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Calcium and the Assays of Human Plasma Clotting Factor XIII

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1. A continuous fluorimetric assay for blood-clotting Factor XIII based on the incorporation of dansylcadaverine into casein was investigated. 2. Hammarsten casein was fractionated to yield the β-casein, which was dephosphorylated and acetylated to give a substrate which itself did not bind calcium and which was not cross-linked by the activated Factor. 3. The modified β-casein was used as substrate for a continuous fluorimetric assay and as substrate for the incorporation of radioactive glycine ethylester. 4. A linked fluorimetric assay for the zymogen is described. 5. The K_m for calcium was redetermined and at 0.2mM was in the physiological range and much lower than the values reported by others using substrates which interact with calcium. 6. The K_m for casein is about 14μM.

In the last step of blood clotting thrombin converts both fibrinogen into fibrin (Bettelheim & Bailey, 1952) and Factor XIII into an enzyme variously known as Factor $XIII_a$ or plasma transglutaminase (Lorand & Konishi, 1964). Factor $XIII_a$ catalyses the calcium-dependent formation of γ-glutamyl–ε-lysyl amide cross-links in the fibrin gel (Pisano *et al.*, 1968) which render the gel clot mechanically tougher and more elastic (Gormsen & Silvertsen, 1964; Tyler, 1969) and decreases the rate of clot fibrinolysis (Bickford & Sokolow, 1961). An essential tool for the study of the activation of the enzyme is a reliable assay which is not sensitive to the effects of calcium on the structure of the substrates.

Early assays for Factor XIII depend on the cross-linked fibrin being insoluble in urea or acid. The assay can be quantified by diluting the Factor to the threshold concentration required to observe clot insolubility (Lorand *et al.*, 1962), although the complex nature of the gel structure (Lorand, 1970) and the difficulties encountered in standardizing the fibrin (Schwartz *et al.*, 1971) make the assay unsuitable for routine use. Non-physiological methods of assay stem from the discovery of Lorand & Jacobsen (1964) that amines can compete for the ε-amino groups of protein lysine residues and become covalently linked to fibrin by reaction with the glutamine residues of the protein. The specificity towards the glutamine substrate is great, and in general only proteins, usually casein, have exactly the environment for the enzyme to act (Matacic & Loewy, 1966). Sampling techniques have been used to measure the rates of incorporation of radioactive glycine ethyl ester (Lorand & Ong, 1966) or radioactive putrescine (Dvilansky *et al.*, 1970) into casein.

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A continuous assay for both Factor XIII and Factor $XIII_a$ is desirable for mechanistic investigations. The present paper reports the development of such an assay, based on the observation of Lorand *et al.* (1971) that incorporation of the dansyl moiety of dansylcadaverine into casein results in an increase in fluorescence. It was necessary considerably to modify the casein to block the lysine residues and thus to prevent casein–casein cross-linking, and to remove protein-bound phosphate to prevent the formation of insoluble calcium caseinates.

Materials and Methods

Simple fluorimetric assay with Hammarsten casein

Hammarsten casein (B.D.H. Chemicals Ltd., Poole, Dorset, U.K.) was dialysed at 25mg/ml against 0.1M-Tris–HCl, pH7.5, for 36h at 0°C and stored frozen in small batches at −15°C until required. Dansylcadaverine was a gift from Chas. Pfizer and Co., Sandwich, Kent, U.K. An approx. 30mM solution in 0.1M-Tris–HCl was standardized spectrophotometrically by assuming the same extinction coefficient as for dansylamide ($4.64 litre \cdot mmol^{-1} \cdot cm^{-1}$ at 326nm, Deranleau & Neurath, 1966). Bovine thrombin(reagent) was from Leo Pharmaceutical Products, Balleny, Denmark. It was dissolved in 50% (v/v) glycerol in 0.1M-Tris–HCl, pH7.5, to give 1000 National Institute of Health (N.I.H.) units/ml and stored at 0°C for up to 2 weeks. The following solutions were pipetted into both the sample and reference cuvettes of the simple fluorimeter (Fig. 1): 0.2ml of casein, 0.1ml of 50mM-2-mercaptoethanol, 0.1ml of 30mM-dansylcadaverine, 20μl of thrombin (1000 N.I.H. units/ml), 1.0ml of 0.1M-Tris–HCl, pH7.5, and 0.1ml of 0.5M-$CaCl_2$. A portion (20μl of approx. 0.6mg/ml) of Factor XIII was added to

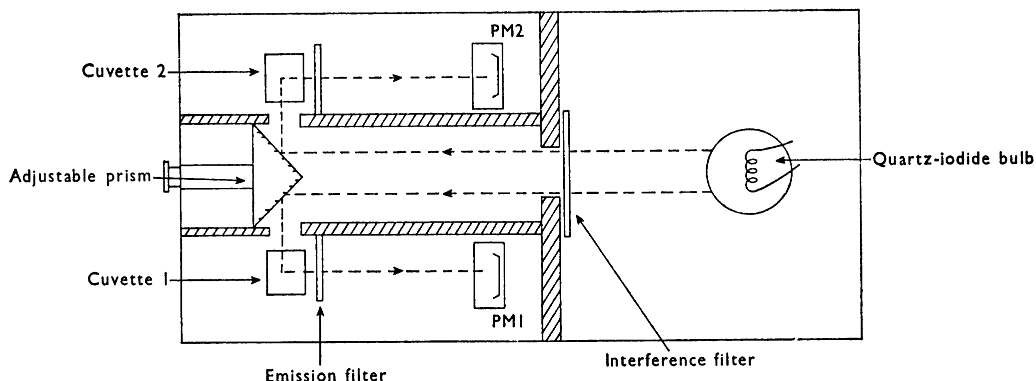


Fig. 1. *Simple ratio fluorimeter used to observe the incorporation of dansylcadaverine into casein*

Light from a 60W quartz-iodide bulb was passed through an interference filter (Balzers B-40, 404nm), split into two beams by the silvered front face of a prism, and used to excite two cuvettes. Radiation at right angles was selected by a Kodak Wratten filter no. 61 (maximum transmission at 520nm) and detected by two photomultipliers (PM1 and PM2). The cell housing was maintained at 25°C. An electrical analogue circuit allowed a record of ratio of the difference in the two photomultiplier signals to the reference signal to be displayed on a chart recorder. One unit of enzyme causes the increase by 1% of the starting fluorescence/min, under the assay conditions chosen (described in the text).

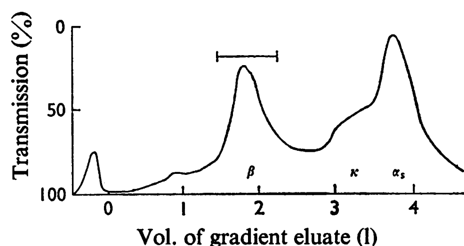


Fig. 2. *Elution of Hammarsten casein from DEAE-cellulose by a linear NaCl gradient*

A column (15cm×10cm) of DEAE-cellulose was equilibrated in 50mM-NaH_2PO_4 containing 4.5M-urea adjusted to pH7.0 with 5M-NaOH (buffer 1). Hammarsten casein (10g) was dissolved in 400ml of buffer 1 and dialysed against 2×20vol. of this buffer for 16h at 4°C. This dialysate was pumped (500ml/h) on to the column, which was then washed with 1 column volume of buffer 1. A linear gradient of 2.5 litres of buffer 1 and 2.5 litres of buffer 1 containing 0.3M-NaCl was used to elute the proteins. The solid line represents the u.v. absorption measured by an LKB Uvicord. The bar represents the pooled fractions.

the test cuvette, and the percentage increase in fluorescence at 25°C was recorded on a chart recorder attached to the fluorimeter described in the legend to Fig. 1.

Several alternative acceptor substrates were tested, by using the technique described above, in which the following proteins (at 10mg/ml) replaced casein: ovalbumin (grade V) and lysozyme (grade I) [Sigma (London) Chemical Co. Ltd., London, S.W.6, U.K.]. Ovotransferrin (a gift from Dr. J. Williams, University of Bristol), horseradish peroxidase and horse myoglobin (Sigma) were freed from cofactor by acid–acetone precipitation. Lysozyme was also succinylated (3-carboxypropionylated) by the method of Klotz (1967), by using a tenfold excess of succinic anhydride over lysine residues.

Standard fluorimetric assay with modified β-casein

A modified β-casein was used in the standard assay to eliminate the difficulties with Hammarsten casein (see the Results section). β-Casein was prepared from Hammarsten casein (a mixture of β- and α_s-casein) by the adaptation of the method of Waugh *et al.* (1962) described in detail in the legend to Fig. 2. Similar elution profiles were reported by Ribadeau-Dumas *et al.* (1964) with urea–imidazole buffers. A 10g portion of Hammarsten casein gave about 3g of β-casein and 4.5g of α_s-casein calculated from the extinction coefficients at 280nm given by Waugh *et al.* (1970), of $E_{280}^{1\%}$ of 4.7 (β-casein) and 10 (α_s-casein). β-Casein rather than α_s-casein was used in the subsequent work because: (1) it is easily resolved on DEAE-cellulose; (2) it is a single protein as opposed to α_s-casein, which consists of two related proteins (Waugh *et al.*, 1962); (3) preliminary experiments showed it to be a better substrate in the fluorimetric

assay, perhaps because of its lower phosphate content (Waugh *et al.*, 1970); and (4) preliminary experiments showed that dephosphorylation of α_s-casein did not prevent the formation of turbid solutions with Ca^{2+} ions, in agreement with Bingham *et al.* (1972), who report that calcium interacts with α_s-casein at negative groups other than phosphates.

β-Casein was dephosphorylated with *Escherichia coli* alkaline phosphatase (a generous gift from Dr. S. Halford, University of Bristol). β-Casein (8g) was incubated at room temperature (18°C) in 200ml of buffer 2 (0.1M-Tris–HCl, pH8, containing 10μM-$ZnCl_2$) containing 6mg of pure alkaline phosphatase. The protein was transferred to dialysis tubing and dialysed against 8 litres of buffer 2 for 2.5h at room temperature and subsequently at 4°C, with buffer changes every 20h. The progress of dephosphorylation was determined by monitoring the appearance of turbidity at 520nm after addition of Ca^{2+} at 66mM for 20min. After 40h dialysis there was no turbidity 20min after addition of Ca^{2+}. The alkaline phosphatase retained 75% of its initial activity after the dialysis, assayed by the method of Halford *et al.* (1969).

The dephosphorylated β-casein was acetylated by the method of Gray & del Valle (1970). Dephosphorylated β-casein solution (15ml, containing 30mg/ml) was mixed with 35ml of 8.5M-urea and 5ml of pyridine and stirred at room temperature. Two 7ml batches of acetic anhydride were added after 5min intervals and after 15min the solution was dialysed against 4×100vol. of water at 4°C for 36h and then freeze-dried. The dephosphorylated, acetylated β-casein was dissolved in 0.1M-Tris–HCl, pH7.5, at 30mg/ml and is referred to as β-substrate. No alkaline phosphate activity (<1%) was detected; presumably it was destroyed by the acetylation.

The standard assay medium consisted of 0.1ml of β-substrate, 0.1ml of 30mM-dansylcadaverine, 1.2ml of 0.1M-Tris–HCl, pH7.5, and 0.1ml of 75mM-$CaCl_2$. In linked assays of the zymogen Factor XIII 10 units of thrombin were included. The simple fluorimeter (Fig. 1) was used to measure the percentage increase of the initial fluorescence as displayed on a chart recorder.

The Factor XIII used in this paper was prepared by the method of Loewy *et al.* (1961) as modified by Lorand *et al.* (1968). The activated Factor (Factor $XIII_a$) was prepared by incubation of Factor XIII (0.7mg/ml) with thrombin (14 N.I.H. units/ml) in 0.1M-Tris–HCl–1mM-EDTA, pH7.5, for 35min at 25°C, when the activation was complete. Activated Factor $XIII_a$ was stored for up to 4h at 0°C until required. Preliminary experiments established that there was no difference in activity between Factor $XIII_a$ preparations that had been treated with 5mM-di-isopropyl phosphorofluoridate before storage (to inhibit thrombin) and untreated controls.

Fluorescence emission spectra

These were recorded uncorrected in a single-ended fluorimeter, which consisted of a 250W xenon arc whose light was selected by a Hilger and Watts D330 monochromator and focused on the centre of a 1cm×1cm quartz cuvette. The emitted light was passed through a Hilger and Watts D192 monochromator and measured on a photomultiplier. After amplification, the photomultiplier current was displayed on a strip-chart recorder. The low transmission of 2mM-dansylcadaverine at its absorption maximum prevented excitation at that maximum (326nm): 390nm was the optimum wavelength for excitation in the apparatus.

Radioactivity-incorporation assay of Factor XIII

A method based on measurement of the amount of radioactive glycine ethyl ester incorporated into either Hammarsten casein or β-substrate was used to determine the K_m values for these substrates. The filterpaper washing and counting technique is based on the method of Dvilansky *et al.* (1970) for measuring the incorporation of labelled putrescine. The assay medium consisted of 3mg of casein substrate/ml (either Hammarsten or β-substrate as required), 5mM-glycine ethyl ester {2.5μCi, prepared by diluting glycine [1-^{14}C]ethyl ester (New England Nuclear Corp., Boston, Mass., U.S.A.) with unlabelled material (B.D.H.)} and 12mM-$CaCl_2$ in 0.1M-Tris–HCl, pH7.5, and was pipetted in 0.5ml batches into tubes in a water bath at 37°C. Factor $XIII_a$ was added and 15min later the reaction was stopped by the addition of 5ml of cold 10% (w/v) trichloroacetic acid containing 0.2M-glycine ethyl ester. The precipitated protein was left for 30min at 0°C, then agitated on a Fisons Whirlimixer and then filtered through 2.5cm diam. discs of Whatman glass-fibre filter paper type GF/D. These papers gave the lowest background counting rates, presumably because their high flow rates permitted much more efficient washing by the more extended washing technique described by Lorand *et al.* (1972*a*). The discs were counted for radioactivity in a Nuclear-Chicago Unilux II liquid-scintillation counter by using a fluid which contained 60g of naphthalene, 37.5mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 2g of 2,5-diphenyloxazole in 500ml of AnalaR dioxan. Assays and blanks were in duplicate. Incorporation was expressed as nmol of radioactive substrate incorporated per min.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

The technique was that described by Tanner & Boxer (1972). Samples were prepared by heating at

100°C for 5min in a solution containing 2mM-phenylmethanesulphonyl fluoride (Bretscher, 1971), 5% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol, 1% 2-mercaptoethanol and 0.01% Bromophenol Blue in 20mM-Tris and 5mM-EDTA adjusted to pH8 with acetic acid. Electrophoresis was in 5% (w/v) polyacrylamide gels at pH7.4 as described by Shapiro *et al.* (1967) until the dye migrated to within 3cm of the end of the gel. Protein was stained with Coomassie Brilliant Blue R-250 (Sigma) by the method of Berg (1969). Logarithms of molecular weights were determined from the distances migrated (Weber & Osborn, 1969), by using ovotransferrin (a gift from Dr. J. Williams, mol.wt. 80000), bovine serum albumin (mol.wt. 67000, Sigma), bovine glutamate dehydrogenase (mol.wt. 56000, a gift from Dr. R. B. Wallis, University of Bristol) and erythrocyte D-glyceraldehyde 3-phosphate dehydrogenase (mol.-wt. 36000, a gift from Dr. M. J. A. Tanner, University of Bristol) as standards.

Results

Simple fluorimetric assay

Addition of Factor $XIII_a$ to cuvettes containing Hammarsten casein, dansylcadaverine and $CaCl_2$ produced a continuous increase in the fluorescence of the solution. The changes were very irregular except in continuously stirred suspensions. Throughout the period there was an increase in turbidity, and because the suspensions started to settle there was imbalance even when the ratio of the signals from two cuvettes was recorded. However, it was possible to measure the dependence of the rate of increase in fluorescence on calcium concentration. The results are shown in Fig. 3. The maximum rate is approached at 50mM-Ca^{2+}, a concentration 20 times that present in plasma (Linder & Blomstrand, 1958), and this suggests that either Factor $XIII_a$ has a very unphysiological requirement for Ca^{2+} or that the high concentrations are due to a reaction between Ca^{2+} and casein, possibly via the serine monophosphate residues of that protein (Bingham *et al.*, 1972). This would mean that the free Ca^{2+} concentrations were less than those added to the cuvettes. The reaction was also complicated by another competing process: the cross-linking of casein, presumably by the formation of glutamyl–lysine cross-links as with fibrin. This hypothesis was confirmed by two experiments described below.

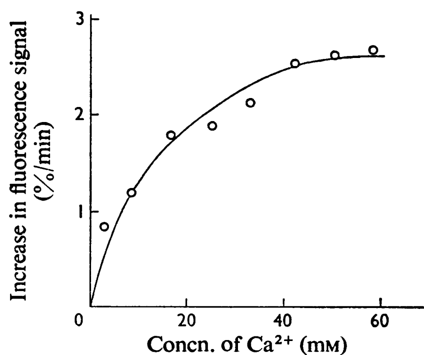


Fig. 3. *Calcium dependence of the simple fluorimetric assay, by using Hammarsten casein and dansylcadaverine*

The assay conditions are described in the text.

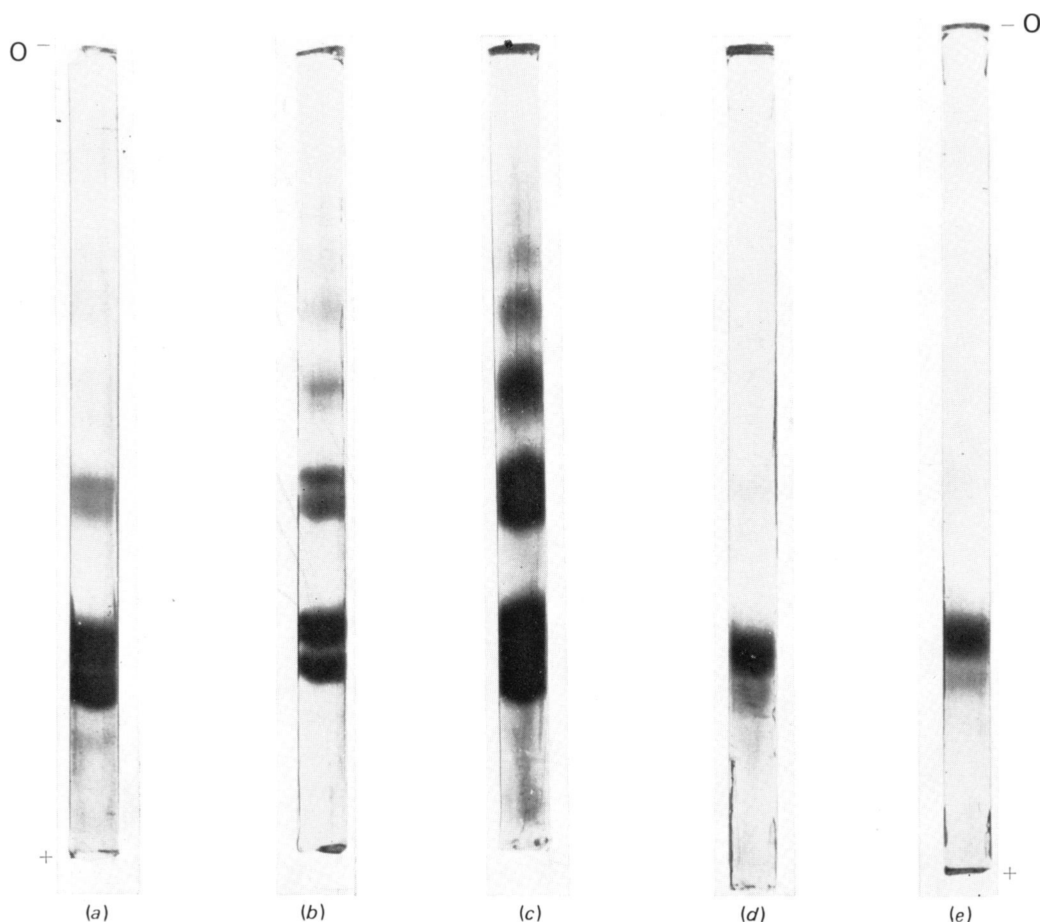
A turbidometric observation of the casein in the assay medium was made. The fluorimeter of Fig. 1 was used except that the excitation filter was changed to 550nm; the emission filter remained at 520nm. The rate of appearance of turbidity was approximately linearly proportional to the enzyme concentration in the test cuvette. The rate of appearance of turbidity was decreased by 30%, but not abolished, if 2mM-dansylcadaverine as well as casein and Factor $XIII_a$ were present in the cuvette. This might be due to the amine competing with the casein cross-linking.

An electrophoretic analysis of casein which had been treated with Factor $XIII_a$ was made (Plate 1). Exposure of casein to the enzyme results in the appearance of high-molecular-weight material with molecular weights corresponding to multiples of the molecular weights of the casein monomers (β-casein, 24000, and α_s-casein, 27000). Dimers, trimers, tetramers and some pentamers are visible on the gels.

Because of the unsatisfactory nature of the simple assay with casein several alternative protein substrates were examined. Neither ovalbumin, ovotransferrin, lysozyme, succinylated lysozyme nor apomyoglobin showed detectable increases in fluorescence in the simple assay. Apoperoxidase gave a rate about 7% of that of casein. These negative results confirm the high specificity that Factor XIII shows towards the glutamine donor (Matacic & Loewy, 1966). The alternative course was to modify the casein, first to remove the phosphate groups which interact with calcium, and secondly to block the lysine ε-amino groups so that casein self-cross-linking is prevented.

Standard fluorimetric assay of Factor $XIII_a$ with β-substrate

Preliminary experiments with dephosphorylated β-casein established that this substrate was still cross-linked by Factor $XIII_a$ and dimers and trimers were visible on sodium dodecyl sulphate–polyacrylamide gels. Also the rates of increase of fluorescence were only linear with time for a short while, presumably because of the build-up of insoluble casein polymers and interference from light-scattering. All further



EXPLANATION OF PLATE 1

Polyacrylamide-gel electrophoretic analysis of casein exposed to Factor $XIII_a$

Electrophoresis was done in 5% (w/v) polyacrylamide gels containing 0.1% sodium dodecyl sulphate and Tris–acetate–EDTA buffer consisting of 40mM-Tris base, 20mM-sodium acetate, 2mM-EDTA, adjusted to pH 7.4 with acetic acid. The gels were run at 8 V/cm for 3 h. Two incubations were set up containing 10 mg/ml solutions of either Hammarsten casein or β-substrate in 10mM-Ca^{2+} at 25°C. Factor $XIII_a$ (2.5 units) (assayed by the standard fluorimetric assay, described in the text) was added to these mixtures. Casein (40 μg) was applied to each gel, except (*c*), which represents an 80 μg sample. (*a*) Hammarsten casein, zero exposure to Factor $XIII_a$; (*b*) Hammarsten casein, 80 min exposure; (*c*) as above, twice the sample size; (*d*) β-substrate, zero exposure to Factor $XIII_a$; (*e*) β-substrate, 80 min exposure.

work used dephosphorylated acetylated β-casein (β-substrate) prepared as described in the Materials and Methods section.

β-Substrate showed no high-molecular-weight polymers after exposure to Factor $XIII_a$ (Plate 1). Acetylated Hammarsten casein was also not cross-linked, suggesting that the acetylation was indeed responsible for the abolition of cross-linking.

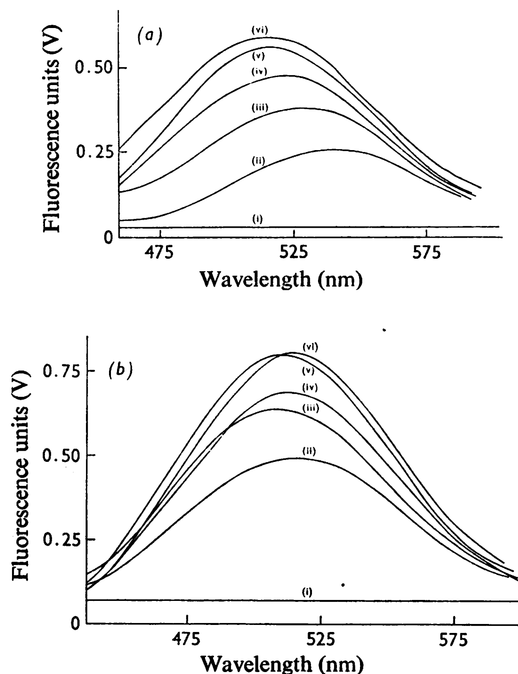


Fig. 4. *Fluorescence emission spectra of solutions containing dansylcadaverine and (a) β-substrate or, (b) Hammarsten casein, and the effect of Factor $XIII_a$ and calcium on these spectra*

(*a*) (i) Tris buffer, Tris with β-substrate (2mg/ml); (ii) dansylcadaverine (2mM) in Tris buffer, i.e. 0.1M-Tris–HCl, pH7.5; (iii) dansylcadaverine (2mM) and β-substrate (2mg/ml); this spectrum is not changed by the addition of $CaCl_2$ (0–60mM); (iv) effect of 3 units of Factor $XIII_a$ (i.e. about 0.01mg/ml) (assayed by the standard fluorimetric assay) on (iii) after 15min exposure in 10mM-Ca^{2+}; (v) effect of Factor $XIII_a$ on (iii) after 30min exposure; (vi) dansylcadaverine (2mM) and β-substrate (4mg/ml). (*b*) (i) Tris buffer, Tris with Hammarsten casein (2.7mg/ml); (ii) dansylcadaverine (2mM) and Hammarsten casein (2.7mg/ml in 50mM-Ca^{2+}); (iii) effect of 3 units of Factor $XIII_a$ (i.e. approx. 0.01mg/ml) on (ii) after 15min exposure; (iv) dansylcadaverine (2mM) and Hammarsten casein (2.7mg/ml in 4mM-Ca^{2+}); (v) effect of Factor $XIII_a$ (0.01mg/ml) on (iv) after 15min exposure; (vi) dansylcadaverine (2mM) and Hammarsten casein (2.7mg/ml) in the absence of Ca^{2+}.

The fluorescence-emission spectrum of dansylcadaverine is enhanced and shifted into the blue by addition of either β-substrate or Hammarsten casein (Fig. 4), presumably because the dye is bound to the proteins. Addition of more protein results in further enhancement of fluorescence. The protein-concentration-dependent enhancement means that this type of assay is unsuitable to measure the K_m for casein, and a radioactive assay was used for that purpose (see below). The emission spectrum of the dansylcadaverine–β-substrate complex was quite insensitive to the presence of Ca^{2+} (Fig. 4*a*), whereas the fluorescence of the same complex with Hammarsten casein was much decreased by the presence of Ca^{2+} (Fig. 4*b*), probably because of decreased penetration of exciting radiation into the turbid suspension. Exposure to the same amount of Factor $XIII_a$ causes the fluorescence of Hammarsten casein to increase by almost the same amount at both 4mM- and 50mM-Ca^{2+}. However, because the initial fluorescence of the complex is lower at the high Ca^{2+} concentration, the relative increase in fluorescence will be greater, and it is relative fluorescence that was measured with the simple fluorimeter of Fig. 1. This offers a partial explanation of the apparent increase in Factor $XIII_a$ activity at Ca^{2+} concentrations from 4 to 50mM. There was no increase in the rate of increase of fluorescence when β-substrate was used when the Ca^{2+} was varied from 5 to 60mM. Thus the two chemical modifications gave a casein substrate which was free of two defects: interaction with Ca^{2+} and self-cross-linking. Use of this substrate in the

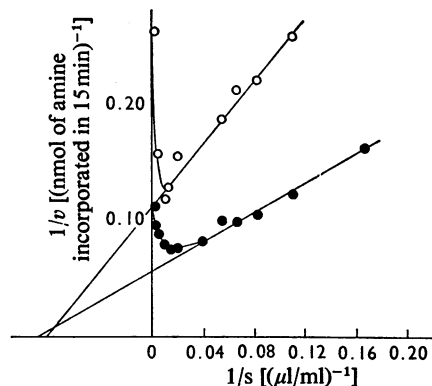


Fig. 5. *Reciprocal plots of velocity against casein concentration*

The procedure for the radioactive assay is described in the Materials and Methods section. The casein concentrations are shown as μl/ml of stock casein solutions (30mg/ml). ○, β-casein; $K_m = 11.8\mu l/ml = 14.1\mu M$; $V_{max.} = 9.1$ nmol of amine/15min. ●, Hammarsten casein: $K_m = 11.1\mu l/ml = 13.4\mu M$; $V_{max.} = 17.9$ nmol of amine/15min.

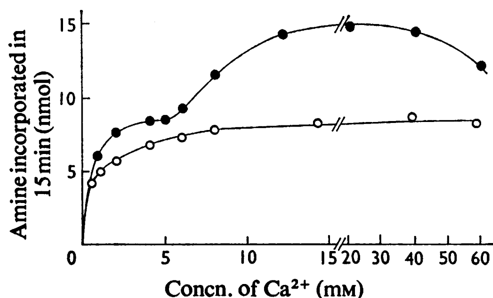


Fig. 6. *Calcium dependence of the incorporation of glycine ethyl ester into Hammarsten casein (9 mg/ml; ●) and β-substrate (9 mg/ml; ○)*

The experimental procedure is given in the text.

standard assay described in the Materials and Methods section gives 'noise'-free traces, which are linear for up to 7% increases in starting fluorescence. The initial rate of increase in fluorescence was proportional to added enzyme until the rate of increase was more than 3%/min. These rates were not altered by the omission of 2-mercaptoethanol from the assay.

Radioactivity assay and the K_m for casein

The fluorescence assay was unsuitable to measure the K_m for casein for the reasons given above. The Materials and Methods section describes an assay based on measuring the amount of radioactive glycine ethyl ester incorporated into casein and counted on glass-fibre filter discs. The incorporation was proportional to added enzyme up to rates of 10 nmol incorporated in 15 min. The K_m values for Hammarsten casein and β-substrate are both about 14 μM (Fig. 5), although the $V_{max.}$ for Hammarsten casein is about twice that for β-substrate. Substrate inhibition is evident at high concentrations of both substrates and about 3 mg/ml gives optimal rates.

The Ca^{2+}-dependence of the radioactivity assay was also investigated. With Hammarsten casein the curve (Fig. 6) showed an unusual inflexion at 12 mM-Ca^{2+}. The rate enhancement in this region was associated with the appearance of turbidity, which suggests that it may reflect interaction between Ca^{2+} and the Hammarsten casein rather than the effect of Ca^{2+} on the intrinsic catalytic activity of the enzyme. Fig. 6 shows, in agreement with this suggestion, that there is no increase in rate with the Ca^{2+}-insensitive β-substrate above 5 mM-Ca^{2+}.

The radioactivity assay was used to study the K_m for casein; however, it is not used as a routine because of the time-consuming washing procedure. It is less reproducible than the fluorescence assay

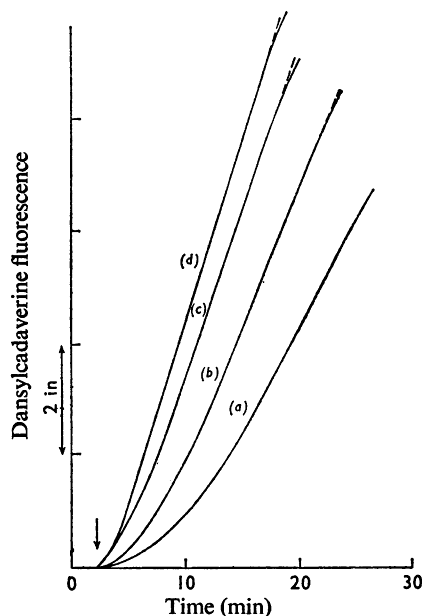


Fig. 7. *Thrombin-dependence of the linked fluorimetric assay for Factor XIII*

The four traces illustrate the relationship between the concentration of thrombin present in the standard assay medium and the assay rate recorded for a fixed amount of Factor XIII zymogen. The thrombin concentrations used were (*a*) 1.66 units/ml; (*b*) 3.34 N.I.H. units/ml; (*c*) 6.65 units/ml; (*d*) 13.35 units/ml. A 1-in increase in the fluorescence scale in 5 min corresponds to an activity of 0.46 unit. The arrow denotes the addition of Factor XIII.

and of course very expensive. A link has been established between the two assays: an amount of enzyme that causes an initial rate of fluorescence increase of 1%/min with the standard assay (β-substrate) incorporates 5 nmol of glycine ethyl ester/min into β-substrate. The 1% fluorescence increase has not yet been converted into mol of dansyl residues introduced into the casein, although this should be feasible after acid hydrolysis.

Thrombin-linked fluorimetric assay of Factor XIII

By including thrombin in the standard fluorimetric assay it should be possible to develop an assay for the zymogen, Factor XIII. The amount of thrombin was varied and four linked assays are shown in Fig. 7. At high concentrations of thrombin the zymogen is quickly converted into Factor $XIII_a$ and the rate is linear with time for about 8 min. At low thrombin concentrations there is a much longer lag phase and even when this is apparently completed (Fig. 7*a*) the steady-state rate is less than maximum. A possible

explanation for this may be that an appreciable fraction of Factor $XIII_a$ is denatured before the activation is complete. For linked assays to respond linearly to the amount of Factor XIII added enough thrombin must be used to keep the lag phase under 5min, that is about 10 units of thrombin/ml of assay medium.

Discussion

The experiments described in this paper have emphasized that the Hammarsten casein normally used to assay Factor $XIII_a$ suffers from two major defects as a substrate. It binds Ca^{2+} and the resultant calcium-caseinate turbidity has deterred all but Lorand *et al.* (1971) from attempting to develop a continuous assay. The calcium-caseinate interaction appears to be responsible for the very varied reports of the Ca^{2+} optimum of an enzyme which in normal plasma would be exposed to about 2.5mM (Linder & Blomstrand, 1958). By using casein Lorand *et al.* (1969) found that 5mM-Ca^{2+} gave optimal rates, whereas others (Tyler, 1970; Dvilansky *et al.*, 1970; Chung & Folk, 1972) have normally used much higher concentrations. Sheltawy *et al.* (1972) have re-examined the Ca^{2+} optimum for dansylcadaverine incorporation into casein by a sampling and washing technique, and found that 12mM was required for optimum rates. The results in Fig. 6 show how these apparently contradictory reports arise. Those groups who only examined Ca^{2+} concentrations above 5mM would report that about 30mM-Ca^{2+} is required for optimum activity, whereas those groups who only examined Ca^{2+} concentrations below 10mM might not suspect that there was a second inflexion in the curve at high Ca^{2+} concentrations. Because the calcium-insensitive substrate (acetylated, dephosphorylated β-casein) shows only a simple calcium-dependence we suggest that the intrinsic activity of Factor $XIII_a$ depends on a K_m of under 1mM, fully compatible with the physiological concentrations of plasma calcium.

The threefold increase in rate of dansylcadaverine incorporation into Hammarsten casein apparently measured by continuous fluorescence assay in the region from 2.5mM to 50mM-Ca^{2+} depends on two distinct factors. First the decrease in initial fluorescence at high Ca^{2+} gives an apparent increase in the relative increase of fluorescence, and secondly the radioactivity-incorporation assay shows that in the region of Ca^{2+} concentrations where turbidity appears there is a twofold real increase in radioactivity incorporated (Fig. 6).

The second defect of Hammarsten casein as a substrate is that it can be cross-linked by Factor $XIII_a$, and this process competes with the incorporation of either fluorescent or radioactive amines. Thus the enzyme activity would depend on a complex relation between the relative concentrations of casein and amine present in the assay. β-substrate is free of this competing reaction.

Several alternative substrates have been described that can replace casein as a source of glutamine residues. Lorand *et al.* (1972*b*) have synthesized low-molecular-weight model substrates which react with dansylcadaverine, although no information is given as to whether there is a fluorescence change during the reaction. Chung & Folk (1972) have shown that labelled amines are incorporated into acetylated oxidized B chains of insulin. This system should also not be susceptible to spurious responses to calcium, although the substrate would be very expensive for routine assays.

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