XI. URICASE AND ITS ACTION.

VII. SOLUBLE URICASE.

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It has been shown in Parts II, III and IV [Truszkowski, 1930] of this series that tissue uricase is associated exclusively with the insoluble fraction of the tissues, and that it is not extractable by aqueous solutions of p_H 6–11, by aqueous glycerol or by other agents generally used for extraction of enzymes.

Ro [1931] described a method of extracting uricase from tissues similar in principle to that of Wiechowski and Wiener [1907], whereby he obtained extremely active extracts. Ro's method consists in the acetone precipitation of an aqueous extract of ox-kidney followed by repeated treatment of the precipitate with acetone, alcohol and ether. The white powder thus obtained yields uricolytic solutions on extraction during an unspecified time with 0.125 % Na_2CO_3 solution.

It was found by the present author that the dry preparation prepared in the above manner yielded active filtrates after extraction during 24 or 48 hrs. at 37° with Na_2CO_3 .

The possibility of obtaining soluble uricase by Ro's method having thus been confirmed, the necessity arose of reconciling this finding with the negative results previously obtained. The differences between the two methods consisted in the use of dry, lipoid-free kidney tissue by Ro, in the presence of water-soluble kidney constituents, and in the use of Na_2CO_3 solution for extraction, instead of the glycine-NaOH buffers used by the present author. With regard to the first point, it seemed improbable that the extractability of uricase could be affected by merely drying the tissues; the necessity of the alcohol-ether treatment should lie rather in removal from the tissues of some substance, probably lipoid, which prevents extraction from fresh tissues. As to the second point, it has been shown (Part IV) that the water-soluble constituents are inactive in the fresh state; experiments described below show that the same applies to this fraction after treatment according to Ro's method. Finally, extractions performed on fresh kidney tissue with aqueous Na_2CO_3 were found to yield inactive filtrates.

EXPERIMENTAL.

Ox-kidney uricase was prepared by the method described in Part IV of this series. Uric acid was dissolved in boiling aqueous NaOH (65 ml. 0.1 N NaOH per g. uric acid); the solutions so prepared had p_H 7.5–7.8. Uric acid was determined by the method of Folin and Denis.

1. Conditions of extraction.

Since Ro's paper gives no details as to the duration and temperature of extraction, the first step was to determine these conditions. 1 g. of dry product prepared according to his method was suspended in 100 ml. of 0.125 % Na_2CO_3 ,

1 ml. of toluene was added to ensure sterility, and the activity of the extract was determined after standing 1, 24 and 48 hrs. at 35° by adding 5 ml. of filtered extract to 10 ml. of 0.144 % sodium urate, with 1 ml. of toluene, and determining uric acid after incubation at 35° during 18 hrs. No diminution in uric acid content was observed in 1 hr. extracts, 24 hr. extracts oxidised 6.1 mg., and 48 hr. extracts oxidised 7.2 mg. of uric acid. The residue left after extraction still contains extractable uricase, as was shown by the following experiment. 1 g. of dry uricase was extracted during 48 hrs. at 35° with 100 ml. of 0.125 % Na_2CO_3 , filtered, the extract stored in a stoppered flask with toluene and the residue extracted as before with a fresh portion of Na_2CO_3 , the second residue being again extracted. 5 ml. of the first extract and 5 ml. of Na_2CO_3 solution, and 10 ml. of the second and third, were left at 35° during 18 hrs. with 10 ml. of 0.13 % uric acid, after which uric acid was determined. 6.3 mg. of uric acid had been oxidised by the first extract, 5.4 mg. by the second and 1.0 mg. by the third, corresponding with an activity of 1.26 mg. per ml. of uricase in the first case, 0.54 mg. in the second and 0.1 mg. in the third.

2. Comparison of activity of products and extracts.

Hashed kidney tissue was shaken with 10 volumes of water, the suspension was filtered through cheese-cloth, and a part of the filtrate was treated according to Ro. The remainder was centrifuged, the centrifugate was filtered and the filtrate treated with acetone, *etc.*, by Ro's method; the residue was shaken successively twice with alcohol and twice with ether and then air-dried at 30°. The various products thus obtained were extracted with 0.125 % Na_2CO_3 , and the activity of the extracts and the suspensions in 0.125 % Na_2CO_3 was determined.

The products examined were: Exp. 1, 1 % suspension of residue (I) obtained after centrifuging the suspension (III) of kidney tissue; Exp. 2, the

Table I. Comparison of activity of products. All flasks contain 10 ml. of 0.104 % sodium urate and 10 ml. of uricolytic suspension, extract or 0.125 % Na_2CO_3 .

No. of Exp.	Product examined	mg. uric acid			
		After 18 hrs. at 35°		After 42 hrs. at 35°	
		Found	Oxidised	Found	Oxidised
Control	0.125 % Na_2CO_3 .	10.4	—	10.4	—
1	1 % suspension of untreated uricase (I).	0.4	10.0	0	10.4
2	Filtered centrifugate (II) of tissue suspension (III).	10.2	0.2	8.2	2.2
3	1 % suspension of dry product (IV) obtained from (II).	10.0	0.4	9.2	1.2
4	0.17 % suspension of dry product (V) obtained from (I).	1.4	9.0	0	10.4
5	0.17 % suspension of dry product (VI) obtained from (III).	1.2	9.2	0	10.4
6	1 % extract of (IV).	10.4	0	10.4	0
7	" " " (V).	0.6	9.8	0	10.4
8	" " " (VI).	0.6	9.8	0	10.4
9	6 % " " (I).	10.0	0.4	9.6	0.8

filtered centrifugate (II); Exp. 3, a 1 % suspension of the dry product (IV) obtained by treating (II) according to Ro; Exp. 4, a 0.17 % suspension of the product (V) obtained by extracting 20 g. of (I) successively with two 200 ml. portions of 96 % alcohol, and with two 100 ml. portions of ether and drying at 35°; Exp. 5, a 0.17 % suspension of the dry product (VI) obtained by Ro's procedure; Exp. 6, 7 and 8, 1 % 24 hr. extracts of (IV), (V) and (VI); Exp. 9, a 6 % extract of (I). 1 % suspensions of (I) were compared with 0.17 % suspensions of (V) and (VI) in view of the fact that the dry content of (I) was about 15 %, as compared with 95 % in (V) and (VI).

It follows from the results obtained (Table I) that active products are obtained exclusively from material containing (I); *i.e.* uricolytic activity is associated exclusively with the insoluble parts of kidney tissue. The slight activity shown by the centrifugate and by extracts of (I) is ascribable to very fine, filter-passing particles of (I). A comparison of Exps. 1, 4 and 5 shows that the activity of (I) is very little affected by treatment with acetone, alcohol, ether and by drying. A comparison of Exps. 1, 7 and 8 shows that only a small part of the original activity of (I) is exhibited by the extracts, since the content of anhydrous material taken for extraction was 6 times greater in Exps. 7 and 8 than that present in Exp. 1. Exps. 4 and 5, 7 and 8 indicate that the uricolytic properties of Ro's product are practically identical with those obtained by treating (I) with alcohol and ether. Finally, Exp. 9 shows that uricolytic extracts cannot be obtained from (I) under conditions comparable with those of extraction of (V) and (VI).

3. *Simplified method of preparation of soluble uricase.*

The experiments described in the preceding section indicate that Ro's technique is unnecessarily complicated and results probably in the inclusion of a certain proportion of inactive matter in his dry products. The steps which appear to be superfluous are: 1. allowing the kidney-tissue suspension to stand with thymol; 2. treating the suspension with acetone. The following procedure was adopted in place of Ro's. Uricase is prepared as described in Part IV of this series, shaken during 2 mins. with 10 vols. of 96 % alcohol and rapidly filtered by suction; the process is repeated once more with alcohol and twice with the same volume of ether, after which the product is allowed to dry at room temperature, or at 37°. It is important that residual ether be allowed to evaporate spontaneously, without expressing on filter-paper, as the product obtained in the latter case is lumpy, black and yields relatively inactive extracts. The final product (this will in future be designated "dry uricase") is a white, impalpable powder, containing 5 % of moisture, and can be stored indefinitely. The yields of dry uricase average about 10 g. per kg. of kidney, as compared with 100 g. obtained by Ro. It is difficult to understand how such high yields could have been obtained, as the average dry content of ox-kidneys does not exceed 15–20 %, and by far the greater part of the raw material remains unsuspended. Moreover, of the solid matter of the suspension filterable through muslin, about 10 % is represented by lipoids, removed during the alcohol-ether extraction, and a further 10 % is present as water-soluble extractives. It must therefore be considered that, whilst somewhat higher yields than those obtained by the present author might be obtained by finer comminution of the original tissues, yet in no case could yields much higher than 1.5 % of dry uricase be expected without considerable contamination by inert matter. Solutions of uricase are obtained under the conditions described in section 1 of this paper.

4. *Significance of lipoid extractives.*

The combined alcoholic extracts from 100 g. of uricase were evaporated to dryness under reduced pressure at 38°, when about 3 g. of a semi-solid, oily, yellow residue, completely soluble in chloroform, were obtained. The combined ethereal extracts, similarly treated, yielded about 2 g. of a dry, white residue, M.P. 52–54°, also soluble in chloroform; 1 g. of each residue was dissolved in 100 ml. of ether, and 1 g. in chloroform. Three 0.2 g. portions of dry uricase were introduced into 100 ml. Erlenmeyer flasks, 5 ml. of the respective ethereal solutions or ether alone were added, and the ether was removed under reduced pressure at > 37°. 20 ml. of 0.125 % Na_2CO_3 and 1 ml. of toluene were then added to each flask, their contents were filtered after 48 hrs., and the uricolytic activity of the extracts was determined as usual, taking 10 ml. of Na urate and 2 ml. of filtrate.

Table II.

No. of Exp.	Solution added to dry uricase	mg. uric acid			
		After 18 hrs. at 37°		After 66 hrs. at 37°	
		Found	Oxidised	Found	Oxidised
1	5 ml. ether	3.4	7.4	0	10.8
2	5 ml. ethereal solution of alcoholic extractives	10.8	0	10.8	0
3	5 ml. ethereal solution of ethereal extractives	10.8	0	10.8	0
4	—	10.8	—	10.8	—

Control: 10 ml. sodium urate, 2 ml. 0.125 % Na_2CO_3 .

The results obtained (Table II) indicate that the extractives removed by alcohol and by ether prevent extraction of uricase by aqueous Na_2CO_3 , *i.e.* uricase is fixed on the tissues by the agency of lipoid substances (Exps. 2 and 3); Exp. 1 shows that the results obtained are not ascribable to inactivation of uricase by ether.

This conclusion as to the rôle of lipoids in the systems in question was further established by the following experiments. Three 0.5 g. portions of dry uricase were placed in three 100 ml. flasks; to one, 5 ml. of a 1 : 1 mixture of the chloroform solutions of the alcoholic and ethereal residues were added, to another

Table III.

No. of Exp.	Solution added to dry uricase	mg. uric acid after 24 hrs. at 37°		Remarks
		Found	Oxidised	
1	5 ml. CHCl_3	0.2	9.0	Lipoids added to filtrate.
2	„	0.2	9.0	
3	5 ml. CHCl_3 solution of combined EtOH–Et ₂ O extractives	8.2	1.0	
4	5 ml. CHCl_3 solution cont. 5 mg. cholesterol	2.6	6.6	Control: 10 ml. sodium urate, 10 ml. 0.125 % Na_2CO_3
5	—	9.2	—	

5 ml. of chloroform containing 5 mg. of cholesterol, and to the third 5 ml. of chloroform. As before the solvent was evaporated at 37°, and the dry residues were extracted by 25 ml. of 0.125 % Na_2CO_3 during 48 hrs. at 37°. 10 ml. of each extract and 1 ml. of chloroform were then added to 10 ml. of sodium urate, and uric acid was determined after 24 hrs. at 37°; in addition, to a second 10 ml. portion of the extract of uricase to which lipoids had not been added, 1 ml. of the combined chloroform solution was added instead of pure chloroform.

The above experiments (Table III) confirm those of Table II; the slight activity shown by the extract in Exp. 3 as compared with the analogous experiment of Table II is probably ascribable to the greater concentration of uricase (10 times greater). Exp. 4 shows that the action of tissue lipoids is not necessarily specific, as the activity of the extract is reduced by 27 % by 5 mg. of cholesterol (50 mg. of lipoids were present in Exp. 3). Finally Exp. 2 proves that the lipoids do not inactivate extracted uricase, *i.e.* that their action consists in preventing extraction and not in inactivating the enzyme.

5. Properties of soluble uricase.

The extracts of soluble uricase are clear or slightly opalescent, straw-coloured liquids, possessing an odour similar to that of aliphatic amines and foaming on shaking. On keeping, they become turbid but do not lose their activity. Quite definite protein reactions are obtained (Millon, Adamkiewicz, biuret), and the N content amounts to 0.08–0.13 %. The p_{H} of the extracts is invariably less than that of the original Na_2CO_3 solution (p_{H} 9.12).

A preliminary survey of the properties of soluble uricase was made, preparatory to more systematic work. The first problem was that of further purification by means of filtration. A suspension of 1 g. of dry uricase in 100 ml. of aqueous Na_2CO_3 was allowed to stand during 24 hrs. at 35°, and 10 ml. of the well mixed suspension (I) were measured into a 100 ml. flask. The remainder of (I) was then filtered through filter-paper, 10 ml. of the filtrate were taken for examination, and the remainder was filtered through a Berkefeld filter; finally, a portion of this filtrate (III) was passed through a Bechhold ultra-filter (filtrate IV). 10 ml. of sodium urate solution and 1 ml. of toluene were added to 10 ml. of the various preparations examined, and uric acid was determined after 18 hrs. at 35°.

Table IV.

No. of Exp.	Nature of preparation	mg. uric acid after 18 hrs. at 35°	
		Found	Oxidised
1	Control	14.4	—
2	Suspension	0	14.4
3	Filtrate (II)	6.6	7.8
4	Berkefeld filtrate (III)	7.0	7.4
5	ditto, 24 hrs. later	7.0	7.4
6	ditto, refiltered	9.0	5.4
7	ditto, 4 days later	9.2	5.2
8	Bechhold filtrate (IV)	9.5	4.9

Filtrates (III) and (IV) were water-clear but (III) became turbid on standing during 24 hrs. at room temperature, without, however, losing any of its activity (Table IV, Exp. 5). Part of the activity is in the precipitate, as is shown by Exp. 6, in which the Berkefeld filtrate of the turbid extract was examined. The clear, second filtrate again became turbid on keeping a further 4 days, with slight inactivation (Exp. 7). The Bechhold ultra-filtrate, obtained using a membrane impermeable to ovalbumin, exhibited moderate activity (Exp. 8). Protein reactions were given by all filtrates, in decreasing order of intensity.

6. *Action of ammonium chloride and of methylamine hydrochloride on uric acid.*

In view of Schittenhelm and Chrometzka's [1927] contention that ammonia acts uricolytically, and of the ammoniacal odour of the extracts, it was thought desirable to investigate the possibility that the activity of the latter might be due to ammonia or methylamine. With this object, a number of systems were set up, consisting of 10 ml. of 0.1 % sodium urate, 10 ml. of 0.125 % Na_2CO_3 , and 10 ml. of 0.08 % NH_4Cl , or of 0.02 %, 0.04 % or 1 % $\text{CH}_3\text{NH}_2\cdot\text{HCl}$. The uric acid content, determined after 24 hrs. and 48 hrs. at 37°, remained constant, pointing to the non-identity of uricase with these substances.

DISCUSSION.

From the biological point of view the most interesting phenomenon of those described above is that of the action of lipoids in preventing extraction of uricase. Whilst it would be premature to enter into a detailed consideration of the mode of action of lipoids in this connection, yet it appears from a number of investigations now in progress in this laboratory that this binding action of lipoids is of considerably wider incidence than in the immobilisation of enzymes on tissues, evidence of its presence having been found in widely different biological processes. For the purposes of this paper, however, the conclusion may be drawn that biologically uricase acts as an insoluble enzyme.

The preliminary survey of the properties of soluble uricase confirms Ro's finding that uricolytic activity is indissociable from traces of alkali-soluble protein. This protein is of relatively small molecular weight, as it passes freely through an unglazed porcelain filter and partly through a membrane impervious to ovalbumin. Further work on soluble uricase will be described in the succeeding parts of this series.

SUMMARY.

1. Ro's method [1931] for the preparation of soluble uricase has been investigated, and his results have been substantially confirmed.

2. The non-extractability of uricase from fresh tissues is attributable to the presence of lipoids; after their removal by the action successively of alcohol and ether, uricase may be extracted by dilute aqueous Na_2CO_3 .

3. A consequence of the above finding is that uricase acts *in vivo* as a contact catalyst.

4. Dissolved uricase passes freely through Berkefeld filters, and partly through Bechhold filters, the membrane of which is impermeable to ovalbumin.

5. Uricolytic activity varies roughly in proportion to the intensity of the protein reactions of the solution.

6. Ammonium chloride and methylamine hydrochloride have no uricolytic action; soluble uricase is therefore not identical with these substances.

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REFERENCES.

- Ro (1931). *J. Biochem.* **14**, 361.
Schittenhelm and Chrometzka (1927). *Z. physiol. Chem.* **162**, 203.
Truszkowski (1930). *Biochem. J.* **24**, 1341, 1349, 1359.
Wiechowski and Wiener (1907). *Beitr. chem. Physiol. Path.* **9**, 247.