CXXXI. STUDIES ON XANTHINE OXIDASE.

II. THE DYNAMICS OF THE OXIDASE SYSTEM.

By MALCOLM DIXON AND SYLVA THURLOW.

From the Biochemical Laboratory, Cambridge University.

(Received July 31st, 1924.)

IN the previous paper we have described a method for obtaining a preparation of the xanthine oxidase in a form suitable for investigation. While a large amount of work on various enzymes has been done, it appears that the thorough systematic investigation of the dynamics of any one oxidase has not been attempted. The chief difficulty in such work is that of obtaining the enzyme in question free from other enzymes and from oxidisable substances. The above-mentioned preparation is satisfactory in this respect and we therefore have employed it in investigating the xanthine oxidase. The methylene blue technique was used throughout.

The reduction velocity-time curve of the reaction with methylene blue was not investigated by Morgan, Stewart and Hopkins [1922], but they believed that the velocity was constant during the course of the reduction. This has been verified by the use of a modification of the colorimetric method used by Dixon and Tunnicliffe [1923]. The amount of methylene blue remaining unreduced at any instant was determined by matching the tint of the reacting mixture against colour standards prepared by adding known amounts of methylene blue to enzyme solution. It was found that, provided traces of oxygen were carefully excluded, the methylene blue disappeared at a constant rate except when reduction was practically complete, when the reduction velocity fell off slightly. If then the "reduction time" is measured from the commencement of the reaction to the time when the colour has reached a very faint standard "end-point," the reciprocal of the reduction time gives a measure of the reduction velocity. In plotting the curves, the reciprocal of the reduction time in minutes has been multiplied, for convenience, by 10,000.

All the curves given in this paper, since they are typical examples of a large number of experiments, can be relied upon. It is not advisable to place too much reliance on the methylene blue technique until the results have been confirmed by repetition.

p_H of Solution.

The reaction velocity was found to be entirely independent of the p_H of the solution within wide limits. Phthalate and acetate buffers markedly inhibit the reaction and therefore could not be used in obtaining this curve,

whereas a solution of the enzyme in either borate or phosphate has a reaction velocity equal to that of an aqueous solution of the same p_H . Borate buffers were therefore used from p_H 8.0 upwards, while phosphate buffers were used between 5.8 and 8.0. On the acid side of p_H 5.8 the p_H of the solutions was adjusted to the required value by the addition of dilute acid to a solution of the enzyme in p_H 7.6 phosphate buffer, the amount of acid required being determined by titration of a sample of the solution. During the addition of

Fig. 1

Fig. 1. p_H curve with hypoxanthine.

Each tube contained: 2.5 cc. enzyme solution in water; 2.5 cc. buffer solution; 0.5 cc. methylene blue; 0.2 mg. hypoxanthine.

Fig. 2

Fig. 2. Enzyme concentration curve with hypoxanthine and xanthine.

Each tube contained: x cc. enzyme solution, $(5-x)$ cc. inactive caseinogen solution (both in p_H 7.6 phosphate buffer); 0.5 cc. methylene blue; 0.2 mg. hypoxanthine or xanthine.

Fig. 3

Fig. 3. Methylene blue concentration curve.

Each tube contained: 5.0 cc. enzyme solution (in 7.6 phosphate buffer); x cc. methylene blue; $(2-x)$ cc. water; 0.2 mg. hypoxanthine.

Fig. 4

Fig. 4. Hypoxanthine concentration curve.

Each tube contained: 5.0 cc. enzyme solution (in 7.6 buffer); 0.5 cc. methylene blue; x cc. hypoxanthine (1 mg. per cc.); $(2-x)$ cc. water.

the acid the solution was thoroughly shaken to maintain the same H ion concentration throughout. Otherwise a partial destruction of the enzyme by the acid might occur. The curve obtained for hypoxanthine is given in Fig. 1.

Those obtained for aldehyde and adenine are similar. The curve shows a constant reaction velocity from p_H 5.8 to 9.0. At 9.0 there is a sudden drop in the reaction velocity, the velocity at p_H 10.0 being only about one-tenth of that at p_H 9.0. The sudden break at 9.0 cannot be due to change of buffer as it comes in the middle of the borate range.

Ohlsson [1921] investigated the p_H curve for the succinoxidase and found a similar break at p_H 9.0. In fact his curve is very similar to that given above for hypoxanthine. The break may possibly be characteristic of this type of oxidising enzyme. Ohlsson found that the position of the break depended on the temperature, the higher the temperature the sooner does the break occur. We have investigated the curve at 15° and at 38°, but at both temperatures the break occurred at p_H 9.0. This break has been found to be due to destruction of the enzyme at reactions more alkaline than p_H 9, since solutions above this p_H do not regain their original activity on readjustment to neutrality. Similar experiments were made to discover the cause of the drop on the acid side of the curve. At p_H 4.2 the activity for hypoxanthine has completely disappeared, but if a solution which has been allowed to stand for 24 hours at p_H 4.2 is then brought back to p_H 7.6 not the slightest loss of activity has occurred. The loss of activity at p_H 4.2 is therefore not due to destruction of the enzyme but to the effect of p_H upon the reaction velocity. The p_H of the solution must be more acid than that at which the reaction can proceed at all before destruction of the enzyme actually occurs. If the solution is brought to p_H 3.5 a complete and irreversible destruction occurs.

Concentration of Enzyme.

With the substrate, methylene blue and caseinogen constant, the concentration of the enzyme was varied. Solutions of equal strength of active and inactive caseinogen were made, and the relative amounts of the two solutions were varied, the total volume being kept constant. The curves obtained with hypoxanthine and xanthine are shown in Fig. 2. Exactly similar curves were obtained with the Schardinger enzyme and the adenase.

It will be seen that the reaction velocity is accurately proportional to the concentration of the catalyst. This shows that, throughout the range of concentrations used in any of our experiments, the catalyst is not present in excess, and its concentration is therefore a limiting factor in the reaction. It was subsequently found that the addition of inactive caseinogen had no effect on the velocity of the reaction, so that the relative amounts of enzyme in various solutions can be estimated by determining the reaction velocity under comparable conditions.

Concentration of Methylene Blue

With the substrate and enzyme concentrations and the total volume kept constant, the amount of methylene blue was varied. The reaction velocity, that is, the amount of methylene blue reduced in a given time, is obtained by multiplying the reciprocal of the reduction time by the amount of methylene blue. It will be seen in Fig. 3 that the reaction velocity is quite independent of the methylene blue concentration except when this is very low.

Concentration of Substrate.

With methylene blue and enzyme kept constant, the concentration of hypoxanthine was varied. The curve obtained, which was of an unexpected form, is shown in Fig. 4. The xanthine curve is similar. It will be observed that as the concentration of hypoxanthine is increased the reaction velocity is decreased, the velocity at high substrate concentrations being extremely small.

Morgan, Stewart and Hopkins (unpublished experiments) found that uric acid inhibited the reaction in the case of both hypoxanthine and xanthine. The obvious explanation of this inhibition would be the application of mass action to a reversible reaction. Since, however, a similar inhibitory effect was obtained with hypoxanthine itself, it would seem that the two inhibitions are due to the same cause, and this cannot be mass action in the case of hypoxanthine. It appeared possible that the effect might be due to adsorption by the enzyme of the inhibitory substance, thus preventing the adsorption of one or both of the reactants. The effect might therefore be expected to be given by other purine substances, and in fact guanine, adenine and xanthine itself were found to produce this inhibitory effect upon hypoxanthine. Some of the curves obtained are shown in Fig. 5. The effect, however, seems to be remarkably specific. Caffeine showed no trace of inhibitory effect. The pyrimidine substances uracil, cytosine and thymine were also tried but showed no inhibition. Histidine, which contains the iminazole ring, was also tried, but had no effect.

If hypoxanthine and uric acid inhibit by the same mechanism, hypoxanthine would be expected to produce no further inhibition if added to a solution which already contained enough uric acid to produce the maximum inhibition, that is, a concentration corresponding to a point on the horizontal part of the inhibition curve. The resulting hypoxanthine concentration curve in presence of uric acid is shown in Fig. 6.

It will be seen that the hypoxanthine concentration curve has entirely changed its shape, and now assumes the form of a typical substrate curve, such, for instance, as that found in the case of succinoxidase by Widmark [1921]. This furnishes a proof that the uric acid and xanthine inhibitions are essentially the same in nature.

Critical Concentration.

The initial parts of the hypoxanthine and xanthine concentration curves were then investigated more thoroughly, and the results are plotted on an extended scale in Fig. 7.

Fig. 5a

Fig. 5b

Fig. 5c

Fig. 6

Fig. 5. Inhibition of hypoxanthine reaction by (a) uric acid, (b) guanine, (c) adenine.

Each tube contained: 5.0 cc. enzyme solution (in buffer); 0.5 cc. methylene blue; 0.2 mg. hypoxanthine; x cc. (a) uric acid (5 mg. per cc.), (b) guanine (1 mg. per cc.), (c), adenine (1 mg. per cc.); $(2-x)$ cc. water.

Fig. 6. Hypoxanthine concentration curve in presence of uric acid.

Each tube contained: 5.0 cc. enzyme solution (in buffer); 0.5 cc. methylene blue; 2.0 cc. uric acid (5 mg. per cc.); x cc. hypoxanthine (1 mg. per cc.); $(2-x)$ cc. water.

It will be observed that once the substrate concentration is sufficient to reduce the methylene blue completely, the velocity is unaffected by change in concentration until a certain critical concentration is reached, after which inhibition suddenly commences. The critical concentration for xanthine is slightly higher under the same conditions than that for hypoxanthine.

It was found that the critical hypoxanthine concentration, while being independent of the methylene blue concentration, varied with varying enzyme concentration. The more concentrated the enzyme solution, the more hypoxanthine is necessary before the critical concentration is reached. The initial flat part of the curve is thus extended. Morgan, Stewart and Hopkins, using a very active milk, that is, one in which the enzyme is at a high concentration, obtained a constant velocity with varying substrate concentration. Our results show that their range of substrate concentrations lay entirely below the critical point for so active an enzyme solution. It was thought that the effect of changing enzyme concentration might be due to changing caseinogen concentration, but it was found that the addition of an equal amount of inactive caseinogen had no effect on the critical concentration, and the change in the position of the critical point must therefore be due to the change in enzyme concentration. The addition of fat was without effect.

On investigating the curve of uric acid inhibition on hypoxanthine, using a stronger enzyme solution and a smaller quantity of hypoxanthine, it was found that this curve also showed an initial horizontal portion and a critical concentration of uric acid. It should be noted that the curve given in Fig. 5 (*a*) was obtained with a dilute enzyme solution and with a moderate quantity of hypoxanthine already present, and consequently does not exhibit the critical concentration effect. On increasing the enzyme concentration, however, the initial flat part of the curve becomes well marked.

With the hypoxanthine concentration such that inhibition is practically complete, the concentration of methylene blue was varied. The curve is shown in Fig. 8.

Whereas with low hypoxanthine concentration the reaction velocity was found to be independent of methylene blue concentration, it will be seen that with high hypoxanthine concentration the reaction velocity increases with increasing methylene blue. The significance of this will be discussed later.

Morgan, Stewart and Hopkins found that the rate of reduction of methylene blue by hypoxanthine was twice the rate of reduction by xanthine. In certain experiments we were unable to obtain this two to one ratio. Our failure to repeat their results led us to investigate the conditions under which the two to one ratio held. As long as the concentrations of hypoxanthine and xanthine remain below the critical values, the ratio remains accurately two to one, but when the concentration exceeds the critical value the ratio begins to approach, and finally reaches, one to one. In very high concentrations the curve for xanthine crosses that for hypoxanthine, so that xanthine actually reduces methylene blue at a higher rate than does hypoxanthine. Typical curves are shown in Fig. 9.

Fig. 7

Fig. 7. *Initial part of hypoxanthine and xanthine concentration curves.*

Each tube contained: 5.0 cc. strong enzyme solution (in buffer); 0.5 cc. methylene blue; x cc. hypoxanthine or xanthine (2 mg. per cc.); $(0.5 - x)$ cc. water.

Fig. 8

Fig. 8. *Methylene blue concentration curve at high hypoxanthine concentration.*

Each tube contained: 5.0 cc. enzyme solution (in buffer); 1.0 cc. hypoxanthine (2 mg. per cc.); x cc. methylene blue; $(2 - x)$ cc. water.

Fig. 9

Fig. 9. *Hypoxanthine and xanthine concentration curves compared.*

Each tube contained: 4.0 cc. enzyme solution (in buffer); 0.5 cc. methylene blue; x cc. hypoxanthine or xanthine (2 mg. per cc.); $(3 - x)$ cc. water.

DISCUSSION.

It will be convenient if the experimental results which must be explained by a complete theory of the mechanism of the enzyme action are summarised here. They are as follows:

1. With high concentration of purines the velocity is diminished.
2. There is, however, an initial range of concentration through which the velocity remains constant.
3. There is a definite concentration of purines at which inhibition suddenly begins.
4. This critical concentration is dependent on the enzyme concentration.
5. At low purine concentrations the reaction velocity is independent of methylene blue concentration.
6. At high purine concentration the reaction velocity depends upon methylene blue concentration.
7. At low substrate concentration, the ratio of the velocities with hypoxanthine and xanthine is two to one, while at high concentrations the ratio becomes one to one.

By making two assumptions it is possible to bring these apparently disconnected facts together to form a coherent and consistent scheme. These assumptions are first, that the enzyme activates purine bases at a constant rate, i.e. one which is independent of their concentrations; and secondly, that as the purine concentrations increases it becomes increasingly difficult for the methylene blue to reach the enzyme surface.

In the light of these two assumptions we shall now consider one by one the above-mentioned experimental facts.

1. The hypoxanthine and methylene blue are in all probability adsorbed on the enzyme side by side, and there react. At low purine concentrations the enzyme is readily accessible to the methylene blue and therefore the reaction proceeds at a velocity which is determined by the rate at which the hypoxanthine is activated by the enzyme. At high purine concentrations, however, according to assumption two, it becomes difficult for the methylene blue to gain access to the enzyme surface, and the rate of methylene blue supply becomes limited. The reaction, therefore, no longer proceeds at its former rate, but with a velocity limited by the rate at which methylene blue can reach the surface of the enzyme.

2. The initial constant velocity follows from the first assumption. As long as the methylene blue has free access to the enzyme surface the reaction velocity will depend on the rate of hypoxanthine activation.

3. According to the above assumptions, as the concentration of purine increases, the methylene blue finds it increasingly difficult to reach the enzyme surface, and a stage is finally reached when its rate of supply to the enzyme is equal to the rate at which hypoxanthine is activated. Any further increase in purine concentration will result in a slowing of the rate of methylene blue

supply below this value, and the reaction velocity will then be limited by this rate of supply and not by the rate of hypoxanthine activation. Any increase of purine concentration beyond that at which the two rates are equal will cause inhibition.

4. The fact that the critical concentration depends on the enzyme concentration indicates that much of the purine in solution is associated with the enzyme, because adding more enzyme causes the purine to be distributed over a larger enzyme surface, and hence so great a resistance is not offered to the passage of methylene blue. Otherwise the addition of more enzyme would not decrease the inhibitory effect of the purine.

5. Since at low purine concentration the reaction velocity depends on the rate of hypoxanthine activation and the methylene blue has free access to the enzyme, the concentration of methylene blue will not affect the reaction velocity.

6. Since at high purine concentrations the access of methylene blue to the enzyme is limited, the reaction velocity will depend upon the rate of access, and it is to be expected that this will depend on methylene blue concentration.

7. When the velocity is limited only by the constant rate of substrate activation which we assume to be the same for hypoxanthine and xanthine, hypoxanthine will reduce methylene blue at twice the rate that xanthine does, since it uses two molecules of methylene blue for its conversion to uric acid whereas xanthine uses but one. Hence the ratio of their rates of reduction of methylene blue is two to one. When, however, inhibition occurs and the velocity comes to depend upon the rate at which methylene blue reaches the enzyme, this rate will be the same for both xanthine and hypoxanthine, and therefore their rates of reduction will be one to one. That the curves show a slight crossing indicates that hypoxanthine is slightly more effective than is xanthine in causing inhibition.

At the present stage of knowledge of enzyme reactions it is extremely difficult to formulate any theory along physico-chemical lines which would explain either the constant rate of hypoxanthine activation by the enzyme or the blocking of the enzyme surface by purines. The temperature coefficient of the reaction, which has been found to have a value of 2.0, indicates that the activation is a true chemical process and one which is not limited by the rate of diffusion of the substrate to the surface of the enzyme. It seems impossible, however, to picture any mechanism by which the rate of activation can remain independent of the substrate concentration. In view of the fundamental importance of obtaining a clearer conception of the nature of activation by enzymes it seems desirable that further work should be done along this line.

In regard to the second of these assumptions, namely that the surface of the enzyme is rendered less accessible to methylene blue by purine in the higher concentrations, there seems to be no doubt from experimental observations that this is actually what occurs, but we are unable to state without further investigation by what precise mechanism such an effect is brought about.

Identity of Enzymes.

The question was raised by Morgan, Stewart and Hopkins as to the possibility of the xanthine oxidase being identical with the Schardinger enzyme. As they pointed out, it seems at first sight improbable that an enzyme showing such marked specificity in the purine group itself should extend its activity to substances differing so widely from purines as aldehyde. They also used as an argument against the identity of the two enzymes the fact that the hypoxanthine reaction proceeds with a much higher velocity than does the aldehyde reaction. This, however, seems to us not to be valid evidence, as several cases are known where one enzyme is capable of bringing about different reactions at widely different rates. We have made an observation which might at first seem to be evidence against the identity of the two enzymes. A solution of enzyme preparation at p_H 4.1 to 4.3 is completely inactive with hypoxanthine, but reacts with aldehyde fairly rapidly. Although this seems to show a destruction of one enzyme and not the other, it has already been shown in previous experiments that the loss of activity for hypoxanthine is merely a p_H effect on the reaction. It seems to us probable that even if the reactions were brought about by the same enzyme they might not be affected by p_H in exactly the same way.

The evidence against the identity of the two enzymes seems therefore to be inconclusive. We have, on the other hand, accumulated a large number of observations which suggest the probability of the enzymes being identical. We do not, however, feel that at present the evidence is sufficient to justify a positive statement to this effect. In the first place we have been unable by any means to effect a separation of the enzymes. Both are completely precipitated from milk with the caseinogen. Neither is removed by the ether extraction. The aldehyde activity of a well-preserved preparation is retained as long as is that with hypoxanthine, but both are very slowly lost as the dry preparation stands in air. As has been mentioned before, the enzyme in a solution exposed to air loses both its aldehyde and hypoxanthine activity. Both enzymes are removed by filter-paper, and are also destroyed in the same degree by heating for a short time.

In the second place, a comparison of a large number of preparations from different milks shows a striking parallelism between the two activities. In no case have we been able to obtain two preparations whose activities varied inversely, one going quicker with hypoxanthine than with aldehyde, the other going quicker with aldehyde. Morgan, Stewart and Hopkins stated that in milk the two activities do not always run parallel with each other. This could be explained by the fact which we have found that fat accelerates the reaction with hypoxanthine but not that with aldehyde. Even if the two processes were brought about by the same enzyme, if the milks contain varying amounts of fat the activities would not be parallel. In our preparation, of course, the fat has been removed.

As stated above, the p_H curves of the two are very similar, but this is not necessarily evidence for their identity. The substrate concentration curve with aldehyde is not of the same form as that given with purines, but this is not to be expected, as the inhibition in all cases so far examined has been found to be a specific property of purines. The aldehyde concentration curve is given in Fig. 10. It can be seen that the velocity is directly proportional to the concentration of aldehyde. This does not necessarily show a difference in enzymes, but merely that the aldehyde does not block the surface of the enzyme as do purines. The purine inhibitory effects, however, are the same for both enzymes, if indeed there be two. The inhibitory effect of uric acid on hypoxanthine has already been shown, and if the enzymes were identical a similar inhibition by purines would be expected in the case of aldehyde. Such inhibition was actually found to occur, as shown in Fig. 11.

Fig. 10

Fig. 11

Fig. 10. *Aldehyde concentration curve.*

Each tube contained: 5.0 cc. enzyme solution (in buffer); 0.5 cc. methylene blue; x cc. 10 % aldehyde (neutral); $(2-x)$ cc. water.

Fig. 11. *Inhibition of aldehyde reaction by uric acid.*

Each tube contained: 5.0 cc. enzyme solution (in buffer); 0.5 cc. methylene blue; 0.1 cc. 10 % acetaldehyde (neutral); x cc. uric acid (5 mg. per cc.); $(2-x)$ cc. water

Caffeine, however, as was the case with hypoxanthine, showed no effect. $N/100$ sodium fluoride and cyanide showed a very slight inhibitory action on both hypoxanthine and aldehyde. In the presence of either alcohol or acetone the activity of both enzymes was markedly and proportionately decreased. In view of this remarkable similarity in behaviour we feel that the balance of evidence is in favour of the two enzymes being identical.

We now turned our attention to the possibility of the oxidation of adenine being due to the same enzyme. In the case of adenine there are two reactions which might occur. The adenine may be deaminated first by adenase, as supposed by Morgan, Stewart and Hopkins, and the resulting hypoxanthine oxidised by xanthine oxidase; or it is possible that xanthine oxidase might oxidise adenine directly, without the adenine undergoing deamination, in

which case it would yield 6-amino-2-8-dioxypurine. Such an oxidation is known to occur in the animal body. If adenase is present it must be in very small quantities, since the reaction with adenine is very slow. Therefore the reaction velocity will be controlled by the speed at which the adenine is deaminated, the hypoxanthine being oxidised as fast as formed.

Since adenase is a deaminase and the xanthine enzyme an oxidase, one would not expect the two enzymes to be very similar in behaviour, e.g. with respect to p_H . As has been stated, however, the p_H curves for hypoxanthine and adenine are very similar, exhibiting the break at p_H 9.0. The substrate concentration curve for adenine, when carried out with a range of adenine concentrations similar to that used for hypoxanthine, is flat, that is, the velocity is independent of the adenine concentration. When, however, a more concentrated adenine solution is used, the velocity is found to decrease with increasing adenine concentration, and the curve resembles that obtained with hypoxanthine and xanthine. The two curves for adenine are shown in Figs. 12 and 13.

Fig. 12

Fig. 12. Adenine concentration curve.

Each tube contained: 5.0 cc. enzyme solution (in buffer); 0.5 cc. methylene blue: x cc. adenine (2 mg. per cc.); $(2-x)$ cc. water.

Fig. 13

Fig. 13. Adenine concentration curve (higher concentration).

Each tube contained: 5.0 cc. enzyme solution (in buffer); 0.5 cc. methylene blue; x cc. adenine (5 mg. per cc.); $(2-x)$ cc. water.

When using adenine as a substrate it was necessary to use very strong enzyme solutions, since otherwise the reduction times were too long for convenient observation. This explains why the critical adenine concentration is

so much higher in these experiments than the critical hypoxanthine concentration. The fact that these curves are of exactly the same type as the hypoxanthine curves is strong evidence in favour of the view that the same enzyme brings about both reactions. Moreover, the effect of inhibiting substances, uric acid, fluoride and cyanide, is the same for adenine as for hypoxanthine, while caffeine again has no effect. The parallelism in activity of various preparations with respect to hypoxanthine and adenine has been observed in every preparation used.

If adenine were directly oxidised, aminodioxypurine would be formed instead of uric acid. This has not yet been settled experimentally. It seems, therefore, at the moment that the evidence in either direction is inconclusive, but with the balance of evidence in favour of identity.

SUMMARY.

1. An investigation of the dynamics of the xanthine oxidase has been carried out by means of the methylene blue technique.

2. The reaction velocity is not affected by p_{H} between p_{H} 5.5 and 9. Above p_{H} 9 and below p_{H} 4 the enzyme is destroyed. Between p_{H} 4 and 5 the reaction velocity is very small but no destruction occurs.

3. The reaction velocity is directly proportional to the enzyme concentration, but is independent of methylene blue concentration provided this is not very small.

4. When the concentration of purine base is increased beyond a certain critical concentration the reaction velocity is decreased. Below this critical concentration the reaction velocity is independent of purine base concentration.

5. Upon addition of purines (e.g. uric acid), other than the substrate the reaction velocity is similarly decreased.

6. The critical concentration of purine base at which inhibition begins increases with increasing enzyme concentration.

7. Below the critical substrate concentration, hypoxanthine reduces methylene blue twice as fast as does xanthine, but as inhibition begins the rates approach one another and become equal when inhibition is complete.

8. When inhibition is complete the reaction velocity is dependent upon methylene blue concentration.

9. The bearing of these facts on the mechanism of the oxidase system is discussed.

REFERENCES.

- Dixon and Tunnicliffe (1923). *Proc. Roy. Soc. Lond.* **94** B, 266.
Morgan, Stewart and Hopkins (1922). *Proc. Roy. Soc. Lond.* **94** B, 109.
Ohlsson (1921). *Skand. Arch. Physiol.* **41**, 77.
Widmark (1921). *Skand. Arch. Physiol.* **41**, 200.