14	109	91	149
15	86	70	131
16	93	83	128
17	108	88	144
18	86	-	115
19	90	-	123

It will be seen that in every case the Hagedorn and Jensen method gives higher results and the Folin method lower results than the new technique.

Influence of Different Deproteinising Agents upon the Determination of Blood Sugar.

The reagent employed for the removal of the proteins from blood prior to the determination of sugar has a marked influence upon the results. Glutathione and certain other substances present in the blood exert reducing actions in many methods and are precipitated by some reagents but not by others in the deproteinising process. It has been shown earlier that the alkaline copper reagent evolved by the present writer is not reduced by glutathione and not appreciably by other known reducing constituents of the blood.

Deproteinising agents employed by other writers may be briefly summarised as follows:-

1. picric acid as used in the Lewis-Benedict method (1915).
2. colloidal ferric hydroxide as used in the MacLean method (1916).
3. zinc hydroxide in the hot (Hagedorn and Jensen 1923) zinc hydroxide in the cold (Somogyi, 1929).
4. sodium tungstate and sulphuric acid (Folin and Wu 1919).
5. mercuric nitrate and sodium hydroxide (Bierry and Moquet 1924; Harned 1925; West, Scharles and Peterson 1929). mercuric sulphate and barium carbonate (West, Scharles and Peterson 1929).
6. Uranyl salts (Conderelli 1924).
7. copper sulphate and sodium tungstate (Somogyi 1931).
8. ferric sulphate and sodium hydroxide (Somogyi 1930.

9./

9. tungstomolybdic acid (Benedict 1931).

Stepp (1921) noted that if phosphotungstic acid filtrates of blood were treated with basic lead acetate, a decrease in reducing values always followed. Bierry and Moquet (1924) and Harned (1925) deproteinsed blood with mercuric nitrate and sodium hydroxide according to the Patein-Dufau method and found that they always obtained lower results than with tungstic acid filtrates using the Folin-Wu method for determining sugar. West, Scharles and Peterson (1929) obtained similar results using a modification of the Shaffer-Hartmann method by Somogyi (1926) for estimating sugar. These writers state that the differences in reducing values of the two types of filtrates is equal to the reducing values of the non-fermentable reducing substances in the tungstic acid filtrates.

Herbert and Groen (1929) compared the sugar values of whole blood, plasma and corpuscles using various reduction methods and methods of deproteinisation. They found that with the MacLean method (1916), tungstic acid filtrates had higher reducing values than filtrates prepared with dialysed iron. They obtained similar results with the Hagedorn and Jensen method (1923), tungstic acid filtrates giving higher reducing values than zinc hydroxide filtrates. They/

They found that these discrepancies were always greater with whole blood than plasma. Herbert, Bourne and Groen (1930) later considered that all the non-sugar reducing substances of the blood could be accounted for as glutathione and state that since this substance is present in the corpuscles only, plasma is free of non-sugar reducing substances. This hardly agrees with their earlier work (1929) since there they found higher values in tungstic acid filtrates of plasma than other types of filtrates using copper methods for determining sugar. The assumption upon which this theory is built is that glutathione is present in the blood to the extent of 50-100 mg. per 100 cc. and calculating the reducing value of glutathione when present in this concentration, values are obtained closely in agreement with the values obtained experimentally for the non-sugar reducing substances of the blood. Hunter and Eagles (1927) from the intensity of the nitroprusside reaction state that glutathione is present in the blood in concentrations of 50-100 mg. per 100 cc, but Benedict (1931) states that using the more recent Mason (1930) method average values of only 20 mg. per 100 cc. have been obtained.

Somogyi (1929,1930) employed zinc hydroxide in the cold for preparing protein free blood filtrates and found that they/

they gave lower values than tungstic acid filtrates. He states that these filtrates are free of uric acid, creatinine, glutathione, ergothioneine and all non-fermentable reducing substances. Somogyi (1930) comparing the reducing values of filtrates prepared by the use of the hydroxides of iron, zinc and mercury obtained very similar results.

An interesting method was evolved by Folin (1930) and Herbert and Bourne (1930) independently. The object of the method was to deproteinise whole blood with tungstic acid without breaking the corpuscles so that the non-diffusible substances of the corpuscles would not appear in the filtrate. This was achieved by addition of sodium sulphate. The composition of such filtrates are very similar to those of plasma.

As a preliminary the reducing values of tungstic acid filtrates and filtrates prepared by the use of mercury salts were compared. The mercuric sulphate method of West, Scharles and Peterson (1929) with slight modifications, was used. Before estimating the reducing values of mercury filtrates all traces of mercury must be removed since this would affect the final results. Bierry and Moquet (1924) using mercuric nitrate and sodium hydroxide as deproteinising agents, employed copper foil to remove the final traces of mercury from the filtrates. Harned (1925), however, states that/

that copper foil in the presence of nitrate tends to form nitrite which would affect the final results. West, Scharles and Peterson employed zinc dust to remove mercury from filtrates prepared by the use of mercuric sulphate. Copper foil will only remove mercury from filtrates having an acid reaction. The present writer employed copper foil for removing traces of mercury from mercuric sulphate filtrates. Before use the copper foil was cleaned with hydrochloric acid, washed with distilled water and dried between pieces of filter paper. The advantage of using copper foil is that one can tell when all the mercury has been removed by adding a fresh piece of foil to a filtrate when no further film of mercury should be deposited.

The method was carried out as follows. 1 volume of blood was laked in 10 volumes of water and then 1 volume of 30% mercuric sulphate in 10% sulphuric acid added. After shaking, solid barium carbonate was added and well shaken until the reaction of the mixture was about pH 7. A clear fluid is obtained upon filtration. To each 25 cc. of filtrate 2 drops of 50% sulphuric acid were added. Barium in solution was precipitated and the filtrate was sufficiently acid for the removal of mercury by means of copper foil. The filtrate was agitated at intervals with copper foil and when the foil became/

became covered with mercury it was replaced by a fresh piece, this being repeated until no further mercury was deposited. The solution was filtered and then placed into a tube which was evacuated 1-2 minutes to remove dissolved carbon dioxide. 10 cc. of the filtrate were used for each estimation. It will be noted that this filtrate represents a 1 in 12 dilution of blood. Filtrates prepared by the above process contain a very low concentration of inorganic salts in contrast to filtrates prepared by means of mercuric nitrate which contain a very high concentration of sodium nitrate. It was found that the reducing values of solutions of pure glucose were unaltered when submitted to the above process.

The tungstic acid filtrates were prepared according to the technique of Folin and Wu (1919) with the exception that normal instead of two-thirds normal acid was employed. During the reduction process cuprous oxide of a light and difficult form to centrifuge was obtained from filtrates prepared by the use of the weaker acid, but using the stronger acid filtrates were obtained from which the cuprous oxide was heavy and easily centrifuged.

Known quantities of glucose were added to blood and it/

it will be seen from the results shown in Table 15 that using either of the two methods of deproteinisation, added glucose could be determined quantitatively.

In Table 16 a comparison is shown of the sugar values of blood as determined in the two types of filtrates.

TABLE 15.

Deproteinisation with sodium tungstate and sulphuric acid:-

Sugar content of Blood. (mg.per 100cc.)	Glucose added. (mg.per 100cc.)	Total Sugar content of Blood. (mg.per 100cc.)	Glucose recovered. (mg.per 100 cc.)
67	50	117	50
67	100	169	102
67	200	266	199

Deproteinisation with mercuric sulphate and barium carbonate.

Sugar content of Blood. (mg.per 100cc.)	Glucose added. (mg.per 100cc.)	Total Sugar content of Blood. (mg.per 100cc.)	Glucose recovered. (mg. per 100 cc.)
103	100	203	100
103	200	301	198
0 (glycolysed)	50	49	49
0 (glycolysed)	100	98	98
0 (glycolysed)	200	201	201

TABLE 16.

	Tungstic Acid Filtrates (mg. per 100 cc. blood.)	Mercury Filtrates. (mg. per 100 cc. blood.)
26	88	72
27	101	76
28	106	84
29	91	75
30	141	125
31	74	68
32	88	76
33	76	73
34	106	81
35	74	68
36	89	78
37	81	73
Average	93	79

It will be seen that there is a difference in the reducing values of tungstic acid and mercury filtrates prepared from the same bloods. This was not to be expected from earlier work where it was shown that none of the known non-sugar reducing substances of the blood reduced the alkaline copper reagent to any appreciable extent. It will also be noticed that the difference between the sugar values as determined in the two types of filtrates are very variable.

Blood was allowed to glycolyse at 37° for 24 hours with constant shaking according to the technique of Hiller, Linder and Van Slyke (1925). After glycolysis the residual reduction was found to be the same, irrespective of the method of deproteinisation. The residual reduction in all cases was almost negligible being of the order 0-3 mg. per 100 cc. The question of glycolysis and yeast fermentation of blood will be discussed more fully later.

It appears from the results that a part of the glycolysable reducing substances present in tungstic acid filtrates is precipitated by treatment with mercuric sulphate and barium carbonate.

Tungstic acid filtrates were treated with mercuric sulphate and barium carbonate and the reducing values of such treated filtrates compared with mercury filtrates prepared directly/

directly from blood. The method of procedure was as follows:-

To 20-25 cc. tungstic acid filtrate were added 1-2 drops of 50% H_2SO_4 and about 0.5g. solid mercuric sulphate. After shaking barium carbonate was added until the reaction of the mixture was about pH 7 . After filtration, mercury and barium were removed from the filtrate as described earlier. The results are shown in Table 17.

TABLE 17.

	Tungstic Acid filtrates. (mg.per 100 cc. blood)	Mercury Filtrates. (mg.per 100cc. blood)	Tungstic acid filtrates treated with mercuric sulphate. (mg. per 100 cc.blood)
38	83	67	69
39	114	89	91
40	100	78	76
41	85	60	63
42	91	66	69
43	114	87	88
44	92	66	67
45	77	58	58
46	91	76	76
47	80	74	72
48	44	37	38
49	59	53	52
50	65	53	53
51	102	80	83
Average	85·5	67·4	68·1

It will have been seen that tungstic acid filtrates treated with mercuric sulphate and barium carbonate have the same reducing values as mercury filtrates prepared directly from blood.

The reducing values of tungstic acid filtrates were compared, as determined before and after treatment with mercuric sulphate. The remainder of these filtrates were saturated with solid benzoic acid, allowed to stand at room temperature several days when the estimations were repeated. The results are shown in Table 18.

TABLE 18.

	Tungstic acid Filtrates before treatment with $Hg.SO_4$. (mg.per 100cc.blood)	Tungstic acid Filtrates after treatment with $Hg.SO_4$. (mg.per 100 cc.blood)
52	149	121
3 days afterwards.	150	148
53	91	75
5 days afterwards.	91	89
54	88	64
4 days afterwards.	88	88
55	76	55
4 days afterwards.	76	74
56	221	194
5 days afterwards.	221	222
57	100	85
5 days afterwards.	100	101
58	83	66
7 days afterwards.	82	80

TABLE 18 (Contd.)

	Tungstic acid Filtrates before treatment with $Hg.SO_4$ (mg.per 100cc.blood)	Tungstic acid Filtrates after treatment with $Hg.SO_4$ (mg.per 100cc. blood)
59	160	155
5 days afterwards.	160	162
60	85	70
6 days afterwards.	85	85
61	100	82
6 days afterwards.	102	101
62	91	74
4 days afterwards.	91	91
63	115	93
4 days afterwards.	116	114

It will be seen that upon standing at room temperature for some days the reducing values of the filtrates were unchanged, but treatment with mercuric sulphate and barium carbonate failed to remove any of the reducing material. Apparently upon standing at room temperature the mercury-precipitable substance or substances present in fresh tungstic acid filtrates are changed to non-precipitable forms with the same reducing power.

At this stage of the work it was found that Fontés and Thivolle (1927) had arrived at almost similar conclusions. They had prepared an alkaline copper reagent very similar to that of the present writer. The amount of cuprous oxide formed during the reduction process was filtered and washed in a special apparatus and then dissolved in the phosphomolybdic acid reagent of Folin and Wu, giving a blue solution. The amount of cuprous oxide that had gone into solution was determined by titration with a standard solution of potassium permanganate, the end point being at the disappearance of the blue colour. Using this method these writers found that tungstic acid filtrates had higher reducing values than filtrates prepared with mercuric nitrate and sodium hydroxide. They found that tungstic acid filtrates when treated with mercuric nitrate and sodium hydroxide had the same reducing values as blood treated directly with these reagents, that is tungstic acid filtrates lost a part/

part of their reducing substances. When, however, these tungstic acid filtrates were heated in a boiling water bath for 3/4 hour with very dilute acid, no change occurred in their reducing values, and treatment with mercuric nitrate and sodium hydroxide did not remove any of their reducing substances. It will be seen then that these results are very similar to those of the present writer.

The hydrolysis technique of Fontés and Thivolle was examined. Tungstic acid filtrates of blood were prepared and their reducing values determined before and after treatment with mercuric sulphate and barium carbonate. The remainder of these filtrates were then put into a boiling water bath with 1/5 volume of N. H_2SO_4 for 45 minutes. After cooling they were made up to their original volumes when the above estimations were repeated. It will be seen from the results shown in Table 19 that the results of Fontés and Thivolle are confirmed.

TABLE 19.

		Tungstic acid Filtrates. (mg.per 100cc.blood)	Tungstic acid Filtrates after treatment with $HgSO_4$ (mg. per 100cc.blood)
64.	before hydrolysis	86	72
	after hydrolysis	86	85
65.	before hydrolysis	100	82
	after hydrolysis	101	100
66.	before hydrolysis	83	66
	after hydrolysis	83	83
67	before hydrolysis	88	68
	after hydrolysis	88	88
68	before hydrolysis	123	105
	after hydrolysis	125	123
69	before hydrolysis	149	121
	after hydrolysis	151	148
70	before hydrolysis	111	100
	after hydrolysis	110	111
71	before hydrolysis	160	155
	after hydrolysis	160	160

At this stage comparisons of the reducing values of tungstic acid and mercury filtrates with the reducing values of filtrates prepared with zinc hydroxide and copper hydroxide were made. These filtrates were prepared as follows:-

Zinc Hydroxide Filtrates:

The method of Somogyi (1929, 1930) was employed. Somogyi stated that such filtrates are free of all non-fermentable reducing substances. This has been confirmed by Herbert, Bourne and Groen (1930).

Solutions:

1. 10% zinc sulphate in 0.25 N. sulphuric acid.
2. 0.75 N sodium hydroxide.

Method:

1 volume of blood was laked with 7 volumes of water and then 1 volume of zinc sulphate solution added. After mixing 1 volume of sodium hydroxide was added and then filtered.

Copper Hydroxide Filtrates:

Somogyi (1930) has stated that when copper sulphate and sodium hydroxide are used as protein precipitants part of the fermentable reducing substances are precipitated, especially when bloods containing high concentrations of glucose are employed. He suggests that there is a tendency for alkaline strata to be formed before the blood and the reagents can be thoroughly/

thoroughly mixed and that in these alkaline strata part of the fermentable sugar tends to be precipitated as a protein complex. Somogyi did not give the exact details of his method. The present writer found the method described below to work very satisfactory. Glucose added to blood could be recovered quantitatively even when added in concentrations of 300 mg. per 100 cc. blood. In several cases the mixture of glycolysed blood and glucose after addition of the deproteinising agents was allowed to stand 1 hour before filtration and in no case was any loss of glucose recorded. After this section of the work had been finished Somogyi (1931) published fuller details of his work. As deproteinising agents he had employed a neutral solution of copper sulphate whereas the copper sulphate used by the present writer contained acid. This would probably account for the results recorded by Somogyi but not experienced by the present writer. If a neutral solution of copper sulphate is employed upon the addition of the requisite amount of sodium hydroxide to precipitate copper hydroxide, there would be a tendency for alkaline strata to be formed, whereas when an acid solution of copper sulphate is employed this would be minimised. Somogyi (1931) in his later work using sodium tungstate in place of sodium hydroxide, obtained much more satisfactory results. He states that copper hydroxide filtrates are preferable to zinc hydroxide filtrates/

filtrates for blood sugar determination, and found that they are free of all non-fermentable reducing substances. The method of preparing copper hydroxide filtrates as used by the present writer was as follows:-

Solutions:

1. 10% copper sulphate in 0.25 N sulphuric acid.
2. 0.75 N. sodium hydroxide.

Method:

1 volume of blood was laked in 7 volumes of water and then 1 volume of copper sulphate solution added. Then 1 volume of sodium hydroxide was added, and the whole well shaken and filtered.

The pH of these copper hydroxide filtrates is about 6.5.

Known amounts of glucose were added to blood already containing sugar and also to glycolysed blood, and the mixtures deproteinised according to one or the other of the two methods to see whether the added glucose could be determined quantitatively. From the results shown in Table 20 it will be seen that added glucose could be determined quantitatively.

The reducing values of zinc and copper filtrates as compared with those of tungstic acid and mercury filtrates of the same bloods are shown in Tables 21 and 22 respectively. It will be seen that the reducing values of copper and mercury filtrates agree but zinc filtrates are higher than these but less than those of tungstic acid filtrates.

TABLE 20.

Deproteinisation with Zinc sulphate and Sodium Hydroxide.

Sugar content of Blood. (mg.per 100cc.)	Glucose added. (mg.per 100cc.)	Total Sugar content of Blood. (mg.per 100cc.)	Glucose recovered. (mg.per 100cc.)
92	100	190	98
92	200	293	201

Deproteinisation with Copper sulphate and Sodium Hydroxide.

Sugar content of Blood. (mg.per 100cc.)	Glucose added. (mg.per 100cc.)	Total Sugar content of Blood. (mg.per 100cc.)	Glucose recovered. (mg. per 100 cc.)
81	100	182	101
81	200	280	199
0 (glycolysed)	100	102	102
0 (glycolysed)	200	198	198
0 (glycolysed)	300	299	299

TABLE 21.

	Tungstic acid filtrates. (mg.per 100cc.blood)	Mercury filtrates. (mg.per 100cc. blood)	Zinc Filtrates. (mg. per 100 cc.blood)
72	77	58	67
73	94	80	88
74	115	103	108
75	91	76	80
76	80	73	78
77	44	37	42
78	59	53	56
79	95	75	83
80	65	53	58
81	102	80	90
82	106	84	98
Average	84.4	70.2	77.1

TABLE 22.

	Tungstic acid Filtrates. (mg.per 100cc.blood)	Mercury Filtrates. (mg.per 100cc. blood)	Copper Filtrates. (mg.per 100 cc.blood)
83	91	69	69
84	91	76	74
85	76	73	73
86	80	74	73
87	44	37	37
88	59	53	53
89	65	53	52
90	102	80	83
91	79	66	64
92	105	83	86
93	68	58	58
Average	78.2	65.6	65.5

The Yeast Fermentable and Glycolysable Sugar of Blood.

A useful criterion of the amount of non-sugar reducing substances that are being included in a blood sugar determination, is to determine the reducing power of blood after fermentation by yeast or after glycolysis. The assumption is naturally made that the reducing substances destroyed during these processes are true sugars and that the reducing powers of the non-sugar reducing substances are unchanged.

Macleod (1926) has raised serious objections to the yeast fermentation method since he states that yeast itself contains or produces reducing substances during fermentation and may even convert the non-sugar reducing substances into non-reducing forms.

It has been shown by numerous workers, including Van Slyke, Hiller and Linder (1925, 1926), Folin and Svedberg (1926), Benedict (1928), and Somogyi and Kramer (1928) that the values of the non-sugar reducing substances vary considerably according to the method employed for determining sugar. Van Slyke, Hiller and Linder (1925, 1926) and Ege and Roche (1930) are however agreed that these values are the same whether determined after glycolysis or after yeast fermentation.

Many/

Many modifications for fermenting blood have been employed. Several workers have fermented blood or protein free filtrates with small quantities of yeast for varying lengths of time. Van Slyke, Hiller and Linder (1925,1926), Folin and Svedberg (1926), Ege and Roche (1930), Bigwood and Wuillot (1927) among others have employed such methods. Somogyi (1927) however stated that if to volume of blood $7\frac{1}{2}$ volumes of a 10% suspension of freshly washed yeast are added, the sugar is almost instantly fermented at room temperature and the mixture could then be deproteinised at once. For the determination of glycolysable sugar, the majority of writers have followed the technique of Van Slyke, Hiller, and Linder, (1925) and Ege and Roche (1930) who incubated blood at 37^{o} for 24 hours with occasional shaking.

The question is as to whether the residual reduction, this term being applied to reducing substances which are not fermentable or glycolysable, presents any systematic variations in different physiological and pathological conditions. Folin and Svedberg (1926) found almost constant figures when the Folin and Folin and Wu methods were used. Van Slyke, Hiller, and Linder (1925) have showed that the values persisted after insulin, while Bigwood and Wuillot (1927) state that the residual reduction of plasma is independent of diet. Ege and Roche (1930)/

(1930) have shown that the residual reduction of blood varies very little from one individual to another and presents only slight variations in the same man during fasting, digestion and exercise. Rabinowitch (1928) has shown that the residual reductions of diabetic blood is within normal limits and is uninfluenced by the administration of insulin or glucose.

Ege and Roche (1930) found higher results for fermentable and glycolysable sugar in the blood using the method of Fontés and Thivolle than when using the method of Hagedorn and Jensen. Folin and Svedberg (1926) obtained higher values for fermentable sugar by the Folin and Wu method than by the Folin method. They found that the difference in values given by the two methods was much higher in diabetic bloods, the differences varying according to the amount of sugar. Oral administration of glucose did not affect this difference but injection of insulin decreased it. Injection of adrenalin at times produced an increase, at other times a decrease in this value. Folin and Svedberg found these differences as appreciable in plasma as in whole blood. These results were confirmed later by Folin and Malmros (1929).

Methods employing yeast fermentation at 37° for definite periods of time have the disadvantage that a certain amount of autolysis of yeast takes place. The present writer found/

that
found when such yeast suspensions were deproteinised with tungstic acid, clear filtrates could not be obtained. When a mixture of blood and yeast were incubated at 37° for a definite period of time, upon deproteinisation with tungstic acid clear filtrates could always be obtained in contrast to the controls. Such a state of affairs would undoubtedly lead to erroneous results. The present writer employed Somogyi's method (1927), and as a preliminary known concentrations of glucose were fermented according to this technique and the mixtures deproteinised according to the various methods already employed. It was found that when 10% suspensions of freshly washed yeast were deproteinised according to any of the methods, the reducing values of the filtrates were always the same as the blank using distilled water - that is the filtrates contained no reducing substances from the yeast. No difficulty was experienced in obtaining clear filtrates when these yeast suspensions were deproteinised by any of the methods. It was found that concentration of glucose of 100 mg. per 100 cc. were completely fermented according to the technique of Somogyi, but in the case of glucose concentrations of 200 mg. per 100 cc. about 10% remained unfermented. When such mixtures were allowed to remain in contact with the yeast for/

for 5 minutes at room temperature prior to deproteinisation, fermentation was always found to be complete.

Blood was fermented according to this technique, but the mixtures of blood and yeast were always allowed to remain in contact for at least 10 minutes prior to deproteinisation. It was found that the residual reduction was found to be of the order of 0-3 mg. per 100 cc. and to be independent of the deproteinising agent employed. Similar results have already been reported for the residual reduction of blood after glycolysis.

The question arises as to whether the differences in reducing values of tungstic acid and copper [and mercury] filtrates of whole blood are observed in the case of plasma. With this in view a series of comparisons of the reducing values of tungstic acid and copper filtrates of plasma were made and these compared with the corresponding values obtained in the case of whole blood. It will be seen from the results shown in Table 23 that copper filtrates of plasma have lower reducing values than those of tungstic acid filtrates and the differences are only slightly less than in case of whole blood.

TABLE 23.

	WHOLE BLOOD.		PLASMA.	
	Tungstic acid Filtrates (mg.per 100cc.)	Copper Filtrates. (mg.per 100cc)	Tungstic acid Filtrates. (mg.per 100cc)	Copper Filtrates. (mg. per 100 cc)
94.	98	84	116	106
95.	91	81	104	98
96.	98	78	106	94
97.	84	73	96	89
98.	121	101	136	121
99.	97	80	106	93
100.	88	77	97	90
101.	108	84	119	98

A series of comparisons were made of the difference in reducing values of tungstic acid and copper filtrates of the blood of normal and diabetic people both before breakfast and 2 hours after an ordinary meal. The figures obtained in the case of normal people are shown in Table 24. It will be seen that the differences are very small in blood taken before breakfast when the people have been without food for 10-12 hours, but 2 hours after a meal the differences are mugh higher. Fontés and Thivolle (1927) have noted that the differences between the reducing values of tungstic acid and mercury filtrates are much higher in bloods taken during digestion than when taken after starvation.

In Table 25 are recorded the differences in reducing values of the two types of filtrates of the bloods of diabetic patients. It will be observed that the differences are very much higher than normal both after starvation and after meals when the patients are not having insulin, but when the patients are having insulin the differences are within normal limits.

TABLE 24.

A. Before breakfast.

	Tungstic Acid Filtrates. (mg.per 100cc.blood)	Copper Filtrates. (mg. per 100cc.blood)	Difference.
	94	94	0
	80	73	7
	88	78	10
	88	81	7
	81	74	5
	76	73	3
	80	74	6
Average	83·9	78	5·4

B. 2 hours after a meal.

	106	84	22
	102	80	22
	95	75	20
	91	74	17
	91	66	25
	92	66	26
	114	87	27
	87	71	16
Average	97·2	75·4	21·9

TABLE 25.

Diabetic Patients.

	Tungstic Acid Filtrates. (mg. per 100cc. blood)	Copper Filtrates. (mg.per 100 cc.blood)	Difference.
A. Starvation and no insulin.			
	170	145	25
	130	115	15
	152	135	17
B. 2 hours after a meal and no insulin.			
	414	351	63
	436	385	51
	182	152	30
	277	231	46
	171	133	38
Average	296	250·4	45·6
C. 2 hours after a meal and with insulin.			
	86	65	21
	88	73	15
	138	111	27
	81	70	11
	123	108	15
	117	95	22
	156	154	2
	217	206	11
	157	133	24
	160	155	5
	115	93	22
Average	130·7	114·8	16

DISCUSSION.

It has been shown that the amount of copper reduced in an alkaline copper reagent by glucose is dependent on the concentrations of tartrate and carbonate in the reagent. An alkaline copper reagent has been prepared in which the concentrations of tartrate and sodium carbonate are such that the amount of copper reduced by glucose is a maximum for this type of reagent. This reagent was not reduced by any of the amino acids examined (Glycine, Aspartic Acid, Glutamic Acid, Tyrosine, Cystine), urea or the Sulphydril compounds Glutathione or Dithioglycollic Acid. Concentrations of these substances higher than present in blood filtrates were used. It has been shown that creatine, creatinine and uric acid in the concentrations that they are present in the blood, have inappreciable reducing values. None of the above mentioned substances affected the reducing values of glucose in any way.

This alkaline copper reagent has been applied to a study of the reducing substances in the blood. Glucose added to both fresh and glycolysed blood could be estimated quantitatively.

It has been found that the residual reduction of blood after yeast fermentation or glycolysis is very low, being/

being of the order of 0-3 mg. per 100 cc. blood. This almost inappreciable value was found to be independent of the deproteinising agents employed (tungstic acid, zinc hydroxide, copper hydroxide and mercuric sulphate + barium carbonate). When, however, the reducing values of different types of blood filtrates were compared, the results were not the same. Mercury and copper filtrates had similar reducing values, while those of tungstic acid filtrates were higher. The reducing values of zinc filtrates were intermediate between those of copper (or mercury) filtrates and those of tungstic acid filtrates. It would appear then that part of the fermentable and glycolysable reducing substances present in tungstic acid filtrates are precipitable or partly precipitable by these metallic reagents. The reducing values of fresh tungstic acid filtrates were found to be reduced by treatment with mercuric sulphate and barium carbonate, to the same values as those of mercury filtrates prepared directly from blood.

Reference has already been made to the work of Fontés and Thivolle (1927). These authors used a method very similar to that used by the present writer and obtained very similar results. They found that the residual reduction both in tungstic acid filtrates and mercury filtrates, as/

as determined by their method, was very low, of the order of that obtained by the present writer. They found, however, that the reducing values of tungstic acid filtrates were always higher than mercury filtrates, that is the former contained more fermentable sugar than the latter. Ege and Roche (1930) using the method of Fontés and Thivolle confirmed the low residual reduction observed in tungstic acid filtrates and found that the fermentable sugar of the blood was higher, using the method of Fontés and Thivolle, for tungstic acid filtrates than when using the method of Hagedorn and Jensen for tungstic acid or zinc filtrates. Reference has also been made to the work of Folin and Svedberg (1926) and Folin and Malmros (1929) who found that the fermentable sugar determined in tungstic acid filtrates was higher using the Folin and Wu method (1919) than using the Folin (1926) method. These authors were unable to identify the substance causing the discrepancy between the two methods.

The present writer has shown that tungstic acid filtrates saturated with benzoic acid and allowed to stand at room temperature several days, showed no change in their reducing values but treatment with mercuric sulphate and barium carbonate failed to remove any of the reducing material. It appears then that under these conditions the mercury precipitable reducing substances present in tungstic acid/

acid filtrates are changed into non-precipitable forms with the same reducing values. It has already been recorded that Fontés and Thivolle found that the mercury-precipitable reducing substances of tungstic acid filtrates were converted into non-precipitable forms of the same reducing power, by weak acid hydrolysis in a boiling water bath for ½ hour. They also noted the ease with which this change took place at room temperature in acid solution.

The present writer and Fontés and Thivolle have found that the differences in reducing values of tungstic acid and copper (or mercury) filtrates of plasma are almost as high as in the case of whole blood. It is interesting that Folin and Svedberg state that the differences in the values of fermentable sugar as determined by the Folin and Wu and Folin methods are as high in plasma as in whole blood.

The present writer has noted that the differences in reducing values of tungstic acid and copper (or mercury) filtrates were very small in blood taken after starvation but were increased considerably in blood taken 2 hours after an ordinary meal. Fontés and Thivolle have recorded similar results.

The differences between the reducing values of tungstic/

tungstic acid filtrates and copper (or mercury) filtrates were observed by the present writer to be much higher than normal in diabetic bloods both after starvation and after meals when the patients were not having insulin, but when the patients were having insulin the results were within normal limits. Folin and Svedberg noted that the differences in values for fermentable sugar obtained by the Folin and Wu and Folin methods were always much higher in diabetic bloods, the difference increasing with the severity of the disease. They found that treatment with insulin reduced the differences within normal limits, but found that administration of glucose had no influence on the differences. Fontés and Thivolle observed that the administration of insulin tended to decrease the differences between the reducing values of tungstic acid and mercury filtrates but glucose had no influence.

It seems that the phenomena observed by the present writer and Fontés and Thivolle are related to the same substance or substances producing the results of Folin and Svedberg.

The substance or substances responsible for the results recorded in this thesis are almost evenly distributed between the corpuscles and the plasma. This substance or substances/

substances are not hexosephosphates or related to glucose. Hexosephosphates are very difficult to hydrolyse and even when hydrolysed show marked increases in their reducing power. Lawaczeck (1924,1925) states that hexosephosphates account for no more than 1% of the reducing power of blood filtrates. The high non-sugar reducing values of tungstic acid filtrates observed when certain other reduction methods have been employed, have been shown to be due almost exclusively to the constituents of the corpuscles, and these values show no systematic variations in starvation, digestion or diabetes, being almost constant under these conditions.

The substance which the present writer, Fontés and Thivolle, and Folin and Svedberg have found to be present in tungstic acid filtrates appears to reduce only very alkaline reagents, e.g. the Folin and Wu (4% sodium carbonate); the reagent of Fontés and Thivolle (9·3% potassium carbonate); the reagent of the present writer (8% sodium carbonate). The Folin reagent (1926) on the other hand is very faintly alkaline.

The present writer up to the present has been unable to isolate or identify this substance. Its isolation is difficult owing to it being so labile and present in such low concentrations in blood except after meals or in diabetes.

Specimens/

Specimens of 'slaughterhouse' blood have been found to contain only traces of the substance - this being probably due to the animals being starved prior to slaughter.

In the past the yeast fermentation technique has been used for determining that part of the reducing value of a blood filtrate which is due to sugar. It is really a very crude method although at present there is no better substitute. Many substances other than sugars and related substances are fermentable by yeast. Neubauer and Fromherz (1911) and Neuberg and Hildesheimer (1911) have found that the following substances are fermented by yeast - exalacetic acid, butyric acid, glycerin phosphoric acid, cystine, phenylamino acetic acid, and p-oxyphenyl pyroracemic acid. The latter two substances being only fermentable in the presence of glucose. It is this last possibility which must not be overlooked when using any yeast fermentation technique or even glycolysis for very little is known about the process. MacLeod (1926) has objected to the yeast fermentation technique. He states that yeast may convert non sugar reducing substances into non-reducing forms.

From the evidence submitted here and in the communications of other workers, the present writer considers that the substance or substances producing the phenomena lately described are more probable of protein rather than carbohydrate origin.

SUMMARY.

1. A study of alkaline copper reagents has been made and a reagent prepared which was not reduced by any of the known non-sugar reducing substances of the blood in concentrations that they are present in the blood.

2. A study of the reducing values of tungstic acid filtrates and filtrates prepared by the use of heavy metals has been made.

The results obtained have been discussed.

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The Enzymatic Synthesis of Proteins

With Special Reference to the Action of Pepsin.

The first report of an enzymatic synthesis of protein from substances of lower molecular weight was made by Danilewski in 1886. Danilewski observed that when an extract of stomach was added to a concentrated solution of the products of a peptic digest of protein, a precipitate formed. He believed that this phenomenon was due to an enzyme since it did not occur if the stomach extract was previously heated to 100°. Okunew in 1895 obtained similar results. The phenomenon was further investigated by Sawjalow (1901) who gave the name plastein to the precipitate he obtained.

The first unchallenged enzymatic synthesis of a protein was obtained by Taylor (1907, 1909). Taylor digested protamine sulphate with a glycerol extract of the liver of the Pacific coast clam (Schizothaerus Nuttalii), containing an enzyme of the autolytic class, a mixture of amino acids being obtained. After concentration of these products and after further addition of the enzyme accompanied by long incubation this observer was able to obtain a substance which was identified as protamine sulphate. This synthesis was not considered of much importance by physiologists on account of the small yield and the long period of incubation necessary - namely, five months.

Regarding/

Regarding the nature of plastein little was known by the earlier workers. The formation of a precipitate was considered sufficient evidence that a more complex substance had been formed. Okunew (1895) stated that his digests contained 2% more water after plastein formation had taken place. Sawjalow (1901) believed that his plastein was a more complex substance than its precursors owing to its greater ease of precipitation by metallic salts. Henriques and Gjaldbäk (1911, 1912) found that there was always a decrease in the formal titrable nitrogen during plastein formation.

The rate of hydrolysis of a protein may be measured by estimating the rate of increase of free amino nitrogen, but a decrease may only be interpreted as a synthesis when the amount of ammonia remains unchanged, and when there is a coincident decrease in the concentration of carboxyl groups. Henriques and Gjaldbäk determined the ratio of free amino nitrogen to total nitrogen of purified specimens of plasteins and found the figures comparable with those of the native proteins. Since Wasteneys and Borsook (1925, 2) found that this ratio was higher for casein than for proteoses prepared from peptic digests/

digests of egg albumin, they do not consider this ratio a fair criterion of the complexity of protein substances.

Morrell, Borsook and Wasteneys (1927) demonstrated that the rate of hydrolysis of a protein by pepsin as measured by the amount hydrolysed in the first half hour, diminishes as the concentration of protein increases. Wasteneys and Borsook (1925, 2) had previously considered that a high concentration of protein disintegration products should favour a reversal of this process, namely a synthesis. These investigators confined their attention to the action of pepsin upon concentrated peptic digests of egg albumin. They found that plastein formation did not occur if the concentration of the digests were less than 2 g. of nitrogen per 100 cc. Wasteneys and Borsook (1925,4) were also the first to accurately determine the optimum hydrogen ion concentration for plastein formation. They obtained the greatest yields of plastein from peptic digests of egg albumin at pH 4.0 and found that the yield decreased very rapidly on either side of this value.

The present writer has further examined this problem, and has extended the investigations to other proteins.

ANALYTICAL METHODS.

The peptic digests were prepared as follows. To 1000cc. of water, 25g. of protein were added followed by sufficient hydrochloric acid to bring the pH to 1.7. This mixture was incubated for about 6 days at 37° with 0.5% pepsin present. After incubation the whole was placed in a boiling water bath for ½ hour to destroy the enzyme and when cool, filtered. The filtrate was concentrated in vacuo at 40° until it had a concentration of 3-4 g. nitrogen per 100 cc.

The amount of plastein formation was determined by the method of Wasteneys and Borsook (1925, 1). They had shown that if to a mixture of protein and its disintegration products, trichloracetic acid was added to a concentration of 2%, protein only was precipitated, such substances as proteoses, peptones and even gelatin remaining in solution. These workers found that plastein which is fairly soluble in concentrated peptic digests, was insoluble in this concentration of trichloracetic acid and could be precipitated from solution in the same way as proteins.

The technique for the determination of the amount of plastein formation was as follows. 5 cc. of the digest

were/

were diluted to 25 cc. and then the nitrogen content was determined in a 5 cc. sample by the Kjeldahl method. To another 5 cc. sample of the digest 15 cc. water were added, then 2.5 cc. of 20% trichloracetic acid and finally the whole was diluted to 25 cc. and mixed. After standing for the specific period the plastein was filtered off and the nitrogen content of 5 cc. of the filtrate was determined. From these two results the percentage plastein formation was calculated.

As a preliminary the effect of the hydrogen ion concentration upon plastein formation in concentrated peptic digests of egg albumin, serum albumin and casein were studied.

INFLUENCE OF HYDROGEN ION CONCENTRATION.

Specimens of concentrated peptic digests of the various proteins were each divided into a series of 10 cc. specimens and the pH of each roughly adjusted by the addition of either dilute HCl or NaOH so that a range of hydrogen ion concentrations from about pH 2.0 to 6.5 was obtained. These specimens were adjusted to the same volume by the addition of the requisite amount of water. 1 g. of pepsin and a few drops of toluol were added to each. They were/

were then stoppered and incubated at 37° for 3 days, being shaken at intervals to facilitate solution of the pepsin. The amount of plastein formation was then determined by the method of Wasteneys and Borsook. The hydrogen ion concentration of each specimen was determined electrometrically after the pepsin had dissolved. The data obtained are shown in Tables 1. (egg albumin), 2 (serum albumin) and 3 (casein).

It will be seen that the optimum pH for plastein formation in all peptic digests of the three proteins examined was a slightly over pH 4.0. This is in agreement with the results obtained by Wasteneys and Borsook (1925, 4) for peptic digests of egg albumin.

TABLE I.

Expt. 1. Concentrated peptic digest of egg albumin.

Total nitrogen - 3.846 g. per 100 cc.

Pepsin - 5 g. per 100 cc.

pH.	% Plastein formation.
3.0	9.3
3.5	14.6
3.9	18.9
4.0	19.8
4.3	20.6
4.7	12.1
5.2	9.8
5.8	6.2
6.4	3.1

Expt. 2. Concentrated peptic digest of egg albumin.

Total nitrogen - 4.431 g. per 100 cc.

Pepsin - 5 g. per 100 cc.

Incubated 3 days at 37°.

pH	% Plastein formation.
2.5	6.8
3.1	9.2
3.8	17.8
4.1	19.2
4.5	14.3
4.9	11.1
5.1	6.3
5.6	6.0

TABLE 2.

Expt. 3. Concentrated peptic digest of serum albumin.

Total nitrogen - 3.961 g. per 100 cc.

Pepsin - 5 g. per 100 cc.

Incubated 3 days at 37°.

pH.	% Plastein formation.
1.4	1.7
2.2	4.9
3.0	8.3
3.7	14.6
3.9	19.4
4.2	21.7
4.5	17.2
5.0	12.2
6.0	3.1

Expt. 4. Concentrated peptic digest of serum albumin.

Total nitrogen - 4.986 g. per 100 cc.

Pepsin - 5 g. per 100 cc.

Incubated 3 days at 37°

pH	% Plastein formation.
2.6	8.3
3.1	12.6
3.5	15.7
3.7	17.9
3.9	19.8
4.1	21.4
4.3	19.6
4.6	15.7
5.6	7.4
6.0	6.2

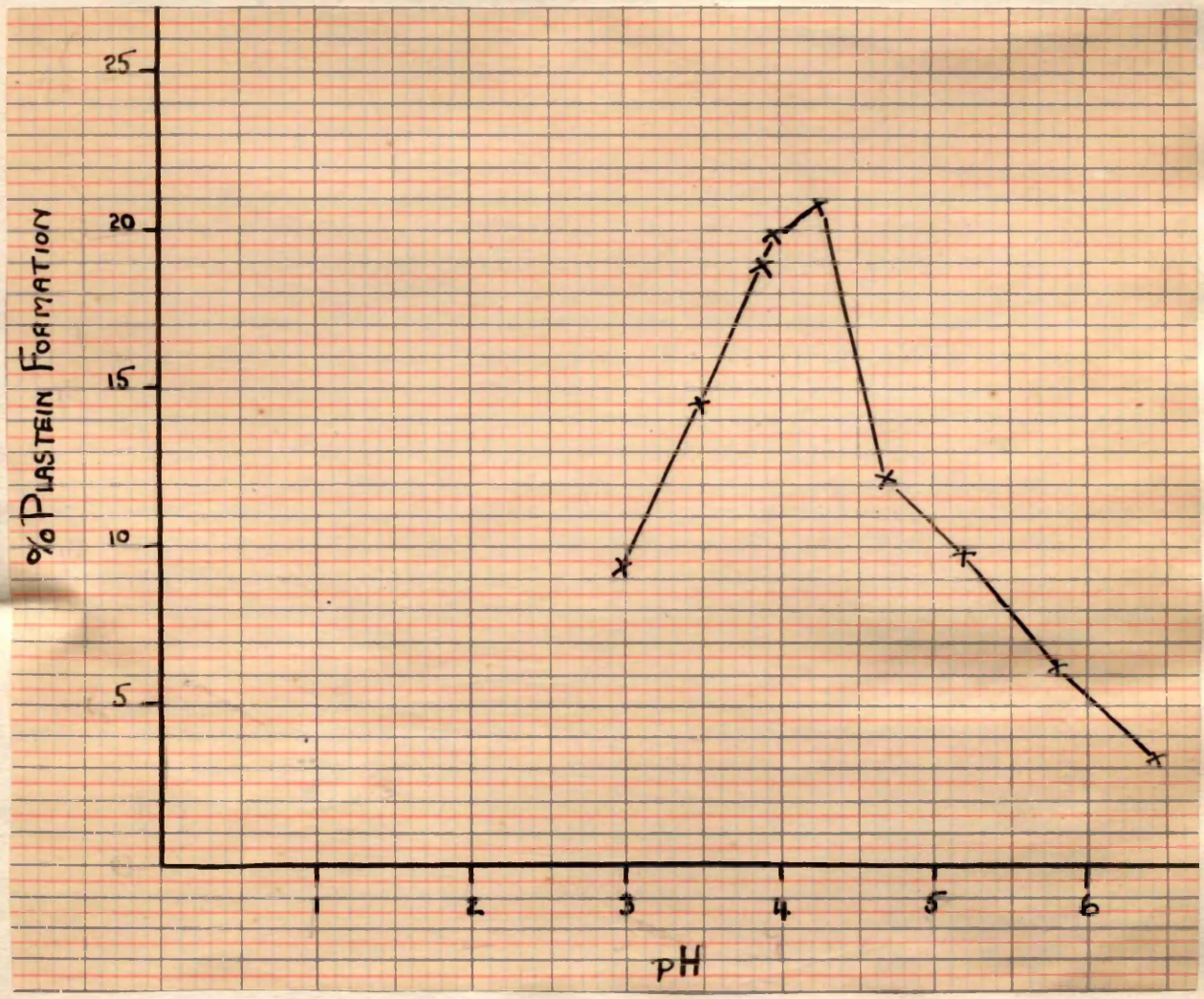
TABLE 3.

Expt. 5. Concentrated peptic digest of casein.

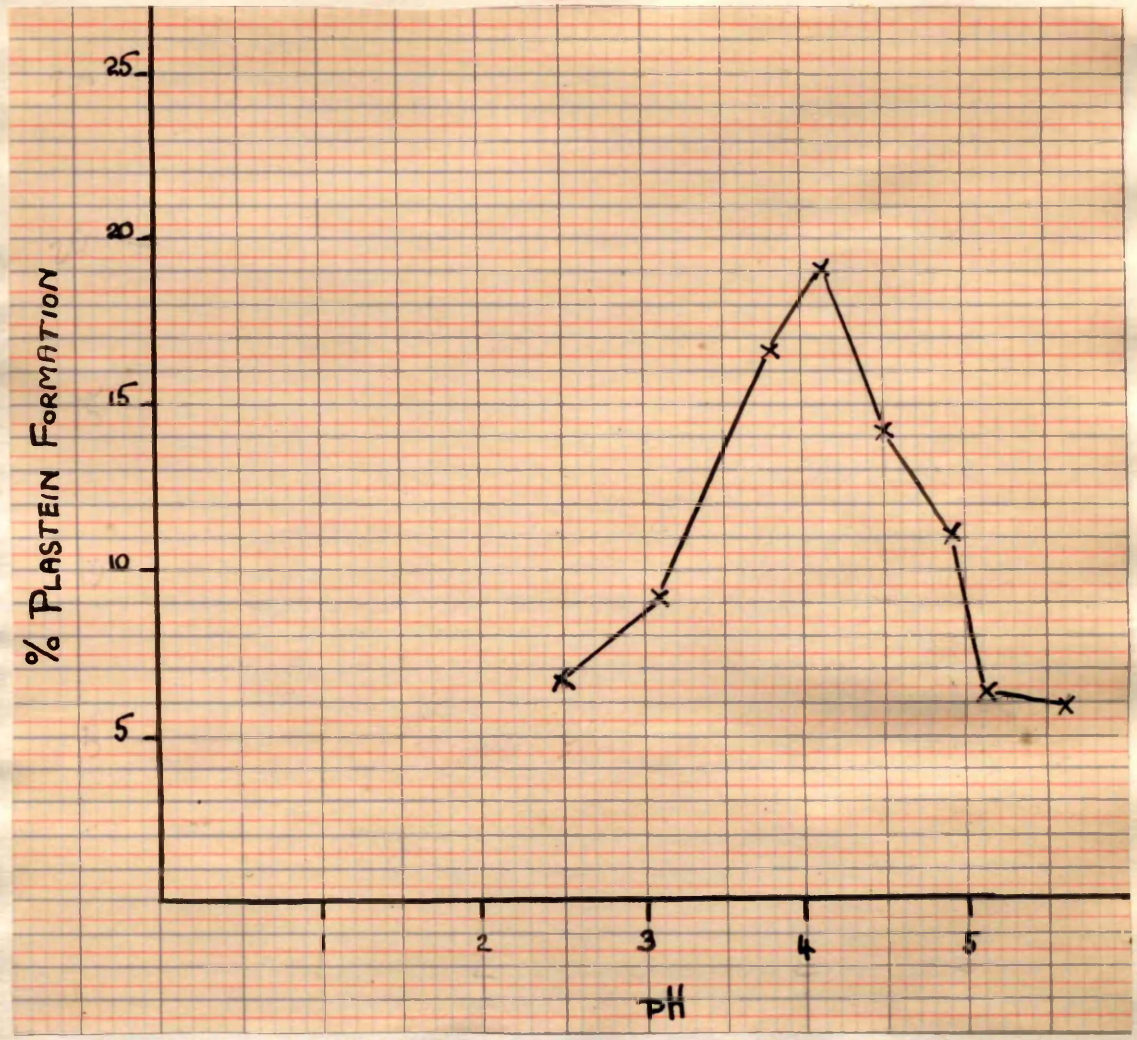
Total nitrogen - 4.634 g. per 100 cc.

Pepsin - 5 g. per 100 cc.

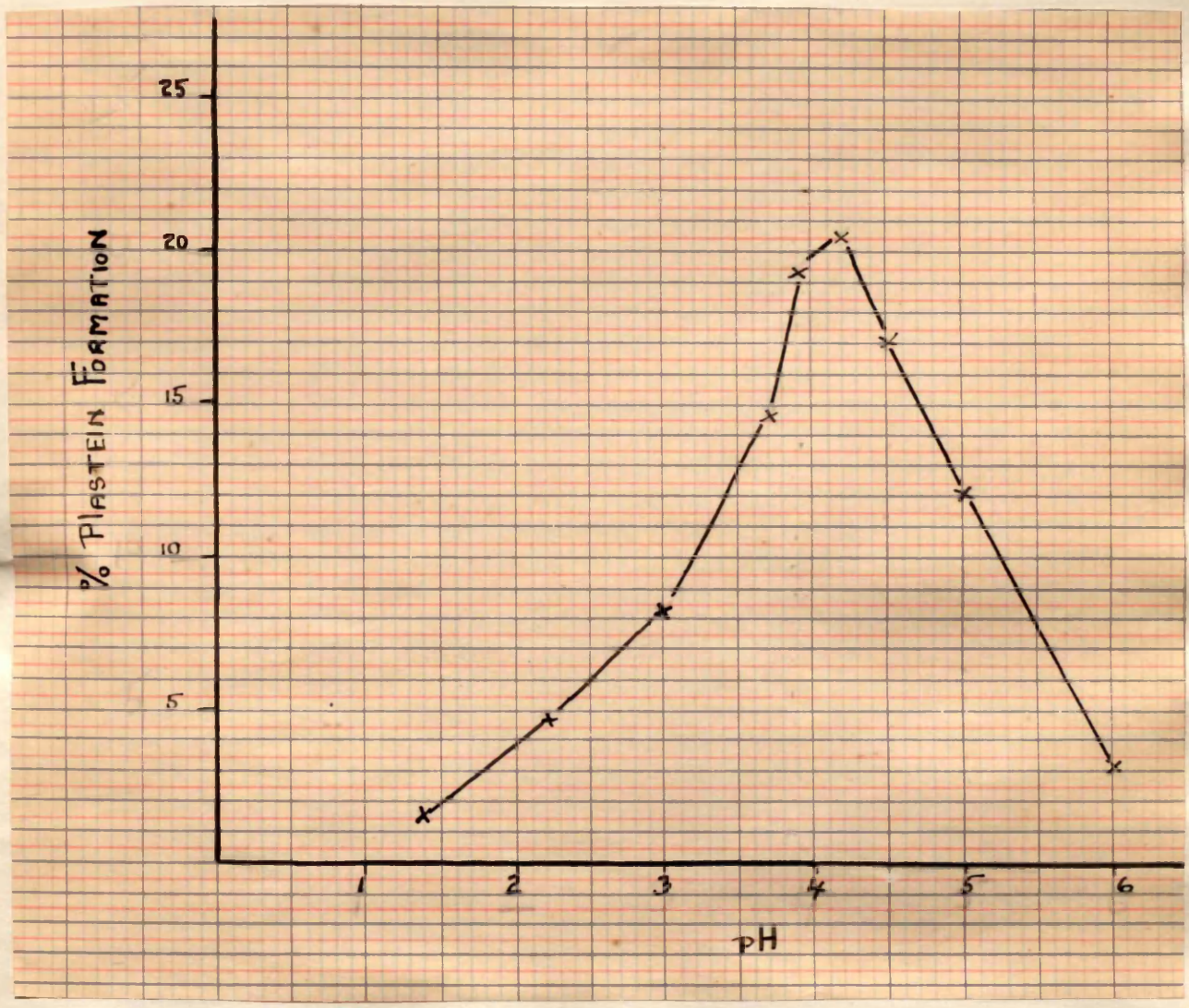
pH.	% Plastein formation.
2.9	4.3
3.4	7.8
3.6	8.1
4.2	9.4
4.4	7.6
4.8	5.4
5.2	3.2
6.1	1.1



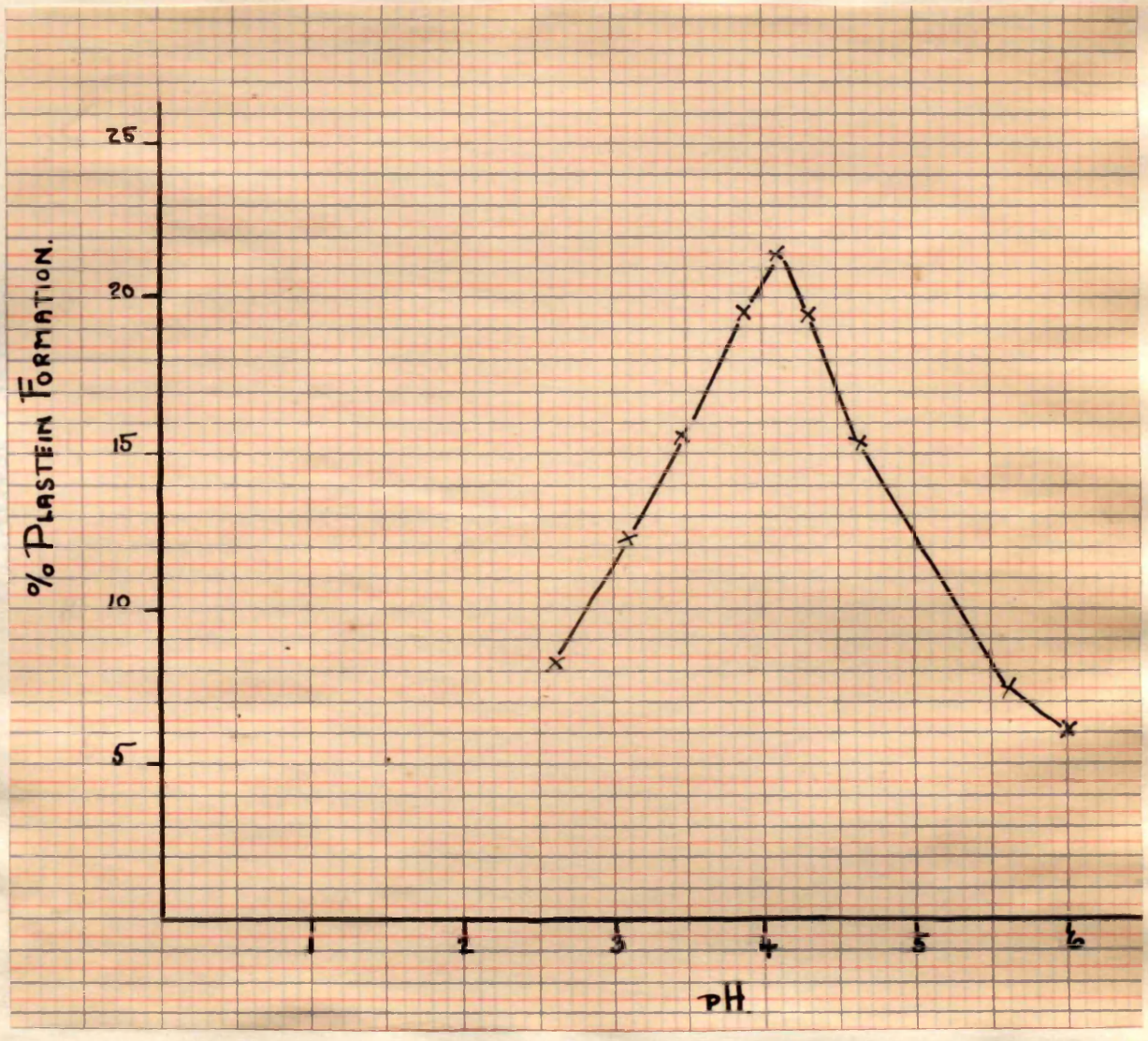
EXPT. I. Influence of Hydrogen Ion Concentration - Concentrated Peptic Digest of Egg Albumin.



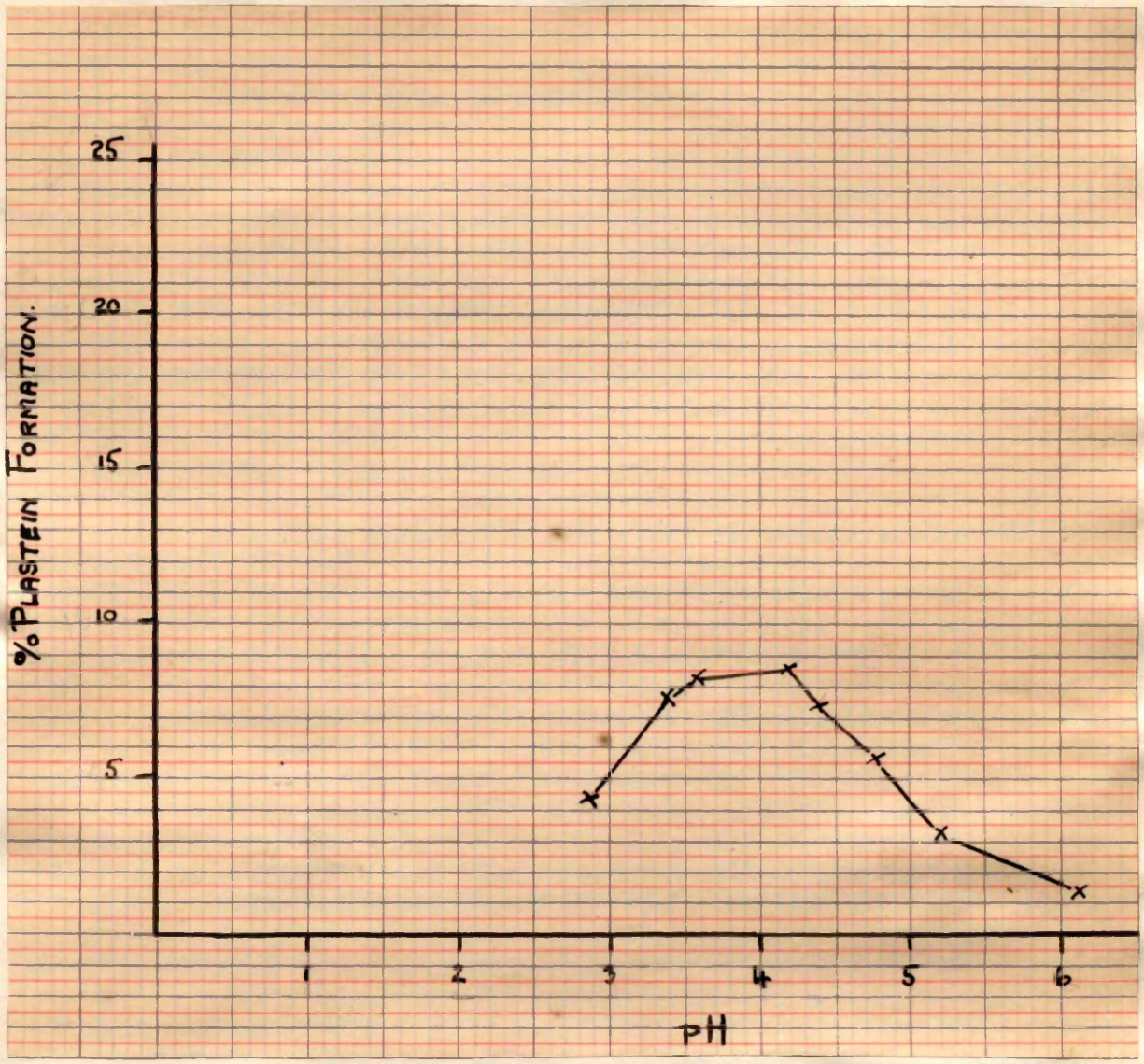
EXPT. 2. Influence of Hydrogen Ion Concentration - Concentrated Peptic Digest of Egg Albumin.



EXPT. 3. Influence of Hydrogen Ion Concentration - Concentrated Peptic Digest of Serum Albumin.



EXPT. 4. Influence of Hydrogen Ion Concentration - Concentrated Peptic Digest of Serum Albumin.



EXPT. 5. Influence of Hydrogen Ion Concentration - Concentrated Peptic Digest of Casein.

THE INFLUENCE OF TEMPERATURE.

Sawjalow (1901) and Henriques and Gjaldbak (1912) had observed from qualitative experiments that an increase in temperature led to an increase in plastein formation. Robertson (1909) and Wasteneys and Borsook (1925, 3) demonstrated this quantitatively.

Plastein formation does not increase indefinitely with temperature, since pepsin is destroyed above 72°. Wasteneys and Borsook (1925, 3) controlling the pH and using concentrated peptic digests of egg albumin, obtained their maximum yield of plastein at 72°, obtaining no plastein at 80°.

This phenomenon has been re-investigated using concentrated peptic digests of egg albumin and serum albumin. The digests were adjusted to pH4.0 before use, and before addition of the enzyme, the digest was allowed to come to the temperature at which the reaction was to be studied. Plastein formation was determined after 24 hours incubation. The results are shown in Tables 4 (egg albumin digest) and 5 (serum albumin digest).

It will be seen from the results that the amount of plastein formation increases rapidly with increase of temperature up to 70°, the amount of plastein at 80° being nil.

TABLE 4.

Expt. 6. Concentrated peptic digest of egg albumin.

Total nitrogen - 3.65 g. per 100 cc.

Pepsin - 5 g. per 100 cc.

pH - 4.0

Time - 24 hours.

Temperature.	% Plastein formation.
24°	8.6
31°	12.2
37°	19.6
50°	23.1
60°	25.7
70°	29.2
80°	0.8

Expt. 7. Concentrated peptic digest of serum albumin.

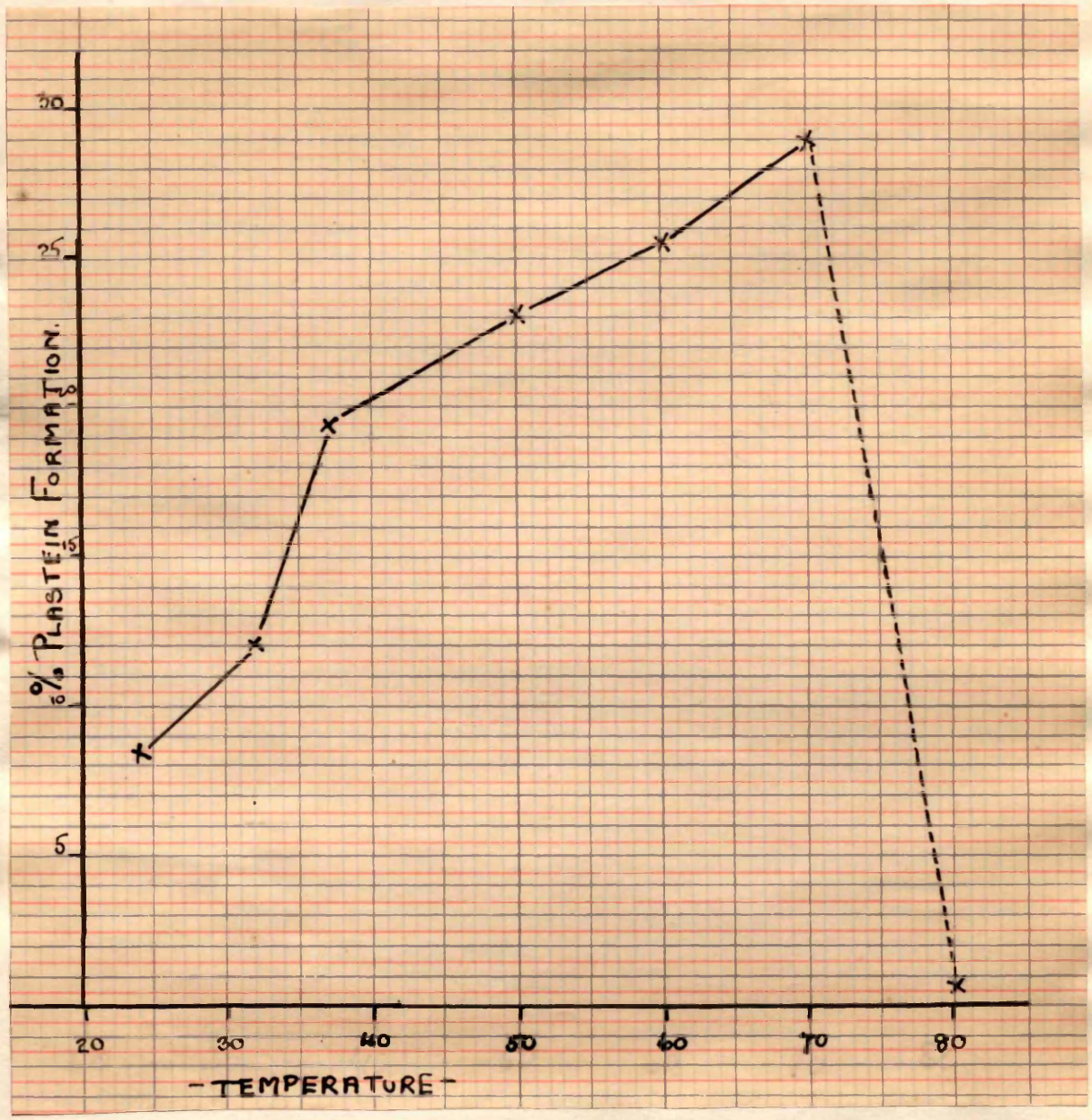
Total nitrogen - 4.02 g. per 100 cc.

Pepsin - 5 g. per 100 cc.

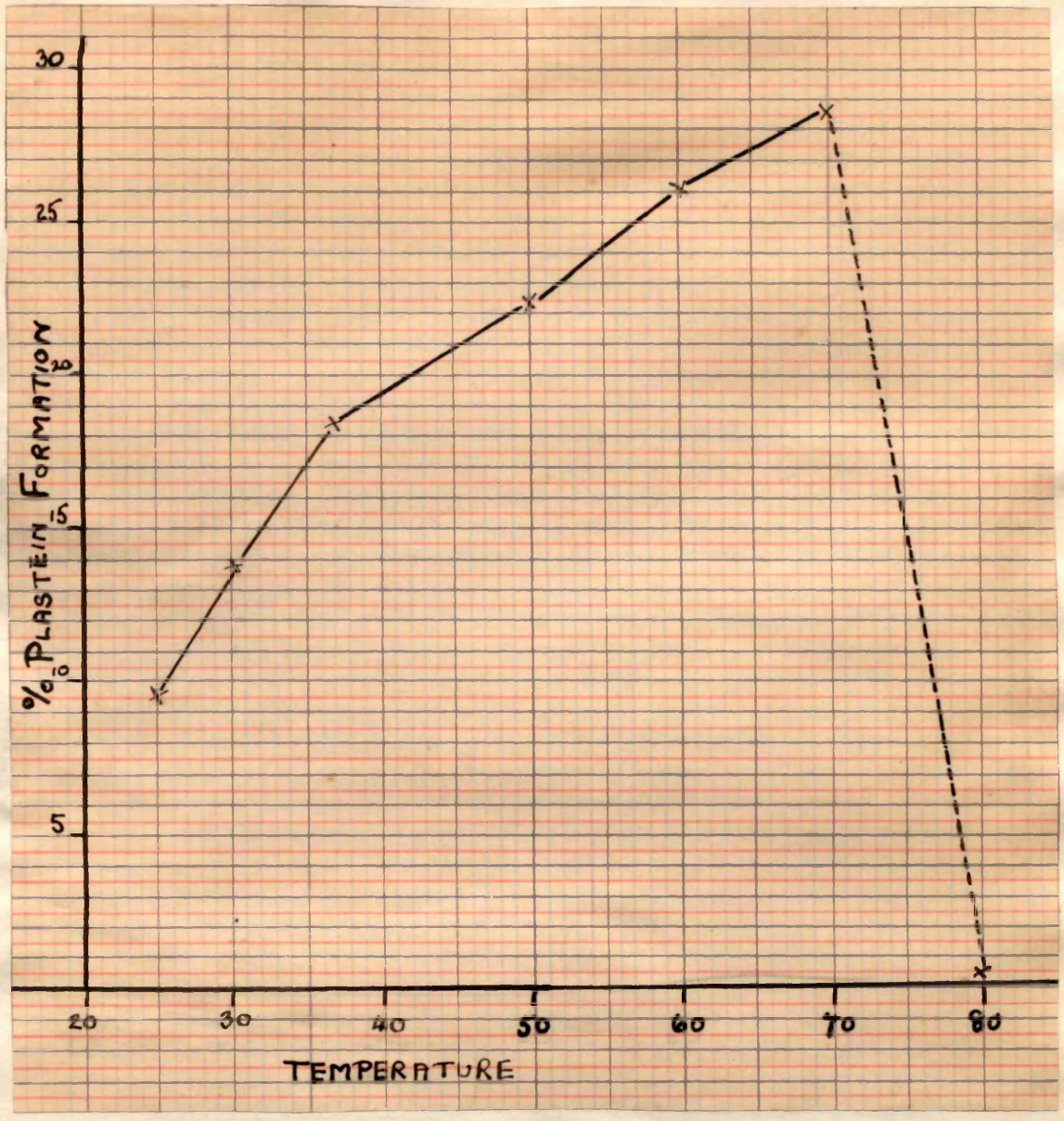
pH - 4.0

Time - 24 hours.

Temperature.	% Plastein formation.
25°	9.8
30°	13.6
37°	18.1
50°	22.2
60°	26.1
70°	28.6
80°	0.6



EXPT. 6. Influence of Temperature Concentrated Peptic Digest of Egg Albumin.



EXPT. 7. Influence of Temperature Concentrated Peptic Digest of Serum Albumin.

THE EFFECT OF ENZYME CONCENTRATION.

Contrary to the classical conception of enzymes the concentration of the enzyme does have a marked influence upon the amount of plastein formation. The data obtained are shown in Tables 6 (egg albumin digest) and 7 (serum albumin digest). It will be seen from these results that increase in concentration of the enzyme leads to increased plastein formation.

TABLE 6.

Expt. 8. Concentrated peptic digest of egg albumin.

Total nitrogen - 3.94 g. per 100 cc.

pH - 4.0

Incubated 3 days at 37°.

Concentration of Enzyme. (g. per 100 cc.)	% Plastein formation.
0.1	7.9
0.5	13.2
1.0	17.8
2.0	20.3
5.0	24.5
10.0	27.4

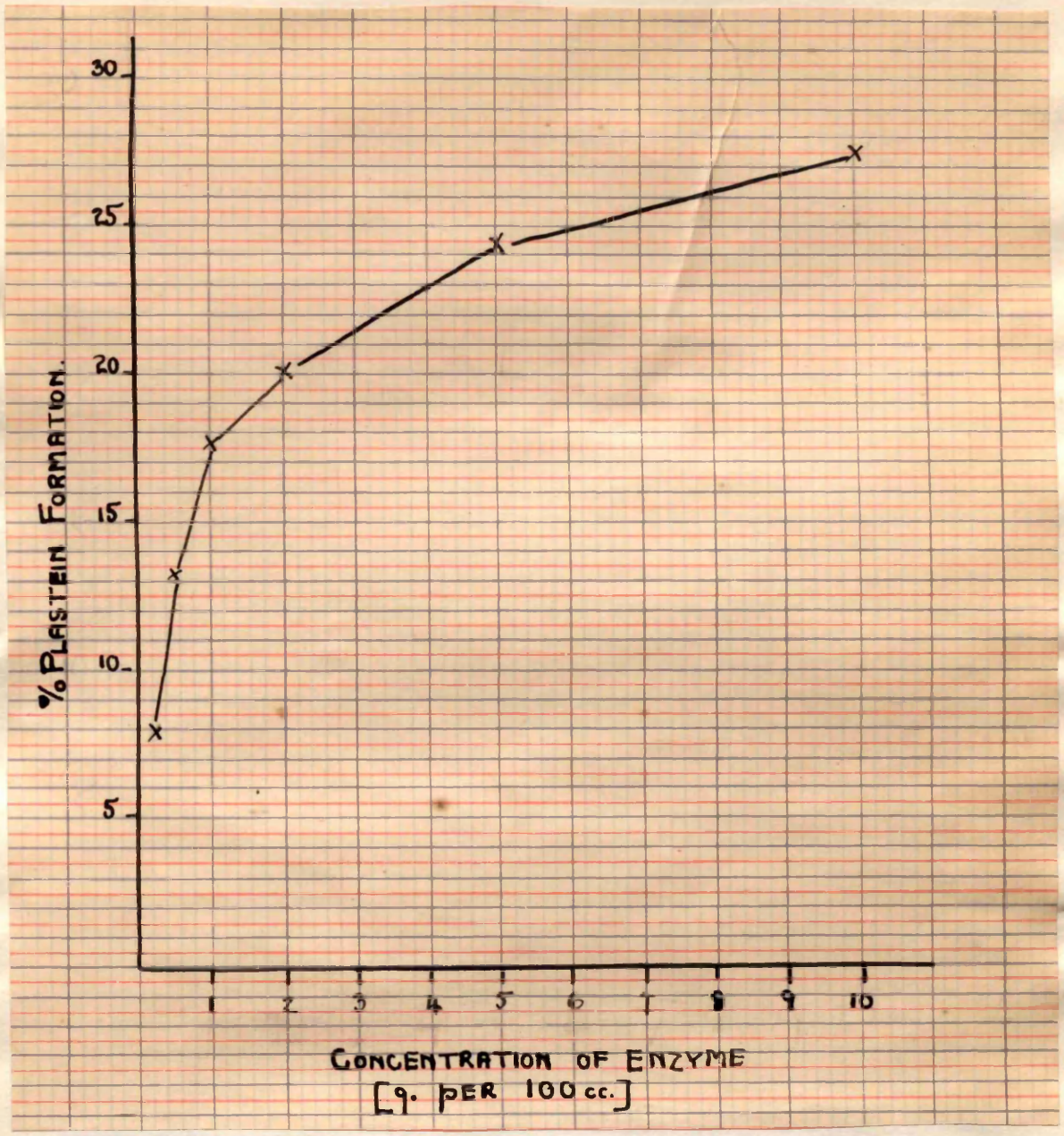
Expt. 9. Concentrated peptic digest of serum albumin.

Total nitrogen - 3.75 g. per 100 cc.

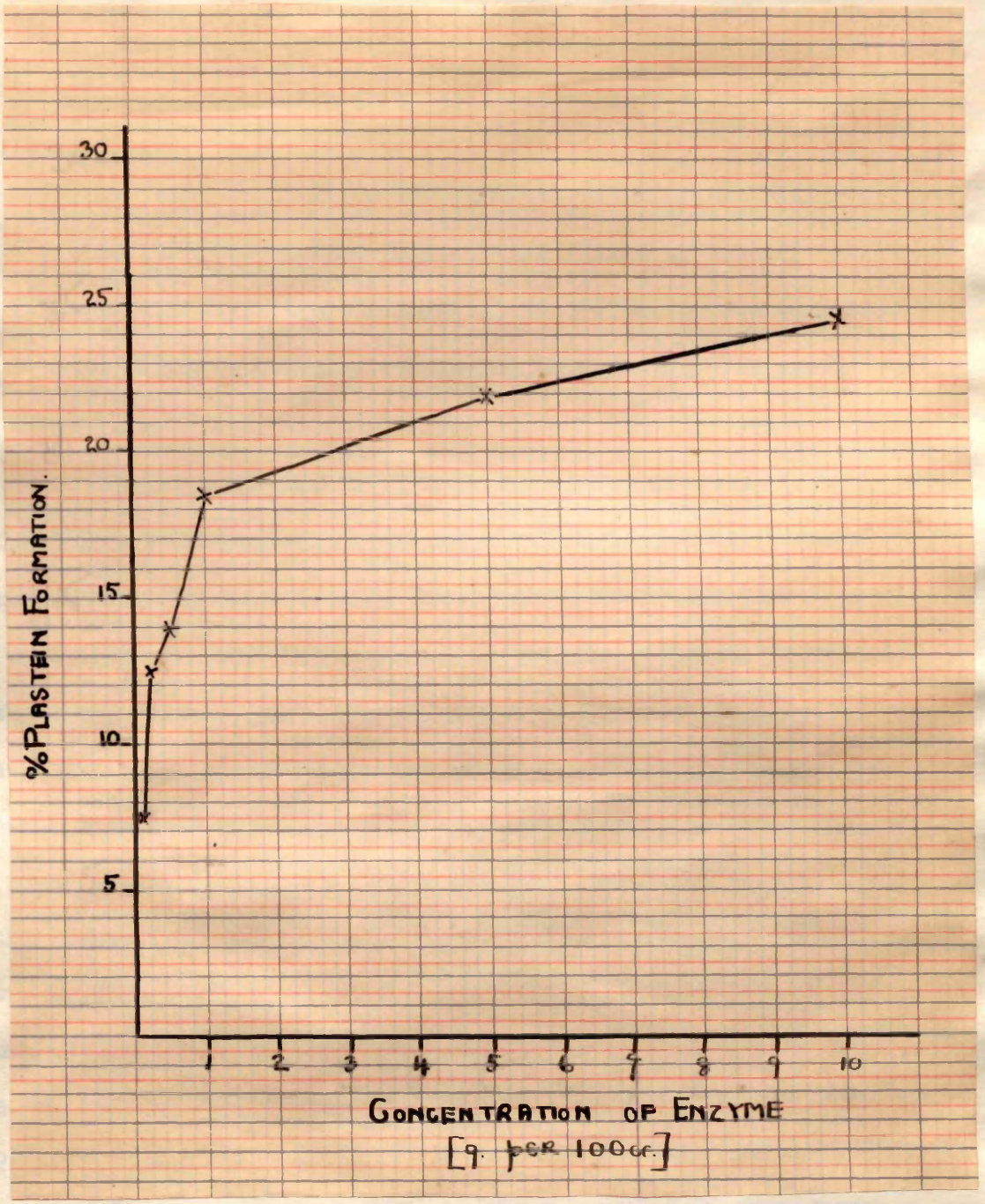
pH - 4.0

Incubated 3 days at 37°

Concentration of Enzyme. (g. per 100 cc.)	% Plastein formation.
0.1	7.9
0.2	12.8
0.5	13.9
1.0	18.7
5.0	21.9
10.0	24.8



EXPT. 8. Influence of Concentration of Enzyme - Concentrated Peptic Digest of Egg Albumin.



EXPT. 9. Influence of Concentration of Enzyme - Concentrated Peptic Digest of Serum Albumin.

So far the only criterion of plastein formation which has been considered, is the property ascribed exclusively to proteins of insolubility in 2% trichloracetic acid. Other confirmatory evidence, therefore, seemed essential.

Henriques and Gjaldbæk (1912) observed a decrease in the formal titrable nitrogen during plastein formation. A later paper by Oda (1926) contested the view that there was any decrease in the free amino nitrogen. Since Oda's measurements were carried out in a micro Van Slyke apparatus, the probability is that a small reduction would be hardly detectable in such an apparatus. A decrease in the free amino nitrogen alone cannot be taken as evidence of the formation of a more complex molecule. It must be shown at the same time that there is no formation of ammonia accompanying such a decrease, otherwise it could be explained as a splitting off of amino groups. Wasteneys and Borsook (1925, 5) found that if either peptic digests of egg albumin, glycine solutions or solutions of glycine and phosphate were incubated at 37° with high concentrations of glucose, a marked decrease in free amino nitrogen was observed when the pH of the mixtures were above 7.0, the effect increasing with/

with increase of pH. They were unable to locate this disappearance of free amino nitrogen as ammonia, urea or cyanic acid. In the case of the peptic digests, although this marked diminution in the free amino nitrogen occurred, they found that it did not involve any formation of more complex products of protein nature. They came to the above conclusions as a result of estimating the amount of proteose, peptone, etc. before and after incubation with glucose. It must be noted that none of these changes were obtained in the region of pH4, the optimum for plastein formation by pepsin.

It has been considered that the action of proteolytic enzymes on the degradation of protein results in the rupture of the - NH - CO - linkages so that free amino and free carboxyl groups should be liberated at the same rate. This has been investigated by many workers. Steudel, Ellinghaus and Gottschalk (1926) and Steudel and Ellinghaus (1927) state that carboxyl groups are liberated at a greater rate than amino groups during hydrolysis of a protein by pepsin, the ratio being about 5: 1. Felix and Harteneck (1927) using histone and Weber and Gesenius (1927) using casein have found that during peptic hydrolysis of these proteins, amino and carboxyl groups are liberated at/

at the same rate, that is in the ratio of 1: 1. Waldschmidt-Leitz and Künster (1927) have also demonstrated in the case of egg albumin, casein, gelatin and histone that amino and carboxyl groups are liberated at the same rate during peptic hydrolysis. Waldschmidt-Leitz (1929) states that the divergencies in the direction of stronger carboxyl liberation as obtained by some workers are due to an incomplete determination of the intrinsic acidity of the unaltered substrate in the initial alkalimetric titration. Sørensen and Katschioni-Walther (1928) also found the ratio to be 1: 1 for the liberation of amino and carboxyl groups during peptic hydrolysis of caseinogen, gelatin and gliadin. Cannan and Muntwyler (1930) found the acid and alkali binding power of gelatin increased in the ratio of 1:1 during peptic digestion. The concensus of evidence is that during peptic hydrolysis of proteins, amino and carboxyl groups are liberated at the same rate.

It is to be expected that during a synthesis of a protein, a reversal of the above process will take place, namely, that the concentration of the amino and carboxyl groups will decrease at the same rate. Rona and Chrometzka (1927) found that although there was a reduction in the free amino nitrogen during plastein formation there were only indefinite variations in the concentrations of the carboxyl/

carboxyl groups. They sought to explain the phenomenon of plastein formation as a deamination, caused by the splitting off of amino groups to form ammonia. They determined ammonia in the digests by aeration with caustic soda or baryta at 70°. Such drastic treatment would presumably lead to ammonia formation so that the results obtained by such a method cannot be relied upon. Rona and Oelkers (1928) re-investigated this aspect of the problem and found that there was a decrease of amino and carboxyl groups in the ratio of 1:1 during plastein formation. They consider that their results support the view that plastein formation is actually a synthesis. They state that the discrepancies between their results and those of Rona and Chrometzka must have been due to methodical errors on the latters' part. Rona and Oelkers found no change in the concentration of ammonia during plastein formation. It was decided to investigate these problems.

RATIO OF DECREASE IN AMINO AND CARBOXYL GROUPS.

Since pepsin takes a considerable time to dissolve in concentrated peptic digests, considerable plastein formation takes place by the time the pepsin has completely/

completely dissolved, the following procedure was employed.

The reaction of the digest to be examined was adjusted so that upon addition of pepsin to a concentration of 10%, the pH of the mixture was 4. 0.5 g. of finely divided pepsin was weighed into each of two 25 cc. volumetric flasks, followed by 5 cc. of digest. The contents of one flask were diluted immediately to 25 cc. and well shaken until all the contents were dissolved. The amino nitrogen, concentration of carboxyl groups and ammonia were determined immediately. Since dilution would stop plastein formation, these values were taken as the initial values before plastein formation. A drop of toluol was added to the contents of the second flask, the flask stoppered and then incubated at 37°. At the end of two days the contents of this flask were diluted to 25 cc., well mixed and the concentration of amino nitrogen, carboxyl groups and ammonia determined. The amount of nitrogen insoluble in 2% trichloracetic acid was determined upon a sample from the second flask. The methods of determining the concentration of amino nitrogen, carboxyl groups and ammonia were as follows.

Amino nitrogen - Linderström-Lang (1928). The solutions/

solutions used were:-

I. 0.1 N hydrochloric acid in 90% alcohol.

2. 0.1% naphthyl red in alcohol.

The determination was carried out upon 1 cc. specimens from the flasks after dilution to 25 cc. 1 cc. of the solution to be examined was placed in a flask together with 8 drops of naphthyl red as indicator. 10 cc. of acetone were added and 0.1.N. alcoholic HCl run in from a microburette until the indicator changed from yellow to a slight reddish tinge. A further 10 cc. of acetone were added and the titration continued. Owing to the insolubility of peptones, proteoses etc. in acetone, considerable errors may arise by the use of this method as described above, since these substances tend to separate out from the acetone in clumps during the titration. Difficulty was experienced in obtaining consistent duplicates. This difficulty was overcome, however. Following the first determination sufficient 0.1N HCl was added prior to the addition of acetone, so that after the addition of acetone, only 1-2 drops of 0.1N acid were necessary to complete the titration. It was found that the titration could be completed before any clumps had settled out, the precipitated substances being still/

still in a finely divided condition. Using this technique consistent results could always be obtained. A blank determination using 1 cc. of distilled water was always carried out and this correction was allowed for.

Carboxyl groups - The method employed was that of Willstätter and Waldschmidt-Leitz (1921). The following solutions were used:-

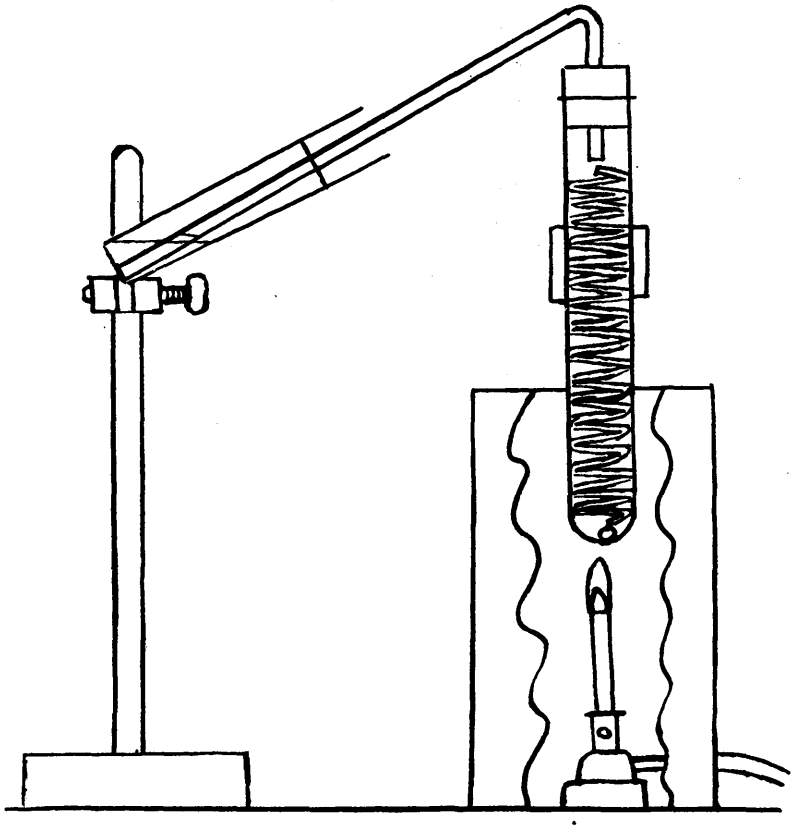
1. 0.2N potassium hydroxide in alcohol.
2. 0.5% thymol phthalein in alcohol.

2 cc. of the solution to be examined were placed in a flask together with 3 cc. of distilled water and 5 cc. of absolute alcohol, after which 1 cc. of thymol phthalein solution was added. The standard alkali was run in from a micro burette until the indicator changed to a greenish blue tint. 40 cc. of absolute alcohol were added, the indicator becoming colourless, after which the titration was continued till the indicator changed to a greenish blue tint again. A blank determination was carried out using 2 cc. of distilled water and this allowed for in the final calculation. No difficulty in obtaining consistent results was encountered.

Ammonia - In such an investigation as this it is necessary to observe whether there is any increased formation of/

of ammonia during the processes being studied. A change in the concentration of ammonia would lead to erroneous results and false interpretations. The method used was as follows:-

Into an 8 x 1 inch Pyrex test tube were placed 1 - 5 cc. of the solution to be examined, the amount depending on the concentration of ammonia. The contents of the tube were diluted to about 5 cc. with distilled water. 2 cc. of saturated borax solution were added followed by a few drops of liquid paraffin and a glass bead to prevent bumping. A spiral of galvanised iron wire was placed in the tube. A delivery tube was fitted into the tube and the end of the delivery tube made to dip under 2 cc. of 0.05 N. HCl, contained in a test tube graduated at 25 cc. The ammonia was distilled over. This method is similar to that for the distillation of the ammonia during the determination of blood urea by the method of Folin as described by Beaumont and Dodds (1925). The distillation was carried out for 4 minutes over a micro-burner. Frothing was prevented by the use of the liquid paraffin and spiral of iron wire. After the distillation, the distillation tube was disconnected and washed into the receiving tube, the contents of which were diluted to about 20 cc. After cooling, the ammonia was determined colorimetrically by the addition of 2.5 cc. of Nessler/



The Determination of Ammonia
in Peptic Digests.

Apparatus used for the Distillation of
Ammonia.

Nessler reagent, dilution to 25 cc., and after mixing comparison with a standard. As a standard 3 cc. of a known ammonium sulphate solution (1cc. equivalent to 0.1 mg. nitrogen) were measured into a 100 cc. volumetric flask, then diluted to about 70 cc. and finally 10 cc. of Nessler reagent added and the whole diluted to 100 cc.

In distilling peptic digests with borax, ammonia from ammonium salts only is liberated, the borax being too weak an alkali to attack the amino groups. This was tested out experimentally with many amino acids.

The results of these investigations are shown in Tables 8 (egg albumin digests), 9 (serum albumin digests) and 10 (casein digests). The concentrations of the amino and carboxyl groups are expressed in terms of cc. normal acid or alkali respectively per 100 cc. digest. The two flasks containing digest and pepsin used for each experiment always contained slightly different amounts of nitrogen, but the amino nitrogen, carboxyl and ammonia figures of the first flask have been corrected to correspond to the nitrogen figure of the second flask. The corrections, however, were very small, being almost negligible.

TABLE 8.

Egg Albumin digest.

Incubated 2 days at 37°C at P_H4.

Total Nitrogen g. per 100 cc.	% Plastein formation.	Amino nitrogen cc.N HCl. %	Carboxyl cc. N. KOH %	Changes in NH_2 and COOH.		Ratio of decrease of $\frac{COOH}{NH_2}$	Concentration of Ammonia. mg. N per 100 cc.	
				NH_2 cc.N.HCl.%	-COOH cc.N.KOH.%			
5.139		81	115.5				36.3	0 hours
5.157	20.7	74.5	109.5	6.5	6.0	0.93	37.1	48 hours
4.419		73.0	107.5				48.3	0 hrs.
4.421	17.2	65.0	98.7	8.0	8.8	1.1	48.7	48 hrs.
4.160		72.0	80.5				25.4	0 hrs.
4.089	10.5	61.5	69.0	10.5	11.5	1.09	27.1	48 hrs.
5.198		94.3	128.5				30.3	0 hrs.
5.211	19.7	80.9	115.5	13.4	13.0	0.97	32.6	48 hrs.

TABLE 9.

Serum Albumin digests.

Incubated 2 days at 37° at P^H4.

Total Nitrogen g. per 100 cc.	% Plastein Formation.	Amino nitrogen cc. N. HCl. %	Carboxyl. cc. N.KOH. %	Changes in NH_2 and COOH.		Ratio of decrease of $\frac{COOH}{NH_2}$	Concentration of ammonia ng. N%	
				NH_2 cc.N. HCl.%	-COOH cc.N. KOH.%			
3.516		70.2	120.9				56.3	0 hours.
3.522	16.4	64.3	115.4	5.9	5.5	0.94	58.1	48 hours.
4.144		84.3	158.5				49.7	o hours.
4.098	21.4	74.7	148.7	9.6	9.8	1.02	50.3	48 hours.
3.917		78.0	144.2				61.3	0 hours.
4.012	18.7	66.6	133.3	11.4	10.9	0.96	63.1	48 hours.
2.961		63.4	110.0				27.4	0 hours.
3.012	12.8	55.1	102.5	8.3	7.5	0.90	27.8	48 hours.

TABLE 10.

Casein digests.

Incubated 2 days at 37° at $_{P}H4$.

Total nitrogen g. per 100 cc.	% Plastein Formation.	Amino nitrogen cc.N/1 HCl %	Carboxyl cc. N/1 KOH %	Changes in NH_2 and COOH		Ratio of decrease of $\frac{COOH}{NH_2}$	Concentration of Ammonia mg. N %	
				NH_2 cc.N/1 HCl.%	COOH cc.N/1 KOH%			
3.722		87.5	111.5				42.4	0 hours.
3.810	10.2	82.1	106.9	5.4	5.6	1.04	43.1	48 hours.
3.019		80.3	102.4				34.6	0 hours.
2.998	8.6	76.1	97.9	4.2	4.5	1.07	34.8	48 hours.
4.526		90.7	124.6				45.7	0 hours.
4.492	11.4	82.4	116.7	8.3	7.9	0.95	46.0	48 hours.
4.259		86.6	105.4				41.6	0 hours.
4.271	9.6	80.1	99.5	6.5	5.9	0.91	41.2	48 hours.

It will be seen from the results that in all cases plastein formation is accompanied by a decrease in the concentration of amino and carboxyl groups. This decrease is in the ratio of 1:1. It appears that plastein formation involves a linking of carboxyl and amino groups to form a peptide linkage. These results further strengthen the view that plastein formation is essentially a synthesis. No appreciable formation of ammonia takes place during plastein formation. The amount of plastein formation as determined by the quantity of nitrogen insoluble in 2% trichloracetic acid does not appear, however, to run parallel to the decrease in concentration of the amino and carboxyl groups. This may be due to the fact that in measuring the former, only one phase of the reaction is being determined. Wasteneys and Borsook (1925, 2) have shown that during plastein formation all the fractions of a protein hydrolysate appear to be involved, there being an increase of certain fractions such as proteoses and peptones from molecules of less complexity.

THE EFFECT OF THE DEGREE OF ENZYMATIC HYDROLYSIS ON THE PROCESS OF RE-SYNTHESIS.

Sawjalow (1901) concluded that the nearer a peptic digest of protein approaches in complexity to the original/

original protein, the more easily does plastein formation take place. Henriques and Gjaldbäk (1912) reached similar conclusions. Wasteneys and Borsook (1930) controlling the hydrogen ion concentration, found that the longer a protein was allowed to digest with pepsin, the lower the yield of pepsin obtained subsequently. The present writer has re-investigated this point using serum albumin.

A peptic digest of serum albumin was prepared and at intervals of 5, 10, 20 and finally 30 days portions were removed, the active enzyme destroyed by heat, the unchanged protein filtered off and the filtrate concentrated in vacuo. The samples were concentrated to approximately the same degree. Pepsin was added to the concentrated digests to 10% concentration and the mixtures brought to pH4. The amount of plastein formation was determined at intervals. From the results shown in Table 11 it will be seen that the data obtained are in agreement with the observations of the above workers.

TABLE 11.

	Duration of hydrolysis.	Duration of Synthesis.	Total Nitrogen.	% Plastein Formation.
1.	5 days.	3 days	3.81	24.3
		10 days	3.86	29.2
2.	10 days.	3 days	3.94	20.2
		10 days	3.98	26.5
3.	20 days.	3 days	3.71	11.2
		10 days.	3.76	17.3
4.	30 days.	3 days	3.85	8.7
		10 days	3.90	12.8

THE CHARACTER OF THE ORIGINAL PROTEIN AND ITS RELATIONSHIP TO PLASTEIN FORMATION.

The amount of plastein obtained under comparable conditions varies considerably according to the protein from which the digest has been prepared. While peptic digests of egg and serum albumin gave the highest yields, peptic digests of casein were found to give much lower yields. Wasteneys and Borsook (1930) found that peptic digests of gliadin gave much smaller yields than peptic digests of egg albumin. So far no investigator has obtained plasteins from peptic digests of gelatin. The present writer has similarly failed. Wasteneys and Borsook (1930) have suggested that this failure may be due to the use of acid in preparing gelatin from collagen since Henriques and Gjaldbäk (1911, 1912) found that partial acid or alkali hydrolysates of proteins yielded no plasteins. With this in view the present writer attempted to form plasteins from a concentrated peptic digest of collagen.

Tendons were first digested with trypsin to hydrolyse proteins other than collagen. The collagen was then washed with water to remove the disintegration products of the foreign proteins. The collagen was digested with pepsin at 37° and pH1.7 for several days. The procedure for preparing/

preparing the concentrated peptic digest of collagen was essentially the same as described earlier in this paper. The concentrated digest of collagen was finally incubated 7 days at pH4 with 10% pepsin, but no evidence of plastein formation was obtained. The results of this experiment do not appear to support the theory advanced by Wasteneys and Borsook to account for their failure to produce plastein from concentrated digests of gelatin.

PROPERTIES AND COMPOSITION OF THE PLASTEINS.

The plasteins prepared from the various peptic digests all gave positive protein biuret tests. They were soluble in strong acids and strong alkalies and were coagulated by boiling from alkaline solution, but not from acid solution. The plasteins were all digested rapidly by pepsin at pH1.7 and from their peptic digests, primary proteoses could be salted out by half saturation with ammonium sulphate. The sulphur, nitrogen and phosphorus content of plasteins from various sources were determined and compared with the original native protein. It will be seen from the data (Table 12), that the content of these elements in the plasteins and the original proteins are of the aame order, with the exception of phosphorus which is almost absent in plasteins derived from casein.

Sulphur was determined by Denis's modification of Benedict's method and phosphorus by the direct weighing of the ammonium phosphomolybdate formed by Neumann's reaction.

TABLE 12.

Source from which plastein prepared.	Sample.	Composition of Plastein. S %	N %	P %	Composition of Protein from which digest prepared. S %	N %	P %
Egg Albumin	1.	1.63	13.41	-	1.62	15.51	-
	2.	1.70	13.82	-			
	3.	1.58	13.56	-			
	Average	1.63	13.59				
Serum Albumin	1.	1.91	14.01	-	1.93	15.93	
	2.	1.83	13.89	-			
	3.	2.10	13.76	-			
	Average	1.94	13.89				
Casein	1.	0.81	14.45	0.11	0.78	15.6	0.81
	2.	0.83	14.02	0.16			
	3.	0.76	14.76	0.21			
	Average	0.80	14.41	0.16			

In Table 13 is shown the comparison of the cystine content and strength of the Molisch a-naphthol test both in the plasteins and in the proteins from whose digests they were derived. The method for the determination of cystine was a modification of the method of Folin and Marenzi (1929) and is described in a subsequent note.

Doubt has often been expressed as to whether the protein molecule really contains a carbohydrate or not. Recently Rimington (1929,1931) and Levene and Mori (1929) have isolated carbohydrate complexes from purified proteins which appear to consist of glucosamine and mannose units and are present to the extent of about 2%.

TABLE 13.

	Cystine. %	Molisch. test.
Egg Albumin	2.29	+ +
Plastein (Egg Albumin digest)	3.07	+ +
Serum Albumin	2.34	+ +
Plastein (Serum Albumin digest)	3.84	+ +
Casein	0.38	faintly +
Plastein (Casein digest)	1.35	+ +
Pepsin.	0.78	+ +

DISCUSSION.

It has been shown that when peptic digests of egg albumin, serum albumin and casein are concentrated to about 3-4 gm. nitrogen per 100 cc., upon addition of pepsin and incubation at 37°, an apparent synthesis of protein results, the maximum synthesis being obtained at pH4.

The amount of protein or plastein as it has been termed is also influenced by the temperature and concentration of the enzyme.

Plastein is insoluble in 2% trichloracetic acid, gives a positive biuret test and during its formation a decrease in the concentration of amino and carboxyl groups in the ratio of 1:1 is observed without any formation of ammonia. It appears then that plastein formation is actually a synthesis of protein from protein decomposition products.

An interesting fact is the difference in the yields obtained from digests of different proteins obtained under apparently optimum conditions. The highest yields were obtained from egg albumin and serum albumin digests, casein digests giving much smaller yields, while gelatin digests produced no plastein at all.

The fact that Henriques and Gjaldbak (1911, 1912) have been unable to obtain plasteins by the action of pepsin upon/

upon partial acid or alkali digests of proteins and that these investigators and also Wasteneys and Borsook were unable to prepare plasteins by the action of trypsin upon tryptic digests of proteins are problems of great interest. It is curious too that the longer pepsin acts upon a protein during the hydrolytic stage, the lower the yield of plastein from the subsequently concentrated digest. The possibility arises that pepsin can only unite those products of protein decomposition containing certain groupings in their structure. While little information was derived from the determination of the percentage of sulphur, nitrogen and phosphorus in the plasteins, the figures obtained being of the order of those of the native proteins from which the plasteins were derived, more information was derived from the analyses of the plasteins for cystine and also by the strength of the Molisch test for carbohydrate. In each case the plasteins contained a higher percentage of cystine than the corresponding native protein. Serum and egg albumin both gave strong Molisch tests as did their corresponding plasteins but the plasteins from casein digests all exhibited stronger tests than the original casein which gave only a faint reaction. The question arises as to the importance of cystine and the carbohydrate group of the protein molecule in the formation of plastein. It is suggestive that serum and egg albumin contain/

contain fairly high percentages of cystine and give strong Molisch tests, while casein contains a very low percentage of cystine and gives a faint Molisch test and gelatin gives no Molisch test and contains no cystine.

The question naturally arises as to what rôle such synthetic processes play in the animal economy. The optimal conditions as defined by these experiments cannot be attained as for example a temperature of 70°. It is possible that certain phenomena which we but dimly appreciate, may bring about such an increased concentration of the initial constituents and such an increased hydrogen ion concentration as are necessary for synthesis.

SUMMARY.

1. The optimum hydrogen ion concentration for the synthesis of 'plastein' from concentrated digests of egg and serum albumins and also casein, incubated at 37° was pH4.

2. The amount of 'plastein' formed from egg and serum albumin digests increased rapidly with temperature reaching a maximum at 70°.

3. Increase in the concentration of enzyme increased plastein formation in concentrated peptic digests of egg and serum albumin.

4. The ratio of the decrease in the amino and carboxyl groups during the process of 'plastein' formation from egg and serum albumin and also casein digests was 1:1, the values being expressed/

expressed respectively in terms of normal acid and alkali.

5. There was no appreciable formation of ammonia during formation of 'plastein'.

6. The longer a protein was allowed to digest the less was the yield of 'plastein'.

7. Peptic digests of egg and serum albumin gave better yields than casein. Gelatin yielded no plastein from the products of digestion.

8. Certain properties of the 'plasteins' are described.

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The Determination of Cystine in Proteins by the Method of Folin and Marenzi.

There have been many modifications of the original method of Folin and Looney (1922) for the determination of cystine in proteins. The principle of their method is that cystine, after reduction to cysteine by sodium sulphite reacts with the phosphotungstic acid reagent of Folin and Denis (1912) to give a blue colour in alkaline solution. Folin and Trimble (1924) made the method more specific for cystine by removing most of the molybdenum compounds from the phosphotungstic acid reagent thereby decreasing its sensitivity towards tryptophan, tyrosine and phenols. Folin and Marenzi (1929, 1) have recently stated that it is necessary to add much more of the reagent than employed by Folin and Looney and also the former recommend adding the sulphite before the alkali since a smaller amount is required. Folin and Marenzi (1929,2) have published the details for the preparation of the phosphotungstic acid or "uric acid" reagent whereby all traces of molybdenum compounds may be removed. Rimington (1930) examining this method encountered two difficulties. He found that a turbidity often formed in the standard and the test solutions, and that the intensity of the colour varied appreciably with slight changes in the concentration of sodium carbonate. Folin (1930) recommended the use of urea for the prevention of this turbidity. Rimington (1930) found this to be quite satisfactory when low concentrations of sodium carbonate were employed but that the higher the concentration of sodium carbonate/

carbonate, the greater was the intensity of the colour and the greater the tendency for turbidity to occur. Hunter and Eagles (1927) have recommended the use of sodium hydroxide in place of sodium carbonate as they believe that the carbon dioxide which is liberated from the carbonate liberates also sulphur dioxide thereby causing a large blank reduction of the phosphotungstic acid reagent.

The present writer found that even when urea was used as recommended by Folin (1930) turbidities often occurred in the test and standard solutions in the Folin-Marenzi method. It was found however that a blue colour developed when sodium bicarbonate solutions were used in place of sodium carbonate. The colour obtained was stronger than that with sodium carbonate and turbidity did not occur even when allowed to stand ½ hour. It was therefore decided to test the possibility of replacing the carbonate by bicarbonate.

The following solutions were used:-

1. Uric acid reagent of Folin and Marenzi (1929,2)
2. 20% sodium sulphite.
3. Saturated sodium bicarbonate solution.
4. Standard cystine solution, containing 1 mg. in 1 cc. N.H_2SO_4.

Into each of three 100 cc. volumetric flasks were/

were measured 2 cc. of standard cystine solution followed by 2 cc. of sulphite solution. After allowing to stand 1 minute to ensure reduction of cystine to cysteine, bicarbonate solution was added as follows. To the first flask 5 cc., to the second 10 cc., and to the third 20 cc., were added. Then 8 cc. of uric acid reagent were added to each and after allowing to stand for 8 minutes, they were diluted to 100 cc. and compared in a colorimeter. It was found that these three solutions compared one with another. Similar results were obtained using 1 cc. and 4 cc. of standard respectively. It was also found that the colour was proportional to the concentration of the cystine in the range tested, i.e. 1 - 4 mg.

Turbidities were never encountered so that it was unnecessary to use lithium sulphate or urea. The colours obtained remained stable without fading for at least ½ hour.

A blank determination was carried out and found to be inappreciable. Solutions of tyrosine, tryptophan glycine, alanine, glutamic acid, aspartic acid and histidine in concentrations greater than present in protein hydrolysates were tested by this method. No colour developed apart from the slight colour which develops during a blank determination. No colour was developed by uric acid.

As/

As a preliminary known amounts of cystine were boiled with 20 cc. of 6 N H_2SO_4 for 18 hours under the same conditions as used in the hydrolysis of the proteins. From the results shown in Table I it will be seen that there was no appreciable loss.

TABLE I.

	INITIAL AMOUNT OF CYSTINE. (mg.)	AMOUNT OF CYSTINE DETERMINED AFTER HYDROLYSIS. (mg.)
1.	10	10.4
2.	20	19.3
3.	40	39.4

For the determination of cystine in proteins, the following hydrolysing technique of Folin and Marenzi (1929, 1) was employed.

1-5g. of protein were boiled with 20 cc. of 6N H_2SO_4 in a 300 cc. Kjeldahl flask under a reflux condenser for 18 hours. After cooling the hydrolysate was filtered and washed into a 100 cc. volumetric flask and then made up to 100 cc.

A volume of 5-10 cc. containing about 1-4 mg. cystine was measured into a 100 cc. volumetric flask, followed by 2 cc. of sulphite solution. After allowing to stand 1 minute 20 cc. of saturated sodium bicarbonate solution was added and then 8 cc. of 'uric acid' reagent. After standing 8 minutes it was diluted to 100 cc. and after mixing, compared with a suitable standard prepared in the same way.

The cystine content of a number of proteins were determined and the results are shown in Table II.

TABLE II.

PROTEIN.	CYSTINE %
Gelatin (British Drug Houses Ltd. Preparation)	Trace
Casein (British Drug Houses Ltd. Preparation)	0.38, 0.41, 0.34 average 0.38
Edestin (British Drug Houses Ltd. Preparation)	1.46, 1.42, 1.48 average 1.45
Egg Albumin (Kahlbaum Preparation)	2.29, 2.29, 2.35 average 2.31
Serum Albumin (Kahlbaum Preparation)	2.29, 2.26, 2.34 average 2.30

SUMMARY.

A modification of the Folin Marenzi method for the determination of cystine in proteins is described in which a solution of sodium bicarbonate takes the place of the carbonate used in the original method.

The colour produced in the presence of bicarbonate is uninfluenced by the concentration of this substance and turbidity has never been experienced .

The cystine content of certain proteins have been determined:- in egg albumin 2.31%, serum albumin 2.30%, edestin 1.45%, casein 0.38%, and in gelatin a trace.

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A METHOD FOR THE DETERMINATION OF THE INORGANIC SULPHATE CONTENT OF BLOOD SERUM OR PLASMA.

Relatively few determinations of the inorganic sulphate content of the blood have been made owing to the difficulties in the estimation of the small concentrations present in the blood. Peters et alia (1929) have stated that sulphate probably accounts for part of the 'undetermined acid' combined with base in cases of nephritis with nitrogen retention. For such an investigation an accurate and convenient method requiring only small volumes of blood is essential.

The methods evolved for the determination of inorganic sulphate in serum or plasma may be summarised as follows:

1. Nephelometric - Denis (1921), Denis and Reed (1926).

In such a method sulphate is precipitated in the form of a cloud as barium sulphate and this is compared in a nephelometer with a cloud prepared under similar conditions from a standard solution of a sulphate. Denis and Reed (1926) claimed an accuracy of 5%. Cope (1931) confirmed this in the case of calf's and sheep's serum, in both of which the actual inorganic sulphate concentration is five or six times as high as in human serum, but found that when applied to normal human serum, the results were much less satisfactory. The pH of the filtrates has a marked influence upon the turbidity obtained, an unfavourable pH tending to inhibit precipitation of barium sulphate. Denis stated that if from a blood filtrate

no/

no precipitate was obtained upon the addition of barium chloride it is not to be concluded that sulphate is absent but it is necessary to determine the pH of the solution. Denis states that the optimum pH for precipitation of barium sulphate is 3.0 to 3.8. but Cope places it 1.8 to 2.4. Furthermore Cope found that a high concentration of trichloracetic acid in a filtrate tends to inhibit the precipitation of barium sulphate. It has been found that these errors are greater when dealing with filtrates containing a low concentration of sulphate than when high.

2. Gravimetric - Loeb and Benedict (1927).

In this method sulphate is precipitated as barium sulphate which is filtered off and weighed. In such a method, however, a large volume of blood and accurate weighing on a micro-balance are required.

3. Sulphide Formation. Lorant (1929).

The sulphate is reduced to sulphide which is determined colorimetrically. Such a procedure is very laborious and ethereal and other sulphur compounds appear to be included in the results of the analyses.

4. Titration. White (1923), Cope (1931).

These investigators precipitated sulphate as benzidine sulphate, the precipitated benzidine being then titrated with a standard solution of NaOH using phenolphthalein as indicator. Great care is necessary to ensure that the standard NaOH is carbonate/

carbonate free. End points in micro titrations are not always easy to determine.

5. Colorimetric - Yoshimatsu (1926), Hubbard (1927,1930) Wakefield (1929,1).

In this type of method the sulphate is precipitated as benzidine sulphate, the precipitated benzidine being determined colorimetrically. The colours obtained are very weak yellows or browns and are very difficult to compare.

The present writer decided to modify the method of Wakefield and Hubbard by applying the series of reactions used by Kahn and Lieboff (1928) for the estimation of inorganic sulphates in urine, viz. diazotisation of the precipitated benzidine sulphate, followed by coupling with phenol in alkaline solution, an azo dyestuff being produced. Since the higher the phenol used, the more intense is the colour obtained, it was decided to test a-naphthol and thymol. The former gives a crimson colour, the latter a red.

TECHNIQUE.

A solution containing 2.0071 g. benzidine hydrochloride in 500 cc. N HCl (1 cc. equivalent to 0.5 mg. sulphur) was prepared. Dilutions were prepared using N. HCl in which 1 cc. was equivalent to 0.001, 0.002, 0.004, 0.005, 0.01, 0.02, 0.04 and 0.08 mg. sulphur respectively. To 2 cc. of each of these dilutions 1 cc. of freshly prepared 0.1% sodium nitrite solution was added and after standing 1 minute 5 cc. of/

of 15% sodium hydroxide were added. After mixing, 5 cc. of 1% thymol in 10% NaOH were added to each. A similar series was prepared using a 1% solution of a-naphthol in 10% NaOH in place of thymol. In both series the colours reached their maximum in 15 minutes and no signs of fading were apparent up to 1 hour. The colours obtained with a-naphthol were relatively much stronger than with thymol. As a result of these tests, it was found that a-naphthol was more suitable for estimating benzidine sulphate containing 0.001 to 0.01 mg. of sulphur per cc. and thymol for higher concentrations,

The following technique was used for the determination of the inorganic sulphate content of serum and plasma.

Reagents:

1. Acetone.
2. 20% trichloracetic acid in water. Commercial trichloracetic acid is purified by distillation with benzidine.
3. 0.5% benzidine in acetone. This should be prepared fresh each day.
4. Benzidine hydrochloride standard solution. 2.0071 g. of benzidine hydrochloride are dissolved in 500 cc. N. HCl. 1 cc. of this solution is equivalent to 0.5 mg. of sulphur and is used as a stock solution. More dilute solutions for use as standards for the determination of inorganic sulphate in blood, being equivalent per cc. to 0.001/

0.001, 0.002, 0.004, 0.01, 0.02, 0.04 and 0.08 mg. sulphur, are prepared by diluting the stock solution with N. HCl.

5. 0.1% sodium nitrite solution - prepared fresh each day.
6. 15% sodium hydroxide solution.
7. 1% thymol in 10% sodium hydroxide.
8. 1% a-naphthol in 10% sodium hydroxide.

Method.

All glass apparatus must be scrupulously clean and where potassium oxalate is used to prevent clotting, it must be shown to be free of sulphate.

To 2 cc. of serum or plasma are added 6 cc. of water and 2 cc. of 20% trichloracetic acid, and the whole well shaken. This mixture is then either centrifuged or filtered. Most brands of filter paper contain traces of sulphate which vitiate the results. The sulphate is insoluble in water but soluble in dilute acid solution, so that before use all filter papers should be washed first with dilute acid, then with distilled water and finally dried in an oven. Into a 10 cc. centrifuge tube 2.5 cc. of the filtrate are measured, followed by 5 cc. of 0.5% benzidine in acetone and well mixed. The tube is capped to prevent evaporation of acetone. The tube is allowed to stand ½ hr. to allow the benzidine sulphate to precipitate. At the end of ½ hr. the tube is centrifuged/

centrifuged and the supernatant fluid removed. The precipitate of benzidine sulphate in the centrifuge tube is washed twice with acetone followed by centrifuging. Finally the centrifuge tube is inverted over a sheet of filter paper until it is dry. The benzidine sulphate is then dissolved in 1 cc. N. HCl, with heat if necessary. After cooling, 0.5 cc. of 0.1% sodium nitrite is added followed one minute later by 2.5 cc. of 15% sodium hydroxide. After thorough mixing 2.5 cc. of alkaline a-naphthol or thymol solution are added and after standing 15 minutes, compared with a suitable standard prepared at the same time. The standard is prepared as follows: 2cc. of one of the dilute standard solutions are measured into a test tube followed by 1cc. of nitrite solution. After standing 1 minute, 5 cc. of 15% sodium hydroxide solution are added, the whole being well shaken, and then mixed with 5 cc. of alkaline a-naphthol or thymol solution. For normal serum or plasma, it is only necessary to prepare the three lowest standards (1 cc. = 0.001, 0.002 and 0.004 mg. S.) With practice one can tell from the amount of precipitated benzidine sulphate what standards to prepare and whether to use alkaline a-naphthol or thymol solution.

As a preliminary, known amounts of ammonium sulphate were analysed in order to determine if the sulphate could be measured quantitatively by this method. The concentrations used were in the region of those found in plasma/

plasma and serum. These determinations were carried out under exactly the same conditions as in carrying out a determination with plasma or serum. From the results shown in Table I, it will be seen that sulphate content could be determined quantitively by this method.

TABLE I.

Determination of sulphate in standard solutions of ammonium sulphate.

	mg.S per 100 cc. taken.	mg.S per 100 cc. determined.
1.	0.2	0.22
2.	0.3	0.31
3.	0.4	0.44
4.	0.5	0.53
5.	0.91	0.90
6.	0.91	0.93
7.	1.83	1.79
8.	2.0	2.04
9.	4.0	3.96

The possible interference of phosphates and chlorides and of potassium oxalate, when plasma is used, must be taken into consideration. It was found, however, that 20 mg. P, 1g. NACl and 1 g. potassium oxalate respectively per 100 cc. of serum or plasma produced no appreciable effect on the determination of sulphate.

From the results shown in Table II, it will be seen that ammonium sulphate added to serum could be determined quantitatively.

TABLE II.

	Inorganic sulphate content of blood serum. (mg.S.per 100 cc.)	Sulphate added. (mg.S.per 100 cc.)	Total Sulphate found. (mg.S.per 100 cc.)	Sulphate recovered. (mg.S.per 100 cc.)
1.	0.23	0.1	0.35	0.12
2.	0.23	0.2	0.45	0.22
3.	1.55	1.14	2.67	1.12
4.	1.55	2.28	3.95	2.40
5.	1.55	4.56	6.22	4.67

The majority of workers have used serum or plasma in preference to whole blood for the determination of inorganic sulphate. Denis and Reed (1927) using a nephelometric method, state that in human blood the inorganic sulphate is exclusively found in the plasma. To test this point three different batches of normal oxalated blood were centrifuged and the cells washed twice with sulphate free 0.85% NACl. 2 cc. of the corpuscular mass were mixed with 6 cc. of distilled water and then 2 cc. of 20% trichloracetic acid solution were added. After filtration the sulphate in the filtrate was estimated. No measurable coloration resulted. In order to make sure that the inorganic sulphur had not dialysed through the red cell membrane the inorganic sulphur content of the whole blood and also of the plasma of nine different bloods were determined. Unfortunately the haematocrit readings were not made but averaging the values and assuming a cellular volume of 40% of the whole blood, the inorganic sulphur content of the cells appears to be about one fifth of an equivalent volume of plasma.

The inorganic sulphate content of the sera of a number of normal healthy subjects were determined. According to Wakefield (1929,2) hospital patients suffering from diseases other than cardiac or renal are generally unsuitable for determining the normal range, since in many cases an increase in inorganic sulphate is observed even where/

where there are no signs of renal inefficiency. It is extremely probable that in view of the extensive catabolic phenomena (Cuthbertson 1930,1931), which result from injury, certain changes will occur in the blood, so that these types of hospital patients must be excluded from any normal survey. The results are shown in Table III. All the cases therein were healthy men and woman between the ages of 20 and 40 years.

In this work it was considered most convenient to express the sulphate content of the blood in terms of S, to preserve the similarity with phosphorus and nitrogen.

TABLE III.

Inorganic sulphate content of normal serum.

Case.	mg.S. per 100 cc.	Case.	mg.S. per 100 cc.
1	0.4	11	0.3
2	0.4	12	0.1
3	0.3	13	0.2
4	0.1	14	0.45
5	0.2	15	0.3
6	0.4	16	0.5
7	0.4	17	0.4
8	0.2	18	0.5
9	0.1	19	0.2
10	0.3	20	0.5
		21	0.45

The inorganic sulphur content of the serum of normal fasting men and women appears to vary from 0.1 to 0.5 mg. per 100 cc. Other workers have obtained normal values expressed as mg. S per 100 cc: Denis (1921) plasma, 0.2 - 0.4; Loeb and Benedict (1927) serum, 0.7 - 1.6; Wakefield (1929) serum, 0.2 - 0.9.

The higher normal values quoted by Loeb and Benedict may perhaps be ascribed to the error of gravimetric estimation of amounts of barium sulphate of the order of 0.2 - 0.7 mg.

SUMMARY.

1. A method has been described for the determination of the inorganic sulphate content of plasma and serum.
2. The normal inorganic sulphate content of human serum is 0.1 - 0.5 mg. S per 100 cc.

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