XXV. STUDIES ON THE INTERACTION OF AMINO-COMPOUNDS AND CARBOHYDRATES.

I. THE ACTION OF UREA ON GLUCOSE, FRUCTOSE AND MANNOSE.

By ALEXANDER HYND.

From the Department of Physiology, University of St Andrews.

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MANY gaps exist in our knowledge of the chemistry of sugar derivatives containing nitrogen, and as, so far, only the more accessible and more stable compounds of this class have been examined from the physiological point of view, very little is known of the behaviour of nitrogenous sugar derivatives in the animal organism.

With the object of furthering our knowledge in this direction, a series of researches was instituted, and glucose ureide was selected as the first compound for investigation. Amongst the reasons which led to the choice of this derivative may be mentioned, firstly, the fact that its preparation had been described by Schoorl [1903], and secondly, that both glucose and urea are important compounds from the physiological point of view. The latter factor acquires a special importance when it is recalled that glucose and urea are present in human blood in equimolecular proportions. Glucose ureide thus promised to be of great biochemical interest, and as the chemistry of the compound appeared fairly complete, the study of its physiological behaviour seemed to depend on the preparation of a sufficient quantity of the compound. Accordingly work was started with this end in view, but it was found that by Schoorl's method considerable difficulty is involved in preparing a large amount of the compound in a state of purity. Alternative methods to effect the condensation were therefore considered, and these led to a study of the action of urea on the simple sugars which forms the subject of the present communication.

EXPERIMENTAL.

The action of urea on glucose in solution in ethyl alcohol.

To an approximately 5% ethyl alcoholic solution of glucose (1 mol.), which had attained its permanent rotation value, excess of urea (4 mols.) was added, and the resulting solution examined polarimetrically at intervals. Only very slight changes in rotation resulted, but after long standing—for a period of nearly four months—a definite decrease had taken place, for, whilst the initial value for α in a 1 dm. tube was $+2.44^\circ$, the value then recorded

was only $+1.73^\circ$. As no material had separated, the solution was evaporated to dryness *in vacuo* and the residue repeatedly extracted with acetone to remove excess of urea. The product thus obtained was slightly yellowish in colour, of a somewhat pasty consistency, contained only traces of nitrogen and had a marked action on Fehling's solution.

A portion of this material was dissolved in water and the concentration of sugar present then examined polarimetrically and also titrimetrically by Benedict's method. Assuming the sugar to be glucose, its concentration by the former method was 3.2 %, by the latter 4.4 %. Consequently the product could not consist entirely of glucose, and it was then found that the solution under examination gave a marked Selivanoff's reaction [1887], whilst a freshly prepared 4 % solution of glucose gave only a very faint colour with this test.

A repetition of the above experiment using equimolecular proportions of glucose and urea gave similar results, the rotation falling from the initial value of $\alpha = +4.23^\circ$ to the constant value of $\alpha = +3.44^\circ$ after six months at room temperature.

Analogous results have also been obtained under somewhat modified conditions, an alcoholic solution of glucose and urea in equimolecular proportions being boiled under a reflux condenser for three successive days. With this treatment the rotation of the solution fell from $+1.67^\circ$ to $+1.41^\circ$, and simultaneously a marked Selivanoff's reaction developed.

The action of urea on glucose in aqueous solution.

The action of urea on glucose was then studied in aqueous solution and to economise space the results obtained are summarised in the following tables.

(a) *Solvent: distilled water.*

1. Glucose : urea = 1 : 1 = 1 mol. : 3 mols.

	A	B	S	R
At beginning of experiment	$+1.89^\circ$	$+1.906^\circ$	—	Neutral
After 6 hours' heating on water bath	0.35	1.916	+++	Alkaline

(A = α observed polarimetrically for $l=1$; B = α calculated from estimations by Benedict's method; S = Selivanoff's test; R = reaction of the solution.)

2. Glucose : urea = 3 : 1 = 1 mol. : 1 mol.

	A	B	S	R
At beginning of experiment	$+1.01^\circ$	$+1.003^\circ$	—	$p_H = 6.3$
After 3 hours at 70°	1.02	.	?—	.
" 2 " 80°	0.96	.	?+	.
" 4 " 80°	0.85	.	+	.
" 3 " 85°	0.55	1.003	+++	$p_H > 8.0$

(b) *Solvent: a phosphate mixture of $p_H = 6.0$ (reaction of normal urine).*

Glucose : urea = 1 : 1 (by weight)				Control without urea		
	A	B	S	A	B	S
At beginning of experiment	$+0.87^\circ$	$+0.997^\circ$	—	$+0.88^\circ$	$+0.971^\circ$	—
After 96 hours at 37°	0.86	.	.	0.88	.	.
" 8 " $80-85^\circ$	0.33	0.982	++	0.85	0.970	—

(c) *Solvent: a phosphate mixture of $p_H = 7.42$ (reaction of normal blood).*

	Glucose : urea = 1 : 1 (by weight)			Control without urea		
	A	B	S	A	B	S
At beginning of experiment	+0.86°	+0.987°	—	+0.86°	+0.945°	—
After 96 hours at 37°	0.86	.	.	0.86	.	.
" 2 " 85°	0.60	.	.	0.60	.	.
" 8 " 85°	0.25	0.982	+++	0.52	0.940	+

Further control experiments with glucose in a buffer of $p_H = 7.42$ showed that no appreciable change in rotation took place after four months' incubation at 37°, or after heating for three weeks at 50°.

(d) *Solvent: a phosphate mixture of $p_H = 7.0$ (absolute neutrality).*

	Glucose : urea = 3 : 1 (by weight).		
	A	B	S
At beginning of experiment	+0.89°	+0.908°	—
After 2½ hours at 85°	0.83	.	.
" 4½ " 85°	0.80	.	.
" 7½ " 85°	0.65	0.908	+++

Experiments at body temperature.

The results of the experiments with glucose and urea, reported in the previous section, indicate that a marked change in the nature of the sugar takes place after heating for a few hours at a temperature of about 85°. Accordingly experiments were then carried out to determine whether any change takes place at body temperature. The results of a typical experiment after 3 months at 37° may be summarised as follows.

(1) The p_H increased from an initial value of 6.0 to over 8.0, whilst in a control experiment with the same amount of urea the p_H increased to 7.9, and in a control with glucose no change in reaction was detected.

(2) The observed rotation decreased from +0.92° to +0.83°; a control experiment with the same amount of glucose showed no alteration in optical activity.

(3) Selivanoff's and Pinoff's [1905] reactions became slightly positive, the coloration produced being equivalent to that of a 0.05 % solution of fructose, whilst in a control experiment with pure glucose neither of these so-called ketose reactions developed.

Experiments were also conducted to ascertain the effect of Winter and Smith's technique [1923] on a mixture of glucose and urea. The results of these are tabulated as follows:

No. of experiment	Glucose : urea	Initial concentration of sugar		Concentration at end of experiment	
		A, %	B, %	A, %	B, %
1	1 : 1	—	—	1.60	1.86
2	1 : 1	0.183	0.18	0.1714	0.18
3	1 : 1	1.83	1.80	1.77	1.89
4	3 : 1	1.73	1.68	1.73	1.74
5	3 : 1	1.73	1.68	2.13	2.20
6	3 : 1	1.73	1.68	1.92	1.91

(A = % calculated from the polarimetric observation; B = % calculated from the copper reduction value estimated by MacLean's method.)

The results of Exps. 1 to 3 seem to suggest that the presence of urea causes the concentration of the sugar as determined by the polarimeter to be less than that determined by copper reduction methods, but Exps. 4 to 6, on the other hand, indicate that the changes observed are probably due to experimental error. This is consistent with the results of the previous series of experiments which show that definite optical changes can be detected only after prolonged incubation at 37°.

Experiments at 50°.

With the object of confirming the fact that glucose and urea interact at temperatures well below 80°, a series of experiments was conducted at 50°. One typical case may be quoted.

Glucose (9 g.) and urea (3 g.) were dissolved in water and the solution made up to 100 cc. The initial rotation of the solution was then determined and the sugar also estimated by Benedict's volumetric process. Thereafter the solution was incubated at 50°, and the rotation of the solution taken periodically. The original colourless solution soon began to assume a slightly yellowish tint, and after 10 days was yellowish brown. Simultaneously an alkaline reaction developed and with Selivanoff's test the solution then gave positive results, which increased in intensity with the duration of the heating. The optical changes noted were as follows:

Duration of heating in hours	0	240	430	800
α in 1 dm. tube	+4.72°	3.15°	2.10°	1.02°

As after 800 hours' heating no further diminution of optical rotation was detected, the sugar content of the solution was determined by both Benedict's and the Wood-Ost methods. The former gave 8.93 %, and the latter 8.91 % of sugar calculated as glucose: so that both results agreed closely with that calculated from the initial rotation value, namely 8.99 %. Assuming, for the moment, that the final mixture contained only glucose and fructose, use was made of the iodometric method described by Judd [1920] to arrive at the proportions of these two sugars present. From the results obtained, the mixture would appear to consist of 80.74 % glucose and 19.26 % fructose. The ratio of these sugars present was also determined by applying the observations of Nyns [1924] that fructose reduces Ost's reagent at 49° whereas other sugars, such as glucose, do not. By this process, the glucose amounted to 82.27 %, the fructose accounting for 17.73 %. It would then seem that approximately 20 % of the original glucose had been converted into fructose; but the rotation data demand the conversion of at least 25 %.

Decomposition on heating aqueous solutions of urea.

As the changes in optical activity of the glucose solutions containing urea recorded above were always accompanied by the development of an alkaline reaction, it seemed that this was the factor responsible for the fall in rotation. Further, as the alkaline reaction could only be brought about by the forma-

tion of ammonia from the urea, attempts were made to determine whether glucose had any effect upon this decomposition.

Working with a 1 % solution of urea, no ammonia could be detected by Nessler's reagent after 5 days at room temperature (15° to 18°). A similar result was obtained with 1 % urea containing 3 % glucose, and no change in the optical power of the solution was observed. Warming similar solutions at 50° for 20 hours caused the production of 4.73 mg. NH_3 %, and 5.00 mg. NH_3 % respectively. After 44 hours the amounts of ammonia were 9.00 mg. and 9.50 mg. %, whilst after 94 hours 20.07 mg. and 25.00 mg. NH_3 % were produced respectively, and the rotation of the glucose-containing solution had then fallen from the initial value of + 2.70° to + 2.51°. At 85° ammonia was rapidly liberated both in the pure urea solution and in that containing glucose, and, as is evident from the following table, the decomposition proceeded at approximately the same rate.

Duration of heating at 85° in hours	1 % urea solution	1 % urea + 3 % glucose solution	
	NH_3 produced mg. %	NH_3 produced mg. %	α
0	—	—	+ 2.70°
2	25	20	—
4	35	30	—
6	53	49	0.64°

On the other hand, experiments in which the change in the p_{H} of the solution was determined indicated that a greater effect resulted on warming at 50° a 1 % solution of urea containing 1 % glucose than a 1 % solution of pure urea, as, in the former the p_{H} increased from 6.5 to 9.3, and in the latter from 7.1 to 8.5.

The action of dilute ammonia on glucose.

A 2 % solution of glucose containing approximately 60 mg. NH_3 showed no optical change at room temperature, but after 24 hours at 50° the rotation fell from + 0.87° to + 0.29°, and a marked Selivanoff reaction was then obtained.

It was thus evident that ammonia caused a marked lowering of the rotation after heating at 50°, and an experiment at 37° with similar solutions showed after 21 hours a fall from + 0.87° to + 0.71°. No further change took place on longer incubation, the mean of a series of readings taken after 65 hours being + 0.72°.

Similar results were obtained when ammonia was added to an aqueous solution of glucose dissolved in an aqueous phosphate mixture of $p_{\text{H}} = 7.40$.

Although ammonia does affect the rotation of a sugar solution it is much less active in this respect than the equivalent amount of caustic soda, as is shown by the results given below:

		Glucose + ammonia	Glucose + NaOH
		α	α
At beginning of experiment		+ 0.58°	+ 0.45°
After 18 hours at 37°		0.47	0.04
" 40 "	37	0.41	0.04
" 18 "	50	0.40	0.03
" 40 "	50	0.16	0.04
" 120 "	50	0.16	0.04

Action of urea on other sugars.

Having shown that urea can produce a fall in the rotation of a solution of glucose, it became of interest to determine what its effect would be on other sugars and more especially on the closely related members of the series, namely fructose and mannose.

As was expected, with fructose a marked diminution of the laevo-rotation was observed, whilst with mannose a fall in the dextro-rotation resulted, and a positive reaction with Selivanoff's test was obtained. The results are tabulated below:

(a) Fructose.

	2 % fructose + 2 % urea	2 % fructose
At beginning of experiment	-1.77°	-1.77°
After 1 hour at 85°	1.52	1.75
„ 3 hours at 85°	1.02	1.76
„ 5 „ 85°	0.47	1.77
„ standing 6 weeks at lab. temp.	0.59	1.69*

* This low value is probably due to a black fungus which had developed in the solution.

(b) Mannose.

	2 % mannose + 1 % urea	2 % mannose
At beginning of experiment	+0.48°	+0.47°
After 20 hours at 50°	0.48	0.47
„ 3 weeks at 50°	0.12 (constant)	0.46

DISCUSSION.

It has been shown [Irvine and Gilmour, 1908, 1909; Irvine and Hynd, 1911] that glucose and other reducing carbohydrates condense readily at laboratory temperature with a variety of amino-compounds in ethyl alcoholic solution, provided the latter have the requisite solubilities. As urea is highly soluble in alcohol, it was hoped that this process might prove applicable to the condensation of glucose and urea and thereby furnish a convenient method for the preparation of glucose ureide. On putting this to the test, it was found that the optical activity of an ethyl alcoholic solution of glucose containing excess of urea diminished after long standing to $\frac{1}{8}$ ths- $\frac{1}{3}$ rds of the initial value.

To account for the decrease in rotation several explanations might be offered. Thus, the change in optical activity observed might be due to the conversion of 25 % of the glucose into the laevo-rotatory glucose ureide, but as glucose ureide is very sparingly soluble in alcohol and as all attempts to isolate from the solution even traces of this compound ended in failure, it seemed that no such condensation had taken place, and accordingly Schoorl's observations are confirmed. The formation of glucose ureide being thus probably excluded, it seemed, from the fact that the mixture now gave a marked Selivanoff reaction, that the alteration in optical activity was due to a change similar to the Lobry de Bruyn transformation [1895, 1, 2; 1896], this having been effected by the interaction of urea in the glucose solution. However, as

during this interaction ammonia would be liberated, the possible production of glucoseimine must not be forgotten, and its formation would furnish another alternative explanation of the fall in dextro-rotation.

The possible importance of this observation in connection with the sugar in urine, blood and other body fluids being recognised, experiments with aqueous solutions were carried out. In these, similar but more marked changes were observed, that is to say no alteration in the copper reducing power of the solution could be detected, but a marked decrease in the rotation of the solution took place, the reaction mixture became alkaline and a strongly positive Selivanoff's reaction developed. These results were obtained when the solvents employed were distilled water or phosphate mixtures of p_H 6.0, 7.42 and 7.0; that is, corresponding respectively to the reactions of normal urine, normal blood and absolute neutrality. Most marked results were recorded when the mixtures were warmed on the water-bath at a temperature of about 80° for a few hours; less marked though quite definite results were obtained when the mixtures were kept in a thermostat at 50° for several days, whilst to obtain definite changes at 37° several months were necessary. It is not surprising, therefore, that, when a mixture of urea and glucose was subjected to a process similar to that employed by Winter and Smith [1923] for obtaining what they considered to be γ -glucose from blood, the slight changes noted could be explained as due to experimental error. Nevertheless, the fact that after a prolonged period definite changes did occur even at body temperature when working with mixtures *in vitro*, suggests that the action of urea and glucose may be of importance *in vivo*, where more than likely the reaction would be catalysed by the presence of a specific enzyme. It might be objected that in the experiment at 37° , the decreased dextro-rotation might be due to the disappearance of a portion of the glucose through the action of micro-organisms. Proof that this was not the case was furnished, not only by no sign of micro-organisms being visible either on naked eye or microscopic examination of the solution, but also by the fact that the final and initial copper reducing powers of the solution were practically identical. Further, later experiments proved that once micro-organisms obtained access to such a mixture of glucose and urea, they developed so rapidly that no further polarimetric examinations of the solutions were possible owing to the turbidity of the latter.

Fructose and mannose have been found to interact with urea in an analogous fashion—the former being more marked in its action than glucose, the latter much less so. As it was thus evident that a similar mechanism was involved in these cases, it seemed futile to duplicate all the experiments, and consequently attention was confined to experiments with glucose with the object of arriving at the actual mechanism of the reaction. This will form the subject of a later paper.

As Schoorl [1903] has observed that fructose, and ketoses generally, do not combine with urea even under the influence of catalysts, the pronounced rise

in rotation observed in the experiment with fructose and urea cannot be due to the formation of fructose ureide. This lends support to the view, already suggested, that ureide formation is not, at any rate, an important factor in accounting for the optical changes observed to occur in alcoholic or aqueous solutions containing glucose and urea. The alternative explanation is accordingly more probable, namely that the ammonia to which the urea gives rise forms glucoseimine or brings about a Lobry de Bruyn conversion.

The latter well-known transformation of glucose into a mixture of glucose, fructose and mannose is known to be brought about by the action of almost any alkali—even a weak alkali such as lead hydroxide. Accordingly it has been suggested by several workers that the glucose of the blood might suffer the change owing to the normal slight alkalinity of this fluid. Though experiments with glucose in a phosphate mixture of $p_H = 7.42$ showed a fall in rotation when heated at 85° , no change was detected after 3 weeks at 50° nor after 4 months' incubation at 37° . On the other hand, the presence of an equimolecular proportion of urea, or the addition of 0.06 % ammonia, brings about a definite change in rotation even at body temperature, and a marked fall after 18 hours at 50° .

These experiments, coupled with the fact already mentioned that the normal ratio of glucose to urea in blood approximates 3 to 1, would seem to furnish a clue as to why polarimetric estimations of sugar in nearly all biological fluids almost invariably give lower values than those obtained by volumetric methods. At the same time it must not be forgotten that there are present in nearly all biological fluids substances other than glucose which contribute both to the optical activity and reducing power of deproteinised filtrates. Consequently, as Denis and Hume [1924] have pointed out, the difference between the results obtained by the two methods is only what might be expected. On the other hand, these same authors have obtained differences between the two methods when employing "artificial" blood filtrates where the only interfering substances present were alanine and urea.

Though the partial transformation of glucose into mannose and fructose would account satisfactorily for the optical changes observed and is supported both by the Willstätter and Schudel [1918] and the Wood-Ost [Wood and Berry, 1904] methods for estimating the reducing sugars present, in no single experiment has it been possible to isolate mannose as its phenylhydrazone or to detect fructose by means of methylphenylhydrazine. This was also the experience of Ling and Nanji [1922] with regard to mannose in their work on the action of ammonia on glucose. Accordingly, apart from the fall in rotation and the reduction of Ost's reagent at 50° , proof that fructose is formed is dependent on the production of positive results with Pinoff's and Selivanoff's reactions. The latter of these tests is probably the more reliable, but this test may simply indicate the presence of a reactive sugar and may not be a specific test for ketoses as generally supposed. In any case, colour tests are not sufficient proof of the actual formation of fructose during

the interaction of glucose and urea, and a characteristic derivative of fructose must be isolated before the presence of this sugar can be established. Experiments with this end in view are in progress, and the part played by glucose-imine is also being investigated.

Incidentally a point of clinical importance may be mentioned with regard to the use of Selivanoff's reaction in testing for the presence of fructose in urine. It is generally recognised that urines containing a fair amount of glucose give positive resorcinol tests. In the light of the experiments reported in this paper, the majority of these positive results are probably due, not to the actual excretion of fructose, but to the interaction of urea and glucose. On the other hand, it was found that the addition of urea to a dilute (0.1 %) fructose solution, which gave a distinct Selivanoff reaction, may mask the reaction completely. Consequently this test is of little value for the detection of fructose in solutions containing urea, and its clinical application to urine should, therefore, be discontinued.

SUMMARY.

1. The addition of urea to an alcoholic or aqueous solution of glucose causes a fall in the optical activity of the solution without altering its copper reducing value. The possible importance of this observation is indicated.

2. The optical changes observed are not due to the action of micro-organisms, and ureide formation is probably also excluded.

3. The decrease in optical activity is accompanied by an increase in the p_H of the solution, and also by the development of a positive result with Selivanoff's test.

4. As similar results are obtained with fructose and mannose, the mechanism of the change is regarded as similar to the Lobry de Bruyn transformation, but further experiments are necessary to establish this point definitely, as, in the experiments with urea and glucose, no derivative of either fructose or mannose has so far been isolated, and, further, imine formation is not excluded.

5. It is questioned whether Selivanoff's reaction is specific for ketoses, and it is also pointed out that this test is of little value for the detection of fructose in urine.

6. Experiments are described which suggest that the presence of reducing sugars accelerates the decomposition of urea.

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