# CXLVI. URICASE AND ITS ACTION.

## II. BACTERIAL NATURE OF THE ACTION OF URICOLYTIC EXTRACTS AND DIALYSATES.

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### Introduction.

The experiments described in Part I of this series [Przyłęcki, 1928, 1930], dealing with the relative activity of variously purified preparations of uricase and with the action of inhibitory substances, led, except in the case of glycerol-chloroform extracts, to results inconsistent with enzyme action, but consistent with bacterial decomposition. The presence of bacteria has now been demonstrated, these bacteria have been identified, and certain of their conditions of viability have been investigated, with a view to a critical application of the observations to the results described in Part I and to those of earlier workers in this field.

### Experimental.

1. *Bacterial content of various preparations of uricase.*

The following preparations of uricase, described in Part I of this series, were examined microscopically for the presence of bacteria: glycerol-chloroform extracts of ox-kidney, of chicken and of human liver, filtered extracts of air-dried precipitate obtained by adding to these extracts a double volume of acetone, or of acetone-alcohol mixture, and dialysates of these extracts, dialysis lasting in some cases one, in others two weeks, in the presence of excess of thymol. In addition to this, extracts prepared by treating kidney tissue by the method of Felix, Scheel, and Schuler [1929], whereby 0·005$N$ disodium phosphate in 50 % glycerol is used for extraction, were examined, as well as dialysates of such extracts. The preparations in question were examined microscopically (Zeiss objective No. 7) after fixing on a slide and staining with methylene blue or methyl violet.

With the exception of fresh glycerol-chloroform extracts and of Felix's 50 % glycerol extract, bacteria were found in every case and were most numerous in the dialysates.

The bacteria were in most cases scattered in pairs over the field of vision; in the case of dialysates they appeared often in large, agglutinated masses.

The microscopic appearance of the bacteria found in all the above preparations, as well as in solutions of uric acid left exposed to the air, was on the whole identical. These bacteria multiplied rapidly at room temperature in all the ordinarily used culture media; in particular, abundant growth was obtained even in the presence of excess thymol in solutions buffered to $p_H$ 7·5 using McIlvaine's phosphate-citrate buffers and containing urea or uric acid.

Dr Brill, of the Bacteriological Institute, Faculty of Veterinary Medicine, who very kindly undertook the identification of the micro-organisms in question, found that *B. mesentericus*, *B. faecalis alkaligenes* and *B. proteus vulgaris* were present in such cultures.

In addition to these organisms, the author of this paper isolated from certain cultures in which a green coloration had appeared other bacteria which were motile, aerobic, non-spore-forming, Gram-negative, and liquefied gelatin, coagulated milk and did not ferment glucose. These reactions in every respect correspond to those of *B. liquefaciens fluorescens*, which has, moreover, been mentioned by Czapek [1920] as being capable of decomposing uric acid.

An examination of the influence of the $p_H$ of solutions on the rate of multiplication of these bacteria, performed by adding 1 cc. of a suspension of such bacteria to a number of flasks containing 40 cc. uric acid solution and buffered to various $p_H$ values from 4·7 to 9·2, and incubating for 48 hrs. at room temperature, showed that growth did not occur below $p_H$ 6, and that maximum growth was obtained at $p_H$ 7·4–8·4. The turbidity of the solutions also serves to a certain extent as a rough index of the intensity of multiplication of the bacteria, those solutions which remain clear usually containing few or no bacteria.

Glycerol-chloroform extracts remained sterile as long as the chloroform had not evaporated. When the solutions used, as well as the flasks, cotton-wool stoppers, etc., were previously sterilised in an autoclave, bacteria did not appear even after 2 weeks, provided that the flasks were not opened; when samples of the solution had been taken, bacterial growth in many cases took place. In the case of Felix's 50 % glycerol extract, profuse bacterial growth commenced on dilution of the extract with uric acid solution; apparently in this case the bacteria present were able to resume growth at concentrations of less than 25 % of glycerol.

### 2. *Action of bacteria on uric acid.*

The uricolytic action of the bacteria usually present in uricase extracts and dialysates was examined by infecting solutions of uric acid buffered to $p_H$ 7·4 (McIlvaine's phosphate-citrate buffers) with a few drops of the given culture. Energetic decomposition of uric acid took place at room temperature; thus, in one case, the uric acid content of 20 cc. of solution fell after 24 hrs. at 15° from 13 to 8·5 mg. in the presence of crystalline thymol, and to 5·4 mg. in its absence.

### 3. *Action of antiseptics on growth of bacteria and on their uricolytic action.*

In the presence of excess toluene or chloroform both the growth of bacteria and their action on uric acid is completely inhibited. Thymol, as has been shown, has only to a certain extent an inhibitive effect on bacterial growth. Methyl alcohol has an inhibitive effect only at relatively high concentrations, and even when the concentration of alcohol is initially sufficient to inhibit the development of bacteria, and hence to prevent bacterial uricolysis, growth is resumed when sufficient alcohol has evaporated.

The experiments with methyl alcohol were carried out in the following way. 1 cc. of a suspension of bacteria was added to each of a series of flasks containing 10 cc. of lithium urate solution and 10 cc. of McIlvaine's phosphate-citrate buffer at $p_H$ 7·5. Methyl alcohol was then added to the flasks in such quantity as to obtain a gradation of 1–10 % alcohol. The uric acid content of all flasks was examined after 48 hrs. at 16°. The entire uric acid present (9·5 mg.) had been destroyed at concentrations of 0, 1, 2, 3, 4, 5 and 6 %; at 7, 8, 9 and 10 % methyl alcohol the entire original content of uric acid remained intact. A microscopic examination of the solutions showed that the bacterial content is roughly the same in 0, 1, 2 and 3 % methyl alcohol; with 4 % alcohol the bacteria appear in long chains and in much smaller quantity, whilst in 6 % alcohol not more than 20 bacteria can be seen in the field of vision (Zeiss No. 7 obj.), as compared to several hundred in the absence of alcohol. In spite of their relatively small number, these bacteria had been able at 16° to destroy 9·5 mg. of uric acid in 48 hrs., so that it would appear that methyl alcohol, whilst depressing the rate of multiplication of the bacteria in question, to a certain extent stimulates their uricolytic activity, as on other occasions much denser cultures had not within a similar time been able to destroy an equal quantity of uric acid.

After the lapse of several days, bacteria also developed in those flasks containing originally 7 and 8 % of methyl alcohol; this retarded development can be ascribed to evaporation of methyl alcohol to below the limiting inhibitory concentration.

The above study of ordinarily used disinfectants shows that only toluene and chloroform are suitable for the purpose; should, however, these agents have been removed by evaporation, re-infection readily takes place should aseptic conditions not be observed.

### 4. *Action of bacteria killed by toluene.*

Quastel and Wooldridge [1927] have shown that *B. coli*, killed by toluene or by other reagents, is still able to catalyse various oxidation reactions. In order to exclude the possibility that the action of uricase preparations in the presence of toluene or of chloroform is due to this agency, 20 cc. of an active, young culture of uricolytic bacteria, grown on a uric acid medium in which

mere traces of uric acid remained, were added to 100 cc. of uric acid solution, the final initial concentration being 0·46 mg./cc., and the system was shaken at 37° in the presence of 4 cc. of toluene during 6·5 hrs., after which the uric acid content was compared with that of other systems of the same composition, except that water had been added in place of the bacterial suspension. The uric acid content was the same in all cases, showing that bacteria are not able in these conditions to destroy uric acid. No difference in the uric acid content of the above systems was observed after they had been allowed to stand for a further 42 hrs. at 16°.

### 5. *Influence of freshness of tissues taken.*

Battelli and Stern [1909] in their important paper upon uricase state that fresh organs contain some substance which inhibits the action of uricase, and which rapidly disappears after the death of the animal from which the tissue in question is taken. They state, further, that this inhibiting substance is also removed by treating the tissue in the following way: 1 vol. of minced tissue is left for 5 mins. with 3 vols. of alcohol, which is then filtered off, the residue is extracted with 3–4 vols. of ether, which is also removed after 2 mins., and the residue is dried first with filter-paper and then for 24 hrs. in the air. Since it appeared to the author of this paper that the apparent activation of the catalyst might in both cases be due rather to development of bacteria, both of those initially present as saprophytes and of air-borne bacteria, such as have been identified above, the following experiment was performed.

The liver was removed from a dog under chloralose narcosis and was immediately passed through a previously boiled mincing-machine. Portions of the hash (10 g.) were left in open 100 cc. flasks for 0, 2, 4, 6 and 28 hrs.; at the end of each period the hash was shaken for 3 mins. with 20 cc. of water, the suspension was filtered through cotton-wool, and 1 cc. was added to 20 cc. portions of uric acid solution (containing 7·1 mg.), previously sterilised in an autoclave at 120° for 1 hr. The uric acid content and the bacterial flora of all flasks were examined after the lapse of 48, 72 and 100 hrs. at 16°, no flask being opened more than once. In none of the flasks had any decomposition of uric acid taken place at 48 and 72 hrs.; after 100 hrs. the uric acid content of the flask to which an extract of tissue had immediately been added was still the same as that of the control flasks. Where the mince had been left 2 hrs. no uric acid remained, and microscopic examination revealed the presence of dense masses of strepto- and diplo-cocci; the result for tissue left 28 hrs. was substantially the same. When it had stood for 4 and 6 hrs. before extraction, 4·4 and 4·1 mg. of uric acid remained respectively. In all cases bacteria were present.

These results to a certain extent confirm our supposition that the apparent activation of tissue uricase after death may be due rather to the development of bacteria than to the destruction of some hypothetical inhibitor.

### 6. *Action of sterile uricolytic preparations.*

Whilst the presence of bacteria in a uricolytic preparation does not necessarily prove that the decomposition of uric acid observed in its presence is due solely to bacterial action, yet results obtained on such material can be of little service for the elucidation of problems connected with the mechanism of biological oxidation of uric acid, as it is not possible quantitatively to distinguish between that part of the uric acid decomposed by micro-organisms, and that oxidised by the enzyme in question. For this reason it is of importance to find such tissue preparations as are able to destroy uric acid under sterile conditions, or in which the bacterial factor can, at least, be reduced to a negligible minimum.

The requisite conditions of sterility can be attained by the use of chloroform or toluene as antiseptics, or by the use of sterile tissues for the purpose.

#### (*a*) *Action of aseptically removed dog's tissues*[1].

The liver and kidneys were removed from a dog under chloralose narcosis, with the use of every precaution to assure asepsis. The organs in question were immediately passed through previously sterilised mincing-machines into tared flasks containing a known quantity of sterile 0·138 % uric acid solution, and the quantity of tissue entering was weighed. In the case of liver, 55 g. of tissue had been added to 200 cc. of uric acid solution, containing 1·38 mg./cc. Since the average water content of dog's liver is about 75 %, it follows that an extra 40 cc. of water had been added to the uric acid solution, bringing the concentration to 1·15 mg./cc. The concentration of uric acid on the following day was 0·76 mg./cc. and the same value was found after leaving for 72 and 120 hrs. at 16°. No bacteria were detected by microscopical examination of smears, even after 120 hrs. Similar results were obtained with kidney tissue, the exception here being that bacteria appeared on the fifth day. On the same day the uric acid content of the solution fell from 0·7 to 0·61 mg./cc.

Under the given conditions, therefore, the only reaction appears to have been one of adsorption, completed in 24 hrs., since in the absence of bacteria, no further removal of uric acid from solution appeared to take place. Quite different results were obtained at 38° with shaking.

Minced liver, removed aseptically, as before, from a dog, was shaken with sterilised uric acid solution. Since 152 g. of mince were taken to 300 cc. of 0·127 % uric acid solution, the volume of the solution had been augmented approximately by 114 cc. and the concentration of uric acid had thus fallen to 0·96 mg./cc. The suspension, after standing for 4 hrs., was shaken and filtered through cotton-wool, and 40 cc. of filtrate were placed in each of a series of flasks. Of these, two were shaken for 6 hrs. and two were allowed to stand at 38°, whilst two were left at 16°. At the end of this time all flasks were examined for bacteria, and their uric acid content was determined, after

[1] The author has great pleasure in acknowledging his gratitude to Dr H. Gnoiński, for his kindness in performing the operations described in this section.

coagulation of proteins by adding acetic acid and heating for 3 mins. at 100°. The uric acid content of the shaken flasks was now 0·07 mg./cc., that of those left without shaking at 38° was 0·39 mg./cc., whilst at 16° it was 0·915 mg./cc. As the original calculated value was 0·96 mg./cc., it follows that with shaking at 38°, 0·79 mg./cc., or a total of 31·6 mg. had been oxidised, at 38° without shaking 12·8 mg., and at 16° 1·8 mg. Microscopic examination showed the presence of numerous strongly staining but somewhat irregular objects to a certain extent resembling bacteria in the shaken flasks, fewer in those left at 38°, and none in those left at 16°.

In order to determine whether these bodies were bacteria or merely cell-debris, and whether, should they be bacteria, they were capable of destroying the amount of uric acid in question, the following experiment was performed. Four flasks containing 1 cc. of the suspension from the flasks shaken at 38° and 20 cc. of 0·127 % uric acid solution were prepared. Of these, two were shaken for 6 hrs. whilst two stood for 6 hrs. at 38°; in addition, two flasks containing 20 cc. of the same uric acid solution and 1 cc. of water were left at 38°—these served as controls. The final uric acid concentration in the shaken flasks was 0·915 mg./cc. and that in the flasks left at 38° was 1·05. This would represent a total of 7·1 mg. oxidised with shaking and 4·4 without, showing that bacteria can in the given case be responsible for only a fraction of the decomposition observed.

The acceleration of reaction due to shaking, nearly three times as much uric acid being oxidised, points to the probability that the uricolytic agent present is in the form of a suspension. This question will be further treated in the following part of this series.

### (*b*) *Action of tissues in the presence of toluene.*

Four flasks were taken, containing 5 g. of dog's liver (minced), 30 cc. of 0·121 % uric acid solution, and 4 cc. of toluene. Two flasks were shaken and two stood for 4 hrs. at 38°, after which their uric acid content was compared with that of solutions of corresponding strength (30 cc. uric acid solution, 3·75 cc. water). No uric acid remained in the two flasks which had been shaken, whilst the concentration in the second pair of flasks was 0·98 mg./cc., as compared with 1·07 mg./cc. in the control flasks.

In this experiment the effect of shaking was much greater than in the previous one, performed on a fine suspension of cell fragments, in place of the relatively large pieces of tissue taken here. At the same time it is evident that toluene does not inhibit uricolysis.

### (*c*) *Felix's glycerol extract and its dialysate.*

This glycerol extract [Felix *et al.* 1929], which is a suspension of cell-fragments in a viscous medium (50 % glycerol), similarly retains its activity in the presence of toluene. These authors do not give complete directions for the preparation of the extract; that used in these experiments was prepared as follows. 300 g.

of hashed ox kidney was left with occasional shaking for 3 days with a solution containing 500 cc. glycerol, 500 cc. water, and 25 cc. of 2 *M* disodium hydrogen phosphate, and the supernatant suspension was then passed through cotton-wool. 10 cc. of the filtrate was left for 96 hrs. at 16° with 20 cc. of uric acid solution, and the final uric acid content was compared with that of other similar systems to which 4 cc. of toluene had been added and with that of control systems to which water had been added in place of extract—toluene was also added to the control flasks. The results, given in Table I, show that the activity of the extract is unaffected by the presence of toluene, and that the extract, on keeping, undergoes gradual loss of activity.

Table I. *Action of Felix's extract.*

| | Uric acid content in mg. | | | | |
|---|---|---|---|---|---|
| | | after 48 hrs. with extract | | after 96 hrs. with extract | |
| Date of exp. | in control | with toluene | without toluene | with toluene | without toluene |
| 1. iv. 30 | 24·0 | — | — | 6·0 | 6·0 |
| 29. iv. 30 | 18·0 | 15·75 | 15·75 | 10·95 | 10·95 |

Thus the fresh extract destroys 18 mg. of uric acid in 96 hrs., whilst a 28 days' old extract destroys only 7·05 mg. in the same time.

It follows that the activity of Felix's extract is not due to bacteria, which may, however, be present. These are not, apparently, in the given case possessed of uricolytic activity.

Felix *et al.* state that, on dialysis of their extract, both the dialysate and the residue are active. The above extract (400 cc.) was placed in Schleicher-Schüll dialysing membranes, which were immersed in 150 cc. of McIlvaine buffer solution at $p_H$ 7·5, and toluene was added, so as to cover both the solution in the dialysers and the dialysate. The dialysate and the residue were taken after 4 days, and their uricolytic activity was tested as usual. After leaving 10 cc. of these preparations for 72 hrs. at 17° with 20 cc. of uric acid solution at $p_H$ 8·5, the final uric acid content of the solutions to which the residue had been added was 5·7 mg., that to which dialysate had been added was 15·9 mg., whilst the control flasks contained 23·1 mg. of uric acid. Both the former systems, however, exhibited profuse bacterial growth (chiefly *B. fluor. liq.*), and this in spite of the presence of toluene during dialysis and at the beginning of the reaction.

This effect is possibly due to the absence of toluene from the lower layers of liquid in the dialyser, as a result of which bacteria remained living, and able, on removal of toluene by evaporation, to resume normal growth.

The above experiment was next repeated in the presence of excess toluene in certain of the reaction flasks, so as to exclude the possibility of growth of bacteria during the reaction. After leaving 20 cc. of dialysate with 20 cc. of uric acid solution for 43 hrs. at 20°, the uric acid contents of the control flasks and of those to which 4 cc. of toluene had been added were identical, viz.

18·8 mg., whilst where toluene had not been added only 17·4 mg. remained, and microscopic examination revealed the presence of bacteria.

It would thus appear that dialysates of Felix's glycerol extract owe their uricolytic action to bacteria, and not to uricase.

Przyłęcki's glycerol-chloroform extract (*cf.* Parts I and III of this series) was also found to be active in sterile solutions. It follows that uricolysis is not inhibited by the action of chloroform.

## Discussion.

The results described show that, with the exception of systems containing chloroform or toluene, or of aseptically taken tissues, all the preparations examined contain bacteria, which also very often appear in originally sterile solutions, unless special precautions are taken to ensure sterility. It follows from this that not only are many of the conclusions drawn in Part I of this series invalid, but also that much previous work on uricolysis cannot, unless these precautions had been taken, be accepted without question.

Thus Battelli and Stern [1909], who measured oxidation of uric acid by production of carbon dioxide, considered that some inhibitory substance is present in fresh but not in old or ether-extracted tissue; as has been shown in this paper this factor may have been merely enhanced bacterial action. These workers did not apparently use any antiseptic in their experiments, so that no hindrance existed to free bacterial growth. Wiechowski and Wiener [1907] used dried, toluene-extracted tissue-powders in their experiments; in this case, the probable point of entry of bacteria was during the process of drying. In most of these workers' experiments thymol was used as the antiseptic; this, as has been shown, gives no guarantee of sterility. Wiechowski and Wiener found that dialysis of suspensions of tissue enhanced their activity; this effect we would ascribe to growth of bacteria during dialysis. The observation that uricase preparations gradually became more acid would also appear to indicate bacterial action. These workers, however, found that excess toluene or chloroform did not inhibit the uricolytic action of the tissue powders used. A further observation indicative of bacterial growth was that tissue powders, if left for a long time with thymol in 0·05 % sodium carbonate solution, gradually gave up their uricase to the solution; thus, after 5 days' dialysis of dog's liver in 0·05 % sodium carbonate solution, an extremely active extract is obtained; according to Wiechowski, thymol retarded extraction, chloroform prevented it, whilst sodium carbonate accelerated this process. These observations are all indicative of the growth of uricolytic bacteria, the optimum reaction for the growth of which is in the alkaline region, whilst the respectively retarding and inhibiting actions of thymol and of chloroform may be due to their antiseptic action.

Felix's glycerol extract is of the nature of a suspension, and the same applies to Przyłęcki's glycerol-chloroform extract. Felix's dialysate is inactive except in so far as it contains bacteria, as is also the case with Przyłęcki's acetone precipitate extracts and dialysates.

The results described in this paper, as well as a review of those obtained by previous workers in this field, suggest that uricase is a contact catalyst, acting exclusively as solid, as opposed to dissolved matter. The next part of this series will be devoted to a closer study of its properties as a contact catalyst.

SUMMARY.

1. Various preparations of "soluble uricase" extracts and dialysates have been shown to contain the following bacteria: *B. proteus vulgaris*, *B. mesentericus*, *B. faecalis alkaligenes* and *B. fluorescens liquefaciens*.

2. These bacteria can survive, multiply, and destroy uric acid at room temperature in the presence of excess thymol or of 6 % methyl alcohol; their growth and action are completely inhibited by chloroform and by toluene.

3. These bacteria do not multiply below $p_H$ 6 or above $p_H$ 9; optimum growth and uric acid consumption is obtained at $p_H$ 7·4–8·4.

4. One or more of the above strains develop spontaneously in solutions of uric acid left exposed to the air.

5. Bacteria killed by the addition of toluene have no uricolytic action.

6. The number of bacteria present in minced tissues increases with time, as does the uricolytic action of a suspension of such tissues; this enhanced activity is possibly due to bacterial action, and not, as Battelli and Stern supposed, to the removal of an inhibitor.

7. Dog's liver and kidney, removed and minced under as far as possible aseptic conditions, decompose hardly any uric acid at 16° unless bacterial growth takes place. At 38°, however, 2½ times as much uric acid is oxidised if the mixture is shaken as if it is allowed to stand, and 7 times as much in the latter case as on standing at 16°.

8. This action is not due to bacteria, as analogous results are obtained in the presence of toluene or of chloroform.

9. Bacteria develop in systems containing Felix's 50 % glycerol extracts, the uricolytic power of which is, however, unimpaired by the addition of toluene; the action of this extract is due probably to suspended particles.

10. Przyłęcki's glycerol-chloroform extract is similarly active in sterile systems; in this case, too, the action of this extract is greatly accelerated by shaking, pointing to the macro-heterogeneous nature of the active factor.

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