In the opinion of Pitt-Rivers (1950) 'the antithyroid action of any compound may be expressed as a function of its reducing power and preferential reactivity with iodine, which inhibits the formation of thyroxine...'. It may be doubted, however, whether this hypothesis can fully account for all the facts established in the present investigation, since, among the compounds which we tested, there is no correlation between the antithyroid action and reducing power. Aromatic compounds containing hydroxyl groups oriented ortho or para with respect to each other (such as catechol, quinol, pyrogallol and hydroxyquinol) are stronger reducing agents *in vitro* than the meta isomers, yet in these experiments most of them have shown no distinctive antithyroid effect.

Differences in antithyroid potency may reflect differential concentration in the thyroid rather than differences in avidity for iodine. Garton & Williams (1949) studied the excretion of quinol, catechol and resorcinol in rabbits, and found that the first two compounds are almost entirely conjugated when excreted, only 2% of the catechol and a trace of quinol being isolated free in the urine. On the other hand, as much as 11–12% of resorcinol was excreted free. This quantitative difference may mean that the resorcinol-treated rats might have a higher blood level of the phenol than rats treated with catechol or quinol.

Dr W. E. Knox, of the National Institute for Medical Research, Mill Hill, brought to our notice a significant correlation between our results and those of Elliott (1932) who catalysed the oxidation of a series of phenols by hydrogen peroxide and milk peroxidase. The compounds tested included phenol, the cresols, catechol, quinol, resorcinol, pyrogallol, gallic acid and β-naphthol. Resorcinol alone was not oxidized by milk peroxidase, the activity of which it inhibited.

Elliott's findings, taken together with Dempsey's (1944) histochemical demonstration of a thyroid peroxidase and the demonstration of such a peroxidase *in vivo* by De Robertis & Grasso (1946), are compatible with the existence of a specific peroxidase of the thyroid gland, similar to milk peroxidase in that it is inhibited by resorcinol, but not by other phenols.

SUMMARY

1. The effect of a representative selection of phenolic substances on thyroid iodine uptake in the rat was studied, using radioactive iodine.

2. With one exception, all the compounds found to be strongly antithyroid could be regarded as derivatives of resorcinol.

3. Possible modes of action of these derivatives on the thyroid are discussed.

It is a pleasure to acknowledge our indebtedness to Dr D. A. Mitchison and Mr J. W. Boag, for statistical assistance, and to Prof. R. T. Williams for advice on the purification of hydroxyquinol.

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The Activity of Enzymic and Acidic Digests of Insulin

By D. M. P. PHILLIPS*
The Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1

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In attempting to determine the nature of insulin and to obtain an active grouping or adjunct from it, many degradations have been carried out using proteolytic enzymes and acids. These studies were summarized by Hill & Howitt (1936) and by Jensen (1938). Proteolytic enzymes were favoured for this work as the mild conditions of their use and their specific action allowed more chance of preserving an active grouping.

The older work is unanimous in declaring that the activity of insulin is readily destroyed by impure pepsin, trypsin and papain, but in general only whole digests were examined for activity. Fisher & Scott (1934), however, examined the activity of the trichloroacetic acid precipitates of a crystalline

* Present address: The Australian National University, c/o The Commonwealth Serum Laboratories, Parkville, Melbourne, N. 2, Australia.

peptic digest, and Banner, Derenvik & Thomas (1942) found that pancreatin and pepsin digests gave ultrafiltrates without any hypoglycaemic activity. No work had been described in which crystalline trypsin and chymotrypsin were used. However, since then, Butler, Dodds, Phillips & Stephen (1948*a*, *b*, 1949) have described the action of crystalline chymotrypsin, trypsin and pepsin on crystalline insulin.

This paper gives the results of tests of hypoglycaemic activity on fractions of peptic, chymotryptic and tryptic digests of insulin, together with some results for acid hydrolyses and the effect of miscellaneous agents on insulin activity.

EXPERIMENTAL

Insulin and crystalline enzymes. The same materials were used as in the previous work (Butler *et al.* 1948*a*, *b*, 1949). The crystalline pepsin used there and here was porcine, the insulin and chymotrypsin bovine. The crystalline insulin assayed 22 units/mg. (Boots Pure Drug Co. Ltd.).

Digestions. Unless otherwise specified, the peptic and chymotryptic digestions were carried out under the same conditions as in the previous papers, generally using 5 mg. insulin/ml. and approximately 4×10^{-4} units of enzyme/ml. The temperature was 25°. The tryptic digest was prepared with a sample of specially purified trypsin (bovine) kindly given by Dr J. H. Northrop and Dr M. Kunitz and used also in previous work (Butler *et al.* 1949).

Insulin activity. The samples tested were aqueous solutions and occasionally suspensions. The largest injection given was 4 ml. All but a few of the injections (noted in the text) were subcutaneous into rabbits of weight 1·5–2·5 kg. The rabbits were starved for 18 hr. before injection and blood-sugar samples were taken from the marginal ear vein immediately prior to and 1, 2 and 3 hr. after injection. All the rabbits used gave good and similar blood-sugar curves with 1 unit of a standard insulin solution, and as little as 0·125 unit consistently gave a depression of the blood-sugar level to about 10% (maximum) below the starving level. The blood sugar was estimated in 0·2 ml. samples by the method of Folin & Wu (1929).

Owing to the large number of samples it was desired to test, most of the estimates of potency were made with single rabbits. In view of the results obtained it is not felt that this approximate method has affected the conclusions. Moreover, some of the results are supported by quantitative assays kindly carried out by Boots Pure Drug Co. Ltd. and reported previously (Butler *et al.* 1948*a*, 1949).

Nitrogen. Nitrogen was determined by the micro-Kjeldahl modification of Ma & Zuazaga (1942). Non-protein nitrogen (N.P.N.) was generally determined by adding trichloroacetic acid to the digest to 0·25 N final concentration and measuring the nitrogen in a sample of the filtrate or supernatant after centrifugation.

RESULTS

Chymotryptic digests

The degradation of insulin by chymotrypsin (Butler *et al.* 1948*a*) gives three fractions: (*a*) undigested insulin, largely precipitable by bringing the digest to pH 5·5, the isoelectric point of insulin; (*b*) the insulin 'core', precipitable in 0·25 N-trichloroacetic acid (for a further description of the properties of this material, see Butler, Phillips, Stephen & Creeth, 1950); and (*c*) a mixture of small peptides, averaging tetra-pentapeptides, which are not precipitated in 0·25 N-trichloroacetic acid (TCA). Fractions (*a*) and (*b*) come down together when TCA is added to the whole digest. Estimates of the hypoglycaemic activity of the whole digest and the three fractions are given in Tables 1–4 and in Fig. 1.

Table 1. *Activity of whole chymotryptic digests of insulin*

(Digestion at pH 8·5 and 25°.)

Non-protein nitrogen (%)	Dose in terms of original insulin units	Approximate activity found (units)	Approximate activity remaining (%)
Start (2 min.)	1·2	0·5	50
—	1·9	>1	>50
30·4	2·4	1	40
40·8	4·8	0·5–1	10–20
47·5	14	0·5	3
—	36	1·5	4
—	144	>1 (convulsed)	>1

Table 2. *Activity of whole chymotryptic digests of insulin prepared at different* pH *values*

pH of digest	Non-protein nitrogen (%)	Dose in terms of original insulin units	Approximate activity found (units)	Approximate activity remaining (%)
7·6	32	2·4	0·3–0·5	12–20
7·7	69	19	1	5
8·4	73	19	1	5
9·8	65	19	Nil	0
10·2	65	19	Nil	0
10·5	40	2·4	0·5	20

Table 3. *Activity of the insulin core (digests C 28 and C 30)*

(The cores were prepared by TCA precipitation at 0·25 N final concentration after removal of the precipitate which forms in the digest at pH 5·5.)

Non-protein nitrogen (%)	Estimated percentage of core	Dose (mg. N)	Activity found/mg. N (units)	Activity of core compared with insulin = 100
22·4	23	0·20	5	3
30·4	30	0·10	5	3
29	37	0·30	1·7	1
40·8	42	0·13	3·7	2
42	44	0·30	1·7	1
47·5	52	0·136	Nil	0

Whole digests. Tables 1 and 2 show that the destruction of insulin activity increases with the formation of N.P.N. and at all digestion pH values tried. No chymotryptic digests have yet been made at pH values acid to 7, as insulin is too insoluble below this pH.

Precipitates obtained at pH 5·5. The correlation between the remaining activity of a whole chymotryptic digest at different times and the amount of precipitate obtainable from it by adjusting samples to pH 5·5 is shown in Fig. 1.

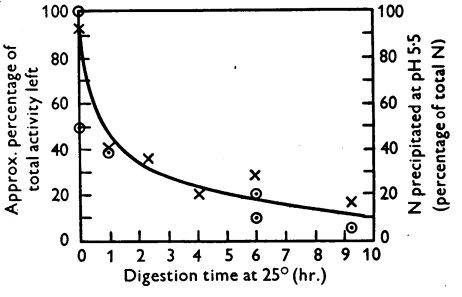

Fig. 1. Correlation between remaining activity of chymotryptic digest of insulin (⊙) and the amount of precipitate obtainable from it at pH 5·5 (×).

With digest C 29, two precipitates were prepared when the N.P.N. values of the digest were 15·4 and 24·4 % of the total nitrogen respectively. Injection of an amount containing 0·0062 mg. N (the nitrogen equivalent of 1 unit of insulin activity in the form of insulin of 25 units/mg.), showed that the earlier precipitate had the full activity and the later one about half the activity of pure insulin. These results show that although the pH 5·5 precipitate contains inactive material, it approximates to the amount expected from the remaining activity of the digest in terms of intact insulin. This suggests that all the activity remaining in a digest is due to the undigested insulin still present. That this is so is shown by the lack of activity in the other two fractions of the digests.

Insulin 'core'. It can be shown that the 1–3 % of activity, compared with insulin, in the insulin 'core' (Table 3), is due to associated insulin which escapes precipitation at pH values near 5·5. Thus an undigested insulin solution adjusted to pH 5·7, and centrifuged and filtered after 4 hr. at 20°, gave a filtrate containing about 1 unit of activity from each 34 units of insulin originally taken, so that approximately 3 % escaped precipitation. The filtrate actually had 0·5 unit of activity/ml. Lens (1948) found that the solubility of pure insulin in pH 5·5, 0·1 M-acetate buffer is 30 mg./l., corresponding to 0·75 unit of activity/ml.

Table 4. *Activity of the small TCA-soluble peptides from chymotryptic digests of insulin*

Non-protein nitrogen when sample was taken (%)	Dose (mg. N)	Approximate activity found (units)	Activity of the same amount of insulin N (units)
15·4	0·056	Nil*	9
24·4	0·10	Nil*	16
30·4	0·063	Nil*	10
36·3	0·025	Nil*	4
—	0·123	Nil	20
40·8	0·256	Nil	41
47·5	0·274	Nil	44

* These results showed hyperglycaemic activity, due to adverse reaction to injection. Bulked samples tested later showed no effect at all (see text).

This insulin remaining in solution would be precipitated by TCA. The efficiency of this precipitant was shown by injecting the neutralized filtrate from 71 units of insulin, after precipitation by 0·25 N final concentration of TCA. The maximum blood-sugar fall was 18 % of the starving level, corresponding to 0·25 unit of insulin remaining in solution. If 5·8 mg. of the peptides derived from insulin by chymotryptic digestion were also present, a maximum blood-sugar fall of 10 % occurred. Under the conditions used in this work, one unit of insulin invariably gave a maximum blood-sugar fall of 40–60 % of the starving level.

Thus the core activity is actually due to traces of insulin in it. In an earlier paper (Butler *et al.* 1948*a*), it was shown by quantitative assays, kindly carried out by Boots Pure Drug Co. Ltd., that TCA pre-

cipitates from whole digests (i.e. containing core and undigested insulin), had an activity equivalent to that expected from the amount of insulin still present, as measured by the amount of precipitate obtainable from the digest samples at pH 5·4.

Small peptides. The injection of up to 16 mg. of peptides (those samples which had shown hyperglycaemia in Table 4 being bulked together and concentrated) had no effect at all on the blood sugar. The largest dose given was derived from the degradation of 750 units of insulin.

Intravenous injection of insulin causes a more rapid effect on the blood sugar than subcutaneous injection, and this route thus allows less time for adverse factors such as tissue enzymes or inhibitors to prevent the display of hypoglycaemic activity. However, the intravenous injection of 54–58 mg. of the peptides from a chymotryptic digest of insulin (C 26, which had digested to a value of 63 % N.P.N.) had virtually no hypoglycaemic effect, one case showing a maximum fall of 18 % of the starving blood-sugar level and other tests showing only considerable hyperglycaemia.

In preparing peptides for injection, the excess TCA used for precipitating the core was removed either by repeated extraction with ether or merely by neutralizing the TCA filtrate. Neither procedure had any effect in itself on the blood-sugar estimations, since intermittent shaking of an insulin solution with ether for 2 hr. had no effect on its potency and the injection of 1 ml. of M-sodium trichloroacetate (183 mg.) produced only a 10 % maximum fall of blood-sugar level below the starving level, and moreover did not prevent 1 unit of insulin from exerting its full effect when admixed with such an amount.

Peptic digests

Like the chymotryptic digests, peptic digests yield three fractions: (*a*) The remaining intact insulin and other insulin-like material, all precipitated at pH 5·5. (*b*) A large fragment corresponding to the chymotryptic 'core' is precipitated either by heating the neutralized digest, by freezing and thawing or by the addition of TCA to N final concentration. (*c*) The small peptides soluble in N-TCA. The N.P.N. of peptic digests in 0·25N-TCA rapidly reaches 100 %, but in N-TCA does not exceed 50 % of the total nitrogen during the digestion.

Tables 5–7 give the activities of whole digests and fractions (*a*) and (*c*).

Precipitates obtained at pH 5·5. The isoelectric precipitates had activities lower than insulin, in accordance with the finding (Butler *et al.* 1948*b*) that adjusting an insulin-free peptic digest to a pH between 4 and 7 resulted in the slow precipitation of insoluble material.

Peptic 'core' of insulin. Two 5 mg. samples of the precipitate formed by freezing digest P 6 had no hypoglycaemic activity when injected subcutaneously into two rabbits. This amount was derived from the digestion of 590 units of insulin, and the same weight of insulin would have had 110 units of activity. Similarly, 4·9 mg. of the precipitate formed by heating digest P 9 (N.P.N. = 100 %) had no influence on the blood sugar.

Dr K. Pedersen of the Physico-Chemical Institute, Upsala, kindly examined this P 9 precipitate. The ultracentrifuge showed the presence of polydisperse material and a slow sedimenting component giving $s_{20} = 1{\cdot}1S$ (average). The best of two diffusion measurements gave the constants $D_A = 20{\cdot}5$ and

Table 5. *Activity of whole peptic digests of insulin*

(The digests were prepared in 0·05N-HCl.)

Digest no.	N soluble in 0·25N-TCA (%)	Dose in terms of original insulin units	Approximate activity found (units)	Approximate activity remaining (%)
30/5	7·8	1·3	1	80–100
P 12	16	1·6	1	60
P 13	22	2·0	1	50
P 12	35	3·0	0·5	20
P 13	39	3·0	0·5–1	20–40
30/5	83	1·3	0·25*	20
P 12	100	81·0	Nil	0

* Maximum fall of blood sugar 21 % of original (starving) level.

Table 6. *Activity of* pH 5·5 *precipitates from a peptic digest*

Total N precipitated at pH 5·5 (%)	Dose (mg. N)	Activity of the same amount of insulin N (units)	Approximate activity found (units)	Approximate activity compared with insulin = 100
75	0·0065	1	0·5	50
49	0·0065	1	0·33	33

Table 7. *Activity of the small peptides soluble in* 1N-*TCA from peptic digests of insulin*

Digest no.	N soluble in N-TCA (%)	Dose (mg. N)	Activity of the same amount of insulin N (units)	Approximate activity found (units)
P12 (i)	9	0·026	4·3	Nil
P12 (ii)	16	0·035	5·7	Nil
P12 (i) + (ii)	—	0·113	18	Nil
P14	5	0·054	9	Nil
P14	9	0·103	17	Nil
P14	13·6	0·036	6	Nil*

* Maximum fall of blood sugar was 16% of the starving level.

$D_M = 21·4$. Combining these, Dr Pedersen concluded that the average molecular weight of the low molecular weight material in P 9 was 4000–5000.

Small peptides. Other digests made at pH values from 1·3 to 2·9 also gave peptide fractions which were inactive. Attempts were made to digest insulin at more alkaline pH values with pepsin, but in 0·1M-citrate-0·1N-hydrochloric acid buffer at pH 3·8, the insulin was almost completely insoluble. Solutions of insulin could be prepared in formate-formic acid at pH 4–4·5, but under these conditions the pepsin and insulin co-precipitated immediately when mixed (Phillips, unpublished).

Intravenous injections. The intravenous injection of a sample of the peptides from a peptic digest (0·15 mg. N, equivalent to 24 units if all insulin N) gave a slight erratic hyperglycaemia, but no fall of blood sugar over the course of 1·5 hr., when measured at 20 min. intervals.

Tryptic digests

The activity of whole digests has been discussed in two previous papers (Butler *et al.* 1948*a*, 1949). Insulin (5 mg./ml.) was digested with 5×10^{-4} units of purified crystalline trypsin/ml. of digest at pH 8·5 and 25°. The N.P.N. (as measured by adding 1 ml. of N-TCA to 3 ml. of the digest) was 7·8 and 15·9% of the total nitrogen after 22 and 45 hr. digestion respectively. After removal of the excess TCA with ether the peptides were tested and none depressed the rabbit blood sugar more than 9% of the starving level. Doses up to 3·4 and 7·2 mg. from the 22 and 45 hr. digests respectively were given subcutaneously. The approximate activities of the whole digest after 22 and 45 hr. digestion were 60 and 30% of the original respectively, which contrasts with the value of about 60% found previously (Butler *et al.* 1949) after 48 hr. digestion. In the present case, however, the enzyme concentration was 3·7 times greater.

The effect of other agents on the activity of insulin

The lack of effect of ether on the potency of an insulin solution has already been mentioned, but in the course of the work the effect of other agents was also tried.

Trichloroacetic acid. An insulin solution (5 ml.) containing 2 units/ml. (0·09 mg./ml.) was precipitated by 5 ml. of 0·3N-TCA, centrifuged, and the precipitate redissolved in 5 ml. of water. Injection of 0·5 ml. of this solution gave a maximum fall of 40% in the blood-sugar level compared with a 35% maximum fall for 0·5 ml. of the original solution with the same rabbit. The potency was thus unaffected by the precipitant and the high efficiency of TCA in precipitating such a weak protein solution was demonstrated. This is supported by the finding already reported that the TCA filtrates from 71 units of insulin had no hypoglycaemic activity.

Urea. By the addition of urea, solutions of insulin can be made at pH 4–7, where it is normally insoluble. As a preliminary to experiments on the digestion of insulin by enzymes between these pH values it was essential to know whether urea would inactivate the insulin.

Clear solutions of insulin were made in 5M-urea at various pH values from 3·2 to 10·1 and kept at 25° for 22 hr. No loss of activity was shown by the pH 3·2, 6·4 and 10·1 samples, and about 50% loss at pH 5·0 and 8·2. Further tests showed no loss after 50 hr. at pH 4·5 and 6·4. These variations must be attributed to the use of an approximate method for estimating the potency, but together implied that 5M-urea could be used safely for a sufficiently long time for enzymic hydrolysis to take place. In a trial experiment it was found that chymotrypsin would digest insulin in 5M-urea at pH 8, and that all the activity had been destroyed by the time the non-protein nitrogen had reached 76%.

It was further found that solutions could be achieved at pH 4·5 and 6 in only 2M-urea, but even at this concentration urea interferes slightly with the precipitation of insulin by TCA. Solutions of 39 and 49 units of insulin were precipitated in the presence of 0·94M- and 0·69M-urea and 0·22N- and 0·17N-TCA respectively (all concentrations refer to final concentrations after the addition of TCA). Only 1% of the insulin activity remained in solution. Even this may be due to the insufficient duration (about 0·5 hr.) of standing before centrifuging the suspension. An injection of 4 ml. of 0·5M-urea,

more than was given in the injections above, caused a 9 % rise in the starving blood-sugar level.

Acid hydrolysis of insulin. The activity of acid hydrolysates of insulin has not been reported in detail. Hot 0·1N-hydrochloric acid forms a reversibly inactive precipitate (Jensen & Evans, 1932; Du Vigneaud, Sifferd & Sealock, 1933). The use of concentrated acids at low temperatures has some promise in this work in that Sanger (1948*b*, 1949) has been able to isolate peptides up to pentapeptide in size by such hydrolyses of insulin.

For potency trials a high ratio of insulin to acid is preferable so that the samples tested can be considerably diluted before injection, and will then contain little acid or salt to cause unfavourable reactions in the rabbit. Dilution is also necessary because a high hydrochloric acid concentration competes with the TCA for the insulin and precipitation is incomplete. In 7·7N-hydrochloric acid at 25°, the N.P.N. values were 60, 77 and 100 % after 0·8, 1·8 and 6 days respectively.

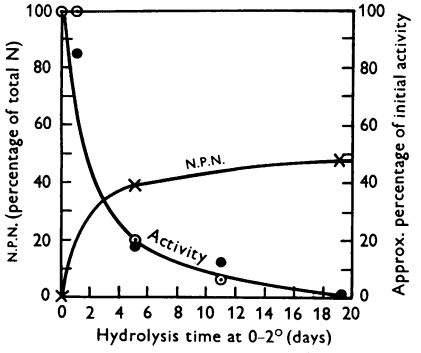

Fig. 2. The formation of N.P.N. (×) (N soluble in 0·25N-trichloroacetic acid) and the activity of insulin digests (●) and of the TCA precipitates obtainable from them (⊙), when insulin is digested in 8N-HCl at 0–2°.

Except for the initial sample none of the whole digests (injecting the equivalent of 8 units of insulin), nor the non-protein fractions (injecting the filtrate from 40 to 50 units of insulin) had any hypoglycaemic activity. The TCA precipitate from 66 units of insulin after 19 hr. hydrolysis, however, had a total activity of about 1 unit, though the subsequent TCA precipitates had none.

On the other hand 0·8N-hydrochloric acid had little effect on insulin, the activity falling to about 50 % only after 6 days at 25°. None of the N.P.N. samples had any definite activity even when derived from 76 units of insulin.

If the hydrochloric acid concentration is much above 0·8N, insulin immediately forms an insoluble precipitate which is not redissolved until the acid concentration is about 6N, though the limit depends upon the concentration of insulin.

Experiments were made with 8N-hydrochloric acid at 0–2°. Fig. 2 shows the formation of N.P.N., and the activity of the whole hydrolysates and the TCA precipitates. The TCA filtrates prepared at various stages of hydrolysis were inactive even when derived from 100 units of insulin. The TCA precipitates, therefore, accounted for all the activity shown by the whole digest samples.

Diisopropyl fluorophosphonate (DFP). DFP has a unique inhibitory effect on chymotrypsin and trypsin (Jansen, Nutting, Jang & Balls, 1949), the former enzyme requiring only 1 mol. DFP/mol. of enzyme of mol.wt. 27,000 for complete inhibition. Such specific inhibition may well lead to the finding of the active grouping or the most important part of these enzymes, and a comparable finding with insulin would thus be valuable.

Insulin was incubated with DFP (4·6–47 mol./mol insulin of weight 12,000) at pH 8 for periods up to 46 hr. at 25° without affecting the potency of the insulin compared with controls. This is illustrated in Table 8. It was confirmed that the DFP retained

Table 8. *Effect of diisopropyl fluorophosphonate (DFP) on insulin at* pH 8 *and* 25°

Treatment of sample	Dose in terms of insulin	Maximum fall of blood sugar level* (%)
18 hr. DFP: insulin ratio = 4·6	1·2	51
As above, then 28 hr. with ratio = 47·0	1·2	50
19 hr. DFP: insulin ratio = 42·0	1·2	50
Control. No DFP	1·2	42
Control, 48 hr. pH 8. No DFP	1·2	55
Control, pH 8·1 buffer with DFP. No insulin	—	14

* As percentage of the starving level = 100 %.

at least 20 % of its initial activity after 24 hr. at pH 8 by testing the extent of inhibition of a chymotrypsin solution (Jansen *et al.* 1949). The pH value 8 was chosen for the tests on insulin as this is near the optimum for the inactivation of trypsin and chymotrypsin by DFP. Furthermore, no evidence of reaction of DFP with any of the individual amino-acids found in insulin could be obtained from paper chromatograms of the acids after incubation with DFP (pH initially 8, but later fell to about 3 owing to the spontaneous hydrolysis of the DFP).

This accords indirectly with the more recent finding of Jansen, Nutting, Jang & Balls (1950), that none of the individual amino-acids of chymotrypsinogen when added to intact chymotrypsin affected the degree of inhibition of that enzyme by DFP.

DISCUSSION

The finding that no product of the digestion of crystalline insulin by crystalline pepsin, chymotrypsin or trypsin has any hypoglycaemic activity

confirms the results on whole digests obtained by earlier workers using impure enzymes.

No fraction or combination of fractions from the digests had any hypoglycaemic activity in large doses, irrespective of the pH of digestion, and all the remaining activity of the digests could be ascribed to the intact insulin still present. This is in spite of the fact that the crystalline proteolytic enzymes give larger protein fragments than the crude enzymes, there being almost no liberation of free amino-acids in the former case (see, for example, Van Slyke, Dillon, MacFadyen & Hamilton, 1941; Winnick, 1943; Butler *et al.* 1948*a*, *b*).

In all degradations of insulin, except the removal of amide groups by boiling 0·1N-hydrochloric acid by Jensen & Evans (1932), the activity is reduced, and in the work of Fisher & Scott (1934) with pepsin, mentioned above, and that of Lens (1949) with carboxypeptidase, the insulin was largely inactivated though very little TCA-soluble nitrogen was formed. The 70% loss of activity during the 45 hr. tryptic digestion reported above, for the formation of only 15·9% N.P.N., also comes into this category.

These inactivations, however, can be interpreted in two ways. Either the whole or only a special part of the molecule is essential for activity. It seems likely (Butler *et al.* 1950) that the core of insulin of mol.wt. 5000, revealed by chymotryptic action, lies at the 'amino' end of the peptide chains of insulin and that the accompanying inactivation is due to the removal of small peptides from the ends of the chains bearing the free α-carboxyl groups. This suggests, like Lens's work, that if only part of the molecule is active, then it lies outside the large core and probably at the carboxyl ends of the peptide chains. One must then assume that all the degradations so far carried out have attacked the essential part.

Even if the whole molecule is required, certain parts will be more intimately involved in the *in vivo* reactions of the hormone. Thus, investigations of insulin derivatives (Sanger, 1948*a*; Fraenkel-Conrat & Fraenkel-Conrat, 1950; Mommaerts & Neurath, 1950) have established the essential nature of the cystine disulphide groups and of some of the tyrosine and carboxyl groups. Nevertheless, although the insulin core formed by the action of chymotrypsin contains all these groupings, no activity was found. Similarly, the tyrosine-cystine dipeptides prepared by Harington & Pitt-Rivers (1944) showed nothing more than a transient slight hypoglycaemic effect of doubtful significance (Prof. F. G. Young & Dr M. P. Whitehouse, private communication). The absence of hypoglycaemic activity in doses of peptides as large as 58 mg., moreover, implies that there are also no fragments present sufficiently like the structure of the hypothetical active group to give even fractional activity. Moreover, the fact that the peptides do not prevent added intact insulin from displaying full activity shows that such 'analogues', if present, are not inhibitory to its action.

In spite of this lack of success with insulin and in degradative studies on the proteolytic enzymes themselves (Northrop, 1932; Northrop & Kunitz, 1932; Northrop, Kunitz & Herriott, 1948), the finding by Li (1948, 1949, 1950) and Cortis-Jones, Crooke, Henly, Morris & Morris (1950) that the activity of certain very highly purified pituitary hormones can be obtained in the form of quite small peptides, though it is uncertain if these were originally attached to the protein by conventional peptide bonds, means that the hypothesis of an active peptide grouping in insulin, attached to a larger protein mass, cannot yet be rejected.

SUMMARY

1. Crystalline chymotrypsin, trypsin and pepsin degrade crystalline insulin to give peptide fractions which show no hypoglycaemic activity.

2. Digestion by purified crystalline trypsin causes considerable loss of activity although little non-protein nitrogen is formed.

3. The remaining activity shown by the whole digests is precipitable at pH 5·5, and is probably due to intact insulin still present.

4. Variation of the pH of digestion affects the rate but fails to give active fractions.

5. Hydrolysis with 8N- and 0·8N-hydrochloric acid at low temperatures similarly destroys the activity, and the non-protein fractions formed during hydrolysis have no activity.

6. Ether, trichloroacetic acid, 5M-urea and di*iso*propyl fluorophosphonate have no appreciable effect on the activity of insulin in periods of contact up to 24 hr.

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Hormonal Factors Influencing Liver Catalase Activity in Mice. Testicular and Adrenal Factors

By D. H. ADAMS
Cancer Research Department, London Hospital Medical College, London, E. 1

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Greenstein and his associates have shown that the presence of a growing tumour in rats and mice is almost invariably associated with a marked depression in liver catalase activity (Greenstein, Jenrette & White, 1941; Greenstein & Andervont, 1942).

As far as the mechanism of the effect is concerned, evidence has recently been obtained suggesting that the tumour exerts its effect through the medium of some toxic material released into the circulation. Nakahara & Fukuoka (1949, 1950) claimed to have produced depressions of liver catalase activity in mice by the injection of ethanol-precipitated fractions obtained from human tumours. Adams (1950*a*, 1951) has shown that mouse tumours in general contain some factor or factors capable of markedly and rapidly influencing liver catalase activity. The way in which such factors could operate is obscure. Greenstein (1943) looked for a direct inhibitor of liver catalase in tumours without success, and finally concluded that in some way tumours must be interfering with the synthesis of catalase in the liver. The purpose of the present work is to examine the mechanisms which control liver catalase activity in the normal animal as a preliminary to further investigation into the effect of tumours on the system.

During the course of the earlier work (Adams, 1950*a*, 1951) a sex difference in normal catalase activity was observed in all three strains of mice used—males having a level some 30% higher than females. It seemed likely, therefore, that there was some hormonal influence on catalase level. A preliminary report (Adams, 1950*b*) has already appeared on the effects of castration, and castration followed by adrenalectomy, in males, and adrenalectomy and ovariectomy in females. Marked falls in liver catalase level were observed within 48 hr. after all these operations, except ovariectomy in females, where there was no significant change.