CCVI. THE IDENTITY OF XANTHINE OXIDASE AND THE SCHARDINGER ENZYME.

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The origin of the problem.

Schardinger [1902] observed that methylene blue was reduced by formaldehyde in the presence of fresh milk. The enzyme concerned in the oxidation of this and other aldehydes became known as "Schardinger's enzyme".

Hopkins [1921] found that certain extracts of yeast and of animal tissues also reduced methylene blue when added to milk. Morgan *et al.* [1922] identified the reducing substance as hypoxanthine and showed that its oxidation was effected by a system similar to that present in tissues. These authors first established that "xanthine oxidase" had many properties in common with the Schardinger enzyme. They found that tissues which were capable of oxidising purines would in all cases also oxidise aldehyde. This striking parallel occurrence of xanthine oxidase and the Schardinger enzyme in milk and in tissues raised the question of their identity. These authors concluded that identity was improbable, because (*a*) the extreme specificity of enzymes towards their substrates argues in general against one and the same enzyme activating substances so different as purines and aldehydes; (*b*) the optimum concentration of purine was only one-hundredth of the optimum concentration of aldehyde; (*c*) the relative activities of the two enzymes varied from one sample of milk to another.

Dixon and Thurlow made a preparation of xanthine oxidase from milk [1924, 1] and studied the dynamics of the enzyme system [1924, 2]. They discussed the following evidence for and against its identity with the Schardinger enzyme. (*a*) Uric acid inhibits both enzymes to a marked extent. The inhibition of an aldehyde oxidase by a purine speaks for identity. (*b*) The slight inhibition by fluoride and cyanide is identical for each enzyme. (*c*) The enzymes cannot be separated: whenever one is precipitated, adsorbed, extracted or destroyed so also is the other. (*d*) The p_H-activity curves with purines and with aldehyde each show a sharp break at p_H 9 which in all cases is due to destruction of enzyme. (*e*) There is a striking parallelism between the activities of the two enzymes in a large number of defatted preparations. The variations in relative activities observed by Morgan *et al.* were never great and could be explained by variations in the fat content of different samples of milk. (Dixon and Thurlow observed that fat accelerated the oxidation of hypoxanthine but not of aldehyde.) (*f*) The disparity in optimum concentrations of the two classes of substrate cannot be used as an argument against identity since several cases are known of one enzyme activating two substrates at very different optimum concentrations. These authors concluded that the balance of evidence was in favour of identity though it was not sufficient to justify a positive statement to that effect.

Morgan [1926] studied the distribution of xanthine oxidase in tissues from many animal species. Wherever xanthine oxidase was found it was invariably

accompanied by the Schardinger enzyme[1] and this concomitance was offered as evidence for identity.

Sbarsky and Michlin [1926] made a purified preparation of the Schardinger enzyme and found that it also had xanthine oxidase activity.

More recently Wieland and co-workers have claimed to have demonstrated the non-identity of these enzymes. Wieland and Rosenfeld [1930] observed a change in the ratio of the activities of the two enzymes after treating the milk preparation with adsorbing agents. This was taken to mean that partial separation had been achieved. Shortly afterwards Wieland and Macrae [1930] found that the velocity of reduction of methylene blue with xanthine and aldehyde both present together was greater than with either alone (method of summation or addition).

On the other hand Sen [1931] found that the oxygen uptake rates with hypoxanthine and aldehyde were not additive. He submitted this effect as evidence for the identity of the enzymes.

In view of Sen's results Wieland and Mitchell [1932] reinvestigated the problem and found that aldehyde slightly reduced the velocity of anaerobic formation of uric acid from xanthine, using methylene blue. But when quinone was used as oxidant they once more obtained evidence for summation with the two substrates.

It is quite clear that this conflicting evidence leaves the question of the identity of the enzymes in an unsatisfactory state. If the two enzymes are identical we have an unusual case of enzyme specificity, namely an enzyme which can activate at once a highly specific purine grouping as well as the aldehyde group. Furthermore, whereas the specificity towards the purine grouping is very great there seems to be no specificity as far as aldehydes are concerned since any aldehyde, aryl or alkyl, can be activated. From the point of view of our knowledge of the mechanism of enzyme reactions the identity of these two enzymes is of great importance.

It would offer a useful example for application—by analogy—to other specificity problems. The question was therefore reinvestigated with a view to clearing up the discrepancies in the literature. In this paper the experimental evidence of Wieland and his co-workers will be considered in detail and it will be shown that their evidence against the identity of the two enzymes is not valid, and fresh lines of evidence will be presented pointing strongly to there being only one enzyme.

Experimental.

The enzyme was prepared from Grade A cow's milk by the method of Dixon and Kodama [1926]. In some experiments the enzyme powder was not defatted. It was dissolved in buffer at the beginning of each experiment.

The concentrations of the more frequently used solutions were as follows: phosphate buffer solution $0{\cdot}25M$, p_H 7·2; methylene blue $0{\cdot}0005M$; benzylviologen $0{\cdot}0005M$; quinone $0{\cdot}001M$; hypoxanthine $0{\cdot}007M$; uric acid $0{\cdot}006M$ (for the inhibition experiments $0{\cdot}03M$ was used); acetaldehyde 20%; salicylaldehyde $0{\cdot}02M$; furfuraldehyde $0{\cdot}1M$; sodium salicylate $0{\cdot}1M$. Purines were dissolved in $0{\cdot}01M$ NaOH, the hypoxanthine solution being freshly made up every few days. Salicylaldehyde was generally preferred to acetaldehyde as being less volatile and therefore more reliable for Thunberg and Barcroft experiments.

[1] There were cases in which aldehydes alone were oxidised but the enzymic nature of the oxidation in these cases was doubtful.

The anaerobic experiments were carried out in Thunberg tubes, the hollow stoppers of which were made large and so shaped as to lessen the risk of the substrate boiling over. Except where stated differently, each tube contained 3 ml. buffered enzyme solution, 1 ml. methylene blue, x ml. substrate and $1-x$ ml. water or another solution. Sets of 4 tubes were evacuated simultaneously with a water pump.

The aerobic experiments were carried out in Barcroft manometers. After equilibration in the bath the substrate was tipped in from Keilin cups.

The temperature of the thermostat in each case was 38°.

Concentration curves.

In order to examine critically the evidence against the identity of the two enzymes certain aspects of the kinetics must be considered.

Morgan *et al.* [1922], using whole milk, found that the velocity of reduction of methylene blue was independent of the concentration of hypoxanthine. Dixon and Thurlow [1924, 2], using the caseinogen preparation, varied the concentration more widely and obtained the substrate concentration curve shown in Fig. 1, *B*. They found that the "critical concentration" (the concentration at which inhibition begins) depended on the enzyme concentration. That is to say that, whereas 0·0006 *M* hypoxanthine may be below the critical concentration with a highly active enzyme solution, the same concentration of hypoxanthine may be above the critical point—and cause inhibition—of an enzyme solution of low activity. With the whey preparation of Dixon and Kodama I have obtained the substrate concentration curve in Fig. 1, *A* (although when milk was used a

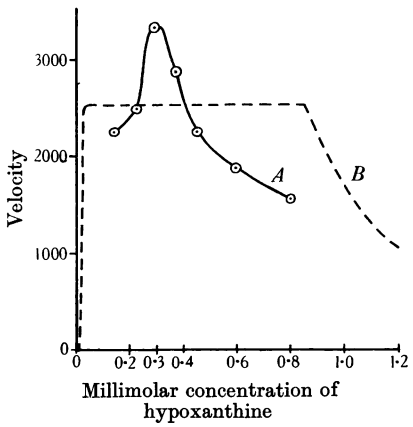

Fig. 1.

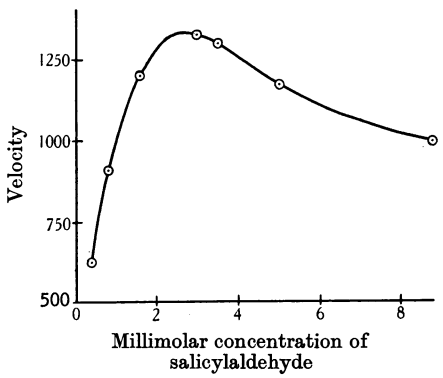

Fig. 2.

Fig. 1. *A*, Substrate concentration curve with hypoxanthine. *B*, Dixon and Thurlow's curve.

Fig. 2. Substrate concentration curve with salicylaldehyde.

curve similar to that of Dixon and Thurlow was obtained). Like Dixon and Thurlow's critical concentration, the optimum concentration was found to vary with enzyme concentration. That is, with a constant substrate concentration the relation between activity and dilution of the enzyme was not linear. For example, with a given concentration of hypoxanthine (optimum for the concentration of enzyme used) the reduction time of methylene blue was 1 min. 30 secs.

Dilution of the enzyme 1 in 3 increased the reduction time not to 4 mins. 30 secs. but to 7 mins. 45 secs. The substrate concentration curve for salicylaldehyde also shows a maximum though it is less marked (Fig. 2). The inhibition of the Schardinger enzyme by excess aldehyde begins at a concentration depending on the enzyme activity and here again the inhibition is more pronounced at lower enzyme activity. Curves showing the variation of enzyme activity with change in dilution are given in Fig. 3. It is noteworthy that neither curve

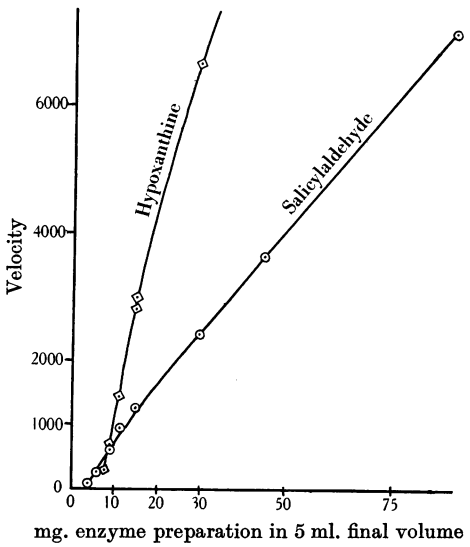

Fig. 3. Enzyme concentration curves with constant substrate concentration. Quantities as given under "Experimental", using 0·2 ml. hypoxanthine and 0·7 ml. salicylaldehyde.

passes through the origin: which means that, at high dilution, activity falls off rapidly owing to inhibition by excess substrate. (In the figures the velocity is expressed as the reciprocal of the reduction time multiplied by 10,000.)

The bearing of this on Wieland and Rosenfeld's adsorption experiments will now be discussed.

Adsorption experiments.

Wieland and Rosenfeld [1930] studied the adsorption of xanthine oxidase and the Schardinger enzyme by alumina Cγ and by calcium oxalate. They determined the ratio of the activities of the two enzymes in their preparation from methylene blue reduction velocities in the presence of xanthine and of salicylaldehyde. They then repeatedly treated the enzyme solution with the adsorbing agent and redetermined the ratio after each adsorption. The ratio was found to change. It was concluded that this change in ratio implied preferential adsorption of one enzyme and hence that the enzymes could not be identical.

These authors defined their unit of activity as the enzyme quantity which reduces methylene blue under given conditions in 5 mins. As it would be laborious to adjust the enzyme quantity in each case to give a reduction time of exactly 5 mins., it seems probable that they actually observed the reduction times and calculated the enzyme units. This calculation involves the assumption that the activity is proportional to the concentration of enzyme. But Fig. 3 shows that such is not the case if the concentration of purine is kept constant.

Now, because the inhibition by excess substrate is more pronounced with purine than with aldehyde, the ratio of the reduction times (and therefore of their reciprocals) should vary with varying enzyme concentration, unless these substrates are present optimally for each particular enzyme concentration. Such variation has been observed experimentally and is shown in Fig. 4. The ratio can be varied widely merely by varying the dilution of the enzyme. Hence in experiments in which this ratio is studied, either (*a*) the enzyme quantity must be adjusted to give a standard reduction time with one substrate and the reduction time with the other substrate observed and compared, or (*b*) the ratio observed must be corrected for dilution of the enzyme by reference to such a curve as in Fig. 4. One of Wieland and Rosenfeld's adsorption experiments was repeated, with the above precautions. The reduction times of an enzyme solution were determined in presence of hypoxanthine and of salicylaldehyde. Ammonium oxalate and calcium chloride were then added and the precipitate centrifuged off. The reduction times (and hence the ratios) were redetermined using the centrifugate in various dilutions. The ratio was found to have changed, but the change was exactly that which would be predicted from Fig. 4 if the adsorption had merely reduced the enzyme concentration. The precipitate was then eluted with phosphate buffer at p_H 8·0, as described by Wieland and Rosenfeld, and the ratios were determined at two dilutions and compared with those read off from the standard curve. There was no significant change. Some of the observed ratios are shown in Table I compared with those predicted for known

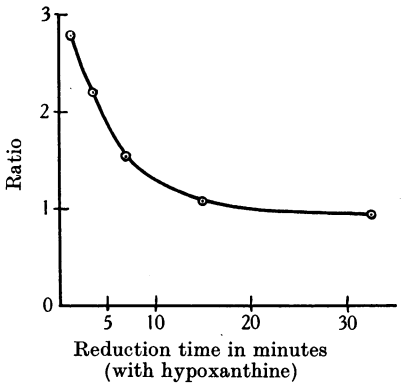

Fig. 4. Dependence of ratio on enzyme concentration.

Table I.

The "predicted ratio" was found for each hypoxanthine reduction time from Fig. 4. Quantities as given under "Experimental", using 0·2 ml. hypoxanthine and 0·7 ml. salicylaldehyde.

	Reduction time		Ratio of reciprocals	Predicted ratio
	Hypoxanthine	Salicylaldehyde		
Unadsorbed material (in centrifugate)	2 mins. 15 secs.	5 mins. 30 secs.	2·5	2·5
	3 ,,	7 ,, 15 ,,	2·3	2·3
	5 ,, 10 ,,	8 ,, 30 ,,	1·6	1·7
Adsorbed and eluted material	2 ,, 30 ,,	6 ,, 30 ,,	2·6	2·4
	7 ,,	10 ,, 45 ,,	1·5	1·5

reduction times with hypoxanthine. Within limits almost any desired ratio could be obtained before or after adsorption or elution by arbitrarily selecting a given dilution of enzyme solution. But the change in ratio does not give any evidence of a separation of the two enzymes.

With regard to Wieland and Rosenfeld's alleged separation it must be emphasised that

(*a*) the separation was only partial and never great;

(*b*) unless performed under more controlled conditions the experiments cited lose their significance;

(*c*) under such controlled conditions there was no indication of any separation.

METHOD OF COMPETITION.

Wieland and Macrae [1930] showed that the velocity of reduction of methylene blue with xanthine and aldehyde present together as substrates was greater than with either alone. I have been unable to confirm this additive effect. For instance, in one experiment the reduction times were: with 0·2 ml. hypoxanthine 5 mins. 30 secs., with 0·7 ml. salicylaldehyde 9 mins. 15 secs., and with 0·2 ml. hypoxanthine + 0·7 ml. salicylaldehyde 6 mins. 30 secs. The two substrates appear to be competing for the same enzyme, the reduction rate with both together being intermediate between the rates with either alone.

Wieland and Mitchell [1932] used benzoquinone as hydrogen acceptor and showed that the rate of its reduction by xanthine and acetaldehyde present together as substrates was greater than that with either alone. The experimental evidence for their conclusions is hardly satisfactory for the following reasons.

(1) The xanthine added was only equivalent to the quinone. Naturally on adding acetaldehyde the rate was increased; if more xanthine had been added instead the rate would have been increased. In this method it is essential that the enzyme be saturated with its substrate throughout the reaction.

(2) Their curves, which are reproduced in Fig. 5, clearly demonstrate that with increasing amounts of aldehyde the rate of reduction of quinone was progressively more rapid. They selected a concentration of aldehyde which did not saturate the enzyme and, as shown by their own curves, the addition of more aldehyde also had an additive effect.

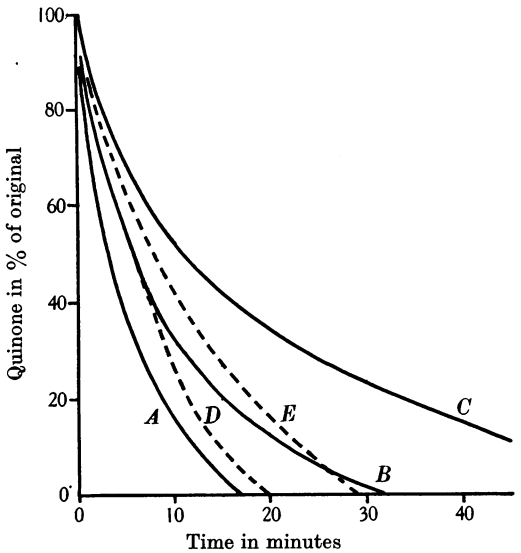

Fig. 5. Wieland and Mitchell's curves. *A*, 2 ml. aldehyde; *B*, 1 ml. aldehyde; *C*, 0·5 ml. aldehyde; *D*, 1 ml. aldehyde + xanthine; *E*, 0·5 ml. aldehyde + xanthine.

(3) Of the two experiments given, in that with the higher concentration of aldehyde (curve *D*)—*i.e.* where the aldehyde concentration approached saturation —the additive effect was patently less, lending support to the above criticisms.

(4) It is noteworthy that in one of the two addition experiments the curves for aldehyde alone (*B*) and for aldehyde + purine (*D*) are coincident for the

greater part of the reaction, and only towards the end, *i.e.* when the xanthine is nearly exhausted, is there an additive effect.

(5) An alternative and as reasonable an explanation is that the destruction of enzyme by quinone proceeds with different velocities in the two cases. There is other evidence that purines protect the enzyme from destruction.

As Wieland and Mitchell's results with quinone were inconclusive the experiment was repeated. The substrates—hypoxanthine and salicylaldehyde—were present in optimum concentrations for the particular enzyme strength used, as determined experimentally. The reduction of the quinone was followed in two different ways. In the first method a series of 3 Thunberg tubes was used. Each tube contained 2 ml. enzyme-buffer solution, 1·0 ml. quinone and 0·75 ml. benzylviologen as indicator; the first tube contained 0·25 ml. hypoxanthine, the second 1 ml. aldehyde and the third contained both. It was assumed that the reduction of quinone did not proceed through benzylviologen as an intermediary. The appearance of the first traces of the blue colour of the reduced form of benzylviologen marks the completion of the quinone reduction. The times were: with hypoxanthine 5 mins. 15 secs., with aldehyde 16 mins. 15 secs., and with hypoxanthine + aldehyde 12 mins. 45 secs. These results give no indication of any additive effect.

It might be objected that the presence of the dye affected the results—that the quinone was not being reduced directly but *via* the dye. This was unlikely from considerations of potential but to meet the possible objection the experiment was carried out in another way which was suggested by an early experiment of Dixon [1926]. Enzyme-buffer solution and quinone in the same proportions as in the above experiment were put into the tube shown in Fig. 6 which was

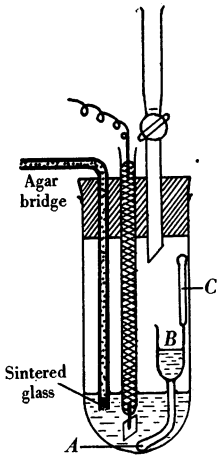

Fig. 6.

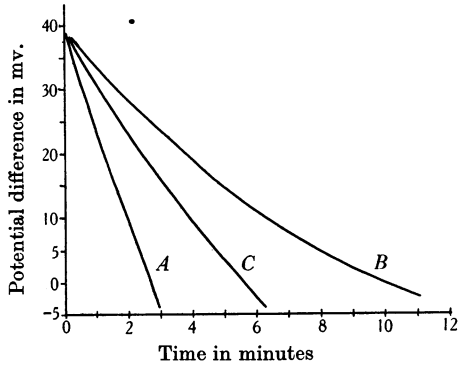

Fig. 7.

Fig. 6. Vacuum electrode vessel.

Fig. 7. Competition of substrates, using quinone. *A*, hypoxanthine; *B*, salicylaldehyde; *C*, hypoxanthine + salicylaldehyde.

evacuated and incubated at 38° for 15 mins., equilibrium with the electrode being then reached. The substrate was added and the potential followed against a saturated calomel half-cell, the latter being at room temperature. The agar in the capillary bridge was prevented from being forced into the evacuated vessel

by sintering a plug of powdered glass in the end: it was then filled with agar-KCl containing a suspension of kaolin. The hypoxanthine was added from the glass cup *B*. The stalk *A* rested on the bottom of the tube, the curved foot keeping the cup against the tube wall, minimising the risk of fouling the gold electrode. On inverting the tube the arm *C* prevented immersion of *B* and allowed the substrate solution to drain out. Mixing was complete after three inversions. Deaerated aldehyde was added through the burette. When no aldehyde was used boiled-out water was added in this way as a control. The results of this experiment are shown in Fig. 7. Competition is clearly indicated by the curves.

Competition was also observed anaerobically when the comparatively negative dye Nile blue was employed.

It thus appears that, whether the hydrogen acceptor is Nile blue, methylene blue, quinone or molecular oxygen, provided that the enzyme is saturated with substrate, competition is always observed. These findings would be unlikely if two enzymes were concerned.

Inhibitors.

Effects of salicylate and formate. Sen [1931] found that vanillin and piperonal inhibit the aerobic oxidation of hypoxanthine by xanthine oxidase. But aldehydes themselves in these experiments behave at once as hydrogen donators and as inhibitors. In order to avoid this complication the oxidation product of salicylaldehyde, namely salicylate, was tested. The results, which are summarised in Table II, show that it inhibits xanthine oxidase and the Schardinger

Table II.

Quantities as given under "Experimental", using 0·2 ml. hypoxanthine, 0·7 ml. salicylaldehyde and 0·1 ml. salicylate.

	Substrate tipped after	Reduction time	
		Hypoxanthine	Salicylaldehyde
Control—no salicylate	2 mins. 30 secs.	5 mins.	11 mins.
Salicylate in stopper	2 ,, 30 ,,	9 ,, 30 secs.	34 ,,
,,	40 ,,	10 ,, 30 ,,	38 ,,
Salicylate in tube	2 ,, 30 ,,	8 ,, 45 ,,	37 ,,
,,	40 ,,	8 ,, 30 ,,	27 ,,

enzyme to approximately the same extent. To determine whether the action of salicylate on the enzyme was one of inhibition or destruction the following experiment was performed. The enzyme was incubated with and without salicylate for 40 mins. before the substrate was tipped in. As shown in Table II there is no indication that the activity of the enzyme is diminished by long contact with salicylate—in fact there is some evidence of increased activity. Neutralised sodium formate also inhibited both enzymes in approximately similar degree. The inhibition of the purine oxidase by the oxidation product of the Schardinger enzyme substrate speaks for identity of the enzymes.

Effect of uric acid. Dixon and Thurlow [1924, 2] found that anaerobically both enzymes were inhibited by uric acid. This fact constitutes strong evidence in favour of identity. While it is reasonable to assume that uric acid is not a general inhibitor of dehydrogenases the evidence would gain in force if the assumption were verified experimentally. The most suitable enzyme on which to test the effect of uric acid would be the aldehyde oxidase from potato which has certain properties in common with the Schardinger enzyme although incapable of activating hypoxanthine [Bernheim, 1928]. Both enzymes activate

aldehydes, both reduce nitrate and oxygen directly and in each case some product of the aerobic oxidation rapidly destroys the enzyme. A crude preparation of the oxidase was therefore made as follows. Potato juice was saturated with ammonium sulphate and the precipitate filtered off, washed several times with saturated ammonium sulphate and dissolved in buffer. This solution was filtered and the precipitation was repeated. The precipitate obtained was dissolved in buffer at p_H 7·0 and the resultant brown solution was used for the experiment. Using acetaldehyde, furfuraldehyde and salicylaldehyde as substrates it was found that anaerobically uric acid had no inhibitory effect whatever on the aldehyde oxidase from potato. Uric acid was also completely without effect on the lactic and α-glycerophosphate dehydrogenases from yeast.

Effect of 3-methylxanthine. Prof. Keilin kindly informed me that 3-methylxanthine strongly inhibits xanthine oxidase aerobically and suggested that the effect of this purine on the Schardinger enzyme should be studied. Experiment showed that the aerobic oxidation of salicylaldehyde as well as of hypoxanthine was definitely inhibited (Figs. 8 and 9). The difference in the shapes of the two curves will be dealt with in a later section, but it is noteworthy that the inhibition is pronounced during the first 5 mins. and after that time the control velocity tails off rapidly, pointing to extensive destruction of enzyme.

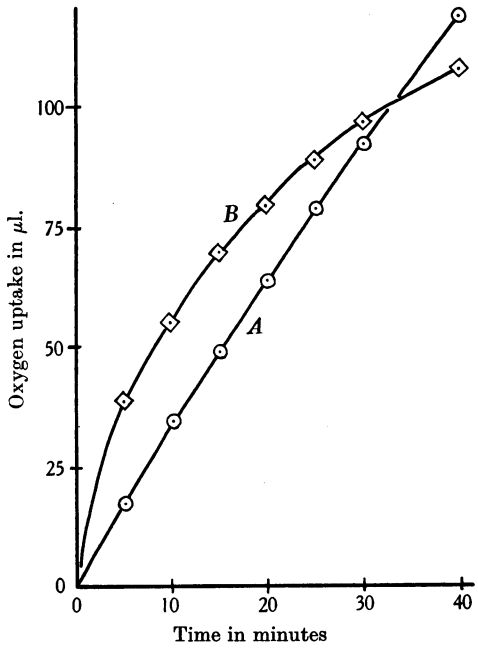

Fig. 8.

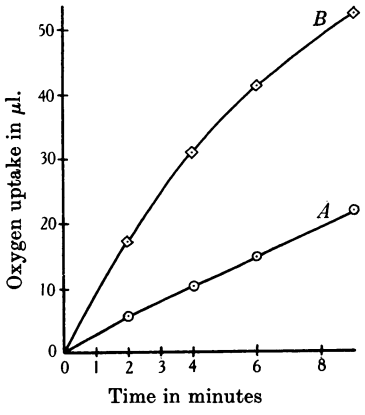

Fig. 9.

Figs. 8 and 9. Inhibition of Schardinger enzyme by 3-methylxanthine.

The right Barcroft manometer cups contained 2 ml. enzyme-buffer solution, and the left (compensating) cups contained 2 ml. buffer. Each cup contained 0·7 ml. salicylaldehyde in a Keilin cup. The right cup of manometer *A* contained 3-methylxanthine. The purine was dissolved in 0·1 *N* NaOH, and 0·6 ml. containing 10 mg. was used. The same quantity was titrated with HCl: it was found that 0·05 ml. of the NaOH had been neutralised by the purine, while 0·55 remained in excess. Therefore 0·55 ml. NaOH was added to the right cup of manometer *B* and to each compensating cup to make the p_H identical. No alkali was put into the pots for absorption of carbon dioxide.

Coombs [1927] showed that 3-methylxanthine, though not itself oxidised, inhibited the enzymic reduction of methylene blue by hypoxanthine. The results in Table III show that 3-methylxanthine also inhibits the enzymic oxidation of various aldehydes by methylene blue.

Table III.

Quantities as given under "Experimental", using 0·0012 *M* 3-methylxanthine and aldehydes in final concentrations shown. Three different enzyme preparations were used for the several aldehydes.

	Reduction time	
	Without purine	With purine
Acetaldehyde (0·01 *M*)	2 mins. 30 secs.	41 mins.
Vanillin (0·01 *M*)	20 ,,	73 ,,
Salicylaldehyde (0·003 *M*)	3 ,, 30 ,,	10 ,,

Preferential inhibition of one enzyme. From time to time cases have been brought to my notice (private communications) of a reagent which strongly inhibits one enzyme although it has little or no effect on the other. But on careful examination, employing suitable controls, these differences largely vanished. In one case the conditions of the experiment were such that the oxidation of aldehyde was not enzymic. In another case it was claimed that the Schardinger enzyme was completely inhibited by a concentration of 2:4-dinitrophenol which had very little effect on xanthine oxidase [Davidson, unpublished experiments]. The inhibition obtained with formaldehyde was certainly greater than with xanthine or hypoxanthine but it was also greater than with other aldehydes, and in no case was the Schardinger enzyme completely inhibited.

I found that neutralised sodium acetate almost completely inhibited xanthine oxidase (using hypoxanthine) although it had but little effect on the Schardinger enzyme (using salicylaldehyde). However, the inhibition, while still marked, was less when xanthine was used or when the hypoxanthine concentration was high. Further, the inhibition of the Schardinger enzyme was greater with acetaldehyde or furfuraldehyde, while with formaldehyde it was even greater than with xanthine.

In view of these experiences it is urged with cogent reason that claims for the non-identity of the enzymes should not be based on preferential inhibition until the matter has been investigated in great detail and all possible controls performed. Indeed it would appear that convincing proof of non-identity can only be provided by complete separation of the enzymes.

Protection of the Schardinger enzyme by purines.

The rate of oxygen consumption by the Schardinger enzyme in presence of an aldehyde falls off very rapidly with time (Fig. 8, curve *B*) and it is in practice impossible to obtain a theoretical uptake before complete destruction of the enzyme sets in. This effect has been attributed to the production of hydrogen peroxide [Dixon, 1925]. Fig. 8, *A* represents the velocity of aldehyde oxidation in presence of 3-methylxanthine. Here the velocity falls off to a much less marked extent, so that after a short time the velocity in presence of the purine is greater than that of the control. The obvious explanation is that the Schardinger enzyme is protected by the purine from destruction by some reaction product.

It was of interest to know whether uric acid exerted a similar protective effect on the Schardinger enzyme. The oxygen uptake by furfuraldehyde in the presence of the enzyme was therefore followed with and without uric acid. The curves in Fig. 10 show that oxidation was still proceeding in the presence of uric acid

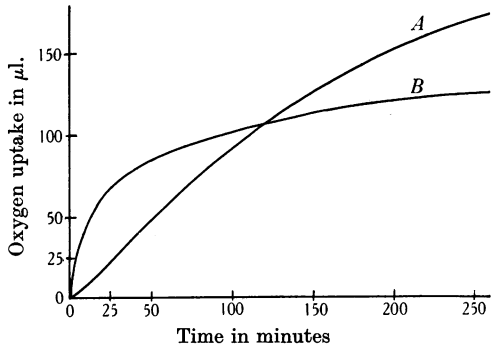

Fig. 10. Protection of Schardinger enzyme by uric acid during aerobic oxidation of furfuraldehyde. *A*, with uric acid; *B*, without. The right Barcroft manometer cups contained 2 ml. enzyme-buffer solution, and the left (compensating) cups contained 2 ml. buffer. Each cup contained 0·2 ml. furfuraldehyde in a Keilin cup. The cups of manometer *A* contained 1·1 ml. 0·03 *M* uric acid. Water was added to all cups to adjust the volume to 3·3 ml. No alkali was put into the pots.

when it had practically ceased in the control. Presumably protection is effected by adsorption and the specific adsorption of purines at the active group of the aldehyde oxidase speaks for its identity with the active group of the purine oxidase.

The liver enzyme.

Xanthine oxidase is found in a number of tissues and is invariably accompanied by the Schardinger enzyme, as shown by Morgan [1926]. If these two enzymes are independent it would indeed be extraordinary if they were always present in the same proportions. The ratios of the activities of the two enzymes from liver were therefore compared with the ratio for milk.

Crude xanthine oxidase preparations were made from ox liver in various ways. Preparations A_1 and A_2 were made by half-saturating an aqueous extract with ammonium sulphate, drying the precipitate and extracting it with ether. *B* was acetone liver. C_1 and C_2 were made by extracting *B* with phosphate

Table IV.

Details as given under "Experimental", using 0·2 ml. hypoxanthine and 0·7 ml. salicylaldehyde. The "predicted ratio" was found for each hypoxanthine reduction time (using the milk enzyme) by reference to Fig. 4.

	Reduction time		Ratio of reciprocals	
Preparation	Hypoxanthine	Salicylaldehyde	Observed	Predicted
A_1	9 mins. 15 secs.	16 mins. 30 secs.	1·8	1·3
A_2	15 ,,	12 ,, 15 ,,	0·8	1·1
B	7 ,,	15 ,,	2·1	1·5
C_1	4 ,, 20 ,,	10 ,, 45 ,,	2·6	2·0
C_2	4 ,, 45 ,,	10 ,, 40 ,,	2·2	1·7
D	4 ,, 15 ,,	6 ,, 30 ,,	1·5	2·0
		Mean	1·8	1·6

buffer, half-saturating with ammonium sulphate and extracting the dried precipitate with ether. *D* was a dialysed aqueous extract. The reduction times of methylene blue with hypoxanthine and salicylaldehyde are shown in Table IV and the ratios are compared with those for milk at corresponding dilutions of enzyme (the criterion of dilution being the reduction time with hypoxanthine) by reference to Fig. 4. The preparations were mostly coloured and had a small reducing blank. The agreement between the ratios found for liver and for milk, though not exact, is striking.

Colostrum. Both enzymes were found in two samples of cow's colostrum. One of the samples was taken a few minutes after parturition.

Embryonic appearance of the enzymes.

Morgan [1930] studied the first appearance of xanthine oxidase in chick embryonic tissues. He found it in the yolk sac on the seventh day and in various other tissues after that time. If the Schardinger enzyme emerged simultaneously with xanthine oxidase in the chick embryo one would have a striking confirmation of identity.

Morgan used Bach's technique in which the tissue is ground in sodium fluoride solution and allowed to stand for a day or more until the reducing blank is negligible. In the present investigation the tissue was washed with water and ground with sand. The fluid was centrifuged off and the residue was suspended in water and again ground with sand. The combined extracts were half-saturated with ammonium sulphate, and after 15 mins. the precipitate was centrifuged off, washed with saturated ammonium sulphate and dissolved in 10 ml. buffer. The experiments were performed within a few hours of opening the eggs. With this technique traces of xanthine oxidase were detected some days earlier than recorded by Morgan. Yolk sac and whole embryo were used for these experiments. It was impracticable to remove the yolk sac earlier than the third day. In order to detect small traces of enzyme both the final volume and the concentration of methylene blue were kept low. The results given in Table V show that the first appearances of the two enzymes are exactly

Table V.

Each tube contained 2 ml. enzyme-buffer solution, 0·15 ml. methylene blue and 0·05 ml. hypoxanthine or 0·08 ml. salicylaldehyde. The blank contained water instead of substrate and in each case the volume was made up to 2·25 ml. with water.

			Reduction time			Enzyme present	
Age in days	Tissue	No. of eggs	Hypo-xanthine	Salicyl-aldehyde	Blank	Xanthine oxidase	Schar-dinger
3	Yolk sac	8	30%*	20%*	∞	+	+
4	,,	5	5 hours	8 hours	∞	+ +	+ +
6	,,	6	20 mins.	50 mins.	3 hours	+ + +	+ + +
7	Whole embryo	3	∞	∞	∞	−	−
8	,,	2	8 hours	8 hours	8 hours	−	−
9	,,	2	12 hours	50%*	∞	+	+

* Percentage reduction in 12 hours.

coincident. If all dehydrogenases made their first appearance at the same time the value of this observation would be small. Five other enzymes were therefore tested for and the following were found in the 8-day whole embryo: succinic, lactic, hexosediphosphate, α-glycerophosphate and glucose dehydrogenases.

Effect of heat.

Xanthine oxidase and the Schardinger enzyme are both more resistant to heat than the majority of dehydrogenases. Dixon and Thurlow [1924, 2] stated that "both enzymes... are destroyed in the same degree by heating for a short time". This observation has been confirmed, an enzyme preparation being heated for varying lengths of time. After heating in a water-bath for an hour at 67° both enzymes were still active although considerable destruction of each had been brought about. More prolonged heating destroyed both. In no case was separation of the enzymes achieved.

Dual destruction.

As a routine procedure during this investigation when for any reason a preparation of xanthine oxidase became inactive towards hypoxanthine its activity towards aldehydes was also tested. In many such experiments there was never encountered a case either of separation of the enzymes or of individual destruction.

Effect of inactive protein on the ratio.

For given conditions the ratio of the activities of the two enzymes is found to vary to some extent in different preparations. The variation is never marked and in general the more protein present the higher the ratio: for instance it is high in a preparation rendered partially inactive through long keeping or by heating. It is reasonable to suppose that with destruction of enzyme inert protein is formed which is still capable of adsorbing the substrate. Thus if adsorption of substrate by inert protein were extensive the concentration remaining in solution might then be suboptimum, and the activity of the enzyme would appear to be low. If the adsorption effects with the two substrates were not identical the ratio would appear to change. This hypothesis was put to experimental test. The reduction time of an enzyme solution with salicylaldehyde was found to increase on addition of boiled enzyme solution. Increasing the aldehyde concentration restored the reduction time to the original value. Increase of the aldehyde concentration of the control had practically no effect. The experiment was repeated using a solution of egg albumin. The results are shown in Table VI. The reduction time with hypoxanthine is almost unaffected by inactive protein whereas that with salicylaldehyde is increased. Consequently the ratio rises as shown in the table.

Table VI.

Quantities: 1 ml. enzyme-buffer solution; 2 ml. buffer, boiled enzyme solution or 4% egg albumin in buffer; others as under "Experimental".

	Reduction time
Enzyme + 0·2 ml. hypoxanthine	3 mins. 15 secs.
,, ,, ,, + boiled enzyme	3 ,,
,, ,, ,, + albumin	3 ,, 15 ,,
,, + 0·7 ml. salicylaldehyde	8 ,,
,, ,, ,, + boiled enzyme	12 ,, 15 ,,
,, ,, ,, + albumin	12 ,,
,, + 1·0 ml. ,,	8 ,, 30 ,,
,, ,, ,, + boiled enzyme	8 ,,
,, ,, ,, + albumin	8 ,,
Boiled enzyme solution + salicylaldehyde	∞
Activity ratio of control	2·5
,, ,, in presence of boiled enzyme	3·7
,, ,, ,, ,, albumin	4·1

Mixed dismutation.

Green *et al.* [1934] showed that isolated dehydrogenase systems cannot react with one another directly: the reaction can only proceed through some intermediary of the type of an oxidation-reduction indicator. They showed, for instance, that, in presence of the respective dehydrogenases, lactate and fumarate would not react anaerobically to form pyruvate unless a suitable oxidation-reduction indicator were added. The indicator was alternately reduced by the lactate system and oxidised by the fumarate system, behaving as a hydrogen carrier. So also formate would only react with nitrate, in presence of the enzymes, to yield nitrite if a carrier were present. Xanthine oxidase + hypoxanthine was linked with the succinic dehydrogenase and with the lactic dehydrogenase systems. Several different preparations of xanthine oxidase (made by the method of Dixon and Kodama [1926]) were used, including one prepared by the author, but in no case was any uric acid produced unless a carrier had been added. These last experiments proved that the xanthine oxidase preparations contained no carrier. Now these findings suggested a possible means of investigating the question of the identity of the Schardinger enzyme and xanthine oxidase, for if the two systems will react without added carrier it is unlikely that two dehydrogenases are concerned.

Accordingly enzyme, salicylate and hypoxanthine were incubated anaerobically. After 5 hours the solution was deproteinised with uranium acetate and tested with Benedict's uric acid reagent and with the phosphotungstic reagent, but no uric acid was detected. Nor was any detected either when the relative concentrations of the reagents were varied or when benzylviologen or methylviologen (indicators with potentials of -359 mv. and -446 mv.) were added as carriers, except traces equivalent to the indicator reduced. The non-production of uric acid even in the presence of a carrier may be due to the irreversibility of the change from acid to aldehyde as catalysed by the Schardinger enzyme. For indeed experiment has shown that the system enzyme-aldehyde-acid-indicator is not reversible regardless of the type of aldehyde tried.

The converse experiment was then performed yielding more promising results. When uric acid and salicylaldehyde were incubated with the enzyme the uric acid almost completely disappeared. Uric acid also diminished when various other aldehydes—acetaldehyde, propionaldehyde, *iso*butylaldehyde, furfuraldehyde, piperonal—were tried, but the disappearance never proceeded to so great an extent as when salicylaldehyde was used. A series of Thunberg tubes was incubated, each tube containing enzyme, uric acid and salicylaldehyde. The uric acid was estimated at intervals, the results being shown in Table VII. Control

Table VII.

Each tube contained 2 ml. enzyme-buffer solution (or buffer only), 0·4 ml. uric acid, 1 ml. salicylaldehyde, with water to make the total volume up to 5 ml. To one tube 0·1 ml. salicylate was added.

	Incubation time	Uric acid mg.
Enzyme + uric acid + aldehyde	0	0·4
,, ,, ,,	1 hour	0·3
,, ,, ,,	2 hours 20 min.	0·2
,, ,, ,,	6 ,, 15 ,,	0·1
,, ,, ,,	10 ,,	0·05
,, ,, ,, + salicylate	6 ,,	0·2
Uric acid + aldehyde + buffer	15 ,,	0·4
Enzyme + uric acid	15 ,,	0·4
Boiled enzyme + uric acid + aldehyde	15 ,,	0·4

experiments were carried out—the aldehyde replaced by water, the enzyme omitted or boiled—but in no case did the concentration of uric acid decrease. The experiments were repeated several times with five enzyme preparations, always with the same result. Salicylate was found to inhibit the reaction between uric acid and salicylaldehyde. That this was due to inhibition rather than to an effect on the equilibrium of the reaction was shown by the following experiment. When uric acid had largely disappeared from a tube salicylate was tipped in from the hollow stopper in a duplicate tube. After incubation overnight no increase in uric acid was observed over that in the control.

The question arose, what is the fate of the uric acid? Is it reduced to xanthine and hypoxanthine? Or does it, when activated by the enzyme, combine with the aldehyde? Into each of four large vacuum tubes were measured 25 ml. very active enzyme-buffer solution, 10 ml. uric acid and 12 ml. salicylaldehyde. A few drops of octyl alcohol were added to reduce foaming during evacuation and to prevent bacterial growth. After 48 hours' incubation more than 90 % of the uric acid had disappeared. The contents of each tube were deproteinised with uranium acetate. The filtrate was made alkaline with ammonia, and ammoniacal silver nitrate was added. The next day the precipitates were centrifuged off, washed, suspended in water and decomposed with hydrogen sulphide. The solutions were filtered repeatedly until clear. Portions of one solution (which contained some uric acid) were shown to reduce methylene blue in presence of the enzyme with production of more uric acid. It was possible that the reduction was due to a compound between aldehyde and uric acid, the former reducing methylene blue and the latter being liberated, but ferric chloride tests failed to show the presence of hydroxybenzene derivatives in any of the solutions. The three other solutions were then heated with alkaline permanganate, acidified and extracted with toluene. This treatment should oxidise any benzene ring compounds likely to be present (*e.g.* salicylaldehyde or complex containing it) to benzoic acid, the latter being the only aromatic acid likely to be extracted by toluene. The extract was washed with saturated sodium chloride, dried by filtering and titrated with alcoholic NaOH. The titrations were negligible.

In the absence of support for the compound hypothesis attention was turned to possible reduction products of uric acid. To an enzyme-buffer solution were added uric acid, salicylaldehyde and a few drops of octyl alcohol. The mixture was put into flasks which were immediately evacuated and incubated at 45° for 3 days with frequent shaking. The flasks were then cooled and opened. Test showed that the uric acid had nearly all disappeared. The proteins were removed with 10 % trichloroacetic acid and the filtrate was concentrated *in vacuo*. The concentrate was filtered, made alkaline with ammonia and filtered again, and ammoniacal silver nitrate was added. The silver precipitate was filtered off, washed with ammonia and then with water and boiled with nitric acid of sp.gr. 1·1. The solution was filtered hot and allowed to cool. The next day a copious precipitate had formed. This was filtered off and recrystallised twice from nitric acid of sp.gr. 1·1. The crystals were suspended in water and decomposed with hydrogen sulphide. The solution was filtered several times, concentrated on a water-bath and filtered hot. Ammonia was added to the hot solution until the p_H was 7·6. The solution was then allowed to cool and the precipitate centrifuged off. The substance was recrystallised from water and dried. It had the following properties:

(1) It was a non-deliquescent white powder.

(2) Its aqueous solution had a slightly alkaline reaction.

(3) Analysis showed 39·7 % N (hypoxanthine has 41·2 %).
(4) With xanthine oxidase it reduced methylene blue rapidly in a vacuum tube.
(5) Similarly it reduced methylviologen.
(6) It took up oxygen in the presence of xanthine oxidase.
(7) Uric acid was produced by incubation with the enzyme and 0·1 % methylene blue.
(8) Uric acid was produced by aerobic incubation with the enzyme.
(9) It formed a silver salt insoluble in cold dilute nitric acid.

The amount of material available was not sufficient for more thorough purification and elementary analysis, but it is improbable that any compound other than hypoxanthine would give these specific biochemical tests. That milk does not contain any hypoxanthine is shown by the fact that methylene blue is never reduced by fresh milk.

These results may be explained in another way. It is known that xanthine, under the influence of the enzyme, is dismuted to some extent to form hypoxanthine and uric acid. Conversely a mixture of hypoxanthine and uric acid reacts to form some xanthine [Green, 1934]. This equilibrium may be represented by the following equation:

$$\text{Hypoxanthine} + \text{Uric acid} \rightleftharpoons 2\ \text{Xanthine}.$$

If xanthine oxidase and the Schardinger enzyme are one a similar reaction between the reductant of one enzyme and the oxidant of the other might occur, the reaction proceeding according to the equation

$$\text{Uric acid} + \text{Aldehyde} \rightarrow \text{Xanthine} + \text{Acid}.$$

Some of the xanthine should then be dismuted, or react with more aldehyde, forming hypoxanthine. Proof that this does occur is furnished experimentally by the disappearance of uric acid and the isolation of hypoxanthine. The term "mixed dismutation" is suggested for the phenomenon. Numerous controls prove that it only occurs in presence of the enzyme. It has been shown [Green *et al.*, 1934] that isolated dehydrogenase systems only react with one another if an oxidation-reduction indicator or carrier is present, and that there is no carrier in the xanthine oxidase preparation. Therefore the phenomenon of mixed dismutation is presented as evidence that only one dehydrogenase is concerned in the activation of purines and of aldehydes.

Conclusion.

In this paper several lines of evidence have been presented in favour of the identity of the two enzymes, and in addition the evidence of Wieland and his collaborators against identity has been shown to be invalid for the most part. The present position may be summarised by the statement that either a set of extraordinary coincidences accounts for the similarity in behaviour and invariable mutual accompaniment of the enzymes or that the two enzymes are identical.

This raises the question: what is meant by identity? Do we mean that one and the same active group (or groups) on the enzyme surface activates both substrates? Do we mean that two kinds of active groups are carried by the same colloid? Or again do we mean a combination of both—one common adsorbing group of low specificity as well as two activating groups, one for certain purines and one for all aldehydes? The first and third seem the more probable suggestions but until more is known about the mechanism of enzyme action it is premature

to discuss these questions. Suffice it to say that the same enzyme appears to activate certain purines and all aldehydes, the term enzyme having its generally understood, though vaguely defined, biochemical meaning.

SUMMARY.

1. The evidence in the literature for and against the identity of xanthine oxidase and the Schardinger enzyme is collated.

2. A study of the kinetics shows that Wieland and Rosenfeld's adsorption experiments—which formerly had been taken to disprove identity—were insufficiently controlled.

3. Competition and never addition is observed when both substrates are present together.

4. Any reagent inhibiting one enzyme also inhibits the other.

5. Purines not only inhibit the Schardinger enzyme but protect it from destruction.

6. Uric acid does not inhibit the aldehyde oxidase from potato.

7. Experimental support is adduced for the contention that preferential inhibition of one enzyme offers no proof of non-identity.

8. Activity ratios for the milk and for the liver enzymes are closely parallel.

9. In yolk sac and in whole embryo the first appearances of the two enzymes are exactly coincident, but (in the whole embryo) are preceded by several other dehydrogenases.

10. Preferential destruction has never been brought about by heat or other agent.

11. Certain observed changes in the activity ratio in the presence of much protein are accounted for by adsorption of one substrate, leaving suboptimum concentration in solution.

12. "Mixed dismutation" occurs between uric acid and aldehyde, with disappearance of uric acid and formation of hypoxanthine, the purine dehydrogenase system reacting directly with the aldehyde dehydrogenase system in the absence of a respiratory carrier.

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