Enzyme Inhibition by Suramin and the Measurement of the Isoelectric Points of some Enzymes

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During a study of the action of the trypanocidal drug, suramin, on various enzymes it was observed that the inhibitory effect of the drug on some enzymes was dependent on the pH of the solution. Thus, whereas $m/3000$ suramin almost completely inhibited urease at pH 5.0, no inhibition occurred at pH 5.3, even when much higher concentrations were used (Wills & Wormall, 1949). In further investigations it was found that with several other enzymes there was a similar sharp drop in the inhibition-pH curve at or near the isoelectric point of the enzyme concerned. As a result of these observations it was suggested that, where an enzyme is inhibited by suramin and where the inhibition shows the above-mentioned marked dependence on the pH of the medium, 'suramin-inhibition' tests might give valuable information about the isoelectric point of the enzyme (Wills & Wormall, 1950*a*).

A study of a wide range of suramin-sensitive enzymes has fully confirmed this view, for there is very good agreement between the values obtained by the suramin-inhibition method and corresponding values recorded in the literature for the isoelectric points of most of these enzymes. Brief preliminary reports of some of these results have been given elsewhere (Wills & Wormall, 1950*b*, *c*). Further results, and full details of the method, are given in this paper.

EXPERIMENTAL

Enzyme preparations. The methods of preparing the various enzymes and the measurement of their activity have been described previously (Wills & Wormall, 1950*a*). The sources of some enzymes are recorded in Table 1. The sources of others were as follows: urease (soya bean and jackbean), peroxidase (horse-radish), ascorbic acid oxidase (cucumber and cabbage), invertase (a commercial preparation from yeast), β -glucosidase (ground almond meal).

Buffer solutions. Acetate buffer was used for the pH range 3.8–5.7, and phosphate buffer for the pH range 5.7–6.5. Both series were prepared to the formulae of Clark and Lubs. The pH of all buffer solutions was measured with the glass electrode before use and the pH of each mixture was checked with indicators at the end of the experiment.

Method

The method consisted of estimating the enzymic activity with and without suramin over a range of pH. The percentage inhibition was then plotted against pH. Where the method is

of value, the curve falls sharply, from about 100 to 10% or less, over a range of 0.3–0.8 of a pH unit, and the midpoint of this range, or probably more accurately that of the range covering 10–90% inhibition, is regarded as the mean isoelectric point of the enzyme. It was usually possible to measure this value to within 0.2, and sometimes to the nearest 0.1 of a pH unit. With some enzymes, the drop in the pH-inhibition curve covered a range of a whole unit of pH or more and it is suggested that in these cases less precision should be given to the '50% inhibition' value. Sometimes, however, this slow fall of the curve can be attributed to the presence of two different but similarly acting enzymes (e.g. α - and β -amylases) in the preparation studied.

$m/1000$ suramin was used in almost every case, since this concentration was found to give the sharply dropping type of curve required. More dilute solutions gave less steep curves, whilst higher concentrations of suramin (e.g. $m/500$), although not exhaustively investigated, appeared to offer no advantage over $m/1000$. The suramin should be left in contact with the enzyme for a standard period of time before the addition of the substrate; a period of 20 min. is satisfactory (Wills & Wormall, 1950*a*).

Since these factors are known to affect the values obtained for the isoelectric point of a protein by conventional methods the type of buffer used and its concentration have been specified in each case. An exhaustive study of the effect of changing the type of buffer and salt concentration has not yet been made, but preliminary experiments have shown that these changes have no marked effect on the results obtained by the suramin-inhibition method, possibly on account of the relative insensitivity of the method.

RESULTS

Effect of pH on the inhibition of various enzymes by suramin

In view of the results obtained with urease (Wills & Wormall, 1949, 1950*a*), similar tests were made with several other enzymes and many were found to give curves of the same type as that given by urease. With many enzymes it has been possible to confirm that the curve drops sharply at the known isoelectric point of the enzyme. In other cases, however, no authentic value for the isoelectric point, as determined by electrophoresis, appears to be available in the literature and the values obtained by the suramin-inhibition method with those enzymes should be regarded as tentative; they are presented here so that they can subsequently be compared with the results which may be obtained by electrophoresis of the crystalline enzymes.

Table 1. *Isoelectric points of some enzymes measured by the 'suramin-inhibition' method and compared with values recorded in the literature*

		Isoelectric point			
		Recorded values			
Enzyme	Buffer* (and concn.)	Suramin method (pH)	For crystalline enzyme (pH)	For non- crystalline enzyme (pH)	Reference
Urease	Acetate	5.1	5.0-5.1	—	Sumner & Hand (1929)
Catalase:					
Ox liver	Acetate or phosphate	5.8	5.7	—	Sumner & Dounce (1937)
Sheep blood	Phosphate	6.2	—	—	—
Rabbit blood	Acetate	5.2	—	—	—
Human blood	Acetate	5.0	—	—	—
Horse liver	Acetate	5.2-5.3	5.4	—	Agner (1942)
Horse blood	Acetate	5.2-5.3	—	—	—
Amylase:					
Human saliva (α)	Acetate (0.03M)	5.3	5.0-5.5†	—	Fischer & Haselbach (1951)
Malt (α)	Acetate (0.02M)	5.0	5.7	—	Daniellson & Sandegren (1947)
Malt (β)	Acetate (0.02M) or phosphate (0.012M)	5.7	—	5.75	Daniellson & Sandegren (1947)
Hog pancreas (α)	Acetate (0.05M)	5.2	5.2-5.6	—	Fischer & Bernfeld (1948)
Carbonic anhydrase:					
Sheep blood	Acetate or phosphate	5.8-6.0	—	—	—
Ox blood	Acetate	5.2-5.4	—	5.3	Petermann & Hakala (1942)
Peroxidase	Acetate	4.9	7.2	—	Theorell (1943)
Tyrosinase:					
Potato	Acetate	4.3	—	5.4	Kubowitz (1937)
Mushroom	Acetate	3.9†	—	Below 5.0	Nelson & Dawson (1944)
Ascorbic acid oxidase	Acetate or phosphate	5.1	—	—	—
Invertase	Acetate (0.05M)	4.1	—	4.42	Dieu (1947)
β -Glucosidase	Acetate	4.4	—	5.7-5.8	Edman & Jorpes (1941)

* 0.067M-Acetate or 0.016M-phosphate, unless some other concentration is indicated.

† Using catechol as substrate.

‡ 'Very probable value' (Meyer, 1949).

Table 1 gives a list of enzymes so far investigated by the suramin-inhibition method and a comparison of the results with those obtained by standard methods for the determination of isoelectric points.

Typical inhibition-pH curves are given in Fig. 1. The '50%-inhibition' point (pH 5.2) for pig pancreatic α -amylase agrees well with the recorded isoelectric point. Human salivary amylase and malt α - and β -amylases gave curves of the same type, and in two cases the pH of the midpoint of the inhibition-pH curve was almost identical with the recorded isoelectric point; in the case of malt α -amylase, however, the suramin inhibition method gave a value of pH 5.0, whereas Fischer & Haselbach (1951) give pH 5.70 as the isoelectric point of this enzyme. It has not so far been possible to explain the difference between the two values.

Invertase also gives a sharply dropping curve, with midpoint about pH 4.1 (Fig. 1), but in this case no comparison with an established authentic isoelectric point is possible, since the crystalline enzyme does not appear to have been prepared.

However, Dieu (1947) as a result of careful electrophoretic studies on purified invertase has concluded that this preparation consisted of two closely associating components. Only one of these was found to possess invertase activity and the isoelectric point of this component was determined as being 4.42, which compares reasonably well with the value found by the suramin method (pH 4.1). Quastel & Yates (1936) obtained a curve similar to that in Fig. 1 in their study of the action, on invertase, of several acidic dyes related to suramin, but they did not extend the investigation to other enzymes.

The inhibition of invertase by suramin in acid solution differs from that of urease in that it can be reversed by adding alkali to change the pH of the mixture from 3.8 to 5.1.

Peroxidase gave a similar sigmoid-type inhibitor-pH curve, but the isoelectric point indicated by the suramin method (pH 4.9) differs appreciably from the value (pH 7.2) quoted by Theorell (1943). It is possible in this case that suramin combines with the haematin group of the enzyme. If inactivation

occurs by such a mechanism it would invalidate any figure obtained for the isoelectric point of the enzyme using the suramin method.

Edman & Jorpes (1941) have partially purified emulsin and state that they obtained an electrophoretically homogeneous fraction which possessed emulsin activity and had an isoelectric point of pH 5.7–5.8; the preparation was not pure β -glucosidase, however, and it showed high phosphatase activity. The value of 5.7–5.8 differs considerably from that found by the suramin method (pH 4.4–4.5), but it is possible that better agreement would be obtained if an electrophoretic study were made on the crystalline enzyme.

Fig. 1. Effect of pH on the inhibition of α -amylase (hog pancreas) and invertase by suramin. 0.001M-Suramin; drug and enzyme left in contact for 20 min. before the addition of substrate. A, invertase; B, α -amylase.

Ascorbic acid oxidase yields a rather unusual type of curve which drops sharply at about pH 5.1, but remains fairly constant at about 40% inhibition as the mixture is made more alkaline (i.e. over the range pH 5.5–8.0). It is, of course, possible that the enzyme preparation used contained two enzymes capable of oxidizing ascorbic acid, and that only one of them is inhibited by suramin.

For the experiments with tyrosinase (monophenol and polyphenol oxidases) two substrates, *p*-cresol and catechol, were used. Potato tyrosinase gave similar but not quite identical inhibition-pH curves with the two substrates, but mushroom tyrosinase gave two different curves (A and B, Fig. 2). It is tentatively suggested that these results indicate that the oxidation of catechol by mushroom extracts is catalysed by one enzyme, whereas that of *p*-cresol may be due to two or more enzymes, only one of which is a true phenoloxidase (cf. Keilin & Mann, 1938).

Catalases gave inhibition-pH curves similar to that obtained with urease. Of the six catalases examined, however, those of horse blood and horse liver gave curves which were almost identical, with a midpoint of pH 5.2–5.3; the other four (those of human, rabbit and sheep blood and ox liver) gave curves which differed amongst themselves, and also from that for horse blood and liver. Recorded values for the isoelectric points of catalases are pH 5.7 for the crystalline ox-liver enzyme (Sumner & Dounce, 1937) and pH 5.4 for the crystalline horse-liver enzyme (Agner, 1942). The suramin-inhibition method gave pH values of 5.8 and 5.2–5.3 respectively, for these two enzymes. Bonnichsen (1947) reported that horse-liver and horse-blood catalases are identical, and it is interesting to note that these two enzymes give essentially the same inhibition-pH curve with suramin.

Fig. 2. Effect of pH on the inhibition of tyrosinase by suramin. Mushroom tyrosinase acting on catechol (A) and *p*-cresol (B). Potato tyrosinase acting on either catechol or *p*-cresol (C).

Fig. 3. Effect of pH on the inhibition of various catalases by suramin. Catalases of human blood (A), rabbit blood (B), ox liver (C) and sheep blood (D).

Evidence of species differences between the carbonic anhydrases, with respect to their inhibition by suramin, was obtained in experiments with the ox- and sheep-blood enzymes. The inhibition-pH curves for these two enzymes do not drop as sharply

as do those of urease, the catalases, etc., mainly because of the difficulty in estimating the activity of the enzyme in acid solution.

Inhibition of crude enzymes by suramin

Only in the case of urease has it been possible to compare the inhibition-pH curves of the crystalline and impure enzymes. Almost identical curves were obtained with crystalline jack-bean urease and the crude enzyme (soya-bean extracts); considerably more suramin is, however, required to give complete inhibition with crude enzyme preparations, owing to the protective action of the protein impurities. Furthermore, it has been found that the addition of unrelated proteins, such as ovalbumin or those of rabbit serum, has no significant effect on the inhibition-pH curve for urease, though here also more suramin has to be used in the tests.

These results led us to suggest that the method might have a special value in that it is applicable to crude enzyme preparations (Wills & Wormald 1950*a*, *b*). Indeed it may sometimes be possible to obtain an indication of the presence of two similarly acting enzymes in an enzyme preparation. With malt extract the amylase inhibition-pH curve slopes gradually over the range pH 4.6-6.3, whereas the separated α - and β -amylases both give sharply falling curves covering the ranges pH 4.6-5.3 and 5.4-6.3 respectively. The curve for the crude malt extract thus appears to be a resultant of the curves for α - and β -amylases.

Fig. 4. Effect of pH on the inhibition, by suramin, of the separated malt α - and β -amylases and the amylases in a crude malt extract. α -Amylase (A), malt extract (C) and β -amylase (B).

DISCUSSION

This suramin inhibition method for the estimation of enzyme isoelectric points, although it has a limited application, may be of value in many enzyme studies. The method has, in fact, already been used by Smith & Mills (1950) to determine the isoelectric point of the β -glucuronidase of *Escherichia coli*. It has not the accuracy, reliability or the

general applicability of the classical physical methods such as electrophoresis, but it has several interesting and important features. (1) It is easily carried out with simple equipment, usually in a period of a few hours. (2) Whereas the standard methods require extremely pure and preferably crystalline enzymes, the suramin inhibition method can be satisfactorily used, where the method is applicable, with crude enzyme preparations. (3) Values obtained with impure enzyme preparations may serve as a guide for the purification of an enzyme by precipitation at various pH values. (4) The slope of the curve may sometimes indicate that two or more similarly acting enzymes are present in the preparation, as, for example, with the amylases and the phenoloxidases. (5) It may sometimes give a value for the isoelectric point of an enzyme which, in a pure state, is unstable at its isoelectric point (e.g. tyrosinase).

It must be emphasized, however, that the suramin inhibition method is not applicable to the following enzymes. (1) Those which are inhibited by suramin independently of the pH of the medium ('group A' enzymes; Wills & Wormald, 1950*a*). (2) Proteolytic enzymes, when protein substrates are used. The drug readily combines with most, or even all, proteins (cf. Bournsell & Wormald, 1939; Town, Wills, Wilson & Wormald, 1950) and this undoubtedly influences the tests for protease inhibition. The use of short-chain peptides and their derivatives might overcome this difficulty, and this possibility is being explored (Town & Wormald, unpublished). (3) Any enzyme whose activity falls sharply on the acid and alkaline sides of its optimum pH.

The possibility that suramin inhibits many enzymes by a 'bridging' effect has been previously discussed (Wills & Wormald, 1950*a*). If this hypothesis is correct and suramin combines with basic groups in the neighbourhood of the active centre of the enzyme, it will be clear that the method described here can only give a measure of the isoelectric point of that part of the molecule near the active centre, whilst standard physical methods give an isoelectric point for the molecule as a whole. Since there is good agreement between the two methods in several cases, this suggests that there is no abnormal arrangement of acidic or basic groupings in the neighbourhood of the active centres of these enzymes.

The literature dealing with enzymes, even that of recent years, usually tends to give an impression that enzymes which carry out the same function, providing they can be isolated completely pure, are chemically identical, irrespective of the source from which they are obtained. That this view is not entirely correct has been demonstrated by the work of Bonnichsen (1947) on catalases and by Meyer

and his colleagues (cf. Meyer, Fischer, Bernfeld & Duckert, 1948) on the amylases, for these investigators have obtained clear evidence of species differences. Additional support for their observations has been provided by the present experiments, where it has been possible to demonstrate species differences with catalases, amylases and carbonic anhydrases (cf. also Wills & Wormall, 1950*b*, *c*). For example, when tested under identical conditions, four catalases gave four distinctly different curves, with '50 % inhibition' or isoelectric points of pH values of 5.0, 5.2, 5.8 and 6.2.

SUMMARY

1. Further details are given of the use of inhibition tests with suramin for the measurement of the isoelectric points of certain enzymes (Wills &

Wormall, 1950*a*, *b*). The method is simple and rapid, and can be carried out with impure enzyme preparations.

2. This suramin-inhibition method has been used to measure the isoelectric points of nineteen enzymes. Where comparison is possible (fourteen enzymes) there is, in general, good agreement between the values obtained and those recorded in the literature for electrophoretic examination of the pure enzymes.

3. The results support the view that there are marked species differences amongst the catalases, amylases and carbonic anhydrases.

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Astaxanthin, Cholesterol and Lipins in Developing Salmon Eggs

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In this paper observations on provitamins and vitamins A and D and the carotenoid astaxanthin in developing salmon eggs are recorded, together with changes in neutral fat, cholesterol and phospholipin content. An adequate discussion of the broader aspects of these matters must await more facts pertaining to more species.

This study shows among other things a marked conservation of astaxanthin during development and a striking lack of evidence that vitamin A (or A₂) is required in more than traces, if at all.

Through the kindness of Dr R. J. Daniel (until recently of the Department of Oceanography in this University), supplies of salmon eggs were made available to us over a total period of some 3 months in one season and at intervals in previous years.

EXPERIMENTAL

Materials

Salmon eggs (*Salmo salar*). The eggs were kept in trays in a shallow tank through which water at 9–10° was kept flowing. The eggs were fertilized early in December; about