CXLVII. URICASE AND ITS ACTION.

III. COMPARISON WITH ACTIVATED CHARCOAL. CONTACT NATURE OF THE ACTION OF URICASE.

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INTRODUCTION.

It has been shown in Part II of this series that the uricolytic action of all uricase preparations studied is due exclusively to the presence of bacteria, with the exception of glycerol and glycerol-chloroform extracts of kidney or liver tissue, which were found under sterile conditions to activate the oxidation of uric acid. These glycerol-chloroform extracts were prepared by macerating 500 g. of comminuted tissue with 50 cc. of chloroform, 150 cc. of glycerol, and 500 cc. of water for 48 hrs., after which the volume was made up to 2 l. and the whole left for a further 24 hrs. The supernatant layer is yellowish-red in colour, and turbid; on prolonged standing partial sedimentation takes place. Such extracts give, on filtration through Berkefeld filters, inactive filtrates [Przyłęcki, 1928], whence it would follow that the uricolytic agent present is associated with the cell fragments in suspension. The active uricolytic agent partakes, therefore, of the nature of a contact catalyst, and comparative experiments with activated charcoal were for this reason performed, with the object of establishing this analogy.

Uric acid solutions were prepared by the solution of uric acid in saturated lithium carbonate solution. Uric acid was in all cases determined by Folin's colorimetric method; p_H was in most cases determined electrometrically, although in some cases Clark and Lubs's colorimetric method was used. Parallel determinations using both methods gave substantially identical results.

All systems were kept at 15–18°.

EXPERIMENTAL.

1. *Influence of hydrogen ion concentration on uricolysis.*

A. *Uricase.*

To a series of 100 cc. Erlenmeyer flasks 20 cc. of buffer solution (McIlvaine's disodium phosphate-citric acid buffers for p_H 5.7–8.0, and Sørensen's boric acid-sodium hydroxide buffers for p_H 7.7 upwards) were added; to one-third

of these flasks, which were to serve as controls, 10 cc. of water were added. A 0.1 % uric acid in lithium carbonate solution was placed in a flask fitted with a Kipp's 10 cc. automatic pipette, and a similar pipette was fitted to an empty flask. All flasks after stopping with cotton-wool were sterilised in an autoclave at 120° for 1 hr. After the flasks had cooled, 10 cc. of uric acid were introduced into each flask, and 10 cc. of extract, from the sterilised flask with the Kipp pipette, into those flasks to which 10 cc. of water had not been previously added. The uric acid was sterilised separately in order to obviate the possibility of different spontaneous decomposition at different p_H . Systems so sterilised remained sterile for at least a week, if the flasks were not opened, as the chloroform present in the extract had practically entirely evaporated after 3-4 days, leaving no hindrance to bacterial infection. The uric acid content of the solutions was determined using Folin's colorimetric method at 72 and 84 hrs.; in some cases at 48 and 114 hrs. In no case were the contents of any flask retained for examination after the flask had once been opened, as vigorous bacterial growth was found then to occur at certain p_H values.

The above experiment was repeated on four different preparations of the

Fig. 1. Action of glycerol-chloroform extracts of ox kidney at p_H 5.7-10. Curves I, Ia. Exp. 1, 72 and 84 hrs. Curves II, IIa. Exp. 2, 72 and 86 hrs. Curves III, IIIa. Exp. 3, 48 and 72 hrs. Curves IV, IVa. Exp. 4, 72 and 144 hrs.

extract, for p_H values of 5.7-12.2. It was not found possible to examine solutions the p_H of which was below 5.7, as uric acid crystallised out, whereby experimental conditions were completely changed. The results (Fig. 1) show that the general effect of increasing p_H is to augment uricolysis. The reaction curves rise sharply at p_H 7.5 and attain a maximum at about p_H 8, above which oxidation becomes less intense; on further alkalisation the oxidation curve in one case again rises. In solutions the p_H of which exceeds 8 uricolysis takes place to a limited extent in the absence of the uricolytic suspension;

the extent of oxidation is in these, as in other cases, taken as the difference between the uric acid content of the control flask and that containing extract.

Felix, Scheel and Schuler [1929] found that the action of pig-liver suspension on uric acid rises to p_H 8.9, then falls to 9.5, again rises to a second maximum at p_H 9.9, after which it again falls. In this case, too, the curve exhibits a break in the alkaline region.

B. *Activated charcoal.*

The action on uric acid solutions of animal charcoal, and of charcoals prepared and activated by a method similar to that of Warburg and Brefeld [1924], was investigated, with the object of comparing the action of these inorganic contact catalysts with that of kidney tissue suspensions. Active charcoal was prepared by igniting to a white heat a paste consisting of 2 g. of Bismarck brown and 0.5 cc. of saturated copper or ferrous sulphate solution. The resultant mass was repeatedly extracted with boiling 2*N* hydrochloric acid, until the extract no longer gave the reactions for copper or iron, and the product was then extracted with water until the extract no longer gave the reaction for chlorine ions. The charcoal was then again ignited to a dull red heat to drive off traces of hydrochloric acid. The uricolytic, and adsorptive power of this charcoal, and the effect on its action of addition of potassium cyanide, alcohols, or sodium pyrophosphate were studied, and finally the p_H curve was constructed as for uricase. The methods used were substantially the same as those described in the first part of this paper.

(i) *Adsorption of uric acid.*

Three flasks were prepared, each containing 30 cc. of uric acid in lithium carbonate solution. To 2 of these flasks 0.1 g. of iron-containing charcoal (this we shall in future designate as C-Fe) was added, one flask was shaken for 1 hr., the contents were filtered, and the uric acid content was determined in 1 cc. (0.62 mg.). The second flask was left for 1 hr. at 16° with 3 cc. of *isoamyl* alcohol, and the uric acid content of the filtrate was determined (0.61 mg./cc.). That of the third flask, to which no charcoal had been added, was 0.615 mg./cc. The above preliminary experiment indicated that C-Fe does not to any demonstrable extent adsorb uric acid at the p_H in question (8.1), but this did not exclude the possibility of adsorption taking place at other p_H values. Experiments at different p_H values were therefore carried out, the systems consisting of 10 cc. of uric acid solution, 20 cc. of buffer solution, and 0.1 g. of C-Fe. The buffers taken were McIlvaine's disodium hydrogen phosphate-citric acid buffers to p_H 8, and Sørensen's glycine-sodium hydroxide buffers above p_H 8. The systems were either shaken for 2 hrs. in closed flasks, or placed in cylinders through which a stream of nitrogen was passed for 2 hrs. In this way the disturbing effects of oxidation during this period were to a large extent excluded.

With shaking, the uric acid content of all flasks, whether with or without

C-Fe, was identical (10.5 mg.) after 2 hrs. at p_H values of 5.94, 6.95, 7.82, 8.35, 9.35, 9.6 and 9.75. Where nitrogen was passed identical results were obtained (uric acid content in all cases 6.1 mg.; p_H values 5.94, 6.95, 7.8, 8.10, 9.35, 9.6, and 9.8).

Hence it follows that the charcoal in question does not adsorb uric acid at any p_H between 6 and 10, and that the uricolytic action of this charcoal does not depend upon adsorption.

(ii) *Uricolytic action of C-Fe at different p_H values.*

Systems consisting of 10 cc. of uric acid solution and 20 cc. of McIlvaine's or Sørensen's buffers were left for 8–48 hrs. at room temperature (16°) with or without 0.1 g. of C-Fe. They were then filtered, and uric acid was determined in the filtrate. It was noticed that uric acid crystallised out below p_H 5.9, so that results are given only for the range p_H 5.94–9.8. The results (Fig. 2) show that the uricolytic action of the charcoal used varies greatly according to the p_H of the solution, twice as much being oxidised at p_H 10 as at 6. As in the case of uricase, the oxidation curves rise sharply at p_H 7.5, and exhibit either a break or a halt, this, however, occurring between p_H 9 and 10, and not between 8 and 9, as for uricase. This effect is best seen in Exp. 4, Fig. 2, as in other cases the final concentration of substrate was too low to give normal values for velocity of oxidation.

Fig. 2.

(iii) *Action of alcohols.*

*iso*Propyl alcohol was added to a number of flasks each containing 0.1 g. of C-Fe and 30 cc. of lithium urate solution at p_H 8. In addition to these flasks, two flasks were prepared, one without alcohol but with charcoal, and the other without charcoal. The uric acid content of all flasks was determined after 24 hrs. at 16°. The results, given in Table I, show that 0.5 % alcohol exerts only a negligible inhibitive effect on oxidation, whilst at concentrations of 1, 1.5, 2, 3, 4 and 5 % inhibition amounts to 37 %; this figure is taken as the maximum possible inhibition attainable by the presence of limiting

concentrations of alcohol. Slightly different results were obtained using *n*-butyl in place of isopropyl alcohol; in this case (Table I) equal inhibition of oxidation took place with 0.5 % as with 10 % alcohol; the limiting inhibitory effect was in this case 25 %. Using 0.2 in place of 0.1 g. of C-Fe, the results obtained using *n*-butyl alcohol were again different, as now no inhibition was observed at concentrations of 0.5 and 1.0 %, whilst inhibition with 10 % alcohol amounted to 38.3 %.

Table I. *Action of alcohols.*

Exp. 1. 0.1 g. C-Fe. All flasks contain 30 cc. of uric acid solution (17.7 mg.) at p_H 8.

isoPropyl alcohol (%)	0	0.5	1, 1.5, 2, 3, 4, 5
Uric acid after 24 hrs. at 16° (mg.)	12.0	12.3	14.1
Uric acid oxidised (mg.)	5.7	5.4	3.6
Maximal inhibition (%)	(5.7-3.6)	100/5.7=37	

Exp. 2. All flasks contain 30 cc. of uric acid solution (19.2 mg.) at p_H 8

<i>n</i> -Butyl alcohol (%)	0	0.5	1.0	3.0	5.0	10.0	} 0.1 g. C-Fe
Uric acid after 22 hrs. at 16° (mg.)	14.4	15.6					
Uric acid oxidised (mg.)	4.8	3.6					
Maximal inhibition (%)	(4.8 - 3.6)	100/4.8 = 25					
<i>n</i> -Butyl alcohol (%)	0	0.5	1.0	3.0	5.0	10.0	} 0.2 g. C-Fe
Uric acid after 22 hrs. at 16° (mg.)	11.4	11.4	11.4	12.6	13.5	14.4	
Uric acid oxidised (mg.)	7.8	7.8	7.8	6.6	5.7	4.8	
Maximal inhibition (%)	(7.8 - 4.8)	100/7.8 = 38.3					

(iv) *Action of potassium cyanide and of sodium pyrophosphate.*

The results obtained with alcohols indicate that the reaction of oxidation by charcoal consists probably of two distinct reactions, one inhibited by small concentrations of alcohol, whilst the other proceeds even at high concentrations. This phenomenon was further analysed by an investigation of the inhibitory action of potassium cyanide and of sodium pyrophosphate. The former agent is well known to be a powerful anti-catalyst, whilst the latter has been shown by Warburg and Sakuma [1923] and by Meyerhof and Matsuoka [1924] respectively to inhibit the reactions of oxidation of cysteine and of laevulose catalysed by iron but not by copper.

In most cases 50 cc. of solution containing from 10.2 to 50 mg. of uric acid dissolved in lithium carbonate and potassium cyanide to $M/1000$ or sodium pyrophosphate to $M/250$ or $M/100$ were taken. The p_H of unbuffered solutions was 8.5. Such systems, with or without 0.1 g. of charcoal, were left for 8-24 hrs. at 37°, and after filtration the uric acid was determined. In one case (Exp. 12) the system had a total volume of 40 cc., and consisted of 20 cc. of uric acid solution containing 29.2 mg. of uric acid, 10 cc. of Sørensen's glycine-sodium hydroxide buffer to p_H 8.1, and 10 cc. of $M/250$ potassium cyanide or of water; this system was kept for 6 hrs. at 16°. The results (Table II) show that cyanide has in every case an inhibitory influence on the action of Kahlbaum's blood-charcoal, on C-Fe, and on C-Cu, although uricolysis is not entirely prevented. Inhibition is, however, greater the earlier the stage at which the reaction was interrupted; this is comprehensible on the basis of the existence

of two parallel reactions, and, in this respect, the results obtained with cyanide confirm those obtained with alcohols. In the case of pyrophosphate, the reaction appears to be accelerated; thus in Exp. 1, 34.5 mg. are oxidised in the presence of $M/250$ pyrophosphate, and 33.75 in its absence; in Exp. 5, 12 mg. are oxidised in the presence of $M/100$ pyrophosphate, and 7.8 mg. in its absence. That this is due exclusively to alkalisation of the solution, with consequent enhanced activity of the charcoal, is shown most clearly by Exp. 8, in which the solutions are buffered to p_H 6.5. When pyrophosphate is added without previous neutralisation, the p_H rises to 8.5, and oxidation amounts to 4.2 mg., whilst, when neutral pyrophosphate is added, only 1.7 mg. of uric acid are oxidised; in the absence of pyrophosphate the same value of 1.7 mg. is found. It follows that pyrophosphate is without influence on the reaction of oxidation of uric acid in the presence of active charcoals. In Exps. 9, 10 and 11 the action of charcoal on the same systems was studied after a lapse of 6 and 24 or 8 and 22 hrs.; in every case it will be seen that oxidation has proceeded further, showing that the action of the charcoal is continuous. On the whole, it will be seen that the action of C-Fe is more intense than that of C-Cu, and that pyrophosphate very slightly activates the latter preparation.

Table II. *Action of potassium cyanide and of sodium pyrophosphate.*

Uric acid (mg.)																
No. of exp.	Variety of charcoal	Total vol. of solution cc.	p_H	Time hrs.	Temp. ° C.	in control	with charcoal									Remarks
							alone		with $M/100$ KCN			with $Na_4P_2O_7$				
							found	oxi- dised	found	oxi- dised	% inhi- bition	found	oxi- dised	conc. $Na_4P_2O_7$		
1	Kahl- baum's blood charcoal	50	8.5	12	37	50	16.25	33.75	26.5	23.5	30	15.5	34.5	$M/250$	Solutions not buffered	
2	C-Fe	"	"	24	"	36	0	36	10.5	25.5	29	0	36	"	"	
3	C-Cu	"	"	24	"	36	1.25	34.75	8.5	27.5	26.4	0.75	35.25	"	"	
4	C-Fe	"	"	8	"	36.5	27.5	9.0	32.5	4.0	44	26.0	10.5	$M/100$	"	
5	"	"	"	16	30	34.5	26.7	7.8	—	—	—	22.5	12.0	"	"	
6	C-Cu	"	"	16	"	34.5	29.2	5.2	—	—	—	29.2	5.2	"	"	
7	C-Fe	"	6.5	5	37	11.7	10.0	1.7	—	—	—	8.0	3.7	"	Phthalate buffers to p_H 6.5. p_H with $Na_4P_2O_7$, 8.5	
8	"	"	"	6	"	10.2	8.5	1.7	—	—	—	8.5	1.7	"	$Na_4P_2O_7$ neutralised	
												6.0	4.2	"	p_H 8.5 $Na_4P_2O_7$ not neutralised	
9	"	"	"	6	"	10.7	8.7	2.0	—	—	—	8.7	2.0	"	$Na_4P_2O_7$ neutralised	
	"	"	"	24	"	10.7	5.3	5.4	—	—	—	5.3	5.4	"	"	
10	"	"	"	8	"	10.6	5.2	5.4	—	—	—	4.5	6.1	"	"	
	"	"	"	22	"	"	0.3	10.3	—	—	—	0.3	10.3	"	"	
11	C-Cu	"	"	8	"	"	8.7	1.9	—	—	—	8.45	2.15	"	"	
	"	"	"	22	"	"	6.6	4.0	—	—	—	5.3	5.3	"	"	
12	C-Fe	40	8.1	6	16	14.7	10.6	4.0	12.2	2.4	40	—	—	—	Sørensen's glycine-NaOH buffers	

(v) *Contact nature of action of charcoal.*

The following experiments were performed in order to exclude the possibility that oxidation in the presence of our charcoals is due not so much to surface action as to dissolved salts extracted from the catalyst; iron and

copper ions have, as is known, a powerful catalytic action on the spontaneous oxidation of uric acid. Systems consisting of 40 cc. of uric acid solution containing 0.322 mg./cc. were left for 2 hrs. at 37° with 0.1 g. of C-Fe or C-Cu, after which they were filtered and uric acid was determined (0.258 and 0.263 mg./cc. respectively). The filtrates were then left for 48 hrs. at 37°, and uric acid was again determined. The values found were identical with those obtained immediately after filtration, showing that the oxidative action of C-Fe and C-Cu is exclusively a contact process.

(vi) *Influence of shaking on velocity of reaction.*

Six flasks each containing 30 cc. of 0.118 % uric acid solution were prepared. To 4 of the flasks 0.1 g. of C-Fe was added, and all flasks were then tightly stoppered and kept at 37°. Two flasks with charcoal were shaken continuously, whilst the remainder were allowed to stand. At the expiration of 6 hrs. all systems were filtered, and uric acid was determined in the filtrates. The results (Table III) show that the reaction proceeds 1.7 times as rapidly with shaking as without. Since analogous results have been obtained with dog-liver (Part II) and with ox-kidney extracts (Part IV), this would support the view that catalytic reaction is in both cases due to contact action on the surface of macroscopic particles.

Table III. *Influence of shaking on velocity of reaction.*

Each flask contains 30 cc. of 0.118 % uric acid solution at p_H 8.5.

System	Uric acid (mg.)		Oxidation %
	found	oxidised	
No C-Fe	35.4	—	—
0.1 g. C-Fe, no shaking	26.4	9.0	25.4
0.1 g. C-Fe, shaking	20.1	15.3	43.2

C. *Action of suspensions of biologically non-uricolytic tissues.*

The researches described in Part II of this series show that it is doubtful whether uricase can be obtained in solutions free from cell debris, and the general similarity of the p_H curves of ox-kidney uricase preparations and of C-Fe suggests the possibility that the reaction is in the former case one of contact catalysis on the surface of fragments of cell constituents. The action of suspensions prepared in the same way as those of ox-kidney, but using tissues generally considered to be devoid of uricolytic action, was next studied, in order to obtain comparative material. The tissues studied were ox-muscle, chicken-liver, and human liver. These were taken as soon as possible after death, in order to minimise the possibility of infection and of *post mortem* changes; the human liver was taken from a 5 months' foetus 30 mins. after delivery. These tissues were treated in the same way as was ox-kidney, and the resulting suspensions were added to sterile solutions of uric acid at various p_H values, as before.

Muscle extracts were clear, and differed visibly in this respect from kidney

or liver extracts. Determinations of uric acid, performed after 5 and 7 days of contact at 17°, showed that oxidation of uric acid had not taken place in any of the systems (Table IV). No uric acid was found in systems consisting of 10 cc. of extract, 20 cc. of buffer solutions, and 10 cc. of water.

Table IV. *Action of ox-muscle, chicken-liver, and human liver glycerol-chloroform extracts on uric acid.*

Each flask contains 20 cc. of buffer solution, 10 cc. of uric acid or water, and 10 cc. of extract or water.

Material	p_H	Uric acid (mg.)				
		in control	in extract	in whole system		
				at beginning	after 120 hrs.	after 168 hrs.
Ox-muscle	5.7, 6.3, 7.4, 8.0, 8.5, 9.0	14.60	0.0	14.6	14.6	14.6
Chicken-liver	6.0, 7.6, 8.4, 8.8, 9.2	10.0	4.4	14.4	after 72 hrs. 14.4	—
Human liver	5.9, 7.4, 9.0	13.8	0.0	13.8	after 84 hrs. 13.8	after 110 hrs. 13.8
„	11.2	11.2	0.0	11.2	11.2	11.2

Chicken-liver extracts differed from the preceding both in appearance—they were turbid, and on standing only very gradually clarified—and in uric acid content. The final uric acid content of the extract may consist partly of preformed uric acid, and partly of uric acid formed during the course of reaction from aminopurines by the action of purine oxidases. Since the activity of these enzymes might be different according to the p_H of the solution, a series of flasks containing 20 cc. of buffer solutions, 10 cc. of water and 10 cc. of extract were prepared, and the final uric acid contents were added to those of the control flasks containing uric acid but no extract. The results (Table IV) show that the final uric acid content of the extract is the same at all p_H values taken, and that the uric acid content of systems containing 20 cc. of buffer solutions, 10 cc. of extract, and 10 cc. of uric acid solution is the same after 72 hrs. as the sum of the two control flasks. It follows that chicken-liver extracts, although macroscopically similar to ox-kidney extracts, have no uricolytic action.

Substantially the same results were obtained with human liver extracts, except that in this case merely traces of uric acid were found in the extract.

DISCUSSION.

The p_H curve of the action of ox-kidney uricase rises sharply at about p_H 7.4 and shows a break at p_H 8. The shape of the corresponding curve using C-Fe is on the whole similar, with, however, the halt at about p_H 9. The curve obtained by Felix *et al.* [1929] for pig-liver powder possesses a similarly

composite form. The fact that our charcoal does not adsorb uric acid indicates that adsorption is not necessarily an essential feature of uricolysis in macro-heterogeneous systems, although in the case of liver tissue extract, Harpuder [1922] has shown that the proteins found in the extract adsorb uric acid more strongly than do those of any other organ extract; it may be that the differences found between Felix's curves and ours are due to this specific adsorption. Thus differential elution at different p_H values may, by increasing the concentration of uric acid in solution, increase the velocity of oxidation, thereby apparently increasing the activity of the enzyme.

Shaking greatly increases the rate of oxidation in the presence both of active charcoal and of ox-kidney extracts (described in Part IV of this series); in this way, too, the action of these catalysts presents points of similarity. The conclusion might be drawn from the results of this experiment, as well as from Przyłęcki's experiments on filtration through Berkefeld filters of active extracts, that the catalyst is in both cases present in close association with macroscopic particles, and not in colloidal solution or as an extractive.

As to the composite nature of contact uricolysis under the influence of the two catalysts studied, as illustrated by the p_H curves, further analysis of the action of charcoal in the presence of commonly used inhibiting substances, such as alcohols, cyanide, and pyrophosphate, showed that inhibition is for the first two agents only partial (the actual value obtained depends, obviously, on the stage of reaction at which measurements were made, since, given sufficient time, the reaction would in all cases proceed to completion), and nil for pyrophosphate. These results point to the composite character of the catalyst; it is probable that, in the case of C-Fe, two types of active centre exist, one containing iron, and inactivated by the agents used, and the other possibly carbon itself—this would, at least on the surface, and at the temperature of ignition applied (white heat) consist largely of graphite. It has been shown (Zylbertal, unpublished work) that birch charcoal free from metals possesses considerable catalytic activity in the reaction in question.

By analogy, and in view of the inactivity of similarly prepared extracts of non-uricolytic organs (muscle, chicken and human liver) it would appear possible that uricolysis catalysed by tissue extracts is due to certain surfaces, whether intra- or extra-cellular, possessing a specific structure, as well as active centres, probably associated with some heavy metal. Whether *in vivo*-uricolysis is catalysed by the same agents, or whether these are artefacts produced by the manipulations to which the tissues in question are subjected, it is not yet possible to state with certainty; the fact, however, that similarly treated corresponding organs of non-uricolytic animals do not exert this influence would on the whole support the former view. Further, the blood is, in all species of animals, devoid of uricolytic power; the absence of uricase and the presence of most other enzymes in the blood would militate against the probability of uricase existing in animal organisms in some soluble form.

SUMMARY.

1. The velocity of oxidation of uric acid in the presence of ox-kidney uricase is greatest at p_H 8.
2. Similarly prepared extracts of non-uricolytic tissues (muscle, chicken and human liver) have no uricolytic action over the p_H range considered.
3. Warburg and Brefeld's active charcoal (C-Fe) does not adsorb uric acid.
4. The action of this charcoal on uric acid is approximately constant up to p_H 7.5; above this value the velocity of oxidation rapidly increases to a maximum value at about p_H 9.
5. The velocity of oxidation in the presence of C-Fe is 1.7 times as great when the systems are shaken as without shaking.
6. *iso*Propyl and *n*-butyl alcohols and potassium cyanide only partially inhibit the uricolytic action of charcoal.
7. Sodium pyrophosphate does not inhibit the uricolytic action of charcoal.
8. The contact action of charcoal catalyst is probably of a compound nature, both active surfaces and centres entering into the process.
9. It is concluded that the activity of extracts (suspensions) is associated with the presence of macroscopic fragments of cell debris; the process of biological uricolysis would thus be one of contact catalysis on certain specific macroscopic surfaces.

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