CXL. ON THE FURTHER PURIFICATION OF THE XANTHINE OXIDASE.

By MALCOLM DIXON (1851 *Exhibition Senior Student*)
AND KEIZO KODAMA.

From the Biochemical Laboratory, Cambridge.

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SINCE the discovery of the xanthine oxidase of milk by Morgan, Stewart and Hopkins [1922] two main attempts have been made to purify this enzyme further; namely, by Dixon and Thurlow [1924, 1], and later by Sbarsky and Michlin [1925].

Dixon and Thurlow obtained an active and stable oxidase preparation by precipitating the enzyme, together with the caseinogen and fat, by half-saturation of the milk with ammonium sulphate. The fat was removed from the dried precipitate by thorough extraction with ether. The resulting preparation is soluble, contains the whole amount of the enzyme, and is free from other milk enzymes such as peroxidase and catalase. Since the caseinogen content of milk is about 3 % it will be clear that this caseinogen preparation is about thirty times as concentrated as milk, *i.e.* 1 g. of the solid preparation contains as much enzyme as 30 g. of milk¹.

Sbarsky and Michlin's method of preparation is essentially similar. They use acetone instead of ammonium sulphate to precipitate the protein, and light petroleum instead of ether to extract the fat. The protein is, however, precipitated in an insoluble form by the acetone, and it is necessary to digest the preparation with dilute acid in order to get the enzyme into solution. The

¹ With regard to the activity of this preparation Sbarsky and Michlin state: "(Dixon and Thurlow) were able to salt out the caseinogen together with a portion of the enzyme. The enzyme content of the precipitate was however so small that they were obliged to use not less than 1.5 g. of the dry powder to bring about the reaction." There is here a complete misunderstanding of the statements of Dixon and Thurlow. They specifically stated that the whole of the enzyme was precipitated by the ammonium sulphate, and that the subsequent ether treatment did not diminish its activity, so that it is clear that the enzyme must have been concentrated to the extent given above. As a matter of fact, a few milligrams of the dry powder are quite sufficient to bring about the reaction actively. A point which seems to have contributed to the misunderstanding is the fact that in Dixon and Thurlow's studies on the dynamics of the enzyme the *strengths* of the enzyme solutions (as distinct from their *activities*) were omitted as having no significance. It may be stated here that the solutions used by Dixon and Thurlow were in general 1 or 2 % solutions, so that the average amount of preparation used in each experiment was about 50 mg., which gave very satisfactory velocities. The mention of "1.5 g." occurs only in connection with an adsorption experiment, in which it was desired to use a very large amount of the preparation.

preparation, moreover, contains the catalase and peroxidase of the milk in addition to the oxidase.

This method naturally gives a solid preparation of the same oxidase content as Dixon and Thurlow's preparation. Sbarsky and Michlin found, however, that in buttermilk the enzyme : protein ratio was several times higher than in milk; and by applying their method to buttermilk they obtained preparations about 240 times as active as the original milk from which the buttermilk was obtained.

The present communication gives the results of further work on the purification of this enzyme, together with a rapid and simple method for obtaining preparations of very greatly increased activity direct from milk.

The problem of further purification resolves itself into that of eliminating the protein without loss of enzyme. Up to the present attempts to purify Dixon and Thurlow's preparation further have not been successful. Precipitation and adsorption methods proved unsatisfactory, cataphoresis experiments at various p_H 's showed that the oxidase always migrated with the caseinogen, and it cannot be dialysed away from the protein. We found, however, that, by making use of the fact discovered by Dixon and Thurlow that when the milk proteins are coagulated by the action of rennin a considerable part of the oxidase remains in the whey, it was possible to get rid of the greater part of the protein at the outset, and so obtain very much more active preparations. The procedure we adopted is as follows.

Rennin method of preparation.

The fresh milk, after its activity had been tested, was warmed to 35° and mixed with a small amount of rennin preparation ("Birks' Junket Powder"). The mixture was allowed to stand for 30 minutes, by which time it had set to a firm clot. This clot was then broken up by stirring, and the insoluble portion or curd was centrifuged off. The whey thus obtained was mixed with an equal volume of saturated ammonium sulphate solution, and allowed to stand. A small amount of precipitate formed, and quickly rose to the surface of the solution, where it formed a compact layer. The liquid was then siphoned off, and the precipitate transferred to a filter-paper, allowed to drain, and squeezed as dry as possible between filter-papers. It was then thoroughly extracted with ether to remove traces of fat. It was again squeezed between filter-papers, and finally dried in a vacuum desiccator. We shall refer to the resulting preparation as "the whey preparation."

It was found that when the milk clots the whole of the oxidase passes into the whey, and the curd, after washing with water, is quite inactive. Thus by this method the caseinogen and fat are got rid of without loss of enzyme.

The whole of the enzyme is precipitated by the half-saturation with ammonium sulphate.

The process of preparation should be carried out as quickly as possible, as the enzyme is much more easily destroyed by oxidation than in the case

of the caseinogen preparation. In particular, the drying must be carried out *in vacuo*. Allowing the preparation to dry in the air is sometimes sufficient to destroy the activity almost entirely, and it always considerably reduces it. The enzyme is perfectly stable in this form once the preparation is completely dry, and it can be kept in the vacuum desiccator for long periods without loss of activity.

The whey preparation is very readily soluble in water, giving a practically clear solution. The yield is about 10 g. from 1.5 l. of milk, and the activity was found by means of the methylene blue technique to be from 500 to 700 times that of the original milk.

It would appear from a comparison of the weights and activities given above that the final preparation contains more enzyme than the whole of the original milk. Since 1.5 l. of milk yield 10 g. of the whey preparation, this should be about 150 times as active as milk, whereas it is actually more than 500 times as active. The facts appear to suggest that the rennin sets free an additional quantity of xanthine oxidase from some inactive precursor in the milk. The rennin itself did not contain xanthine oxidase. Further work is necessary on this point.

The rennin method of preparation has been found most useful for general work, as it gives a means of obtaining, without difficulty, a soluble preparation of very high activity.

Methods for further purification.

(a) *Charcoal.* It was stated by Dixon and Thurlow that charcoal does not adsorb the oxidase from a solution of the caseinogen preparation. This observation was confirmed with the whey preparation. It was found, however, that in this case the charcoal did remove a considerable proportion of the protein. This suggested a method for further purification. The details are as follows.

1.5 g. of the whey preparation was dissolved in 150 cc. of distilled water. To the solution were added 3 g. of charcoal (Merck's medicinal), and it was then shaken for a few minutes and filtered through a Buchner funnel, using a Whatman filter-paper. A clear filtrate was obtained. After the enzyme activity and the relative protein content of this filtrate had been determined, a further quantity of charcoal was added and the above procedure repeated. This process was carried out repeatedly until the whole of the protein had been removed from the solution by adsorption. The results obtained are given in Table I.

For the estimation of the enzyme activity the following quantities were taken: 1 cc. of the filtrate + 1 cc. phosphate buffer (p_H 7.6) + 0.5 cc. methylene blue (1 : 5000) + 0.2 mg. hypoxanthine. The reduction took place at room temperature (15°), and by the activity we mean here the reciprocal of the reduction-time (in minutes) $\times 100$ [cf. Dixon and Thurlow, 1924, 2].

The protein was estimated as follows: to 1 cc. of the filtrate were added

5 cc. of 2 % sulphosalicylic acid, and the resulting turbidity was compared in a nephelometer with that given by 1 cc. of the original solution treated similarly. Calling the protein content of the original solution 100 units, it is a simple matter to calculate the protein content of any filtrate in these units from the nephelometer readings. Sufficient time was always allowed for the turbidity to develop to its full extent before taking the readings.

Table I. *The adsorption of xanthine oxidase and the protein by charcoal.*

No. of adsorptions	Amount of charcoal used g.	Reduction time	Enzyme activity of filtrate	Relative protein content
0	0	1' 30"	66.7	100
1	3	1' 20"	75	67
2	3	1' 15"	80	45.5
3	3	1' 20"	75	30.7
4	2.4	1' 35"	63	19.2
		1' 40"		
5	2	2' 10"	46.2	12.8
		2' 15"		
6	2	5' 0"	20	5.6
7	2	15' 0"	6.5	2.1

It will be seen from the above results that at each stage the charcoal adsorbs 33 % of the protein in solution, except in the final stages, where the proportion is higher (about 50 % and 65 % in the sixth and seventh stages respectively). It will also be seen that the enzyme is not adsorbed by the charcoal until about 80 % of the protein has been removed, after which the enzyme is adsorbed together with the protein. The slight rise in activity after the first two adsorptions has been observed in every case where charcoal was used as adsorbent, and is quite definitely not due to experimental error. We have as yet no explanation of this curious effect.

The charcoal method, therefore, enables us to concentrate the whey preparation a further five times. The filtrate after four charcoal adsorptions is a clear, colourless solution of very high activity; and by half-saturation of this with ammonium sulphate, filtering off the precipitate, and drying *in vacuo*, we obtained the whole of the enzyme in the form of a preparation which was, as we expected, five times as active as the whey preparation, or 2500 times as active as milk.

(b) *Kaolin*. It was found that kaolin could also be used for the further purification of the whey preparation. In order to ascertain the best conditions for its use the factors influencing the adsorption of the enzyme by kaolin were studied.

The following experiments show how the adsorption depends on the p_H of the solution. 1 g. of a moderately active whey preparation was dissolved in 100 cc. of distilled water. The reduction time, under the conditions given above in the description of the charcoal experiment, was 2' 40". 10 cc. of this solution were measured into each of a series of test-tubes, and 1 cc. of acetic acid or sodium hydroxide of the strength given in Table II was added. The p_H of each solution was then determined colorimetrically, using a few drops

only. To each test-tube 0.5 g. of kaolin was added, the mixture was shaken thoroughly for 1 minute, and then filtered through a small Whatman filter-paper by suction. The enzyme and protein contents of each clear filtrate were determined in the manner previously described. The results are given in Table II.

Table II. *Adsorption of xanthine oxidase by kaolin at different p_H 's.*

Enzyme solution	Acetic acid or NaOH	p_H	Reduction time	Enzyme activity	Relative protein content
10 cc.	1 cc. N/10 acid	3.4	29'	3.4	43.6
"	" N/20 "	4.2	43'	2.3	43.2
"	" N/40 "	4.7	66'	1.5	40.8
"	" N/80 "	5.0	91'	1.1	44.8
"	" water	5.6	59'	1.7	45.8
"	" N/100 NaOH	6.8	23'	4.3	56.0
"	" N/40 "	7.6	5'	20.0	62.0
"	" N/20 "	8.6	4'	25.0	63.5

Enzyme activity of original solution 37.5.

In slightly acid solution the xanthine oxidase is almost completely adsorbed by the kaolin, while nearly half the protein is left in solution. The most favourable p_H for the adsorption of the enzyme is 5.0. In slightly alkaline solution, on the other hand, only a small proportion of the enzyme is adsorbed. This marked change in the degree of adsorption with p_H in the case of the enzyme is in sharp contrast with the behaviour of the protein, as the adsorption of the latter is only slightly affected by the change in p_H . This effect is of interest as suggesting that the xanthine oxidase may contain a dissociable group which is responsible for the change.

Having determined the optimum p_H for adsorption we have next to find the minimum amount of adsorbent to effect complete adsorption. It was found that treatment of 50 cc. of a solution of the whey preparation (containing 1 g. of the latter, having an enzyme activity of 100, and adjusted to p_H 5.0) with 3 g. of kaolin removed about 85 % of the enzyme, and treatment of the resulting filtrate with a further 2 g. of kaolin brought the amount adsorbed up to 97 %. It was therefore concluded from this experiment that 5 g. of kaolin is sufficient for 50 cc. of a 2 % solution.

The data presented in Table II suggested that the adsorbed enzyme might be liberated from the kaolin by extraction at an alkaline p_H . This actually proved to be the case, and it was found that extraction with 1 % sodium carbonate solution gave the best results. Unfortunately, however, it was not found possible to extract more than 50 to 60 % of the enzyme adsorbed. Table III gives the results of extracting the 5 g. of kaolin obtained in the experiment described in the previous paragraph.

Table III. *The liberation of the adsorbed enzyme by 1 % Na_2CO_3 .*

No. of extraction	Na_2CO_3 used cc.	Reduction time	Enzyme activity	Enzyme recovered %
1	20	0' 51"	118.0	47.0
2	10	3' 5"	32.5	6.5
3	10	10' 0"	10.0	2.0

Although the kaolin treatment thus resulted in a loss of about 45 % of the enzyme the fall in the protein content was found to be considerably greater in proportion, having been reduced to less than one-eighth of its original value by this treatment; and on making a solid preparation in the usual way by half-saturation of the filtrate with ammonium sulphate it was found that it had an activity of between four and five times that of the whey preparation¹.

We may say, then, that the charcoal method gives a means of purifying the whey preparation without loss of enzyme, but can only be used for the removal of 80 % of the protein. On the other hand, a single kaolin adsorption and extraction is sufficient to give this degree of purification, and a repeated application of this treatment would undoubtedly give preparations of a very much higher activity, but unfortunately the 50 % loss of enzyme at each stage renders the method unsatisfactory.

The most active preparations we have obtained have been made by a combination of both methods. The following is a brief account of the procedure.

5 g. of the whey preparation were dissolved in 250 cc. of distilled water. This solution was treated four times in succession with 5 g. of charcoal in the manner described above. The filtrate thus obtained was slightly alkaline (p_H 7.8), and was therefore adjusted to p_H 5.0 by adding dilute acetic acid. To this solution were added 5 g. of kaolin, and after thorough mixing it was filtered by suction. This treatment with kaolin was repeated once more. The 10 g. of kaolin were transferred to a mortar and triturated with 100 cc. of 1 % Na_2CO_3 solution. On filtering, a clear solution was obtained. To this an equal volume of saturated ammonium sulphate solution was added, and after standing for 30 minutes the whole solution was centrifuged. The supernatant fluid was poured off and the precipitate collected on a hard filter-paper. (A hard paper should be used, as the material at this stage is very sticky.) After being squeezed well, it was dried in the vacuum desiccator over sulphuric acid. The yield was about 0.23 g.

In the process of preparation it is important to avoid prolonged contact with air as far as possible, as the purified enzyme is very easily destroyed in the moist state by oxygen. The dry preparation is perfectly stable, however, and can be kept in the desiccator for long periods.

The final preparation is very readily soluble in water, producing a perfectly clear solution. The activity is from 8 to 10 times that of the whey preparation, or about 4000 times that of milk. 1 mg. of the preparation gave a reduction time of 1' 30" at 13°, corresponding to 4 cc. of the fresh milk.

¹ Since the work described in this paper was completed a paper by Sbarsky and Michlin [1926] has appeared describing the further purification of their buttermilk preparation by a method essentially similar to that given here. The method consists in the digestion of their solid preparation for several days with dilute acid, the adsorption of the enzyme from the acid digest by kaolin, extraction of the enzyme from the kaolin by very dilute ammonia, and finally evaporation to dryness *in vacuo*. The activity of the final preparation was 2000 times that of milk. The figures show that here also there was a loss of about 45 % of the enzyme.

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