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THE CHARACTERIZATION OF *n*-BUTANOL-PSEUDOSOLUBILIZED AND TRYPSIN-SOLUBILIZED PORCINE THYROID IODIDE PEROXIDASE

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Summary

Porcine thyroid peroxidase (Iodide: hydrogen-peroxide oxidoreductase, EC 1.11.1.8) was solubilized by proteolytic and non-proteolytic procedures. A kinetic and physical study was undertaken to ascertain the catalytic properties of the peroxidase prepared by the two purported solubilization procedures. Where possible, the properties of the two enzyme preparations were compared with the original microsomal preparation.

The *n*-butanol-solubilized thyroid iodide peroxidase is not truly soluble, but exists as a large molecular weight lipoprotein aggregate. The trypsin-solubilized thyroid iodide peroxidase is truly soluble, active, and contains lipids. The microsomes, butanol-pseudosolubilized enzyme, and trypsin-solubilized enzyme have similar kinetic properties such as pH optima, K_m for iodide and H_2O_2, sigmoid character of the saturation curves, substrate inhibition, and inhibition by 3,5-diiodotyrosine. Since the proteolytic solubilization procedure produced a soluble peroxidase with catalytic properties similar to the microsomal preparation, trypsin-solubilized peroxidase can be studied with reasonable assurance that its properties are essentially unaltered and are not artifacts of the solubilization procedure.

Introduction

Proteolytic enzymes, in combination with detergents, have been utilized to effect solubilization of thyroid iodide peroxidase from microsomal membranes [1—5]. Unfortunately, fragmentation of the thyroid iodide peroxidase molecule may occur during the solubilization procedure and result in irreversible modification of catalytic and physical properties of the enzyme. For example,

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minor variations in proteolytic solubilization procedures have produced a wide spectrum of different thyroid peroxidase species with different molecular weights and different physical and kinetic properties [1,6]. Similarly, fragmentation of cytochrome b_5 and NADH-cytochrome b_5 reductase occurs when proteolytic enzyme solubilization procedures are employed [7—9].

Since the observed properties of the trypsin-solubilized thyroid iodide peroxidase may be artifacts of the solubilization procedure, a reevaluation of this procedure is warranted. Non-proteolytic solubilization methods involving *n*-butanol were recently reported to solubilize an intact thyroid iodide peroxidase molecule from the membrane [10,11]. In the present study, the properties of thyroid iodide peroxidase prepared by these two purported solubilization methods were compared. Where possible, the properties of both types of enzyme preparations were also compared to the properties of the original membrane fragments. To our knowledge, this is the most complete comparative study of the catalytic and physical properties of thyroid iodide peroxidases solubilized by proteolytic and non-proteolytic methods.

Methods

1. Enzyme preparation. A thyroid microsomal fraction was isolated from frozen porcine thyroid glands (Armour Pharmaceutical Co., Kankakee, Ill., U.S.A.) according to the procedure of Neary et al. [12]. All buffers used in the procedure contained 1 mM phenylmethylsulfonylfluoride (Sigma Chemical Co.) as a proteolytic enzyme inhibitor. The microsomes were resuspended in buffer and stored at —20° C.

Microsomal suspensions were thawed and thyroid iodide peroxidase was pseudosolubilized by *n*-butanol according to the procedure of Davidson et al. [11]. The protein concentration of the micrsomal suspensions was usually 4—5 mg/ml, as determined by Lowry et al. [13]. The pseudosoluble peroxidase was stored frozen at —20° C. Some protein aggregation occurred during the freeze-thaw cycle and was removed by centrifugation at 10 000 × *g* for 30 min with little loss in peroxidase activity. The 10 000 × *g* supernatant was optically clear and readily passed through a 100-nm pore filter (Millipore Filter Corporation).

Thyroid iodide peroxidase was solubilized by trypsin (bovine pancreas, twice crystallized, Sigma) and purified using the procedure of Pommier et al. [4], except 1.0% sodium cholate (A grade, Calbiochem) was substituted for digitonin. This modification in the procedure caused the soluble thyroid peroxidase to precipitate between 35—65% $(NH_4)_2SO_4$ saturation rather than 0—35% saturation [4]. All other properties of the enzyme remained unchanged from the original procedure of Pommier et al. [4], who also reported that further purification efforts resulted in an unexplained inactivation of the peroxidase. Hence, purification beyond the Sephadex G-100 step was not attempted in this study. Most preparative steps were performed at 3° C.

2. Peroxidase assay procedures. Thyroid peroxidase activity was measured using the guaiacol assay of Hosoya [14,15] and was standardized according to the definitions of Davidson et al. [11], where the Guaiacol Unit (G.U.) is defined as the amount of peroxidase that will catalyze the formation of 1.0 μmol oxidized guaiacol/min at 25° C. The molar extinction coefficient for the

oxidized guaiacol is 5570 $M^{-1} \cdot cm^{-1}$ [16]. The assay was performed at pH 8.2 instead of pH 7.4, the value originally used by Hosoya [14].

Thyroid peroxidase activity was also measured using the triiodide assay of Alexander [17] and the Iodide Unit (I.U.) is defined as the amount of peroxidase that will catalyze the formation of 1.0 μmol triiodide ion/min at 25° C. The molar extinction coefficient for the triiodide ion is 22 900 $M^{-1} \cdot cm^{-1}$ [17]. The linearity of the assay is seriously affected by the concentration of H_2O_2 in the cuvette and the absorbance of the triiodide ion does not obey Beer's Law at low iodide concentrations due to the iodide/iodine/triiodide equilibrium [18]. Therefore, initial velocities were corrected for the equilibrium constant, Kd, according to Eqn. 1 [19]:

$$V_0 = (\Delta A/\text{min}) \frac{(K_d + C)}{C} \tag{1}$$

where K_d = 1.35 mM and C = iodide concentration.

Inhibition studies using 3,5-diiodotyrosine (Nutritional Biochemical Co.) were performed by the addition of 0.10 mM diiodotyrosine (in distilled water) to the sample cuvette in place of the water. All assays were performed at 25° C. The concentration of the H_2O_2 was determined by monitoring the absorbance of H_2O_2 at a wavelength of 230 nm (molar extinction coefficient, 72.4 $M^{-1} \cdot cm^{-1}$), according to George [20]. Optimum H_2O_2 concentrations for various concentrations of guaiacol and iodide were routinely performed. Velocity-enzyme concentration assays were performed to assess linearity of rate with respect to enzyme concentration. Control assays were performed in which either peroxidase or H_2O_2 was omitted from the assay. Non-enzymatic rates were not encountered for the guaiacol assay, whereas the triiodide assay exhibited a minor non-enzymatic rate when peroxidase was omitted from the assay system. This difficulty was remedied by using appropriate blanks.

Saturation curves for H_2O_2 and iodide were fitted to Eqn. 2 using the least-squares fit by the gradient search method. The initial estimates for parameters were determined visually from plots of raw data. A program utilizing routines for raw data, function plotting, and the gradient least-squares fit translated into WANG BASIC from the FORTRAN programs of Bevington [21] was written for the Wang 2200 computer with output writer and plotter (Cook, P.F., unpublished data). "Goodness of fit" was determined by minimization of Chi-square. All other plots were fitted visually.

$$v = \frac{VA^n}{Ka + A^n} \tag{2}$$

3. Physical studies on thyroid peroxidase

(a) Temperature stability. 1-ml aliquots of resuspended microsomes, butanol pseudosolubilized, and trypsin-solubilized thyroid peroxidase were incubated in capped test tubes at various temperatures (0—75° C) for 1 h. Aliquots were removed from the tubes and immediately assayed at 25° C for peroxidase activity using the guaiacol and triiodide assays. The activities were compared to the activity of the control which was held at 0° C for 1 h.

(b) Lipid analysis. Lipids were extracted according to the procedure of Bligh

and Dyer [22], and were subjected to thin-layer chromatography according to the procedure of Freeman et al. [23]. Fatty acid methyl esters of the lipid samples were prepared for gas-liquid chromatography using the procedure of Stoffel et al. [24], except during derivatization 3% H_2SO_4 was used in place of 1% HCl. The methyl esters were resolved using a polar column containing 10% diethylene glycol succinate on Chromosorb W.

(c) Polyacrylamide gel electrophoresis. Electrophoresis was performed at 3° C according to the procedure of Davis [25] using 5% gel at constant current (4 mA/gel). The protein bands were detected using Coomassie Brilliant Blue [26]. The butanol thyroid peroxidase was detected by incubating the gels in 3,3-diaminobenzidine (ICN Pharmaceuticals Inc.) according to the procedure of Novikoff et al. [27]. The trypsin-solubilized thyroid peroxidase was detected by the guaiacol staining procedure of Taurog [5].

Results

1. n-Butanol pseudosolubilization of thyroid peroxidase

Thyroid peroxidase was pseudosolubilized by *n*-butanol and was obtained from the crude homogenate in yields of 50% with a 10-fold increase in the specific activity. The preparation could oxidize 0.46 μmol guaiacol/min per mg protein. The results compare favorably with the original results of Davidson et al. [11].

2. Trypsin solubilization of thyroid peroxidase

Thyroid peroxidase was solubilized by treatment of membrane fragments with trypsin and then with sodium cholate as described earlier. The enzyme was purified 860-fold from the crude homogenate with a 16% recovery and could oxidize 45.3 μmol guaiacol/min per mg protein. These results compare favorably with the original results of Pommier et al. [4]. Polyacrylamide gel electrophoresis demonstrated that the enzyme is about 20% pure. The trypsin-solubilized thyroid peroxidase was passed through a Bio-Gel A-5m column to assess the molecular size of the peroxidase preparation. The elution profile (unpublished data) clearly demonstrated that the trypsin/cholate-solubilized thyroid peroxidase was retarded. Thus, this preparation can be regarded as representing a true molecular solution of active thyroid peroxidase molecules.

3. Kinetic studies on thyroid peroxidase

(a) The guaiacol assay. The pH optimum of all three peroxidase preparations using the guaiacol assay was approx. pH 8.2 (unpublished data). This value corresponds to the pH optimum reported by other investigators [10,15].

The butanol-pseudosolubilized and trypsin-solubilized thyroid peroxidase preparations exhibited similar H_2O_2 saturation curves that can be described as hyperbolic, with an optimum H_2O_2 concentration range between 40–70 μM, and with marked substrate inhibition occurring at H_2O_2 concentrations greater than 100 μM. The saturation curves were run at pH 8.2 and at a guaiacol concentration of 0.5 mM. These conditions were found to yield valid kinetic parameters for H_2O_2. The results are summarized in Table I. The differences in the $K_m(H_2O_2)$ values were not held to be significant.

TABLE I

SUMMARY OF KINETIC PARAMETERS OBTAINED FROM THE GUAIACOL AND TRIIODIDE ASSAYS

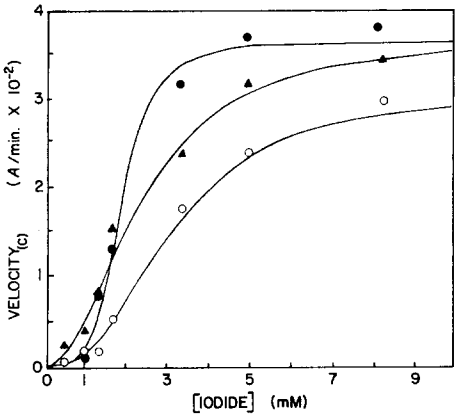
Assay system and enzyme preparation	Kinetic parameters derived from H_2O_2 saturation curves			
	V	K_m (μm)	Hill number	
Guaiacol assay	nmol/min			
B-TPO * (0.20 mg)	16.5	4.3	—	
T-TPO ** (2.4 μg)	22.5	7.0	—	
Triiodide assay	Iodide units/min			
Microsomes (0.19 mg)	0.015	26	2.6	
B-TPO (0.16 mg)	0.039	40	1.6	
T-TPO (2.4 μg)	0.035	42	1.1	
	Kinetic parameters derived from iodide saturation curves			
	V	K_m (mM)	Hill number	K_I (μM)
Triiodide assay	ΔA/min			
Microsomes (0.12 mg)	0.031	3.0	2.6	—
B-TPO (0.19 mg)	0.036	1.9	4.6	46
T-TPO (2.4 μg)	0.037	2.3	2.1	46

* B-TPO, butanol-pseudosolubilized peroxidase.
** T-TPO, trypsin-solubilized peroxidase.

(b) The triiodide assay. The pH optimum of all three enzyme preparations used in the triiodide assay was found to be 7.6 (unpublished data). Mahoney and Igo [28] reported a pH optimum of 7.5 for thyroid peroxidase using the triiodide assay.

Iodide saturation curves were run using the three enzyme preparations and the results are shown in Fig. 1 and are summarized in Table I. The results clearly demonstrate the nearly identical sigmoid character of all three enzyme preparations with Hill numbers ranging between 3 and 5 and apparent K_m (iodide) (equivalent to $S_{0.5}$) values ranging between 2—3 mM. Pommier et al. [4,19,29] observed sigmoid iodide saturation curves for a trypsin-solubilized thyroid peroxidase and noted apparent K_m (iodide) values between 1—6 mM. The butanol-pseudosolubilized peroxidase has not been previously characterized using the triiodide assay.

A comparison was made between the microsomes, butanol-pseudosolubilized, and trypsin-solubilized thyroid peroxidase preparations with respect to H_2O_2 saturation and the results are summarized in Table I. The microsomes and butanol pseudosolubilized peroxidase exhibit sigmoid H_2O_2 saturation curves, while the trypsin peroxidase preparation exhibits a distinctly hyperbolic curve with a Hill number of 1.1. It should be noted that the microsomes and butanol pseudosolubilized peroxidase contained considerable extraneous protein and that the time course exhibited distinct lag-times at low concentrations of H_2O_2.



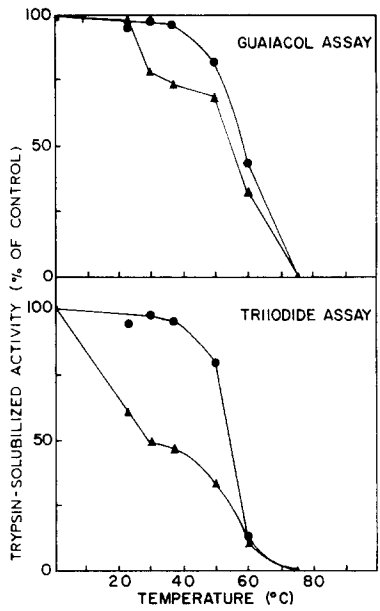


Fig. 1. The Triiodide assay: iodide saturation curves microsomes, 0.012 Iodide Units (0.12 mg)/assay, (○); butanol-pseudosolubilized thyroid peroxidase, 0.038 Iodide Units (0.19 mg)/assay, (●); trypsin-solubilized peroxidase, 0.034 Iodide Units (2.4 μg)/assay, (▲).

Fig. 2. Temperature stability of butanol- and trypsin-solubilized thyroid peroxidase. Initial rates were obtained and the peroxidase activity was expressed as % activity of the control (enzyme incubated at 0°C for 1 h). Butanol-pseudosolubilized thyroid peroxidase, 0.14 Guaiacol Units (0.30 mg)/guaiacol assay and 0.04 Iodide Units (0.20 mg)/triiodide assay, (●); and trypsin solubilized peroxidase, 0.11 Guaiacol Units (2.4 μg)/guaiacol assay and 0.03 Iodide Units (2.4 μg)/triidode assay, (▲).

The trypsin-solubilized peroxidase contained very litte extraneous protein and did not exhibit a lag-time. The non-enzymatic iodination of protein by iodine at neutral pH was found to produce marked lag-times. Clearly, if large amounts of extraneous protein are present in the assay, then at low concentrations of H_2O_2 nearly all of the H_2O_2 will be consumed in the oxidation of iodide to iodine and this iodine will then iodinate the extraneous protein. Thus, marked lag-times will be generated and any rate at 353 nm corresponding to the production of the triiodide ion will not be the true initial rate. At low concentrations of H_2O_2 very low, non-steady state rates could be produced and this could generate a pseudosigmoid H_2O_2 saturation curve, an artifact of the triiodide assay. Experiments in which boiled butanol-pseudosolubilized peroxidase was added to the trypsin-solubilized peroxidase assays, produced significant lag-times and decreased the apparent rates.

Finally, both the butanol-pseudosolubilized and trypsin-solubilized thyroid peroxidase are inhibited to the same extent by 3,5-diiodotyrosine. The K_I was calculated and found to be 46 μM.

4. Physical studies on thyroid peroxidase

(a) Temperature stability. The butanol-pseudosolubilized and trypsin-solubilized thyroid peroxidase were incubated at various temperatures for 1 h

and then assayed for peroxidase activity using the guaiacol and triiodide assays. The results are shown in Fig. 2. The butanol-pseudosolubilized peroxidase exhibits a monophasic temperature inactivation curve with 50% inactivation at 57—59°C in both assays. The microsomes exhibited temperature inactivation curves very similar to the butanol-pseudosolubilized peroxidase. The trypsin-solubilized peroxidase exhibited a biphasic temperature inactivation curve for both the guaiacol and triiodide assays. The guaiacol assay demonstrated an initial inactivation in the lower temperature range.

(b) Lipid analysis on thyroid peroxidase. Thin-layer chromatographic analysis demonstrated that the three enzyme preparations contain considerable neutral and polar lipids. The presence of lipids in the microsomes and butanol-pseudosolubilized peroxidase preparations corroborates the results of Neary et al. [10,11,30]. The presence of substantial quantities of lipids in the trypsin-solubilized peroxidase preparation was somewhat of a surprise, as this preparation was expected to contain very little or no lipid. Methyl ester derivatives of fatty acids obtained from the three enzyme preparations were analyzed by gas-liquid chromatography. The results are summarized in Table II. All three preparations contain relatively large amounts of palmitic acid (16 : 0), oleic acid (18 : 0), and linoleic acid (18 : 1). This is the first relatively complete analysis and comparison of the lipids associated with thyroid peroxidase preparations and is also the first report of lipids associated with a proteolytically-solubilized thyroid peroxidase preparation.

(c) Polyacrylamide gel electrophoresis of thyroid peroxidase. Polyacrylamide gel electrophoresis was performed on the butanol-pseudosolubilized and trypsin-solubilized thyroid peroxidase preparations and separate gels were stained for protein and peroxidase activity. The butanol-pseudosolubilized peroxidase exhibited two major bands as judged by Coomassie-staining and

TABLE II

SUMMARY OF GAS-LIQUID CHROMATOGRAPHIC ANALYSIS OF THYROID PEROXIDASE FATTY ACIDS

Fatty acids were identified by comparison of retention time with authentic standards. The areas under each peak were measured and expressed as percentages of the total area.

Fatty acid	Microsomes (%)	Butanol peroxidase (%)	Trypsin peroxidase (%)
12 : 0	— *	—	4.6
12 : 1	2.4	—	4.5
14 : 0	4.2	4.6	7.2
14 : 1	2.6	1.8	3.7
16 : 0	22.8	23.8	25.3
16 : 1	3.6	tr	tr
16 : 2	1.1	tr	1.5
18 : 0	10.6	17.3	7.2
18 : 1	44.2	47.7	42.1
18 : 2	7.6	4.5	3.9
20 : 0	tr **	tr	—
21 : 0	tr	tr	—

* — = not detected.

** tr = trace.

both bands stained positive for peroxidase activity. The trypsin-solubilized peroxidase exhibited three major and five minor bands as judged by Coomassie-staining. The gel pattern was very similar to those obtained by Pommier et al. [4] and by Taurog [5] who employed 7.5% gels at room temperature with 4 mA/gel.

Discussion

The *n*-butanol solubilization procedure was reported to solubilize an intact thryoid peroxidase molecule from the membrane [10,11]. Unfortunately, this procedure may be generating a very large molecular weight lipoprotein aggregate which is not amenable to further purification by traditional biochemical methods. Specifically, we have found that the treatment of the butanol thyroid peroxidase preparation with concanavalin A, protamine sulfate, ammonium sulfate, and ammonium sulfate in the presence of 0.5% sodium cholate irreversibly aggregated the thyroid peroxidase and usually resulted in the co-precipitation of 90—100% of the protein with the peroxidase activity. The peroxidase could not be eluted from the Sepharose 2B-concanavalin A affinity column despite repeated efforts using a number of elution buffers containing α-methyl-D-mannoside and detergents. Davidson et al. [11] reported a purification step using Whatman DE-52 Cellulose chromatography and employing a pH gradient (pH 10.3—12.7) to elute the peroxidase from the column. Efforts to duplicate these results proved unsatisfactory because the peroxidase activity peak coincided with the main protein peak. In addition, the eluted peroxidase did not "activate" [11] upon standing, and hence, little or no increase in the specific activity was observed. We suspect the "activation" phenomenon [11] was an artifact of the guaiacol assay since a non-linear velocity versus enzyme concentration relationship was observed for thyroid peroxidase using the guaiacol assay. Unusually low velocities were encountered at low enzyme concentrations, but the velocities significantly increased at slightly higher enzyme concentrations. The butanol thyroid peroxidase preparation was applied to a number of gel filtration columns (Sephadex G-100, G-200, Bio-Gel A-5m, and Bio-Gel A-15m). The peroxidase activity eluted with the void volume in each case with no significant purification.

Davidson et al. [11] and Neary et al. [10] reported that the butanol "solubilization" procedure yielded a truly soluble thyroid peroxidase molecule since their preparation obeyed Razin's solubility criteria [31]. Some of their observations, however, are difficult to explain. First, Neary et al. [10] reported that their butanol thyroid peroxidase preparation was retarded on Sephadex G-G-200 and possessed an apparent molecular weight of 350 000. The material that was applied to the column, however, was initially treated with protamine sulfate and ammonium sulfate. These workers caution against the use of such treatments as they cause aggregation and induce the formation of large molecular weight aggregates of peroxidase. The observed retardation on the column may result from physico-chemical interactions on the surface of the Sephadex beads and may not be due to true gel filtration. Secondly, while these workers did not observe any membranous structures by electron microscopy, they detected particles of 50—100 nm diameter [11].

The microsomes, butanol-pseudosolubilized, and trypsin-solubilized thyroid peroxidase preparations are very similar with respect to the basic physical and kinetic properties such as presence of lipid, pH optima, K_m for iodide and H_2O_2, sigmoid character of the saturation curves, presence of substrate inhibition, and inhibition by 3,5-diiodotyrosine. Many of the kinetic properties were very similar to values reported in the literature [4,10,15,19,28,29]. Minor differences in kinetic properties were not held to be significant.

The difference between the butanol-pseudosolubilized and the trypsin-solubilized peroxidase preparations with respect to temperature inactivation, (Fig. 2) may result from differences in the lipid content and physical structure of the two molecules. The butanol peroxidase preparation was shown to be a very large lipoprotein aggregate. Thyroid peroxidase within such an aggregate would not be expected to denature as readily because of restraints imposed upon it by the physico-chemical properties of the aggregate. The microsome preparation (unpublished data) exhibited a similar temperature inactivation curve. This observation supports our contention that the membrane may impose physical stability to the thyroid peroxidase molecule. The trypsin-solubilized peroxidase, while it contains lipid, is a truly soluble preparation and does not possess the physical restraints of the butanol and microsomal preparations. One could envision that upon heating, a portion of the polypeptide chain(s) of the trypsin peroxidase unfolds and causes partial enzyme inactivation to occur. This would result in the first phase of the biphasic temperature inactivation curve, followed by further unfolding and denaturation at higher temperatures.

The discovery that trypsin-solubilized peroxidase contains considerable lipid is interesting and not unreasonable. While trypsin may "nick" the thyroid peroxidase molecule at specific points, detergents are required to disrupt the lipid bilayer and thereby solubilize peroxidase from the hydrophilic portion of the membrane. Solubilized preparations of this type frequently possess a "shell" of lipid and detergent molecules around the protein. Neary et al. [10,30] have reported that preparations that were depleted of lipid lost peroxidase activity.

In summary, the proteolytic solubilization procedure produced a soluble peroxidase with catalytic properties similar to the microsomal preparation. Hence, the trypsin-solubilized peroxidase can be studied with the knowledge that the catalytic properties are essentially unaltered and are not artifacts of the solubilization procedure.

References

1 Alexander, N.M. (1977) Endocrinology 100, 1610–1620
2 Danner, D.J. and Morrison, M. (1971) Biochim. Biophys. Acta 235, 44–51
3 Hosoya, T. and Morrison, M. (1967) J. Biol. Chem. 242, 2828–2836
4 Pommier, J., dePrailaune', S. and Nunez, J. (1972) Biochimie 54, 483–492
5 Taurog, A. (1970) Rec. Prog. Horm. Res. 26, 189–247
6 Ljunggren, J. and Åkeson, Å. (1968) Arch. Biochem. Biophys. 127, 346–353
7 Spatz, L. and Strittmatter, P. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1042–1046
8 Strittmatter, P., Rogers, M.J. and Spatz, L. (1972) J. Biol. Chem. 247, 7188–7194
9 Spatz, L. and Strittmatter, P. (1973) J. Biol. Chem. 248, 793–799
10 Neary, J.T., Davidson, B. and Maloof, F. (1973) Ann. N.Y. Acad. Sci. 212, 183–194

11 Davidson, B., Neary, J.T., Schwartz, S., Maloof, F. and Soodak, M. (1973) Prep. Biochem. 3, 473—493
12 Neary, J.T., Davidson, B., Armstrong, A., Maloof, F. and Soodak, M. (1973) Prep. Biochem. 3, 495—508
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Hosoya, T., Kondo, Y. and Ui, N. (1962) J. Biochem. (Tokyo) 52, 180—189
15 Hosoya, T. (1963) J. Biochem. (Tokyo) 53, 381—388
16 Hosoya, T. (1960) J. Biochem. (Tokyo) 47, 794—803
17 Alexander, N.M. (1962) Anal. Biochem. 4, 341—345
18 Morrison, M., Bayse, G.S. and Michaels, A.W. (1971) Anal. Biochem. 42, 195—201
19 Pommier, J., Deme, D. and Nunez, J. (1973) Eur. J. Biochem. 37, 406—414
20 George, P. (1953) Biochem. J. 54, 267—276
21 Bevington, P.R. (1969) Data Reduction and Error Analysis for the Physical Sciences, pp. 219—222, McGraw-Hill, San Francisco
22 Bligh, E.G. and Dyer, W.J. (1959) Can. J. Biochem. Physiol. 37, 911—917
23 Freeman, B.A., Sissenstein, R., McManus, T.T. Woodward, J.E., Lee, I.M. and Mudd, J.B. (1976) J. Bacteriol. 125, 946—954
24 Stoffel, W., Chu, F. and Ahrens, E.H. (1959) Anal. Chem. 31, 307—308
25 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
26 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
27 Novikoff, A.B., Beard, M.E., Albala, A., Sheid, B., Quintana, N. Biempica, L. (1971) J. Microsc. 12, 381—404
28 Mahoney, C.P. and Igo, R.P. (1966) Biochim. Biophys. Acta 113, 507—519
29 Pommier, J., Tourniaire, J., Deme, D., Chalendar, D., Bornet, M. and Nunez, J. (1974) J. Clin. Endocrinol. Metab. 39, 69—80
30 Neary, J.T., Davidson, B., Armstrong, A., Maloof, F. and Soodak, M. (1976) Fed. Proc. Abstr. 35, 1629
31 Razin, S. (1972) Biochim. Biophys. Acta 265, 241—296