Some Physico-Chemical Aspects of Immobilised Trypsin

by

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A thesis submitted to the University of St. Andrews in application for the degree of Master of Science.

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DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that this thesis is my own composition and that no part of it has been presented previously for a higher degree. The research was carried out in the Department of Biochemistry of the University of St. Andrews under the direction of Dr. W. E. Hornby.

CAREER

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CERTIFICATE

I hereby certify that Philip Burford spent four terms in research studies between October 1972 and October 1975 under the direction of Dr. W.E. Hornby and has fulfilled the conditions of Resolution of the University Court, 1974, No. 2 and that he is qualified to submit this thesis for the degree of Master of Science.

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Dr. W.E. Hornby and Dr. W. Ledingham, Department of Biochemistry, University of St. Andrews, who supervised the work.

ABSTRACT

The supposition that an immobilised protein is covalently bound to its inert support was investigated by comparison of the entropy change on thermal denaturation of the protein before and after its immobilisation. If the bonding was covalent the protein structure on immobilisation would be more constrained than that of the free protein and on denaturation would not be able to relax to the same degree as the structure of the unbound protein, this would cause a reduction in the entropy change of denaturation for the immobilised protein compared to that of the free protein.

Trypsin was chosen as the subject of the investigation since its thermal denaturation is reversible and can readily be followed by measuring the loss of enzyme activity.

The original investigation of the reversible thermal denaturation of trypsin was performed in 1933 using a colorimetric estimation of the intensity of blue colour produced by the action of a phenol reagent on the soluble products of the tryptic digestion of a haemoglobin substrate as a measure of tryptic activity. The reproducibility of these findings was first tested using a more modern spectophotometric technique where the absorbance at 280nm of the tyrosine/tryptophan content of trichloracetic acid soluble products from trypsin hydrolysed denatured haemoglobin was used as a measure of tryptic activity. The values determined for the entropy change on reversible denaturation of soluble trypsin were within 5% of those calculated from the 1933 investigations, this showed favourable comparability of the methods used.

Trypsin immobilised on cellulose powder and subjected to thermal denaturation was found to give no consistency of results when assayed spectrophotometrically using haemoglobin as substrate, this

was probably due to the partial absorption of the haemoglobin hydrolysate products by the cellulose support medium which caused erratic absorbance readings at the wavelength of the assay.
An alternative titrimetric assay technique was developed to follow the thermal denaturation of both soluble and immobilised trypsins, this used N Benzoyl-L-Arginine Ethyl Ester HCl as substrate. Values for the entropy change of thermal denaturation for soluble trypsin as assayed by the titrimetric technique were found to be 44% lower than those determined by the spectrophotometric technique. To determine the reason for this and to confirm the validity of the titrimetric assay method as a means of following the denaturation process titrimetric assays were performed in the presence of soluble carboxymethyl cellulose which had similar size and charge to the haemoglobin/hydrolysed haemoglobin molecules found in the spectrophotometric assay solutions. As the concentration of carboxymethyl cellulose in the assay mixture was increased the entropy change of denaturation values for the thermal denaturation of soluble trypsin also increased but did not attain their original high values. Values within 4% of those determined from the spectrophotometric assay procedure were obtained however when the entropy of denaturation was calculated from the results of the thermal denaturation of soluble trypsin assayed titrimetrically using BAEE as substrate in the presence of 1% enzymically inactive trypsin hydrolysed haemoglobin. This showed the titrimetric assay technique to be a valid technique to use, and any entropy change of denaturation values for immobilised trypsins would be compared to that determined titrimetrically for soluble trypsin using BAEE as substrate.

Trypsins immobilised on cellulose by various chemistries were subjects to reversible thermal denaturation, the progress of the

denaturation being followed by the titrimetric assay technique, and the entropy change of denaturation values were calculated. The calculated values were found to be 33 to 41% lower than that for soluble trypsin determined using the same assay technique. This finding supports the original theory that the bonding between the protein and its support material is covalent.

The same trends were not evident when nylon was used as a support material however since it was not possible to determine entropy change of denaturation values due to the thermal denaturation losing its reversible nature.

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1. INTRODUCTION

Proteins may be insolubilised by cross linking with a bifunctional reagent or by association with an insoluble support medium, usually a polymer.

Polymer support materials are available in many different configurations such as mesh, powder, bead, membrane and tube, so facilitating various modes of immobilisation.

When polymers are used as the support matrix a variety of interactions between protein and polymer (1) may be used to insolubilise the protein, these include

(a) adsorption on a polymer support

e.g. Ribonuclease on Dowex 50 cation exchange resin (2).

β Galactosidase on DEAE cellulose (3).

(b) inclusion in a polymer

e.g. Carbonic anhydrase and urease in nylon microcapsules (4), (5). Catalase in collodion membrane capsules (6).

(c) covalent bonding to a polymer surface

e.g. Ficin to carboxy methyl cellulose (7).

β Fructofuranosidase to polystyrene beads and tubes (8).

Adsorption of a protein on a polymer is not usually favoured as an immobilising method owing to its reversibility and the consequent protein desorption.

Inclusion of a protein in a polymer is often considered advantageous (9) as the included protein will usually be in a structural configuration very close to that of the "native" soluble protein since little or no covalent bonding occurs between the protein and its support, as for example in the microencapsulation of enzyme derivatives (10).

The third insolubilising method relies on the protein being covalently bound to its support by the reaction of suitably reactive groups on the protein molecule, such as the terminal amino and carboxyl groups and the substituent groups of some

amino acid residues e.g. arginine (guanidyl substituent), lysine (amino substituent), aspartic acid (carboxyl substituent), tyrosine (phenol substituent), cysteine (sulphydryl substituent), serine (hydroxyl substituent), histidine (imidazoyl substituent), with active groups on the support medium e.g. the amino groups of aminated polystyrene, and the carboxyl groups of carboxy methyl cellulose or the aliphatic amino and carboxyl groups of partly hydrolysed nylon. Examples of such immobilisations being the coupling of β Fructofuranosidase to aminated polystyrene tubes and beads (8), ficin to carboxy methyl cellulose (7), the enzymes urease (12) and aparaginase (13) to the amino groups on the inside surface of partly hydrolysed nylon tubing as well as for the immobilisation of uricase on nylon powder (14).

Covalent coupling of the protein to the carboxyl groups of its support may be achieved directly via the acid azide derivative (Fig. 1.1) or indirectly by using the carboxyl group as a site for attachment of some other reactive center. For example benzidine, an aromatic diamine, has been condensed with the carboxyl group using a carbodiimide and the resulting product diazotised and used for the coupling of protein (Fig. 1.2) The enzyme trypsin has been immobilised on nylon tube by this method (11).

Direct coupling of protein to carboxyl groups on a polymer support medium

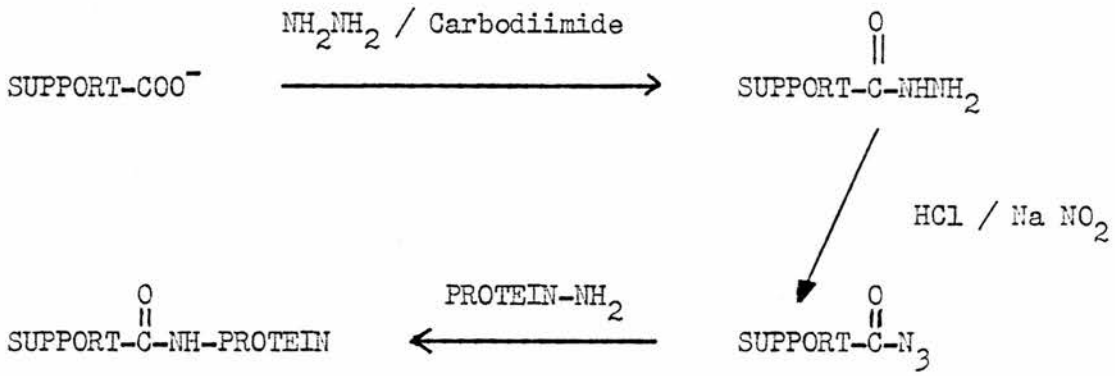


Fig. 1.1

Indirect coupling of protein to carboxyl groups on a polymer support medium

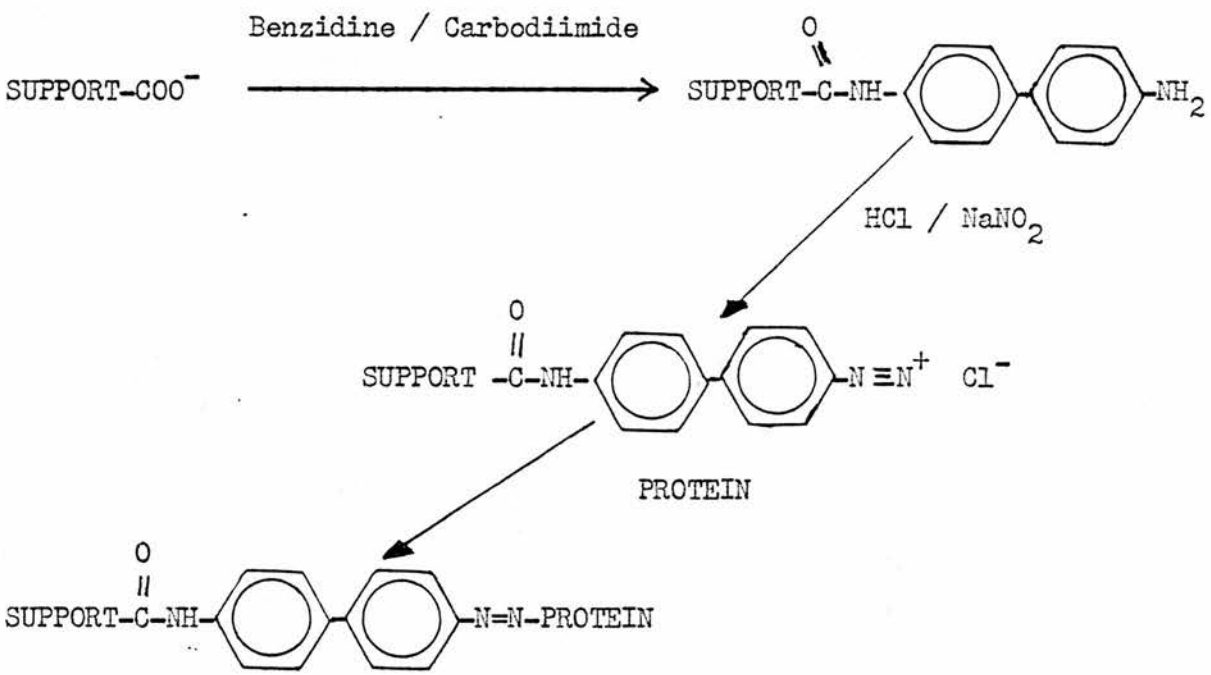


Fig. 1.2

Chemical attachment of a protein to a polymer support via free amino groups on the support surface is conveniently accomplished by the use of a bifunctional reagent, a variety of which are available, for example glutaraldehyde (Fig. 1.3) or the bis-imido ester ethyl adipimidate (Fig. 1.4)

Coupling protein to the amino groups of a polymer support via glutaraldehyde

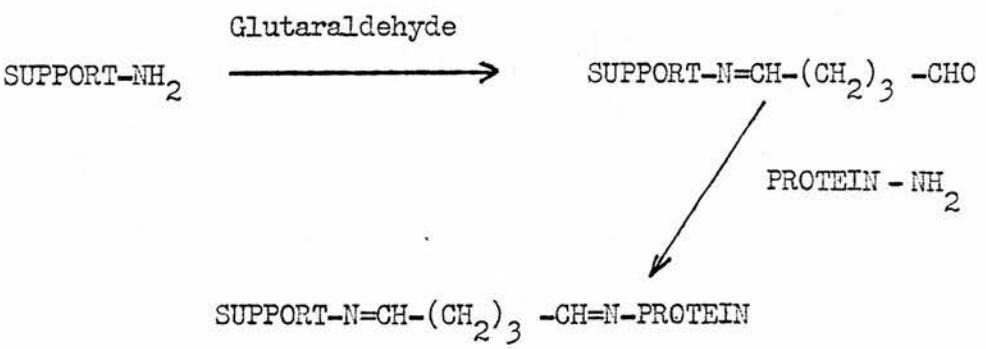


Fig. 1.3

Coupling of protein to the amino group of a polymer support via ethyl adipimidate

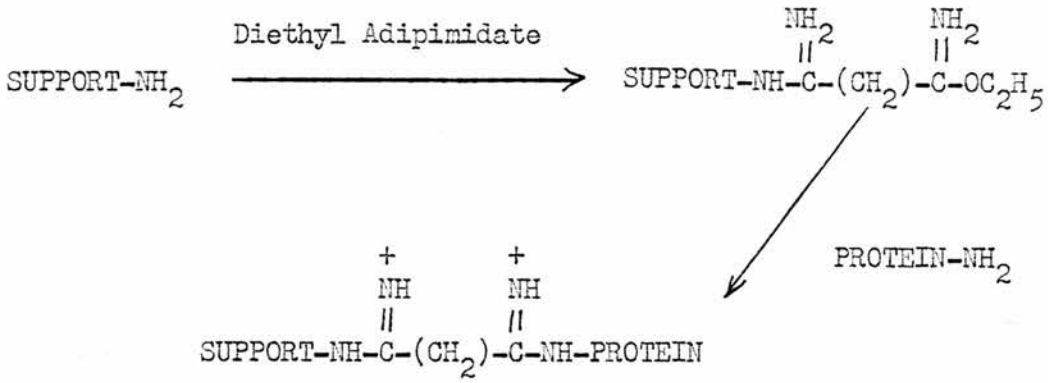


Fig. 1.4

When a polymer support such as nylon requires activation by cleavage of the polymeric backbone for the release of the coupling groups, the degree of cleavage must be limited to a level which does not impair the mechanical strength of the polymer. The consequent restriction of the number of active groups released limits the protein binding capacity, a higher degree of protein binding can be obtained by the use of a chemistry which does not involve peptide bond cleavage. The secondary amide of the peptide bond can be converted to an imido ester by alkylation with an alkylating reagent such as dimethyl sulphate or triethyloxonium tetrafluoroborate (Fig. 1.5), the imidates so formed react readily with amino groups under mildly alkaline conditions producing amidines (Fig. 1.6). proteins can be bound directly to the imidate salt of the nylon or a bifunctional amine spacer molecule such as lysine or hexamethylene diamine can be covalently bound to the support and the protein then coupled to the free amino group of the spacer molecule using a cross linking reagent such as glutaraldehyde or ethyl adipimidate (Fig. 1.7).

Formation of the imidate salt by alkylation of the peptide bond

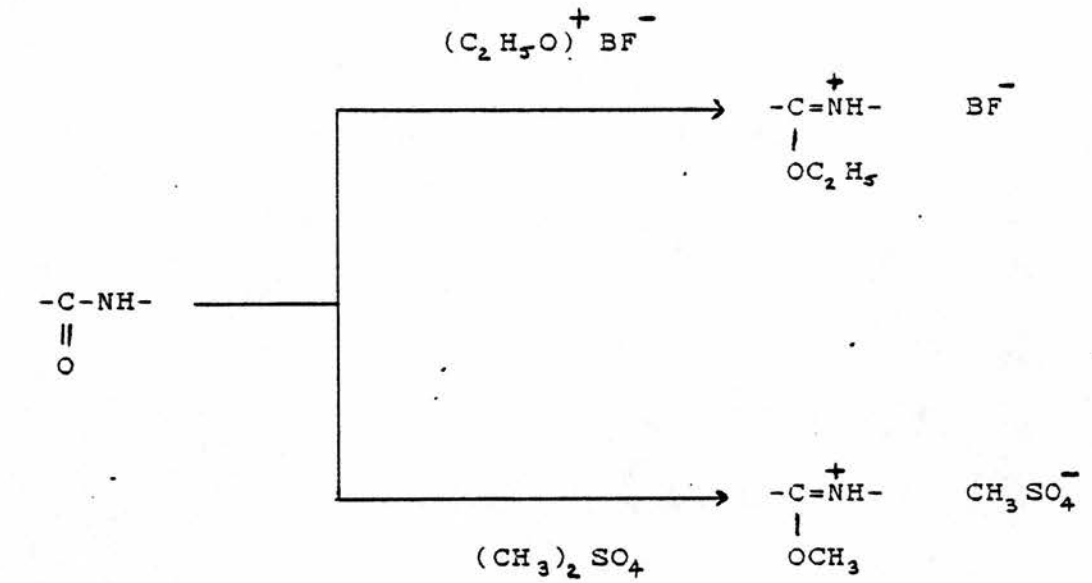


Fig. 1.5

Coupling of amines to the imidate salt

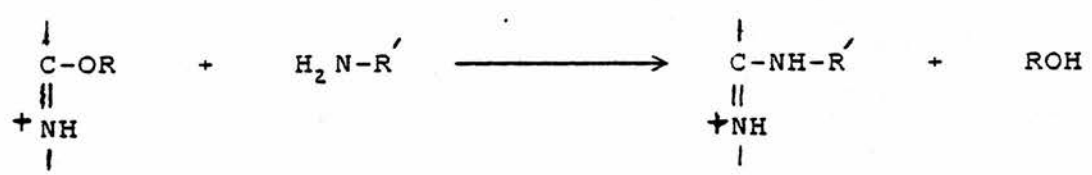


Fig. 1.6

Binding of the spacer molecule to alkylated nylon

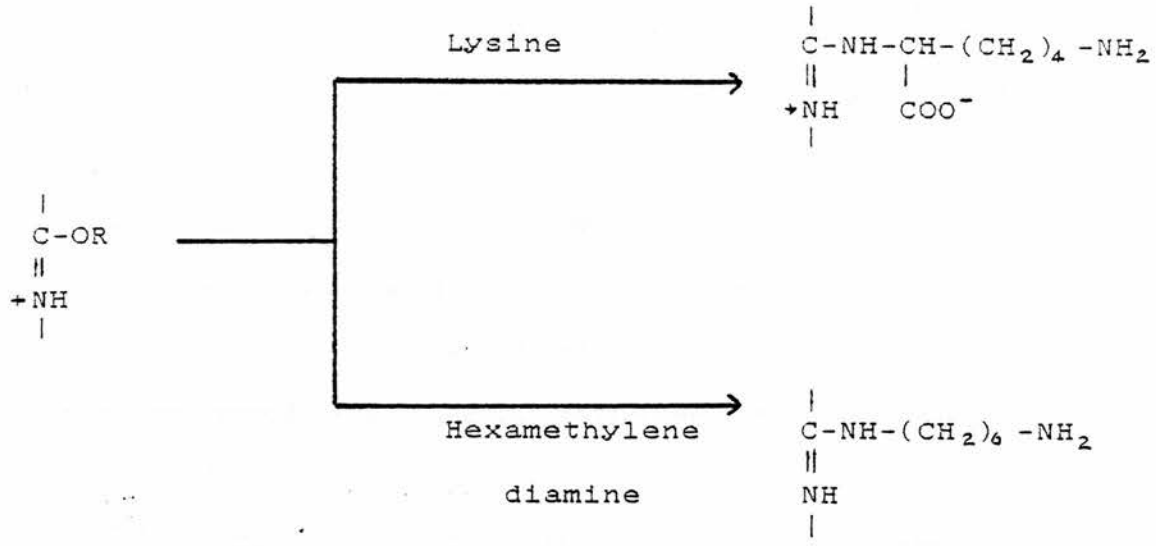


Fig. 1.7

Covalent binding of the types described might be expected to stabilise the conformation of the protein, this would account for the stability towards denaturation of the enzyme trypsin insolubilised by coupling to cyanogen bromide activated Sephadex (15).

Gabel (16) has described how Sephadex immobilised trypsin is active and stable in 8M urea and how this stability to denaturation diminishes, tending towards that of the soluble enzyme, as the number of amino groups per molecule coupled to the support decreases.

Such conformational stabilisation of the protein should, by reducing the freedom of the structure to unfold, cause a reduction in the entropy change on denaturation.

To test the validity of this supposition it was proposed that the entropy change on denaturation of an immobilised protein be compared to that of the same protein in its soluble form. If there was a reduction in entropy change for the denaturation of the immobilised

protein compared to the soluble protein the original supposition would be valid.

An entropy change of this nature can be most easily measured if:-

(i) the protein used is an enzyme, since the denaturation process can be conveniently followed by measuring the loss of enzymic activity.

(ii) the denaturation is a reversible one, since the entropy change can be assessed from measurement of the enthalpy change for the denaturation

The enzyme trypsin (EC 3.4.21.4) satisfies both these requirements in that it is:-

(i) a proteolytic enzyme with esterase activity and

(ii) undergoes a reversible thermal denaturation (17).

Trypsin has been shown by Anson & Mirsky to behave as though there was an equilibrium between active trypsin (T_n) and enzymically inactive trypsin (T_d), the equilibrium being represented as

$$T_n \rightleftharpoons T_d$$

The equilibrium constant (K) for the denaturation being

$$K = \frac{[T_d]}{[T_n]}$$

Anson & Mirsky developed a method for the estimation of active native trypsin in the presence of inactive denatured trypsin (16) using urea to freeze the equilibrium and so prevent the conversion of T_d into T_n. This enabled the concentration of native trypsin to be ascertained at various temperatures during the thermal denaturation process and hence the percent denaturation could be found.

Some of the Anson Mirsky data are given in Table I (18).

From such data it is possible to calculate the value for the equilibrium constant, K, for the equilibrium $T_n \rightleftharpoons T_d$ (Table II)

and hence the enthalpy change ΔH, for the denaturation by the use of the Van't Hoff equation (19). This equation relating the equilibrium constant, K, to the enthalpy change, ΔH, the gas constant, R, and the absolute temperature, T, is of the form

$$\frac{d \ln K}{dT} = \frac{\Delta H}{RT^2}$$

which may be integrated to

$$\ln K = -\frac{\Delta H}{RT} + \text{constant}$$

and used in the form

$$\log_{10} K = -\frac{\Delta H}{2.303RT} + \text{constant}$$

From the gradient of the straigt line obtained by plotting $\log_{10} K$ against the reciprocal of the absolute temperature (Fig. 1.8) the enthalpy change for the denaturation, ΔH, is calculated to be 287.79 kJ mol^{-1}.

For such a reaction at equilibrium the free energy change, ΔG, is zero and hence the entropy change, ΔS, calculated from the relationship

$$\Delta G = \Delta H - T\Delta S$$

is $+ 907\ JK^{-1}\ mol^{-1}$

Since the value of ΔH and hence ΔS depends on the accurate determination of the slope of the $\log_{10} K \vee \frac{1}{T}$ plot, the gradient of the best fit straight line, its intercept on the x axis ($T_{\frac{1}{2}}$), and its coefficient of determination (r^2), were calculated for all such plots using a statistical linear regression analysis (Appendix 1).

Using bovine haemoglobin as enzyme substrate a revised Anson and Mirsky assay technique was to be used to verify the original experiment (3.1.1), then various immobilised trypsin samples were to be denatured and their entropy changes on denaturation compared to that for the denaturation of soluble trypsin.

Cellulose was chosen as a suitable support polymer on which to immobi-

lise the enzyme since a well proven immobilisation technique using the bifunctional reagent gluteraldehyde was available (3.2.1 to 3.2.3) and assay of the immobilised trypsin as a suspension in the substrate solution would be relatively uncomplicated.

Table I Reversible thermal denaturation of Trypsin

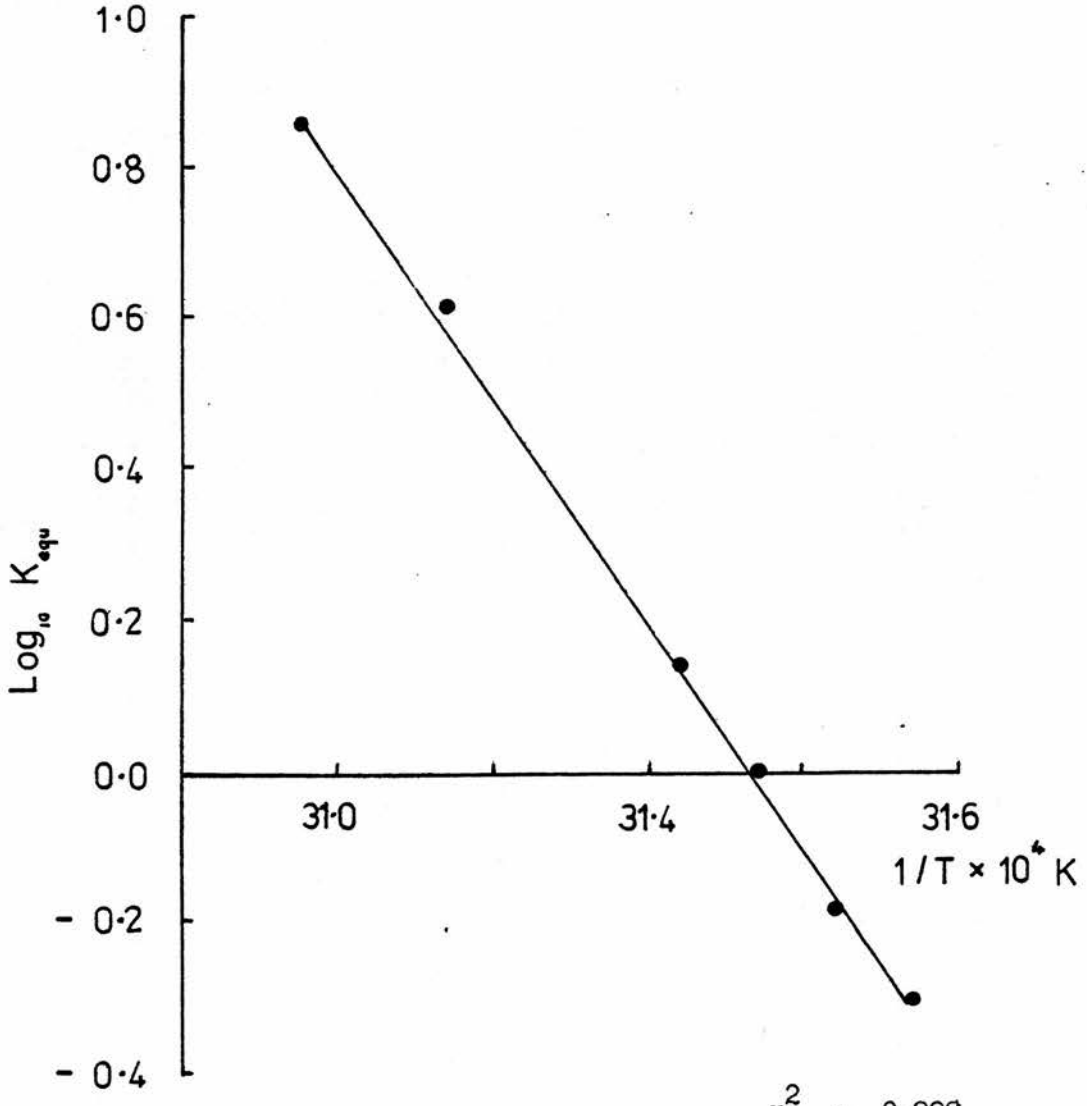
Temperature °C	Percentage denaturation
42	32.8
43	39.2
44	50.0
45	57.4
48	80.4
50	87.8

Table II K_{equ} for the denaturation equilibrium of Trypsin

Temperature °C	Temperature °K	% Denaturation	% Native	K	$\log_{10}K$	$\frac{1}{T} \times 10^4$
42	315	32.8	67.2	0.488	-0.3115	31.75
43	316	39.2	60.8	0.645	-0.1906	31.65
44	317	50.0	50.0	1.000	-0.0000	31.55
45	318	57.4	42.6	1.348	0.1259	31.45
48	321	80.4	19.6	4.100	0.6130	31.15
50	323	87.4	12.2	7.196	0.8571	30.96

Calculation of ΔH for the thermal denaturation of trypsin

(A + M 1933)



r^2 = 0.998

ΔH = +287.79 kJ mol^{-1}

ΔS = +907 JK^{-1} mol^{-1}

$T_{\frac{1}{2}}$ = 317.1 K

Fig 1.8

2. MATERIALS

2.1. General laboratory reagents

Adiponitrile

Boric Acid

Chloracetic acid

Hydrated calcium chloride

Hydrochloric acid

Sodium borohydride

Sodium carbonate

Sodium chloride

Sodium hydrogen carbonate

Sodium hydroxide

Sodium nitrate

Sodium tetraborate

2.4.6. Trinitro benzene sulphonic acid

Trichloracetic acid

Urea

These reagents were of Analar quality supplied by B.D.H. Chemicals Ltd., Poole, Dorset.

2.2. Solvents

Dioxan

Dichloro methane - Redistilled and dried over molecular sieve. (Type 3A potassium alumino silicate pellets, (B.D.H.)

Absolute Ethanol

Methanol

These reagents were of Analar quality supplied by B.D.H. Chemicals Ltd., Poole, Dorset.

2.3. Enzyme Support Materials

2.3.1. Cellex A.E. Anion exchange cellulose supplied by Bio Rad Laboratories, 32nd and Griffin, Richmond California.

2.3.2. Carboxy methyl Cellulose (C.M.C.) supplied by Whatman Biochemicals Limited Springfield Mill, Maidstone, Kent.

2.3.3. Nylon tube: Type 6 nylon, extruded from poly-caprolactam. Bore 1mm. Wall thickness 0.3mm

Source: Portex Ltd., Hythe, Kent.

2.4. Enzyme and Substrate Materials

2.4.1. Trypsin Type III 2 x crystallised from bovine pancreas. Dialysed and lyophilized. Activity 1000-1500 BAEE units/mg.

2.4.2. Haemoglobin Type I 2 x crystallised. Dialysed and lyophilized.

2.4.3. α-N-Benzoyl-L-Arginine Ethyl Ester HCl (BAEE)

These materials supplied by Sigma London Chemical Co. Ltd., Norbiton Station Yard, Kingston upon Thames, Surrey.

2.5. Support activation and coupling materials

2.5.1. N. Ethyl morpholine (NEM): B.D.H. Chemicals Ltd. Poole, Dorset.

2.5.2. Malonimidate : Aldrich Chemical Co. Ltd. The Old Brickyard, Gillingham, Kent.

2.5.3. Glutaraldehyde: 25% for electronmicroscopy B.D.H. Chemicals Ltd., Poole, Dorset.

2.5.4. Hydrazine hydrate: B.D.H. Chemicals Ltd. Poole, Dorset.

2.5.5. 1 cyclohexyl-3-(2 morpholino ethyl)-carbodiimide metho-p-toluene Sulphonate (CDI) : Aldrich Chemical Co. Ltd., The Old Brickyard, New Road, Gillingham, Kent.

2.5.6. Triethyloxonium tetrafluoroborate : donated by Dr. W. E. Hornby, prepared by the method of Meerwein (20).

2.6. Laboratory prepared reagents

2.6.1. Soluble O-Carboxymethyl cellulose (3.7)

2.6.2. Diethyl adipimidate (3.9)

2.6.3. Hydrolysed haemoglobin (3.8)

3. METHODS

3.1 Determination of tryptic activity

3.1.1. Spectrophotometric method for soluble trypsin using haemoglobin as substrate

The assay used was a modification of a standard trypsin assay reported in Bergmeyer (21)

Haemoglobin was denatured in alkaline urea solution. The tyrosine/tryptophan content of trichloracetic acid (TCA) soluble products from trypsin hydrolysed denatured haemoglobin was determined spectrophotometrically.

Optimum conditions for the assay were found to be

(i) pH of 7.0 to 8.0

(ii) a substrate concentration of 6-7mg ml^{-1} haemoglobin in the reaction mixture

(iii) the reaction mixture to be 0.02M with respect to Ca^{++} ions to prevent the formation of enzymically inert protein

Solutions used:-

Boric acid Solution I : 6.18 g boric acid and 0.29 g NaCl were dissolved in water and made to 100ml total volume with water.

Haemoglobin solution II : 2.0g haemoglobin were dissolved in 50ml distilled water, 36g urea and 8ml 1M NaOH were added, the solution diluted to about 80ml with water and allowed to stand at room temperature for 30 to 60 min. 10ml boric acid solution I were added and the mixture shaken before adding 4.4ml of 5% (w/v) $CaCl_2$. The pH of the solution was adjusted to 7.5 with 1M HCl and the total volume made to 100ml with water.

This solution was used as the substrate in the assay for trypsin activity.

Assay: Reagents Trypsin 2 to 20 mg ml^{-1} in 0.01M HCl
Substrate - haemoglobin solution II
5% (w/v) Trichloracetic acid

0.1ml of enzyme in 0.01M HCl was mixed with 5ml haemoglobin substrate solution II and the mixture incubated in a water bath at 25° for 5 min. After this time the reaction was stopped by the rapid addition of 5ml 5% T.C.A., the mixture being well shaken immediately the T.C.A. addition was complete. The solution was allowed to stand at room temperature for 30 min. and was then centrifuged (600 x g, 15 min.) and the supernatent filtered.

The absorbance of the filtrate was determined at 280nm, using a Beckmann D.B.G.T. grating spectrophotometer, against a control produced as above but using 0.1ml of 0.01M HCl in the reaction mixture in place of 0.1ml enzyme solution.

3.1.1.1. Modification of spectrophotometric method for soluble trypsin for assay of thermally denatured samples of soluble trypsin

A 0.1ml sample of the stirred incubated solution of trypsin from the thermal denaturation (3.10.1) was transferred rapidly to 5ml haemoglobin substrate solution II. by a 0.1ml blow out pipette, preheated to the same temperature as the trypsin solution. The assay was performed as in 3.1.1.

3.1.1.2. Modification of spectrophotometric method for soluble trypsin for assay of thermally denatured samples of trypsin immobilised on cellulose

A 0.1ml sample of the rapidly stirred incubated suspension

of cellulose immobilised trypsin from the thermal denaturation (3.10.2) was transferred to 5ml haemoglobin substrate solution II by a 0.1ml constriction pipette, preheated to the same temperature as the suspension. The assay was performed as in 3.1.1.

3.1.2. Titrimetric method for soluble trypsin using BAEE as substrate

The principle of the method is that trypsin hydrolyses BAEE to produce free acid groups which are titrated at constant pH against a standard alkali solution, the rate of consumption of alkali indicates the rate of production of free acid groups and hence the rate of hydrolysis of the ester.

Reagents:-

Aqueous $CaCl_2$, 0.02M with respect to Ca^{++}

Trypsin solution 0.125-2.0 $mgml^{-1}$ in 0.01M HCl

Titrant - 0.05M NaOH

Apparatus used consisted of:-

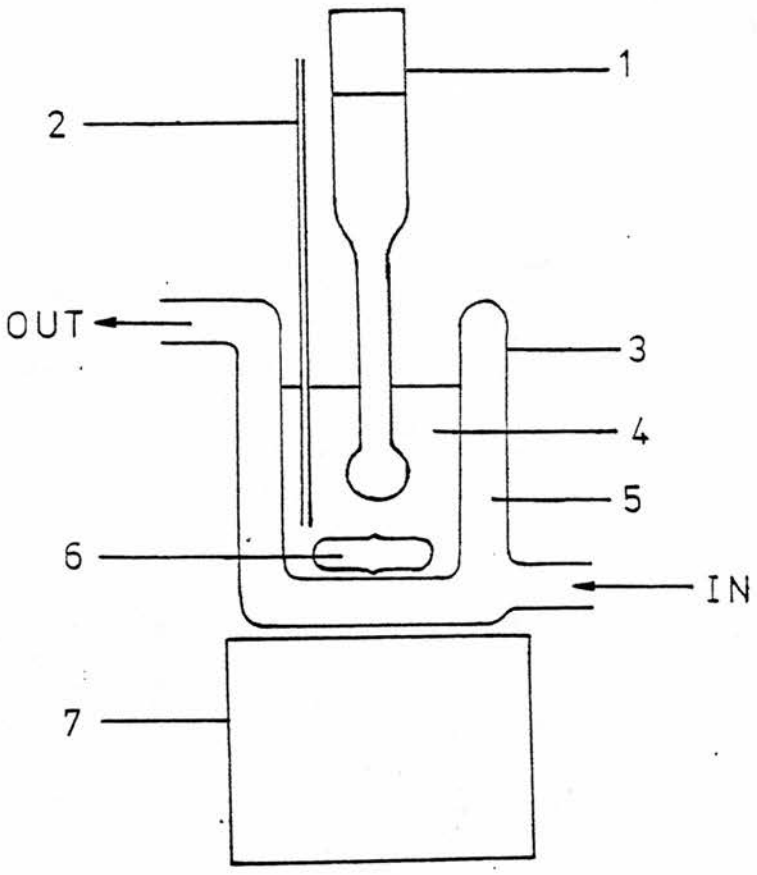
(i) A Radiometer titrator type TTT.1 operating in its pH stat mode, with a 0.5ml micrometer syringe burette for titrant addition. The titrant reservoir was protected from atmospheric CO_2 absorption by a Carbabsorb tube.

(ii) An Activion pH electrode: combined electrode type

(iii) A water jacketed, constant temperature (25°), reaction vessel of 20ml capacity with stirring facility (fig. 3.1)

Assay:- 14.5ml 0.02M $CaCl_2$ and 0.1ml trypsin in 0.01M HCl were pipetted into the reaction vessel, and the mixture constantly stirred. The pH was adjusted

Reaction vessel used in soluble tryosin assays



(fig. 3.1)

1. Activion miniature combined pH electrode.
2. Capillary tube for titrant delivery from syringe burette of Radiometer titrator.
3. Constant temperature, double walled, 20ml capacity reaction vessel.
4. Assay mixture.
5. Recycling water from constant temperature (25^{o}) water bath.
6. Magnetic follower.
7. Magnetic stirrer.

to 8.0 first by addition of small volumes of 1M NaOH from a micrometer syringe and finally by addition of titrant using the instrument itself. When a steady base-line had been established at pH 8.0, the assay was started by the addition of 0.5ml BAEE.

The activity of the enzyme was determined by measuring the rate of NaOH consumption and converting this to μ mole BAEE hydrolysed per min.

3.1.3. Titrimetric method for soluble trypsin using BAEE as substrate in the presence of 6M urea

The assay procedure was identical to that described in 3.1.2. except that the aqueous 0.02M $CaCl_2$ solution was made 6M with respect to urea.

3.1.3.1. Modification of Titrimetric method for soluble trypsin using BAEE as substrate in the presence of 6M urea for assay of thermally denatured samples of soluble trypsin

A 0.1ml sample of the stirred incubated solution of trypsin from the thermal denaturation (3.10.1) was transferred rapidly to 14.5ml 0.02M $CaCl_2$ solution 6M w.r.t. urea in the reaction vessel, by a 0.1ml constriction pipette preheated to the same temperature as the solution.

3.1.3.2. Modification of the titrimetric method for soluble trypsin using BAEE as substrate in the presence of 6M urea and 1% (w/v) soluble carboxymethyl cellulose

The assay procedure was identical to that described in

3.1.3. except that the aqueous 0.02M $CaCl_2$ solution 6M w.r.t. urea was made 1% (w/v) w.r.t. soluble carboxymethyl cellulose (3.7).

3.1.3.3. Modification of the titrimetric method for soluble trypsin using BAEE as substrate in the presence of 6M urea and 1% (w/v) hydrolysed haemoglobin

The assay procedure was identical to that described in 3.1.3. except that the aqueous 0.02M $CaCl_2$ solution 6M w.r.t. urea was made 1% (w/v) w.r.t. hydrolysed haemoglobin (3.8).

3.1.3.4. Modification of titrimetric method for soluble trypsin using BAEE as substrate in the presence of 6M urea for the assay of thermally denatured samples of trypsin immobilised on cellulose

A 0.1ml sample of the rapidly stirred, incubated suspension of cellulose immobilised trypsin from the thermal denaturation (3.10.2) was transferred to 14.5ml 0.02M $CaCl_2$ solution 6M w.r.t. urea in the reaction vessel, by a 0.1ml constriction pipette preheated to the same temperature as the suspension. The assay was performed as in 3.1.3.

3.1.3.5. Modification of the titrimetric method for soluble trypsin using BAEE as substrate in the presence of 6M urea for the assay of nylon tube immobilised trypsin

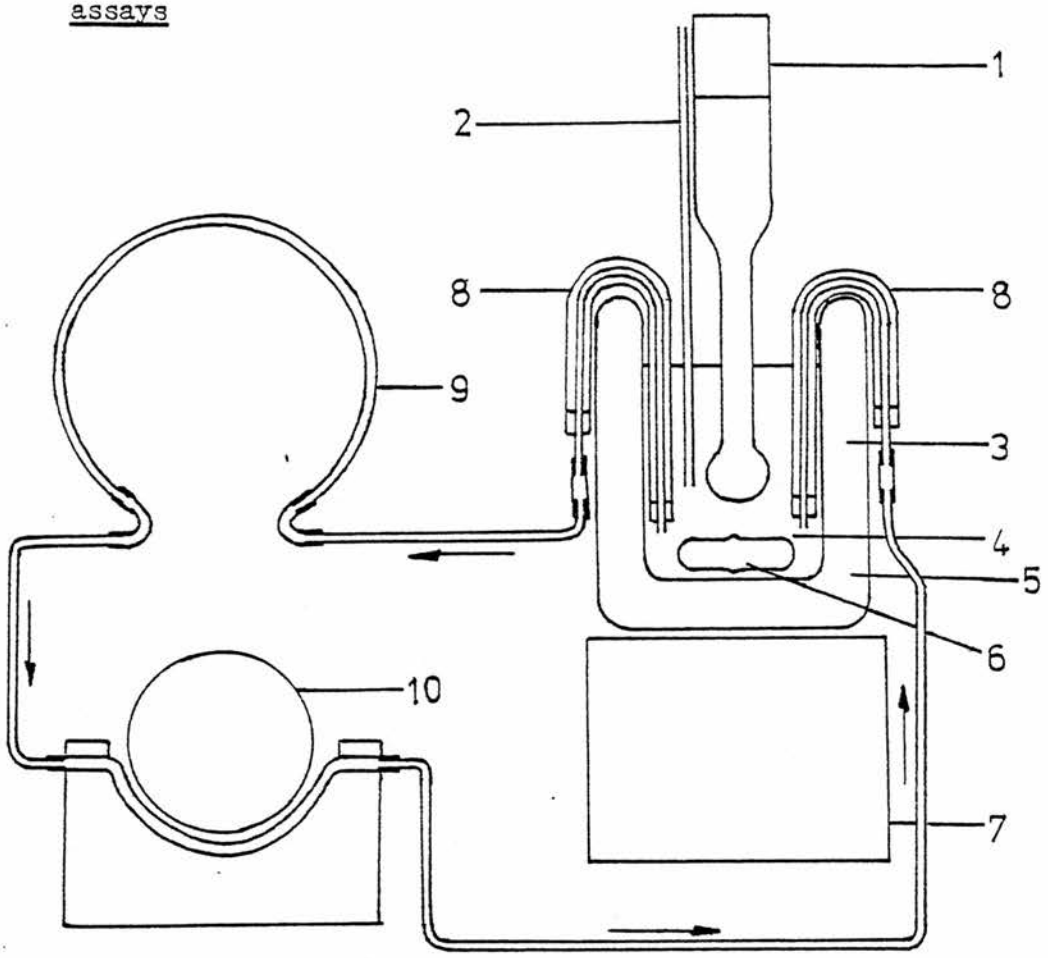
Tube-immobilised trypsin was assayed using a recycling system (fig. 3.2) similar to that used by Ford (22)

A Watson and Marlow pump adjusted to give a flow-rate of 26ml min^{-1} was used. The tube runs between the pump and the reaction vessel were kept to a minimum.

25cm lengths of the tube immobilised trypsin were found to give satisfactory performance in the assays i.e. conversion was no more than 2% per cycle.

The assay procedure used was identical to that used in 3.1.3. except that the 0.02M $CaCl_2$/6M urea solution was rapidly cycled through the nylon tube whilst its pH was adjusted to 8.0 by the addition of sodium hydroxide from the titrator syringe burette. Once a steady base-line had been established the assay was started by addition of 0.5ml BAEE.

Reaction vessel used in nylon tube immobilised trypsin assays



(fig. 3.2)

1. Activion miniature combined electrode.
2. Titrant delivery tube.
3. Constant temperature double walled, 20ml capacity vessel.
4. Assay mixture.
5. Recycling water at 25°.
6. Magnetic follower.
7. Magnetic stirrer.
8. Entry and egress points for recycling assay mixture.
9. Nylon tube immobilised trypsin sample.
10. Pump.

3.2 Preparation of Trypsin - Glutaraldehyde - Amino ethyl cellulose

3.2.1. Pretreatment of Aminoethyl cellulose

A weighed quantity of Cellex A.E. (2.3.1.) was stirred into 15 volumes (volume solution/dry weight cellulose) of 0.5M HCl and the stirring continued for 30 min. The slurry was filtered and the treated cellulose washed in the buchner funnel until the effluent was at pH 4.0. The Cellex was stirred into 15 volumes of 0.5M NaOH for a further 30 min. filtered and water washed until the effluent was at neutral pH.

The treated cellulose was then suspended in water and stored at 4°. The weight of cellulose per unit volume of stirred slurry was determined.

3.2.2. Activation of A.E. Cellulose

0.5g of pretreated Cellex A.E. was suspended with stirring in 50ml 0.1M Borate buffer pH 8.5 at room temperature. To the stirred suspension was added 10ml 20% (w/v) glutaraldehyde solution and the stirring was continued for a further 5 min. The suspension was filtered on a buchner funnel and washed five times with 50ml aliquots of 0.1M Borate buffer pH 8.5.

3.2.3. Coupling of activated A.E. Cellulose to Trypsin

25mg trypsin were dissolved in 25ml 0.1M Borate buffer pH 8.5 which was held in an ice bath. To this solution was added 0.5g of activated Cellex A.E. and the suspension was stirred in the ice bath for 2 h. The suspension was filtered and washed twice with 50ml aliquots of ice cold 0.1M Borate buffer pH 8.5. The cellulose was resuspended and stirred in 25ml water at 0°, this suspension was made 25mM w.r.t.

sodium borohydride, the pH being adjusted to between six and seven. Stirring was continued for 20 min. then the suspension made 50mM w.r.t. sodium borohydride and the pH brought to between six and seven. Stirring continued for a further 20 min. at 0°. The cellulose derivative was filtered and washed with at least 2 ℓ of water at 0° and stored as a slurry in 10ml 0.01M HCl at 4°.

3.3 Preparation of Trypsin - Adipimidate - A.E. cellulose

Cellex A.E. was pretreated as described in 3.2.1. 300mg of pretreated Cellex A.E. in aqueous suspension were filtered on a buchner and washed twice with 10ml aliquots of absolute ethanol. The washed Cellex A.E. was stirred into a solution of 100mg of diethyl adipimidate (3.9) in 8ml absolute ethanol and 2ml Nethyl morpholine (NEM) and the stirring was continued for 25 min. at room temperature. The suspension was filtered and washed with 0.1M Na_2CO_3/ $NaHCO_3$ buffer pH 10 and then stirred in 200ml of the same buffer for 1 h. The washed suspension was filtered and the activated cellulose stirred into a solution of 30mg trypsin in 10ml 0.1M Na_2CO_3/$NaHCO_3$ buffer pH 10. Stirring was continued for 1 h, the suspension was filtered and the solid washed with 500ml 0.1M Na_2CO_3/$NaHCO_3$ buffer pH 10, 500 ml water, then resuspended in a small volume of 0.01M HCl and stored at 4°.

3.4 Preparation of Trypsin - Malonimidate - A.E. cellulose

The preparation of the malonimidate immobilised trypsin derivative followed the same procedure as for the adipimidate immobilised derivative described in 3.3 except that 300mg absolute ethanol washed Cellex A.E. was stirred into a solution of 56mg malonimidate (2.5.2.) in 8ml absolute ethanol and 2ml NEM.

3.5 Preparation of Trypsin - Azide - Carboxymethyl cellulose

3.5.1. Preparation of carboxymethyl cellulose hydrazide

1g carboxymethyl cellulose (CMC) (2.3.2.) was added with stirring to 10ml water and 3ml hydrazine hydrate (50 x excess). The suspension formed was titrated down to pH 4.0 with 1M HCl. 3g of 1 cyclohexyl-3-(2 morpholino ethyl) - carbodiimide metho-p-toluene sulphonate (CDI) (2.5.5.) were added and the suspension was stirred in an ice bath for 2 h. During this time the pH of the suspension was monitored. The reaction was judged to be complete when a pH rise of at least one unit had occurred and a steady pH reading was maintained. The preparation was washed six times with water and filtered.

A sample of the preparation was tested for the presence of hydrazide by adding it plus a few crystals of 2,4,6 trinitrobenzene sulphonic acid to a saturated solution of sodium tetraborate in water, a positive hydrazide test being shown by the support turning crimson.

3.5.2. Preparation of CMC azide from the hydrazide derivative

1g CMC hydrazide was suspended in 150ml of 2% (w/v) HCl, 9ml of 3% (w/v) $NaNO_2$ solution was slowly stirred into the suspension over a period of 20 min. and the mixture was left stirring for a further 20 min. in an ice bath.

The insoluble derivative was filtered, washed with 150ml dioxan and then with water until the effluent was at pH 7.0.

3.5.3. Coupling of trypsin to CMC Azide

1g CMC Azide was stirred into 50ml 0.1M Borate buffer pH 8.5 in which 50mg trypsin had been dissolved. The suspension was stirred on ice for 2 h. and then filtered. The Trypsin-(Azide) - CMC derivative was washed with water, then

repeatedly with 0.5M NaCl and finally six times with 50ml aliquots of water. The derivative was suspended in 25ml 0.01M HCl and stored as a slurry at 4°.

3.6 Preparation of nylon-tube immobilised trypsins

3.6.1. Preparation of Trypsin - Glutaraldehyde - Nylon tube

A 5m length of nylon tube (2.3.3.) was filled with a 15% (w/v) solution of triethyloxonium tetrafluoroborate (2.5.6.) in dichloromethane and allowed to stand at room temperature for 30 min. to allow alkylation to proceed (23). The tube was then washed for 10 s. with dry dichloromethane and filled with 0.5M hexamethylene diamine in methanol. After standing at room temperature for 30 min. the hexamethylene diamine was washed out with water and the washing continued for 4 h.

The alkylated nylon tube was activated by recycling for 10 min. with 5% (w/v) glutaraldehyde solution in 0.1M Borate buffer pH 8.5 and was then washed with water for 15 min.

Coupling with enzyme was achieved by filling the tube with a solution of 2mg ml^{-1} trypsin in 0.1M borate buffer, pH 8.0. The tube was left at room temperature for 2 h. then washed with water, perfused overnight with 0.5M NaCl and finally washed with water.

3.6.2. Preparation of Trypsin - Adipimidate - Nylon tube

A 5m length of nylon tube was alkylated and amine substituted by treatment with triethyloxonium tetrafluoroborate and hexamethylene diamine as in 3.6.1

The water washed tube was dried by perfusion with dry methanol for 10m, drying being carried out to prevent hydrolysis of the bisimidate.

The tube was activated by recycling for 10 min at room temperature with a freshly prepared 4% (w/v) solution of diethyl adipimidate (3.9.) in 20% Nethyl morpholine in dry methanol.

After recycling the tube was rapidly washed with dry methanol and immediately filled with a solution of trypsin (1mg/ml solution) in 0.1M N ethyl morpholine buffer, pH 8.5. The tube was left at room temperature for 2h, then washed with water, perfused overnight with 0.5M NaCl and finally washed with water.

3.7 Preparation of soluble O-Carboxymethyl cellulose

15g of cellulose fibres were dispersed in 400ml of propan-2-ol and the suspension stirred vigorously while 40ml of 30% (w/v) aqueous NaOH were added over a period of 30 min. at room temperature. The stirring was continued for a further 1h, then 18g chloracetic acid were added to the mixture in a 1 ℓ beaker over a period of 30 min. The beaker was covered with aluminium foil and left overnight at room temperature.

The liquid was drained from the still fibrous product and the latter was stirred in 70% (v/v) methanol while sufficient 90% (v/v) acetic acid was added to neutralise the excess alkali. The product was drained, washed with 70% methanol, with absolute methanol and finally dried at 60°.

The resulting product was readily soluble in water.

3.8 Preparation of hydrolysed haemoglobin

3.8.1. Preparation of haemoglobin solution prior to hydrolysis

144g urea and 1.8g $CaCl_2 . 2H_2O$ were dissolved in water and made to 350ml with water in a 600ml beaker. 5g haemoglobin

were slowly stirred into the solution and when this had completely dissolved the volume of solution was made to 390ml with water. The pH of the solution was adjusted to pH 8.0 with 0.05M NaOH.

3.8.2. Preparation of titrator control base line for haemoglobin hydrolysis

A control was prepared by adjusting the pH of 400ml water to pH 8.0 in an identical beaker to that used in 3.8.1. The stirred contents at pH 8.0 were titrated against 0.05M NaOH using the Radiometer titrator over a period of several hours.

The titration curve obtained gave a base line curve for the uptake of NaOH titrant due to absorption of atmospheric CO_2 by the solution.

3.8.3. Enzymic hydrolysis of haemoglobin solution using the titrator

10ml trypsin solution, concentration 20mg ml^{-1}, pH 8.0, were added to 390ml haemoglobin solution from 3.8.1. The mixture was slowly stirred and autotitrated at pH 8.0 against 0.25M NaOH using a Radiometer titrator. Autotitration was continued until the rate of alkali consumption was equal to the rate of alkali consumption in the titrator control blank (3.8.2). This process took approximately 36h.

3.8.4. Preparation of 1% hydrolysed haemoglobin solution

The hydrolysed haemoglobin (3.8.3) was incubated at 75° to inactivate the trypsin.

Inactivation was judged to be complete when a 1ml sample of the haemoglobin solution indicated no tryptic activity when tested using the titrator assay technique (3.1.2).

The hydrolysed haemoglobin solution was made to 500 ml with an aqueous solution 6M w.r.t. Urea, 0.02M w.r.t. Ca^{++}

3.9. Preparation of Diethyl adipimidate

Diethyl adipimidate was prepared by the Pinner synthesis (24). 21.6g adiponitrile (2.1.) and 18.4g (23.2ml) of dry ethanol were dissolved in 250ml dry dioxan. The solution was cooled in a bucket of crushed ice until its temperature was below 4°. This temperature was maintained while dry HCl gas was bubbled through the solution. The HCl addition was continued until 24g of gas had been added, this quantity being in excess of the stoichiometric requirement (14.6g).

The flask was sealed and left refrigerated at 4° for 110h. The imidate was precipitated by the addition of cold dry ether, the precipitate was filtered, washed with cold dry ether and stored in a dessicator under vacuum.

The yield was 48.8g (89% of maximum theoretical yield).

3.10 Thermal denaturation of trypsin samples

3.10.1 Thermal denaturation of soluble trypsin

The sample of trypsin solution was held in a tall form, thin walled, polythene capped, 10ml capacity glass specimen tube. The tube was filled to no more than half its capacity and was clamped in a water bath so that it was submerged to over three quarters of its length in the bath. The solution was stirred continuously by a miniature glass coated magnetic follower driven by a submersible stirrer.

The temperature of the water bath was adjusted to the required value and the tube containing the trypsin solution stirred at that temperature for 10 min. to allow for temperature stabilisation before any samples were assayed.

Samples for assay were removed by a preheated 0.1ml blow out pipette.

3.10.2 Thermal denaturation of trypsin immobilised on cellulose

The method used was identical to that in 3.10.1 but the rate of stirring was increased to ensure even distribution of the suspended particles.

Sampling was done by a 0.1ml preheated constriction pipette since this was found to be less prone to blocking by the solid particles in the suspension.

3.10.3 Thermal denaturation of trypsin immobilised on nylon tube

The nylon tube segments were filled with 0.1M HCl from a syringe and formed into loops which were closed by silicon sleeving. The loops were clamped below the surface of a water bath and held at the desired temperature for at least 10 min.

When required for assay they were rapidly perfused with 6M urea solution in an attempt to freeze the active trypsin/ denatured trypsin equilibrium and then connected to the recycling assay system illustrated in fig. 3.2

4. RESULTS

4.1 Verification of the work of Anson and Mirsky

4.1.1. Construction of a standard curve for soluble trypsin concentration v absorbance

A standard curve of soluble trypsin concentration v absorbance was constructed (fig. 4.1) using the trypsin assay method described in 3.1.1.

A stock trypsin solution, 1mg/ml in 0.01M HCl solution, was used to prepare dilutions of trypsin giving a concentration range from 0.25 to 1.0mg/ml 0.01M HCl solution and a final trypsin concentration in the assay ranging from 0.005 to 0.02mg/ml of assay mixture.

4.1.1. Standard curve for (Soluble Trypsin) v Absorbance

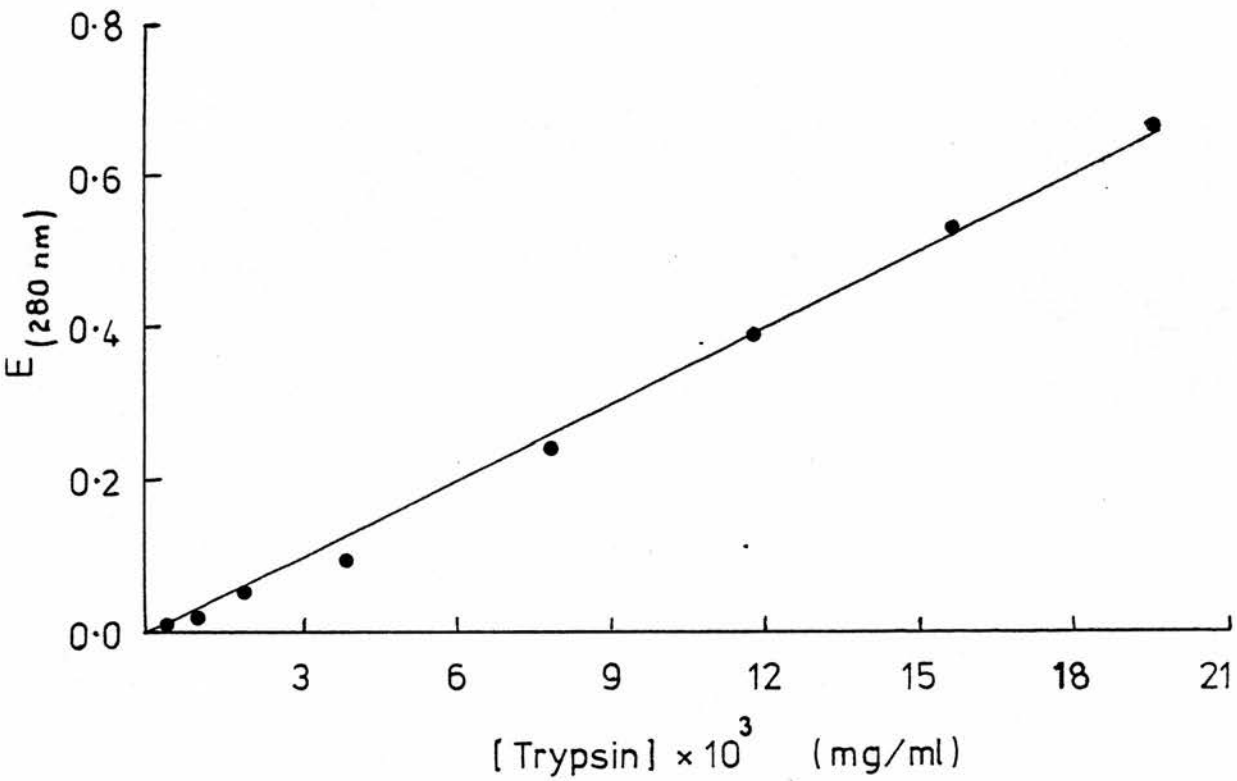


Fig. 4.1

4.1.2. Thermal denaturation of soluble trypsin using the haemoglobin substrate assay technique

The thermal denaturation of soluble trypsin was performed using the method described in 3.10.1. The active trypsin remaining at each temperature was determined as described in 3.1.1.1

The results were plotted (fig. 4.2)

The stock trypsin solution used was of concentration 4mg trypsin/ml 0.01M HCl solution.

RESULTS

r^2 = 0.957

$T_{\frac{1}{2}}$ = 318.6°K

ΔH = +304.3 kJ mol^{-1}

ΔS = +955.13 J mol^{-1} K^{-1}

4.1.2. Thermal denaturation of soluble trypsin using the haemoglobin substrate assay technique

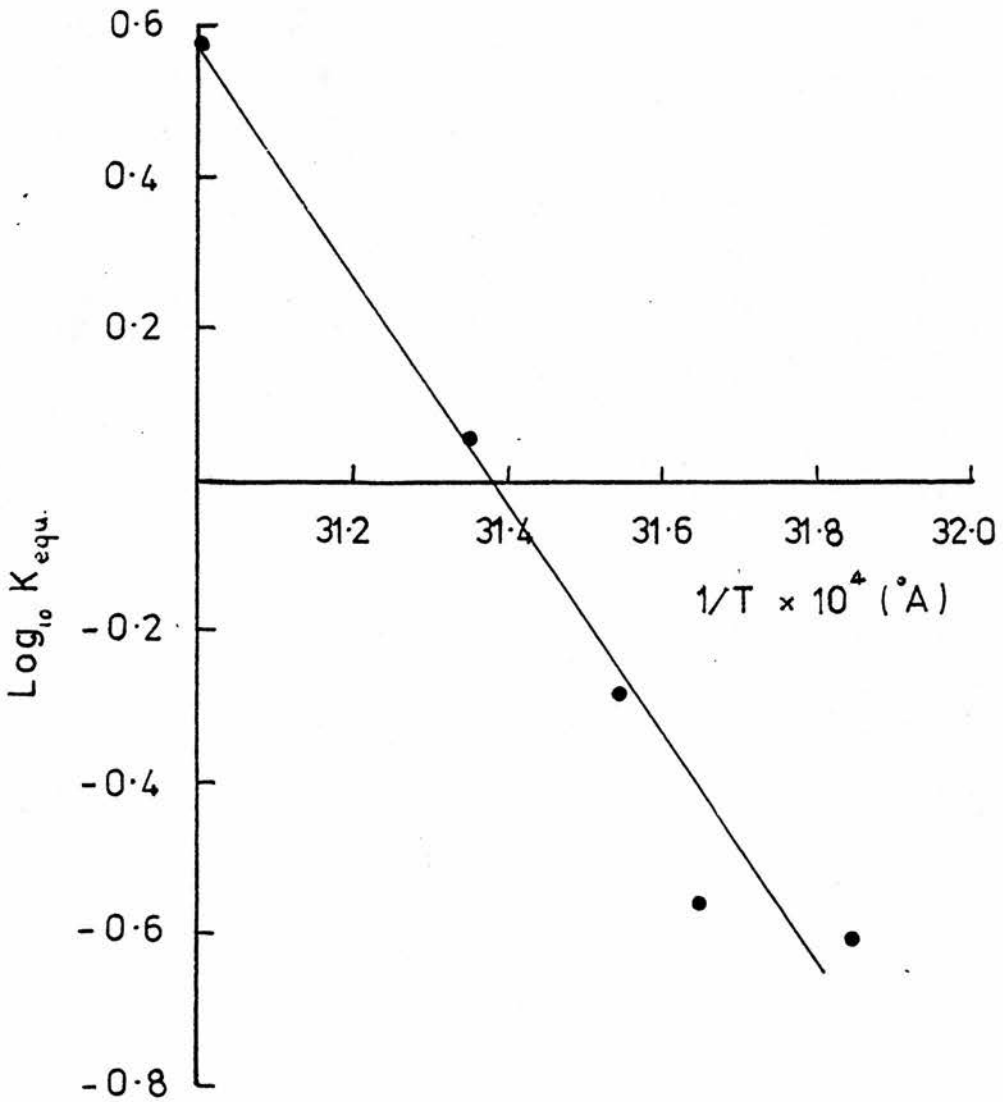


Fig. 4.2

4.1.3. Thermal denaturation of immobilised trypsin derivatives

Three aminoethyl cellulose immobilised derivatives were checked for tryptic activity using the method described in 3.1.1.2

The immobilised derivatives were

(i) Trypsin - Glutaraldehyde - Cellex AE (3.2)

(ii) Trypsin - Adipimidate - Cellex AE (3.3)

(iii) Trypsin - Malonimidate - Cellex AE (3.4)

Thermal denaturation of each derivative was attempted and in all cases the absorbance measurements for the samples held over the various temperatures of the thermal denaturation range (25° to 50°) were of a random nature. Also when the heated samples were cooled and equilibrated at 25° there appeared to be no reversibility of denaturation.

4.2 Standard curves for soluble trypsin concentration determination using the titrimetric assay with BAEE as substrate

Standard curves (fig. 4.3) were plotted for

(i) Concentration of soluble trypsin in 0.02M Ca^{++} solution v Rate of hydrolysis of BAEE

(ii) Concentration of soluble trypsin in 6M Urea/0.02M Ca^{++} solution v Rate of hydrolysis of BAEE

using the methods described in 3.1.2 and 3.1.3 respectively.

4.2. Standard curves for soluble trypsin concentration determination using the titrimetric assay with BAEE as substrate

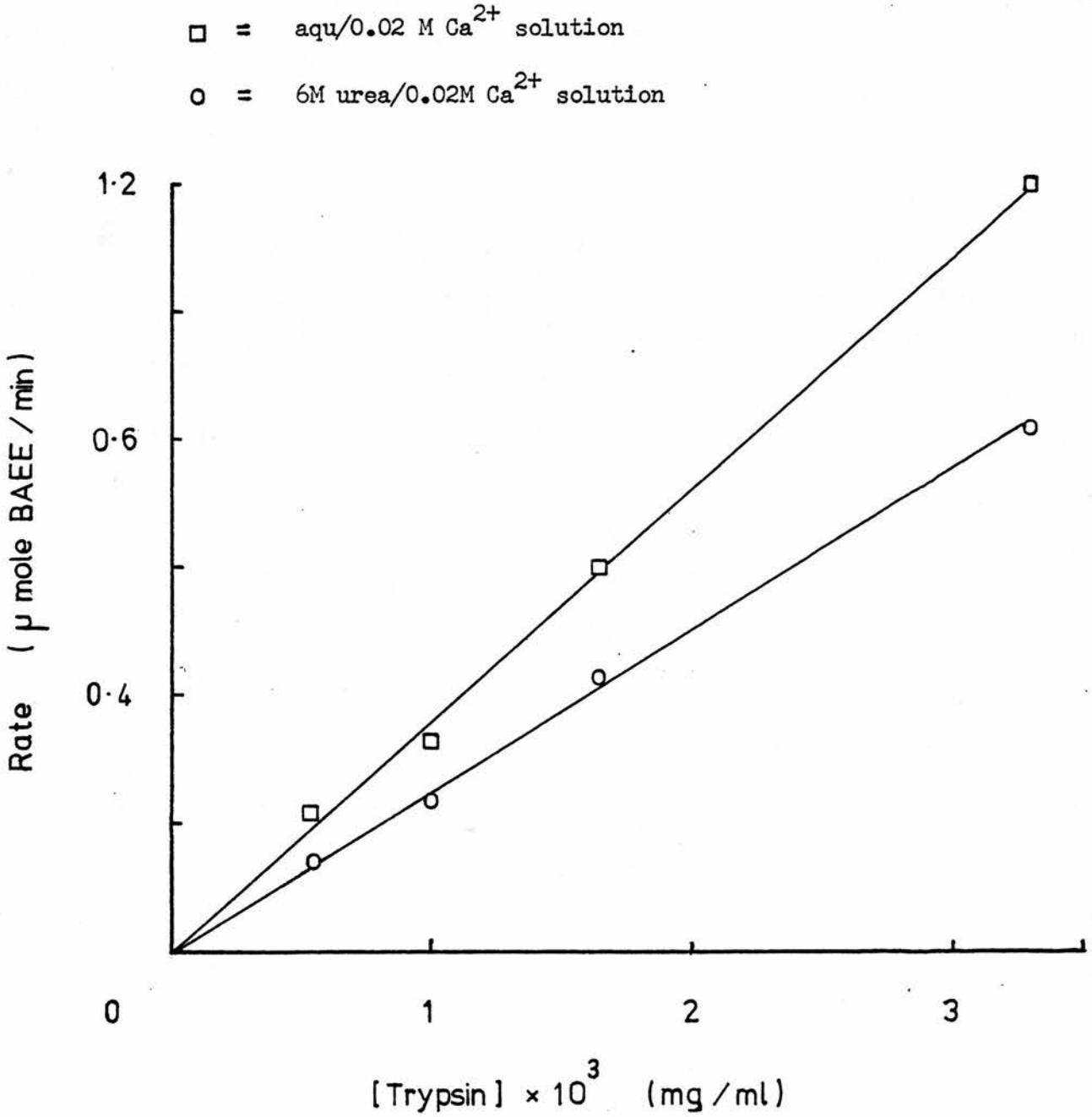


Fig. 4.3

4.3 Thermal denaturation of soluble trypsin using the BAEE substrate assay technique

The thermal denaturation of soluble trypsin was performed using the method described in 3.10.1.

The active trypsin remaining at each temperature was determined by the assay described in 3.1.3.1

The results were plotted (fig. 4.4)

RESULTS

r^2 = 0.986

$T_{\frac{1}{2}}$ = 306.067°K

ΔH = +165.53 kJ mol^{-1}

ΔS = +539.76 J mol^{-1} K^{-1}

4.3. Thermal denaturation of soluble trypsin using the BAEE substrate assay technique

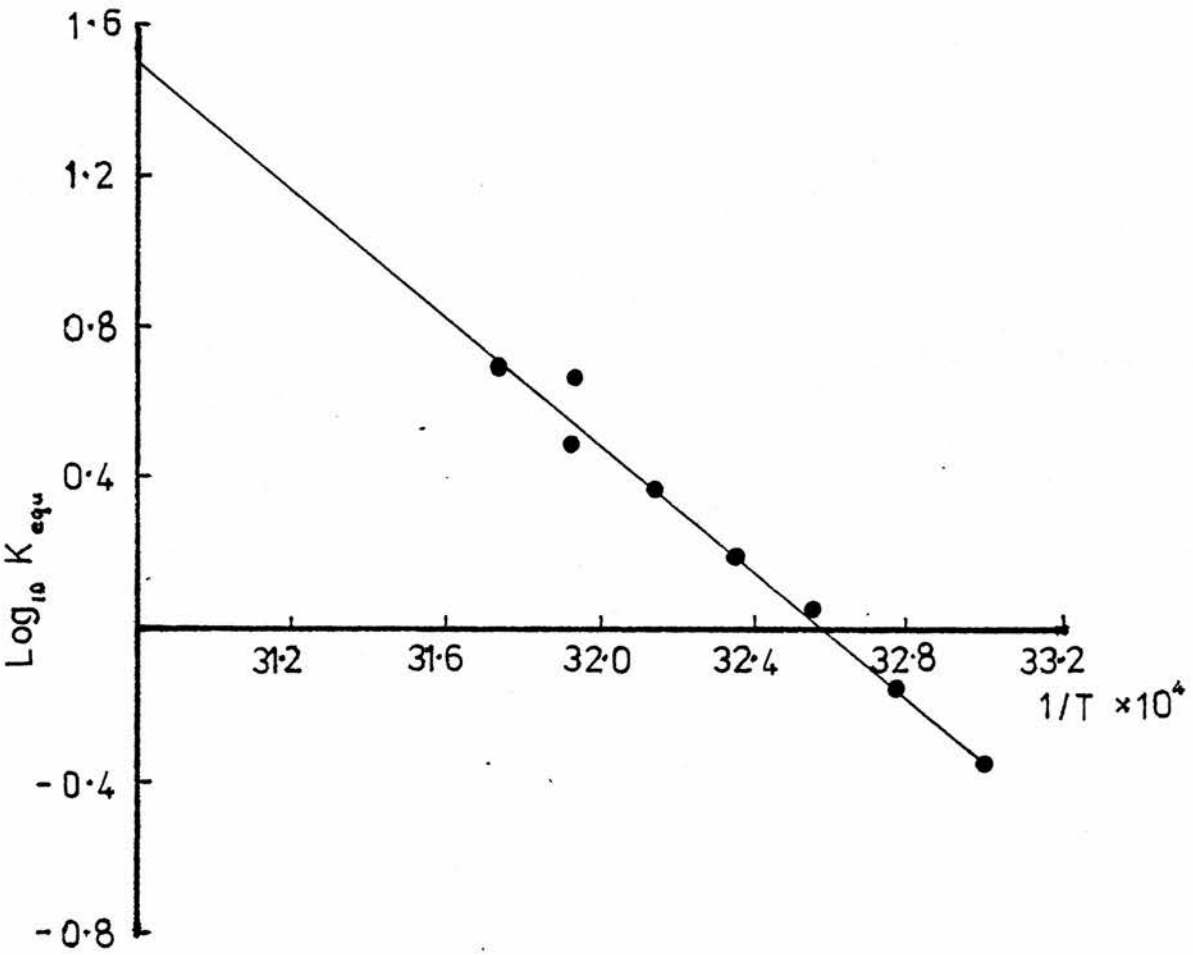


Fig. 4.4

4.4 Thermal denaturation of soluble trypsin assayed in the presence of soluble carboxymethyl cellulose

The thermal denaturation of soluble trypsin was repeated as described in 3.10.1

The active trypsin remaining at each temperature was assayed in the presence of 1% (w/v) soluble carboxymethyl cellulose (3.6) using the method described in 3.1.3.2

The results were plotted fig. (4.5)

RESULTS

r^2 = 0.998

$T_{\frac{1}{2}}$ = 317.6°K

ΔH = +244.11 kJ mol^{-1}

ΔS = +768.61 kJ mol^{-1} K^{-1}

4.4. Thermal denaturation of soluble trypsin in the presence of 1% soluble carboxymethyl cellulose

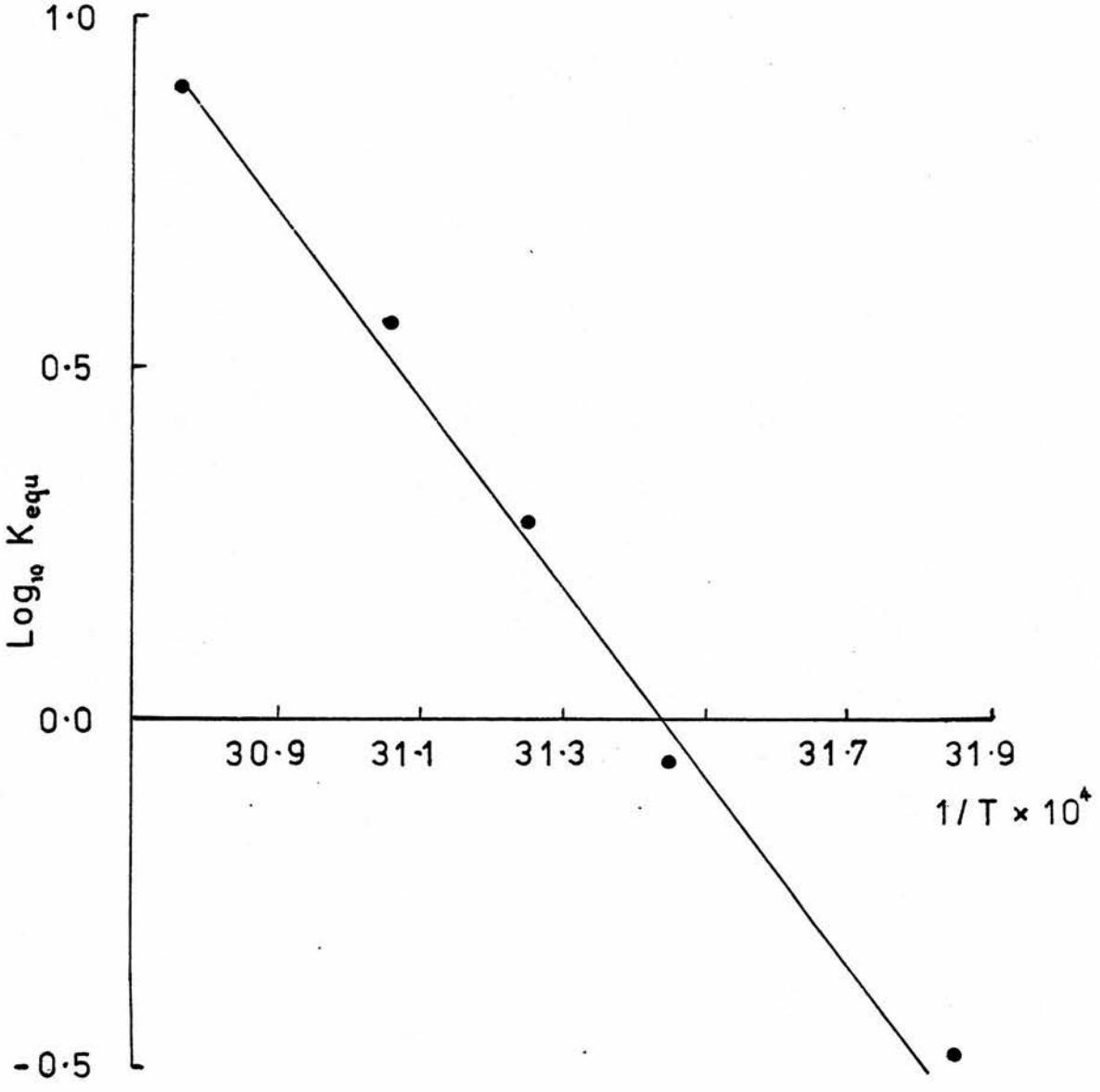


Fig. 4.5

4.5 Thermal denaturation of soluble trypsin assayed in the presence of hydrolysed haemoglobin

The thermal denaturation of soluble trypsin was repeated as described in 3.10.1

The active trypsin remaining at each temperature was assayed in the presence of 1% (w/v) hydrolysed haemoglobin (3.8) using the method described in 3.1.3.3

The results were plotted (fig. 4.6)

RESULTS

r^2 = 0.998

$T_{\frac{1}{2}}$ = 318.02°K

ΔH = +292.18 kJ mol^{-1}

ΔS = +918.76 J mol^{-1} K^{-1}

4.5. Thermal denaturation of soluble trypsin assayed in the presence of 1% hydrolysed haemoglobin

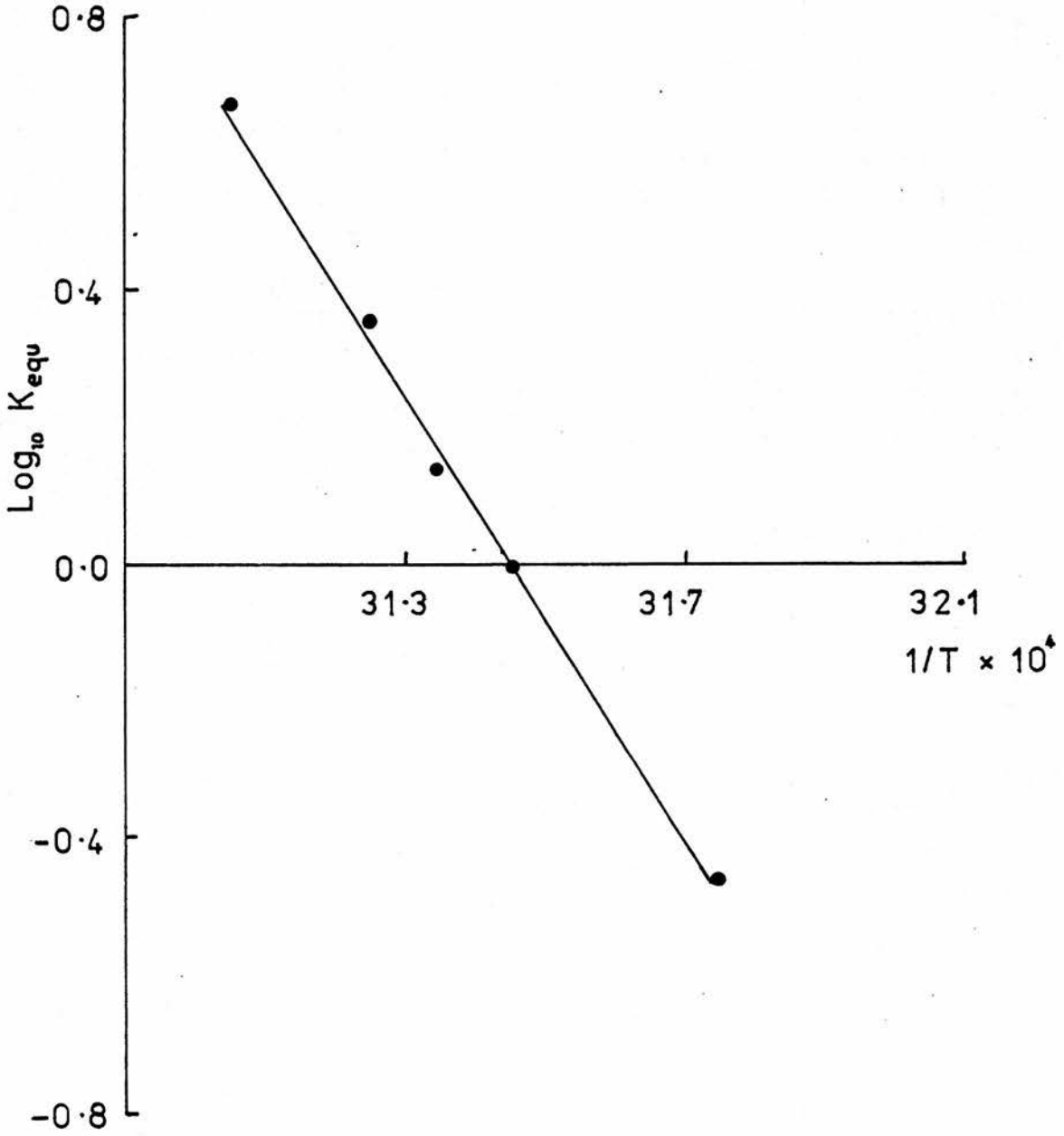


Fig. 4.6

4.6 Thermal denaturation of Trypsin - Glutaraldehyde - Cellex AE (T - G - C)

Thermal denaturation of T - G - C (3.2) was carried out using the method described in 3.10.2

The active trypsin remaining at each temperature was assayed using the method described in 3.13.4

The results were plotted (fig. 4.7)

RESULTS

r^2 = 0.979

$T_{\frac{1}{2}}$ = 319.4°K

ΔH = +125.4 kJ mol^{-1}

ΔS = +393.09 J mol^{-1} K^{-1}

4.6. Thermal denaturation of T-G-C

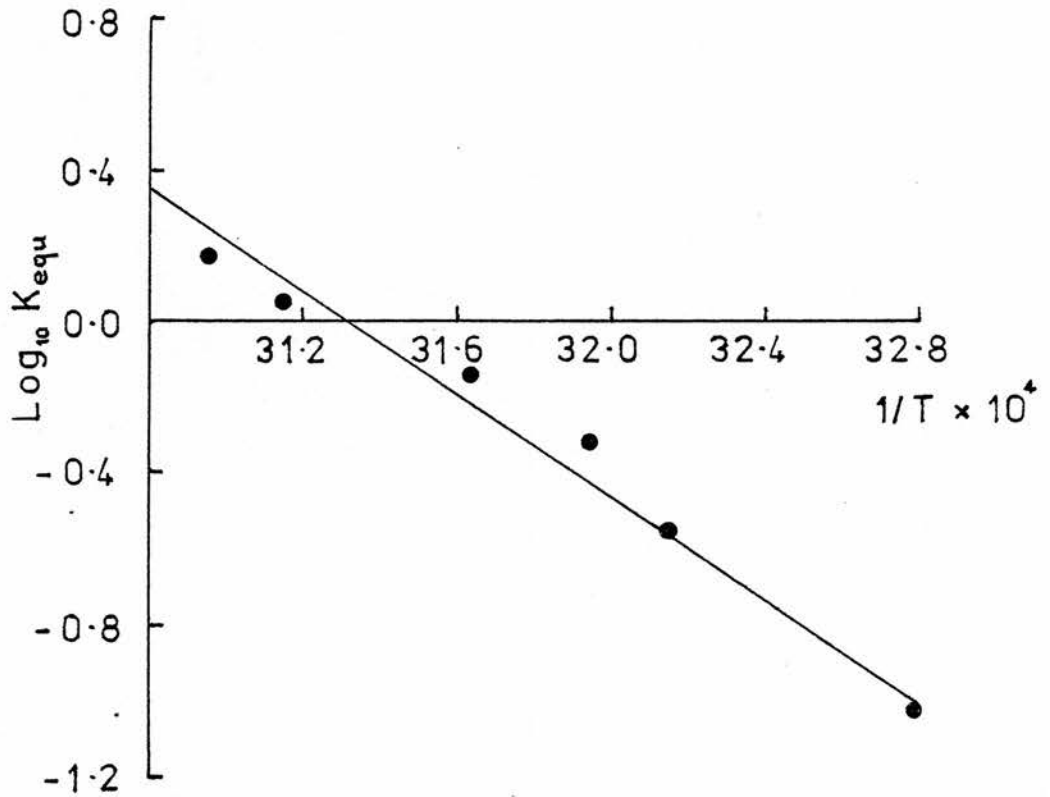


Fig. 4.7

4.7 Thermal denaturation of Trypsin - Azide - Carboxy methyl cellulose (T - Az - CMC)

Thermal denaturation of T - Az - CMC (3.5) was carried out using the method described in 3.10.2

The active trypsin remaining at each temperature was assayed using the method described in 3.1.3.4

The results were plotted (fig. 4.8)

RESULTS

r^2 = 0.99

$T_{\frac{1}{2}}$ = 335°K

ΔH = +119.34 kJ mol^{-1}

ΔS = +356.24 J mol^{-1} K^{-1}

4.7. Thermal denaturation of T-Az-CMC

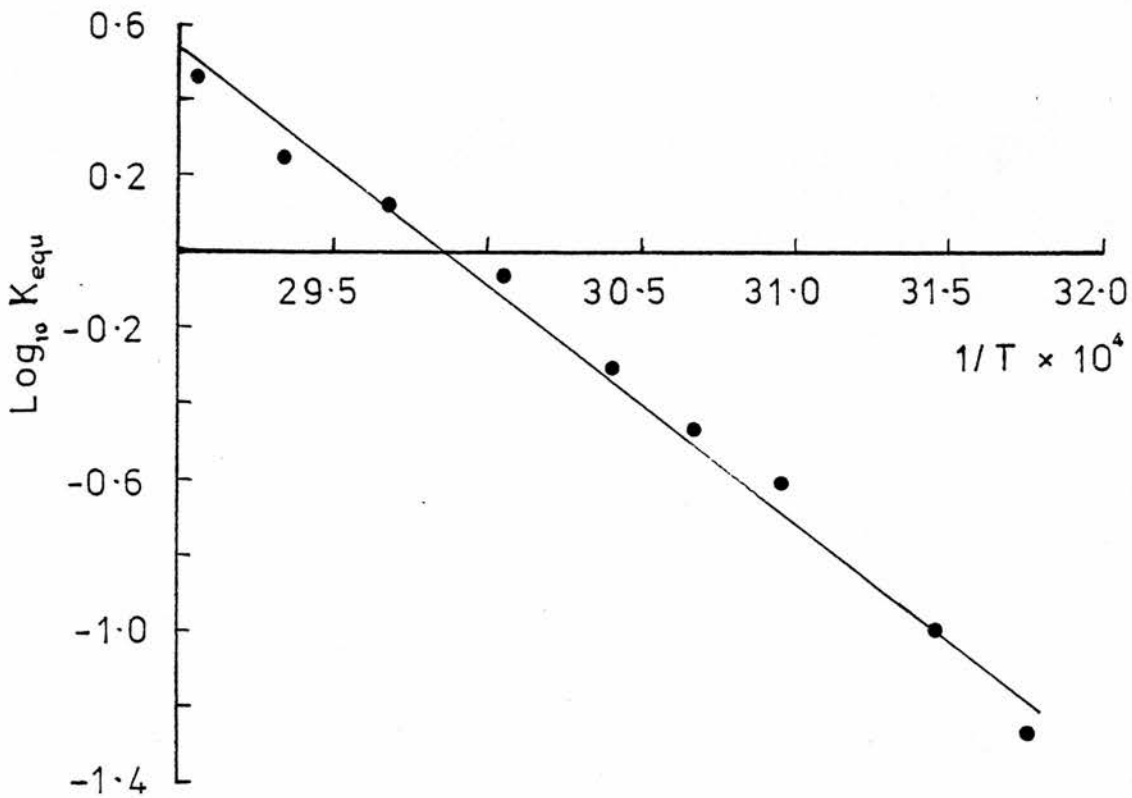


Fig. 4.8

4.8 Testing nylon tube immobilised trypsin for uniformity of binding

The activity of various lengths of trypsin immobilised nylon tube 3.6.1, 3.6.2 were tested for tryptic activity using the recycling titrator assay technique as described in 3.1.3.5 The optimum length of tube to give an acceptable rate of substrate hydrolysis under the conditions of the assay was found to be 25cm.

Four 0.5m lengths of tube were cut into 25cm segments and labelled. Each segment was assayed in turn and the tryptic activity was found to be the same for all the tube segments to within 2%.

4.9. Thermal denaturation of nylon tube immobilised trypsin

Thermal denaturation was attempted on several samples of nylon tube immobilised trypsins using the method described in 3.10.3.

Tryptic activity of the samples decreased as the equilibrium temperature increased, but on equilibrating the tube at 25° after each thermal denaturation the tryptic activity was not restored to its original pre heat treatment value. The activity was found to remain at the decreased level.

If the same tube was once more raised to a higher equilibrium temperature the reduced activity level was found to reduce further and to remain at the lower level after equilibrating once more at 25°.

Thus nylon tube immobilised trypsin samples showed no reversibility of thermal denaturation but underwent a progressive irreversible denaturation.

Summary of entropy and enthalpy changes associated with the thermal denaturation of soluble and immobilised trypsins

State of Enzyme	Assay Type	ΔS J mol^{-1} K^{-1}	ΔH kJ mol^{-1}	$T_{\frac{1}{2}}$ K
Soluble (A and M results)	Spectrophotometric	907	+287.8	+317.1
Soluble	Spectrophotometric	955.13	+304.3	+318.6
Soluble	Titrimetric	539.76	+165.5	+306.1
Soluble/1% CMC	Titrimetric	768.6	+244.1	+317.6
Soluble/1% Hydrolysed Haemoglobin	Titrimetric	918.8	+292.18	+318.6
Immobilised Trypsin/Glutaraldehyde/Cellex AE	Titrimetric	393.1	+125.4	+319.4
Immobilised Trypsin/Azide/Carboxy methyl cellulose	Titrimetric	356.2	+119.3	+335

Table 4.1.

5. DISCUSSION

The modified Anson and Mirsky method for the determination of tryptic activity using haemoglobin as substrate (3.1.1.) was found to give values for the enthalpy change, ΔH, and the entropy change, ΔS, for the thermal denaturation of soluble trypsin (4.1.2.) which were comparable with those of the original Anson and Mirsky method.

Trypsin immobilised on AE cellulose using glutaraldehyde or the bis-imidates, adipimidate or malonimidate, showed no reversibility of denaturation in that, after heating and then cooling a sample of immobilised trypsin, the trypic activity did not return to its original value as was the case with the soluble enzyme.

It was conjectured that the haemoglobin or the products of haemoglobin hydrolysis might have been adsorbed on the cellulose support material thus altering the A_{280nm} reading which was used to measure tryptic activity from a standard curve.

As a result of the failure of the haemoglobin substrate method to give reliable determinations of the activity of trypsin immobilised on a cellulose support an alternative assay procedure was sought. A titrimetric method using a small ester molecule, BAEE, as substrate was adopted (3.1.3.). Standard curves were produced to relate trypsin concentration to the rate of ester hydrolysis for the assay performed both in aqueous solution and in high urea concentration (4.2.). The standard curves showed linear proportionality over the range of trypsin concentrations used.

Thermal denaturation was performed on soluble trypsin using this alternative assay method (4.3.), the denaturation was found to be reversible in that when a trypsin sample was heated its

activity decreased due to its partial denaturation, the original activity was totally regained after the same sample was cooled to 25°C and re-assayed at 25°C. in the absence of urea.

Using the titrimetric assay procedure the ΔH and ΔS values for the thermal denaturation of soluble trypsin were found to be approximately 50% of the values obtained using the spectrophotometric assay with haemoglobin as substrate.

A theory to account for the decrease in values was that the presence of haemoglobin in the assay solution of the original spectrophotometric assay method may have caused some protein-protein stabilization of the enzyme structure which was not present in the assay using the small ester molecule. To test this theory it was proposed that the thermal denaturation of trypsin be performed in the presence of a large molecular weight substance which,

(1) had a similar charge to the haemoglobin at the pH of the assay

(2) was not hydrolysable by the enzyme

(3) was at the same concentration as the haemoglobin in the original assay mixture

The material chosen was soluble carboxy methyl cellulose (3.7.).

The values for ΔH and ΔS for the thermal denaturation of soluble trypsin in the presence of 1% CMC (4.4.) were found to be consistently higher (i.e. 83% of the original haemoglobin substrate values), than those for the denaturation without CMC. This would indicate that stabilization of the enzyme structure had occurred in the original haemoglobin substrate assays. If stabilization could be produced to the same degree as in the original assay with haemoglobin the ΔH and ΔS values for the thermal denaturation of

the soluble trypsin should be restored to their original values. Thermal denaturation of soluble trypsin in the presence of 1% (w/v) solution of trypsin hydrolysed haemoglobin (4.5.), previously checked for lack of tryptic activity, were performed. The ΔH and ΔS values were found to be within 96% of those determined using the original haemoglobin substrate assay.

The titrimetric assay using BAEE as substrate was thus validated as a method for obtaining ΔH and ΔS values for the thermal denaturation of soluble trypsin. These were the values which were subsequently used for comparison with those obtained from thermal denaturations of immobilised trypsins.

The values of ΔS for the thermal denaturation of cellulose immobilised trypsins using two different chemistries of immobilisation, namely glutaraldehyde (3.2.) and azide (3.5.) coupled protein, were always considerably lower, 41% and 33% respectively, than the value for soluble trypsin (Table 4.1.). This indicated that the immobilised trypsin molecules had greater conformational stability than the soluble molecules, most probably brought about by the covalent binding of the enzyme protein to the support material via. the spacer molecules. Binding of this type would restrict the freedom of the protein to relax its structure as thermal denaturation occurred. Such a reduction in randomness would thus be reflected in a smaller increase of entropy accompanying the denaturation process.

In the case of nylon tube immobilised trypsin using glutaraldehyde (3.6.1.) or adipimidate (3.6.2.) as spacer molecules the tube samples, after coupling, were found to show tryptic activity when assayed using BAEE as substrate in a

recycling titrator assay technique (3.1.3.5.) at 25°C. Activity was not affected by the presence of urea in the assay mixture and provided the tubes were stored at ambient temperatures or below, was maintained for several weeks without noticeable activity loss.

Any active tube which was subjected to the heating and perfusion with 6M urea treatment, required to test for denaturation of the enzyme, was found to show a diminution in tryptic activity, the extent of which increased with increase in temperature until all activity was lost at temperatures of 60°C and above.

Full tryptic activity was not regained when heat treated tubes, after removal of urea from the assay mixture, were re-assayed at 25°C to check the reversibility of the denaturation. In all cases the activity on re-assay at 25°C was found to be the same as that at the higher temperature. This indicated that for these nylon tube immobilised trypsins thermal denaturation was no longer the reversible process shown by the immobilised trypsins using cellulose as a support medium or by soluble trypsin. Thermal denaturation of the nylon tube immobilised trypsin had become irreversible.

Reheating of a tube showing partial activity loss in a previous heating/urea perfusion treatment resulted in further activity loss. If such reheating was carried out several times all activity for a particular tube was eventually lost. This confirmed the irreversibility of the thermal denaturation.

This behaviour was probably due to the attraction of the denatured enzyme protein to the charged nylon structure of the activated tube, the attraction being strong enough to prevent the relaxed structure of the thermally denatured enzyme protein

regaining its original conformation when urea was removed and the temperature lowered.

Although the results from nylon tube immobilised trypsins were inconclusive those from the various cellulose immobilised examples clearly showed the predicted decrease in entropy change shown by the immobilised enzyme compared to the soluble enzyme on thermal denaturation. This would indicate that the enzyme protein was actually covalently bound to the support matrix rather than held at the surface of the support by physical adsorption.

These results are paralleled by those of Moore and Greenwood (25) in the investigation of the effect of temperature on the bleaching of the 695nm band of ferricytochrome c, insolubilised on agarose gel. The insolubilisation results in a species of ferricytochrome c which retains the 695nm band of the soluble species. The band is very sensitive to conformational changes within the molecule and disappears with increasing molecular denaturation, being completely bleached before any major conformational changes occur. In all experiments with insoluble cytochrome c the 695nm band is found not to be as sensitive to temperature as that of soluble cytochrome c and the entropy change for the denaturation process is found to be decreased by more than 20% on insolubilisation.

If trypsin were cross linked using gluteraldehyde to form higher molecular weight agglomerates, this should have the effect of stabilizing the conformation of the cross linked units. Provided such agglomerates underwent reversible thermal denaturation a reduction in entropy change on denaturation would be expected. Thus a further avenue of investigation might be to compare the entropy changes on thermal denaturation of soluble trypsin with those of cross linked trypsin and cross linked trypsin immobilised on a variety of support material.

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7. APPENDIX

Since the Van't Hoff equation,

$$\text{Log}_{10} K = -\frac{\Delta H}{2.303RT} + \text{constant},$$

is of the form $y = a_1 x + a_o$, where a_1 and a_o are constants, linear regression by the method of least squares was the technique used to find values for a_1 and a_o which gave closest agreement between experimental data and the straight line equation.

The program illustrated (Table 6.1.) was input into an HP25 Programmable scientific calculator. Paired values of data (x_i, y_i), $i = 1, \ldots\ldots, n.$ were input and the regression constants a_1 and a_o calculated. A third value, r^2, the coefficient of determination, was also found. The value of r^2 lies between 0 and 1, and indicates how closely the equation fits the experimental data, the closer r^2 is to 1 the better the fit.

For the equation $y = a_1 x + a_o$

Regression constant:

$$a_1 = \frac{\Sigma xy - \frac{\Sigma x . \Sigma y}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

$$a_o = \bar{y} - a_1 \bar{x} \qquad \text{Where } \bar{y} = \frac{\Sigma y}{n}$$

$$\bar{x} = \frac{\Sigma x}{n}$$

Coefficient of determination:

$$\frac{\left[\Sigma xy - \frac{\Sigma x \Sigma y}{n}\right]^2}{\left[\Sigma x^2 - \frac{(\Sigma x)^2}{n}\right]\left[\Sigma y^2 - \frac{(\Sigma y)^2}{n}\right]}$$

The gradient of the line corresponds to the value of a_1.

Thus ΔH can be found:

$$\text{Gradient} = a_1 = -\frac{\Delta H}{2.303R}$$

The intercept of the line on the x axis corresponds to the value of a_2, where $a_2 = a_o/a_1$

Thus $t_{\frac{1}{2}}$ can be found:

$$\text{Intercept} = a_2 = T_{\frac{1}{2}}$$

Hewlett-Packard HP-25 Program

DISPLAY LINE	DISPLAY CODE	KEY ENTRY	X	Y	Z	T	COMMENTS
00			y	x			Steps 1-7 for summation
01	31	f	y	y	x		
02	15 02	g x^2	y^2	y	x		
03	23 51 02	STO + 2	y^2	y	x		Σy^2
04	22	R↓	y	x		y^2	
05	21	x⇄y	x	y		y^2	
06	25	Σ+	n	y		y^2	n, Σy, Σxy, Σx^2, Σx
07	13 00	GTO 00	n	y		y^2	
08	24 05	RCL 5	Σxy				
09	24 07	RCL 7	Σx	Σxy			
10	24 04	RCL 4	Σy	Σx	Σxy		
11	61	x	Σx Σy	Σxy			
12	24 03	RCL 3	n	Σx Σy	Σxy		
13	71	÷	Σx $\Sigma y/n$	Σxy			*
14	41	-	C				$C = \Sigma xy - (\Sigma x\ \Sigma y/n)$
15	24 06	RCL 6	Σx^2	C			
16	24 07	RCL 7	Σx	Σx^2	C		
17	15 02	g x^2	$(\Sigma x)^2$	Σx^2	C		
18	24 03	RCL 3	n	$(\Sigma x)^2$	Σx^2	C	
19	71	÷	$(\Sigma x)^2/n$	Σx^2	C	C	
20	41	-	D	C	C	C	$D = \Sigma x^2 - [(\Sigma x)^2/n]$
21	71	÷	a_1	C	C	C	$a_1 = C/D$
22	23 01	STO 1	a_1	C	C	C	
23	24 07	RCL 7	Σx	a_1	C	C	
24	61	x	a_1 Σx	C	C	C	
25	32	CHS	$-a_1$ Σx	C	C	C	
26	24 04	RCL 4	Σy	$-a_1$ Σx	C	C	
27	51	+	$\Sigma y - a_1$ Σx	C	C	C	
28	24 03	RCL 3	n	$\Sigma y - a_1$ Σx	C	C	
29	71	÷	a_0	C	C	C	$a_0 = \bar{y} - a_1 \bar{x}$
30	23 00	STO 0	a_0	C	C	C	
31	74	R/S	a_0	C	C	C	Halt to display a_0
32	24 01	RCL 1	a_1	a_0	C	C	
33	74	R/S	a_1	a_0	C	C	Halt to display a_1
34	21	x⇄y	a_0	a_1	C	C	
35	22	R↓	a_1	C	C	a_0	
36	61	x	a_1 C	C	a_0	a_0	
37	24 02	RCL 2	Σy^2	a_1 C	C	a_0	
38	24 04	RCL 4	Σy	Σy^2	a_1 C	C	
39	15 02	g x^2	$(\Sigma y)^2$	Σy^2	a_1 C	C	
40	24 03	RCL 3	n	$(\Sigma y)^2$	Σy^2	a_1 C	
41	71	÷	$(\Sigma y)^2/n$	Σy^2	a_1 C	a_1 C	
42	41	-	E	a_1 C	a_1 C	a_1 C	$E = \Sigma y^2 - [(\Sigma y)^2/n]$
43	71	÷	r^2	a_1 C	a_1 C	a_1 C	$r^2 = a_1$ C/E
44	13 00	GTO 00	r^2	a_1 C	a_1 C	a_1 C	
45							
46							
47							
48							
49							

REGISTERS

R 0 — a_0
R 1 — a_1
R 2 — Σy^2
R 3 — n
R 4 — Σy
R 5 — Σxy
R 6 — Σx^2
R 7 — Σx

Load Data/Execute Program

STEP	INSTRUCTIONS	INPUT DATA/UNITS	KEYS				OUTPUT DATA/UNITS
1	Key in program						
2	Initialize		f	REG	f	PRGM	
3	Perform for i = 1,..., n:						
	Input x-value and y-value	x_i	↑				
		y_i	R/S				i
4	Compute regression constants		GTO	08	R/S		a_0 *
			R/S				a_1 *
5	Compute coefficient of deter-						
	mination		R/S				r^2
6	To calculate a projected y-value,						
	input the x-value	x	RCL	1	x	RCL	
			0	+			ŷ
7	Perform step 6 as many times as						
	desired						
8	For a new case, go to step 2.						
	* The contents of the stack						
	should not be disturbed at these						
	points.						

Table 6.1.