5. Biochemistry of a Cystitis Organism. 1 and 2

By Hugh Kirkman King, *From the Biochemical Laboratory, Cambridge*

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1. NUTRITION OF THE ORGANISM

The biochemistry of the organisms causing cystitis presents a problem in nutrition, since they maintain themselves indefinitely in anaerobic conditions utilizing as sources of both C and N some urinary constituent. The problem is more interesting as it is well known that anaerobic conditions severely restrict the range of C compounds capable of supporting growth.

The primary object of the present investigation was to ascertain which constituents of urine supported the anaerobic growth of the organism.

EXPERIMENTAL

The organism was isolated from the urine of a male case of cystitis. It grew very profusely in this urine, and also in normal male urine. It was identified as *Bact. coli** and the strain described as 'P2'. When cultured on broth it had the typical rod-shape, but on urine assumed a highly contracted form. Another *Bact. coli* ('stock') strain gave very much poorer growth on urine.

Culture. To avoid 'adaptive' changes, the organism was cultured anaerobically on urine. The urine was taken to pH 9 with NaOH, filtered through kieselguhr for partial removal of phosphates, restored to pH 7 with HCl and passed through a Seitz filter to sterilize. A urine-agar medium for plating out was prepared by adding 2% of agar to urine and autoclaving. Subculture was carried out at 2-day intervals.

GROWTH EXPERIMENTS

Various compounds, including some constituents of urine, were supplied to the organism as C-source to ascertain which could support growth. The substance under test was added to the basal medium (Stephenson [1939], Medium VII, p. 318; N supplied as NH_4 salts), which was then autoclaved. Experiments were performed in duplicate and a positive result was claimed only after maintenance of growth through four subcultures. Results are given in Table 1.

Table 1

C source	Aerobic	Anaerobic	C source	Aerobic	Anaerobic
Lactate	+	Not tested	Hippuric acid (0.1%)	(+)	—
Creatinine (0.08%)	—	—	Ascorbic acid (<i>M</i> /100)	—	—
Urea (1.5%)	—	—	Urea, creatinine, and uric acid	—	—
Uric acid (0.035%)	—	—	Urea, creatinine, uric and hippuric acids	(+)	—

+ growth; — no growth; (+) poor growth.

The concentrations of urea, creatinine, and uric acid are approximately as in normal urine.

None of the urinary compounds tested support growth, though the ability of the organism to use ammonium lactate as sole source of C and N indicates that this was not due to lack of any 'growth factor'. The nature of the substances supporting growth was now investigated by simple fractionation of urine. A sample was extracted with ether, first neutral and then at pH 1.5 (HCl added). Another sample was acidified with H_3PO_4 , distilled *in vacuo*, and both distillate and neutralized residue were tested (Table 2).

* Typed by Dr Carruthers, of the Pathological Laboratory, Cambridge.

Table 2

	Aerobic	Anaerobic		Aerobic	Anaerobic
Ether extraction:			Distillation at acid reaction:		
'Neutral' extract	—	—	Distillate	—	—
'Acid' extract	—	—	Residue	+	+
Extracted residue	+	+			

+ growth; — no growth.

A concentrate of the non-fermentable sugar of urine (see below) supported growth however. It was prepared as follows: 1000 ml. urine were evaporated *in vacuo* to 100 ml. and 25 % basic Pb acetate was added till no further precipitation took place; the precipitate was decomposed with H_2S , and the resulting filtrate evaporated *in vacuo* at low temp. to 6 ml.; an equal vol. of alcohol was added and the precipitate (mainly inorganic) discarded. The filtrate, diluted to 50 ml. with water, was treated with Ag_2CO_3 , filtered, the filtrate freed from Ag by passing H_2S (excess removed by evaporating slightly *in vacuo*), and fermentable sugar was removed by yeast (see below). The concentrate, containing 20 % of the non-fermentable sugar of the original urine, diluted to give a sugar concentration of *ca.* 40 mg. per 100 ml. and with addition of inorganic requirements as above, supported both aerobic and anaerobic growth through four subcultures.

REMOVAL EXPERIMENT

To ascertain which constituents disappeared during growth of the organism, a sample of urine was assayed for most of the well-recognized constituents before and after growth had taken place.

Urea, uric acid, creatinine, hippuric acid, total amino-acids (as α -amino-N), reducing sugar, and total carbohydrate were assayed. As urine contains much other little-known organic material, total C and N were estimated and a balance-sheet was constructed for these elements. Since most of the C and N is accounted for by urea, this (together with NH_3) was removed before assay of these elements, the figures for which would otherwise have had little significance.

Urea [Cole, 1937].

Analytical methods

Amino-acid N [Peters & Van Slyke, 1933, p. 385]. Urea and NH_3 , which interfere with this assay, were removed. Other interfering substances were allowed for by a 'blank' described by these authors. For removal of urea and NH_3 , 25 ml. urine, 25 ml. *M*/15 phosphate buffer pH 7, and 0.4 ml. (the smallest quantity effecting complete removal) of a 10 % solution of the 'pure' urease preparation described by Peters & Van Slyke [1933, p. 545] with two drops of 0.04 % bromothymol blue, were heated to 40–45° and *N* HCl was added to neutralize the NH_3 as formed. When the reaction was apparently complete, acid was added until the indicator was yellow, and the vessel fitted with a bung and capillary was evacuated to remove CO_2 , the evacuation being stopped to add more acid when necessary. The solution was then neutralized with *N*/10 NaOH and kept 1 hr. at 45°. The NH_3 was distilled off according to Peters & Van Slyke [1933, p. 927], save that CO_2 was finally removed with HCl instead of acetic acid.

The enzyme preparation introduced some C and N and to allow for this in the estimation of these elements, a 'blank' was set up as above but with 1.5 % urea solution in place of urine.

Creatinine [Folin, 1914].

Hippuric acid [Henriques & Sørensen, 1910].

Uric acid [Blauch & Koch, 1939]. The uricase destruction described by these authors was omitted as the error due to interfering substances was found to be small.

Sugar. The reducing substances of urine, assumed to be mainly sugar, were estimated according to Harding & Selby [1931]: figures should be read 'as glucose'.

Removal of fermentable sugar by yeast. Harding & Selby [1931] describe a method for the removal of fermentable sugar by treatment with washed yeast for 8 min. at 37°. I found however that fructose is not quantitatively removed unless the time of treatment is extended to 60 min. The small amount of fermentable sugar in urine being entirely glucose [West & Steiner, 1932], treatment for 8 min. only was adequate. The yeast has no appreciable reduction blank.

'Total carbohydrate' was estimated by the orcin method [Pirie, 1936]. The figures should be read 'as glucose'. The values obtained for total carbohydrate in urine were usually about twice the reducing values (Table 3). This accords with the findings of Fleury & Ambert [1930].

Table 3. Comparison of 'total' and 'reducing' carbohydrate in three samples of urine

	mg. per 100 ml. 'as glucose'	
	Reducing	'Total'
I	58	105
II	49	99
III	47	95

Total N (exclusive of urea and NH_3) was estimated [Cole, 1933, p. 332] on the fraction from which these had been removed, with correction for urease 'blank'.

Total C (exclusive of urea). 0.5 ml. urea-free urine containing 2-3 mg. C, was evaporated to dryness and the estimation proceeded with as described by Van Slyke *et al.* [1933] with correction for urease 'blank'.

Analytical results

Table 4 gives the complete analysis of a sample of urine. The C and N contents of each constituent are given (cols. 2 and 4) and also the percentages of the total C and N for which they account (cols. 3 and 5). The 'undetermined' C and N are obtained by difference: the

Table 4. Urine collected 5 p.m. to 9 a.m. Vol. 990 ml.

Substance	g./l.	C		N	
		g./l.	% of total	g./l.	% of total
Urea	16.8	—	—	—	—
Creatinine	0.94	0.354	11.5	0.31	36.5
Uric acid	0.47	0.167	5.5	0.156	18.7
Hippuric acid	0.224	0.133	4.3	0.017	2.0
Amino-acids	0.092*	0.395†	12.9	0.129†	15.2
Reducing sugar	0.58	(not included, as duplicates 'total carbohydrate')			
Total carbohydrate	1.05	0.42	13.7	—	—
Undetermined C and N	—	1.59	52.0	0.24	27.6
Total C and N	—	3.06	100.0	0.85	100.0

* Amino-N figure.

† Estimates based on the amino-N figure and the composition of the 'average' amino-acid.

'total' C and N are exclusive of urea and NH_3 . The undetermined C is disappointingly high, but there is no evidence that this fraction is of importance in the metabolism of the microorganism.

This urine (250 ml.) after sterilization by Seitz filtration, was sown with organism, incubated for 5 days in an atmosphere of N_2 , during which the pH rose from 5.7 to 8.2, the (inorganic) precipitate and the crop of organism were centrifuged off, and the supernatant assayed as before. The dry weight and C content of the crop of organism were determined (Table 5).

Table 5

Substance	C		N	
	g./l.	% of total	g./l.	% of total
Urea	12.3	—	—	—
Creatinine	0.88	0.325	0.284	43.7
Uric acid	0.039	0.014	0.013	2.0
Hippuric acid	Nil	—	—	—
Amino-acids	0.080*	0.334	0.112	17.3
Reducing sugar	0.080	(not included, as duplicates 'total carbohydrate')		
Total carbohydrate	0.54	0.204	—	—
Organism	0.058	0.027	Not assayed	
Undetermined C and N	—	1.74	0.24	37.0
Total determined C and N	—	0.914	0.409	63.0
Total C and N	—	2.65	0.65	100.0

* Amino-N figure.

In Table 6 the analyses before and after anaerobic growth are compared.

Table 6

	Before g./l.	After g./l.	Change	
			g./l.	% of total
Urea	16.8	12.3	-4.5	-27
Creatinine	0.94	0.88	-0.50	-6.4
Uric acid	0.47	0.039	-0.43	-92
Hippuric acid	0.224	Nil	-0.224	-100
Amino-acids	0.092*	0.080*	-0.012	-13
Reducing sugar	0.58	0.08	-0.50	-86
Total carbohydrate	1.05	0.54	-0.51	-48
Organism	—	0.058†	0.058	—

* Amino-N figure. † Dry wt.

Only a small fraction of the creatinine and amino-acids had disappeared. The hippuric acid has all been removed, but this may indicate hydrolysis to glycine and benzoic acid. (Hippuric acid could not be used as a hydrogen donor, see below, and supported growth very poorly.) Most of the uric acid was removed, but this is a somewhat unstable compound and also gave negative results in both the growth and dehydrogenase experiments (below). Total N decreased by 27%, but this could be accounted for by conversion into NH_3 of the N of the lost creatinine, uric, hippuric and amino-acids.

Total C shows a rather large fall. This may be partly explained by the conversion of non-volatile compounds (e.g. carbohydrates) into volatile fermentation products, whose C could not be assayed quantitatively by the methods used. Adding this loss of 410 mg./l. to the increase in 'undetermined' C gives a minimum figure for C metabolized of 555 mg./l.; as 58 mg./l. organism with a C content of 27.4 mg. were obtained, the growth efficiency was 5%, a normal figure for anaerobic conditions.

Nearly all the 'reducing' but only half the 'total' carbohydrate was metabolized. The actual amounts removed—0.50 and 0.51 g. respectively—were the same, suggesting that urine may contain two substances assaying as 'total carbohydrate', one reducing, one non-reducing, only the former being utilized by the organism.

The decomposition of 27% of the urea indicated that the organism possessed a powerful urease. It was thought that this might be an adaptation to life in the bladder. M. Stephenson and J. H. Waelsch, however, in a private communication quoted by kind permission, state: 'Twelve strains of *Bact. coli* isolated from the bladder of cystitis patients... were investigated, and... four contained urease and eight did not. Three stock strains of *Bact. coli*, not containing urease, were cultivated through nine subcultivations in urine: none of these developed any trace of urease. There therefore seems to be no reason to postulate the possession of urease as an adaptation to life in the bladder.'

DEHYDROGENASES OF THE ORGANISM

A micro-organism can use as a source of C only compounds which it is capable of dehydrogenating [Quastel *et al.* 1925]. The C source of the organism was now investigated by studying the ability of the washed organism to dehydrogenate various substrates, including urine and some of its constituents.

Preparation of washed suspensions. The organism was grown semi-anaerobically on sterile urine (1 l.) for 24 hr. The crop of organism was centrifuged down and washed three times on the centrifuge with distilled water. The considerable inorganic precipitate formed during growth was removed by differential centrifuging. The suspension was used the same day.

The washed organism was suspended in 10–15 ml. water and the yield estimated turbidometrically. 1 l. of urine normally yielded 50–100 mg. (dry wt.), about half of which was C. This is an informative figure, since it indicates that not less than, say, 250 mg. of the substance(s) supporting growth must be present in 1 l. of urine.

Hollow stoppered Thunberg tubes were used. In the hollow stopper was 0.5 ml. washed suspension, containing about 3 mg. (dry wt.) of the organism. The actual amount is stated in each case.

The tube contained:

Methylene blue, 1/5000; 1 ml.

Phosphate buffer, *M*/15, pH 7.0; 1 ml.

The substrate under test, usually 1 ml., neutralized.

Water to give total vol. (incl. organism) 5 ml.

The tubes were evacuated, placed in the thermostat (37°) for a few min. to equilibrate and to demonstrate that no reduction took place in the absence of the organism. The tubes were then tipped to add the suspension to the rest of the contents, and the time required for complete reduction of the methylene blue noted. 'Blanks' with no substrate present gave in most cases reduction times of over 100 min.

Fig. 1. Urine as hydrogen donor. Two samples of urine, used with organism suspensions containing: for urine A 4.9 mg., urine B 4.1 mg., dry wt. per tube.

Results with various substrates

Urine. The reduction times with urine at various dilutions are shown in Fig. 1. The two curves A and B represent separate experiments with different samples of urine and of washed suspension.

Constituents of urine, and some other substances. Of the substances tested, only lactate and formate are active. But, as Fig. 1 shows, urine gives reduction in about 5 min. at a dilution of 1/50: lactate gives reduction in the same time only at a concentration of 0.2%. If therefore the active substrate in urine were solely lactate, the concentration of the latter would have to be of the order of 10%. Formate is excluded since the urinary hydrogen-donor is not volatile in acid solution (see below).

Table 7. *Dehydrogenation of constituents of urine, and some other substances*

Substrate	Organism mg.	Reduction time	
		With urine	With substrate
Creatinine, 0.016%	1.7	7 min. 55 sec.	>2 hr.
Uric acid (few crystals of solid)	1.7	7 min. 55 sec.	>2 hr.
Urea, 0.3%	1.7	7 min. 55 sec.	>2 hr.
Lactic acid: 0.004%	1.7	7 min. 55 sec.	>2 hr.
0.02%	4.0	3 min. 50 sec.	24 min.
0.2%	4.0	3 min. 50 sec.	5 min. 15 sec.
Formic acid, 0.01%	3.7	5 min. 55 sec.	7 min. 0 sec.
Hippuric acid, 0.1%	3.7	5 min. 55 sec.	>1 hr.
Glycine, 0.02%	2.0	3 min. 0 sec.	>1½ hr.
Ascorbic acid, M/100	2.2	4 min. 40 sec.	>1½ hr.

The first column gives mg. (dry wt.) of organism per tube. The second column gives, for comparison, the reduction time when 1 ml. normal urine was supplied as substrate.

Investigation of the fractions obtained by ether extraction and by distillation at acid reaction (see above) shows (Table 8) that the active substrate in urine is not ether-extractable or volatile in acid solution.

Table 8. *Dehydrogenation of urine fractions*

Substrate	Organism per tube, mg.	Reduction time	Substrate	Organism per tube, mg.	Reduction time
Untreated urine	2.3	7 min. 5 sec.	Untreated urine	2.8	5 min. 55 sec.
'Acid' ether extract	2.3	>2 hr.	'Acid distillate'	2.8	2 hr. 10 min.
'Neutral' ether extract	2.3	>2 hr.	Residue	2.8	6 min. 10 sec.
Residue	2.3	7 min. 10 sec.	Blank (no substrate)	2.8	>2 hr.

Sugars. The sharp falling-off in activity at a certain concentration, and the maintenance of full activity down to very low concentration (about 1/100 dilution of urine) suggested in view of the results of Quastel & Whetham [1925] that the active substrate in urine might be a sugar or related compound. The failure of the substrates of Table 7 to account for the hydrogen-donor activity of urine, and the results given in Table 8, were consistent with this speculation.

The sugar dehydrogenases of the organism were now investigated: the results for glucose, fructose, lactose and galactose are given in Fig. 2, and for arabinose, rhamnose, glucuronic and galacturonic acids in Table 9.

Comparison of Figs. 1 and 2 shows that urine gives a curve intermediate between those for fructose and glucose. With both these sugars, hydrogen donor activity falls off sharply at a concentration of about $M/40,000$.* The corresponding concentration with urine is the dilution 1/100. Were the hydrogen donor in urine either of these sugars, this would indicate a concentration of the sugar in urine of $(M/40,000)/(1/100)$, i.e. $M/400$, or 0.45 g./l. Now, as Table 10 shows, the fermentable sugar content of urine is usually of the order of 1/10 this amount, or less. But the mean content of the non-fermentable sugar

* This may be due either to the enzyme being 'saturated' only above this concentration, or to there being stoichiometrically insufficient sugar at lower concentrations to effect complete reduction of the methylene blue.

of the ten urines recorded in Table 10 is 0.56 g./l. It will be recalled that the yield of washed suspension of organism from a given volume of urine indicated a lower limit of 0.25 g./l. for the substance(s) supporting growth.

Fig. 2. Dehydrogenation of sugars. Dry wt. washed suspension, per tube: glucose 1.9 mg., fructose 2.0 mg., lactose 2.7 mg., galactose 2.2 mg.

Table 9. *Dehydrogenation of some sugars*

Substrate	Organism mg.	Reduction time	
		With 1 ml. urine	With substrate
Glucuronic acid, 0.001 M	1.9	9 min. 45 sec.	33 min.
Galacturonic acid, 0.0025 M	1.9	9 min. 45 sec.	> 1½ hr.
Arabinose, 0.01 M	2.2	4 min. 40 sec.	12 min. 45 sec.
Rhamnose, 0.01 M	2.2	4 min. 40 sec.	> 2 hr.

Table 10. *Fermentable and non-fermentable sugar content of ten urines*

Subject	Sex	mg. per 100 ml. (as glucose)		Subject	Sex	mg. per 100 ml. (as glucose)	
		Fermentable	Non-fermentable			Fermentable	Non-fermentable
MCAC	F.	0	79	DJDH	M.	6	65
EFG	M.	9	59	RvH	F.	0	31
AN	M.	41	103	HKK	M.	2	60
FMM	F.	0	22	DH	M.	3	7.4
JFD	F.	0	47	IDPW	M.	0	44

Fermentable and non-fermentable sugar estimated according to Harding & Selby [1931]. All normal healthy subjects, ages 20–35.

Table 10 shows considerable variation in non-fermentable sugar content: this is to be expected, as the urines were not collected under standard conditions. Now if the hydrogen-donor activity of urine is attributable to this non-fermentable sugar, activity should be proportional to its concentration. Fig. 3 shows that this is in fact the case: here the relation between velocity of reduction and sugar concentration is plotted for five of the urines of Table 10. The corresponding curve for glucose is included for comparison. Fig. 3 differs from Fig. 1 in that abscissae are sugar concentrations, not urine concentrations; also the curves in Fig. 3 are corrected for the 'blank' (no substrate) reduction time.

This proportionality between hydrogen donor activity and reducing sugar content was observed also in concentrates of this sugar prepared during attempts at its isolation.

(Details are given in Part 2.) Fig. 4 shows the results, with a curve for a normal urine sample for comparison. Concentrate 'A' was prepared by precipitating the urine with basic lead acetate and recovering the sugar from the precipitate, and 'B' by further purification of 'A' by precipitation of the sugar with baryta from methyl alcoholic solution.

Fig. 3. Dehydrogenation of urinary sugar. Dry wt. washed suspension, per tube: glucose 1.9 mg., other curves 2.2 mg. Glucose: $\otimes$ --- $\otimes$. Urines (see Table 10): RvH ∇ — ∇ , DH $\square$ — $\square$, HKK $\odot$ — $\odot$, DJDH $\triangle$ — $\triangle$, IDPW $\times$ — $\times$.

Fig. 4. Dehydrogenation of sugar concentrates. Dry wt. washed suspension, per tube: urine and concentrate A 2.1 mg.; concentrate B 1.9 mg. Urine, $\square$ — $\square$; concentrate A, $\times$ — $\times$; concentrate B, $\triangle$ — $\triangle$.

Table 11 gives further support to the speculation that the active substrate of urine is the sugar. The reduction times for normal urine are compared with those for a sample after anaerobic growth of the organism.

Table 11

Dilution of urine	Reduction times	
	Normal urine	Urine after anaerobic growth
1/5	3 min. 50 sec.	1 min. 0 sec.
1/50	4 min. 0 sec.	13 min. 0 sec.

Anaerobic growth *decreases* reduction time at 1/5 dilution but on increasing the dilution ten times an almost proportional increase in reduction time occurs. This is consistent with

the conversion of the original sugar into numerous fermentation products, many of which are actively dehydrogenated by the organism. None of these however has as high an affinity for its enzyme as the parent sugar, hence at the higher dilution their reducing power is decreased to a relatively greater extent.

Urine on which aerobic growth had taken place showed, as was to be expected, no hydrogen donor activity.

The active substrate in urine was also shown to be completely dialysable.

Although the above evidence renders it very improbable that the active substrate in urine is yeast-fermentable, direct confirmation of this was desired. Fig. 5 gives the curves for yeast-treated urine and for an untreated control: there is no essential difference. The yeast itself was shown to introduce no 'blank'.

Fig. 5. Effect of yeast treatment on hydrogen-donor activity of urine.

For this experiment the urine was treated with yeast for 8 min. This does not remove fructose completely, but Table 12 gives the results for a sample of urine which had been yeast-treated for 60 min. This removes fructose quantitatively.

Table 12. Effect of yeast treatment for 60 min. on H-donor activity of urine

Substrate	Reduction time
Untreated urine, dil. 1/50	2 min. 45 sec.
Yeast-treated urine, dil. 1/50	3 min. 30 sec.
Blank (no substrate)	2 hr.

DISCUSSION AND SUMMARY OF PART I

The results described imply that the 'sugar' of urine is the source of carbon in the nutrition of *Bact. coli* in cystitis. The evidence is summarized as follows:

1. The organism shows vigorous anaerobic growth on urine, but not on media containing its well-known constituents—urea, uric acid, hippuric acid—separately or together, as C-source. But a concentrate of the urinary sugar, prepared from urine by a basic Pb acetate method, supported anaerobic growth.

2. During anaerobic growth the organism removes from urine the reducing sugar, and uric and hippuric acids. Only small quantities of creatinine and amino-acids are utilized and there was no evidence suggesting utilization of the compounds not investigated, which account for about half the total C, exclusive of urea.

3. Study of the dry weight of organism obtainable from a given volume of urine indicated a lower limit of 0.25 g./l. for the substance(s) supporting growth.

4. The organism, though possessing a powerful dehydrogenase for some substrate present in urine, cannot attack urea, uric acid, glycine, hippuric acid, ascorbic acid, glucuronic acid, galacturonic acid.

5. The substrate is not ether-extractable or acid-volatile. It is completely dialysable.
6. The dehydrogenase maintains its maximal activity even at great dilutions of the substrate, suggesting that the latter might be a sugar or related compound. Study of the effect of anaerobic growth of the organism on the hydrogen-donor activity of the urine gave results consistent with this suggestion.
7. The hydrogen-donor activities of several urines, and of concentrates of sugar prepared from urine, were quantitatively proportional to the reducing sugar contents.
8. Evidence is adduced to indicate that the small amount of fermentable sugar found in normal urine cannot account for these results.

2. INVESTIGATION OF THE SUGAR OF NORMAL URINE

Though the presence of a sugar in normal urine has long been recognized, and the isolation and identification of a compound occurring to the extent of 0.5 g./l. does not appear superficially to be a difficult problem, yet little is known about this sugar, despite the efforts of numerous investigators. These have hitherto followed one of two lines: (1) the indirect method of studying the effect of various treatments, e.g. Br oxidation [Everett *et al.* 1934] or boiling with acid [Harding *et al.* 1936], on the reducing power of the urine; and (2) more frequently, direct attempts have been made to prepare and identify an osazone of the sugar.

The earlier work on osazones [cf. Everett & Sheppard, 1931] has often been invalidated by failure to recognize sources of error such as that described by Jaffe [1896] who showed that urea forms 1-phenylsemicarbazide under the conditions normally employed in the preparation of osazones. Harding *et al.* [1936] prepared N-free sugar extracts from urine and studied the effect of boiling with N acid: they found that a small amount of fermentable matter was liberated by this treatment, including a little galactose, which they detected by the differential fermentation methods developed by Harding & Nicholson [1933] and Nicholson [1936]. Nicholson & Archibald [1939] decided, from a study of the NaIO oxidation of the sugar, that 75% behaved as aldose—in contrast to Everett *et al.* [1934] who concluded from a study of Br oxidation that the sugar was mainly ketose. Nicholson & Archibald's [1939] differential fermentation experiments yielded no further information as to the nature of the bulk of the material.

In the present investigation, sugar concentrates were prepared containing up to 25% of the dry wt. as sugar. These were investigated by means of colour reactions and osazone formation.

EXPERIMENTAL

Concentration of urinary sugar

The urine was concentrated $\times 10$ by evaporation.* Methods for preliminary separation of the sugar involved (1) 'clearing' of the concentrated urine followed by (2) precipitation with a sugar precipitant. Numerous methods were tried which will be briefly mentioned before describing that finally adopted.

Preliminary trials. The technique of Harding *et al.* [1936] resulted in loss of 40% of the sugar during the basic Pb acetate and 70% during the strongly acid HgSO₄ treatments. Nearly all the rest of this acid- and alkali-labile sugar was destroyed by the highly alkaline copper-lime reagent.

Hg acetate proved unsatisfactory as a 'clearing' agent (loss 50%, probably due to oxidation), and though 5% Fe(SO₄)₃ followed by BaCO₃ gave clear colourless filtrates with up to 70% of the original sugar, neutral Pb acetate was finally adopted, as more convenient in view of the subsequent treatments (see below).

* All evaporations were performed *in vacuo*.

The sugar itself was completely precipitated from the urine concentrate by basic Pb acetate. For regeneration of the sugar, H_2S , H_2SO_4 followed by BaCO_3 , and oxalic acid were tried in turn, but abandoned in favour of CO_2 . The concentrate used in the dehydrogenase test of Fig. 4 (curve for 'Concentrate A'), however, was obtained by oxalic acid regeneration (10 % soln. added till pH fell to 2.5, followed by concentration *in vacuo* and removal of excess oxalate as the Ba salt; recovery 40 % of the original sugar).

Basic Pb acetate gave very variable results however in respect both of the percentage of sugar precipitated and the percentage regenerated by CO_2 . This was partly due to differences between different samples, but mainly to the precipitation of the sugar being apparently effected by adsorption on the precipitate formed with other substances present in the urine concentrate, the nature and amount of which were very variable. As a result of this, only partial precipitation could be achieved from 'cleared' solutions, and this led to the abandonment of basic Pb acetate as such for sugar precipitation.

The technique was therefore adopted of making the basic Pb acetate *in situ* by addition of the neutral salt followed by NH_3 [cf. Rimington, 1929; 1931]. The composition of the precipitate, now mainly PbO or Pb(OH)_2 , could be controlled by adjustment of the pH, and by repeating the process the sugar could be completely precipitated from 'cleared' solutions. As the most successful procedure for separating the sugar was based on this method, full experimental details of this alone will be given.

Procedure finally adopted. 250 ml. urine concentrate (containing 1.17 g. sugar*) were treated with 250 ml. 30 % neutral Pb acetate, the precipitate discarded, aqueous NH_3 added to raise the pH to 9.0, the resulting 'basic Pb' precipitate filtered off, 100 ml. neutral Pb acetate added and NH_3 as before, the fresh precipitate filtered off, and the precipitation repeated. The final filtrate was discarded, and the three 'basic Pb' precipitates combined, washed with 250 ml. water, suspended in 250 ml. water, mechanically stirred, and a brisk current of CO_2 passed in. When the reaction was complete ($\frac{1}{2}$ –1 hr.) the mixture set to a semi-solid paste. This could be filtered, however, and the bulky residue was washed twice by suspending in 250 ml. water and passing in CO_2 with mechanical stirring. The combined filtrate and washings were then freed from lead by passing H_2S , and the resulting filtrate (after evaporation *in vacuo* to remove H_2S) contained 0.59 g. sugar, a recovery of 51 %. The various residues were decomposed with H_2S and assayed for sugar (Table 13). 81 % was accounted for.

Table 13. *Separation by neutral-ammoniacal lead acetate precipitation*

Further purification of the sugar concentrates

The sugar concentrates thus obtained were not an adequate starting point for the identification of the sugar, which accounted for only 10 % of the total solid matter. Two contaminants, urea and fermentable sugar, required specific removal.

* All figures for sugar in this and subsequent sections refer to non-fermentable sugar.

Urea was removed by urease. The concentrate, containing about 0.5 g. sugar, was evaporated to about 100 ml. and 100–200 mg. 'pure' urease (see Pt. I) and a drop of 0.04 % bromothymol blue added. The solution was heated to 37° and $N H_2SO_4$ added to neutralize the NH_3 as formed. When the reaction was complete, the solution was shaken with a little charcoal and filtered through kieselguhr.

Fermentable sugar was removed at this stage by treatment with washed yeast for 15 min. at 37°. Most of the yeast was removed by centrifuging, the remainder by filtration through kieselguhr. Blank experiments showed that neither yeast nor urease added any reducing substances to the solution.

The filtrate was now evaporated till solid began to separate and excess alcohol was added. A heavy precipitate, consisting of some inorganic salts and the ammonium sulphate formed from the urea, was filtered off, the filtrate evaporated to small bulk and excess alcohol was again added. The concentrate was now ready for further purification, and two methods were tried.

(1) This was essentially the same as that found by Neuberger [1938] to give 95 % recovery in the somewhat similar problem of the isolation of a carbohydrate derivative from egg-albumin.

The solution was evaporated to dryness and acetic anhydride and dry pyridine were added. After standing 48 hr. at room temperature, the mixture was poured into ice-water and extracted with benzene, the extract dried and the solvent evaporated off. Attempts to recrystallize the oily residue proving unsuccessful, it was deacetylated by treatment with dry methyl alcohol containing a trace of NaOEt, but the deacetylated product contained only 2.5 % of the sugar originally present. It was presumed that the reagents employed had proved too drastic for this very labile compound.

(2) Recoveries of 40–70 % were obtained, however, by the method of Baba [1935] involving precipitation of the sugar by $Ba(OH)_2$ from methyl alcoholic solution. The use of a non-aqueous solvent much reduces the danger of destruction of a labile sugar by the $Ba(OH)_2$, but, as Baba points out, speed in manipulation is vital for success.

The alcoholic solution, from which urea and fermentable sugar had been removed, and which contained about 0.5 g. sugar, was evaporated to dryness and the residue taken up in 25 ml. methyl alcohol. Ether was added until the solution was just turbid (5–10 ml. required), and sufficient $N Ba(OH)_2$ added to effect complete precipitation (20–30 ml. required). The heavy precipitate was centrifuged down and washed twice with methyl alcohol containing a little ether, then suspended in water and neutralized by CO_2 . The precipitate of $BaCO_3$ was filtered off and the filtrate freed from traces of Ba by careful addition of H_2SO_4 . The entire operation from addition of the $Ba(OH)_2$ to neutralization with CO_2 was complete in 25 min.

A sugar concentrate thus prepared had the same hydrogen-donor activity for the organism, relative to its sugar content, as urine (see Pt. I, Fig. 4, 'Concentrate B').

INVESTIGATION OF THE SUGAR CONCENTRATES

Colour reactions

These were performed under the conditions described by Cole [1933] on a 1 % solution of the sugar in 70 % aqueous alcohol.

'*Rapid furfural*.' Here Cole's [1940] modified method was used. The reaction was positive, the urine sugar giving the same reaction as fructose.

Foulger's test for ketoses gave a negative result.

Tests for pentose. Bial's test was negative, but the phloroglucinol test was indecisive: arabinose gave a rose tint, turning to a rich red-brown; the urinary sugar gave a yellow colour turning to a red-brown very like that given by arabinose.

Tollens' naphthoresorcinol test for uronic acids and uronides was negative.

Reduction of Fehling's solution

The reduction of Fehling's solution by the urinary sugar was qualitatively compared with the reduction by other sugars. The results are given in Table 14. 1 ml. 1 % sugar solution was taken and 5 ml. Fehling's solution.

Table 14. *Reduction of Fehling's solution*

	At 60°	At 40°
Urine sugar	Reduction commenced after 2 min., strong reduction, 5 min., complete in 10 min.	No definite signs of reduction after 20 min.
Fructose	Similar to urine sugar but slightly more rapid	Reduction commenced 2½ min., but not complete even after 20 min.
Glucose	Only traces of reduction after 20 min.	Not investigated
Arabinose	Slight reduction after 3 min.: still only slight after 10 min.	Not investigated

Preparation of an osazone

In spite of numerous and varied attempts, great difficulty was experienced in obtaining a clean crystalline product, presumably owing to the very variable nature of the impurities accompanying the sugar.

The main difficulty, the separation of the osazone as an oil or tar, could be overcome by carrying out the reaction in alcoholic solution: but the product then failed to separate on cooling. The best results were obtained by using a mixture of alcohol and water in such proportions that the product did not separate during the heating but crystallized out on cooling.

To 15 ml. water and 5 ml. alcohol, containing 0.19 g. sugar, were added 1 g. recrystallized phenylhydrazine hydrochloride and 2 g. sodium acetate. The solution was heated to dissolve the reagents, filtered and heated on the water bath for 2 hr. and allowed to cool slowly, being finally left overnight in the ice chest. Some oil was formed, but also a crop of crystals. The latter was filtered off and dried, giving 0.205 g. or 54 % of the theoretical yield. A sample of this crude product was analysed (Table 15, 'Urinary osazone 1').* The osazone was recrystallized from aqueous alcohol, giving a constant but not very sharp M.P. 160°. (All M.P.s taken with the same apparatus and the same thermometer; uncorr. unless otherwise stated.) This product was also analysed (Table 15, 'Urinary osazone 2').

8.4 mg. in 3 ml. dry pyridine gave $\alpha_D - 0.39^\circ$, $l = 2$ dm.; $[\alpha]_D - 70^\circ$.

Sometimes a product with a much higher and sharper M.P. was obtained. Thus in an experiment with a concentrate containing 0.67 g. sugar, heated with phenylhydrazine acetate and sodium acetate in aqueous solution, but with addition of sufficient alcohol at the end of the reaction to dissolve all the oil formed, 0.84 g. crude osazone was obtained (63 % yield), M.P. 190–192° (recryst. from aqueous alcohol), M.P. 206° (corr.; recryst. from alcohol). A second recrystallization from alcohol gave M.P. 215° (corr.) sharp and constant after further recrystallization. Some authentic osazones were now prepared by standard methods, and the following M.P. and mixed M.P. taken (corr. values, Table 16). The material was also analysed (Table 15, 'Urinary osazone 3').

The analysis of the recrystallized osazone (Table 15, 2, 3) though close to the theoretical figures for a hexosazone do not agree exactly. Much better agreement is obtained with the figures for a hexosazone with the elements H₂O added. This, however, may not be significant.

Although the osazone was obtained in good yield, much was lost during recrystallization, and there was a danger that the purified fraction was not representative. An analysis

* Analyses by Dr G. Weiler.

Table 15. *Analysis of osazones*

Found:	C %	H %	N %	Theoretical:	C %	H %	N %
Urinary osazone: 1	60.5	6.2	14.4	Hexosazone	60.3	6.15	15.7
2	57.8	6.4	14.9	Hexosazone + H ₂ O	57.7	6.4	14.9
3	58.6	6.6	14.8				

Table 16

	M.P. or mixed M.P.		M.P. or mixed M.P.
<i>d</i> -Glucosazone	205°	Urine sugar and <i>dl</i> -xylose	216°
<i>d</i> -Galactosazone	193°	Glucose and <i>dl</i> -xylose	207-8°
<i>dl</i> -Xylosazone*	215°	Glucose and galactose	186°
Urine sugar and glucose	203°	Urine sugar and galactose	197°

* Prepared by Dr D. J. Bell.

was therefore made of the crude product (Table 15, 'Urinary osazone 1'). Agreement for a hexosazone is good for C and H but the N figure is low.

An attempt was made to prepare the acetyl derivative of the osazone following the technique of Maurer & Scheidt [1935] for acetylglucosazone, viz. treatment with acetic anhydride in pyridine solution. The material separated as an oil, however, and all attempts to obtain a crystalline product failed.

Preparation of osazones for comparison. In addition to the 'authentic samples' described above the following phenylosazones were prepared: *d*-maltose, M.P. 193°; *l*-arabinose, M.P. 159°, *d*-xylose, M.P. 160°; *l*-rhamnose, M.P. 189° (corr.). Some specific rotations determined (*c* = 5 % in dry pyridine) are listed in Table 17.

Table 17

	[α] _D	
	Initial	Final
<i>d</i> -Glucosazone	- 84°	- 35°
<i>d</i> -Galactosazone	+ 160°	+ 94°
<i>l</i> -Rhamnosazone	+ 111°	+ 61°

DISCUSSION

In Part 1 of this paper, evidence is adduced that the non-fermentable sugar of normal urine is the principal carbon source of *Bact. coli* in cystitis. In attempts to isolate this sugar, the concentrates obtained contained only a fraction of that originally present. That this fraction, however, is representative of the whole is indicated by the experiment illustrated by Fig. 4, where the same proportionality as in urine is shown to exist between activity as substrate for the dehydrogenases of the organism and copper reduction.

The extreme lability of the sugar was constantly in evidence and much enhanced the difficulties of isolation. This problem does not appear to have confronted other workers to so great an extent.

The colour reactions confirm that the sugar is one of the more labile members of the group; it is unwise to draw further conclusions, as much other material was present. The negative reaction to the test for uronic acids should, however, be noted.

The sugar reduces Fehling's solution rather less readily than fructose, rapid reduction being observed at 60° but not at 40°. This probably excludes *l*-xyloketose, the sugar of pentosuria [Levene & LaForge, 1913; 1914], which it was thought might be present in normal urine in small amounts, especially in view of Enklewitz & Lasker's [1935] suggestion that its precursor in the body is glucuronic acid. These authors state, however, that this sugar is characterized by very rapid reduction of Fehling's solution even at 40°.

The evidence of the osazone must be interpreted with caution. Thus, Table 18 gives values for the M.P. of galactosazone taken from various sources:

Table 18. *M.P. of galactosazone*

Author	M.P.	Author	M.P.
Schleiber [1884]	170–171°	Fischer & Tafel [1887]	196°
Fischer [1887]	182°	Levene & LaForge [1915]	201°
Tollens [1887]	184–186°		

It is noteworthy that when Fischer [1888] first prepared mannosazone he failed to recognize its identity with glucosazone. Moreover, as two osazones having the same M.P. frequently give no depression on mixing, to the difficulty of purifying an osazone is added the doubtful validity of M.P. as a criterion of purity, or of mixed-M.P. as a criterion of identity. In some cases too confusion has been caused by the recording of polarimeter readings, α_D as specific rotations $[\alpha]_D$ [e.g. Micheel, 1939; Levene & LaForge, 1915]. The figures for elementary analysis are more reliable when it is certain that the osazone is pure. But though on the whole I agree with the conclusion of Everett *et al.* [1934] who, after investigations more extensive than these, including the use of substituted phenylhydrazines, speak of 'the failure of osazones to provide an answer to this problem... (of the urinary sugar)', it is probably safe to conclude that the osazone is derived from a hexose or closely related compound.

In trying to narrow the field further, the chemical evidence must be considered in conjunction with the results of the growth and dehydrogenase experiments. Thus, the analysis of the osazone excludes a pentose. This accords with the negative results of the dehydrogenase experiments with xylose and arabinose. If the sugar is a hexose, it is unlikely to be a simple aldose on account of its lability. It may be a ketose, though fructose is excluded. The analysis of the osazone rules out a desoxy-sugar, and the organism failed to dehydrogenate rhamnose. Glucuronic acid and uronides have sometimes been mentioned in connection with the problem of the urinary sugar [e.g. Grimbert & Bernier, 1909] but the evidence against identity is considerable, viz. (1) analysis of the osazone; (2) failure to act as an active H-donor; (3) negative naphthoresorcinol reaction of the concentrates. The suggestion that the compound is ascorbic acid, whilst explaining its lability, has the following facts against it: (1) The daily excretion, about 1.0 g., is much too high; (2) ascorbic acid cannot support growth of the organism; (3) the organism cannot dehydrogenate ascorbic acid; (4) the sugar concentrates contained no material reducing 2 : 6-dichlorophenolindophenol.

SUMMARY

1. The nutrition of *Bact. coli* isolated from a cystitis case was investigated, with normal urine as medium under anaerobic conditions.
2. Experiments involving growth of the organism on synthetic media, study of the removal of certain substances during anaerobic growth on urine, and investigation of the dehydrogenases of the organism, indicated that the main source of carbon during anaerobic growth is the non-fermentable sugar of normal urine.
3. Attempts to isolate this sugar from urine resulted in the preparation of syrups, on which certain colour reactions were performed, and from which an osazone was prepared giving analytical figures approaching those for a hexosazone.
4. The nature of the sugar is discussed in the light of both the chemical and bacteriological evidence, and, though no positive suggestion can be made as to its identity, a considerable number of sugars can be excluded.

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