64. ENZYMIC DECOMPOSITION OF AMINO-SUGARS

By CECILIA LUTWAK-MANN¹

From the Biochemical Laboratory, Cambridge

(Received 31 May 1941)

CONTINUING the study of the biochemical properties of cartilage and of other tissues which make up the joint [Lutwak-Mann, 1940, 1], experiments were carried out with chondroitin and mucoitin sulphuric acids, both of which occur in joint tissues (cartilage, tendons, mucous membranes). However, preliminary experiments have shown that comparatively little progress could be made in this field unless more information was available about the components of these complex substances and in particular, about the amino-sugars. In this paper results are given which describe the behaviour of amino-sugars under the influence of enzymes in animal tissues, yeast and bacteria. A preliminary account was given to the Biochemical Society [Lutwak-Mann, 1940, 2].

Materials

The following substances were used as substrates: glucosamine HCl, *N*-acetylglucosamine made according to White [1940], chondrosamine (galactosamine) HCl obtained by acid hydrolysis of chondroitin sulphuric acid, glucosaminic acid prepared from glucosamine by oxidation with HgO [Pringsheim & Ruschmann, 1915], chondroitin sulphuric acid from cartilage and from tendons made according to the method of either Levene & La Forge [1913] or Meyer & Smyth [1937].

For most experiments the animal tissues were used in the form of tissue slices but in some instances the tissues were minced and reduced to a pulp. There were also a few experiments where amino-sugars were fed or injected into animals (rats).

Yeast and bacteria were used as washed suspensions and only occasionally in the form of cell-free extracts.

Methods

Several methods were used to study the changes which amino-sugars undergo in the presence of various tissues and cells. Changes in the gas uptake or output were measured manometrically, using Barcroft differential manometers with side bulbs and gas outlets. In addition, the rate of decomposition of amino-sugars was estimated colorimetrically by the method of Elson & Morgan [1933]. The reducing sugar values were determined by the method of Hagedorn & Jensen [1923]. In all experiments the deamination of the amino-sugars was also studied and the $\text{NH}_3\text{-N}$ was determined using the Parnas & Heller apparatus [1924]. The content of amino-N was occasionally estimated in the Van Slyke apparatus. It may be stated here that glucosamine HCl, chondrosamine HCl and *N*-acetylglucosamine were tested and found to yield no NH_3 in the presence of alkalis like borax, MgO, or NaHCO_3 , which were used for the distillation of NH_3 .

For the most part, rat and rabbit tissues were used, but some experiments were done with tissues of other animals (pig, horse, cat, guinea-pig).

¹ Fellow of Newnham College, Cambridge.

EXPERIMENTAL

A. *Behaviour of amino-sugars under the influence of animal tissues.* Increases in O_2 uptake and formation of NH_3 were invariably observed when amino-sugars were added to tissue slices from the following organs: kidney (cortex), testis, brain (cortex). Ovaries, retina and lung also showed certain rather small effects. On the other hand, negative results were obtained with liver, muscle (skeletal and heart), mucous membranes (intestine, joint), cartilage, spleen, red cells, plasma and synovial fluid. In Table 1 experiments are given which show the activities of a number of tissues towards glucosamine HCl, chondrosamine HCl and *N*-acetylglucosamine.

Table 1. *Oxidation and deamination of amino-sugars by animal tissues*

120 mg. wet wt. tissue slices. Ringer-phosphate pH 7.6; 10 mg. substrate. Barcroft manometers filled with O_2 ; KOH in inner tube.

Tissue	Exp.	Time hr.	Glucosamine HCl		Chondrosamine HCl		<i>N</i> -acetyl- glucosamine	
			μ l. O_2 *	μ g. NH_3 -N	μ l. O_2	μ g. NH_3 -N	μ l. O_2	μ g. NH_3 -N
			O_2 *	NH_3 -N	O_2	NH_3 -N	O_2	NH_3 -N
Testis (rat)	1	$\frac{1}{2}$	72	22	60	10	80	8
	2	1	190	38	88	19	110	12
	3	1	150	32	100	22	170	20
		2	240	48	170	30	208	28
	4	4	—	128	—	42	—	48
Kidney cortex (rabbit)	1	$\frac{1}{2}$	40	16	30	3	36	Trace
	2	1	160	28	80	12	120	12
		3	240	68	160	28	220	28
		4	410	140	270	48	—	46
Brain (rat)	1	1	68	20	42	13	18	Trace
		2	108	30	96	26	42	12
Brain (rabbit)	2	4	—	78	—	—	—	—
Retina (pig)		2	190	28	78	16	190	4
Ovaries (cat)		2	—	32	—	—	—	16
Lung (rabbit)		2	—	29	—	12	—	12

* Values for O_2 -uptake in this and all other tables refer to the 'extra'- O_2 uptake due to the substrate.

It can be seen from Table 1 that there are differences in the behaviour of the various tissues towards the three amino-sugars. In kidney and in testis there is comparatively little difference in the O_2 uptakes due to glucosamine HCl and *N*-acetylglucosamine. However, the deamination of the latter compound is slower than that of glucosamine. The enzyme system present in brain cortex shows little activity towards *N*-acetylglucosamine. It may be mentioned here that when experiments were carried out with mixtures of glucosamine and acetylglucosamine, the rates of deamination of both substances remained unaltered. The decomposition of chondrosamine HCl in all tissues examined is less rapid than that of glucosamine.

Before describing in detail the properties of the enzyme system involved in the breakdown of amino-sugars, it should be stated that in animal tissues their deamination was found to occur only aerobically. In the absence of O_2 the amino-sugars appeared to be stable in the presence of animal tissues; yeast and bacteria, on the other hand, were found to be active anaerobically as well (*v. inf.*). A distinct fall in pH due to acid production accompanied the O_2 uptake; no free CO_2 appeared to be formed in the course of the reaction.

Optimum pH. The breakdown of amino-sugars depends greatly on the pH which affects the oxidation and the deamination equally, so that it was impossible to separate the two processes by altering the acidity. The optimum pH was found to lie between 7.4 and 7.8. At pH 6.5 the reaction was very slow and below pH 6.0 it did not occur. Besides the phosphate buffer, pH 7.6, which was used throughout most of this work, borate, veronal and bicarbonate + CO₂ buffers were also tried. In all these buffers the reaction was slightly slower than in phosphate. In the presence of bicarbonate alone, both the O₂ uptake and the NH₃ production, though very rapid at the beginning, fell off nearly completely in the course of 30 min.

Comparison between the rate of O₂ uptake, NH₃ production and glucosamine disappearance. The experiments with animal tissues indicated that the O₂ uptake preceded the NH₃ formation, and similar results were obtained with yeast and bacteria, as will be seen later.

The results in Table 1 show that considerable amounts of O₂ were taken up in the course of amino-sugar breakdown, before any appreciable quantities of NH₃ could be detected. More detailed information can be gathered from the data in Tables 2-4. The experiments in Table 2 show that glucosamine added to

Table 2. *Rate of glucosamine disappearance and NH₃ formation*

Tissue	Glucosamine HCl added	Aerobic incubation time hr.	mg. glucosamine HCl found		$\mu\text{g. NH}_3\text{-N}$ formed
			By determination of reducing sugar	By colorimetric method	
Testis (rat)	4.8 mg. ($\equiv 312 \mu\text{g. NH}_3\text{-N}$)	2½	2.5	3.0	62
		3½	2.0	1.7	85
Testis (rat)	3.0 mg. ($\equiv 195 \mu\text{g. NH}_3\text{-N}$)	2	1.7	—	70
		3	1.38	—	85
Kidney (rabbit)	4.14 mg. ($\equiv 269 \mu\text{g. NH}_3\text{-N}$)	1½	—	2.5	55
		3	—	1.8	100
Kidney (rabbit)	3.8 mg. ($\equiv 247 \mu\text{g. NH}_3\text{-N}$)	¾	2.4	—	16
		1½	1.4	—	56
		3½	1.1	—	88
Kidney (rabbit)	4.0 mg. ($\equiv 260 \mu\text{g. NH}_3\text{-N}$)	½	—	3.1	24
		2	—	1.9	70
		3½	—	1.42	104

tissue slices under aerobic conditions disappears fairly rapidly during the first 60-90 min., as shown both by the colorimetric and reduction values. The NH₃ liberation is very slow during this initial phase. In the later stages of the reaction the rate of amino-sugar disappearance falls considerably while the NH₃ production increases. However, even after prolonged periods of incubation, when more than ¾ of the substrate added has disappeared, the NH₃ values correspond only to about ½-¾ of the amino-sugar used up during this period. In Table 3, in addition to the amounts of glucosamine used up and the NH₃ liberated, data are also given for the amount of O₂ taken up, while Table 4 illustrates the effect of pH on the course of glucosamine decomposition.

These experiments suggested the possibility of an oxidation product being first formed from the amino-sugar and then undergoing deamination. The decrease in pH which, as was mentioned previously, accompanied the O₂ uptake made it probable that this intermediary oxidation product would be an acid. It was thought that glucosaminic acid might be the substance formed under

Table 3. *O₂ uptake, glucosamine disappearance and NH₃ production*

Tissue	Glucosamine HCl added	Aerobic incubation time hr.	O ₂ -uptake due to glucosamine μ l. O ₂	mg. glucosamine HCl found	μ g. NH ₃ -N formed
Testis (rat)	2 mg. ($\equiv$ 130 μ g. NH ₃ -N)	1½	140	0.78	40
Brain (rabbit)	2 mg. ($\equiv$ 130 μ g. NH ₃ -N)	1½	180	0.67	28

 Table 4. *Effect of pH on decomposition of glucosamine*

Testis (rat). Added 5 mg. glucosamine HCl ($\equiv$ 358 μ g. NH₃-N). Aerobic incubation.

Time (hr.)	pH	mg. glucosamine-HCl found		μ g. NH ₃ -N formed
		By determination of reducing sugar	By colorimetric glucosamine estimation	
1½	7.6	3.7	3.3	55
	6.6	4.1	3.7	29
3½	7.6	2.5	2.2	130
	6.6	3.5	3.3	46

these circumstances. To test this possibility glucosaminic acid was prepared from glucosamine, but when tested with animal tissues it was found to be completely stable, both with regard to NH₃ formation and to O₂-uptake. The addition of glucosaminic acid to any of the amino-sugars tested in no way interfered with their usual course of breakdown. These results seemed to disprove the assumption that glucosaminic acid was the product responsible for the NH₃ formation from amino-sugars; other experiments which will be referred to below make its formation unlikely in the course of amino-sugar decomposition by the enzyme system described in this paper.

Effect of various substances on the oxidation and deamination of amino-sugars.

It was stated before that, except in brain cortex, there is little difference in the rate of O₂-uptake due to glucosamine and *N*-acetylglucosamine, but that the deamination of the acetylated compound is generally very slow. It was thought therefore that this difference in the rates of deamination of the free and the acetylated glucosamine, could be utilized to prove the enzymic conversion of glucosamine into an acetyl (or other) derivative.

With this possibility in mind, the rate of NH₃ formation from glucosamine was examined in the presence of several substances which were considered likely to yield derivatives able to block the NH₂ group. Table 5 shows the effect of several organic acids, aldehydes, alcohols and sugars on the deamination. It was found that some of the substances used decreased not only the deamination of the amino-sugar but also the 'blank' NH₃ formation, which means that they have an inhibitory effect upon deaminases generally.

Among the acids tested, oxaloacetic acid showed a fairly strong inhibition of the NH₃ production from glucosamine, especially if used in high concentration. On the other hand, acetic, lactic and pyruvic acids caused not more than c. 10% decrease. Butyric and β -hydroxybutyric acids showed a somewhat more marked effect, while oxalic acid had no influence.

The addition of acetaldehyde and methylglyoxal depressed the deamination to a moderate degree, but with *dl*-glyceraldehyde a much more pronounced effect was obtained, particularly if kidney cortex slices were used as the source of enzyme (Table 6).

Table 5. *Effects of various substances on the deamination of glucosamine*

Tissue	Exp.	Time hr.	$\mu\text{g. NH}_3\text{-N}$ formed			
			No additions		With additions	
			Tissue alone	Tissue + glucosamine	Tissue alone	Tissue + glucosamine
Kidney	1	2	28	80	22	Acetate <i>M</i> /10 70
	2	2½	28	100	28	Butyrate <i>M</i> /20 60
		2½	28	100	28	β -Hydroxybutyrate <i>M</i> /20 85
	3	3	36	130	35	Oxaloacetate <i>M</i> /40 62
		3	36	130	28	Oxaloacetate <i>M</i> /15 32
		3	36	130	32	Oxalate <i>M</i> /20 110
Testis	1	1	15	65	13	Pyruvate <i>M</i> /20 60
		1	15	65	12	Lactate <i>M</i> /20 52
Brain	1	2	30	56	40	Formate <i>M</i> /20 60
		2	30	56	30	Citrate <i>M</i> /20 48
Kidney	1	2	26	90	20	Acetaldehyde <i>M</i> /20 78
		2	26	90	18	Methylglyoxal <i>M</i> /30 50
		2	26	90	15	Glyceraldehyde <i>M</i> /20 30
Kidney	1	3	38	122	20	Glycerol <i>M</i> /40 77
	2	2	22	90	22	Menthol <i>M</i> /50 78
		2	22	90	26	Borneol <i>M</i> /50 90
Testis	1	3	30	112	20	Glucose <i>M</i> /20 42
	2	3	37	96	8	Glucose <i>M</i> /5 20
Kidney	1	1½	22	60	18	Glucose <i>M</i> /5 30
	2	3	32	125	20	Glucose <i>M</i> /20 56
		3	32	125	26	Cori ester <i>M</i> /10 70
		3	32	125	27	Fructose <i>M</i> /10 73
		3	32	125	36	Galactose <i>M</i> /10 120
Brain		3	32	125	36	Sucrose <i>M</i> /8 140
	1	2	15	49	4	Glucose <i>M</i> /8 22
	2	2½	40	80	41	Glucuronate <i>M</i> /8 76
		2½	40	80	44	Glucurone <i>M</i> /10 78

Table 6. *Effect of glyceraldehyde on glucosamine deamination in various tissues*

Tissue	Exp.	Time hr.	$\mu\text{g. NH}_3\text{-N}$ formed			
			No additions		With glyceraldehyde	
			Tissue alone	Tissue + glucosamine	Tissue alone	Tissue + glucosamine
Kidney	1	2	22	84	13	Glyceraldehyde <i>M</i> /50 37
	2	1½	20	66	18	Glyceraldehyde <i>M</i> /100 30
			20	66	16	Glyceraldehyde <i>M</i> /30 22
Testis	1	2½	36	85	27	Glyceraldehyde <i>M</i> /50 48
	2	1½	15	47	10	Glyceraldehyde <i>M</i> /60 31
Brain	1	1½	26	40	23	Glyceraldehyde <i>M</i> /50 32

When various sugars were examined for their ability to influence the deamination of glucosamine, it was found that glucose alone among several sugars tested strongly interfered with the amino-sugar deaminase. It was possible, by using higher concentrations of glucose, to prevent the deamination of glucosamine nearly completely (Tables 5 and 7). Except for glucose-1-phosphoric acid (Cori ester), none of the other sugars could be compared with glucose in its effect upon the deaminating enzyme.

It seemed desirable to investigate more closely the behaviour of amino-sugars in the presence of glyceraldehyde and glucose, not only in connexion with their

effects upon the deamination but also with regard to the O_2 -uptake. The colorimetric estimation of glucosamine disappearance was, however, impossible in the presence of glyceraldehyde, owing to the fact that the presence of this aldehyde prevented the formation of colour in the method of Elson & Morgan. Similarly, it was impracticable to attempt to assess the reducing sugar values for the amino-sugar in the presence of glucose; in the colorimetric method glucose caused no interference.

It can be seen from Table 7 that glucose had a more pronounced effect on glucosamine deamination than glyceraldehyde but that glyceraldehyde inhibited the O_2 -uptake more than glucose. The same table contains also data obtained with lactate and pyruvate, which had little effect upon either the oxidation or the deamination of glucosamine.

Table 7. *Effects of glucose, glyceraldehyde, lactate and pyruvate on the O_2 uptake and NH_3 formation*

Tissue	Time		$\mu l.$ O_2 taken up	$\mu g.$ NH_3 -N formed
Testis	2 hr. 30 min.	No addition	290	78
		Glucose $M/15$	205	29
Kidney	1 hr. 30 min.	No addition	172	48
		Glucose $M/12$	119	12
Testis	1 hr. 30 min.	No addition	210	42
		Glyceraldehyde $M/60$	38	21
Testis	50 min.	No addition	120	30
		Lactate $M/20$	90	28
		Pyruvate $M/20$	148	33

In some of these experiments the content of amino-N was estimated in the samples at the beginning and end of the experiment, both without and with the addition of substances like glyceraldehyde, glucose, oxaloacetate, lactate and pyruvate. But the results obtained completely failed to produce any evidence that the glucosamine amino-group was blocked, as might have been expected if acetylation or a similar process had taken place during the incubation of glucosamine with these substances.

To ascertain whether the effect of glucose on the glucosamine deamination was due to glucose itself or to its breakdown products, experiments were carried out in which the tissue slices were incubated for a time with glucose, either in air or anaerobically, before the amino-sugar was added. From the data in Table 8 it appears that the presence of glucose oxidation products had practically the same effect on the deamination of glucosamine as glucose alone; there was, however, a small increase in the amount of O_2 used up. When the tissue was kept with glucose in N_2 , before the addition of glucosamine, the results did not differ

Table 8. *Effect of glucose and of its products on glucosamine oxidation and deamination*

Tissue slices (testis) incubated for 1 hr. with glucose in O_2 (c) or in N_2 (d), before addition of glucosamine.

		$\mu l.$ $O_2/1$ hr.		$\mu g.$ NH_3 -N/1 hr.	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
(a)	Glucosamine	156	160	52	61
(b)	+ glucose (no incub.)	100	118	20	13
(c)	+ glucose (O_2 incub.)	180	195	18	10
(d)	+ glucose (N_2 incub.)	94	100	20	4

from those obtained when glucose and glucosamine were added simultaneously to the tissue slices.

Interesting results were obtained when glucose and galactose were compared in their effects on the oxidation and deamination of chondrosamine. The experiment in Table 9 shows that while galactose had comparatively little effect on the deamination of this amino-sugar, it interfered more with its oxidation than glucose.

Table 9. *Effects of glucose and galactose on chondrosamine breakdown*

Tissue		$\mu\text{l. O}_2$ taken up in 90 min.	$\mu\text{g. NH}_3\text{-N}$ formed in 90 min.
Kidney	Chondrosamine	280	52
	+ glucose <i>M</i> /10	210	20
	+ galactose <i>M</i> /10	170	40

Poisons and inhibitors. With the intention of obtaining some indication as to the type of reaction involved in the enzymic decomposition of amino-sugars, several substances were tested with regard to their effects on the enzyme system and the results are collected in Table 10. The experiments in this table were carried out using kidney cortex slices, but analogous results were obtained with other active tissues.

Table 10. *Effects of poisons and inhibitors on glucosamine oxidation and deamination*

	$\mu\text{l. O}_2$ taken up/100 min.	$\mu\text{g. NH}_3\text{-N}$ formed/100 min.
Without additions	230	70
With		
Iodoacetate <i>M</i> /100	0	0
Fluoride <i>M</i> /50	156	44
Arsenate <i>M</i> /75	130	33
Cyanide <i>M</i> /250	56	52
Cyanide <i>M</i> /75	32	29
Azide <i>M</i> /75	190	58
Malonate <i>M</i> /100	200	67
Cinchophen <i>M</i> /10	0	0
Salicylate <i>M</i> /50	180	43
Toluene	0	0
Sulphapyridine	248	75
Merthiolate 1:20000	0	0

It was interesting to discover that the reaction under investigation is extremely sensitive to iodoacetic acid (IA). Both oxidation and deamination of amino-sugars were abolished by this poison. Smaller effects were obtained with NaF and arsenate, while malonate and azide caused no inhibition at all. KCN largely prevented the oxidation, but had comparatively little effect on the NH_3 formation. Salicylic acid was found to be a mild inhibitor, but cinchophen (phenyl cinchoninic acid) suppressed completely the activity of the amino-sugar enzyme system. Organic solvents like alcohol, acetone, ether and toluene destroyed the enzyme, even in low concentrations. It was also inactivated in the presence of merthiolate. On the other hand, sulphanilamide and sulphapyridine had no influence on the reaction. On several occasions the tissues used for the experiments with amino-sugars, were removed from animals which had received sub-lethal doses of either sulphapyridine or sulphanilamide; yet the enzyme system responsible for the decomposition of amino-sugars remained entirely unaffected.

The effect of semicarbazide and dimedone upon the rate of glucosamine breakdown was also tested; no significant changes, however, were observed, as might have been the case if ketones or aldehydes had been produced in the course of the reaction. The lack of effect with these 'trapping' substances agreed well with the negative results obtained for bisulphite-binding substances (*vide infra*).

Tissues other than kidney, testis and brain. It was mentioned previously that a slight O_2 uptake and deamination of amino-sugars could be observed with ovaries, retina and lung tissue. Among other animal tissues and fluids which were tested in various ways and found to contain no enzymes capable of either the oxidation or deamination of amino-sugars, were liver, muscle, intestine, cartilage, blood (whole and haemolysed), serum and synovial fluid.

Particular attention was paid to liver as this tissue is well known to possess enzymes which might be related to the system dealing with amino-sugars, such as glucose oxidase and amino-acid oxidase. However, in all experiments with liver tissue, used either in the form of slices or pulp, neither oxidation nor deamination of amino-sugars took place. The absence of deaminase activity was not due to substitution of the amino-group, because it was found after prolonged incubation of glucosamine with liver that the amino-group remained free even when such substances as acetate, lactate, pyruvate etc., were added.

It may be of some interest to mention here that the addition of amino-sugars greatly interfered with the oxidation of glucose by liver slices (Table 11). A similar effect of amino-sugars upon glucose oxidation was also observed in testis slices; in this instance there was, of course, a mutual interference of glucose and amino-sugar as described on p. 615.

Table 11. *Effect of amino-sugars on glucose oxidation in liver*

	$\mu l. O_2$ taken up/70 min.
Glucose	205
+ glucosamine	100
+ chondrosamine	130
+ <i>N</i> -acetylglucosamine	84
Glucosamine	0
Chondrosamine	0

It appeared fairly probable from the results obtained with liver that this organ is devoid of enzymes acting on amino-sugars. It seemed, however, worth while to investigate whether the apparent stability of the amino-sugars in liver was not due to the presence of substances capable of suppressing their decomposition.

Liver slices were incubated aerobically for 1–2 hr. in Ringer-phosphate; the tissue was then removed and the liquid was heated for 5 min. to 80° . When this heat-inactivated fluid was added to kidney cortex slices, it considerably retarded the deamination of glucosamine. Similar effects were obtained by adding the extract from liver to brain or testis slices. If, however, the liver slices were incubated anaerobically instead of aerobically, the effect of the heat-inactivated extract was much less conspicuous. Further experiments of this kind showed that the liver factor responsible for the suppression of amino-sugar deamination, is dialysable and heat-stable and also that it is not formed aerobically at pH below 6.0 (Table 12). On the other hand, its formation in the liver was not prevented by KCN, phloridzin or semicarbazide. Heat-inactivated extracts from kidney or testis slices, or boiled yeast juice, failed to produce any marked effect on the NH_3 production from amino-sugars.

Table 12. *Effect of heat-inactivated liver extract on the deamination of glucosamine by kidney cortex*

		$\mu\text{g. NH}_3\text{-N produced/1 hr. 30 min.}$
Glucosamine		68
	+ liver extract, aerob., pH = 7.4	22
	+ liver extract, dialysed	50
	+ liver extract, aerob., pH = 4.8	60
	+ liver extract, anaerob., pH = 7.4	42

In experiments with muscle or mucous membranes, it was necessary to mince the tissues and to use them in the form of a pulp. As stated previously, negative results were obtained with these tissues, except for some very slight anaerobic oxidation of glucosamine by washed heart muscle mince in presence of cozymase and methylene blue (Table 15). In this connexion it should be stated that when active kidney cortex or testis slices were broken up by grinding in Ringer-phosphate and made into a thick pulp, their activity towards amino-sugars was considerably reduced (Table 13). It would seem therefore that the enzyme system dealing with amino-sugars depends largely upon the tissue structure being preserved intact. Completely negative results were obtained with a kidney cortex acetone powder preparation, although this preparation was very active towards *D*-alanine. Also a preparation of testicular diffusing factor (obtained through the kindness of Dr Bacharach) appeared devoid of any power to oxidize or to deaminate amino-sugars.

Table 13. *Decomposition of amino-sugars by tissue slices and by pulp*

Tissue	Preparation	Substrate	$\mu\text{l. O}_2/2 \text{ hr.}$	$\mu\text{g. NH}_3\text{-N/2 hr.}$
Kidney cortex	Slices	Glucosamine	260	79
		<i>N</i> -acetylglucosamine	190	21
	Pulp	Glucosamine	56	38
		<i>N</i> -acetylglucosamine	Not measured	Trace
Testis	Slices	Glucosamine	190	88
		<i>N</i> -acetylglucosamine	142	34
	Pulp	Glucosamine	Very small	52
		<i>N</i> -acetylglucosamine	Very small	18

Attempts to identify the reaction products. The distinct fall in pH accompanying the breakdown of amino-sugars, made probable the formation of an acid in the course of the reaction. Estimations of lactic and acetic acids were negative. The presence of succinic acid was made improbable by experimental results described below.

Estimations of bisulphite-binding substances [Clift & Cook, 1932] gave negative results. No substances were formed which would yield derivatives with 2:4-dinitrophenylhydrazine.

A special series of experiments was carried out to investigate more thoroughly the formation of glucosaminic acid. It was mentioned already that glucosaminic acid was found to be remarkably stable in the presence of animal tissues and therefore it could hardly be regarded as the (hypothetical) oxidation product from glucosamine which undergoes deamination. Nevertheless, it seemed important to examine whether glucosaminic (or chondrosaminic) acid was formed from the amino-sugars under the experimental conditions described in this paper.

This was done in the following manner. It was found that glucosaminic acid yields CO_2 readily in the presence of chloramine-T at pH 4.6, in quantities roughly proportional to the amount of the acid, while glucosamine causes only a very slow gas production under similar conditions. The CO_2 evolution can easily be measured manometrically (Table 14).

Table 14. *Behaviour of amino-sugars and glucosaminic acid in the presence of chloramine-T*

	$\mu\text{l. CO}_2/20 \text{ min., } 38^\circ, \text{pH}=4.6$
Glucosamine HCl, 2 mg.	15
Chondrosamine HCl, 2 mg.	12
Glucosaminic acid, 2 mg.	120
Glucosaminic acid, 4 mg.	195

The technique adopted for the estimation of glucosaminic acid largely followed the method described by Cohen [1940]. It was found necessary to remove NH_3 formed from glucosamine in the course of the enzymic reaction, in order to avoid N_2 formation by the chloramine-T treatment. This was conveniently accomplished by distillation in the Parnas-Heller apparatus.

Tissue slices (250 mg. wet wt., total volume of solution 3 ml.) were incubated with a known quantity of glucosamine in the usual manner. O_2 uptake and NH_3 formation were estimated and at various intervals the reaction was stopped by heating the sample to 90° for 3 min. After the removal of NH_3 and the precipitation of proteins by tungstic acid, the filtrate was condensed at 100° to half its original volume. The amount of fluid taken for the final chloramine-T experiment was chosen so as to contain between 2 and 4 mg. of glucosaminic acid. Numerous controls were carried out to ascertain that glucosaminic acid added to the tissue under the conditions referred to could be recovered without loss in spite of the prolonged preparatory manipulations.

However, from a series of experiments carried out with glucosamine and chondrosamine HCl, both alone and in the presence of glucose, it appeared certain that glucosaminic acid was not formed under these conditions, at least in any appreciable quantities. Similar negative results were also obtained using *B. coli* instead of the animal tissues.

The following experiments were carried out in yet another attempt to identify the reaction products formed during the breakdown of amino-sugars. Minced and thoroughly washed muscle pulp was prepared, such as is known to contain several dehydrogenases. Using this enzyme preparation, the effect was studied on the methylene blue reduction time of the addition of a heat-inactivated extract from tissue slices which had been allowed to act for a considerable time on amino-sugars. Table 15 gives the results of some of these Thunberg experiments. It can be seen that (a) glucosamine itself is very slowly oxidized by heart muscle enzymes in the presence of cozymase, and (b) in the tissue slices extract containing the reaction products from amino-sugars, there is a substance which in the presence of cozymase definitely accelerates the reduction of methylene blue. There are two substrates which do not require cozymase for their anaerobic oxidation with methylene blue by muscle enzymes: succinic and α -glycerophosphoric acids. These two can therefore be excluded as possible intermediary or end-products of amino-sugar breakdown. The formation of α -glycerophosphate was already unlikely, because separate experiments showed no evidence of phosphorylation being involved in the amino-sugar decomposition.

Table 15. *Effect of muscle dehydrogenases on the reaction products of amino-sugar breakdown*0.3 g. muscle dehydrogenase preparation, 0.6 mg. cozymase, 1 ml. *M*/2000 M.B., pH=7.4.

	Reduction time, min.
Heart muscle prep.	∞
+ cozymase	95
+ glucosamine	∞
+ cozymase + glucosamine	28
+ cozymase + chondrosamine	70
+ extract from tissue slices	80
+ cozymase + extract from slices	45
+ extract from slices incubated with glucosamine	55
+ cozymase + extract from slices incubated with glucosamine	12
Skeletal muscle prep. + cozymase	50
+ cozymase + <i>N</i> -acetylglucosamine	40
+ cozymase + extract from slices	38
+ cozymase + extract from slices incubated with <i>N</i> -acetylglucosamine	14

Experiments with chondroitin sulphuric acid. Comparatively few experiments were carried out using this compound as substrate for tissue or bacterial enzymes; in fact, their variability was the main cause why they were abandoned for the time being and the research on amino-sugars was taken up instead.

Chondroitin Na-sulphate was added to tissue slices (kidney, testis) and incubated for 2, 4 and 6 hr. at 37°, at pH values varying between 6.0 and 8.0. Afterwards the supernatant fluid was examined for the presence of amino-sugars. Traces of amino-sugar could usually be detected but the amount varied considerably and it appeared probable that this was due to a further decomposition of the amino-sugar set free from chondroitin sulphuric acid by the tissue enzymes. This assumption was confirmed by the results of the work reported in this paper.

Attempts were also made to assess the rate of amino-sugar liberation during acid hydrolysis of chondroitin sulphuric acid. Use was made of the enzymes present in tissue slices to indicate the presence of amino-sugar. For this purpose, samples of chondroitin sulphuric acid were hydrolysed in sealed tubes at 100° in *N* HCl for 1, 2, 4 and 12 hr. They were carefully neutralized and freed from NH₃. They were then added to testis or kidney cortex slices and incubated aerobically in the usual manner. Production of NH₃ indicated the presence of amino-sugar, as chondroitin sulphuric acid itself gave rise to no NH₃ formation. In this way it was found that after 60 to 90 min. of acid hydrolysis chondroitin sulphuric acid yields a considerable amount of amino-sugar in a form in which it can be attacked by the tissue enzymes. An inconvenient complication of these experiments was caused by the rather high NaCl concentration of the hydrolysed and neutralized samples. It was ascertained in separate experiments that *N* NaCl or KCl completely inactivates the system acting on amino-sugars. It was therefore necessary to dilute the hydrolysed samples very considerably to diminish the salt concentration and thus only comparatively small fractions of the amino-sugar present were used for the final enzymic experiment.

Experiments with animals. One of the main obstacles, so far, to the study of amino-sugar metabolism is due to the difficulty of quantitative estimation of amino-sugar in urine. It appears probable that urea is the substance which interferes with the colorimetric estimation of amino-sugar in urine, as it was

found that small amounts of urea give the same orange (instead of pink) colour with the Ehrlich reagent as is obtained with dilute urine.

As no successful and simple way could be found to apply the Elson & Morgan method to estimations in urine, it was decided to study instead the effect of amino-sugars on the $\text{NH}_3\text{-N}$ content of the urine in animals which were known to possess organs able to deaminate amino-sugars. Glucosamine HCl (1 mg./g. body weight) in Ringer was injected subcutaneously into rats (180 g.) while Ringer alone was injected into control animals. The following method of urine collection was adopted. The food was removed early on the day of the experiment and urine was collected for some 10 hr. Then followed the injection and a further collection was made for 10–12 hr. Food was then restored and urine was collected for a further 4–6 hr. In all samples the total volume, pH and the $\text{NH}_3\text{-N}$ were then estimated. Table 16 gives the results of some of these experiments. It can be seen that there were considerable variations both in the volume of urine excreted in a given time and in the $\text{NH}_3\text{-N}$ content of the urine samples, even before the injection. In the samples following the injection there was a slight increase in the $\text{NH}_3\text{-N}$ content of the urine in the animals which received glucosamine HCl, but this was very small indeed if compared with the $\text{NH}_3\text{-N}$ content of the amino-sugar injected. There were no significant changes in the pH of the urine samples after the injection of the amino-sugar. It would seem that, at least in rats, the amino-sugars were prevented from complete deamination, although *in vitro*, in the presence of rat tissues like kidney or testis, they would have lost their amino-group in the course of prolonged incubation.

Table 16. *Effect of subcutaneous injection of glucosamine on the NH_3 content of urine in rat*

Urine sample	Volume (ml.)			pH			mg. $\text{NH}_3\text{-N}$ in total urine		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Fasting animals:									
7 hr. before injection of saline	3	15	1	6	6.9	5.9	6.00	4.95	1.40
7 hr. before injection of glucosamine	7	18	1.3	5.9	6.6	6.0	6.16	9.00	0.80
Fasting animals:									
12 hr. after injection of saline	2.6	8	3	6	6.6	5.8	2.47	2.80	4.20
12 hr. after injection of glucosamine	3.4	6	9	5.9	6.5	6.2	3.40	6.00	7.20
Fed animals, 10 hr. after above collection:									
Saline-injected animals	11	8.5	—	7.3	7.5	—	2.53	3.65	—
Glucosamine-injected animals	16.5	6.5	—	7.0	6.9	—	2.55	3.30	—

It seems worth while to report briefly certain other experiments, which although interesting cannot at present be extended owing to the difficulties in obtaining large quantities of dried egg white. Male albino rats were kept on the 'egg white injury'-producing diet [György, 1939]. After a period of about 120 days they developed the typical skin and spectacled eye condition characteristic of egg white injury. Some of these animals were given 20 mg. of glucosamine HCl per day for a week, during which period they began to show a definite improvement such as growth of hair on the most affected skin areas as well as a distinct clearing up of the eye exudates. Their weight was maintained at a constant level. Chondrosamine HCl tested under similar conditions failed to produce any effect at all. However, after some 8 days of improvement due to glucosamine its effect faded and a relapse followed, which was not relieved by further doses of glucosamine HCl.

In a different series of experiments the effect of glucosamine feeding was investigated on B₂-deficient rats. Here however, no visible results of any kind were obtained. The tissues of both egg white-fed and B₂-deficient rats possessed the enzyme system concerned with the breakdown of amino-sugars.

B. *Yeast enzymes.* Several experiments were carried out using suspensions of baker's or brewer's yeast as the source of enzymes acting on amino-sugars. An enzyme system was found to exist in yeast which very slowly deaminated glucosamine and chondrosamine HCl in aerobic as well as in anaerobic conditions. The enzymes showed very little activity towards *N*-acetylglucosamine (Table 17). No free CO₂ appeared to be formed either in air or in N₂. As in animal tissues, the rate of oxidation exceeded the NH₃ formation; there was also the formation of acid reaction products which have not yet been identified.

Table 17. *Decomposition of amino-sugars by baker's yeast*

12 mg. yeast (dry wt.), 10 mg. substrate, pH=7.6.

	Oxygen uptake μl. O ₂ /1 hr.	Aerobic NH ₃ formation μg. NH ₃ -N/2½ hr.	Anaerobic NH ₃ formation μg. NH ₃ -N/2½ hr.
Glucosamine-HCl	120	60	28
Chondrosamine-HCl	72	34	11
<i>N</i> -acetylglucosamine	50	28	8
Glucose	1340	—	—

The yeast enzymes showed the same behaviour towards poisons, e.g. iodoacetate and KCN, as described previously in connexion with animal tissues. The specificity of the glucosamine deaminase in yeast was proved by the fact that while it was completely inhibited in the presence of iodoacetate, this poison did not affect the deamination by yeast of other substrates such as adenosine or adenylic acid.

Cell-free extracts from fresh baker's yeast, as well as Lebedew maceration juice from dried brewer's yeast, were found to be very weak in activity towards amino-sugars.

C. *Bacterial enzymes.* For most experiments *B. coli* was used in washed suspensions, but a few experiments were also done using suspensions of *Str. faecalis* and *Proteus vulgaris*.

B. coli was found to contain a powerful enzyme system which at pH 6.8–7.8 rapidly oxidized and deaminated glucosamine HCl and, a little more slowly, chondrosamine HCl and *N*-acetylglucosamine (Table 18).

Table 18. *Decomposition of amino-sugars by bacteria*

2 mg. suspension (dry wt.), 10 mg. substrate, pH=7.6.

	Glucosamine HCl	Chondrosamine HCl	<i>N</i> -acetyl- glucosamine
<i>B. coli</i> :			
μl. O ₂ /40 min.	180	110	130
μg. NH ₃ -N/40 min.: aerob.	98	62	80
μg. NH ₃ -N/40 min.: anaerob.	40	28	38
<i>Str. faecalis</i> :			
μl. O ₂ /50 min.	90	45	—
μg. NH ₃ -N/50 min.: aerob.	160	130	—
μg. NH ₃ -N/50 min.: anaerob.	140	41	—
<i>Proteus vulgaris</i> :			
μl. O ₂ /60 min.	68	90	—
μg. NH ₃ -N/60 min.: aerob.	18	29	—

The oxidation of amino-sugars was accompanied by acid formation. Also in anaerobic conditions *B. coli* deaminated the amino-sugars and produced acid reaction products from them. There was no CO₂ evolution in the course of either of these reactions.

Except for the ability to decompose the amino-sugars anaerobically, the bacterial enzyme system behaved in all respects like the animal system, i.e. the same substances were found to act as inhibitors and poisons in bacteria as described above for kidney slices or testis (Table 19).

Table 19. *Effects of glyceraldehyde, glucose, cyanide and iodoacetate on the decomposition of glucosamine by B. coli*

	$\mu\text{l. O}_2/1 \text{ hr.}$	$\mu\text{g. NH}_3\text{-N}/1 \text{ hr.: aerob.}$
Glucosamine	290	160
+ glyceraldehyde <i>M</i> /60	85	100
+ glucose <i>M</i> /20	188	36
+ cyanide <i>M</i> /100	60	130
+ iodoacetate <i>M</i> /150	0	0

The sequence of the processes in *B. coli* which cause the breakdown of amino-sugars appeared to be similar to that demonstrated for the animal enzymes, that is to say oxidation preceded deamination. By adding glucose to the bacterial suspension it was possible to diminish considerably the NH₃ liberation without much affecting the O₂ uptake. On the other hand, the addition of glucose to the culture medium of *B. coli* did not influence the amino-sugar deaminase in any marked way.

B. coli was also found to be capable of rapidly oxidizing the amino-sugars under anaerobic conditions in the presence of methylene blue as hydrogen acceptor. Table 20 A illustrates the results of experiments carried out with the Thunberg technique. It shows the effects of various poisons on the anaerobic oxidation, which are analogous with those obtained in aerobic conditions. Treatment of the bacterial suspension with toluene completely destroyed the enzyme activity.

Further experiments of this kind confirmed the assertion previously made that considerable amounts of the amino-sugar are oxidized before any large-scale deamination sets in. In the B section of Table 20 the results show that in the course of 16 min. roughly 3 mg. of methylene blue were reduced (or c. 2 mg. glucosamine HCl oxidized), but that the amount of NH₃ produced during this

Table 20. *Anaerobic oxidation of glucosamine by B. coli*

1 mg. bacterial suspension (dry wt.); 1 ml. *M*/2000 M.B.; pH=7.4; total volume, 3 ml.

A.	Reduction time, min.
Blank	60
Glucosamine	4
Glucosamine, pH=6.0	28
Glucosamine + iodoacetate <i>M</i> /100	49
+ fluoride <i>M</i> /70	37
+ cyanide <i>M</i> /100	5
+ toluene	65

B. 4 mg. bacterial suspension (dry wt.) in 3 ml. total volume.

	Reduction time, min.	$\mu\text{g. NH}_3\text{-N found}$
Blank + 2 mg. M.B.	70	24
Glucosamine + 1 mg. M.B.	4	40
+ 2 mg. M.B.	9	49
+ 3 mg. M.B.	16	80

period (0.056 mg. $\text{NH}_3\text{-N}$) corresponded to not more than half the amount of the substrate oxidized.

Various attempts were made to isolate the reaction products of glucosamine breakdown in *B. coli*, on lines similar to those adopted for animal tissues. It was established that there was no lactic or glucosaminic acid formation. The latter was found to be completely stable in the presence of bacteria.

Suspensions of *Str. faecalis* were found to be active towards the amino-sugars, but their oxidizing enzymes were less strongly developed than those of *B. coli*, while their deaminase was rather powerful, both in O_2 and in N_2 .

Suspensions of *Proteus vulgaris* acted very slowly on glucosamine HCl both as regards O_2 uptake and deamination; they appeared, however, slightly more active towards chondrosamine HCl (Table 18).

DISCUSSION

From the experimental data presented above some information can be gathered about the enzyme system capable of amino-sugar decomposition. Its fairly wide distribution, as shown by the fact that it occurs in animal tissues, yeasts and bacteria, is of particular interest when one bears in mind that amino-sugars form an essential part of various complex polysaccharides which seem to play an important part in many physiological processes.

There is evidence from the experiments described that the enzyme system concerned in the decomposition of amino-sugars is strictly specific for these substances. Its non-identity with the glucose oxidase was easily proved by the results obtained with liver. This organ was incapable of attacking the amino-sugars although it rapidly oxidizes glucose. Moreover, amino-sugars were found to interfere with the glucose oxidase; this phenomenon may perhaps be regarded as an instance of competitive inhibition.

Furthermore, there appears to be no relationship between the amino-sugar enzyme and the amino-acid oxidase; this can be simply shown using acetone kidney cortex preparations, which, though strongly active towards *D*-alanine, fail completely to attack the amino-sugars. It was also found that while iodoacetic acid completely stopped the breakdown of glucosamine in kidney cortex slices, it had comparatively little effect on the decomposition of *L*-alanine. The amino-sugar enzyme is also distinct from the tyramine oxidase, as shown by experiments with intestinal mucosa which oxidizes and deaminates the amine but does not act on amino-sugars.

By applying iodoacetic acid to yeast it was possible to show that the glucosamine deaminase is distinct from the enzyme which deaminates adenine derivatives.

The enzyme system concerned with the amino-sugars seems to be composed of two closely linked enzymes of which one is responsible for the oxidation and the other for the deamination of the substrate. The evidence for this view is based on the observation of the behaviour of the enzyme system in the presence of various substances. For instance, by the addition of glyceraldehyde or KCN, one can largely depress the oxidation without much affecting the deamination; on the other hand, glucose or oxaloacetate considerably diminish the amount of NH_3 produced, but have less effect on the O_2 uptake. The fact that the enzyme largely depends on the integrity of cell structure, makes it difficult to resolve this system into its components. As the experiments with cell-free extracts from tissues or from other sources were few, it was not possible to determine whether this enzyme required any coenzyme or what the nature of this coenzyme might be.

Generally speaking, of the three amino-sugars tested, glucosamine is oxidized and deaminated more rapidly than either chondrosamine or acetylglucosamine; this difference in the rates of breakdown exhibited by the enzyme preparations tested may be due to a greater affinity for glucosamine than for galactosamine. The presence of the acetyl group in *N*-acetylglucosamine proves an obstacle to the deaminase but interferes comparatively little with the oxidation. In spite of many attempts to bring about an enzymic acetylation of glucosamine, no positive results have been achieved so far. But the difference in the rates of deamination of glucosamine and acetylglucosamine may be very useful as an indication of the blocking of the NH_2 group.

The nature of the reaction products in addition to NH_3 formed from amino-sugars has yet to be discovered. So far, it seems certain that there is no lactic, acetic or succinic acid among the breakdown products; substances of aldehyde or ketone character are also absent. With regard to the mechanism of the enzymic decomposition it appears probable that the amino-sugars undergo first an oxidation and that it is this oxidation product which subsequently gives off NH_3 . The experimental results indicate that glucosaminic acid is not formed in the course of glucosamine oxidation and it can therefore be excluded as the reaction product which undergoes deamination. It was interesting to note the considerable stability of glucosaminic acid in the presence of enzymes from various sources.

From the experiments with the whole animal it follows that considerable amounts of amino-sugar can be injected without causing any marked change in the NH_3 -N excretion. It is quite likely that in the intact animal the amino-sugars undergo enzymic processes different from those occurring in isolated tissues. The remarkable effect of glucosamine feeding in egg white injury, although transient and probably unspecific, shows that much more study is necessary in the field of amino-sugar metabolism, especially with regard to the nutritional requirements of the organism for these substances. At present no data seem to be available as to whether the amino-sugars can be formed within the organism, or whether they must be added with other exogenous foodstuffs.

Yeast and bacteria act on the amino-sugars both aerobically and anaerobically. The yeast enzyme is rather weak but the enzyme in *B. coli* decomposes amino-sugars very rapidly. The effects of various inhibitors on these enzymes are the same as in animal tissues.

Much attention has recently been given to amino-sugars in connexion with work on the diffusing factor and mucinase, which hydrolyse certain polysaccharides and liberate from them amino-sugars. It is unlikely that the sensitive enzyme system described in this work could play any part or be present in the preparations of testicular diffusing factor, which apart from being stable in the presence of antiseptics like merthiolate, are known to exert their maximum activity at a low pH, at which the enzyme causing the oxidative deamination of amino-sugars would no longer be active. In connexion with this it may be interesting to report that bee venom ('Lyovac', Mulford Laboratories) was found to oxidize and deaminate both glucosamine and chondrosamine HCl.

SUMMARY

An enzyme system which decomposes glucosamine HCl, *N*-acetylglucosamine and chondrosamine HCl, was found to exist in animal tissues, yeast and bacteria.

In animal tissues the addition of amino-sugars was found to cause an increase in the O_2 uptake, accompanied by acid formation and production of NH_3 . No free CO_2 was formed in the course of the reaction. In yeast and bacteria, the

same processes occur but, in addition, the amino-sugars are attacked also in the absence of O_2 .

The enzyme examined is specific for amino-sugars; it bears no relation to either the glucose oxidase or the amino-acid oxidase or the amine oxidase. Its activity was found to depend greatly on the cell structure being preserved. The optimum pH was found to lie between 7.4 and 7.8.

From quantitative estimations of the amino-sugar disappearing and the O_2 uptake as well as the NH_3 produced, it appears that the amino-sugars are first converted by oxidation into some product, probably an acid, which in turn undergoes deamination. It was found that glucosaminic acid cannot be regarded as the oxidation product responsible for the NH_3 accumulation. Glucosaminic acid was found to be stable in the presence of animal tissues, yeast and bacteria.

Attempts were made to identify the reaction products. So far, it has been established that there is no formation of lactic, acetic or succinic acid, that substances of ketonic or aldehyde character are absent, and that the products formed from amino-sugars are oxidized by muscle dehydrogenases in the presence of cozymase.

Iodoacetic acid, cinchophen and antiseptics completely abolish the activity of the amino-sugar enzyme. KCN and glyceraldehyde inhibit the oxidation, but have little effect on the NH_3 liberation. Glucose, unlike other sugars, considerably depresses deamination but interferes comparatively slightly with oxidation.

Part of the expenses of this research were defrayed by a grant from the Empire Rheumatism Council which the author wishes gratefully to acknowledge. Mr S. W. Williamson and Miss R. Leader rendered valuable assistance.

REFERENCES

- Clift & Cook (1932). *Biochem. J.* **26**, 1788.
Cohen (1940). *J. biol. Chem.* **136**, 565.
Elson & Morgan (1933). *Biochem. J.* **27**, 1824.
György (1939). *J. biol. Chem.* **131**, 732.
Hagedorn & Jensen (1923). *Biochem. Z.* **135**, 46.
Levene & La Forge (1913). *J. biol. Chem.* **15**, 69.
Lutwak-Mann (1940, 1). *Biochem. J.* **34**, 517.
— (1940, 2). *Chem. Ind.* **59**, 348.
Meyer & Smyth (1937). *J. biol. Chem.* **119**, 507.
Parnas & Heller (1924). *Biochem. Z.* **152**,
Pringsheim & Ruschmann (1915). *Ber. dtsh. chem. Ges.* **48**, 680.
White (1940). *J. chem. Soc.* p. 428.