CLXI. THE INTERACTION OF FREE AMINO-NITROGEN AND GLUCOSE.

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In the course of an investigation into the influence of added glucose upon the action of tissue proteases, an interaction of glucose with the free amino-group was observed. In alkaline solution in the presence of this sugar, the free amino-nitrogen of glycine and of other more complex derivatives of protein, such as a peptic digest of albumin, Witte's peptone, and Parke-Davis' peptone, was found to decrease. Three preparations of glycine, two commercial and one obtained in this laboratory, when allowed to react separately with each of four specimens of glucose from different sources, gave in each case qualitatively and quantitatively similar results. The reaction was found to proceed unchecked in solutions of tested sterility and to be unaffected by preliminary boiling or autoclaving of the reagents.

When a peptic digest in which the above reaction had occurred was submitted to the method of fractional analysis for incomplete protein hydrolysates devised by the authors [1925], the reduction in free amino-nitrogen was found to have been unaccompanied by a synthesis of the lower fragments into more complex derivatives; nor was any loss in total nitrogen, formation of ammonia, urea or cyanic acid discovered even, in the case of glycine, with an 85 % diminution of the free amino-nitrogen.

Boiling in weak acid solutions when phosphate is present restores but slightly the free amino-nitrogen which has disappeared during the incubation with glucose; in the absence of phosphate this procedure restores in large part the lost free amino-nitrogen.

On the other hand, the incubated solution of protein derivative and glucose was found to have acquired the property of reducing methylene blue in neutral or even slightly acid solutions in the absence of any added enzyme. This reduction was usually carried out at 56°, to ensure sterility, but it also occurs, more slowly, at 37°. With glycine and peptone, and probably therefore with other protein derivatives, the reduction of methylene blue is greatly accelerated by the presence of phosphate. Unhydrolysed albumin after incubation with the sugar rapidly reduced methylene blue without any added phosphate. The addition of washed muscle to an incubated mixture of peptone and glucose accelerated the reduction of methylene blue.

Acetamide, urea, or creatine added to glucose did not reduce methylene blue at a lower p_H or at a greater rate than the sugar alone.

It has not been found possible as yet to complete an investigation of the nature of the products occurring in the preliminary incubation and the subsequent oxidation. In the meantime, it was deemed of value to publish the results of a preliminary qualitative survey of the facts discovered, in view of the recently published observations of Raper and Wormall [1925], Happold and Raper [1925], Robinson and McCance [1925] and Onslow and Robinson [1925] on the induction of oxidative deamination of amino-acids by certain phenols, and of the discovery of a new oxidation-reduction system composed of glycine, acetaldehyde and phosphate by Haehn and Pülz [1925].

The increased oxidation of protein and amino-acids by alkaline permanganate in the presence of glucose has been observed by Fosse [1921] and also in the presence of formaldehyde by Fearon and Montgomery [1924]. The latter workers suggested, in explanation of the effect of formaldehyde, a destabilisation of the amino-group by its preliminary combination with the aldehyde group.

In incubated solutions of glucose and glycine, of which details are given below, the sugar content, analysed by copper reduction in alkaline solution, showed no change.

The accelerating influence of phosphate on the oxidation of glucose by H_2O_2 has been studied by Löb and his collaborators [1910, 1911, 1912; Beysell and Löb, 1915] and by Witzemann [1920]. Harden and Henley [1922] showed that phosphate, though accelerating the oxidation of glucose by H_2O_2, was not indispensable. The influence of phosphate on the reduction of methylene blue by glucose and glycine or peptone, described above, is directly analogous.

Warburg and Yabusoe [1924] observed the oxidation of fructose by atmospheric oxygen in the presence of phosphate, but found glucose to be unaffected. In view of these experiments Meyerhof and Matsuoka [1924] again brought forward the hypothesis first proposed by Neuberg [1924] that glucose is converted to fructose before undergoing oxidation *in vivo*. Glucose in the presence of glycine undergoes caramelisation at 37°, even at reactions less alkaline than p_H 8, which suggests that significant changes are effected by glycine in the reactivity of the glucose molecule, of which possibly one may be an increased susceptibility to oxidation. This increased oxidisability certainly exists in the amino-acids. The yield of ammonia from the oxidation of glycine by hydrogen peroxide and ferrous sulphate was increased from 8 % of the total possible when it was oxidised alone, to 25 % when the oxidation was carried out in the presence of glucose and phosphate. Neither urea nor cyanic acid was found even when the oxidation resulted in 25 % deamination.

Experimental.

The first observations on the effect of glucose in reducing the free amino-N of protein derivatives were made during a repetition, in modified form, of

the experiments of Kostytcheff and Brilliant [1914] on the influence of glucose on the enzymic synthesis of protein. The following is a typical result.

Table I. *Effect of glucose in reducing free amino-N.*

Exp.	p_H	Concentrated peptic digest of albumin cc.	Glucose g.	Hog liver powder g.	Free amino-N after 48 hours' incubation at 37° mg.
1	8·4	10	4	0·1	56·5
2	8·4	10	—	0·1	154·6
3	8·4 (+4·0)	10	4	0·1	114·0
4	8·4 (+4·0)	10	—	0·1	166·9
5	4·0	10	4	0·1	160·1
6	4·0	10	—	0·1	161·0

Pfanstiehl's "C.P." glucose was used. The liver powder, as will be shown later, was not a factor here. The free amino-nitrogen was estimated by the method of Van Slyke in the macro-apparatus. The p_H of Exps. 3 and 4 was changed from 8·4 to 4·0 after 24 hours' incubation. The dilution was maintained constant in all. Toluene and chloroform were employed as preservatives.

The small reduction in free amino-nitrogen in Exp. 2 was probably due to a synthetic reaction, as analysis showed some increase in the protein content.

The interaction between glucose and the amino-group takes place only in alkaline solution. This effect of the hydrogen ion concentration is shown in Table II.

Table II. *Effect of c_H on the interaction of glucose and the amino-group.*

p_H	Amino-N after incubation with glucose. % of total nitrogen
Control (p_H 7·0)	18·5
5·0	18·3
6·3	17·5
7·1	15·1
7·8	12·8
8·4	12·4

To 10 cc. of a concentrated peptic digest of albumin 4 g. Pfanstiehl "C.P." glucose were added with chloroform and toluene, and incubated at 37° for 48 hours. The liver powder, also added to these solutions, effected some small amounts of synthesis, which accounts for the slight reductions in the free amino-nitrogen in the acid solutions. The free amino-nitrogen was estimated by formaldehyde titration.

In Table III the results of fractional analysis of a peptic digest after incubation with glucose show quite definitely that there are no indications of a synthetic reaction of sufficient extent to account for the large reduction in free amino-nitrogen.

Table III. *Fractional analysis of peptic digest after incubation with glucose.*

Exp.	Concentrated peptic digest of albumin p_H 8·5 cc.	Glucose g.	Hog liver powder g.	Free amino-N of mixture	Protein-N	Proteose-N	Peptone-N	Sub-peptone-N
				Percentage of total nitrogen				
1	30	12	0·6	9·6	6·4	42·2	27·9	24·0
2	30	12	—	9·8	5·4	43·8	29·9	20·8
3	30	—	0·6	17·2	3·8	52·0	23·9	21·3
4	30	—	—	17·4	3·6	53·1	23·9	19·4

Pfanstiehl glucose was used. The differences between Exps. 1 and 2, and 3 and 4, in the values for the proteose and peptone fractions, are due to the increased solubility of proteose in the presence of glucose, which prevents a maximum salting out. This increased solubility has been observed also with albumin, Witte's peptone and Parke-Davis' peptone, and is probably the result of the affinity of the glucose for the amino-group. No increase in ammonia was found (the titrations were carried out with $N/14$ acid and alkali) in any of the experiments, nor was any urea or cyanic acid detected.

A comparison of the free amino-nitrogen values in Exps. 1 and 2 in Table III shows that the decrease in free amino-N is as great in the absence of enzyme as when it is present. This is again demonstrated in Table IV. The peptic digest of albumin was boiled for half an hour. Two specimens of glucose were used, one, Baker's "C.P.," the other prepared from technical glucose by two recrystallisations according to the method of Hudson and Dale [1917].

Table IV. *Action of glucose in the absence of enzyme.*

Exp.	Glucose used	Free amino-N in 10 cc. of digest after incubation mg.	p_H Before incubation	p_H After incubation
1	—	66·6	8·3	8·3
2	"C.P."	37·1	8·3	7·2
3	Recrystallised	37·1	8·3	7·2

In Exps. 2 and 3, 10 cc. of the digest and 4 g. of sugar were used. No. 1 was without sugar. The mixtures were incubated for 48 hours at 37°. The fall in p_H in Exps. 2 and 3 is to be expected from the removal of the amino groups.

The dependence of this interaction of glucose and amino-nitrogen on excess of hydroxyl ions, shown in Table II, is found again when glycine is substituted for a peptic digest as the carrier of the amino-group. In Table V are given the results after incubation of a number of 1 % glycine solutions in $M/2$ phosphate at various hydrogen ion concentrations with 13·2 % glucose (Baker's "C.P."). Chloroform was added to these mixtures; the flasks were tightly stoppered and incubated at 37°: electrometric p_H determinations and free amino-nitrogen estimations (Van Slyke's method) were made from time to time. As a control, a parallel series of glycine solutions at the same hydrogen ion concentrations containing identical amounts of phosphate, but without sugar, was observed. The free amino-nitrogen values given are corrected for the change in volume due to the added glucose.

Table V. *Effect of c_H on the interaction of glucose and glycine.*

Time hours	p_H.	Free amino-N in 10 cc. With glucose mg.	Free amino-N in 10 cc. Without glucose mg.
0	7·8	9·4	9·5
20	7·8	8·5	9·5
46	7·8	8·1	9·4
0	7·9	9·4	9·4
22	7·9	8·4	9·4
46	7·9	7·7	9·4
0	8·3	9·4	9·4
16	8·3	8·1	9·4
48	8·3	7·5	9·4
0	8·7	9·5	9·5
19	8·7	7·7	9·4
48	8·7	7·4	9·4
0	9·2	9·0	9·2
19	9·2	5·8	9·2
46	9·2	5·6	9·2

To carry the p_H range further, another series of 1 % glycine solutions in $M/2$ phosphate with and without sugar was prepared, and incubated for 48 hours at 37°. The p_H values given in Table VI are those of the phosphate solutions before the addition of glucose or glycine. The free amino-nitrogen values, as in Table V, are corrected for the volume changes due to dissolved sugar.

Table VI. *Effect of c_H on the interaction of glucose and glycine.*

p_H	Free amino-N (in 10 cc. of 1 % glycine after incubation at 37° for 48 hours) With glucose mg.	Without glucose mg.
9·2	6·7	9·6
10·0	6·0	9·5
10·6	3·9	9·5
11·0	2·5	9·3

After incubation with glucose in alkaline solution and suffering reduction in free amino-nitrogen, glycine, peptic digest, peptone and albumin acquire the property of reducing methylene blue without the aid of an enzyme. In these experiments the preliminary incubation and the subsequent treatment with methylene blue were performed with an aseptic technique, controlled by tests for organisms and spores. The reagents were heated for half an hour in a boiling water-bath, and chloroform and toluene were added to the final mixtures. In Table VII are stated the details of the mixtures and the results.

The peptic digest, free from material precipitable by trichloroacetic acid, was prepared from a 3 weeks' peptic digest of 3 % albumin. It was neutralised to litmus and concentrated over a boiling water-bath to one-tenth of the original volume. The glycine solution contained 14·4 g. Pfanstiehl C.P. glycine in 100 cc. of $M/2$ K_2HPO_4. The concentrated digest and the glycine solutions were adjusted to p_H 8·5. The glucose employed was Baker's C.P.

Table VII. *Reduction of methylene blue by incubated mixtures of glucose and glycine or peptone.*

Exp.	Concentrated peptic digest of albumin cc.	14·4 % glycine cc.	$M/2$ K_2HPO_4 cc.	Glucose g.	p_H after incubation and dilution	Free amino-N before heating in acid solution mg.	Free amino-N after heating in acid solution mg.	Time to decolorise 0·05% methylene blue before heating in acid solution	Time to decolorise 0·05% methylene blue after heating in acid solution
1	10	—	—	4	8·0	25	63	48 hrs.	Partial in 24 hrs.
2	10	—	—	4	8·0	30	62	48 hrs.	Partial in 24 hrs.
3	—	—	10	4	9·7	—	—	No decolorisation	No decolorisation
4	—	10	—	4	7·5	193	198	15 mins.	19 mins.
5	—	10	—	4	7·5	192	198	15 mins.	19 mins.
6	—	10	—	—	8·4	254	251	No decolorisation	No decolorisation
7	10	—	—	—	8·4	45	62	No decolorisation	No decolorisation
8	10	—	—	4	—	—	—	Partial in 48 hrs.	—
9	—	2·5	—	1	—	—	—	24 hrs.	—

After 48 hours' incubation in a water-bath at 37° sterility tests were made on 1 cc. removed aseptically from each of the above solutions, and the remainder was diluted to 100 cc. with $N/280$ NaOH.

Solutions nos. 8 and 9 were prepared at this time. In solution no. 8, 4 g. sugar were dissolved in 10 cc. $N/280$ NaOH and 10 cc. peptic digest, and the resulting solution diluted to 100 cc. with $N/280$ NaOH. Solution no. 9 consisted of 1 g. glucose and 2·5 cc. of the control 14·4 % glycine solution (no. 6 in Table VII) diluted to 25 cc. with $N/280$ NaOH.

For the determination of the time required to decolorise methylene blue, 10 cc. of the solutions described above and in Table VII were pipetted into 10 cc. of 0·05 % methylene blue, in 25 cc. test tubes. These were tightly stoppered with rubber corks and placed in a water-bath at 56°. To 40 cc. (except nos. 8 and 9) 1 cc. of conc. HCl was added and the mixtures heated for 1 hour in a boiling water-bath and then left overnight in an ice-chest. The added acid was then neutralised, and the resulting solution made up to 50 cc. with distilled water. Methylene blue was added to 10 cc. of these as in the unboiled solutions. The free amino-nitrogen of both boiled and unboiled mixtures was estimated by the Van Slyke method, and the results are expressed in terms of 10 cc. of original undiluted peptic digest and glycine respectively. The p_H values given in Table VIII are those of the incubated solutions after dilution with $N/280$ NaOH.

At the end of the 48 hours' incubation all solutions were found to be free from organisms or spores. Solutions 4 and 5 had become dark reddish brown and possessed the odour characteristic of solutions containing caramel. The results of the treatment of solutions 4 and 5 show also that the compound, which arises during incubation of glucose with glycine, and which reduces methylene blue, is not destroyed by heating with acid. The result with solution 9 compared with 4 and 5 shows that the preliminary 48 hour incubation reduced the time required to decolorise methylene blue from 24 hours to 15 minutes.

In the case of the peptic digest, experiments 1 and 2, the reduction of methylene blue was more sluggish, and the amino-nitrogen lost during incubation was restored by heating in acid solution. These differences between peptic digest and glycine are in large part due to the absence of phosphate from the peptic digest mixtures in 1 and 2, as is clearly shown in Table VIII.

Table VIII. *Effect of phosphate on the reduction of methylene blue by peptone and glucose.*

Exp.	Digest	Glucose g.	$M/2$ K_2HPO_4 p_H 8·5 cc.	14·4 % Glycine cc.	Free amino-N before heating with acid mg.	Free amino-N after heating with acid mg.	p_H	Time to decolorise 0·05 % methylene blue before heating with acid	Time to decolorise 0·05 % methylene blue after heating with acid
1	With phosphate	—	—	—	73·5	85·6	8·2	No decolorisation	No decolorisation
2	,,	8	—	—	24·6	62·8	7·5	22 mins.	23 mins.
3	,,	8	—	—	24·4	64·9	7·5	18 mins.	19 mins.
4	Without phosphate	8	—	—	44·8	107·2	7·4	Partial in 24 hrs.	None in 24 hrs.
5	,,	8	—	—	44·4	105·3	7·4	Partial in 24 hrs.	None in 24 hrs.
6	,,	—	—	—	73·5	108·8	8·5	No decolorisation	No decolorisation
7	—	8	8	—	—	—	9·1	Slight in 24 hrs.	Slight in 24 hrs.
8	—	2	2	5	207·8	—	7·4	18 mins.	—

Two 30 % solutions of Witte's peptone were made up, one with $N/14$ NaOH, the other with 0·75 M K_2HPO_4. The reaction in both was adjusted to p_H 8·5. The solutions described in Table VIII were made with the same aseptic technique as those in Table VII, and incubated, as there, for 48 hours in a water-bath at 37°. Solutions 1, 2 and 3 contain, however, more total N than the corresponding ones in Table VII, because no withdrawal of solution was made for sterility tests. Solutions 2 and 3 were much less turbid than 1 on account of the increased solubility of peptone in the presence of glucose. Solution 8 served as a control for the activity of the whole system.

After 48 hours all except solution 8 were diluted with $N/280$ NaOH to 100 cc. and number 8 was diluted to 50 cc. The free amino-nitrogen estimation, the methylene blue reduction, the p_H determinations, and the heating in acid and subsequent neutralisation and dilution were carried out in exactly the same manner as in the experiments described in Table VII.

In the presence of phosphate the reduction of methylene blue by peptone and glucose is as rapid as the reduction by glycine, glucose and phosphate. Less of the amino-nitrogen lost by the peptone during incubation is restored by boiling with acid when phosphate is present. The increased value for free amino-nitrogen after boiling with acid in solutions 4, 5 and 6, as compared with solutions 2 and 3, is probably accounted for by the increased hydrolysis in the greater acidity due to the absence of buffer.

With protein, phosphate is not as essential for the rapid reduction of methylene blue after incubation with sugar. The reduction in free amino-nitrogen in the preliminary incubation with glucose, if any, is quite small.

Two suspensions of egg albumin were prepared, of approximately 30 % concentration, one in $M/4$ K_2HPO_4, the other in dilute NaOH, both with a

final p_H of 8·5. The protein was more soluble in the phosphate than in the NaOH, and both protein solutions were considerably clearer after addition of glucose.

A number of solutions were made, and incubated in a water-bath at 37° for 48 hours. The details of the solutions and the results are noted in Table IX.

Table IX. *Reduction of methylene blue by albumin and glucose.*

Exp.	Albumin with K_2HPO_4 cc.	Albumin without K_2HPO_4 cc.	Glucose g.	K_2HPO_4 p_H 8·5 cc.	Free amino-N before heating with acid mg.	Free amino-N after heating with acid mg.	p_H	Time to decolorise 0·1 % methylene blue before heating with acid	Time to decolorise 0·1 % methylene blue after heating with acid
1	20	—	8	—	1·4	1·7	8·1	55 mins.	—
2	—	20	8	—	1·5	1·2	9·1	38 mins.	40 mins.
3	20	—	—	—	1·8	1·7	9·2	Partial. 0·01 % in 24 hrs.	No decolorisation
4	—	20	—	—	1·5	1·9	10·0	Partial. 0·01 % in 24 hrs.	No decolorisation
5	—	—	8	20	—	—	9·1	0·01 % in 46 mins.	0·01 % in 46 mins.

The treatment after incubation was the same as in the previous experiments.

The free amino-nitrogen values are uncertain. The faster reduction of methylene blue in the solution containing phosphate was due more probably to the higher alkalinity than to the phosphate.

An attempt was made to ascertain whether a similar power to reduce methylene blue was possessed by acetamide, urea and creatine after incubation with glucose. No attempts were made to measure any changes that might have occurred in the NH_2 group. It was found that acetamide, urea and creatine after this treatment, did not reduce methylene blue, even in the presence of phosphate, at a rate appreciably faster than glucose alone, or at a less alkaline p_H.

Table X. *Effect of washed muscle on reduction of methylene blue by peptone and sugar.*

Exp.	Digest cc.	Inc. sugar	Sugar g.	$M/2$ K_2HPO_4 p_H 8·5 cc.	Washed muscle (enzyme) g.	Time to decolorise 0·01 % methylene blue
1	10	+	—	—	2	3½ hrs.
2	10	+	—	—	—	10 hrs.
3	10	—	+	—	2	Nearly complete in 24 hrs.
4	10	—	+	—	—	Partially in 24 hrs.
5	—	—	1	10	2	No decolorisation
6	10	—	—	—	2	No decolorisation
7	—	—	1	10	2	Slight decolorisation in 24 hrs.
8	—	—	—	10	2	No decolorisation

The decolorisation of methylene blue by peptone after incubation with glucose is accelerated by the addition of washed muscle prepared by the method of Thunberg [1917]. A concentrated peptic digest of albumin was incubated at an initial p_H of 8·3 with 10 % glucose for 24 hours at 37°. The solution was then diluted with an equal volume of $M/2$ K_2HPO_4 and heated at 56° for 1 hour to drive off the chloroform and to sterilise the mixtures. At the

same time 50 cc. of digest without sugar were similarly treated and, after heating, glucose to a concentration of 10 % added. The p_H of both solutions was finally 7·9. In Table X "inc. sugar" designates digest incubated with glucose for 24 hours, and "sugar" the digest to which glucose was added immediately before the addition of the washed muscle.

Preliminary incubation with glucose increases the yield of ammonia after treatment with H_2O_2 and ferrous sulphate. A typical result is given in Table XI.

Table XI. *Effect of glucose on the oxidation of glycine by* H_2O_2.

Exp.	1 % glycine in K_2HPO_4 cc.	Glucose g.	30 % H_2O_2 cc.	H_2O cc.	Ammonia-N mg.
1	25	3·1	8	2	5·8
2	25	2·0	6	4	5·7
3	25	1·25	4	6	2·2
4	25	0·75	2	8	2·9
5	25	0·25	2	8	2·5
6	25	—	2	8	2·2
7	25	3·1	—	10	0·4

A 1 % glycine solution in $M/2$ K_2HPO_4 at p_H 8·8 was used. All the solutions were incubated for 24 hours at 37°. At the end of this period there still remained some undecomposed H_2O_2 which was removed by freshly precipitated manganese dioxide. The ammonia was determined by aeration in a strongly alkaline solution, and the urea by precipitation with xanthhydrol. The cyanic acid was estimated by incubation with excess of ammonium chloride and precipitation of the resulting urea with xanthhydrol. The ammonia formed in Exps. 1 and 2 corresponds to 17·5 % of the total nitrogen. No cyanic acid or urea was found.

The dependence of the oxidation of glycine and glucose by H_2O_2 on the reaction is shown in Table XII.

Table XII. *Effect of* p_H *on the oxidation of glycine and glucose by* H_2O_2.

Exp.	p_H	Ammonia-N formed; percentage of total N
1	9·5	19·1
2	8·7	17·6
3	8·0	17·2
4	7·7	14·1
5	7·2	9·5
6	6·9	7·6
7	6·3	4·4
8	5·9	5·7
9	3·8	2·4

1 % glycine solutions were made up in $M/2$ K_2HPO_4 at various p_H values and the final p_H in each case determined electrometrically. To 30 cc. of each of these solutions 3·75 g. glucose (Pfanstiehl C.P.) and 10 cc. of neutralised 30 % H_2O_2 and one crystal of ferrous sulphate were added. After 24 hours' incubation at 37°, the solutions were neutralised and the excess H_2O_2 was

decomposed with freshly precipitated manganese dioxide. The ammonia was determined by aeration and the urea and cyanic acid by xanthhydrol. Neither urea nor cyanic acid was found.

SUMMARY.

1. In alkaline solution, in the absence of any enzyme, an interaction occurs between glucose and protein derivatives which results in a reduction of the free amino-nitrogen. The reduction is not the result of a synthetic process and neither urea, cyanic acid nor ammonia is formed.

2. Simultaneously with the reduction in free amino-nitrogen the mixture of glucose and protein derivative acquires the property of reducing methylene blue in approximately neutral solution.

3. With glycine and peptone this reduction of methylene blue is accelerated by phosphate. With albumin an equal velocity of reduction is obtained without phosphate.

4. The reduction of methylene blue by incubated peptone and glucose is accelerated by washed muscle.

5. Preliminary incubation with glucose increases the yield of ammonia after oxidation with hydrogen peroxide.

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