The Effect of some Thiol Compounds upon Trypsin, Chymotrypsin and Chymotrypsinogen

By R. A. PETERS AND R. W. WAKELIN, *Department of Biochemistry, Oxford*

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WITH AN ADDENDUM ON

Examination of Chymotrypsin in the Ultracentrifuge

By R. CECIL, *Department of Biochemistry, Oxford*

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In the course of a research with another objective during the war in 1943–4, we found that thiol-containing compounds inactivated trypsin and chymotrypsin. This was not reported then. In the interval, Grob (1945–6) has published work upon trypsin done independently in 1943 and with somewhat similar observations. The work which we have now completed was carried out upon crystalline chymotrypsin and trypsin and with a different criterion for digestion; some observations have been added upon the behaviour of chymotrypsinogen. Our results for trypsin are in substantial agreement with those of Grob, though our interpretation of the phenomenon is different.

EXPERIMENTAL

Trypsin and chymotrypsin were prepared from ox pancreas by the methods of Kunitz & Northrop (1935–6) and stored in a desiccator at 2°. The trypsin used was crystalline, but had not been recrystallized; it contained 32.6% enzyme protein, accepting the usual figure for the N content of trypsin as 16%. The chymotrypsin had been once recrystallized and had a protein content of 73.7% (N, 15.5%). Ultracentrifugal examination by Mr R. Cecil (see Addendum) showed that the specimen was homogeneous. The chymotrypsinogen investigated had been reprecipitated (protein content 60.2%). It was part of the specimen used in preparation of chymotrypsin; no attempt was made to separate different forms of chymotrypsin (Kunitz, 1938–9). The activity of both enzymes was estimated by determining the extent of digestion of casein using micro-Kjeldahl estimations of N not precipitable by trichloroacetic acid (N.P.N.).

For preparation of the casein substrate, the method essentially followed that of Northrop & Kunitz (1932–3); Casein (Glaxo A/E, 50 g.) was stirred into 0.1 M-phosphate buffer, pH 7.6 (11.), approx. 5 N-NaOH (3–3.5 ml.) was added and the mixture heated on the water bath for 30 min. with additions of 5 N-NaOH to maintain the pH at 7.6. After cooling and removal of undissolved casein by centrifuging, the solution was kept in the refrigerator, thymol being added. Values for the preformed N.P.N. varied from 1 to 2 mg./6 ml. casein substrate; different batches of casein substrate showed variations in digestibility.

Experiments were in all cases planned to show differences due to the action of added thiol compounds. Each was set up with a control, weighed sample of the same enzyme. As no attempt was made to homogenize the enzyme preparation, this procedure controlled possible variations in the salt content of samples taken. Usually the enzyme (1–2 mg.) was incubated in 0.1 M-phosphate buffer, pH 7.6 (1.0 ml.), for 45 min., after which 6 ml. casein substrate were added. After digestion for 40–50 min. at 38°, 1 ml. 25% (w/v) trichloroacetic acid was added; after standing for 30 min. at room temperature the precipitate was filtered and washed twice with 5 ml. 2.5% (w/v) trichloroacetic acid. After adjusting the volume to 25 ml., 5 ml. were taken for ashing by micro-Kjeldahl. Most incubations were done in narrow test tubes in air; results in N₂ were not appreciably different. Glass-distilled water was used to reduce contamination by metals to a minimum.

In the earlier experiments the thiol compound was added directly to the digestion mixture; all later experiments were done by a previous incubation of thiol with the enzyme before addition to the casein. The results were qualitatively similar, but the latter procedure gave a much more marked effect. Several experiments were made with crude trypsin (liquor pancreaticus, Bengel) which are not included, as they are difficult to interpret (see below).

The results are expressed as the difference in N.P.N. determined by the micro-Kjeldahl method in the digests with untreated enzyme and with enzyme incubated with the substance under test, the usual unit being mg. N.P.N./6 ml. casein substrate. In the experiments of 1943–4, control values after digestion with 1 mg. chymotrypsin were usually 14–15 mg. N.P.N., and with 2 mg. trypsin approx. 8 mg. N.P.N. For trypsin a control was done with the specific substrate N¹-benzoylarginine amide.

Control experiments with suitable samples to which no thiol was added showed that during the periods of incubation less than 10% of trypsin or 5% of chymotrypsin was inactivated. There was no evidence that SH compounds had an appreciable effect on the course of digestion; the thiol concentration during digestion was one seventh of that in the incubation mixture, i.e. incubation with 0.1 M-thiol was followed by digestion at 0.014 M; the rate of inactivation is lower for the enzyme in absence of substrate at this reduced concentration of thiol (see Fig. 1), and with casein present the rate is still lower, and not more than 1–2% extra

inhibition was found in a 30 min. digestion period. If the concentration of thiol was maintained at 0.1M throughout the incubation and digestion periods by addition of extra MTEG with the casein, it was found that extra inhibition occurred, not exceeding 10%, interpreted as due to further inactivation by thiol of the enzyme itself. In one experiment upon trypsin, in which the thiol was removed by oxidation prior to digestion, the results were in agreement with this view. Hence we have no evidence that thiol compounds were inhibiting the course of digestion in these experiments, and it seems logical, therefore, to describe our effects as due to inactivation.

RESULTS

Inactivation of the proteinase activity of trypsin or chymotrypsin, or of both, has been shown by the following SH compounds: monothioethyleneglycol (MTEG), cysteine ester hydrochloride, glutathione,

Fig. 1. Effect of previous exposure to varying concentrations of thiol compounds on the digestion of casein substrate by crystalline chymotrypsin. Addition of 1 mg. enzyme preparation to 6 ml. casein solution, pH 7.6, 38°. Digestion time 45 min. Results expressed as decrease in mg. of N.P.N. formed.

2:3-dimercaptopropanol (BAL), sodium sulphide and thiosalicylic acid; slight inactivation (14%) was produced by sodium cyanide (0.2M) at pH 7.8; no inactivation was produced by oxidized monothioethyleneglycol (0.25M), cystine ester hydrochloride (0.07M), thiosulphate (0.2M on chymotrypsin), sodium diethyldithiocarbamate (0.09M) or pyrophosphate (see below); also, in a different category, no inactivation was produced by lewisite oxide (0.65 mm), allantoin (0.038M), cetyltrimethylammonium bromide (CTAB) (1 mg./ml.) or heparin (1 mg./ml.). MTEG and cysteine also had a special action upon chymotrypsinogen.

Figs. 1 and 2 show in detail the effect of increasing molar concentrations of some thiol compounds acting for 45 min. at 38° upon chymotrypsin and trypsin. At approx. 0.1M under these conditions,

there was an almost maximum inactivation. The degree of inactivation induced was approximately the same for each thiol compound; this suggests that the thiol group is the active agent.

Fig. 2. Effect of previous exposure to thiol compounds on the digestion of casein substrate by crystalline trypsin. Addition of 2 mg. enzyme preparation to 6 ml. casein solution, pH 7.6, 38°. Digestion time 45 min. Results expressed as a decrease in mg. of N.P.N. formed.

Fig. 3. Effect of time upon the inactivation of chymotrypsin activity by 0.127M-monothioethylene glycol. Casein digest, pH 7.6. Results expressed as decrease in mg. of N.P.N. formed.

With sodium sulphide on chymotrypsin the results varied much with the alkalinity; working in tightly stoppered vessels to avoid escape of hydrogen sulphide and at pH 7.6, decreases in N.P.N. with 1 mg. chymotrypsin were observed as follows: for 0.082M-sulphide, 4.05 mg. and for 0.125M, 5.5 mg. N.P.N.

The relation between inactivation of 1 mg. chymotrypsin due to 0.127M-MTEG and the time of action is shown in Fig. 3, and with this concentration the action was rapid.

The effect of varying the pH with chymotrypsin and MTEG is shown in Fig. 4, where it is clear that the action was much increased as the pH rises above 7.0; this would be expected in the case of a thiol effect.

Fig. 4. Effect of varying pH on the inactivation of chymotrypsin activity by 0.127M-monothioethyleneglycol. Digestion time 45 min. for 1 mg. enzyme added to 5 ml. casein solution, pH 7.6. Results expressed as decrease in mg. N.P.N. formed.

The inactivating effect of thiols upon the action of trypsin upon the specific substrate *N*¹-benzoyl-arginine amide (Bergmann, Fruton & Pollok, 1939) was also proved in two experiments, of which one is quoted in Table 1.

Table 1. Inactivating effect of monothioethyleneglycol (MTEG) and 2:3-dimercaptopropanol (BAL) upon the digestion of *N*¹-benzoylarginine amide

(Volumes of 0.01 N-ethanolic HCl required by 0.5 ml. substrate titrated in 90% acetone (Linderström-Lang technique) before and after digestion at 38° for 2 hr. and containing initially 0.051 M-benzoylarginine amide and 0.4 mg. trypsin.)

Addition	Start (ml.)	2 hr. (ml.)	Diff. (ml.)
None	+0.08	+1.27	+1.19
MTEG (0.128M)	+0.06	+0.05	-0.01
BAL (0.031M)	+0.43	+0.51	+0.08

Table 3. Attempted reactivation of chymotrypsin (1 mg.) and trypsin (2 mg.) with cystine ester or oxidized glutathione, respectively, after inactivation by sodium sulphide or monothioethyleneglycol (MTEG)

	Inactivating compound	Attempted reactivation by	Decrease in N.P.N. found (mg.)
(a) Chymotrypsin	0.082M-Na ₂ S	—	4.05
	0.125M-Na ₂ S	—	5.5
	0.082M-Na ₂ S	0.29M-cystine ester*	5.1
	0.125M-Na ₂ S	0.29M-cystine ester*	6.4
(b) Trypsin	0.64M-MTEG	—	3.25
	0.64M-MTEG	0.13M-oxidized glutathione	3.25

* Reckoned on S content.

These phenomena were so much like those already well known for insulin (cf. Abel & Geiling, 1925; du Vigneaud, 1927), where reduction of a S-S group leads to inactivation, that they led to attempts to get evidence (i) that S-S compounds had no effect on these enzymes; (ii) that there was no reactivation upon reoxidation with S-S compounds; and (iii) that a SH group was present after treatment with the thiol. The results were as follows:

(i) Neither cystine ester hydrochloride (30 mg./ml.) nor oxidized MTEG (20 mg./ml.) produced any effect (Table 2).

Table 2. Effect of oxidized MTEG, and of cystine ester HCl upon the activity of chymotrypsin

(All results expressed for 1 mg. enzyme in 1 ml. added to 6 ml. casein solution. Inhibitors added at start of digestion; 38° for 45 min.)

Addition	N.P.N. formed (mg.)	Decrease in N.P.N. formed (mg.)
None	14.0	—
0.256M-MTEG	—	4.5
0.256M-MTEG (oxidized)	—	0.15
0.127M-Cystine HCl	—	2.8*
0.162M-Cystine ester HCl	—	0.4*

* In arriving at these figures, a blank has been subtracted for the N present in the cystine or cysteine.

(ii) An attempt to reactivate chymotrypsin and trypsin, after exposure to sodium sulphide and MTEG and subsequent removal of unchanged thiol, is shown in Table 3 (a) and (b); relevant details of the experiments are given below. No reactivation was found either with cystine (as ester) or with oxidized glutathione.

The treatment of chymotrypsin in (a) was as follows: After exposure for 45 min. to the sulphide at pH 7.6 in a stoppered tube to prevent escape of H₂S the unchanged H₂S was removed *in vacuo* at pH 6.0 (HCl) until none was detectable by smell. Cystine ester (0.29M, reckoned on S content) was added, the pH readjusted, and the whole incubated for a further 45 min. Casein substrate (6 ml.) was added and digestion carried on for 45 min. Trichloroacetic acid (25% w/v; 1 ml.) was then added, and the whole left to stand for 30 min. The precipitates were washed three times

with 2.5% trichloroacetic acid (5 ml.) and the filtrates made up to 25 ml., 5 ml. being taken for analysis. The precipitates were left standing with 5% trichloroacetic acid (5 ml.) for 18 hr., after which the supernatant fluid gave no nitroprusside test for cystine, indicating complete extraction. The control enzyme was incubated for 1.5 hr., NaCl (12 mg./ml.) being added equivalent to that formed from Na_2S in the experimental tube.

The treatment of trypsin in (b) was as follows: After exposure to MTEG for 2 hr. and cooling, the solution of inactivated enzyme was saturated with MgSO_4 and allowed to stand in the cold. The precipitate was removed by a small filter, washed with saturated MgSO_4 until the washings were free from SH, and then taken up in phosphate buffer, pH 7.6. A sample was removed for estimation of activity, and the remainder treated with oxidized glutathione and incubated for 2 hr. at 38°. Further samples were then taken to estimate the activity.

(iii) Evidence of the appearance of SH groups was sought by titrating the enzymes with porphyrindin solution (Kuhn & Desnuelle, 1938) before and after treatment with monothioethyleneglycol. Mirsky & Anson (1934-5) used thioglycolic acid, but MTEG has the advantage that no COOH group is present to form possible salts. Some difficulty was experienced in making certain that any effect observed was not due to traces of MTEG absorbed on the precipitates or filter papers. After treatment with the thiol, trypsin was clearly much altered; it was no longer precipitable by 5% trichloroacetic acid, which prevented use of this reagent in washing. Two procedures were finally adopted, viz. precipitation by ammonium sulphate, and treatment with 90% acetone (Stern & White, 1937), both at acid reaction, to minimize oxidation of any thiol group. Examples of these are given below, Exp. 1 for chymotrypsin, Exp. 2 for trypsin and Exp. 3 for the parent protein chymotrypsinogen.

In these experiments, the ratio of thiol to enzyme was reduced for experimental convenience. The relation of thiol concentration to rate of inactivation has not been systematically investigated, but control experiments showed that the concentrations used in the experiments below were adequate to inactivate substantially within 30 min.

Exp. 1 (7. viii. 47). Chymotrypsin (50 mg. containing 36.8 mg. ash-free enzyme) was dissolved in 2 ml. 0.05M-phosphate buffer, pH 7.6; 100 mg. MTEG were added and the pH again adjusted. After incubation at 38° for 120 min., the mixture was cooled, N-HCl was added to bring the pH to 3.0, and saturated $(\text{NH}_4)_2\text{SO}_4$ added to 60% of saturation. The precipitate was removed by centrifuging (2500 r.p.m.) and then transferred to a very small pressure filter (filter paper 8 mm. diam.); the precipitate was washed on the filter with saturated $(\text{NH}_4)_2\text{SO}_4$ solution until the filtrate gave no nitroprusside reaction for thiol; it was then suspended, with the paper, in 0.001N-HCl, reprecipitated with $(\text{NH}_4)_2\text{SO}_4$ and again washed on a fresh filter paper. This operation was repeated once again. The washed precipitate of 'thiol-treated' enzyme was then suspended in phosphate buffer,

pH 7.3, and immediately titrated with porphyrindin (0.5 mg./ml. standardized against cysteine ester hydrochloride) until fresh additions of 0.025 ml. did not decolorize in 30 sec. The total amount used was 1.05 ml. porphyrindin, equivalent to 0.69 mg. cysteine ester HCl. One earlier experiment gave 87% of this value.

Reckoning the S content of chymotrypsin as 1.85% (Kunitz & Northrop, 1934-5), this means that approximately one fifth (17.4%) of S had appeared as SH groups. In a control experiment run at the same time, a solution of 25 mg. of chymotrypsin, not treated with thiol during the incubation, was acidified; MTEG was then added, and the protein immediately precipitated with $(\text{NH}_4)_2\text{SO}_4$ and the washing procedure carried out as above. When dissolved in phosphate buffer this control chymotrypsin reduced less than 0.025 ml. porphyrindin solution.

In another experiment with chymotrypsin and MTEG done by the method described under Exp. 3, the chymotrypsin washed free from MTEG gave a marked nitroprusside reaction for SH upon addition of nitroprusside and ammonia.

Exp. 2 (9. viii. 47). Trypsin (50 mg.) was treated with 2.0 ml. phosphate buffer, pH 7.8; the precipitated magnesium phosphate was removed by centrifuge, and the resulting solution placed in a closed vessel (Stern & White, 1937); N_2 was passed at 38°. After 5 min. MTEG (100 mg.) was quickly added; the resultant pH was 7.6. After action for 60 min. in the N_2 atmosphere, 0.3 ml. N-HCl was added, the mixture cooled and 9 vol. of acidified acetone (0.55 ml. N-HCl to 250 ml.) added. After centrifuging, the precipitated protein was washed 6 times with the acidified acetone and dried in the centrifuge tube *in vacuo*. There was no smell of thiol. The protein was dissolved in 1 ml. phosphate buffer, pH 7.3, and immediately titrated with porphyrindin, using an amount of dye equivalent to 0.106 mg. cysteine ester hydrochloride. Reckoning 1.1% as the S content of trypsin (Kunitz & Northrop, 1934-5) this gives 10.2% of S reduced, a value which should be increased by at least one tenth to allow for losses during the initial washing.

A previous experiment with trypsin, carried out on the lines of Exp. 1 with $(\text{NH}_4)_2\text{SO}_4$ fractionation, gave 8.2% of S reduced, which is of the same order as that in Exp. 2. Trypsin (50 mg.), not treated with thiol until the washing stage, reduced less than 0.02 ml. dye.

Exp. 3 (20. viii. 47). Chymotrypsinogen (50 mg.) was treated with 2.0 ml. phosphate buffer, pH 7.8, and MTEG (100 mg.). Rapid precipitation took place, most of the protein coming out of solution in 10 min. After 30 min. at 38°, the solution was cooled on ice, the precipitated protein was removed by centrifuging, and suspended in 0.02N-HCl (2.0 ml.); 0.2 ml. N-HCl was added and 9 vol. of cold acidified acetone. After removing the precipitate by centrifuge, it was resuspended in cold 0.02N-HCl, 9 vol. of acid acetone added, and the precipitate washed with acetone; the resuspension and washing were repeated, and the precipitate was finally dried *in vacuo*. There was no smell of thiol. The dried product was suspended in phosphate buffer, pH 7.3 (1.0 ml.), transferred to a small test tube and titrated with porphyrindin until small additions were not decolorized in 30 sec.; the porphyrindin used was equivalent to 0.397 mg. cysteine ester HCl. Reckoning chymotrypsinogen S as 1.48% (Brand & Kassell, 1941-2), this corresponds to a reduction of 15.4% of the S present. Before thiol treatment, chymotrypsinogen showed no SH content by porphyrindin titration.

These experiments have convinced us that reducing thiol groups are formed in these proteins by the action of MTEG; they accounted for one tenth to one fifth of the total S stated to be present; but the exact amounts of thiol which can be formed must be decided by future, more accurate experiments. In this connexion Brand & Kassell (1941-2), in a very pure specimen of chymotrypsinogen, have found the content of methionine S to be 0.262 % and of cystine + cysteine S, 1.225 %; it is not known how the S content changes upon conversion to chymotrypsin, but they record the odd point that hydrolysis with hydrochloric acid yields 4 cysteine groups/molecule; they state that no reactive SH was present in chymotrypsinogen as determined by nitroprusside and ammonia, with which our titration values agree.

Relation to denaturation. The appearance of thiol groups in these experiments is not similar to the well-known unmasking of thiol groups in the denaturation of egg albumin; in the controls mentioned, which had been treated with acid acetone, the chymotrypsin and chymotrypsinogen were denatured in the sense that the protein became insoluble, without liberation of thiol. Similarly, neither boiling the three proteins in water to which a trace of acetic acid had been added to slow down oxidation of any possibly liberated thiol, nor treatment with purified guanidine hydrochloride (6M) (Greenstein, 1938) led to a liberation of thiol; subsequent addition of sodium nitroprusside and ammonia gave no thiol reaction, which readily appeared upon addition of minimal amounts of cyanide. The denatured proteins had a disulphide grouping and behaved exactly as denatured serum albumin (Walker, 1925).

All the evidence so far available suggests that the behaviour of trypsin and of chymotrypsin, as well as of chymotrypsinogen, to treatment with thiols is similar to that of insulin.

Attempted inactivation by pyrophosphate. In view of the statement by Grassmann, Dyckerhoff & Schoenebeck (1930) that trypsin kinase was inactivated by pyrophosphate as well as by cysteine, this

has been investigated. No effect was found in two experiments; details of one are given in Table 4.

Table 4. *Effect of pyrophosphate (pH 7.6) upon activity of crystalline trypsin (2 mg.)*

(Incubated for 3 hr. with and without sodium pyrophosphate (0.224M); casein substrate then added and digestion carried out for 40 min.)

	N.P.N. formed (mg.)
Control	3.80
Pyrophosphate	4.00

Relation to metals. As might be expected, there was no change in the degree of inactivation due to thiol produced in our experiments by addition of manganese or zinc (1 mg.) together with the thiol. The action of thiols was not an effect on metals. The activation of papain by cyanide and thiol compounds is well known, and also the pioneer experiments of Hopkins & Morgan (1938) upon the reversible inactivation of succinodehydrogenase by S-S compounds. As noted by Grob (1945-6), the only earlier reference to an inactivating action of thiol compounds upon trypsin, by Grassmann *et al.* (1930), referred to inactivation of trypsin kinase at pH 8.9 in 0.2M-ammonia-ammonium chloride buffer solution. Using amounts of cysteine up to 0.02M, it was found that trypsin alone was substantially inactivated; after adding intermediate amounts of kinase, the extra digestion due to kinase was inhibited, even by 0.001M-cysteine; addition of extra kinase practically abolished the inhibition. Pyrophosphate behaved in a similar way. It is to be noted that they were using an ethanolic titration as a method of estimation. Since we found no effect of pyrophosphate, in our opinion their results would be better interpreted as being due to the action of a peptidase, present in the trypsin preparation, which was activated by some divalent metal added with the kinase. It might be expected that the effect of the metal would be removed by combination with a thiol or with pyrophosphate.

We have tried the effect of adding thiol upon the manganese activation of the peptidase which splits

Table 5. *Increasing effect of 0.288M-MTEG upon the activation by manganese of aminopeptidase in a Fruton-type extract of rat skin as the manganese concentration is reduced. Substrate, L-leucylglycine*

(Samples of 0.5 ml., containing 4.67 mg. L-leucylglycine and 0.2 ml. skin extract, taken after digestion for titration in 90% acetone by the Linderström-Lang technique. Results given are increases in 0.01N-ethanolic HCl titrated as a result of the digestion.)

Exp.	Digestion time (38°) (hr.)	Addendum	Manganese addition		
			Nil (ml.)	0.0037M (ml.)	0.00037M (ml.)
4	3.5	Nil	0.33	2.16	—
		MTEG	0.23	1.63	—
5	2	Nil	0.38	—	1.85
		MTEG	0.01	—	1.00

L-leucylglycine in skin (Fruton, 1946; Neville-Jones & Peters, 1948), and have found a decreasing effect with the addition of larger amounts of manganese, a result much in keeping with the results of Grassmann *et al.* (Table 5).

DISCUSSION

Using smaller amounts of crystalline trypsin than in these experiments Grob found with approx. 0.01M-cysteine, 59% inhibition; with 0.04M-sodium thioglycollate, 26%; with 0.003M-glutathione, 17%; and with one fifth saturated H_2S solution, 7%. These inhibitions are less complete than those noted by us. He also found a 50% inactivation with 0.05M-cyanide. Working at pH 7.6–7.8 we have only found slight inhibition with 0.2M-cyanide. It is to be noted that Blatherwick, Bischoff, Maxwell, Berger & Sahyun (1927) found that, at pH 7.0, 0.4M-cyanide took 18 hr. to inactivate insulin completely. Grob considered that his results showed a sensitivity to oxidation-reduction in the protease molecule; since we have found that there is no reactivation by exposure to S-S compounds and that the molecule seems definitely to come to pieces, we think the phenomenon is irreversible. The precipitation of chymotrypsinogen by SH action is the first case known to us in which such a change of state can be produced by reduction alone; in this it seems uniquely interesting.

It is interesting also that at least three active proteins from the pancreas are inactivated by reduction, in addition to the similar action on chymotrypsinogen. It is natural to think that inactivation by reduction may have biological importance, but

for the present we have no evidence of this and must point out that the concentrations of thiol agents used here were relatively very high.

Note added 21 October 1947. Since the completion of this manuscript our attention has been drawn to the statement by E. C. Webb & R. van Heyningen that 0.0042M-BAL had no effect upon a commercial specimen of trypsin (Webb & van Heyningen, 1947). The result does not conflict with our statements, as the concentration used was relatively small and the time of exposure to the enzyme alone not more than 15 min.

SUMMARY

1. Several thiol compounds, monothioethylene-glycol, cysteine, glutathione, 2:3-dimercaptopropanol, thiosalicylic acid and sodium sulphide irreversibly inactivate crystalline trypsin and chymotrypsin.

2. This action, like the well-known effect on insulin, increases on the alkaline side of the neutral point.

3. The inactivating effect is not reversible by exposure to S-S compounds, and is accompanied by the appearance of SH groups. This is not an unmasking of thiol groups by ordinary denaturation.

4. SH groups also appear in chymotrypsinogen upon treatment with some thiol compounds, and the protein is precipitated.

Our thanks are due to Mr A. Harrison for help with the preparation of the crystalline enzymes; to Dr L. A. Stocken for cystine and cysteine ester hydrochloride and for porphyridin, and to Dr Whittaker for monothioethyleneglycol.

ADDENDUM

Examination of Chymotrypsin in the Ultracentrifuge

By R. CECIL, *Department of Biochemistry, Oxford*

A sample of chymotrypsin was examined in the Svedberg oil turbine ultracentrifuge by the method of Philpot (1938). The concentration of protein was 0.74% (refractive increment at $546\text{ m}\mu = 0.00134$) in buffer containing 0.2M-NaCl, 0.058M- KH_2PO_4 , and 0.008M- Na_2HPO_4 . The speed was 1020 rev./sec.

The sedimentative diagram (Fig. 5) showed a single homogeneous component of $S_{20}(\text{corr.}) = 3.09 \times 10^{-13}$. Integration of the area of the boundary accounted for 75% of the total refracting material.

Fig. 5. Sedimentative diagram of chymotrypsin, showing the boundary 70 min. after reaching full speed.

REFERENCES

- Abel, J. J. & Geiling, E. M. K. (1925). *J. Pharmacol.* **25**, 423.
 Bergmann, M., Fruton, J. S. & Pollok, H. (1939). *J. biol. Chem.* **127**, 643.
 Blatherwick, N. R., Bischoff, F., Maxwell, L. C., Berger, J. & Sahyun, M. (1927). *J. biol. Chem.* **72**, 57.
 Brand, E. & Kassell, B. (1941-2). *J. gen. Physiol.* **25**, 167.
 du Vigneaud, V. (1927). *J. biol. Chem.* **75**, 393.
 Fruton, J. S. (1946). *J. biol. Chem.* **166**, 721.
 Grassmann, W., Dyckerhoff, H. & Schoenebeck, O. V. (1930). *Hoppe-Seyl. Z.* **186**, 183.
 Greenstein, J. P. (1938). *J. biol. Chem.* **125**, 501.
 Grob, D. (1945-6). *J. gen. Physiol.* **29**, 219.
 Hopkins, F. G. & Morgan, E. J. (1938). *Biochem. J.* **32**, 611.
 Kuhn, R. & Desnuelle, P. (1938). *Hoppe-Seyl. Z.* **251**, 14.
 Kunitz, M. (1938-9). *J. gen. Physiol.* **22**, 207.
 Kunitz, M. & Northrop, J. H. (1934-5). *J. gen. Physiol.* **18**, 433.
 Kunitz, M. & Northrop, J. H. (1935-6). *J. gen. Physiol.* **19**, 991.
 Mirsky, A. E. & Anson, M. L. (1934-5). *J. gen. Physiol.* **18**, 307.
 Neville-Jones, D. & Peters, R. A. (1948). *Biochem. J.* (in the Press).
 Northrop, J. H. & Kunitz, M. (1932-3). *J. gen. Physiol.* **16**, 313.
 Philpot, J. St L. (1938). *Nature, Lond.*, **141**, 283.
 Stern, K. G. & White, A. (1937). *J. biol. Chem.* **117**, 95.
 Walker, E. (1925). *Biochem. J.* **19**, 1082.
 Webb, E. C. & van Heyningen, R. (1947). *Biochem. J.* **41**, 74.

Quantitative Determination of Glutamine and Glutamic Acid

By H. A. KREBS, *Unit for Research in Cell Metabolism (Medical Research Council),
 Department of Biochemistry, The University, Sheffield*

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Washed suspensions of *Clostridium welchii*, strain S.R. 12, as shown by Gale (1945, 1947), specifically decarboxylate L-glutamic acid and L-glutamine and are thus a specific reagent for the determination of these two substances. It is shown in this paper that the decarboxylation of glutamine is accompanied by the formation of an equivalent amount of ammonia, and that the glutaminase responsible for this reaction is highly specific. No other compound has so far been found to yield ammonia when incubated with washed suspensions of the organism at pH 4.9. This glutaminase preparation is more convenient to make than Archibald's (1944) glutaminase preparation from kidney and is more specific.

The following method for the quantitative determination of glutamic acid and glutamine rests on these facts, and combines the principles used by Gale (1945) and by Archibald (1944). The sum of the substances is determined according to Gale's procedure, slightly modified, by manometric determination of the carbon dioxide evolved on decarboxylation. The ammonia is then estimated in the solution treated with *Cl. welchii*, and in a sample not so treated. The difference represents the glutamine.

Preliminary experiments indicated that the rate of CO₂ evolution from glutamine is under some conditions considerably slower than the CO₂ evolution from glutamic acid, and a detailed study of the conditions favourable for the quantitative decarboxylation of glutamine was therefore required.

EXPERIMENTAL

Bacteria. The strain S.R. 12 of *Cl. welchii* as recommended by Gale (1945) was used for most experiments. In a few cases, a strain of the same species supplied by the Department of Bacteriology of this University and containing a histidine- and a glutamic-decarboxylase was used. The organisms were grown at 38° in the medium suggested by Gale except that 50 ml. of yeast extract were added to 1 l. medium (prepared by autoclaving baker's yeast with an equal volume of water and filtering). The washed cells from about 1 l. medium were suspended in about 15 ml. of 0.45% (w/v) NaCl. The dry weight of the bacteria in the suspension was determined in a 0.2 ml. sample. The yield from 1 l. of medium was 1.1-1.5 g. of dry bacteria. All data on bacterial weights in this paper refer to dry weight. The suspension was kept in a refrigerator where its activity did not appreciably deteriorate within a month.

Chemical determinations. CO₂ was measured manometrically with air in the gas space of the vessels, NH₃ according to the method of Parnas & Heller (see Parnas, 1934).

Chemicals. The L-glutamine used was prepared from mangel-wurzels. In spite of frequent recrystallizations from ethanol-water and water, its purity was only 93% as judged by amide-N content and CO₂ evolution. Among the impurities were about 1.3% arginine (see Archibald, 1945). The L-glutamic acid preparation was 96% pure. The impurity was probably its optical enantiomorph, as the amino-N content agreed with the theoretical value whilst Gale's method yielded 96%.