CXXVII. URICASE AND ITS ACTION.

PART I. PREPARATION.

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Two main tendencies may be distinguished in the development of modern enzymology. One of these consists in the chemical analysis of the enzyme and the other in the study of the action of the enzyme under natural physiological conditions.

The consensus of opinion now is that an enzyme must possess two characteristic properties—a definite physico-chemical state, whose change involves changes in the activity of the enzyme, and an active and specific group or centre.

In the present state of our knowledge we cannot yet state in what depends the specificity of an enzyme, except that no doubt exists that the colloidal carriers and active centres may be very different. It may be as a result of the specific binding with the substrate that a given enzyme acts upon any particular substance. As to the active groups it would appear that even in one group of enzymes, such as, for example, the oxidases, there are purely organic groups as well as others containing some metal, which is not in every case the same. In some cases this is iron, whilst in others, *e.g.* uricase, copper is present, as will be shown in Part III of this series.

It appears to us that the researches of Warburg [1928], as well as the application of micromethods to purified enzyme preparations, will, at least for the oxidases, allow the nature of this active group to be to a considerable extent elucidated.

It is not known whether only one or several active groups are present, or whether these are identical; this applying in particular to such complex enzymes as succinoxidase. This problem is of the utmost importance for those enzymes which influence reversible reactions of synthesis from two different substrates. Not only the carrier, but also the active group, may possess a specific character. Further research upon the structure of enzymes should therefore take both these factors into account, analysing each separately.

At the present stage of development of biochemistry it would appear that the study of the active group is likely to yield more fruitful results than the study of the colloidal carrier, in particular in those cases where the active

group contains some metal. As for the carrier, comparative research might be carried out upon it, such as the effect of changing p_H and temperature, and of inhibitory agents upon the action of the same enzyme derived from different organisms.

Certain of the features possessed by the carrier might, however, be elucidated at the present juncture, such as whether or not the same enzyme derived from different animals or plants possesses the same colloidal carrier. Such investigations should be carried out upon an enzyme acting specifically only upon one compound, in order to exclude as far as possible the danger of working upon mixtures of several enzymes, or upon different enzymes of closely related but not identical functions.

Such experiments might include determinations of the velocity of diffusion through various membranes, of solubility, of the action of temperature, of the precipitating effect of various salts, etc., as well as possibly the effect of changing p_H and the investigation of phenomena of cataphoresis, although here the active group also may be involved.

Similar research would also be desirable for the investigation of the active group. It is possible that several groups having a similar function might act in a similar or even identical manner upon a substrate, whence it might appear that two enzymes are identical, although both their active groups and their colloidal carriers may be different. This is all the more important and interesting in that the same substrate may be decomposed by a given organism under the influence of various catalytic factors.

Due consideration should, of course, be given to the purely energetic aspect of oxidations catalysed by various substances, *i.e.* to the relationship between energy absorbed during an endothermic reaction and evolved during a coincident exothermic reaction. This factor, whilst of first-rate, and for some reactions of oxidation decisive, importance, is not the only one concerned—questions of chemical affinity also play an important rôle. A number of other factors might, however, be involved, entirely distinct from the question of affinity for oxygen.

The first of these is adsorption. A second factor is that of the possibility of some chemical change occurring in the substrate under the influence of specific adsorption. It is quite probable that the introduction of certain compounds leads to such changes in the structure of the substrate as would facilitate its combination with oxygen under the influence of certain chemically-active groups which are, however, distinct from those directly responsible for oxidation.

Such sensitisation might, however, also be brought about by the agency of ordinary non-specific adsorption, and in view of the possibility of the existence of different types of adsorption of the same substance upon different adsorbents, the same active group may act differently according to the circumstances.

The specific action of the various oxidases may therefore depend upon a number of factors. These may be due not only to fundamental differences in

the active group but equally well to the concentration of the substrate within the close vicinity of peroxides, and to changes in the substrate brought about by adsorption or by supplementary groups of the adsorbent, distinct from those responsible for transference of oxygen or activation of hydrogen. These factors are all functions of the carrier, and support the view that both the carrier and its active group are concerned in the specific action of a given enzyme.

The carrier may be supposed to play a more or less important rôle in producing chemical change of the substrate, in accordance with the nature of the action of the given ferment, but the necessity of the existence of some spatial configuration favourable to oxidation must be emphasised. This consideration applies equally to reactions of synthesis, in which the combination of two different substances depends upon the active groups being so placed that the substrates are able to come into contact [cf. Euler, 1926].

Many of the above problems are at present capable of solution. In the research of which this is the first communication they will be considered, using as experimental material uricase, a widely distributed and unusually specific ferment, acting exclusively upon uric acid.

The experimental programme in view was first to prepare as pure as possible samples of uricase from different sources, such as bovine kidneys, frogs and wheat seeds, and to compare the carriers and the active groups of these preparations with a view to establishing ultimately their identity or otherwise. Before commencing these investigations, however, it was necessary to establish whether uricase is a dehydrogenase or a true oxidase.

At the same time work is proceeding in this laboratory upon the oxidation or decomposition of uric acid through the agency of a biocatalyst, and of various purely chemical catalysts possessing an oxidising or dehydrogenating influence, as it is our opinion that only a very wide programme of research will here lead to the elucidation of the rôles of the colloidal carrier and the active group.

EXPERIMENTAL.

I. *The purification of frog uricase.*

The enzyme was purified by precipitation with acetone, alcohol or ammonium sulphate, or by dialysis.

The precipitation by means of acetone was carried out according to Przylecki [1925]. 300–500 g. live weight of frogs were comminuted in a mincing-machine, and the hash was left for 48 hours in a mixture containing 50 g. of chloroform, 100 g. of glycerol and 500 cc. of water. Water was then added in amount equal to ten times the weight of tissue taken and the mixture left for 24 hours with occasional shaking, after which it was filtered through cotton-wool.

Two volumes of acetone were added to one of the extract and the flocculent precipitate was filtered after 30–60 minutes through a fluted filter-paper and

allowed to dry on the paper. The filtrate contained no uricase, which was present in its entirety in the residue.

Uricase may be precipitated by acetone and left in contact with the latter for several days and washed, after filtering, with 10–20 litres of 65 % acetone without any impairment of its activity, as is shown by the results given in Table I. The apparent diminution in the yields of uricase previously reported by the author was due, not to any deleterious effect of acetone upon the enzyme, but to its incomplete removal from the precipitate before solution in water or physiological saline solution. As a result of this, acetone was present in the reaction mixture and able to exert its inhibitory influence, possessed in common with alcohols, upon the reaction of uricolysis. This inhibitory action is, as will be shown later, reversible under certain conditions.

Table I. *mg. of uric acid found in 1 cc. of solution consisting of equal volumes of sodium urate solution at p_H 7.5 and of acetone-precipitated uricase.*

Serial no. of preparation	Uric acid at beginning of experiment	Material used as source of uricase	After 12 hours' action of extract of acetone precipitate								
			Residue left on evaporation of acetone filtrate	Acetone precipitate washed with 30 litres of acetone	Time in hours intervening between addition of acetone and filtration						
					$\frac{1}{2}$	1	6	12	24	51	72
5	0.44	Bovine kidneys	0.43	0.09	0.09	0.08	0.09	0.08	0.08	0.09	0.09
7	0.71	Bovine kidneys	0.71	0.10	0.12	0.11	0.11	0.11	0.13	0.11	0.12
10	0.34	Wheat seed	0.35	0.07	0.08	0.07	0.07	0.06	0.08	0.08	0.06
12	0.50	Wheat seed	0.50	0.13	0.13	0.14	0.13	0.15	0.13	0.14	0.13
14	0.43	Frogs	0.42	0.09	0.10	0.09	0.10	0.10	0.10	0.09	0.10
15	0.65	Frogs	0.64	0.13	0.12	0.12	0.12	0.13	0.13	0.12	0.13

The filter-paper coated with the dried residue was cut into narrow strips and extracted by prolonged shaking with water or 1 % salt solution. Where larger masses of residue, hardened by keeping, were to be dissolved, they were first powdered in a mortar before extraction. The extract was filtered after 24–26 hours, when a clear filtrate was obtained, giving flocks of coagulated protein upon boiling. The amount of precipitated protein so obtained was small in comparison with the volume of filtrate, and it was noticed that this was especially so for those extracts prepared from uricase which had been left for over a day after precipitation in contact with acetone.

A comparison of the uricolytic power of the glycerol extract and of the second extract, at equivalent dilutions (*i.e.* the residue obtained after adding 2 litres of acetone to 1 litre of glycerol extract is dissolved in 1 litre of 1 % salt solution or water), shows that in most cases that of the original extract was less than that of the purified product. The values found are given in Table II.

Table II. *Frog uricase. Decomposition of uric acid under the influence of uricase from the same glycerol extract purified by different methods. Uricolytic action is expressed in percentages of the original quantity of uric acid decomposed in 10–16 hours at 37°.*

In most cases the successive precipitates from a litre of glycerol extract were extracted with an equal volume of water or saline solution, except in the case of dialysed enzyme preparations, where the acetone residue extract was made up to a litre and poured into the dialysers, which were surrounded by a litre of water. In all cases equal volumes of uricase and of urate solutions at a constant concentration (this was 50–100 mg. per 100 c.c.) were taken.

Serial number of preparation	Crude glycerol extract	Extract purified by			
		acetone	acetone and dialysis	acetone and alcohol	acetone, ammonium sulphate, alcohol
4	30	49	47	38	29
6	50	83	91	49	38
11	58	90	99	53	50
13	45	78	84	41	36
16	54	81	80	52	43

This enhanced activity of uricase after precipitation with acetone or alcohol has been noticed by Wiechowski and Wiener [1907] and by Batelli and Stern [1909]. This may be due to various causes. In the first place it would appear that uricase is exclusively in adsorption both in the cell and in the glycerol extract. This view is supported by the absence of this ferment in the free state from mammalian blood. Acetone precipitation has the effect of irreversibly coagulating many of the colloidal constituents of the cell, from which free uricase may then be extracted. It is further possible that concentrated acetone solutions elute uricase from the colloids precipitated by this agent, and that the latter are at the same time so denaturated that uricase is no longer adsorbed upon them, even after the removal of the acetone. The fact that acetone-purified uricase is easily dialysable, whilst that of the crude extract or of the tissue juices does not diffuse through a similar membrane, or at least does so very slowly, would appear to support this explanation.

Adsorbed uricase may act more feebly than unadsorbed for several obvious reasons.

A number of observations indicate that intracellularly uricase is present almost exclusively in the adsorbed state. This is shown by the difficulty with which it can be extracted and by the fact that a large excess of chloroform has to be employed for its quantitative extraction. Wiechowski and Wiener found that extraction was more complete in a slightly alkaline medium, and this we have been able to confirm.

The greater activity possessed by extracts of the acetone precipitate may also be due to the separation of the enzyme from inhibitory substances which are probably present in the glycerol extract.

Uricase purified by one acetone precipitation still contains a comparatively large quantity of impurities which are able to reduce methylene blue. Thus 10 cc. of the second extract without the addition of uric acid are able in 24–36 hours to decolorise an equal volume of 0.1 % methylene blue solution.

Uricase was further purified by dialysis or ultra-filtration, as a second acetone precipitation gave very poor yields of enzyme unless much greater quantities of acetone than were used in the first precipitation were added. The best results were given by dialysis, filtrates from Berkefeld candles or certain ultrafilters being less pure. Dialysis was carried out by placing uricase solution in paper dialysing tubes (No. 579, manufactured by Schleicher and Schüll), which were kept immersed in water or 1 % sodium chloride solution for 3 days, with the addition of 1 % thymol solution to prevent infection. The dialysate so obtained possessed a very strong uricolytic action, whilst at the same time it was almost free of substances reducing methylene blue. Such dialysates had often a greater uricolytic action than their parent preparations of uricase, in spite of the dilution being doubled. The results obtained are given in Table II.

The dialysates were clear, colourless liquids, which on boiling with sodium chloride gave only an insignificant opalescence. They were from 1000 to 3000 times as active as the frog tissues from which they were originally derived, *i.e.* 1 g. of dry dialysed uricase achieved decomposition of a quantity of uric acid 2000 times greater than that decomposed within the same time by 1 g. of the original tissue (*cf.* Table III), using in both cases different volumes of extract with the same volume of uric acid solution. A very similar degree of purity was obtained by using fairly large pore ultrafilters or collodion membranes supported on sintered glass. The filtrates thus obtained were also powerfully uricolytic, and barely gave traces of precipitate on boiling.

A third method applied to the purification of uricase consists in reprecipitating a solution of the first acetone residue with ethyl alcohol, or with a mixture of ethyl alcohol with acetone. The addition of these reagents in inverse order gave not only a lower yield of uricase but also a more impure product.

The acetone precipitate was dissolved in 1 % sodium chloride solution or water and 5–10 volumes of 96 % alcohol were added, the mixture was well agitated, and left for 15–30 minutes at a temperature of 0°, after which it was poured into a fluted filter paper. The residue was dried as quickly as possible, using an electric fan. The white powder so obtained rapidly darkened on exposure. Such a preparation of uricase may be kept apparently indefinitely in a stoppered bottle without loss of activity. The powder is extracted with water or 1 % sodium chloride solution when required and filtered, most of the impurities still present remaining on the filter-paper, whilst the filtrate is colourless, does not foam on shaking, is not opalescent, and forms scarcely any precipitate on boiling.

Further purification was effected in the following way. Twenty volumes of alcohol were added to the filtrate when a white precipitate appeared, which was filtered off after decantation of most of the supernatant fluid. The residue was washed with 95 % alcohol at 0° when a white residue remained, which in the same way as the previous preparation darkened on exposure to the atmosphere.

The product of double precipitation with acetone and alcohol successively yielded on dialysis an exceptionally pure product, although the yield of active substance was considerably less than that obtained by dialysis after acetone precipitation alone. The former dialysate contains an infinitesimal solid content, coupled with an unusually intense uricolytic action.

The fifth method applied was to salt out the aqueous extract of the first acetone residue with concentrated ammonium sulphate and to precipitate the extract of the residue with ten times its volume of alcohol, the further procedure being the same as for ordinary alcohol precipitation. This method also yields preparations of great activity calculated on unit weight of dry enzyme. The results are given in Tables II and III. Uric acid was in all cases determined by the colorimetric method of Folin and Denis.

Table III. *Estimation of the degree of activity of frog uricase at different degrees of purity.*

(Mean values in heavy type.)			
Type of preparation	Solid content (mg. per litre)	Mg. uric acid decomposed by 1 g. in 1 hour*	Ratio of activity of 1 g. of given pre- paration to that of 1 g. of tissue
Tissue pulp (calculated on glycerol extract)	—	7.0, 8.7, 6.5, 9.1, 7.82	—
Dialysed acetone precipitate extract	83.2, 67.0, 43.1, 35.0	10,800, 15,400, 20,900, 26,000, 15,700	2010
Filtered dialysate of acetone precipitate extract	31.0, 24.0, 29.1, 19.5?	15,500, 23,700, 17,800, 38,150, 26,300	3370†
Dialysate of extract of product obtained by successive precipitation with acetone, ammonium sulphate and alcohol	23.1, 17.6?, 23.4	13,200, 19,300, 11,600, 14,700	1884

* Calculated from 12 hours' action.

† 4240 maximum.

II. *Purification of uricase derived from mammalian kidney.*

It has been shown by Batelli and Stern [1909] and Wiechowski and Wiener [1907] that uricase from mammalian kidneys may be purified by acetone or alcohol precipitation, or by the elimination by dialysis of certain dialysable constituents of the tissues (uricase does not pass through the membrane under these conditions). The use of combinations of these methods, which were not applied by these workers, shows that very concentrated preparations of uricase may be obtained both by double precipitation and by precipitation and dialysis.

The chief factor for the preparation of powerfully uricolytic products from mammalian organs is, as for frogs, the addition of sufficiently strong solutions of glycerol and chloroform to the finely comminuted tissue. Usually the following procedure was applied. To 100 g. of well-minced tissue 25 cc. of chloroform, 75 cc. of glycerol and 100 cc. of water were added, and the mixture was left for 48 hours with frequent shaking. A litre of water was then added,

and the whole left a further 24 hours with occasional shaking, after which the mixture was filtered through cotton-wool or muslin. The uricolytic power of the extract so obtained differs little from that found by Batelli and Stern, also for bovine kidneys, namely, 100 g. of tissue decomposed in 1 hour 1.06 g. of uric acid, whilst our extract of 100 g. of tissue decomposed 0.98 g. in the same time. It would hence appear that the uricase present in the tissues can be almost quantitatively eluted from them.

The same measures of purification were carried out on the above extract as for that from frogs—the results are given in Tables IV and V.

Table IV. *Bovine kidney uricase.*

(Procedure as in explanation to Table II.)

Serial number of preparation	Crude glycerol extract	Extract purified by			
		acetone	acetone and dialysis	acetone and alcohol	acetone, ammonium sulphate, alcohol
23	70	89	91	64	55
24	53	85	90	50	42
25	47	70	85	39	39
26	63	82	87	51	42

Table V. *Estimation of the degree of activity of bovine kidney uricase at different degrees of purity.*

(Mean values in heavy type.)

Type of preparation	Solid content (mg. per litre)	Mg. uric acid decomposed by 1 g. in 1 hour*	Ratio of activity of 1 g. of given preparation to that of 1 g. of tissue
Tissue pulp (calculated on glycerol extract)	—	12.0, 13.1, 11.3, 12.2	—
Acetone precipitate extract	74, 65.1, 49.3	15,450, 14,950, 25,000, 18,500	1512
Dialysed acetone precipitate extract	38	26,700	2220
Filtered dialysate of acetone precipitate extract	39.4, 26.9, 31.7	24,140, 31,470, 32,000, 28,900	2370
Dialysate of extract of product obtained by successive precipitation with acetone, ammonium sulphate and alcohol	30.3, 24.0, 19.4	21,800, 24,100, 25,100, 23,700	1943

* Calculated from 12 hours' action.

III. *Purification of uricase derived from wheat seed.*

The same treatment was applied to wheat seed as to frog or kidney tissue, and the results obtained show that the same methods are applicable to the preparation and purification of wheat uricase as in the two former cases. The acetone precipitate can similarly be dialysed, yielding an extremely pure product. The results obtained are given in Table VI.

Table VI. *Wheat seed uricase.*

(Procedure as in explanation to Table II.)

Serial number of preparation	Crude glycerol extract	Extract purified by		
		acetone	acetone and dialysis	acetone and alcohol
32	35	59	65	41
34	51	71	—	—
35	57	66	76	—
37	43	74	92	—

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