XXXII. URICASE AND ITS ACTION.

V. FURTHER EXAMINATION OF OX-KIDNEY URICASE.

By RICHARD TRUSZKOWSKI.

*From the Biochemical Laboratory, Medical Faculty,
The University, Warsaw.*

(Received January 25th, 1932.)

INTRODUCTION.

It was shown in Part IV of this series [Truszkowski, 1930] that no uricolytic preparation could be separated from ox-kidney tissue by the action of aqueous solutions at p_H 6–11.5, by dissolution in alkali with subsequent reprecipitation by acid, or by elution with *n*-butyl alcohol; in all cases either the activity of kidney tissue was destroyed, or the extracts were inactive. It is, however, obvious that the conclusion that the whole of the insoluble constituents of kidney tissue are identical with uricase cannot be drawn on the basis of these experiments.

It is reasonably certain that all water-soluble kidney constituents are inactive, as was shown by experiments on centrifuged extracts. Both the fine particles in suspension and the exhaustively extracted residue of coarsely divided kidney tissue are active, the former much more so than the latter. The problem of the further purification and possible closer identification of uricase thus resolved itself into the problem of fractionating a mixture of insoluble substances.

There are three possible modes of attack of this problem—mechanical, chemical or biological. To the first belong methods such as sedimentation of suspensions or separation by dissection. Of the second type, the action of various factors which might dissolve inactive material, leaving a residue of more active uricase, or *vice versa*, will be described in this paper. The third method of attack would consist in administering to experimental animals poisons acting selectively on the glomeruli or tubules, and examining the kidneys in the usual way for uricase. It being impossible to use oxen as experimental animals, the success of this mode of attack depends on finding a laboratory animal possessing uricase in its kidneys, and of sufficient size to yield a workable amount of material. This limits the choice to dogs, cats or rabbits; as will be shown in the present paper, the kidneys of none of these animals contain appreciable quantities of uricase, so that, in the case of kidney-uricase, this method had to be abandoned. Work is, however, now in hand on the localisation of liver-uricase by an analogous method.

EXPERIMENTAL.

Uricase was prepared from ox-kidney by the methods described in Part IV of this series. Uric acid was determined colorimetrically by Folin's method. Solutions of uric acid were prepared by dissolving the acid in boiling saturated lithium carbonate, and making up to the desired volume with distilled water. The p_H of such solutions was 7.5–8.0.

(1) *Sedimentation of uricase suspensions.*

A litre of uricase suspension in chloroform water was left 24 hours in a cylinder at room temperature; 7 successive portions, every one of about 140 cc., were removed from the cylinder by means of a siphon and centrifuged, and 0.2 g. of the residue from every portion was shaken for 1 hour at 37° with 30 cc. of 0.05 % uric acid solution. The results, given in Table I, indicate that

Table I. *Sedimentation of uricase suspensions. Control systems contain 14.7 mg. uric acid.*

Successive portions from above down	Mg. uric acid		
	Found	Oxidised	Oxidised per g. uricase per hr.
1	6.98	7.72	38.6
2	7.35	7.35	36.7
3	6.98	7.72	39.6
4	6.6	8.1	40.5
5	7.5	7.2	36.0
6	6.98	7.72	38.6
7	8.62	6.08	30.4

only the lowest layer of suspension gives a comparatively less active preparation of uricase than the remaining six layers, the activities of the suspensions from which were very similar in all cases; since the lowest layer consisted chiefly of coarse particles, the relatively feeble action of this preparation might be due as much to the smaller surface of contact offered by unit weight of substance as to its higher content of inactive constituents. Further work in this direction was abandoned in view of the negative results obtained.

(2) *Preparation of uricase from isolated medullary and cortical tissue.*

On section of ox-kidney the medullary portions are visible as brownish-red patches, surrounded by greyish-yellow cortical tissue. The cortex was removed, practically free of medullary tissue, and the residue was as far as possible freed of cortical tissue. All fragments which appeared to contain both types of tissue were rejected, and the cortex and medulla were minced and shaken with chloroform water for 30 minutes; the suspensions were roughly filtered through wire gauze and centrifuged. The residue left by cortical suspensions was greyish-white in colour, whilst medullary uricase was brownish-yellow. 10 cc. of 1 % suspensions of both preparations were added to 20 cc. portions of uric acid, 2–3 cc. of toluene were added, and the flasks were left for 44 hours at 30°, after which

uric acid was determined. As is shown in Table II, cortical uricase is about 25 % more active than is medullary.

Table II. *Action of medullary and cortical tissues.*

Uricolytic preparation taken	Total volume of system cc.	Temp. and duration of exp.	Mg. uric acid		
			Initially present	Found at end of reaction	Oxidised
0.1 g. centrifuged medullary suspension residue	30	30° Stood 44 hrs.	24.6	4.35	20.25
0.1 g. centrifuged cortex	30	do.	24.6	0.42	24.18
2 g. washed extracted medullary tissue	25	34° Shaken 2 hrs.	33.1	11.7	21.4
2 g. washed extracted cortex	25	do.	33.1	3.6	27.5

The residues of coarsely divided kidney tissue (3–4 mm. cubes) left after filtering the suspensions were thoroughly washed in running water, and 2 g. of cortical and of medullary tissues were added to 25 cc. of uric acid, and the systems were shaken for 2 hours at 34°, after which uric acid was determined. As with kidney extract suspension residues, the activity of washed cortical tissue is about 25–30 % greater than is that of medullary tissue.

Whilst it is realised that it is not possible to effect complete separation of cortical from medullary tissue, or *vice versa*, by the rough methods employed, yet the results obtained are strong evidence that uricolytic properties are possessed by the cells of both parts of the kidney. The somewhat greater activity of cortical tissue may be due to its greater friability, as a result of which the dispersion of the catalyst is increased relatively to a greater extent during the reaction, as well as to the greater content of connective tissue elements in the medulla.

(3) *Action of ethyl alcohol and ether.*

Three 4 g. portions of uricase were left 24 hours at 37° with 30 cc. of alcohol, ether and water respectively, the suspensions were centrifuged, and 0.2 g.

Table III. *Action of C_2H_5OH and of $(C_2H_5)_2O$.*

Preparation taken	Mg. uric acid	
	Found	Oxidised
Water-extracted uricase	3.37	5.33
Ether-extracted uricase	5.4	3.3
Alcohol-extracted uricase	6.6	2.1
Ether and alcohol extracts	8.7	0
Control	8.7	—

portions of the washed residues were shaken 90 minutes at 37° with 30 cc. of uric acid solution. The filtered ethereal and alcoholic centrifugates were evaporated at 18° under reduced pressure, and the colourless or faintly yellow oily residues were similarly shaken 90 minutes with 30 cc. of uric acid. The

results, given in Table III, indicate that the lipoidal constituents of kidney tissue are inactive, and that the activity of uricase is greatly reduced by contact with ether or alcohol.

(4) *Action of H^+ .*

It was shown in Part IV that uricase is soluble in alkaline solution, but that it is at the same time irreversibly inactivated. The possibility remained that either uricase or inactive constituents could be dissolved in acid solutions; before work could be done in this direction it was necessary to determine which concentrations of H^+ did not inactivate uricase. 10 cc. portions of McIlvaine's buffer solutions (p_H 2-7.6) were added to a number of centrifuge-tubes containing 10 cc. of 1 % uricase suspension, and the systems were centrifuged after keeping 1 hour at 37° , the centrifugates were taken for the determination of p_H (1.98, 2.67, 3.67, 4.28, 5.29, 6.56, 7.59), and the residues were suspended in 20 cc. portions of distilled water, and again centrifuged. 20 cc. portions of uric acid solution were added to the washed residues, as well as to an extra tube which had been previously wetted with water. Uric acid was determined in all systems after keeping for 15 hours at 37° , in the presence of toluene. 36.8 mg. were found in the control, and in all other systems except that treated with buffers at p_H 7.59, which contained 17.6 mg. (19.2 mg. oxidised). The p_H of all the final systems varied from 7.6 to 7.8.

The above result explains the initial rise in the p_H -oxidation curves, from p_H 6 to 7.4 [1930, Fig. 1, p. 1360]—the diminished oxidation of uric acid with diminishing p_H below 7.4 is due to increasing inactivation of the catalyst by H^+ ; in the same way with p_H above 9.5 the rapid decline in activity is ascribable to inactivation by OH^+ .

A further conclusion to be drawn from the above experiment is that neither solutions more acid than p_H 6.56 nor more alkaline than p_H 9.5 can be used for the purification of uricase.

(5) *Action of oxidising agents, poisons, heavy metals, etc.*

Since the separation of uricase from inactive material cannot, as a result of its instability, be effected by the methods ordinarily applied to the separation of solid mixtures, the following experiments were performed, with the object of obtaining additional evidence as to its chemical nature. To 10 cc. portions of a 2 % suspension of cortical uricase in a number of 30 cc. centrifuge-tubes, 10 cc. of the following solutions (brought to p_H 7 by addition of N HCl or NaOH) were added: 0.5 % KCN, 0.01 N iodine, 0.1 N iodine, 3 % H_2O_2 , 1 % $FeSO_4$, 1 % $CuSO_4$, 1 % $HgCl_2$, and water. The tubes were left 1 hour at 15° , centrifuged (10 minutes), the centrifugates were decanted off, and the residues were shaken three times with 15 cc. of distilled water, the suspension being centrifuged after each addition of water. 20 cc. of uric acid solution were added to every tube, as well as to a wet centrifuge-tube, taken as a control, and all tubes were left with toluene for 15 hours at 37° ,

when uric acid was determined. The control system contained 24.0 mg. of uric acid, as did also the systems containing uricase previously treated with KCN, 0.01 and 0.1 *N* iodine, H_2O_2 , FeSO_4 and HgCl_2 . That containing uricase treated with CuSO_4 contained only 18.0 mg., whilst where water only had been added to uricase 16.4 mg. remained.

It follows that uricase is irreversibly inactivated by all the above reagents, with the exception of CuSO_4 . In this case, two interpretations may apply—either uricase is not inactivated by CuSO_4 , or it is inactivated, but the copper bound by protein or other material present enters into a new uricolytic complex. In view of the action of other heavy metals (Fe, Hg) the latter explanation appears to be more probable, and work on its experimental verification is now in hand.

(6) *Specificity of action of uricase.*

Since certain light might be thrown on the chemical nature of uricase by elucidating its action in oxidations other than that of uric acid, its action on oxalic acid and glycine was next studied. Should its action not be specific for uric acid, it might be possible to include it amongst the types of catalyst postulated by Quastel and Wooldridge [1927] or by Warburg [1928] in biological oxidations in general.

In the oxidation of glycine ammonia is evolved, and the rise in ammonia content of a system containing glycine and uricase would thus be an index of oxidation. 20 cc. of 5 % glycine solution were shaken for 5 hours at 37° with 10 cc. of 5 % uricase suspension, and the coloration given by the filtrate with Nessler's reagent was compared with that given by the filtrate of 20 cc. of glycine solution and 10 cc. of uricase suspension shaken separately, and mixed immediately before filtration. No difference was observed, indicating that under the given conditions no oxidation of glycine had taken place. In a second experiment air was aspirated for 48 hours at 37° through a number of pairs of cylinders, of which the first contained 10 cc. of 5 % uricase, 10 cc. of water, 20 cc. of borate buffer (p_{H} 9.5) and 25 cc. of 0.1 *N* H_2SO_4 respectively, the second the same, substituting 10 cc. of alkaline 5 % glycine for uricase, and acid, and the third glycine and uricase together. The acid in the second cylinder of every pair was then titrated, 11.2 cc. of 0.1 *N* acid being found to have been neutralised in the first and third cylinders, and none in the second. These results indicate that an alkaline gas is evolved by uricase in alkaline solution, and that uricase has no oxidative or deaminative action on glycine.

In the case of oxalic acid, three systems containing respectively 10 cc. each of 2 % uricase suspension and of 0.5 % ammonium oxalate, 10 cc. of uricase and of water, and 10 cc. of oxalate and of water were left for 48 hours at 27°, and filtered, and the number of cc. of 0.05 *N* KMnO_4 required to oxidise 10 cc. of the filtrates was determined. 16.9 cc. of KMnO_4 were used in the case of oxalate alone, 1.0 cc. in that of uricase alone, and 17.8 cc. where both were present together. It follows that the oxidation of oxalic acid is not catalysed by uricase.

(7) *Temperature of inactivation of uricase.*

1 % suspensions of cortical uricase were kept for 5 minutes in water at 20, 30, 40, 50, 60, 70, 80, 90 and 100°, and 20 cc. of uric acid solution were added to the cooled suspensions, which were then kept with toluene for 15 hours at 37°. The control system contained 20.7 mg. of uric acid, as did also those containing uricase heated at 80–100°, whilst 0.15 mg. was oxidised at 70°, 1.35 mg. at 60° and 14.4 mg. at 20–50°, indicating that the inactivation temperature lies between 50 and 60°. This result is in agreement with Batelli and Stern's [1909] findings for ox-kidney uricase.

(8) *Action of KCN and of Na₄P₂O₇.*

It has been shown in Part III of this series that the action of Fe-containing active charcoal on uric acid is largely inhibited by KCN but not by Na₄P₂O₇. Warburg and Sakuma [1923] and Meyerhof and Matsuoka [1924] have, however, shown that the oxidation of cysteine and of laevulose by traces of metal is inhibited by KCN and Na₄P₂O₇ when Fe is present, but only by KCN when Cu is present. Whilst, therefore, the differentiation of these metals by the use of KCN and Na₄P₂O₇ is not universally applicable, yet it is of interest to examine the effects of these reagents on uricase.

With this object, a number of systems were set up, containing 10 cc. of uric acid, 10 cc. of 2 % uricase suspension, and 10 cc. of *M*/166 KCN, 0.21 *M* Na₄P₂O₇, or water (all at *p*_H 7.5), and uric acid was determined after shaking for 90 minutes at 30°. 23.8 mg. were found in the control systems, containing 10 cc. of uric acid solution and 20 cc. of water, 23.7 mg. in the presence of KCN, and 17.25 mg. in the systems containing Na₄P₂O₇ or water.

(9) *Uricolytic action of kidneys of dog, rabbit and cat.*

The uric acid content of systems containing 20 cc. of uric acid and 0.1–1 g. of the residues from kidney extract centrifugates (medulla and cortex taken separately) was determined before and after shaking for 3–5 hours at 40°; in no case was any difference observed. Uricase is not therefore present in appreciable quantity in the kidneys of these animals.

DISCUSSION.

The above experiments demonstrate the impossibility of the purification or concentration of ox-kidney uricase by any of the methods commonly used; the unusual sensitiveness of uricase to H⁺ and OH⁻, oxidising agents, heavy metals, *etc.*, renders any but the mildest chemical methods impracticable. At the same time, sufficient material is available to allow of certain conclusions as to the chemical nature of uricase.

Both the extreme instability of uricase and the results obtained with ether and alcohol extracts make it improbable that the active substance is a lipid.

The insolubility of the active factor excludes a whole series of extractive substances, leaving only proteins to be considered.

Uricase is, therefore, in all probability a protein of highly specific and delicate surface configuration. The inhibitory action of KCN points to the presence of a heavy metal in this protein, and the fact that sodium pyrophosphate does not inhibit reaction suggests that this metal may be copper. The inactivation temperature (50–60°) is one at which many other enzymes and proteins undergo denaturation.

As to the site of action of uricase in the kidney, little definite information is available. It is present in both the cortex and the medulla, that derived from the former being more active, whence it might be concluded that it is a constituent of the epithelium of the renal tubules.

SUMMARY.

1. Ox-kidney uricase suspensions do not yield fractions of different activity on sedimentation.
2. Both medullary and cortical tissues are active, the action of the latter being, however, about 25 % greater than that of the former.
3. Alcohol and ether extracts of uricase are inactive; the residues are less active than is the unextracted material.
4. Uricase is irreversibly inactivated by contact with solutions of p_H 2–6.56, but not of p_H 7.59.
5. Uricase is irreversibly inactivated by neutral solutions of KCN, iodine (0.01 and 0.1 N), H_2O_2 , $FeSO_4$ and $HgCl_2$, but not by $CuSO_4$.
6. Uricase has no action on acid and alkaline glycine solutions, or on alkaline oxalate solutions.
7. Uricase is completely inactivated by heating for 5 minutes at temperatures above 70°, almost completely inactivated at 70°, partly at 60°, and not at all at 50° or less.
8. KCN completely inhibits uricolysis by ox-kidney-uricase, whilst $Na_4P_2O_7$ has no effect.
9. Dog, rabbit and cat kidneys do not contain uricase.

The author has much pleasure in acknowledging his gratitude to Prof. St J. Przyłęcki for his constant interest in the prosecution of this research.

REFERENCES.

- Batelli and Stern (1909). *Biochem. Z.* **19**, 241.
Meyerhof and Matsuoka (1924). *Biochem. Z.* **150**, 1.
Quastel and Wooldridge (1927). *Biochem. J.* **21**, 1224.
Truszkowski (1930). *Biochem. J.* **24**, 1340.
Warburg (1928). Über katalytischen Wirkungen d. lebendigen Substanz J. Springer, Berlin),
— and Sakuma (1923). *Pflüger's Arch.* **200**, 203.