CXXX. STUDIES ON XANTHINE OXIDASE.

I. PRÉPARATION AND PROPERTIES OF THE ACTIVE MATERIAL.

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MORGAN, STEWART and HOPKINS [1922] have recently demonstrated the existence in milk of an enzyme capable of oxidising the purine bases hypoxanthine and xanthine to uric acid. It has long been known that an enzyme with this property exists in most living tissues, and the work of these authors on several points in connection with the mode of action of the enzyme in milk left little doubt that it is identical with that in the tissues. As is the case with most other oxidising enzymes, the xanthine oxidase¹ is capable of using various oxidising agents to bring about the reaction. The technique mainly used hitherto for the study of oxidising enzymes consists in the measurement of the rate of disappearance of the oxidising agent used, for example, the rate of absorption of oxygen or of decolorisation of added methylene blue.

The investigation of the properties of any individual oxidising enzyme in preparations obtained from tissues is usually complicated by the presence of other enzymes. Many of these are able, with the aid of the above-mentioned oxidising agents, to oxidise various substances present and they thus tend to mask the action of the enzyme under investigation. This effect renders it necessary to control every experiment by appropriate "blank" tests, and makes it a matter of some difficulty to determine the velocity of the single reaction brought about by the enzyme in question.

For the study of the xanthine oxidase milk offers special facilities as a source of the enzyme, as not only are most of the usual interfering enzymes absent, but there is also normally a complete absence of any substances which can be oxidised by those enzymes present in it. A systematic study of an individual oxidising enzyme appears only to have been made in the case of the enzyme converting succinic acid to fumaric acid—the succinoxidone of Batelli and Stern, studied by Ohlsson [1921] and Widmark [1921].

In this class of work one has a choice of two main experimental techniques, both of which were employed by Morgan, Stewart and Hopkins, namely the

¹ Although the enzyme is generally spoken of as the xanthine oxidase, it is not strictly an oxidase, but rather an oxydase in the sense of Batelli and Stern. The usual name has however been retained here.

measurement of the absorption of oxygen by means of the Barcroft apparatus, and the reduction *in vacuo* of methylene blue to its leuco form. The latter method, first used for the study of oxidising enzymes by Thunberg [1920], is much the easier, and it was the only method used by Ohlsson and Widmark. It has been largely used in the present work.

The procedure of Morgan, Stewart and Hopkins was adopted with little modification. The experiments were all carried out in Thunberg vacuum tubes which were exhausted by a water-pump to a pressure of a few mm. of mercury, refilled with nitrogen and again exhausted, the procedure being repeated two or three times. The methylene blue was made up to a strength of 1/5000 in distilled water, and the solutions of the purine bases were made up to a strength of 2 mg. per cc. in very dilute NaOH, as it was found that they could not easily be kept for any length of time in neutral solution. If neutralised and allowed to stand several days a change is found to take place, especially if phosphate is present. They become altogether incapable of oxidation by the enzyme, and at the same time are no longer precipitable by ammoniacal silver nitrate. Although no definite growth of bacteria could be observed, the change appears to be due to bacterial action, since it did not take place if special precautions were taken to keep the solutions absolutely sterile. This observation is obviously of considerable practical importance in carrying out investigations on this enzyme. The addition of the slightly alkaline purine solutions in the small quantities used in most experiments did not appreciably change the p_H of the enzyme solution. When it was necessary to add larger quantities, the purine solutions were so far neutralised that, although there was no precipitation, no appreciable change in the p_H of the enzyme solution occurred when they were added.

The reductions took place in a thermostat kept at 37°.

There are several disadvantages in the employment of milk for the investigation of the properties of the xanthine oxidase. Firstly, different milks vary enormously in their oxidase activities, so that it is difficult from day to day to obtain comparable experiments. Secondly, the activity of any one milk is not usually constant for more than a few hours at a time; the activity falls off fairly quickly, and the milk if sterile usually becomes inactive in the course of one or two days. Thirdly, the milk, if not perfectly sterile, invariably develops a marked power to reduce methylene blue by itself in the absence of purine substances, owing to the growth of bacteria.

Attempts were therefore made to obtain a preparation from which the various complex constituents of milk had as far as possible been removed, and which would contain the oxidase in a stable form.

Upon removal of the fat by centrifuging there was a marked fall in activity of the xanthine oxidase, and this led to the belief that the enzyme was adsorbed on the fat surface. Further investigation, however, showed that the effect was probably due to the catalytic action of the large surface presented by the fat. On adding boiled cream, which of course is itself inactive, to a solution

containing the enzyme, a large increase of oxidase activity was produced. Fat, however, had no effect upon the Schardinger enzyme.

On further investigation it was found that the oxidase was precipitated completely, with the caseinogen, on half saturation with ammonium sulphate. This naturally led to the following routine method of preparation of the active material.

The activity of the milk is first tested in the usual way with a small trace of hypoxanthine and methylene blue. It is important to use as active a milk as possible, as the activity of the final preparation depends entirely upon that of the milk employed. The milk is then mixed with an equal volume of saturated ammonium sulphate solution, and allowed to stand for a few minutes. The precipitated caseinogen and fat are then filtered off and dried as far as possible by pressing between filter-papers. The ease of subsequent manipulation depends to a great extent upon the thoroughness with which this is carried out. The mass is now broken up and extracted thoroughly with cold ether, the process being hastened by frequent shaking. The ether is drained off and the material again pressed between filter-papers so as to remove as much ether and water as possible, after which it is spread out and dried in a vacuum desiccator for about 12 hours. After this it is again extracted with ether, dried thoroughly *in vacuo*, and powdered in a mortar.

The resulting preparation is very convenient to use, consisting of a fine white powder readily soluble in water forming a slightly opalescent solution. It was the only preparation used in this work. The powder is extremely active, and if kept in a well stoppered bottle retains for long periods its activity unimpaired. Preparations usually exhibit a good activity after being kept six months or more, and in all cases seem to remain quite free from any trace of bacterial contamination. The active material consists of caseinogen together with small amounts of ammonium sulphate.

It was found that the preparation contained not only the xanthine oxidase but also the Schardinger aldehydeoxidase. The small amount of adenase found in milk by Morgan, Stewart and Hopkins is also present in the final preparation. Reduction was not obtained in presence of any substance other than hypoxanthine, xanthine, adenine and aldehydes.

Many methods were tried in an endeavour to separate the enzyme from the caseinogen¹. The concentration of ammonium sulphate used for precipitation was varied, in the hope that the caseinogen might be precipitated first; but as soon as the caseinogen is completely precipitated the remaining solution from which the precipitate has been centrifuged off is quite inactive. (In preparing the active material the addition of at least an equal volume of saturated ammonium sulphate is advisable in order that the final product may be easily soluble in water.) It was then thought that the enzyme might be separated from the caseinogen by adding to the solution a substance more

¹ We have very recently found that when milk is clotted by rennin a considerable part of the enzyme remains in the whey.

strongly adsorbing than caseinogen. Little of the enzyme is removed from neutral solution by strongly adsorbing substances, such as animal charcoal and kaolin. Willstätter [1923] found alumina very useful in the purification of certain enzymes, which were adsorbed by the alumina and could then be washed off. Preliminary experiments of this nature have been carried out with the xanthine oxidase. A suspension of freshly prepared alumina was thoroughly mixed with the enzyme solution and then removed by centrifuging. The resulting solution was practically inactive. On testing the alumina with hypoxanthine in the Barcroft apparatus there was found to be a steady but rather slow uptake of oxygen. This shows that the enzyme is still active on the alumina, but as yet we have not been successful in removing it from the adsorbent. Further experiments with this object in view are in progress.

It was observed that by filtering the enzyme solution through filter-paper the activity was greatly reduced. A moderately dilute solution was rendered completely inactive, while a concentrated solution lost about 50 % of its activity. There was, however, no visible residue on the filter-paper. In order to determine the weight of the material removed from solution, a filter-paper was dried in the vacuum desiccator and weighed. Through it was filtered twice a solution of a very active caseinogen preparation containing 1.5 g. in 30 cc. of p_H 7.6 phosphate buffer. The filter-paper was then thoroughly washed with distilled water, dried in the vacuum desiccator and weighed. The increase of weight was 0.0012 g. The filtrate and washings were found to be inactive, while a filter-paper treated in a similar manner was found in the Barcroft apparatus to take up oxygen in presence of hypoxanthine as rapidly as an unfiltered solution. The presence of filter-paper renders the methylene blue technique inapplicable, as the methylene blue becomes adsorbed on the filter-paper and is then reduced only with extreme slowness.

The adsorption of the enzyme by filter-paper affords an explanation of the discrepancy between the results of Rohde and Jones [1909] and those of Morgan, Stewart and Hopkins on tissues of the rat. Rohde and Jones, having filtered their tissue extracts, stated that xanthine oxidase was not present in the tissues of the rat, while Morgan, Stewart and Hopkins, working with unfiltered extracts, found that it was present.

The effect of various organic solvents was then tried. Exposure for an hour of the solid preparation to both alcohol and acetone caused a marked decrease in activity, while ether, chloroform, toluene and glycerol had no effect.

It was noticed that dry preparations kept tightly corked up and undisturbed retained their activity better than those which were frequently opened and shaken up. This led to the belief that the enzyme might be undergoing a slow oxidation process brought about by atmospheric oxygen. It has already been observed that milk upon standing loses its activity. This is also true of a solution of the preparation kept in air, whereas a solution kept in an evacuated tube retains its activity unimpaired as long as it can be kept free from bacteria.

SUMMARY.

1. A method for obtaining from milk a stable, water-soluble caseinogen preparation containing the xanthine oxidase in a form suitable for systematic investigation has been described.
2. The enzyme is adsorbed from a neutral solution of the preparation only slightly by animal charcoal, but almost completely by alumina and filter-paper.
3. The enzyme when adsorbed on alumina or filter-paper still retains its activity.
4. The presence of fat in a finely divided state, as in milk, greatly increases the rate of oxidation of purine bases by the enzyme.
5. The enzyme is slowly destroyed by atmospheric oxygen.
6. The enzyme is destroyed by alcohol and acetone but not by ether, chloroform, toluene or glycerol.
7. Hypoxanthine and xanthine on standing in neutral solution become incapable of oxidation by the enzyme.

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