Thrombokinase of the Blood as Trypsin-Like Enzyme

J. H. MILSTONE

From the Department of Pathology, Yale University School of Medicine, New Haven

ABSTRACT Thrombokinase of the blood, while resembling enterokinase in its role of activator, is more closely analogous to trypsin in its intrinsic origin. It probably arises from a plasma precursor; but it is different from plasmin (fibrinolysin). Like trypsin, thrombokinase can activate prothrombin without the aid of other factors; however, it is potentiated by platelets plus calcium. Unlike certain tissue "thromboplastins," it does not sediment appreciably in 2 hours at 85,000 *g*. Like trypsin, it hydrolyzes *p*-toluenesulfonylarginine methyl ester (TAMe). Chromatography on DEAE–cellulose separated thrombin from thrombokinase. The TAMe esterase associated with the thrombokinase fractions was largely suppressed by soybean trypsin inhibitor, while that associated with the thrombin fractions was not. Highly purified thrombokinase was used as starting material; and thrombokinase was eluted in the last major protein band. Under these conditions stepwise elution was as effective as gradient in leading to further purification. The product of 199 liters of bovine plasma was chromatographed in 1 day; and the specific activity was comparable to that attained previously by repeated electrophoretic fractionations. The assembled data suggest that the thrombokinase protein may be approaching homogeneity.

For more than half a century, students of blood coagulation have been inspired and guided by advances in the knowledge of digestive enzymes. In 1899 Schepowalnikow (1), working in Pawlow's laboratory, discovered enterokinase. And in 1904, Morawitz (2) introduced the term, thrombokinase, to suggest that the action of tissue juice on prothrombin was analogous to that of enterokinase on trypsinogen. In the following year, Morawitz (3) recognized that not only tissue juice, but also the blood itself, could activate prothrombin. The responsible factor, intrinsic to the blood, he also designated as thrombokinase. In this usage, he was followed by Mellanby (4), by Collingwood and MacMahon (5), and by Dale and Walpole (6).

That pancreatic extract could give rise to an intrinsic activator of its own enzymes had been suggested by Vernon in 1901 (7). This and Vernon's later work (8) further indicated that the activator was distinct from the rennet enzyme. However, this was not widely accepted until it was corroborated and

clarified in the 1930's by Northrop and Kunitz. Having crystallized the enzymes and their precursors, they showed that the more important proteinase, trypsin, was the activator, while chymotrypsin had the rennet activity (9–14).

Thus it appears that thrombokinase of the blood, while resembling enterokinase in its role of activator, is more closely analogous to trypsin with respect to its intrinsic origin. Moreover, in slight modification of Eagle's analogy (15), thrombokinase of the blood activates prothrombin as trypsin activates chymotrypsinogen.

Not only are these activators analogous, but trypsin can replace thrombokinase as an activator of prothrombin. It had been known since 1916–17 (6, 16, 17) and reiterated in 1928 (18) that trypsin had a blood-clotting action. In 1932, Northrop and Kunitz reported that their crystallized trypsin accelerated the clotting of blood (11). Using their crystallized trypsin, Eagle and Harris (19) found that it could activate partially purified prothrombin, apparently without the aid of calcium. However, Ferguson and Erickson (20), who also used crystalline trypsin supplied by Northrop and Kunitz, added the important observation that cephalin and calcium acted *synergistically* with trypsin. This removed, in 1939, the difficulty which Eagle (15) had sought to avoid in 1937,—that of considering the unlikely possibility that cephalin plus calcium had an action *equivalent* to that of trypsin. Since then, trypsin has been found to activate prothrombin which had been purified in a variety of ways (21–24).

These developments intensified the search for a blood enzyme with a trypsin-like clotting action (25, 26). The idea was entertained that this enzyme was identical with the fibrinolytic enzyme called plasmin by Christensen and MacLeod (27); but it was reported in 1946 (28) that suitably purified fibrinolytic preparations did not activate prothrombin.

The present writer, working in Northrop's laboratory, noted in 1942 (29) that a certain plasma globulin precipitate contained thrombokinase. This merely confirmed an observation made in 1930 by Mellanby (30). Mellanby's work (31) was further corroborated in that the prothrombin fraction, under appropriate conditions, became active "spontaneously" without the addition of extraneous activators. Moreover, the activation could occur in the presence of 0.026 M oxalate. Clearly, if contaminating thrombokinase were responsible for this activation, it must be capable of functioning in the presence of oxalate. And it should be possible to prepare such thrombokinase from this or related fractions. These considerations led to the purification and study of blood thrombokinase in this laboratory during the past fifteen years. By 1947 (32) it could be reported that certain preparations of thrombokinase did not show conspicuous fibrinolytic activity.

Early results indicated that thrombokinase was probably an enzyme which arose from a precursor in the plasma (33). Like trypsin, it was found to activate

prothrombin without the addition of calcium, and even in the presence of oxalate (34). Like trypsin, it could activate prothrombin apparently without the help of any other biologic factor (21, 35). But, when thrombokinase was present as prime activator, production of thrombin was much faster if platelet material or cephalin was included. On the other hand, if thrombokinase was absent, platelets plus calcium were ineffective (36). Although ionic calcium was not required for the action of thrombokinase, it was necessary for the accessory effect of platelets or cephalin:

Basic reaction			Accessory factors
Prothrombin	$\xrightarrow{\text{Thrombokinase}}$	Thrombin	Ca^{++}, platelets (or cephalin)

This is reminiscent of the idea put forth many years ago by Leathes and Mellanby (37) and later by Macfarlane (38) that cephalin was a coenzyme for thrombokinase. The mechanism of the accessory effect is still not well understood. However, the effect is a large one, consistent with the well known practical importance of platelets and calcium ions.

In favor of the view that thrombokinase of the blood might be a molecule about the size of trypsin, rather than a macromolecular particulate like some tissue "thromboplastins," it was found that thrombokinase did not appreciably sediment in 2 hours at 85,000 *g* (39). Its further resemblance to trypsin was suggested by the fact that soybean trypsin inhibitor suppressed the production of thrombin by a mixture of prothrombin and thrombokinase. The general "anti-thromboplastic" effect of the soybean inhibitor had long been known (40–43).

Meanwhile, it had been noted, in 1948 (44) that alpha *p*-toluenesulfonyl-L-arginine methyl ester was a very sensitive substrate for trypsin. And in 1954, *p*-toluenesulfonylarginine methyl ester (TAMe) was reported to be a good substrate for plasmin (45) and for thrombin (46). Sherry, Troll, and Glueck (47) recognized the possibility that TAMe might also be a substrate for the natural activator of prothrombin. And in 1959, Arscott, Koppel, and Olwin (48) reported that thromboplastin-generating systems containing plasma and serum components showed TAMe esterase characteristics.

The method now used in this laboratory for preparation of purified thrombokinase yields only about 1.2 mg/liter of plasma; but the activity of this material is easily measurable when diluted back to the volume of parent plasma (49). Repeated fractionations by continuous flow electrophoresis have led to material which gave a single peak (50), with values for protein, thrombokinase, and TAMe esterase closely parallel. More recently (51), purification comparable to that achieved by repeated electrophoresis has been accomplished more efficiently by chromatography on DEAE-cellulose (52).

The present report describes further investigations of the chromatographic

procedure, and further observations on the trypsin-like nature of thrombokinase.

EXPERIMENTAL

Materials and Methods

DEAE-CELLULOSE *N,N*-Diethylaminoethylcellulose, Eastman Organic Chemicals Rochester, New York. 40 gm was washed on a Buchner funnel with 1 liter of normal sodium hydroxide, followed by many liters of 0.05 M NaCl: 0.02 M Na_2HPO_4 until the pH was below 9.0. It was then dispersed in the foregoing solution to make 1 liter of suspension. A new shipment was used for the column of Fig. 2. With this new batch, packing was more difficult, and flow was faster.

SOYBEAN TRYPSIN INHIBITOR Crystallized five times by Worthington Biochemical Laboratory, Freehold, New Jersey, according to the method of Kunitz (53). Stock solution dialyzed against the Tris buffer used for assay of TAMe esterase.

ESTIMATION OF PROTEIN Method of Lowry *et al.* (54).

ASSAY OF THROMBOKINASE Working assay, as described (49), but with addition of simultaneous tests on a working standard, stored at −18°C. Although assays were performed in the presence of calcium ions, the capacity of chromatographed thrombokinase to function in the presence of 0.01 M oxalate was verified by tests on the peak fraction of Fig. 2. This corroborated past experience with chromatographic fractions (51).

ASSAY OF THROMBIN Essentially as described (49).

ASSAY OF TAME ESTERASE Method of Sherry and Troll (46). In experiments with soy inhibitor the enzyme was incubated with the inhibitor for 30 minutes at 37°C before the substrate was added. There was a control preliminary incubation with buffer, in a test to which no inhibitor was added. Control activity was usually lower with this preincubation than without it.

Gradient Elution of Thrombokinase

The routine preparation of thrombokinase in this laboratory yields an isoelectric precipitate which is dissolved in weakly buffered salt solution (49). Continuous flow electrophoresis of this material at pH 8.6 has revealed three major protein bands, of which the kinase band migrated most rapidly (55). This suggested that thrombokinase would be among the last proteins to be eluted from DEAE-cellulose; for Sober *et al.* had observed a general trend toward higher electrophoretic mobility in successive fractions eluted by decreasing pH and increasing salt gradient (56). It so happened that thrombokinase did appear in the last major protein band to be eluted by a series of

phosphate buffers, applied in stepwise fashion (51). It remained to be seen whether gradient elution would result in greater resolution.

A column, 19 mm in diameter and about 14 cm high, was prepared with a suspension of 6 gm DEAE-cellulose. At 4°C automatic collection of fractions at 60 drops per fraction was begun, as 40 ml of thrombokinase, at the stage of redissolved isoelectric precipitate, was introduced into the column. Immediately after the kinase, fluid was led into the column from a 500 ml centrifuge bottle used as mixing chamber containing 300 ml 0.05 M NaCl: 0.02 M Na_2HPO_4. This in turn received fluid from a 1 liter Erlenmeyer flask containing 900 ml 1.0 M phosphate, pH 8.1. Under pressure, the effluent emerged at 11.4 ml per hour.

As seen in Fig. 1, protein appeared in three major bands and the kinase was associated with the third band. At its peak the kinase activity was 29.5 times that of the working standard; and the ratio of kinase activity to protein concentration was 118. This ratio for the five best kinase fractions was 114, 118, 95, 108, and 107, respectively, with an average value of 108. These figures were in the same range of values as those which were being attained at that time by stepwise elution. Gradient elution therefore offered no obvious advantage in resolution.

The concentration gradient was reflected in the specific conductance of the fractions, which began to rise conspicuously with fraction 36, at 179 ml. These conductance values were converted to molarity of phosphate by use of an empirical graph. From these data, it could be estimated that kinase began to emerge when the phosphate was about 0.10 M and that elution was practically complete when it was 0.40 M. Independently, stepwise elution on several columns had set these limits at 0.12 M phosphate and 0.40 M phosphate, when the pH was maintained near 8.0.

Stepwise Elution of Thrombokinase

Previously (51), stepwise elution had given good results even though there were only two chief kinase fractions. As will be seen below and in Fig. 2, resolution was not significantly improved by dividing the kinase band into more fractions.

A column 19 mm in diameter and about 22 cm high, was prepared with a suspension of 6 gm DEAE-cellulose. Flow rate was maintained at 80 ml per hour by pressure; and the entire run was completed in 1 day at 4°C. Manual collection of 10 ml fractions was begun as 199 ml of thrombokinase, representing the product of 199 liters of plasma, at the stage of redissolved isoelectric precipitate, was introduced into the column. This was followed by 200 ml 0.12 M phosphate, pH 8.0, and then 200 ml 0.40 M phosphate, pH 8.0.

As shown by Fig. 2, there was a good separation of thrombin from thrombokinase. But the separation was not perfect. A trace of kinase, too small to show in the figure, began to appear at 239 ml, as eluant B began to emerge. A small amount of thrombin also appeared in some of the kinase fractions. In varying degree, this separation was previously achieved by ammonium sulfate precipitation (34) and by continuous electrophoresis (55). But the chromatographic separation is the most efficient yet tried.

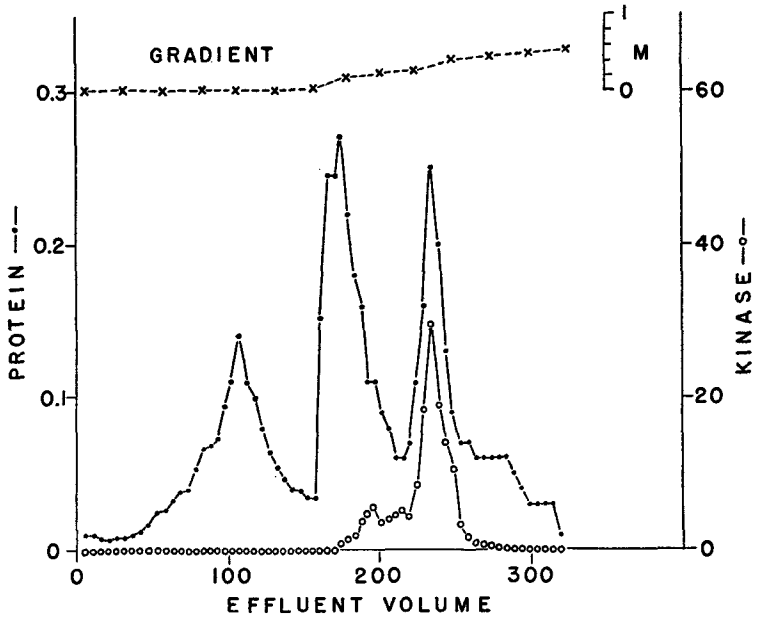

FIGURE 1. Gradient elution of thrombokinase from DEAE-cellulose. Effluent volume, milliliters. Protein, milligrams per milliliter. Kinase, activity per milliliter, relative to working standard. *M*, molarity of phosphate.

For the seven best fractions, the ratio of kinase activity to protein was 121, 125, 132, 140, 146, 133, and 132, respectively, with an average value of 133. This represented a twofold purification, since the ratio for the starting material was 68. This ratio did not vary enough among the seven fractions to indicate significant resolution within that band. Therefore, the spur on the left side of the kinase peak in Fig. 2 is attributed to an irregularity in column flow, rather than to another component. This interpretation is substantiated by the parallelism of the values for protein, kinase, and esterase in this region. The seven best fractions together contained 71 per cent of the original kinase activity in 35.5 per cent of the original volume; *i.e.*, thrombokinase was concentrated by the procedure. These results offer the basis for a simple chromatographic step in the purification of thrombokinase.

TAMe Esterase Activity of Thrombokinase Fractions

The close association of TAMe esterase with thrombokinase through repeated electrophoretic fractionations has been demonstrated (50, 55). The five main electrophoretic fractions of the purest material had, respectively, 329, 359, 338, 333, and 325 TAMe esterase units per milligram protein, with an average

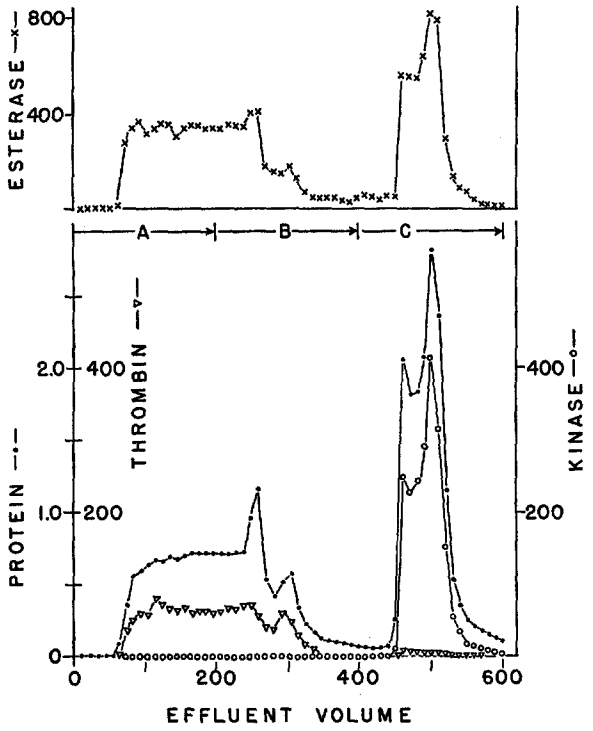

FIGURE 2. Stepwise elution of thrombokinase from DEAE-cellulose. *A*, thrombokinase in 0.05 M sodium chloride: 0.02 M phosphate, pH 7.8; *B*, 0.12 M phosphate, pH 8.0; *C*, 0.40 M phosphate, pH 8.0. Effluent volume, milliliters. Protein, milligrams per milliliter. Thrombin, NIH units per milliliter. Kinase, activity per milliliter, relative to working standard. Esterase, TAMe units per milliliter.

value of 337. The two chief kinase fractions yielded by a previous chromatographic procedure averaged 347 units per milligram (51). The seven main kinase fractions of Fig. 2 had an average value of 296 TAMe esterase units per milligram protein. In view of the comparable levels reached by different methods and the experience with repeated electrophoretic fractionations, it seems possible that practically all the esterase activity of the kinase band belongs to the kinase protein. These data also suggest that the protein may be

approaching homogeneity, as do the data on specific kinase activity of the fractions.

How much of the TAMe esterase of the thrombin band belongs to the thrombin is left open. However, there is an interesting difference between the esterase activity of the two bands of Fig. 2. In Table I, it is seen that the TAMe esterase activity of a thrombin fraction (F15) was unaffected by soybean trypsin inhibitor in a concentration of 2.0 mg per ml. This is in accord with the results obtained with thrombin by Sherry and Troll (46).

On the other hand, the esterase activity of the peak kinase fraction (F48) is mostly suppressed by the soy inhibitor at 0.2 mg per ml and still more so by 2.0 mg per ml. This is in harmony with the fact that the soy inhibitor suppressed the direct, unaided activation of prothrombin by thrombokinase (39). Moreover, the results of Table I make it unlikely that the soy inhibitor exerts its effect by acting on the substrate; for there the substrate is still available to the thrombin fraction. The most plausible interpretation is that the soy inhibitor reacts with the enzyme, thrombokinase.

TABLE I

EFFECT OF SOYBEAN TRYPSIN INHIBITOR ON HYDROLYSIS OF TAME

Soy inhibitor	F15 (Thrombin)	F48 (Kinase)
mg. per ml.	*ml. 0.02 N NaOH*	*ml. 0.02 N NaOH*
0.0	1.10	1.19
0.2	1.14	0.35
2.0	1.07	0.17

Values in the body of the table represent formol titrations of groups liberated by hydrolysis. The concentration of inhibitor is given in milligrams per milliliter of buffer–enzyme–inhibitor–substrate mixture.

DISCUSSION

While evidence is accumulating that thrombokinase bears similarities to trypsin, there are also differences. Preliminary tests for casein digestion in this laboratory (57) have suggested that the proteolytic activity of thrombokinase may be slight compared to that of trypsin.

Trypsin frequently does not act appreciably on native proteins (58); and this may prove to be true for thrombokinase. A solution of bovine plasma albumin is used as a diluent in the assay of bovine thrombokinase (49). Albumin actually enhances the assay, which indicates that it does not act as an effectively competing substrate for the kinase. This is appropriate physiologically; for thrombokinase must activate prothrombin in a natural environment of albumin molecules which are in great numerical preponderance.

Correspondingly, it might be physiologically advantageous for thrombokinase to be relatively insusceptible to much of the antitryptic power of plasma. It was reported ten years ago (34) that some preparations of serum protein depress the activation of prothrombin by trypsin, but exert little influence on the activation of prothrombin by thrombokinase.

Then again, there are degrees of similarity in the response to inhibitors. In different amounts, and under different conditions, the crystalline trypsin inhibitor from pancreas (14) has exerted no (29), little (59), or moderate (60) effect on the activation of prothrombin. Against certain prothrombin-activating systems, the soy inhibitor has been effective although the pancreatic inhibitor has not (22). Soybean trypsin inhibitor largely blocks the action of purified thrombokinase, whether the substrate is prothrombin or TAMe.

The capacity to hydrolyze TAMe is a property shared not only with trypsin, but also with a few enzymes from the plasma (61).

Many years ago (32, 33), results with partially purified materials led to the working hypothesis that thrombokinase arose autocatalytically from a precursor, as in the case of trypsin. Certain subsequent observations (34, 35, 49) have been in accord with this hypothesis. This is one of the many aspects of the trypsin analogy which remain to be studied in detail.

The writer was assisted by Nadia Oulianoff, Harriet F. Brown, and Thomas R. Saxton.
This investigation was supported by Research Grant H-3906 from the National Heart Institute, Public Health Service.

REFERENCES

1. SCHEPOWALNIKOW, N. P., quoted from NORTHROP, J. H., KUNITZ, M., and HERRIOTT, R. M., Crystalline Enzymes, New York, Columbia University Press, 2nd edition, 1948, 127.
2. MORAWITZ, P., *Beitr. chem. Physiol. u. Path.*, 1904, **5**, 133.
3. MORAWITZ, P., *Ergebn. Physiol.*, 1905, **4**, 307.
4. MELLANBY, J., *J. Physiol.*, 1909, **38**, 28.
5. COLLINGWOOD, B. J., and MACMAHON, M. T., *J. Physiol.*, 1912, **45**, 119.
6. DALE, H. H., and WALPOLE, G. S., *Biochem. J.*, 1916, **10**, 331.
7. VERNON, H. M., *J. Physiol.*, 1901, **27**, 18.
8. VERNON, H. M., *Biochem. J.*, 1914, **8**, 494.
9. NORTHROP, J. H., and KUNITZ, M., *Science*, 1931, **73**, 262.
10. NORTHROP, J. H., and KUNITZ, M., *J. Gen. Physiol.*, 1932, **16**, 267.
11. NORTHROP, J. H., and KUNITZ, M., *J. Gen. Physiol.*, 1932, **16**, 295.
12. KUNITZ, M., and NORTHROP, J. H., *Science*, 1933, **78**, 558.
13. KUNITZ, M., and NORTHROP, J. H., *J. Gen. Physiol.*, 1935, **18**, 433.
14. KUNITZ, M., and NORTHROP, J. H., *J. Gen. Physiol.*, 1936, **19**, 991.
15. EAGLE, H., *Medicine*, 1937, **16**, 95.
16. DOUGLAS, S. R., and COLEBROOK, L., *Lancet*, 1916, **2**, 180.

17. HEARD, W. N., *J. Physiol.*, 1917, **51**, 294.
18. WALDSCHMIDT-LEITZ, E., STADLER, P., and STEIGERWALDT, F., *Naturwissenschaften*, 1928, **16**, 1027.
19. EAGLE, H., and HARRIS, T. N., *J. Gen. Physiol.*, 1937, **20**, 543.
20. FERGUSON, J. H., and ERICKSON, B. N., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 265.
21. MILSTONE, J. H., *Yale J. Biol. and Med.*, 1950, **22**, 675.
22. TRAVIS, B. L., and FERGUSON, J. H., *J. Clin. Inv.*, 1951, **30**, 112.
23. KLEINFELD, G., and HABIF, D. V., *Proc. Soc. Exp. Biol. and Med.*, 1953, **84**, 432.
24. ALEXANDER, B., *in* Symposium X, Blood-Clotting Factors, (E. DEUTSCH, editor), *Proc. 4th Internat. Congr. Biochem., Vienna*, New York, Pergamon Press, 1959, **10**, 37.
25. FEISSLY, R., *Schweiz. med. Woch.*, 1942, **72**, 516.
26. TAGNON, H. J., *J. Lab. and Clin. Med.*, 1942, **27**, 1119.
27. CHRISTENSEN, L. R., and MACLEOD, C. M., *J. Gen. Physiol.*, 1945, **28**, 559.
28. SEEGERS, W. H., and LOOMIS, E. C., *Science*, 1946, **104**, 461.
29. MILSTONE, H., *J. Gen. Physiol.*, 1942, **25**, 679.
30. MELLANBY, J., *Proc. Roy. Soc. London, Series B*, 1930, **107**, 271.
31. MELLANBY, J., *Proc. Roy. Soc. London, Series B*, 1933, **113**, 93.
32. MILSTONE, J. H., *Science*, 1947, **106**, 546.
33. MILSTONE, J. H., *J. Gen., Physiol.*, 1948, **31**, 301.
34. MILSTONE, J. H., *J. Gen. Physiol.*, 1951, **35**, 67.
35. MILSTONE, J. H., *Yale J. Biol. and Med.*, 1952, **25**, 19.
36. MILSTONE, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 225.
37. LEATHES, J. B., and MELLANBY, J., *J. Physiol.*, 1939, **96**, 39P.
38. MACFARLANE, R. G., *J. Physiol.*, 1947, **106**, 104.
39. MILSTONE, J. H., *J. Gen. Physiol.*, 1955, **38**, 757.
40. TAGNON, H. J., and SOULIER, J. P., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 440.
41. MACFARLANE, R. G., and PILLING, J., *Lancet*, 1946, **2**, 888.
42. CROXATTO, H., *Rev. Soc. arg. biol.*, 1946, **22**, 477.
43. GLAZKO, A. J., *J. Clin. Inv.*, 1947, **26**, 364.
44. SCHWERT, G. W., NEURATH, H., KAUFMAN, S., and SNOKE, J. E., *J. Biol. Chem.*, 1948, **172**, 221.
45. TROLL, W., SHERRY, S., and WACHMAN, J., *J. Biol. Chem.*, 1954, **208**, 85.
46. SHERRY, S., and TROLL, W., *J. Biol. Chem.*, 1954, **208**, 95.
47. SHERRY, S., TROLL, W., and GLUECK, H., *Physiol. Rev.*, 1954, **34**, 736.
48. ARSCOTT, P. M., KOPPEL, J. L., and OLWIN, J. H., *Nature*, 1959, **183**, 753.
49. MILSTONE, J. H., *J. Gen. Physiol.*, 1959, **42**, 665.
50. MILSTONE, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1960, **103**, 361.
51. MILSTONE, J. H., *Nature*, 1960, **187**, 1127.
52. PETERSON, E. A., and SOBER, H. A., *J. Am. Chem. Soc.*, 1956, **78**, 751.
53. KUNITZ, M., *J. Gen. Physiol.*, 1946, **29**, 149.
54. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., *J. Biol. Chem.*, 1951, **193**, 265.
55. MILSTONE, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1959, **101**, 660.

56. SOBER, H. A., GUTTER, F. J., WYCKOFF, M. W., and PETERSON, E. A., *J. Am. Chem. Soc.*, 1956, **78**, 756.
57. SISSON, J. A., and MILSTONE, J. H., 1960, unpublished data.
58. NORTHROP, J. H., KUNITZ, M., and HERRIOTT, R. M., Crystalline Enzymes, New York, Columbia University Press, 2nd edition, 1948, 146.
59. FERGUSON, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1942, **51**, 373.
60. GROB, D., *J. Gen. Physiol.*, 1943, **26**, 423.
61. WEBSTER, M. E., and PIERCE, J. V., *Proc. Soc. Exp. Biol. and Med.*, 1961, **107**, 186.