and it is likely that the agent responsible for producing it is the C_2H_4OH radical. There is insufficient evidence available to be able to write equations explaining the production of DPNH by this radical, but in any case the alcohol dehydrogenases cannot work by this particular reaction because they are not inhibited by dissolved oxygen.

Theorell & Bonnichsen (1951) have shown that liver alcohol dehydrogenase (ADH) forms a complex with DPN, both in the oxidized and in the reduced form. This complex reacts with ethanol as follows (Theorell & Chance, 1951):

There is much recent evidence that enzymic oxidations and reductions are free-radical reactions (George, 1952; Parravano, 1951), and as there are many points of resemblance between the enzymic and the γ -ray reductions of DPN, it seems likely that reaction (5) might be found to take place by a free-radical mechanism. Work is in progress to obtain further information on the radiation chemistry of this system.

SUMMARY

1. Gamma radiation acts on aqueous ethanol to produce acetaldehyde. The effect of dose and ethanol concentration on the yield is described for air-saturated solutions.

2. The yield of acetaldehyde in oxygen-free solutions is smaller than in the presence of air, and when DPN^+ is added the yield increases and DPNH appears. DPN^+ solutions do not produce DPNH when irradiated alone in air, or in the absence of oxygen, or in the presence of hydrogen.

3. When DPN^+ is added to air-saturated ethanol solutions the acetaldehyde yield is almost the same as in the absence of DPN^+ and DPNH does not appear.

4. It is considered that the free C_2H_4OH radical is responsible for the production of DPNH. This reaction is inhibited by dissolved oxygen and does not occur as a step in the action of the alcohol dehydrogenases.

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Pancreatic Trypsin Inhibitor

1. PREPARATION AND PROPERTIES

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Two specific trypsin inhibitors are known to be present in pancreas; one, soluble in 2.5% trichloroacetic acid, is a dialysable polypeptide which was crystallized by Kunitz & Northrop (1936). The other inhibitor is non-dialysable, and precipitated by trichloroacetic acid; it was obtained as a hetero-

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geneous product in only small amounts (Kazal, Spicer & Brahinsky, 1948). The polypeptide inhibitor of Kunitz & Northrop formed a crystalline compound with trypsin, devoid of either inhibitor or trypsin activity. Its molecular weight was reported to be about 6000, but no evidence for purity of the final product was presented, and the analytical figures were admitted to be of uncertain significance.

The object of the present investigation was to prepare this polypeptide inhibitor in amounts sufficient to establish its homogeneity, and then to obtain data on its amino-acid composition and mode of combination with trypsin.

The low inhibitor content of pancreas (yielding only 1 mg./kg. of fresh pancreas) rendered impractical the preparation of the desired amounts of inhibitor by the method of Kunitz & Northrop, which starts with the residues of trypsin preparation from fresh pancreas. Industrial pancreas residues from which insulin had been extracted (kindly made available by British Drug Houses Ltd.), proved to be a suitable source of inhibitor. Deproteinized extracts of these residues were about one-fifth as active as the starting material used by Kunitz & Northrop, so considerable preliminary purification had to be effected. The steps used in this preliminary purification are described in the present paper, together with tests for homogeneity and analytical data on the inhibitor. The following paper (Green & Work, 1953) describes kinetic data on its combination with trypsin, and a further paper describes its ultraviolet-absorption spectrum (Beaven & Green, 1953). A preliminary report of the work has been already made (Green & Work, 1951).

METHODS

Estimation of protein concentration. Protein concentration was estimated by measuring, with a Beckman photoelectric spectrophotometer, the optical density, E , at 280 m μ ., using a 1 cm. cell. Trypsin was determined in 0.0025 N-HCl (pH 3), using the factor 0.585 to convert E to mg. trypsin/ml. (Kunitz, 1947). Inhibitor was measured in 0.01 M-phosphate buffer at pH 7, the factor 0.83 being used to convert E to mg. inhibitor/ml.; this factor was determined on three salt-free lyophilized inhibitor preparations dried at 80° *in vacuo*. During purification of inhibitor, E_{280} was checked at intervals against N content.

Estimation of trypsin activity. This was done by a modified version of the haemoglobin digestion method of Anson (1938), the amounts of material, time and temperature of reaction being reduced to conserve inhibitor and to avoid non-specific inhibition by split products.

Trypsin solution. Trypsin was prepared by the method of McDonald & Kunitz (1946), recrystallized twice and dried at -10°. The stock trypsin solution contained about 1 mg. crystalline trypsin/ml. of 0.0025 N-HCl and was stable for about 1 week at 3°. The exact concentration of trypsin was calculated from the value of E_{280} , and the solution was diluted approx. 1 in 10 with water immediately before use to give a solution containing about 4 μ g. protein/ml. (E_{280} = 0.075); the dilute solution lost 2-4% of its activity in 30 min. at 25°.

Substrate solution. Dried crystalline carboxyhaemoglobin (3.6 g.), prepared by the method of Jope (1948), was dissolved in 116 ml. of a 33% (w/v) aqueous solution of urea (recrystallized from 95% ethanol). N-NaOH (12.0 ml.) was added with stirring and the solution was left at 25° for 30-60 min. The pH was adjusted to 7.5 with 18.5 ml. of a solution made from urea (40 g.) and α -KH₂PO₄ (100 ml.).

This solution, containing 3.2 mg. protein N/ml., was stable for several weeks at 3°.

Digestion. This was carried out at 25° for 5 min. in 10 ml. conical flasks (up to 20 in number); volumes were measured with semi-automatic pipettes which worked on the principle of the micropipettes of Linderström-Lang & Holter (1931). Trypsin solution (44 μ g./ml.) was measured into the flasks and the volume made up to 1 ml. with water; after a few minutes at 25°, substrate solution (1 ml.), also at 25°, was added to successive flasks at regular time intervals (15 sec. or more), and after exactly 5 min., each digestion was stopped by 5% (w/v) trichloroacetic acid (2 ml.). The contents of each flask were centrifuged after 15 min. and the E_{280} of the supernatant liquid was measured, using as a blank the supernatant fluid from one digestion tube which was prepared by mixing trichloroacetic acid with trypsin before the substrate was added (Kunitz, 1947). E for the blank was checked against water and was usually between 0.16 and 0.21; if it was higher, fresh substrate solution was made.

Fig. 1. Calibration curve for determination of trypsin units (TU) from E_{280} of digest; the two quantities are identical below E_{280} = 0.78. Concentration of trypsin solution 44 μ g./ml.

Trypsin unit. One trypsin unit (TU) was defined as the amount of trypsin giving digestion products with E_{280} = 1.0 under the conditions of estimation. A standard trypsin calibration curve (Fig. 1), plotting E against trypsin added, was linear (i.e. TU = E) up to about E = 0.8. Above this value, a correction for non-linearity was made from the curve, which was constructed afresh for each new specimen of trypsin used. From the curve, 1 TU was contained in 0.43 ml. of trypsin solution, equivalent to 19 μ g. of trypsin protein, i.e. 1 TU/ml. = 19 μ g./ml.

Estimation of inhibitor activity. The unit of inhibitor activity (IU) was defined as the amount of inhibitor which inactivated 1 unit of trypsin at pH 7. Specific activity, used to express inhibitor strengths, was defined as inhibitor units/ml. of solution of unit optical density at 280 m μ . and pH 7 (IU/ml., E = 1).

For estimation of activity, the inhibitor, dissolved in 0.01M-phosphate buffer pH 7, was measured into the digestion flasks and the volume was made up to 0.5 ml. with buffer. Trypsin (0.5 ml. to give final trypsin concentration 1.0 TU/ml.) was added and, after 5 min. standing at 25° to allow combination with inhibitor to take place, the subsequent procedure followed that used for trypsin estimation, duplicate inhibitor-free controls being run in every case. The blank was not affected by the inhibitor unless very impure samples were tested; in such cases a correction was applied afterwards, calculated from the E_{280} of the original inhibitor solution and its final dilution in the supernatant digest. At least three different amounts of inhibitor solution were added to a constant amount of trypsin. The slope of the straight line resulting from plotting volume of inhibitor added against trypsin activity remaining (from trypsin standard curve if necessary) represented the value IU/ml. (Fig. 3, —).

Electrodialysis. In the preliminary experiments a four-compartment cell (Work, 1951) with cellophan (no. 400) membranes was used; subsequently, the apparatus was changed to that depicted in Fig. 2. The solution to be dialysed was placed in a 2 l. jar containing the electrodes; the cathode (2) was a silver tube, through which cooling water was passed, and the anodes (3) were two platinum spirals wound on glass tubes and connected through Hg contacts at the bottom of each tube. The electrodes were each enclosed in a cellophan sac (5). Permeability to inhibitor of different samples of cellophan tubing varied considerably and a suitable batch was found only by trial and error. Outside the cathode sac was an annular glass ring (4) which could be moved by hand up and down the cellophan surface to remove any precipitated material; outside this (not shown) was a glass cooling coil. The main compartment was stirred mechanically. Mains voltage (220 V. d.c.) was applied and the current kept below 1.6 amp. by means of a variable resistance, in order to maintain the temperature of the cathode compartment below 30°. The entire contents of anode and cathode compartments were changed whenever the pH of the catholyte rose above 11.

Precipitation curves. These were constructed by varying either the amount or the pH of the precipitant added to constant amounts of inhibitor; the filtrates (suitably diluted) were examined for both protein content (E_{280}) and inhibitor activity, corrections being made for the effects of the precipitants. The curves were analysed by the specific property solubility test of Falconer & Taylor (1946, 1947). Precipitants were added to inhibitor solutions contained in Emich microfiltration vessels (porosity 3) which were then shaken gently for 14 hr. at 37°; subsequent filtration through the sintered-glass disk was carried out under a positive pressure of N_2 .

Precipitants. (a) Buffered NaCl. Weighed amounts of NaCl (A.R.) were added to inhibitor dissolved in 5 ml. of McIlvains citrate-phosphate buffer (0.067M). Since addition of NaCl lowered the pH of the buffer, the requisite pH for each NaCl concentration had to be predetermined. (b) 3M-Phosphate buffer, pH 6.5. Different amounts of stock buffer (prepared by half neutralization of KH_2PO_4 (A.R.) with KOH (A.R.), Butler & Montgomery, 1933) were added to aqueous inhibitor solutions.

Analytical methods. Salt-free inhibitor was obtained by dialysis at 2° against distilled water, using collodion membranes, made by Dr H. Gutfreund, which were im-

permeable to inhibitor. The salt-free material was freeze-dried and finally dried at 80° *in vacuo* before determining E_{280} /mg./ml. Amide N was estimated after 2 hr. hydrolysis at 100° in 6N-HCl, the NH_3 produced after alkalization being distilled in a Conway cell into 0.001N-HCl. Total N was estimated by Dumas or by Conway distillation.

Amino-acid analyses. These were mostly carried out on hydrolysates obtained by 24 hr. boiling with 6N-HCl, using two-dimensional paper chromatography, first solvent phenol/water (NH_3 atmosphere), second solvent collidine/lutidine/water (Dent, 1948). Size and strength of each ninhydrin spot at several intensities near the minimum

Fig. 2. Electrodialysis apparatus. 1, hard-board support; 2, cathode; 3, anodes; 4, annular stirrer; 5, cellophan sacs.

visible level were compared by eye with spots of similar intensities produced with measured amounts of each amino-acid (Lindan & Work, 1951), 50–500 μ g. of inhibitor being used. Methionine was estimated as methionine sulphone after molybdate-catalysed peroxide oxidation (Dent, 1948). Cystine was examined as cysteic acid on one-dimensional phenol chromatograms run on oxidized hydrolysate, but estimation proved difficult owing to variable destruction by H_2O_2 ; cystine was therefore estimated by electrometric titration of unhydrolysed inhibitor as described later. Leucine and isoleucine were separated by *tert.*-amyl alcohol/water with diethylamine atmosphere (Work, 1949); tyrosine and histidine, separated by the butanol/acetic acid mixture described by Campbell, Work & Mellanby (1951), were developed with Pauly reagent (Dent, 1948), 750 μ g. of hydrolysed inhibitor being used to show the absence of histidine. Tryptophan and tyrosine were estimated spectro-

photometrically (Goodwin & Morton, 1946) on unhydrolysed inhibitor (see Beaven & Green, 1953).

Cystine estimation. Unhydrolysed inhibitor was dissolved (0.5 mg./ml.) in 0.3N- H_2SO_4 containing 8M-urea. This solution was reduced at a stirred Hg cathode (described by Dohan & Woodward, 1939) contained in a 30 ml. beaker; 0.5 ml. samples were taken at intervals and the liberated SH groups were titrated amperometrically against 0.01N- $AgNO_3$ by the method of Kolthoff & Harris (1946), the $AgNO_3$ being standardized against cystine reduced by the same method. The presence of urea accelerated reduction (Fraenkel-Conrat, Mohammad, Ducau & Mecham, 1951) and, although it rendered the electrode sluggish, it had no effect on the end-point.

End-group and lysine assay. Inhibitor (15 mg.) was treated with fluorodinitrobenzene according to Sanger (1949) and the derivative hydrolysed with 6N-HCl at 105° for 8 hr. The ethereal extract of the hydrolysate was examined for amino-acid dinitrophenyl (DNP) derivatives by paper chromatography (Biserte & Osteaux, 1950). DNP-lysine and DNP-arginine in samples of the aqueous extract were estimated spectrophotometrically (Sanger, 1945) after separation on silica-gel columns using the solvent system ethyl methyl ketone:ether (66:34 by vol.) equilibrated with 2M- Na_2HPO_4 (Blackburn, 1949). Corrections were applied for losses on the column and for breakdown of the DNP-lysine and arginine by carrying out control hydrolyses.

Filter-paper electrophoresis. This was carried out on 150 μ g. of inhibitor in the apparatus described by Durrum (1950) using buffers of pH 8-10.

RESULTS

Shape of inhibition curve

When partially purified inhibitor was being assayed, a straight-line relationship existed between amount of inhibitor added and trypsin units remaining. With crystalline inhibitor, a departure from linearity was observed at low inhibitor concentrations with certain trypsin preparations (Fig. 3). The magnitude of the effect, although dependent on the particular sample of trypsin used, was not directly connected with the trypsin activity of the sample, nor was it changed by recrystallization. It was also unaffected by change in trypsin and substrate concentrations. The trypsin sample (called standard trypsin) finally chosen for estimation purposes was one which gave imperceptible falling off (—.—).

Production of inhibitor from crude pancreas residues

Preliminary experiment on purification. Damp acid-ethanol-containing pancreas residues from insulin preparation were extracted with 0.25N-sulphuric acid and the extract 80% saturated with ammonium sulphate. The precipitate dissolved in water had no proteolytic activity, but after removal of protein with hot trichloroacetic acid and saturation with magnesium sulphate at pH 3, a precipi-

tate was obtained which had inhibitory activity corresponding to an inhibitor content of 1-2%. Subsequent experiments showed that preliminary heating of the damp pancreas residues to 70° to remove ethanol before extraction doubled the inhibitor activity of the magnesium sulphate precipitate. This product, containing about 50% magnesium sulphate and 5% nitrogen, was the starting material for subsequent preparations, but could not be directly purified by the method of Kunitz & Northrop (crystallization of inhibitor-trypsin compound) since the impurities rendered crystallization impossible.

Fig. 3. Inhibition curves using different samples of trypsin and inhibitor. ●—●, trypsin sample A, prepared in laboratory (30 TU/ml., $E=1$) + crude inhibitor (4.5 IU/ml., $E=1$); ○—○, trypsin sample A + crystalline inhibitor prepn. (i) (220 IU/ml., $E=1$); □—□, standard trypsin, prepared in laboratory (30 TU/ml., $E=1$) + crystalline inhibitor prepn. (i); △—△, commercial trypsin (20 TU/ml., $E=1$) + crystalline inhibitor prepn. (i); ×—×, commercial trypsin (30 TU/ml., $E=1$) + crystalline inhibitor prepn. (i); ---, indicates degree of departure from linearity.

Salt fractionation. Optimal conditions for salt fractionation were determined by constructing salting-out curves and pH-solubility curves using sodium chloride as precipitant. Fig. 4 shows the effect of pH on the solubility of crude inhibitor in saturated sodium chloride solution, both total protein concentration (E_{280}) and inhibitor activity of filtrate being plotted against pH. At pH 6, the precipitate contained 28% of the total peptide but no inhibitor; at pH 3 all the inhibitor was precipitated, but only 82% of total peptide. Fig. 5 shows the effect of increasing salt concentration at pH 2.5. There is a single break in the curve; the less soluble component was without inhibitor activity; the more soluble component, although impure as shown by its low activity, behaved as a single phase ($\log E$ plotted against ionic strength gave a straight line). Either type of fractionation was suitable as a

method of purification; the former was chosen, being slightly more convenient, the fraction precipitated in saturated sodium chloride solution between pH 5.8 and pH 3 being retained.

Fig. 4. Effect of pH on solubility of crude inhibitor in saturated NaCl solution. ●—●, total protein concentration in filtrate measured by E_{280} ; ○—○, inhibitor units in filtrate.

Fig. 5. Solubility of crude inhibitor in NaCl solution at pH 2.5. △—△, total protein concentration in filtrate (E_{280}); ○—○, inhibitor units in filtrate.

Electrodialysis. Salt-precipitated inhibitor was substantially freed from sodium chloride by dialysis through cellophan for a short period during which practically no inhibitor was lost. The remainder of the sodium chloride was removed during the first stages of electrodialysis, when little peptide moved from the centre compartment. During the next few hours, about 5% of the total peptide, containing

60–70% of the inhibitor, migrated to the cathode but negligible amounts went to the anode. The pH of the centre compartment had little effect on inhibitor mobility; even at pH 12 little peptide material with negligible inhibitor activity went to the anode. At pH as low as 1, little further peptide moved to the cathode, beyond that which moved at neutral reactions. Repeated electrodialysis of the catholyte effected no further purification. In all experiments, about 20% of the original inhibitor activity remained in the centre compartment, and was presumably due to some other substance which inhibits trypsin.

Electrodialysis was adopted as a preparative step after salt fractionation, and following these processes the compound of the inhibitor with trypsin could be crystallized.

Large-scale inhibitor preparation

The scheme (see p. 262) for the preparation of inhibitor from pancreas residues is given and experimental details of a typical preparation follow. All weights refer to damp filter cakes; activities are expressed as IU/ml., $E = 1$.

Extraction of crude inhibitor. Oven-dried pancreas residue (100 kg.) from insulin extraction was suspended in 0.25N H_2SO_4 (140 l.) for 24 hr. with occasional stirring. The liquid, separated by centrifugation, was 80% saturated with $(NH_4)_2SO_4$, and the centrifuged precipitate (1.3 kg.) was dissolved as far as possible in water (3.2 l.). After straining to remove lumps, the solution was heated to 60° in batches (1.5 l.) and mixed with an equal volume of boiling trichloroacetic acid (50% w/v). After standing for 4 min. at 80°, the mixture was cooled rapidly to 30° by pouring through cooling coils, then centrifuged; the supernatant was adjusted to pH 3 and saturated with $MgSO_4$. The precipitate, containing inhibitor, was collected in a Sharples centrifuge (250 g., activity 10). Any solid found floating on the centrifugate was combined with the main bulk.

(Since the activities of different batches of pancreas were variable, it was found advisable to run pilot extractions, with a few kg. of pancreas residues, as far as this stage.)

Salt fractionation. The crude inhibitor (250 g.) was dissolved in water (5 l.) and the pH adjusted to 6.5. NaCl (1.7 kg.) was added and the pH was adjusted to 5.8; after 3 hr., the precipitate was removed in the Sharples centrifuge; the supernatant liquid was acidified to pH 3, when a yellow gummy precipitate was formed, containing most of the inhibitor. The pH 5.8 precipitate was redissolved in water and refractionated as above. The combined pH 3 precipitates (50 g., activity 18) were dissolved in a minimum of water (approx. 50 ml.) at 40°, and partly freed from NaCl by dialysis in a rotating cellophan sac against 1 l. of distilled water (at 40°, to prevent the solution from setting to a gel). The water was changed at hourly intervals until the dialysate no longer tasted salty.

Electrodialysis. The inhibitor solution was diluted to 1.2 l. and electrodialysed in two portions as described in Methods. The various catholyte fractions were combined into two batches according to their activities, and concentrated tenfold *in vacuo*; inhibitor was precipitated by saturating the

solution with NaCl at pH 3. (Yield 10 g., activity 110 ± 0.7 g., activity 60).

Subsequent steps in producing crystalline inhibitor followed the method of Kunitz & Northrop (1936) except that the inhibitor-trypsin compound was recrystallized from 0.7 sat. MgSO_4 , not from sat. MgSO_4 (see scheme).

Recrystallization of inhibitor. Crystalline inhibitor (0.6 g.) was dissolved in 0.1 M-acetate buffer pH 6.5 (6 ml.) and the solution saturated with MgSO_4 . A little partially crystalline material precipitated and was filtered off. The filtrate was

preparation (iii) rose to 140 units, a small amount of peptide material being detected in the early dialysates of preparation (iii).

Homogeneity of inhibitor

Precipitation curves with increasing concentrations of phosphate buffer, pH 6.5, were constructed for crystalline inhibitor preparation (ii) (activity 130) and, for purposes of comparison, a sample

acidified to pH 3; a precipitate which formed immediately crystallized rapidly and was filtered off after standing overnight. (Yield: 0.3–0.4 g. after two recrystallizations.) The fraction precipitated at pH 6.5 was recrystallized separately and yielded a slightly less active material.

Activity of inhibitor preparations

Three main batches (i, ii and iii) of inhibitor were prepared, final specific activities of crystalline materials being 220, 130 and 120, respectively. A small amount prepared from fresh pancreas (Kunitz & Northrop, 1936) had activity 180. Removal of salt by dialysis caused a fall in activity of preparation (i) to 160 units, while the activity of

(activity 70) purified only as far as electrodialysis. Plotting inhibitor activity or total peptide concentrations against ionic strength of buffer gave smooth curves (Fig. 6) for crystalline inhibitor which were straight lines when plotted semi-logarithmically (Fig. 7). The impure material gave broken lines (Fig. 6, ---) suggesting the presence of about four components, none of which was closely associated with inhibitor activity. Application of the specific property solubility test (Falconer & Taylor, 1946, 1947) showed that precipitation of inhibitor was directly proportional to precipitation of peptide for the crystalline inhibitor (Fig. 8), indicating that the inhibitor was behaving as a

homogeneous substance under the conditions of test.

Filter-paper electrophoresis. When tested by filter-paper electrophoresis using buffers in the pH range 8–10, crystalline inhibitor (ii) appeared to be homogeneous, migrating at all pH's to the cathode as a single sharp spot. The impure specimen gave a long streak. The magnitude of the electro-endosmotic effect prevented a determination of the isoelectric point, but the inhibitor proved to be at least as basic as lysine.

Fig. 6. Salting-out curves of two inhibitor samples in potassium phosphate buffer, pH 6.5. ○, total protein concentration in filtrate (E_{280}); ●, inhibitor units in filtrate. —, Crystalline inhibitor (prepn. (ii), activity 130 IU). ---, Electrolysed inhibitor (activity 65 IU).

Properties of inhibitor

General. The crystalline salt-free inhibitor was a white solid, soluble in water, 70 % (w/v) methanol and ethanol, and 50 % (v/v) acetone. It showed a solubility minimum at pH 3, both in strong inorganic salt solutions and in aqueous solution; it could in fact be recrystallized by changing the pH of a concentrated aqueous solution from 5.5 to 3. Its solubility in organic solvents was low in relation to its water solubility, as shown by distribution experiments in butanol/acetic acid and in chloroform/methanol/water, and by its failure to move on paper chromatograms with the following solvent systems: collidine/lutidine/water; *n*-butanol/*N*-ammonia; *n*-butanol/20 % acetic acid; *sec*-butanol/3 % acetic acid; tetrahydrofurfuryl alcohol/water (80:20). In phenol/water (acetic acid atmosphere) it ran with the solvent front.

The molecular weight of preparation (ii), determined by Dr H. Gutfreund by osmotic pressure

measurements, was 9000 ± 500 . Values of E_{280} /mg./ml. for preparations (i), (ii), and (iii) were 0.84, 0.82 and 0.837, respectively.

Fig. 7. Salting-out curve of crystalline inhibitor prep. (ii) in potassium phosphate buffer, pH 6.5, expressed semi-logarithmically.

Fig. 8. Relation between total protein and inhibitor activity left in solution with increase in ionic strength of potassium phosphate buffer, pH 6.5. ○—○, Crystalline inhibitor (prepn. (ii), activity 130 IU); ●—●, electrolysed inhibitor (activity 65 IU).

The inhibitor was stable in organic solvents and in dilute acids at 100°, but was slowly inactivated in alkaline solutions at room temperature (Table 1). It was precipitated by sodium picrate, but some activity was lost on regeneration of the free base.

Chemical composition. The mean analytical results on the three samples are as follows: C, 47.6; H, 6.7; N, 16.5; amide N, 0.75 %. The results were identical except in the case of nitrogen which was 16.0, 17.0 and 16.5 % for preparations (i), (ii) and

(iii), respectively. The sulphur content was not determined because of shortage of material, but from the results of the determination of amino-acid content, given below, would be about 2.5%. The Fiske and Subbarow test for phosphate, carried out on the hydrolysed inhibitor, was negative. The inhibitor gave a negative Molisch test for carbohydrate and positive ninhydrin, biuret, Sakaguchi and Pauly diazo reactions.

Table 1. *Alkali stability of inhibitor*

(Percentage of inhibitor activity left after standing at 25° under the conditions shown. 0.14% (w/v) solution.)

Time (hr.) ...	1	2	5	24	72
pH 9.3	—	—	—	84	46
pH 10.0	—	—	—	79	61
pH 10.8	—	—	—	53	—
pH 11.9	—	—	82	49	—
0.1 N-NaOH	—	—	—	29	—
N-NaOH	66	27	—	—	—

Amino-acid composition. All amino-acid analyses were carried out in parallel on preparations (i) and (ii), but no difference between the two samples was found, except that the first hydrolysate prepared from preparation (ii) showed on chromatograms a molybdate/peroxide-stable spot in the position of methionine sulphoxide (Green & Work, 1951); this was not found in subsequent hydrolysates from the same material, so it was presumed to be a peptide which had resisted hydrolysis.

Electrolytic reduction of inhibitor in acid solution, carried out to reduce cystine S—S linkages before amperometric titration of sulphhydryl groups, caused 80% decrease in activity after 30 min. reduction. (Reduction of cystine under the same conditions was not complete even after 2 hr., but addition of urea completed the reduction.) A steady titration value, indicating the presence of 7.2% cystine, was obtained with inhibitor after 45 min. reduction in the presence of urea. Unreduced inhibitor gave a zero titration, showing the absence of free sulphhydryl groups.

Table 2 shows the results of all amino-acid analyses. An amino-acid mixture resembling the hydrolysed inhibitor gave paper chromatograms similar to those from hydrolysed inhibitor.

End-group analysis and lysine content. Preparations (i) and (ii) were treated in parallel and no significant difference was found between them. The ethereal extract from the hydrolysate of the DNP-inhibitor was only a very pale yellow; paper chromatograms gave two spots, with R_f values different from those of any known DNP-amino-acid but identical with those of dinitroaniline and dinitrophenol, presumably decomposition products from hydrolysis of basic DNP derivatives. The end group, if any, was therefore a basic amino-acid and, since there was no histidine in the inhibitor, was

probably arginine. Samples of the aqueous layer were applied to ethyl methyl ketone: ether columns; in front of the DNP-lysine band was a faint band. This was proved to be DNP-arginine by its R_f value on the column relative to lysine, by paper chromatography, and by the value of E_{350}/E_{390} . Contamination of the arginine band by colourless *o*-DNP-tyrosine (indicated by absorption at 300 m μ .) prevented an estimation of DNP-arginine from E_{350}/E_{390} .

Table 2. *Approximate amino-acid composition of salt-free lyophilized inhibitor, estimated ($\pm 15\%$) by paper chromatography, except where stated otherwise*

Amino-acid	g./100 g. of inhibitor	Residues/mol. of inhibitor
Aspartic acid	8	6
Glutamic acid	8	6
Cystine*	7.2	3
Serine	3.5	3
Glycine	7.5	12
Threonine	4.5	4
Alanine	13	18
Valine	3	2
Leucine	3	2
Isoleucine	1	1
Proline	6	5
Phenylalanine	8	5
Tyrosine†	9	5
Tryptophan†	0	0
Methionine	1.6	1
Histidine	0	0
Lysine‡	7.3	5
Arginine	15	9
Amide N	0.74	5
Total N	16.5	
End group (arginine)	—	1

* Estimated amperometrically.

† Estimated spectrophotometrically (Beaven & Green, 1953).

‡ Estimated through DNP derivative.

When further columns were run, a third faint band was always observed just in front of DNP-arginine; its strength relative to the arginine band was 0.4:0.6. This band was possibly due to an arginine-containing peptide which resisted acid hydrolysis, since the products of 1 hr. alkaline hydrolysis (Mills, 1950) gave spots on chromatograms similar to those given by control hydrolysates of DNP-arginine, except for the presence of an additional unidentified purple spot. The quantitative values showed that this band plus the arginine band added up to one end group per molecule, but this cannot be taken as an accurate assay.

DISCUSSION

Activity measurements. Measurements of inhibitor activity must be carried out under conditions which are completely reproducible and where non-specific inhibition of trypsin by proteolysis products

is at a minimum. The use of synthetic substrates should achieve this, but with benzoylarginine amide as substrate the digestion rate was so slow as to be inconvenient for routine testing. As digestion rates with denatured protein substrates are faster, the substrate chosen was carboxyhaemoglobin, crystallized to ensure reproducibility, trypsin activity being measured by a slight modification of Anson's (1938) method. The reaction velocity during the 5 min. digestion period was constant to within 10 %, thus the digestion values were approximate measures of initial reaction velocities. Duplicate estimations of trypsin activity were repeatable to within 2 %, while duplicate inhibition curves agreed within 5 %.

The departure from linearity of the inhibitor curve at low inhibitor concentrations with certain trypsin samples might be explained by the presence in the trypsin of some impurity which reacted with the inhibitor. A likely contaminant is denatured trypsin (Bier & Nord, 1951) but experiments described in a following paper (Green & Work, 1953) showed that heat- or alkali-denatured trypsin does not combine with inhibitor to any significant extent. Considerable evidence is accumulating which suggests that trypsin is not homogeneous, either enzymically (Werle, Kehl & Koebke, 1950; Pope & Stevens, 1951) or electrophoretically (Anderson & Alberty, 1948). Green (unpublished) has found that the activity of trypsin can be raised by passing it through a Celite partition column (Martin & Porter, 1951).

Properties of inhibitor. The three different preparations of inhibitor had identical elementary analyses and optical properties, and the two samples examined had similar amino-acid compositions; but inhibitory activities were slightly different. The activities were not altered by recrystallization, even after change of pH and of precipitating salt, but in two cases they were affected by the final dialysis carried out in order to remove salts. The origin of these differences appeared to be in the original pancreas residues, which showed great and inexplicable variations of inhibitory activity; the most active starting material yielded the most active crystalline inhibitor, but many batches of pancreas residues showed negligible inhibitor activity.

Homogeneity of the least active sample was investigated by precipitation curves, and the specific property solubility test indicated that the inhibitor behaved as a single entity under the conditions of test. The variation of activity must be attributed to some small variation in structure undetectable in this large molecule by the analytical methods employed, or to a difference in arrangement of amino-acids. The inhibitor as prepared may consist of a family of peptides of slightly differing bio-

logical activity but with chemical properties so similar as to prevent separation by any of the methods used (Synge, 1949).

Amino-acid estimations were mainly carried out by paper chromatography and were consequently only approximate, but they enabled a rough picture of the amino-acid composition to be obtained. All the amino-acids commonly occurring in proteins were present with the exception of tryptophan (Beaven & Green, 1953) and of histidine; the methionine content was only one residue/mole. Amperometric titration showed the absence of sulphhydryl groups, but after electrolytic reduction there were six sulphhydryl residues/mole showing that the inhibitor contained three cystine residues. This figure differs from the preliminary figure (one residue) quoted previously (Green & Work, 1951) which was determined by rough paper chromatography. Subsequently more careful chromatography with peroxide-treated controls gave the same value as that obtained electrometrically. Arginine was found to constitute the sole end group; an accurate end-group assay was not obtained, but it was possible to say that there was not more than one end group per molecule. The high arginine content (about nine residues per mole) accounts for the basicity of the molecule as a whole; five of the twelve acidic amino-acid residues were present as amides and another five acidic charges would be neutralized by the free amino groups of the five lysine residues.

The basic nature of the inhibitor, originally revealed by electrodialysis, was unexpected, since Kunitz crystallized the inhibitor at pH 5.5. In this respect, the inhibitor resembles lysozyme, a basic protein of mol.wt. 15 000 which crystallizes at pH 4 (Alderton & Fevold, 1946). The exact isoelectric point of the inhibitor has not been determined, but it is certainly higher than pH 10. The analytical results on the inhibitor differ from those given by Kunitz & Northrop (1936) whose N value (11.25 %) is unusually low for a peptide; the molecular weight of 9000 found for our preparation is also different from the value of 6000 found by Kunitz & Northrop.

Comparison with other trypsin inhibitors. In addition to the pancreatic inhibitor, specific inhibitors for trypsin have been identified in soya bean, lima bean, egg white, colostrum and *Ascaris* (Kunitz, 1947; Fraenkel-Conrat, Bean & Lineweaver, 1949; Fraenkel-Conrat, Bean, Ducay & Olcott, 1952; Schmitz, 1939; Laskowski & Laskowski, 1951; Collier, 1941); they are all stable proteins, with the exception of ovomucoid inhibitor which contains 25 % carbohydrate. Only pancreatic, soya bean and colostrum inhibitors have been obtained crystalline, but preparations of ovomucoid and lima bean inhibitors each gave a single boundary

on electrophoresis. Molecular weights of soya bean and ovomucoid inhibitors are 24 000 and 28 000 and one pancreatic inhibitor is also of relatively high molecular weight; the other inhibitors have molecular weights of 10 000 or less. The isoelectric points of soya, lima and ovomucoid inhibitors lie between 4 and 5.

Complete amino-acid analysis has been carried out only on lima bean inhibitor; it bears no resemblance to pancreatic inhibitor except for the absence of tryptophan, also noted in colostrum inhibitor.

SUMMARY

1. Crystalline pancreatic trypsin inhibitor was obtained from industrial pancreas residues remaining after insulin extraction. Three separate preparations yielded materials of similar composition but of slightly differing activities.

2. The homogeneity of one preparation was investigated by the specific property solubility test.

3. The properties of the inhibitor were those of a basic peptide; it migrated during electrodialysis through cellophan to the cathode when the pH of the centre was below 10.

4. Elementary analysis was C, 47.6; H, 6.7; N, 16.5 %.

5. The molecular weight was 9000 ± 500 .

6. The amino-acid composition was determined mostly by paper chromatography. Histidine was absent and there was only one methionine residue/mol. of inhibitor. Cystine was determined amperometrically on unhydrolysed inhibitor after electrolytic reduction in the presence of urea.

7. End-group assay showed arginine to be the terminal amino-acid.

8. The inhibitor was slowly inactivated in alkaline solution at room temperature, but was stable in hot dilute acid.

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