but in addition to azelaic acid there was also produced some suberic acid.

2. Oxidation with potassium permanganate in acetone solution of methyl hexadecatrienoate isolated from rape-leaf glycerides yielded pimelic,

oxalic and propionic acids. This evidence in association with the spectroscopic results before and after isomerization show that this acid has the formula

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Further Observations on the Action of Chymotrypsin on Insulin

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(Received 30 January 1951)

The action of crystalline chymotrypsin on insulin has recently been described (Butler, Dodds, Phillips & Stephen, 1948) and the biological activity of the products has been investigated (Phillips, 1951). The degradation of the hormone by this enzyme gave a large fragment, the core, of molecular weight about 5000 (Butler, Phillips, Stephen & Creeth, 1950) and a mixture of small peptides which have been partially separated by paper chromatography (Phillips, 1949).

In this paper the degradation has been further studied: (a) to see whether the products of proteolysis cause any inhibition of the enzyme, (b) to locate the pH optimum for the reaction (this has been found to have two optima) and (c) to define the general character of the reaction, which has been found to be of the 'all-or-none' type of Tiselius & Eriksson-Quensel (1939).

EXPERIMENTAL

Insulin. Crystalline bovine insulin from Boots Pure Drug Co. was used, assaying 22–23 units/mg.

Chymotrypsin. Part of a sample of α -chymotrypsin kindly given by Dr M. Kunitz was used. The digestions were carried out in a similar way to the previous work, using approximately 3×10^{-4} t.u. (trypsin units) of enzyme/5 mg. insulin

in 1 ml. of buffer at 25°. The trypsin unit referred to here is the quantity of enzyme which will liberate 1 mmol. of tyrosine from haemoglobin in 1 min. at 35.5°. According to Northrop, Kunitz & Herriott (1948) 0.038 trypsin unit is equivalent to 1 mg. of protein N.

Nitrogen. Amino N, non-protein N (N.P.N.) and total N were determined as in the previous papers. N.P.N. is expressed as % of total N.

Enzyme activity. This was determined by the method of Anson (1938).

Tyrosine. The colour given with the phenol reagent of Folin & Ciocalteu (1927) was measured, there being no tryptophan in insulin to interfere with it.

Insulin activity. This was determined by measuring the blood sugar levels (Folin & Wu, 1929) immediately prior to and 1, 2 and 3 hr. after subcutaneous injection into 1.5–2.5 kg. rabbits which had been starved 18 hr., and which had recently been shown to give a 40–60% maximum fall when given 1 unit of insulin.

RESULTS

The effect of the products of digestion of insulin by chymotrypsin on the activity of the enzyme

This effect has been estimated in three ways: by the remaining enzyme activity in the digest when tested against a standard substrate (haemoglobin); by digestion of insulin in the presence of urea; and

by measuring the digestion of insulin in the presence of various amounts of whole digest and the separated products of digestion.

There is a spontaneous loss of activity in chymotrypsin solutions which are allowed to stand at 25°. For example, a solution containing 7.8×10^{-4} T.U./ml. in phosphate buffer, pH 8.3, lost 20–30% of its activity in 20 hr., although a 50% decrease required 90 hr. Measurements in the presence of insulin were lacking in precision, since the medium being assayed already contained tyrosine-bearing peptides when added to the standard blood substrate. As these peptides were soluble in trichloroacetic acid (TCA), a high 'blank' value resulted. In spite of this it has been possible to show that there is residual chymotryptic activity in insulin digests. Thus the activity of three digests which started with 3.8, 5.0 and 4.7×10^{-4} T.U./ml. became 4.0, 3.3 and 5.1×10^{-4} T.U./ml. after 24, 24 and 10 hr. respectively.

The enzyme assay was done in a solution 5.55M with respect to urea, and it is thus possible that enzyme-inhibitor complexes, if present, will be broken rapidly in this medium. Insulin was therefore digested in the presence of urea to see whether the breakdown was faster in such a medium.

Insulin (4.8 mg./ml.) was digested with 3×10^{-4} T.U. of chymotrypsin/ml. at pH 8.3 and 25° in three parts: (i) control without urea; (ii) in M-urea and (iii) in 5M-urea. The N.P.N. was determined by precipitating 1 ml. with 5 ml. of 0.3N-TCA and measuring the colour of the filtrate with the Folin-Ciocalteu phenol reagent. Fig. 1 shows the increase

mination in the 20 hr. control digest gave the value 77% N.P.N. (point A). Insulin is not, however, digested more rapidly in the presence of urea, the increased N.P.N. formation in Fig. 1 being due to the solubilizing effect of the urea lowering the efficiency of TCA. This was shown by adding urea to the TCA used to precipitate the control digest. By adding sufficient to simulate the conditions in the 5M-urea experiment, the 'N.P.N.' of the control was raised as is shown in one instance by the arrow in Fig. 1. Addition of urea after filtration had no effect on the colour produced. In some tentative experiments to reduce the urea content of digest samples for injection, it was found that urea could be extracted readily from aqueous solution by *tert.*-amyl alcohol.

Addition of a solution of intact insulin to a chymotryptic digest which had reached 78% N.P.N. (1 ml. digest to 5 ml. 0.3N-TCA) lowered the N.P.N. value to 39%, and was quickly followed by digestion which restored the N.P.N. value to 77%. Thus a high degree of enzyme activity remained. Moreover, the addition of peptides (derived from a chymotryptic digestion of insulin) to a solution of insulin did not affect its rate of digestion at pH 8 and 25° by chymotrypsin when compared with a control. Two experiments with added peptide: insulin ratios (by weight) of 0.28 and 0.72 were done.

If extra enzyme is added to a digest which has already become apparently stationary as regards N.P.N. formation, further formation of N.P.N. occurs at a slow rate. This is ascribed to the digestion of the core (mol.wt. 5000) which is normally precipitated by TCA (see Butler *et al.* 1950). It is clear from these experiments that the earlier opinion of Butler *et al.* (1950), that chymotrypsin is inhibited by an insulin digest, can no longer be sustained.

The effect of pH on the rate of digestion of insulin by chymotrypsin

The pH optimum. Insulin (2.15 mg./ml.) was digested with 3×10^{-4} T.U. of enzyme/ml. at 25° in buffers at various pH (measured by glass electrode), and samples taken at intervals for N.P.N. estimation (1 ml. digest to 5 ml. 0.3N-TCA). The results are shown in Fig. 2. The optimum is near pH 8.6, but there is a second optimum near pH 9.5; series of digestions confirmed this observation. Resolution of the curve after 1.5 hr. digestion into two symmetrical curves gives the optima at about pH 8.2 and 9.7.

The effect of buffer. After 1.5 hr. digestion only a 1% difference in N.P.N. value was observed between a digest in pH 8.3 phosphate-citrate buffer and another in borate-hydrochloric acid buffer. Moreover, the buffers used over the whole pH range in Fig. 2 were borate-HCl or borate-NaOH, and in phosphate-citrate buffer it was shown that the digestion rate was lower at pH 9.0 than at pH 8.2 or 8.3.

Enzyme activity. Solutions of chymotrypsin at pH 7.6 and 9.9 in borate buffers as used above were found to have the same activity initially and after 6 hr. at 25°, and no absolute loss of activity was observed. Thus the double optimum is not related to any differential loss of activity at different pH by this enzyme.

Samples of digests prepared at pH 7.6 and 9.9 (on the opposite limbs of the pH-digestion curve, Fig. 2) were

Fig. 1. Digestion of insulin by chymotrypsin. Control ($\times$ — $\times$), in M-urea ($\bullet$ — $\bullet$) and in 5M-urea ($\circ$ — $\circ$), as measured by colour with Folin reagent. A = point checked by nitrogen estimation.

during digestion of TCA-soluble material giving colour with this reagent. It has been found in separate experiments (see below) that the intensity of the blue colour developed in this way is directly proportional to the N content. An N deter-

coupled with 1-fluoro-2:4-dinitrobenzene according to the method of Sanger (1945), and a qualitative examination of the end groups by paper chromatography with *tert.*-amyl alcohol-water was made. The pattern, position and intensity of the yellow spots of dinitrophenylamino-acids were the same in both cases. Moreover, both digests had lost about 80% of their insulin activity after 5 hr. digestion at 25°.

Fig. 2. Digestion of insulin by chymotrypsin at various pH values. Formation of N.P.N. after 1.5 and 3.5 hr. digestion at 25°.

As the activity of insulin is unaffected in 17 hr. at pH 9.9 and 25° as measured in a few standardized rabbits, it is unlikely that the second optimum is due to the presence of denatured or inactive insulin, especially as the two optima are more apparent after 1.5 hr. digestion than after 3 hr.

Enzyme specificity in the digestion of insulin by chymotrypsin

End groups. Chymotrypsin favours the fission of peptide, amide or ester bonds liberating the carboxyl group of tryptophan, tyrosine, phenylalanine or methionine (Bergmann & Fruton, 1937; Fruton

& Bergmann, 1942; Kaufman, Neurath & Schwert, 1949; Neurath & Schwert, 1950). Such rules have not yet been proven for proteins, though a start has been made with pepsin by Desnuelle & Casal (1948), using Sanger's (1945) fluorodinitrobenzene method. This, however, can only identify amino-acids which provide the —NH— group of the peptide bond which is split. This method, together with that of Mills (1950) and paper chromatography of the dinitrophenyl (DNP) amino-acids with *tert.*-amyl alcohol-water, have been used qualitatively on chymotryptic digests of insulin.

In the TCA-precipitable cores (see Butler *et al.* 1950) the following end groups were found: (1) DNP-glycine, 2–3 residues; (2) DNP-valine, 1 residue. DNP-leucine has also been found. In the TCA-soluble peptides the DNP derivatives of leucine, valine, phenylalanine, alanine, glycine, glutamic acid, threonine and tyrosine were found, the proportions decreasing in that order, the tyrosine being present only as a trace. Now insulin has two glycine and two phenylalanine terminal groups per mol.wt. 12,000 (Sanger, 1945), so that these would be expected in the end groups of the products. These findings support the view of Bergmann & Fruton that chymotrypsin splits bonds on the carboxyl side of aromatic amino-acids.

The 'all-or-none' character of the degradation of insulin by chymotrypsin

From electrophoretic studies of peptic digests of ovalbumin, Tiselius & Eriksson-Quensel (1939) postulated that no products intermediate between ovalbumin and the final products appeared during the digestion, i.e. the reaction for a given molecule was completed apparently in one operation.

In the digestion of insulin, this has been studied by measuring (a) the average number of bonds broken per degraded molecule; (b) the tyrosine released in the form of TCA-soluble peptides; (c) the proportions of TCA-precipitable core and TCA-soluble peptides formed; and (d) the activity

Table 1. Number of bonds broken during the chymotryptic digestion of insulin

Percentage of intact insulin remaining (A)	Percentage of molecules degraded (100 – A)	Average number of bonds broken in all molecules (Van Slyke) (B)	Average number of bonds broken per degraded molecule (100B/100 – A)
100	0	0	—
92–93*	7–8	0.8	10–11.4
55–60*	38–44	5.3	12–13.9
30–35*	61–70	7.0	10–11.5
34	66	8.3	12.4
26	74	10.4	14.0
11.5	88.5	12.3	13.9
4.4	95.6	14.2	14.8

* These values were obtained by measurement of the N.P.N. of the digest, and the knowledge that the amount of core formed is 75–100% of the amount of N.P.N. The other values were obtained by quantitative assay of activity (by Boots Pure Drug Co. Ltd.) of the TCA precipitates at stages during digestion, and agree well with the amount of precipitate formed on bringing the digest to pH 5.4, the isoelectric point of insulin.

remaining in the digest, and comparing it with that expected in a random fission of the enzyme-specific bonds, assuming that any single fission inactivates the hormone. This assumption has not been proven but, at least, none of the products of this digestion has any hypoglycaemic activity (Phillips, 1951).

Number of bonds broken. Amino nitrogen determinations were made at intervals during the digestion by Dr J. M. L. Stephen. Table 1 shows the relation of this measurement to the number of molecules degraded. Every molecule attacked has therefore had almost the full number or bonds broken in it irrespective of the time of sampling.

Table 2. *Experimental and calculated loss of insulin activity in samples of insulin digested by chymotrypsin*

Percentage insulin activity remaining	Average number of bonds split per molecule (<i>n</i>)	Calculated percentage of molecules still intact
100	0	100
92	1	37
84*	2	13.5
77*	3	5
34	8	0

* These are interpolated from the experimental values from the known shape of the isoelectric precipitate-time of digestion curves, and the knowledge from the assays that this precipitate accounts for all activity remaining in the digests. Interpolation was necessary to keep *n* small compared with 15, the maximum fission.

If the assumption is made that the fission of a single peptide bond in insulin by chymotrypsin causes inactivation of that molecule, then a random degradation process, which splits say one bond in each of fifteen molecules, will achieve far more inactivation than a process splitting fifteen bonds in one molecule before proceeding to deal with another molecule. How does the fall in activity of the digest compare with the calculated rate for the random process? It is assumed too that there are fifteen specific bonds per molecule (this being the total number of aromatic amino-acid residues and approximately the number of bonds split in chymotryptic digests of insulin reaching 60–70 % N.P.N.). Then the number of intact molecules remaining, when the number of bonds broken per mol. is still small compared with fifteen, is given by the Poisson distribution

$$P = \frac{n^m}{m!} e^{-n},$$

Table 3. *Tyrosine content of the TCA-soluble peptides after acid hydrolysis*

N.P.N. as percentage of total N	Total peptide N taken (mg.)	Tyrosine N in peptides (μg.)	Tyrosine N × 100 Total peptide N
30.4	1.57	118.4	7.55
36.3	0.615	45.0	7.3
40.8	0.56	45.2	8.0
43.7	0.50	32.0	6.4
47.5	0.43	31.2	7.2

where *m* = actual number of bonds split in a given molecule (when *m* = 0, the molecule is intact and active), and *n* = the average number of bonds split in all the molecules.

Table 2 shows this value as a percentage for a series of degrees of digestion from *n* = 0 to *n* = 8. The loss of activity in the random process is far more rapid than occurs in practice (column 1), so that with the assumption made, the process is far from random and a much smaller proportion of the molecules is attacked. Once attacked, each molecule is totally disrupted.

Tyrosine released. The tyrosine in the TCA-soluble peptides at various stages during the digestion was determined in 6 N-hydrochloric acid hydrolysates and in unhydrolysed samples. Table 3 shows the results of the former analyses and Fig. 3

Fig. 3. Correlation of N.P.N. formed (●) and the colour given by the N.P.N. samples with the Folin-Ciocalteu reagent (×) during the chymotryptic digestion of insulin.

the parallelism between the latter analyses and the measured N.P.N. at the same stages of digestion. These results show that the tyrosine released per molecule attacked is, like the amino groups, a constant throughout the digestion.

Core and peptide proportions. The relative proportions of the two classes of products of the digest—the core and the TCA-soluble peptides—remain nearly constant throughout the digestion, as shown in Fig. 4. The undigested insulin was estimated by adjusting the pH of the digest to the isoelectric point of insulin and centrifuging off the precipitate.

The core in the clear centrifugate was then precipitated with TCA. The relation alters towards high values owing to the appreciable secondary digestion of the core. Alternatively, a plot of the percentage of core or of peptides formed against the percentage of insulin remaining in the digest (as measured by the amount of isoelectric precipitate) also gives a straight line.

Fig. 4. The relation between the amount of core nitrogen and TCA-soluble peptide nitrogen, during the chymotryptic digestion of insulin.

DISCUSSION

The results show that very little enzyme activity is lost during the chymotryptic digestion of insulin, and that the rapid slowing down of this digestion when the N.P.N. is 40–50 % (see Butler *et al.* 1948) is not due to the spontaneous inactivation of the enzyme, nor to its inhibition by the products. In this connexion many cases of inhibition of an enzyme by the products of its action have been reported, e.g. urease by ammonia (Laidler & Hoare, 1949).

The double optimum of digestion described above is most simply interpreted as due to chymotrypsin containing more than one enzyme or due to insulin being more than one protein. Kunitz & Northrop (1935) digested casein with α -chymotrypsin, and if a smooth curve is drawn through their formol titration points a double optimum results, which is not entirely eliminated by adding the points from nitrogen determinations. It is known, however, that casein consists of two major and one minor component (Gordon, Semmett, Cable & Morris, 1949) and the net charge per molecule on the major components will be considerably different at pH between 7 and 10. On the other hand, Rocha e Silva & Andrade (1943) found a very marked double optimum in the digestion of a single substrate, *N*-benzoyl-L-arginineamide by activated papain, but in this case it is known that papain consists of more than one enzyme.

The chymotrypsin used here was found (by Dr J. M. Creeth) on electrophoresis at pH 8 to have an isoelectric point lower than pH 8 and to have about 25 % of a minor component, migrating faster and also having an isoelectric point on the acid side of pH 8. When titrated in the presence of sodium dodecyl sulphate a precipitate formed at a pH lower than 4.9, suggesting that the isoelectric point is the same as that determined by Kunitz & Northrop (1935) from the migration of coated collodion particles. Neurath & Schwert (1950), however, accept the values of 9.5 for the isoelectric point of α -chymotrypsinogen and 8.1–8.6 for α -chymotrypsin measured by Anderson & Alberty (1948) and Kubacki, Brown & Laskowski (1949). They also report that α -chymotrypsinogen precipitates at pH 5.4 in the presence of dodecyl sulphate and suggest the isoelectric point may be influenced by a dimerization. However, it is curious to note that the chymotrypsin *B* and its zymogen prepared by Brown, Shupe & Laskowski (1948) had isoelectric points at 4.7 and 5.2, respectively, and had a specificity indistinguishable from that of chymotrypsin (Fruton, 1948). It seems that if the insulin double optimum is to be explained by the presence of two components in the chymotrypsin, then the minor enzyme components would have to be very much more active than the major component in order to achieve the similar rates of degradation actually observed.

Some samples of crystalline insulin contain a hyperglycaemic factor (Sutherland & Cori, 1948), but the material used here has not been tested for this. However, the usual quantity of the factor is estimated at not more than 5 % of the whole, and as the optima indicate the liberation of more than 50 % of the nitrogen as N.P.N. at both optima, the presence of such a small amount could not account for the result. On the other hand, some evidence of the inhomogeneity of insulin has been obtained (Phillips, unpublished) by chromatography of the 2:4-dinitrophenyl derivative of insulin, containing the theoretical number of 19 added groups per mol. Three components soluble in alkaline methyl ethyl ketone were obtained, accounting for 40 % of the insulin. These components had different proportions of dinitrophenyl groups in them. While this is yet inconclusive it seems the most likely explanation of the double optimum observed in the digestions.

The specificity of chymotrypsin towards synthetic peptide substrates is now quite well defined (Neurath & Schwert, 1950), and as there is no tryptophan or methionine in insulin, the only specific residues involved are those of tyrosine and phenylalanine (assuming these rules apply to proteins). The specificity revealed in the chymotryptic digestion of insulin indirectly supports the results with synthetic peptides and suggests that leucine

and valine, with long aliphatic side chains, are favoured components on the 'unspecific' side of the peptide bond split. However, the complete description of the specificity towards a protein must await either an elucidation of the amino-acid sequence in the neighbourhood of these aromatic residues or the introduction of a reagent for the identification of the other partner of the specific peptide bond.

The results show that every molecule attacked by the enzyme undergoes thirteen or fourteen fissions even though there may be a vast excess of intact insulin molecules present. All this degradation may be achieved within the lifetime of the first enzyme-insulin complex formed, or the affinity between intermediates (i.e. molecules of size intermediate between insulin and the core) and the enzyme is so much greater than the insulin-enzyme affinity so that the intermediates are preferentially attacked. This is likely on the grounds that denatured proteins are more readily degraded than the native forms. The measurements made here cannot definitely decide whether the fourteen bonds are split in one catastrophic process or as a result of successive approaches of enzyme to partially degraded material.

Although any fission of insulin (except the removal of amide groups) causes inactivation and suggests that the whole of the insulin molecule is essential for activity, it is more likely, in view of the essential nature of only some of the tyrosine groups, a few of the carboxyl groups and the different rates of inactivation per bond split by different enzymes, that there is a specially active group in the molecule. Thus far no degrading agent has come to light which avoids this grouping. From this one infers that many bonds might be broken without affecting the activity, and in such a degradation even a random fission of bonds would cause a much slower rate of inactivation than in the case calculated in Table 2, where the fission of any single bond is assumed to cause inactivation. However, in any random case, the presence of intermediates should be demonstrable. No exhaustive search has been made for such intermediates, and it has been assumed that protein precipitating at the isoelectric point of insulin and having a weight activity approximately equal to that of insulin is actually intact insulin. Moreover, it has been shown that the core is homogeneous (Butler *et al.* 1950). A complication follows here in that insulin may have a molecular weight of only 6000 (Fredericq & Neurath, 1950). If this is so, one has to assume either that the core is an association of smaller units to give a mol.wt. of 5000, or that insulin contains two types of molecule, one being only slightly degraded to give the core and the other completely degraded to trichloroacetic acid-soluble peptides, averaging pentapeptides. The core is

probably derived from that end of the peptide chains of insulin bearing the free α -amino groups, and the fact that it is inactive (Phillips, 1951) and that insulin which has lost only one or two alanine residues from the carboxyl ends of the peptide chains (Lens, 1949) is also inactive, strongly suggests that the end bearing the free carboxyl groups of insulin must be left intact if activity is to be retained.

However, if it is assumed that the fourteen fissions occur within the lifetime of the enzyme-insulin complex, some sort of lability even in this combination seems to be required, since there is only one site on each enzyme molecule responsible for causing fission. At least, this is the simplest interpretation of the finding by Jansen, Nutting, Jang & Balls (1949) that one molecule of diisopropyl fluorophosphonate (DFP) inactivates one molecule of chymotrypsin of mol.wt. 27,000. This may merely mean that an essential anchorage is denied the enzyme while its active centres remain unaffected. However, the fact that the fission of a substrate as small as L-tyrosine ethyl ester is also prevented by treatment of the enzyme with DFP implies that such anchorage is very near if not coincident with the active point of the enzyme.

SUMMARY

1. Throughout the digestion of insulin by chymotrypsin, the enzyme remains almost fully active, and the digestion rate is unaffected by the presence of urea or the products of the digestion.

2. The pH-digestion rate curve for this proteolysis has two optima, at pH 8.6 and 9.5.

3. The double optimum is not due to inactivation of part of the insulin or the enzyme, and the bonds split at pH 7.6 and 9.9 appear to be the same.

4. Insulin is inactivated at the same rate at pH 7.6 and 9.9 by chymotrypsin.

5. The amino groups revealed by the proteolysis belong mostly to amino-acids other than tyrosine and phenylalanine, and together with the distribution of tyrosine and phenylalanine among the products give indirect support to the specificity rule suggested for this enzyme by Bergmann & Fruton (1937).

6. The degradation has the character of an 'all-or-none' reaction as postulated by Tiselius & Eriksson-Quensel (1939). Every molecule degraded has undergone the fission of thirteen or fourteen bonds irrespective of the number of intact insulin molecules present.

I wish to thank Prof. E. C. Dodds, F.R.S., for his help and interest in this work, Mrs S. Simpson and her assistants for the assays of hypoglycaemic activity and Dr J. M. L. Stephen and Dr J. M. Creeth for permission to quote certain results. Thanks are also due to Messrs Imperial Chemical Industries Ltd. for financial assistance. The work reported in this paper formed part of a Ph.D. thesis submitted to the University of London.

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On the Cyanide Inactivation of Succinic Dehydrogenase and the Relation of Succinic Dehydrogenase to Cytochrome *b*

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(Received 8 February 1951)

Unlike most oxidizing enzymes, the dehydrogenases were considered to be insensitive to cyanide, until Dixon & Keilin (1936) discovered that xanthine dehydrogenase is slowly but irreversibly inactivated by cyanide. In fact, the cyanide inactivation of xanthine dehydrogenase is in a class of its own. The usual type of cyanide inhibition of oxidizing enzymes is instantaneous, readily reversible and only partial, a definite percentage inhibition to each cyanide concentration. In the case of xanthine dehydrogenase, the inhibition is irreversible and it proceeds to completion if, prior to the addition of substrate, the enzyme is allowed to remain with cyanide for a sufficiently long time.

The treatment which Dixon & Keilin (1936) used with xanthine dehydrogenase was applied to a number of enzymes, including succinic dehydrogenase, by Leloir & Dixon (1937) and no inactivation by cyanide was obtained. This was later confirmed

by Potter (1941), who found that the enzyme system responsible for the reduction of cytochrome *c* by succinate was not affected by incubation with cyanide. On the other hand, using a higher cyanide concentration, Banga & Porges (1938) and recently Slater (1949*b*) reported some inhibition of succinic dehydrogenase by cyanide.

In the present investigation it is shown that succinic dehydrogenase is slowly and irreversibly inactivated by cyanide. It is also shown that the enzyme can be effectively protected from the action of cyanide by succinate and sodium dithionite; dicarboxylic acids other than succinic are, however, incapable of giving any protection. A preliminary account of some of these findings has appeared elsewhere (Tsou, 1951*a*).

MATERIALS AND METHODS

Enzyme preparations. The heart-muscle and kidney preparations (Keilin & Hartree, 1947; Slater, 1949*b*) containing the succinic dehydrogenase-cytochrome system were prepared as previously described (Tsou, 1951*b*).

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