The Calcium-Induced Dissociation of Human Plasma Clotting Factor XIII

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1. Large quantities of human Factor XIII were prepared from ethanol precipitates of outdated human plasma. 2. Material homogeneous after chromatography on DEAE-cellulose was further resolved into two proteins, A and B, after filtration on Sepharose 6B. 3. Protein A has a molecular weight of 350000 and a subunit structure a_2b_2 and is activated by thrombin and calcium. Protein B is inactive and probably has a subunit structure b_2 . 4. Calcium causes protein A, after thrombin cleavage, to fragment to give protein B and a protein, containing only a' subunits, which is catalytically active. The latter protein slowly forms a misty precipitate which is still active and not cross-linked covalently. This confirms the suggestion of Schwartz *et al.* (1971) that catalytic activity is only associated with a' subunits. 5. Iodoacetate, which inhibits the enzyme, does not inhibit dissociation and aggregation of protein A. 6. The existence of two proteins and the fragmentation are possible explanations for the wide range of molecular weights given for Factor XIII in the literature.

A description of the mechanism of action of human plasma clotting Factor XIII requires not only a knowledge of the subunit structure of the Factor, but also information about the possible changes which occur when the zymogen is cleaved by thrombin and activated with calcium. The usually accepted pure form of Factor XIII is homogeneous after chromatography on DEAE-cellulose (Loewy *et al.*, 1961a; Lorand *et al.*, 1968) and after filtration on Sephadex G-200 (Bannerjee *et al.*, 1971), and yet there are widely different reports of the molecular weight covering the range 70000 (Bannerjee *et al.*, 1971), 156000–195000 (Lorand *et al.*, 1968), 200000 (Lewis, 1964), 290000 (Bohn, 1970), 330000 (Kiesselbach & Wagner, 1967) to 340000 (Loewy *et al.*, 1961b; Schwartz *et al.*, 1971, 1973). In an attempt to understand these differences we have re-examined the method of preparation of Factor XIII and have found that the material eluted from DEAE-cellulose actually consists of two proteins with different molecular weights.

Schwartz *et al.* (1971) have shown that Factor XIII contains two sorts of subunit, a and b , which both have molecular weights close to 80000 and which are just separated on sodium dodecyl sulphate-polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol. Only the mobility of the a subunit is changed when Factor XIII is cleaved with thrombin and thus Schwartz *et al.* (1971) suggested that catalytic activity was associated with the a and not the b subunit. In the present paper we show that the subunit structure of protein A is a_2b_2 and that of protein B is probably b_2 . Further it is shown that there are no gross changes in the subunit composition

of protein A after cleavage with thrombin, but that addition of calcium causes rapid fragmentation to give inactive protein B and a catalytically active protein, containing only a' subunits, which itself slowly aggregates without much change in catalytic activity.

Materials and Methods

Sepharose 6-B and Sephadex G-200 were from Pharmacia Fine Chemicals, Uppsala, Sweden. DEAE-cellulose was microgranular DE-52 from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. Pig heart lactate dehydrogenase and bovine liver glutamate dehydrogenase were also from Whatman. Bovine thyroglobulin was from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.

Factor XIII was assayed in the linked assay with thrombin as described by Cooke & Holbrook (1974) which continuously measures the increase in fluorescence as dansylcadaverine is incorporated into dephosphorylated, acetylated β -casein. One enzyme unit is the amount of enzyme that gives 1% increase in the starting fluorescence/min at 25°C.

Sodium dodecyl sulphate – polyacrylamide-gel electrophoresis was performed as described by Cooke & Holbrook (1974) except that 2-mercaptoethanol was omitted from the sample preparation buffer and the gels were 7.5% polyacrylamide (a 30:1 weight ratio of gel monomers). The omission of reducing agent produces a much clearer separation of the subunits (Schwartz *et al.*, 1973), because of the enhanced mobility of the b chains.

Sedimentation velocity was measured in a Beckman model E ultracentrifuge at 20°C with an An-D rotor at

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58780rev./min, by photographing the schlieren pattern every 8min in two sector cells, one with a 1°-positive wedge window. The sedimentation constants are reported uncorrected. Diffusion constants were determined in a valve-type synthetic-boundary centrepiece by the method of Schachman (1957).

Human plasma, which was out-dated, was fractionated with ethanol by the procedure of Cohn *et al.* (1946) after a precipitate that forms in the plasma after freezing and thawing had been removed by centrifugation. The Cohn-I precipitate from 100 litres of cryosupernatant was a gift from the Scottish National Blood Transfusion Association in Edinburgh, U.K. It was stored as a frozen paste at -15°C until a sample had been shown to be free of Australia antigen. This test was kindly performed in the laboratory of Professor A. J. Zuckerman of the London School of Hygiene and Tropical Medicine by using electron microscopy. One batch in five was discarded. The Cohn-I pastes were stored for between 1 and 5 months before preparation of Factor XIII. Thrombin-cleaved Factor XIII (Factor XIII_a) was prepared as described in the preceding paper (Cooke & Holbrook, 1974).

Results

Preparation of Factor XIII

The Cohn-I paste from 100 litres of cryosupernatant was thawed and cut with a bread knife into 2cm cubes and was stirred slowly with 3 litres of 0.5M-KCl at room temperature (18°C). Dissolution was facilitated by raising the temperature to 37°C for 15min. The pH was maintained at 7.5 with 10% (v/v) NH₃. After 2h (or 4h if the Cohn-I paste had been stored for 4 months) the mixture was treated with saturated (NH₄)₂SO₄ to give 16% saturation while the pH was maintained at 7 with 10% (v/v) NH₃. After stirring for 1h at 4°C the precipitate was recovered by centrifuging the suspension for 10min at 2000g_{av.} in a Mistral 6L centrifuge. The 16%-satd.-(NH₄)₂SO₄ precipitate, which contains fibrinogen and most of the Factor XIII, was dissolved in 3 litres of 0.15M-KCl and was heated in 1-litre portions in a 2-litre stainless-steel beaker in a water bath at 58°C so that the liquid inside reached 56°C for 3min. After cooling, the rubbery ball of denatured fibrinogen was squeezed out to remove included liquid. The Factor XIII was precipitated from the clear liquid by addition of saturated (NH₄)₂SO₄ to 40% saturation and was recovered by centrifugation as described above. The precipitate was dissolved in buffer A (50mM-Tris-1mM-EDTA, adjusted to pH7.5 with 11M-HCl) to give a final volume of 400ml and was dialysed against 2×100vol. of buffer A overnight at 4°C before being pumped on to a column of DEAE-cellulose, which had been equilibrated at room temperature with

Fig. 1. Chromatography of Factor XIII on DEAE-cellulose

A column (15cm×10cm) of DEAE-cellulose was equilibrated in buffer A (50mM-Tris-1mM-EDTA adjusted to pH7.5 with 11M-HCl). A linear gradient of 4 litres of buffer A and 4 litres of buffer A containing 0.25M-NaCl was used to elute the proteins. The solid line represents the u.v. absorption measured by an LKB Uvicord instrument. The bar represents the pooled fractions showing Factor XIII activity (assayed by the method of Cooke & Holbrook, 1974).

buffer A as described in detail in the legend to Fig. 1. Factor XIII was usually the second protein peak to be eluted after the breakthrough peak, and was recovered by adjusting the eluate (about 1.4litres) to 40% saturation with saturated (NH₄)₂SO₄ as described above and then centrifuging at 10000g for 10min.

The sediment was redissolved in and dialysed against 0.1M-Tris-1mM-EDTA adjusted to pH7.5 with 11M-HCl to give a clear solution of Factor XIII, which was stored at 4°C. The half-life for loss of enzyme activity was about 8 weeks. The final yield of activity was 45% of that in the Cohn-I paste and by using $E_{280}^{1\%} = 13.8$, given by Schwartz *et al.* (1973), the final yield was 0.43 g of protein. The purification is summarized in Table 1. Although the number of units of enzyme recovered is less than would be recovered from an equivalent volume of fresh plasma, the use of the Cohn-I precipitate from large volumes of out-dated plasma is the only way to get enough enzyme to perform protein-chemical studies. Samples containing (NH₄)₂SO₄ were dialysed before assay. We found that as little as 1% (NH₄)₂SO₄ in the assay gave 50% inhibition. Chung & Folk (1972) have shown that NH₃ inhibits the enzyme by competition with the amine donor. The yield of Factor XIII varied considerably, depending on how long the Cohn-I paste was stored before the preparation was started.

Table 1. *Preparation of Factor XIII*

One unit is arbitrarily defined as that quantity of enzyme required to bring about a 1% change in fluorescence/min of the dansylcadaverine under the standard conditions of the assay (Cooke & Holbrook, 1974). The $E_{280}^{1\%}$ of Factor XII is 13.8 (Schwartz *et al.*, 1973).

Fraction	Vol. (ml)	Protein absorbance at 280 nm in 1 ml	Total units	Sp. activity (units/protein absorbance)	Purification relative to Cohn-I paste
Cohn I in 0.5M-KCl	4100	140000	160000	1.14	1
16%-satd.-(NH ₄) ₂ SO ₄ precipitate in 0.15M-KCl	3000	90000	144000	1.60	1.4
Above fraction after heat step	2500	8000	100000	12.50	11
40%-satd.-(NH ₄) ₂ SO ₄ precipitate of heat step	400	6000	95000	15.90	14
Active fraction from DEAE-cellulose after precipitation and dialysis	81	600 (0.43 g of protein)	75000	125	110

Fig. 2. *Resolution of proteins A and B on Sepharose 6-B*

A 1.5 ml sample of Factor XIII prepared as described in the Materials and Methods section was applied to a column (43 cm $\times$ 1.7 cm) of Sepharose 6-B equilibrated in 0.1M-Tris-1 mM-EDTA adjusted to pH 7.5 with 11M-HCl. The flow rate was 3.6 ml/h and 1 ml fractions were collected. The dashed line indicates the Factor XIII activity distribution across the elution profile, determined by the linked-assay procedure described by Cooke & Holbrook (1974).

Resolution of Factor XIII into proteins A and B

In a preliminary experiment a sample of Factor XIII was filtered through a column of Sepharose 6-B. Two peaks of protein were eluted with elution volumes corresponding to mol.wts. of 580000 and 300000 on a column calibrated with thyroglobulin (mol.wt. 750000), glutamate dehydrogenase (mol.wt. 335000 in dilute solution) and lactate dehydrogenase (mol.wt. 144000). The elution profile from a column described in detail in the legend to Fig. 2 showed that the higher-molecular-weight component (protein A) was enzymically active in the linked assay, whereas the smaller protein (protein B) was enzymically inert. Plate 1 shows the sodium dodecyl sulphate-polyacrylamide-gel electrophoretic patterns of samples from across the elution profile. Protein A contains the two subunits labelled *a* and *b* by Schwartz *et al.*

(1971), whereas protein B contains only *b* subunits. In agreement with those workers we also confirm that only the migration of the *a* subunit alters after cleavage with thrombin and that the molecular weight of both sorts of subunit is approx. 80000. About 20 mg of protein A could be recovered from the Sepharose column. The amount of protein B varied from about 20% of protein A in samples of the DEAE-cellulose material that had been prepared from fresh plasma to 70% in material that had either been stored for some time or been prepared from very old samples of Cohn-I pastes. More detailed chemical studies show that the *b* subunits in proteins A and B are really the same protein (Cooke, 1973). The immunochemical studies of Israels *et al.* (1973) and Bohn *et al.* (1973) show that fresh human plasma contains a protein of only *b*-type subunits. This suggests that, although the B protein in our preparation can arise from decay of A protein, protein B is a normal constituent of human plasma.

Sedimentation analysis of Factor XIII

Ultracentrifugation of the material eluted from DEAE-cellulose showed two components with uncorrected sedimentation constants of 9S and 4.8S (Fig. 3). Similar analysis of material in protein A showed only a 9S particle, whereas protein B contained only 4.8S material. The diffusion constant of protein A from a synthetic-boundary cell was $21 \times 10^{-8} \text{ cm}^2 \cdot \text{s}^{-1}$. The partial specific volume calculated from the amino acid analysis was 0.72 ml/g, in agreement with the value measured by Schwartz *et al.* (1973) of 0.73 ml/g. The molecular weight of protein A calculated from these values is 350000. Since protein A contains subunits *a* and *b* of mol.wt. 80000, the molecular formula of protein A is a_2b_2 . This is in agreement with the gels of protein A (Plate 1) which display two bands of about equal intensity i.e. equal amounts of the *a* and *b* subunits (assuming equal staining intensity). Protein B would

Fig. 3. Sedimentation-velocity analysis of Factor XIII

The sedimentation of 8 mg of Factor XIII/ml (as prepared in the Materials and Methods section) in an analytical ultracentrifuge is shown in the lower trace (plane window). The upper trace (wedge window) is a sample of Factor XIII_a (8 mg of Factor XIII/ml, exposed to 12 units of thrombin/ml for 45 min). The buffer used was 0.1 M-Tris-HCl (containing 1 mM-EDTA), pH 7.5. The three photographs shown here were taken at 16-min intervals. The direction of sedimentation is from left to right.

Fig. 5. Ultracentrifugal analysis of the calcium-induced fragmentation of Factor XIII_a

An 8 mg/ml solution of the same preparation used in Fig. 3 was utilized. The lower trace (plane window) is Factor XIII_a in 0.1 M-Tris-HCl (containing 1 mM-EDTA), pH 7.5. The upper trace (wedge window) is the calcium supernatant obtained by exposing Factor XIII_a to 60 mM-Ca²⁺ for 1 h at 20°C and centrifuging (10000g for 10 min at 15°C) the resultant turbid solution. The three photographs shown here were taken at 12-min intervals. The direction of sedimentation is from left to right.

Fig. 4. Calcium-induced fragmentation of Factor XIII_a

The calcium-dependent changes in the E_{280} of the solutions were measured at 25°C with a Hilger-Gilford recording spectrophotometer. The Factor XIII solution (0.43 mg/ml in 0.1 M-Tris-HCl, pH 7.5) was activated by thrombin (10 units/ml). No increase in turbidity was recorded during this thrombin activation (approx. 30 min required at 25°C). CaCl₂ was added to the cuvette at the time indicated (final concentration 33 mM), causing the slow appearance of turbidity. The solid line represents the behaviour of protein A, the dashed line protein B and the dotted line corresponds to centrifugation time.

have a molecular formula of b_2 if its diffusion coefficient and partial specific volume were the same as for protein A (giving a mol.wt. of 150000). However, these uncertainties make the subunit structure ascribed to protein B tentative. The sedimentation coefficient of protein A was 9S between 0.5 and 5 mg/ml.

Change in the molecular structure of Factor XIII_a induced by Ca²⁺

Factor XIII is converted into Factor XIII_a by cleavage with thrombin. There was no measurable change in the sedimentation coefficients of proteins A or B during the cleavage. The slight change in the molecular weight owing to a subunits being converted into a' subunits (Schwartz *et al.*, 1971) might not be seen in the sedimentation coefficient of a 9S particle (Fig. 3). Factor XIII_a, prepared by treating either pure protein A or the mixture of proteins A and B with thrombin, was a clear protein solution that could be stored at 4°C for 4 h and still exhibit full enzyme activity when mixed with CaCl₂ in the assay medium.

When Factor XIII_a (prepared from protein A) was treated with calcium as described in the legend to Fig. 4 the solution became turbid. This process was monitored by measuring the scattering of light at 280 nm, and took about 60 min to complete. During this period there was, at high protein concentration (5 mg/ml), no loss of enzyme activity and only a slight (30%) loss at the protein concentration (0.4 mg/ml) used to measure the turbidity. The clear supernatant obtained by centrifuging the misty suspension at 10 000g for 10 min was enzymically inactive. The resuspended sediment contained the activity. The supernatant contained half the initial protein absorbance (Fig. 4). Gel electrophoresis (Plate 2) showed that the sediment contained a' subunits and that the supernatant contained b subunits. Sedimentation analysis of a sample of Factor XIII_a, containing 75% protein A and 25%

EXPLANATION OF PLATE I

Resolution of proteins A and B on Sepharose 6-B

The sodium dodecyl sulphate-polyacrylamide gels (7.5% gels) of the elution profile shown in Fig. 2 are shown. Some 95% of the Factor XIII activity and 80% of the protein was recovered from the Sepharose column. A third protein was visible on gels of the material applied to the Sepharose column and absent from proteins A and B eluted. This material might be the 20% of the applied protein not recovered. For preparation purposes, a larger Sepharose column was used (42 cm $\times$ 3.3 cm), enabling about 20 mg of protein A to be recovered. (i) Column applicant; (ii) protein A; (iii) protein B; (iv) reference gel. *a* and *b* show the position of subunit *a* and *b* respectively. The molecular weights of the reference compounds are shown.

EXPLANATION OF PLATE 2

Sodium dodecyl sulphate-polyacrylamide-gel analysis of the calcium-induced fragmentation of Factor XIII_a

Electrophoresis was done in 7.5% polyacrylamide gels, in the absence of 2-mercaptoethanol (see the Materials and Methods section). About 10 μ g of protein was applied to each gel. The samples were: (A), Factor XIII; (B) Factor XIII_a; (C), supernatant obtained by centrifuging the turbid solution described in Fig. 4 [excess of EDTA (pH 7.5) was added to C with the gel buffer to prevent precipitation of the sodium dodecyl sulphate]; (D), precipitate obtained as above; (E) a reference gel. The bands correspond to molecular weights of 80000, 67000, 56000 and 36000.

protein B, which had been treated with Ca^{2+} and allowed to become fully turbid (Fig. 5), revealed that the original 9S component (protein A) had disappeared and that the area under the 4.8S peak (protein B) had increased by about half the area of the original 9S peak. Scheme 1 presents a model to account for these findings.

If turbidity formation was allowed to go to completion it could not be reversed by the addition of excess of EDTA over Ca^{2+} either before or after centrifugation (i.e. with or without protein B). However, if the EDTA was added after 10min (i.e. before turbidity had reached its maximum) there was a partial decrease in turbidity. This suggests that the final stages of aggregation are practically irreversible, whereas the early stages may be an equilibrium between soluble protein containing a' subunits and protein B. There was no appearance of turbidity if Mg^{2+} was substituted for Ca^{2+} nor if the Ca^{2+} was added to Factor XIII that had not been treated with thrombin. 2-Mercaptoethanol had no effect on the extent or rate of appearance of turbidity. Iodoacetate (2mM), which gave 75% inhibition 5min after addition of Ca^{2+} , also had no effect on the extent or rate of appearance of turbidity. Addition of more thrombin, over and above that required to convert Factor XIII into Factor XIII_a, did not affect the appearance of turbidity.

Discussion

To prepare enough Factor XIII to study its mechanism of action by protein-chemical techniques we have developed a procedure which starts from an ethanol fraction from out-dated, cryoprecipitated, pooled human plasma. After DEAE-cellulose chromatography the material contains two proteins, A and B. The proportion of the inactive protein B increases if the ethanol fraction from the pooled plasma is stored for a long time before the preparation is started or if the purified Factor XIII is stored for many months. Protein B can thus arise from protein A, and it might be asked whether there is any protein B at all present in fresh human plasma. Immunochemical experiments of Israels *et al.* (1973)

and Bohn *et al.* (1973) suggest, however, that protein B is a normal constituent of plasma.

Proteins A and B can be resolved by gel filtration on Sepharose 6-B and in sedimentation-velocity experiments, but not by gel filtration on Sephadex G-200. The molecular weight of protein A was 350000 and it was estimated that protein B had a molecular weight of 150000 (without separate measurement of D and $\bar{v}$). Sephadex G-200 might just not resolve two such proteins, but with Sepharose 6-B the elution volumes were those corresponding to globular proteins of much higher molecular weights. This finding has not been unambiguously explained, but glycoproteins [the b subunits of both protein A and B contain 8.5% carbohydrate (Bohn *et al.*, 1973)], do sometimes interact anomalously with carbohydrate gels.

Scheme 1 accounts for the ultracentrifugation and gel-electrophoretic analysis that we have made of the process by which Ca^{2+} causes Factor XIII_a to dissociate. Some points require further comment. The subunit structure of protein B might be b or b_3 should the molecular weight turn out to be 80 000 or 240 000. The subunit structure of protein A is more firmly established. We have no evidence as to the molecular formula of the soluble active protein containing only a' subunits, which is initially formed after adding calcium. It is shown as a'_2 only because this is the remainder after subtracting b_2 from a'_2b_2 . Both Bohn (1970) and Takagi & Doolittle (1973) have noted the misty appearance of Factor XIII_a in the presence of Ca^{2+} and both groups suggested that the active enzyme could cross-link itself. Bohn (1970) gave no evidence in support of his suggestion, but Takagi & Doolittle (1973) did observe the appearance on sodium dodecyl sulphate-polyacrylamide gels of a band with about twice the mass of the a' subunit. We have observed this band in samples of purified Factor XIII that have been stored for several months at 4°C. It might be the result of cross-linking of denatured enzyme protein, but it can be removed by filtration on Sepharose 6-B (Plate 1) and is not formed on addition of calcium to Factor XIII_a within a period at which maximum turbidity appears. Gels of turbid suspensions (Plate 2) show only the

Scheme 1. Calcium-induced dissociation of Factor XIII_a

The evidence for the various molecular structures is given in the text. Schwartz *et al.* (1973) showed that a subunits are converted into a' subunits when thrombin acts on Factor XIII. The aggregation of soluble a' subunit containing protein to give an active precipitate is a slow process. The a' -protein precipitate is not covalently cross-linked.

two subunits *a'* and *b*. The turbidity formed over 1 h cannot be the result of enzyme covalently cross-linking itself, since enzyme inactivated with iodoacetate also becomes turbid.

Although the model accounts for experiments at protein and calcium concentrations higher than in blood it may also apply at more physiological concentrations. For example, Loewy & Edsall (1954) showed there was no Factor XIII_a activity in the soluble serum remaining after removal of the clot (consistent with the precipitation of *a'* subunits), whereas Bohn (1972) showed there was a protein containing only *b* subunits remaining in the serum. Bohn (1972) speculated that Factor XIII must dissociate after cleavage by thrombin. From the results given in the present paper it is clear that it is calcium and not thrombin which induces Factor XIII_a to fragment (at least at protein concentrations above 0.5 mg/ml where molecular weights can be measured). Although the model shows that calcium is required to fragment Factor XIII_a this does not preclude the possibility that calcium has another role in the mechanism as well.

Extreme conditions [freezing at -20°C (Schwartz *et al.*, 1973), low pH or heating at 58°C for 3 min] increase the fraction of protein B in Factor XIII preparations. The variable proportions of proteins A and B so produced would explain the wide range of molecular weights given for Factor XIII, as noted in the introduction. Storage of pure protein A for 5 weeks at 4°C results in some turbidity, some loss of activity and the appearance of some protein B. Schwartz *et al.* (1973) have denatured Factor XIII by freezing at -20°C for 2 months and found an inactive white precipitate containing *a'* subunits and a supernatant containing *b* subunits. These workers report a considerable loss of enzyme activity in such frozen and thawed samples. We could detect no activity after this treatment.

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References

- Bannerjee, D., Morton, R. O., Delaney, R., Cahill, C. & Hampton, J. W. (1971) *Fed. Proc. Fed. Amer. Soc. Exp. Biol.* **30**, 340, Abstr. 829
- Bohn, H. (1970) *Thromb. Diath. Haemorrh.* **23**, 455–468
- Bohn, H. (1972) *Ann. N.Y. Acad. Sci.* **202**, 256–272
- Bohn, H., Becker, W. & Trobisch, H. (1973) *Blut* **26**, 303–311
- Chung, S. I. & Folk, J. E. (1972) *J. Biol. Chem.* **247**, 2798–2807
- Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M. & Taylor, H. L. (1946) *J. Amer. Chem. Soc.* **68**, 459–475
- Cooke, R. D. (1973) Ph.D Thesis, University of Bristol
- Cooke, R. D. & Holbrook, J. J. (1974) *Biochem. J.* **141**, 71–78
- Israels, E. D., Paraskewas, F. & Israels, L. G. (1973) *J. Clin. Invest.* **52**, 2398–2403
- Kiesselbach, T. H. & Wagner, R. H. (1967) *Thromb. Diath. Haemorrh.* **26**, 155–165
- Lewis, J. H. (1964) *Proc. Soc. Exp. Biol. Med.* **116**, 120–122
- Loewy, A. G. & Edsall, J. T. (1954) *J. Biol. Chem.* **211**, 829–838
- Loewy, A. G., Dunathan, K., Kriel, R. & Wolfinger, H. L., Jr. (1961a) *J. Biol. Chem.* **236**, 2625–2633
- Loewy, A. G., Dahlberg, A., Dunathan, K., Kriel, R. & Wolfinger, H. L., Jr. (1961b) *J. Biol. Chem.* **236**, 2634–2643
- Lorand, L., Downey, J., Gotoh, T., Jacobson, A. & Tokura, S. (1968) *Biochem. Biophys. Res. Commun.* **31**, 222–230
- Schwartz, M. L., Pizzo, S. V., Hill, R. L. & McKee, P. A. (1971) *J. Biol. Chem.* **246**, 5851–5854
- Schwartz, M. L., Pizzo, S. V., Hill, R. L. & McKee, P. A. (1973) *J. Biol. Chem.* **248**, 1395–1407
- Schachman, H. K. (1957) *Methods Enzymol.* **4**, 32–103
- Takagi, T. & Doolittle, R. F. (1973) *Biochem. Biophys. Res. Commun.* **51**, 186–191