CCLXXII. STUDIES ON XANTHINE OXIDASE.

XII. THE OXIDATION OF NUCLEOSIDES.

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ACCORDING to the orthodox view of purine metabolism the oxidation of hypoxanthine and xanthine to uric acid takes place (under the influence of xanthine oxidase) only after they have been liberated from the nucleosides. On this view uric acid is produced in the body in the free state. The evidence hitherto available does not however exclude the alternative possibility, namely that the purines are deaminated and oxidised while still combined as nucleosides, so that the nucleoside of uric acid is first produced and free uric acid subsequently liberated from this by hydrolysis.

This alternative view indeed gains some probability from the fact that Davis, Newton and Benedict [1922] have isolated the nucleoside of uric acid (uric acid *d*-riboside) from the blood of a number of animals, including man. It is not easy to account for its existence in the body on the orthodox view of purine metabolism.

For this reason it is important that xanthine oxidase should be tested with respect to its power to oxidise hypoxanthine and xanthine while still combined in the form of nucleosides. If the second view is the correct one, the oxidase should be capable of directly oxidising inosine (= "hypoxanthosine") and xanthosine with the production of Benedict's nucleoside, while if the accepted view is correct it should only be able to oxidise the free purines.

Such an investigation is also of interest from the point of view of enzyme specificity, since this oxidase has been found [Coombs, 1927] to show remarkable specificity within the purine series.

We have therefore investigated the action of the xanthine oxidase of milk on the following substances: inosine, xanthosine, adenosine, guanosine, inosinic acid, muscle adenylic acid, cozymase.

METHODS.

Since much difficulty was experienced in preparing the nucleosides by following the published methods, the reasons for failure were investigated in some detail. The results of this work, together with the methods finally adopted for the preparation of the pure nucleosides, are given in an Appendix to this paper.

The inosinic acid and muscle adenylic acid used were prepared by the methods of Ostern [1932], and the cozymase preparation used was obtained by the method of Euler and Myrbäck [1928]. The inosinic acid was in the form of the pure crystalline barium salt, and solutions were made up from this as required by suspending weighed amounts in water containing Na_2SO_4 in slight excess. On warming to about 40° the inosinate went into solution, and BaSO_4 was precipitated and filtered off. The other substances were made up in water and adjusted to p_H 7.5 if necessary.

All the experiments were carried out at 37°, and the p_H of the solutions was maintained at 7.5 by phosphate buffer. For the majority of the tests the methylene blue technique was used, and the refinements described by Green and Dixon [1934] were employed throughout. The measurements of oxygen uptake were carried out in Barcroft manometers by the direct method, as described by Dixon [1934].

EXPERIMENTAL OBSERVATIONS.

Exp. 1 shows the results of a preliminary survey of the action of a xanthine oxidase preparation from milk on the nucleosides by means of the methylene blue technique. The oxidase preparation used in this experiment was the "whey preparation," made as described by Dixon and Kodama [1926, p. 1105] except that the ether extraction was omitted. (The significance of this will be seen later.) In what follows we shall refer to this as "whey preparation A."

Exp. 1. The following quantities were used: 2 ml. of 2.5 % xanthine oxidase solution in $M/5$ phosphate buffer p_H 7.5, 1.0 ml. 0.00068 M methylene blue and 1.0 ml. of $M/200$ solution of the substance stated.

Tube no.	Substance	Time of reduction of methylene blue
1	Inosine	22 mins. 45 secs.
2	Xanthosine	∞
3	Inosinic acid	30 mins. 30 secs.
4	Adenosine	2 hrs. 30 mins.
5	Adenylic acid	2 hrs. 10 mins.
6	Guanosine	∞
7	Hypoxanthine	1 min. 15 secs.
8	Control	∞

The sign ∞ here denotes that the methylene blue was not reduced in 36 hours.

It will be seen that four of the substances, inosine, inosinic acid, adenosine and adenylic acid, were oxidised, though much more slowly than hypoxanthine itself. Xanthosine and guanosine, on the other hand, were not oxidised¹. Inosine and inosinic acid, being compounds of hypoxanthine, might be expected to be oxidised. The results with adenosine and guanosine are also not unexpected, since the oxidase slowly oxidises adenine but is quite unable to oxidise guanine. The difference between xanthosine and inosine is however unexpected and remarkable. The failure of the enzyme to oxidise xanthosine is not due to any inhibitory action of the xanthosine, for on adding hypoxanthine (after some hours) to a duplicate of tube 2 the methylene blue was reduced in 1 min. 20 secs. (*cf.* tube 7). We shall return to this point later.

The behaviour of the individual nucleosides will now be considered in greater detail.

Inosine.

The oxidation of inosine has been obtained with a number of different xanthine oxidase preparations and with milk. Some further results are shown in Exp. 2, in which inosine is compared with hypoxanthine and with the same solution of inosine after hydrolysis with acid. For this hydrolysis enough

¹ After this paper was written our attention was drawn to the fact that Gulland and Macrae [1933], in an investigation of the constitution of the nucleosides, had already attempted to bring about the oxidation of xanthosine and guanosine by means of the xanthine oxidase of milk. Using the methylene blue technique, they found that the oxidase was incapable of oxidising either of these nucleosides.

concentrated HCl was added to a portion of the inosine solution to make the concentration of acid $M/2$, and it was heated under reflux for 1 hour, after which it was neutralised with NaOH and made up to the correct volume.

Exp. 2. The following quantities were used: 3 ml. milk or oxidase solution, 1 ml. 0.00068 M methylene blue, 0.3 ml. $M/152$ inosine *etc.* The reduction times were as follows:

Substrate	Milk		Caseinogen		Whey (1)		Whey (2)	
	mins.	secs.	mins.	secs.	mins.	secs.	mins.	secs.
Inosine	17	45	60	0	22	45	82	0
Hydrolysed inosine	1	10	12	15	—	—	—	—
Hypoxanthine	1	10	12	25	1	15	1	25

The results with the caseinogen preparation were obtained at 15°, the remainder at 38°. The caseinogen preparation was made as described by Dixon and Thurlow [1924, 1]. Whey preparation (2) had been extracted with ether a day or two before the experiment (see below).

As was to be expected, the hydrolysed inosine behaves exactly like hypoxanthine. It will be seen that although inosine is oxidised by all the preparations of oxidase, the activities of the different preparations towards inosine and hypoxanthine do not run parallel: the values of the ratio of the velocity with hypoxanthine to the velocity with inosine for these four preparations are 15.2, 4.8, 18.2 and 58 respectively. This is in marked contrast with the constancy of the ratio of the velocities with hypoxanthine and xanthine, for instance, and gives the impression that some other factor in addition to the xanthine oxidase is concerned in the oxidation of inosine. It will be shown below that this is in fact the case.

The velocity with inosine comes somewhat nearer that with hypoxanthine if its concentration is increased. The oxidase has a very high affinity for hypoxanthine and becomes saturated at an extremely low concentration of the latter. The velocity is then not increased by increasing the hypoxanthine concentration further [Dixon and Thurlow, 1924, 2]. With inosine however the velocity increases with increasing concentration and reaches its maximum value only with considerably higher concentrations.

Exp. 3. The following quantities were used: 3 ml. oxidase solution (caseinogen preparation in phosphate), 0.5 ml. 0.00068 M methylene blue, the stated volumes of $M/68$ inosine, water to 4.5 ml. Temperature 37°.

Amount of inosine ml.	Time of reduction of methylene blue	
	mins.	secs.
1.0	10	5
0.5	10	0
0.2	13	0
0.05	17	55
0.0	∞	

The total amount of methylene blue reduced by a given amount of inosine was determined as follows.

Exp. 4. In a vacuum-tube (Keilin type) were placed 3 ml. xanthine oxidase solution (caseinogen preparation) + 3.5 ml. 0.00068 M methylene blue, and 0.3 ml. inosine solution (1 mg./ml.) was placed in the hollow stopper. The tube was then alternately evacuated and filled with oxygen-free nitrogen several times, as described by Green and Dixon [1934], in order to remove every trace of oxygen. The solutions were then thoroughly mixed and the tube was placed in the thermostat at 38°. After 1½ hours the greater part of the methylene blue had been reduced, and no further change in colour took place during the next

20 hours. The amount of methylene blue remaining unreduced was determined by visual comparison with a series of tubes containing oxidase solution and varying amounts of methylene blue, and by this means it was found that 0.25 ml. of the methylene blue solution had remained unreduced. The amount reduced by 0.3 mg. of inosine was thus 3.25 ml., while 3.30 ml. should theoretically be reduced by this amount of inosine on the assumption that the hypoxanthine it contains is oxidised to uric acid. It is clear that this is actually the case, and that the reaction is due to the inosine itself and not to any hypothetical impurities in it.

The oxidation of inosine occurs equally well with oxygen instead of methylene blue, and the total amount of oxygen taken up corresponds with the amount necessary to oxidise its hypoxanthine to uric acid, as the following experiment shows.

Exp. 5. In the left-hand flasks of two Barcroft manometers were placed 2.0 ml. xanthine oxidase solution (2.5 % whey preparation in p_H 7.5 phosphate) + 1.0 ml. water. In the right-hand flasks were placed 2.0 ml. oxidase solution + 1.0 ml. $M/200$ inosine or hypoxanthine respectively. The latter were placed in "Keilin-tubes" which could be shaken down into the oxidase solution after temperature equilibration. KOH was placed in the receptacles provided in order to absorb any traces of CO_2 . The course of the oxygen uptake is shown in Fig. 1.

It will be seen that although the oxidation of the inosine is much slower than that of the hypoxanthine, they both ultimately take up the theoretical amount of oxygen. An experiment with hydrolysed inosine (see Exp. 2) gave the same results as with hypoxanthine. The somewhat curious form of the hypoxanthine curve has been met with many times with xanthine oxidase preparations. It is due to the fact (first discovered by Thurlow [1925] and later confirmed by Wieland and Rosenfeld [1930]) that H_2O_2 is formed in this reaction. This accounts for the excess oxygen uptake, and the subsequent fall in the curve is due to the decomposition of the peroxide with the liberation of the excess oxygen. When the oxygen uptake is slow this effect is not usually observed, as the peroxide then decomposes almost as fast as it is formed.

Inosine shows no tendency whatever to take up oxygen in the absence of the oxidase.

While these experiments leave no doubt that inosine undergoes oxidation when added to preparations of xanthine oxidase, they do not definitely prove that the xanthine oxidase is able to oxidise inosine as such. The possibility remains that the inosine first undergoes hydrolysis by some other catalyst present in the oxidase preparations (inosine does not hydrolyse spontaneously in neutral solution), and that the free hypoxanthine thus formed is then oxidised by the xanthine oxidase, giving free uric acid.

Attempts were first made to determine whether the product of the reaction was free uric acid or Benedict's compound by making use of the fact that Benedict's uric acid reagent gives a much weaker colour with the nucleoside than it does with free uric acid (according to Davis *et al.* [1922] about one-fifth as strong). At the end of Exp. 5, when the oxygen uptake was complete, the solutions were removed from the flasks of the Barcroft manometers and tested with the uric acid reagent. If the inosine had been converted into Benedict's nucleoside the colour obtained should be five times as strong with the products from the hypoxanthine as with those from the inosine. Actually the intensity of the colour was exactly the same in both, showing that the reaction product was free uric acid in both cases.

This is however still not conclusive, for the inosine might have been oxidised to the nucleoside, and the latter subsequently hydrolysed during the experiment with the production of free uric acid.

The matter was finally decided in the following way. If the inosine has to be hydrolysed before it can be attacked by the xanthine oxidase, the rate of reduction of methylene blue by the inosine must in fact measure the rate at which the inosine is being hydrolysed, for the hypoxanthine is oxidised rapidly in comparison. If this is correct, previous incubation of the inosine with the enzyme preparation before adding the methylene blue (and in complete absence of oxygen) should cause hydrolysis of the inosine with accumulation of free hypoxanthine, and on adding the methylene blue at this stage the reduction time should be much shorter, becoming equal in fact, if the incubation time is sufficiently long, to that obtained with hypoxanthine itself. That this is actually the case is shown by the following experiment (Exp. 6).

Fig. 1.

Fig. 2.

Fig. 1. Oxygen uptake of inosine (curve I) and of hypoxanthine (curve H) in presence of unpurified xanthine oxidase.

Fig. 2. Curve H gives the activity of the preparation with hypoxanthine (scale on right); curves Ia and Ib give the activity with inosine (scale on left). The portion of the preparation which was extracted with ether at the time shown by the arrows gave curves H and Ia. Curve Ib was given by the untreated part. "Relative velocity" is defined as 10,000/reduction time in minutes [Dixon and Thurlow, 1924, 2]. The observations at "2 days" were made after the ether extraction.

Exp. 6. In four vacuum-tubes (Keilin type) were placed 2 ml. xanthine oxidase solution (2.5 % whey preparation A in p_H 7.5 phosphate), together with 1 ml. $M/200$ inosine in two of the tubes and 1 ml. $M/200$ hypoxanthine in the other two. In the hollow stoppers of all the tubes was placed 1 ml. 0.00068 M methylene blue. The tubes were freed from all traces of oxygen as before, and placed in the thermostat at 38°. After a few moments to allow the temperature

to become constant, the solutions were mixed in one of the inosine and one of the hypoxanthine tubes and the reduction times noted. The other two tubes were incubated in the thermostat for 3 hours before mixing the methylene blue with the other solutions. The reduction times were as follows:

Substrate	Methylene blue added			
	immediately		after 3 hours	
	mins.	secs.	mins.	secs.
Inosine	47	0	1	15
Hypoxanthine	1	35	1	30

It will be seen that previous incubation of the inosine with the enzyme preparation has increased the velocity of the reaction almost forty-fold, and that it has become practically the same as for hypoxanthine. Incubation of hypoxanthine with the enzyme has no effect.

There is therefore a catalyst present in the oxidase preparations which hydrolyses inosine. The following experiment shows that this is a thermolabile enzyme.

Exp. 7. A portion of the same oxidase solution as that used in the previous experiment was boiled for 5 minutes. 2 ml. of this + 1 ml. *M*/200 inosine were placed in two vacuum-tubes, and 1 ml. unboiled oxidase solution + 1 ml. methylene blue were placed in each of the stoppers. The experiment was then carried out as before, one tube being tested immediately and the other after 5½ hours' incubation at 38°.

Substrate	Methylene blue added	
	immediately	after 5½ hours
	mins.	mins.
Inosine	85	80

Thus incubation with the boiled oxidase before the unboiled oxidase and the methylene blue are added has no effect. (The reduction time is longer than in the previous experiment because there is now only 1 ml. of active oxidase.)

Attempts to isolate the "inosinase" have not up to the present been carried very far, but it has been found that a preparation of xanthine oxidase almost entirely free from it can be obtained by thorough treatment with ether. This has no immediate effect, but appears to make the inosinase unstable, so that it becomes inactive on standing 2 or 3 days in the solid form, while the activity of the xanthine oxidase remains unaffected. Fig. 2 (p. 2069) shows the change in activity towards inosine and hypoxanthine of a portion of whey preparation A which was extracted three times with ether and dried. It was kept in the solid form in an evacuated desiccator, and solutions were made up from it each day for testing. The activity towards hypoxanthine remained the same as in the unextracted part and fell off only slightly during the course of several weeks. The activity with inosine fell off rapidly and became almost zero within 3 days after extraction. The activity of the unextracted part with inosine also fell off somewhat, but only slowly in comparison. After 2 days the reduction time with inosine had become very long, presumably owing to inactivation of the hydrolysing enzyme. If this were the case, previous incubation of the inosine with the oxidase preparation should have little effect. The incubation experiment was therefore repeated with the ether-treated portion which had been kept for 2 days after the extraction (which for convenience may be called "whey preparation B").

Exp. 8. This was performed exactly as *Exp. 6*, except that whey preparation B was used instead of preparation A. The reduction times were as follows:

Substrate	Methylene blue added	
	immediately	after 3 hours
Inosine	7 hrs.	3 hrs.
Hypoxanthine	1 min. 50 secs.	1 min. 45 secs.

It will be seen that now previous incubation has only a slight effect. The enzyme which hydrolyses inosine is evidently quite distinct from the xanthine oxidase.

It will also be noticed that the disappearance of the hydrolysing enzyme is accompanied by a corresponding loss of the power of oxidising inosine. Inosine is oxidised by this preparation at only 1/240th of the rate of hypoxanthine, and even this slow oxidation is clearly due to hydrolysis, for the sum of the incubation time and the reduction time in the second tube is of the same order as the reduction time in the first tube. We are therefore justified in the important conclusion that xanthine oxidase cannot oxidise inosine at all; it can only oxidise free hypoxanthine liberated by its hydrolysis. The orthodox view of purine metabolism is therefore supported, and the remarkable specificity of xanthine oxidase is once again brought out.

As mentioned above, the rate of reduction of methylene blue by inosine is in reality a measure of the rate of hydrolysis of the inosine by the nucleosidase, and this gives us a convenient method of investigating the properties of the latter. The true significance of *Exp. 3* is thus that it gives an indication of the affinity of inosine for the nucleosidase, and not of that of inosine for the xanthine oxidase. The velocity falls to half its maximum value at a substrate concentration of roughly $M/7000$, which indicates an affinity unusually high for a hydrolysing enzyme. It is hoped to separate the inosinase and to study its properties in more detail, but this lies outside the scope of the present investigation.

Inosinic acid.

As shown in *Exp. 1*, inosinic acid, like inosine, is oxidised by the crude oxidase preparation in presence of methylene blue. Like inosine too it is oxidised by oxygen, giving results very similar to those shown in *Fig. 1*. In view of the behaviour of inosine with this enzyme preparation, it appeared probable that this oxidation also was due to the production of free hypoxanthine from the inosinic acid, probably by the same enzyme. The incubation experiment was therefore repeated with inosinic acid.

Exp. 9. This was carried out in the same way as *Exp. 6*, using whey preparation A, and with $M/200$ sodium inosinate instead of the inosine. The reduction times were as follows:

Substrate	Methylene blue added			
	immediately		after 5½ hours	
	mins.	secs.	mins.	secs.
Inosinic acid	47	15	1	15
Hypoxanthine	1	25	—	

It is clear from this experiment that free hypoxanthine is first produced by hydrolysis of the inosinic acid, and that it is this, and not the inosinic acid as such, which is oxidised by the xanthine oxidase.

Xanthosine.

It was shown in Exp. 1 that xanthosine, unlike inosine, is not oxidised by the enzyme preparation. This is the case even after incubation of the xanthosine with the enzyme, as the following experiment shows.

Exp. 10. This was carried out in the same way as Exp. 6, using whey preparation A, and with *M*/200 xanthosine instead of the inosine.

Substrate	Methylene blue added	
	immediately	after 3 hours
Xanthosine	∞	∞
Hypoxanthine	1 min. 35 secs.	—

Two things are shown by this experiment: (*a*) xanthine oxidase is quite unable to oxidise xanthosine¹; (*b*) inosinase is quite unable to hydrolyse xanthosine.

With regard to (*a*), it may be mentioned that the results in this experiment were completely negative. After 24 hours not even 10 % of the methylene blue had been reduced, so that the reduction time (if indeed there was any reduction at all) could not have been less than about 300 hours. In other words xanthosine is oxidised, if at all, with a velocity less than 1/10,000th part of that of hypoxanthine. We are therefore justified in saying that the oxidase can only oxidise hypoxanthine and xanthine in the free state.

With regard to (*b*), the mere fact that there is no oxidation of the substrate shows that there is no hydrolysis, for any free xanthine formed would at once be oxidised. A comparison of Exps. 6 and 10 shows that, since an amount of inosine equivalent to the methylene blue is hydrolysed in 47 minutes, while this amount of xanthosine is not hydrolysed in 300 hours, and since hypoxanthine reduces twice as much methylene blue as xanthine, xanthosine can only be hydrolysed, if at all, at about 1/200th of the rate of hydrolysis of inosine. In view of the very close relationship of the two nucleosides the difference in behaviour is remarkable. It is now known that the carbohydrate is the same in both, and that it is attached through its reducing group to the purine in the same position (7) in each case; in fact it is practically certain that xanthosine is identical with inosine except for the extra oxygen atom in position 2 of the purine. The fact that the inosinase can hydrolyse only one of these nucleosides shows that it has a surprisingly high degree of specificity, and throws a new light on the nature of the nucleosidases. This point will be discussed more fully in a later section.

Adenosine.

This is oxidised, though more slowly than inosine, by the unpurified preparation of xanthine oxidase. Free adenine is itself oxidised by xanthine oxidase, though more slowly than hypoxanthine, and in view of the results obtained with the other nucleosides it appeared probable that the adenosine underwent hydrolysis before oxidation. The incubation technique was therefore applied to adenosine, but led to a somewhat unexpected result.

Exp. 11. This was carried out in the same way as Exp. 6, with whey preparation A, and with *M*/200 adenosine and adenine.

Substrate	Methylene blue added	
	immediately	after 5 hours
Adenosine	2 hrs. 25 mins.	2 mins. 15 secs.
Adenine	2 hrs. 20 mins.	1 hr. 10 mins.
Hypoxanthine	1 min. 40 secs.	—

¹ See footnote on p. 2066.

Similar results were obtained with adenosine using fresh milk instead of the whey preparation, as shown in the following experiment.

Exp. 12. This was similar to Exp. 11, except that milk was used instead of the oxidase preparation.

Substrate	Methylene blue added		
	immediately	after 2½ hours	after 5 hours
Adenosine	2 hrs. 20 mins.	18 mins.	1 min. 53 secs.
Hypoxanthine	1 min. 48 secs.	—	—

It will be seen in these experiments that after incubation the reduction time with adenosine has become, not that of adenine, but (within a few seconds) that of hypoxanthine, some 60 times shorter (*cf.* also Exp. 14). This can only mean that the adenosine is not only hydrolysed but also deaminated. It will be noticed however that free adenine still reacts very slowly even after incubation¹. The deamination must therefore take place while the adenine is still in the combined state. It is in fact due to an "adenosine-deaminase" which has little or no action on free adenine. It is clear that the deamination must precede the hydrolysis; if the latter occurred first, adenine would be formed, and this, as Exp. 11 shows, reacts only slowly with the enzyme preparation.

By using other oxidase preparations, particularly those which had been kept for 2 or 3 weeks, the presence of traces of adenase could be avoided (adenase appears to be somewhat less stable than the other enzymes), and we were then able to show that the adenosine-deaminase has no action at all on free adenine, as the following experiment shows.

Exp. 13. This was carried out with another oxidase preparation (A') made in the same way as preparation A and kept for 19 days.

Substrate	Methylene blue added		
	immediately	after 2 hours	after 5 hours
Adenosine	2 hrs. 31 mins.	33 mins.	3 mins. 45 secs.
Adenine	1 hr. 58 mins.	—	1 hr. 45 mins.
Hypoxanthine	2 mins. 15 secs.	—	—

It may be mentioned that in Exps. 12 and 13 the tubes which had been incubated for only 2 hours showed almost complete reduction in less than 3 minutes; the reaction then slowed down very greatly, and the times for complete reduction were as shown in the tables. This is to be expected on the assumption that during the short incubation the amount of hypoxanthine liberated is rather less than equivalent to the methylene blue added.

It follows from the above results that the oxidation of adenosine involves the co-operation of three enzymes: it is first converted into inosine by the adenosine-deaminase, the inosine is then hydrolysed by the nucleosidase, and the resulting hypoxanthine is oxidised by the xanthine oxidase.

It is interesting to note that the existence of an adenosine deaminase has been inferred by Jones [1911] from the fact that hypoxanthine is formed during the autolysis of animal tissues which do not contain adenase, but hitherto it does not appear to have been directly demonstrated, and Jones's observations could presumably be explained equally well by the existence of the now well-

¹ The slight effect of incubation on adenine is to be ascribed to the presence of a trace of adenase (*cf.* Exp. 13). Dr D. E. Green informs us that he has found some adenase in milk and in crude xanthine oxidase preparations. It is absent from purified preparations, which can nevertheless oxidise adenine, probably to 6-amino-2:8-dioxypurine, as suggested by Dixon and Thurlow [1924, 2].

known adenylic acid deaminase of the tissues. The comparative scarcity of adenase in animal tissues has led Jones to the view that in the metabolism of nucleic acid the deamination of adenine normally occurs while it is still in the combined state. Guanase on the other hand is very plentiful in animal tissues.

Guanosine.

This is not oxidised at all even by the crude oxidase preparations. This fact cannot however be taken as evidence either that it is not deaminated or that it is not hydrolysed. If it were deaminated, xanthosine would result, and as we have shown this is neither hydrolysed nor oxidised by our enzyme preparations. If on the other hand it were hydrolysed, guanine would be produced, and this is not oxidised by xanthine oxidase. This question can therefore only be decided by further work.

Muscle adenylic acid.

This is oxidised by the crude xanthine oxidase preparations, the velocity being of the same order as in the case of adenosine. It also behaves similarly on previous incubation, as shown by the following experiment, in which adenylic acid is compared with adenosine and cozymase.

Exp. 14. This was carried out in the same way as Exp. 6, but with a 4 % solution of whey preparation A', with $M/200$ adenylic acid and adenosine, and with a 0.3 % solution of cozymase preparation. The reduction times were as follows:

Substrate	Methylene blue added	
	immediately	after 4 hours
Adenylic acid	1 hr. 0 min.	1 min. 12 secs.
Adenosine	1 hr. 17 mins.	1 min. 58 secs.
Hypoxanthine	1 min. 38 secs.	—
Cozymase	1 hr. 17 mins.	2 mins. 7 secs.

It is thus clear that adenylic acid, like adenosine, is deaminated and hydrolysed by the enzyme preparation before the oxidation takes place. (It may be mentioned that with this enzyme solution inosine gave a reduction time of 30 minutes, showing that the deamination is not a very rapid process.)

It was thought that it would be of interest to compare cozymase with adenylic acid, as it is believed to be an adenylic acid closely related to, but not identical with, muscle-adenylic acid. As is shown in Exp. 14, it behaved in a very similar way, giving a reduction time of the same order and showing the same effect of previous incubation. It is not certain however that the effects were due to the cozymase itself, and not to other substances of similar nature present in the preparation, for, though very active, it was not one of the very highly purified preparations more recently obtained by Euler and Myrbäck. (The cozymase preparation did not reduce methylene blue at all in the absence of the oxidase.)

(The whey preparation A' used in Exps. 13 and 14 contained slightly less xanthine oxidase than preparation A and was therefore made up in somewhat stronger solution for both these experiments, so as to give the same oxidase activity as before. Apparently however it contained about the same amount of the deaminase as did preparation A, so that this enzyme was now present in rather larger amounts than previously, and the velocity with adenosine was correspondingly higher. The difference between the velocities in the two experiments is due to the fact that Exp. 14 was done when preparation A' was freshly made and Exp. 13 after it had been kept for about 3 weeks. In this connection

it may be mentioned that Exps. 1, 5, 6, 9, 10 and 11 were all done with the same preparation within a few days of one another, and it should therefore be legitimate to compare them with one another.)

DISCUSSION.

It is clear from the foregoing results that the question which was the starting-point of the investigation, namely whether xanthine oxidase is able to oxidise nucleosides as such without previous hydrolysis, must be answered in the negative. The purified oxidase can oxidise none of the nucleosides or nucleotides tested, and the oxidation of these substances by milk or by crude preparations of the oxidase is due, as we have shown, to the presence of other enzymes which first liberate free hypoxanthine from them. Thus the oxidation of inosine requires the successive action of two enzymes, while three are necessary for the observed oxidation of adenosine. The inability of xanthine oxidase to oxidise hypoxanthine or xanthine when combined with ribose illustrates still further the high degree of specificity of this enzyme which was shown by the previous investigations. It also provides the evidence which was lacking in favour of the accepted view of purine metabolism.

On the other hand it makes it difficult to account for the existence of Benedict's uric acid nucleoside. It would seem that this can only arise in two ways: (a) by the oxidation of the purine bases while still combined with ribose, or (b) by synthesis from free uric acid. If (a) is the case, the oxidation cannot be due to ordinary xanthine oxidase, but must be due to a similar oxidase capable of acting on combined purines. This is not impossible in view of the great specificity of enzymes concerned with the purine group, but there is no evidence of the existence of such an enzyme. If, on the other hand, (b) is the true explanation, it is rather curious that the uric acid nucleoside should contain *d*-ribose, the sugar with which the purines were originally combined before their oxidation.

The properties of the nucleosidase which we have shown to be present in milk seem to throw a new light on the nature of nucleosidases. Very little is known at present about these enzymes. They are believed, though apparently on very little evidence, to be relatively unspecific ribosidases. If this view were correct one would expect that they would be capable of hydrolysing other ribosides, *e.g.* methyl-*d*-riboside. It has however been shown by Levene *et al.* [1912] that the nucleosidases are totally unable to hydrolyse either α - or β -methyl-*d*-riboside, so that even if they are ribosidases they show some specificity towards the non-carbohydrate part of the molecule. On the other hand they are considered to be quite unspecific within the purine series by Levene [1931], who speaks of "nucleosidase" (in the singular), and says (though without conclusive evidence) "All known purine nucleosides...are hydrolysed by the same enzyme." The pyrimidine nucleosides are however not hydrolysed by this enzyme [Levene and Medigreceanu, 1911] but by a distinct nucleosidase [Deutsch and Laser, 1929], although the carbohydrate part of the molecule is the same. Thus the few facts available seem to indicate rather that the nucleosidases are specific for the non-carbohydrate part of the molecule, and indeed the evidence that they are ribosidases at all is very slight.

The properties of the nucleosidase which we have shown to be present in milk strongly support this view. It is evidently highly specific towards the purine group and appears to be adapted for liberating hypoxanthine from its compounds. Such a slight change as the introduction of an oxygen atom in

the 2 position in the purine (*i.e.* in the ring to which the carbohydrate is not attached) completely prevents its action, as we have shown, and this strongly suggests that the enzyme "fits" the purine part of the molecule.

The question whether this nucleosidase is capable of liberating hypoxanthine from inosinic acid as well as from inosine cannot yet be answered. As shown above, inosinic acid behaves like inosine with our enzyme preparations, but this is not conclusive, as it may first be actually converted into inosine by a liberation of the phosphoric acid group. Demuth [1925] has shown that milk contains a very active phosphatase, which would certainly bring about this reaction, and until we can obtain the other enzymes completely free from phosphatase the point cannot be decided. The same applies to the case of adenylic acid. This behaves like adenosine, but it does not follow that the adenosine deaminase can act also on adenylic acid, as the latter may first be converted into adenosine by the phosphatase. If this is actually the case the observed oxidation of adenylic acid must be due to the successive actions of four distinct enzymes.

With regard to the adenosine deaminase which we have found in milk, it is interesting to note that there must be three distinct deaminases for adenine, corresponding with the three stages of hydrolysis of the nucleotide. There is first the adenylic acid deaminase present in muscle, which, as Schmidt [1928] has shown, is quite unable to deaminate adenosine. Secondly there is the adenosine deaminase of milk, which is unable to deaminate free adenine. And lastly there is adenase itself. These are all specific for adenine compounds and cannot deaminate the corresponding guanine compounds.

APPENDIX.

Preparation of the nucleosides.

Adenosine and guanosine. The procedure of Levene [1931, p. 163] for the preparation of adenosine and guanosine from yeast nucleic acid did not give satisfactory results when applied to the yeast nucleic acid obtained from British Drug Houses. Only a small part of the guanosine came out of solution after heating the ammoniacal solution of nucleic acid, and this amount was not appreciably increased by allowing to stand or by seeding with guanosine crystals. Consequently the adenosine obtained from the picrate still contained guanosine, and further amounts of guanosine were also lost in the mother-liquors from the picrate. Moreover, a good deal of the nucleosides was lost in the baryta precipitation, whereas the nucleotides were not completely removed, so that picric acid gave amorphous resinous precipitates. Adenosine picrate could be obtained from these by fractional crystallisation, as the other picrates are either insoluble or more easily soluble than adenosine picrate, but much of the latter was lost.

The following procedure was finally adopted.

50 g. yeast nucleic acid (B.D.H.) were dissolved in 40 ml. concentrated ammonia and 200 ml. water, and this solution was heated in four sealed tubes for 3½ hours at a temperature of 135–145°. (The temperature of 175–180° as given by Levene for heating in the autoclave is too high [Jones and Germann, 1916; Levene and Jacobs, 1916].) After cooling, the undissolved guanosine was filtered off, and the filtrate was made slightly acid with acetic acid. The nucleotides were precipitated by lead acetate and the precipitate filtered off and washed with water. The nucleosides were then precipitated from the filtrate by lead acetate and ammonia. The precipitate was washed, finely dispersed in water and, after the addition of some calcium carbonate, decomposed by H₂S with heating. After filtering, the solution was concentrated *in vacuo* to a small volume and slightly acidified with sulphuric acid. The picrates were then precipitated by adding an excess of cold saturated picric acid solution. A second fraction of the picrates, mixed with some picric acid, separated when the mother-liquor was concentrated to a small volume. The mixed nucleosides were set free from the united picrates by Levene's procedure. The resulting solution was evaporated in a vacuum-desiccator over sulphuric acid, and the residue was treated with 100 ml. of cold

water. The guanosine remained undissolved and was added to the pure, but not yet recrystallised, guanosine. The adenosine solution was concentrated to a small volume and again precipitated by picric acid solution. After one recrystallisation the picrate was analytically pure. For the preparation of the free adenosine from the picrate, and for the purification of the crude guanosine, the procedure of Levene was followed.

Xanthosine. Attempts to obtain xanthosine from guanosine, following Levene's [1931, p. 174] method exactly, proved unsuccessful. The guanosine which separated on cooling the hot nitrite solution could not be brought completely into solution by heating for 5 mins. after the addition of acetic acid. In consequence a mixture of xanthosine and unaltered guanosine resulted, whereas if the heating in the nitrous acid mixture were continued for a longer time the guanosine went into solution, but no xanthosine crystallised out afterwards.

It was found that it is essential to heat the guanosine with the nitrite solution for some time before the acetic acid is added. The solution can then be cooled, and the guanosine still remains in solution. We cannot say definitely what is the reason for this, but it is possible that some double salt is formed with the nitrite.

The following procedure was finally adopted.

0.1 g. guanosine was suspended in 0.3 ml. water, and a solution of 0.25 g. NaNO_2 in 0.7 ml. water was added. The mixture was heated just to the boiling-point until all the guanosine was completely dissolved and no longer precipitated when the solution was cooled under the tap. 0.2 ml. glacial acetic acid was added. The solution was allowed to stand for a few hours, and 0.7 ml. water was then added. Xanthosine crystallised readily from the ice-cold solution on standing for 24 hours. After one recrystallisation it was completely pure, forming large prisms. The yield was 70 % of the theoretical.

Inosine. The procedure adopted for the preparation of xanthosine proved satisfactory also for the preparation of inosine from adenosine. The method is thereby simplified, and the necessity for preparing the triacetyl derivative of inosine is avoided.

0.5 g. adenosine was boiled with a solution of 1 g. NaNO_2 in 3 ml. water until it was completely dissolved, and the solution was cooled under the tap. 1 ml. of glacial acetic acid was then added. After 3 hours' standing the mixture was cooled in ice and made just acid to Congo red with sulphuric acid (a few ml. of 2N H_2SO_4 being sufficient). After the addition of 4 volumes of absolute alcohol the solution was placed in a freezing mixture for 2 hours, after which it was sucked off from the Na_2SO_4 and the latter was washed with alcohol. The filtrates were exactly neutralised with a few drops of ammonia and evaporated *in vacuo*. The solid residue was taken up in as little cold water as possible. This solution was seeded with inosine crystals, and the walls were rubbed with a glass rod until crystallisation began. On standing in a refrigerator overnight 0.17 g. inosine crystallised out. It was sucked off on a Willstätter knob, washed with a little cold water and recrystallised from 80 % alcohol.

It is occasionally difficult to get a crystallisation of inosine by this method. In this case the isolation must proceed by way of the triacetyl derivative as described by Levene but with the following slight modifications. Very dilute ($N/10$) baryta is to be used for the saponification, and instead of boiling for 30 mins. under reflux the mixture is heated to 80° for only a few minutes after solution is complete. The addition of a small quantity of silver sulphate, as recommended by Levene, was not found to be necessary. The lead salt is decomposed by H_2S , and the filtrate evaporated *in vacuo*. The solid product which separates cannot be crystallised immediately from 80 % alcohol, but it is necessary first to dissolve it in a very little water, from which it crystallises on standing. This crystalline product is now recrystallised from 80 % alcohol, from which fine shining needles of pure inosine separate. A further quantity can be obtained in the same way from the mother-liquors from the first crystallisation.

(It may be mentioned that in Levene's description of the preparation of inosine starting from adenosine picrate there is no reference to the removal of the picric acid at any stage. Mention of this necessary step seems to have been overlooked.)

All the nucleosides were found to have the exact melting-points given by Levene.

SUMMARY.

1. A study has been made of the action of the xanthine oxidase of milk on the following substances: inosine, inosinic acid, xanthosine, adenosine, muscle adenylic acid, guanosine, cozymase.

2. All these substances, with the exception of xanthosine and guanosine, are oxidised by milk and by crude xanthine oxidase preparations. The rate of oxidation is however smaller than with the free purine bases.

3. This oxidation is due to the presence of other enzymes which liberate free hypoxanthine from the substances named, and it is this which is oxidised by the xanthine oxidase. The oxidase can be obtained free from the other enzymes and is then incapable of oxidising any of the above substances.

4. The oxidation of inosine is due to the presence of a nucleosidase which liberates free hypoxanthine from it. If the inosine is incubated anaerobically with the enzyme preparation before the oxidation is allowed to take place, the rate of oxidation is found to be increased some 40 times, becoming equal to that of free hypoxanthine. Incubation with purified (or with boiled crude) xanthine oxidase has no effect.

5. The nucleosidase is extremely specific towards the purine part of the molecule and is unable to hydrolyse even such a similar substance as xanthosine. The bearing of its behaviour on the nature of nucleosidases is discussed.

6. Previous incubation of adenosine with milk or with crude oxidase preparations also produces a great increase in the rate of oxidation. The rate then becomes equal, not to that of free adenine, but to that of free hypoxanthine, which is much higher. It is concluded that adenosine is first deaminated by a third enzyme, giving inosine, which is then hydrolysed and oxidised as before.

7. Incubation of free adenine with the oxidase preparations has little or no effect, showing that this enzyme is an "adenosine deaminase" which can act only on combined adenine and not on adenine itself. The deamination must therefore precede the hydrolysis.

8. Inosinic acid and adenylic acid behave exactly like inosine and adenosine respectively, but it is possible that this is due to the presence of phosphatase, which would convert them into the nucleosides.

9. Improved methods for the preparation of the nucleosides are given.

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