ANALYTICAL BIOCHEMISTRY 83, 346–356 (1977)

A Simplification of the Protein Assay Method of Lowry *et al.* Which is More Generally Applicable

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Received September 17, 1976; accepted July 29, 1977

Some recent modifications of the protein assay by the method of Lowry, Rosebrough, Farr, and Randall (1951, *J. Biol. Chem.* **193**, 265–275) have been reexamined and altered to provide a consolidated method which is simple, rapid, objective, and more generally applicable. A DOC–TCA protein precipitation technique provides for rapid quantitative recovery of soluble and membrane proteins from interfering substances even in very dilute solutions (<1 μg/ml of protein). SDS is added to alleviate possible nonionic and cationic detergent and lipid interferences, and to provide mild conditions for rapid denaturation of membrane and proteolipid proteins. A simple method based on a linear log–log protein standard curve is presented to permit rapid and totally objective protein analysis using small programmable calculators. The new modification compared favorably with the original method of Lowry *et al.*

Recent modifications have been proposed to improve various aspects of the original Folin–Ciocalteu reagent method (1) as given by Lowry and coworkers (2) for estimation of total protein. Bensadoun and Weinstein (3) introduced a deoxycholate–trichloroacetic acid (DOC–TCA) procedure for the quantitative precipitation of bovine serum albumin (BSA) at concentrations down to <1 μg/ml. This procedure permits analysis of very dilute protein solutions together with removal of most interfering substances. Wang and Smith (4) and Dulley and Grieve (5) found that sodium dodecylsulfate (SDS) prevents the usual precipitate formation resulting from nonionic and cationic detergents. These detergents are commonly used in research on membrane proteins. Lees and Paxman (6) also found that SDS in the presence of alkali is an effective solubilizing agent for proteolipid proteins. High temperature and prolonged exposure to alkali have been used to provide adequate solubilization and denaturation of proteolipid proteins and membrane proteins, particularly from acid precipitates (7–9). However, such conditions cause serious oxidation of lipids, which subsequently react (10) with the assay of Lowry *et al.* (2). SDS does not interfere with the assay, and in the presence of NaOH permits immediate solubilization, leaving the lipids transparent and noninterfering (6). Bates and McAllister (11) and Stauffer (12) have shown that the nonlinear Lowry protein standard curve is transformed into a linear function by plotting the

ISSN 0003-2697

log of the absorbance vs the log of the protein concentration. The protein calculations can thus be made quickly and objectively with small programmable calculators. Finally, Schacterle and Pollack (13) among others have modified the Lowry reagents to obtain a stable alkaline copper reagent, which facilitates the simplicity of the assay.

In this study, I have reexamined these modifications in some detail, confirmed and extended the original findings, and modified them as appropriate to produce a consolidated method. The result is a simplified and more generally applicable protein assay, modified from the method of Lowry *et al.* (2).

METHODS

A. Stock Reagents

1. Copper–tartrate–carbonate (CTC). A solution of about 20% sodium carbonate is added slowly while stirring to a solution of copper sulfate–tartrate to give final concentrations of 0.1% copper sulfate (pentahydrate), 0.2% potassium tartrate, 10% sodium carbonate. This solution is stable for at least 2 months at room temperature (20°C).

2. 10% Sodium dodecyl sulfate (SDS). Sigma's SDS (approximately 95% SDS) is comparable in the required qualities to Pierce's Sequanal grade SDS, and is less expensive. It has an absorbance (at 750 nm) of about 0.005 in a 1% final concentration in the assay, which is controlled by an appropriate reagent blank.

3. 0.8 N Sodium hydroxide.

4. Folin–Ciocalteu phenol reagent. Obtained from Fisher Scientific as a 2 N solution.

B. Working Solutions

1. 0.15% Sodium deoxycholate (DOC).

2. 72% Trichloroacetic acid (TCA).

3. Bovine serum albumin (BSA) (0.5 mg/ml). Sigma's crystallized and lyophilized BSA has about the same extinction as their BSA which is further prepared as "essentially fatty acid-free" BSA. Either preparation is mixed in quantity together with 1 mg/ml of sodium azide, as a noninterfering antibacterial agent, and stored frozen (−90°C) in small aliquots. Thawed aliquots are stored in the refrigerator and discarded after 5 days.

4. Reagent A. Mix equal parts of stock CTC, NaOH, SDS, and H_2O. This keeps at least 2 weeks at room temperature (20°C). The presence of a small amount of dark precipitate, or a white flocculent precipitate (SDS), does not effect color development if shaken well before use. SDS will come out of solution more readily at colder temperatures, but redissolves upon warming or dilution. Fresh Reagent A is prepared when the dark precipi-

tate accumulates or when the viscosity becomes excessive (the solution may gel after several weeks).

5. Reagent B. One volume of Folin–Ciocalteu phenol reagent is mixed with five volumes of distilled water. It is stable at room temperature (20°C) in an amber bottle.

C. Assay Procedure

1. Standard assay. a. Precipitation step. Bring sample containing between 5 and 100 μg of protein to a total volume of 1.0 ml with distilled water. Add 0.1 ml of 0.15% DOC, mix, and allow to stand for 10 min at room temperature (20°C). Add 0.1 ml of 72% TCA, mix, and centrifuge at 3000*g* for 15 min. The supernatant to be discarded is efficiently removed by immediately decanting after centrifugation and turning the tube upside down over absorbent paper in a rack. The remaining fluid is removed from the rim and sides of the tube by aspiration prior to returning the tube to the upright position. The pellet is saved for step 1.b.

This step can be omitted if interfering substances are absent, in negligible quantities, or appropriately controlled for [see Refs. (3), (14)–(18)]. If protein is too dilute, multiples of the proportions outlined here can be utilized to augment the total protein recovered in the precipitate. However, longer centrifugation times may be necessary in such cases to assure complete pelleting [see Ref. (3)].

b. Spectrophotometric step. Bring sample containing between 5 and 100 μg of protein to a total volume of 1.0 ml with distilled water. This is either the pellet obtained above in step 1.a., or some aliquot if the precipitation step is unnecessary. Add 1.0 ml of Reagent A, mix, and allow to stand for 10 min at room temperature (20°C). Solubilization of the protein–DOC–TCA pellets is immediate after addition of Reagent A. Add 0.5 ml of Reagent B and mix immediately. After 30 min, read absorbance at 750 nm. Read within 2 hr unless standards are included with the group of tubes. Color loss is 1–2% per hour at room temperature (20°C).

2. Microassay. a. Precipitation step. This step is the same as in the standard assay (1.a.), except that the total protein per sample lies between 1 and 20 μg.

b. Spectrophotometric step. The procedure is the same as for the standard assay except that all quantities are reduced by a factor of 5 to give 1 to 20 μg of protein in a total volume of 0.5 ml.

3. Standard curve. Linear transformation of the Lowry protein standard curve permits simple determination of the curve for routine analysis by two sample points. Three replicates of 0.05 and 0.2 ml of 0.5 mg/ml BSA are utilized to determine the standard curve. More accurate curves are obtained by using 10 different standard concentrations, and the linear regression is fitted by the method of least-squares analysis of the log–log data.

The two-point method is reproducible and thus satisfactory in our experience for routine analysis. The unknown protein is calculated from the following equation after Stauffer (12):

$$\text{Protein } (\mu g) = (I \times A_{750})^S, \quad [1]$$

where I is the reciprocal of the intercept (i.e., A_{750} at 1 μg of protein) and S is the reciprocal of the slope of the line. These parameters may be estimated according to the "eyeball" method described by Stauffer (12), or by a calculator. With the two-sample point standard method, the following equations apply:

$$S = \log (h/l)/\log (A_{750}{}^h/A_{750}{}^l), \quad [2]$$

$$I = \text{antilog } ((\log h)/S - \log A_{750}{}^h), \quad [3]$$

where h and l refer to the high and low protein standards, respectively, and $A_{750}{}^h$ and $A_{750}{}^l$ to their corresponding absorbance values at 750 nm. The log–log curve is linear up to a protein concentration of 40 μg/ml (final concentration in the assay), after which a second linear portion of lesser slope is found. This break corresponds to 20 μg of total protein in the microassay and 100 μg in the standard assay. If desirable this second portion may be calibrated with appropriate standards and the equations above to extend the range of the protein assay.

D. Experimental

1. Optimum conditions for assay. Optimum concentrations of copper sulfate, potassium tartrate, sodium carbonate, and sodium hydroxide were examined. Sodium hydroxide was varied between 0.016 and 0.20 N (final concentration), and different ratios of copper sulfate and potassium tartrate at 0.01 and 0.04% final concentrations of copper sulfate were examined in the presence of 0.4, 1.0, 2.0, and 4.0% final concentration of sodium carbonate. The development of color and the absorption spectrum between 400 and 900 nm were examined with respect to the time of exposure to Reagent A before addition of Reagent B, and to the time after addition of Reagent B. All measurements were made through a 1-cm light path with a Zeiss spectrophotometer.

2. Effects of SDS on the assay. The presence of up to 5% final concentration of SDS was tested for its effect on the reagent blanks, absorption spectra, standard curve, optimum concentrations of Reagent A constituents, and rate of color development after addition of Reagent A or Reagent B.

3. Application of DOC–TCA precipitation to membrane proteins. Bensadoun and Weinstein (3) investigated their DOC–TCA precipitation method with respect to the recovery of BSA. The modified DOC–TCA

precipitation procedure outlined above was tested for the recovery of BSA and of membrane proteins by both the standard and microassay procedures. Membrane proteins were isolated from the brine shrimp by centrifuging a 5000*g*, 10-min supernatant fraction of brine shrimp naupliar homogenates at 48000*g* for 30 min. The resulting pellet was rehomogenized in distilled water and centrifuged at 48000*g* for 30 min. The washed pellet was resuspended in distilled water and filtered through Miracloth, and its protein concentration was determined relative to BSA by direct analysis (i.e., without a precipitation step).

Application of the DOC–TCA precipitation method was further tested by precipitating BSA and membrane proteins in the presence of sucrose, the nonionic detergent Lubrol WX, and both together. These are common reagents used in membrane protein research and might be expected to produce harsh test conditions for quantitative precipitation of membrane proteins. Bensadoun and Weinstein (3) examined sucrose effects on BSA recovery, but did not examine any nonionic detergents.

4. Comparison with the method of Lowry et al. (2). The method of protein analysis as described above was compared with the original method of Lowry *et al.* (2) for several different proteins and interfering substances, both with and without a precipitation step. All proteins were examined at 50 μg in a final volume of 2.5 ml. The proteins investigated included bovine hemaglobin (2× crystallized, Nutritional Biochemicals Corp.), bovine liver catalase (purified powder, Sigma), BSA fraction V (Cohn fraction V powder, Sigma), crystalline BSA (crystallized and lyophilized, Sigma), fatty acid-free BSA (crystalline BSA further prepared "essentially fatty acid free," Sigma), and brine shrimp membrane protein (calibrated against crystalline BSA). Interfering substances included 0.1 ml of either 0.5% (w/v) Lubrol WX, 60% (w/v) sucrose, or 0.1 M Tris base, and were examined in the presence of crystalline BSA and the brine shrimp membrane proteins.

For the method of Lowry *et al.* (2) the protein sample, including any interfering substance, was contained within 0.2 ml, to which was added 0.1 ml of 2 N NaOH followed by 2.0 ml of their Reagent C. After 10 min, 0.2 ml of the Lowry *et al.* Reagent E was added and mixed, and the absorbance was read at 750 nm after an additional 30-min incubation at room temperature (20°C). For comparison with the DOC–TCA precipitation method above, additional protein samples (0.2 ml) were precipitated with 0.5 ml of 10% TCA for 20 min at 20°C. The precipitate was collected by centrifugation as described above for the DOC–TCA precipitation method. The pellets were dissolved after the method of Hess and Lewin (8) to assure applicability to even stubborn proteolipid proteins. This involved adding 0.2 ml of water and 0.1 of 2 N NaOH to the pellets, sealing, and incubating for 18 hr at 37°C. The samples were then analyzed by the method of Lowry *et al.*

RESULTS

The optimum concentrations of Reagent A constituents (excluding SDS) are similar to those found by Lowry *et al.* (2). The optimum ratio of copper sulfate to potassium tartrate is 2:1. The total amount of copper sulfate–potassium tartrate can be varied, but with little or no advantage in sensitivity or resistance to sucrose interference (19). Sodium hydroxide is best at a final concentration of 0.08 N in the presence of 1% sodium carbonate, regardless of the copper sulfate–potassium tartrate concentrations. Lower concentrations of sodium carbonate produce a sharper peak in the pH profile, whereas higher concentrations, while producing a flatter pH profile, cause a loss of sensitivity. The absorbance maximum is nearly flat between 650 and 750 nm. The 750-nm wavelength is preferred since it permits use of a narrower slit width. Color development requires 5–10 min with Reagent A and 20–30 min with Reagent B. Color loss after maximum development is about 1% per hour at room temperature (20°C), and 0.05 and 5% per hour at 0 and 37°C, respectively.

SDS has no effects on any of the optimum reagent concentrations for the assay, on the absorption spectra, on the time for color development, or on the standard curve. SDS does produce a small absorbance (0.005), which is controlled by the reagent blank.

Membrane proteins as well as BSA are quantitatively recovered by the DOC–TCA precipitation procedure outlined in Methods, even from solutions of less than 1 μg/ml of protein. Figure 1 shows the microassay of 0.1

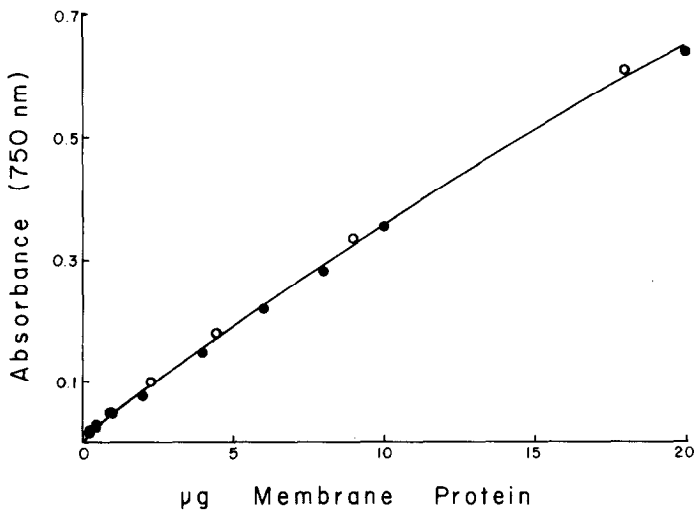

FIG. 1. Standard curve for the microassay using brine shrimp membrane protein calibrated against BSA. Open circles are samples processed through the DOC–TCA precipitation step prior to color reaction. Closed circles are samples processed directly for color reaction without prior precipitation.

to 20 μg of brine shrimp membrane proteins with and without the DOC–TCA precipitation step.

Table 1 demonstrates the recovery of 50 μg of brine shrimp membrane protein in the presence of various amounts of 1.6% Lubrol WX and/or 2.0 M sucrose. Up to 0.9 ml of the Lubrol WX (0.58% final concentration), 0.1 ml of the sucrose (80 mM final concentration), or 0.1 ml of their combination can be tolerated without loss of recovery of the membrane proteins. Similar recoveries were obtained using 5, 10, and 100 μg of membrane protein.

Figure 2 shows the standard curve for the standard assay method for 10–200 μg of BSA as plotted on a log–log scale. One linear portion is obtained below 100 μg of BSA ($r^2 = 0.999$, $n = 10$), and a second portion of lesser slope is obtained above 100 μg of BSA ($r^2 = 0.996$, $n = 10$). The method of least-squares analysis for the first linear region (10–100 μg) produces an estimation of the constants of Eq. [1] of 1.165 and 81.91 for S and I, respectively. The two-sample point method using 20 and 80 μg as the standards results in the values of 1.156 and 84.53 for S and I, respectively. This difference produces an error in the estimate of protein between 2.5 and 0.1% for protein between 5 and 100 μg, respectively. Similar error estimates are obtained from calculations of this type for the second linear portion (110–200 μg).

Table 2 shows the results of the comparison of the original method of Lowry *et al.* (2) to that of this paper. By direct analysis (i.e., without a precipitation step) the method of Lowry *et al.* shows a slightly higher extinction for the various proteins examined (105.2 ± 3.0%, $\bar{X}$ ± SD, $n = 6$). Lubrol interferes with the method of Lowry *et al.* by formation of

TABLE 1

EFFECTIVENESS OF THE DOC–TCA PRECIPITATION STEP ON RECOVERY OF BRINE SHRIMP MEMBRANE PROTEINS (50 μg) IN THE PRESENCE OF DIFFERENT AMOUNTS OF INTERFERING LUBROL WX AND SUCROSE[a]

Volume of interfering substance (ml)	Absorbance (750 nm)[b]		
	1.6% Lubrol WX	2.0 M Sucrose	Lubrol WX + sucrose
0	0.384	0.384	0.386
0.05	0.380	0.387	0.356
0.10	0.386	0.370	0.352
0.20	0.382	0.356	0.389
0.50	0.378	0.305	0.540[c]
0.90	0.376	0.133	1.218[c]

[a] Assayed as described in Methods using the standard assay procedure.

[b] Values represent the average of triplicates read against appropriate reagent blanks.

[c] These samples produced a yellow precipitate. After centrifugation the A_{750} was 0.456 and 0.468 for 0.50- and 0.90-ml samples, respectively.

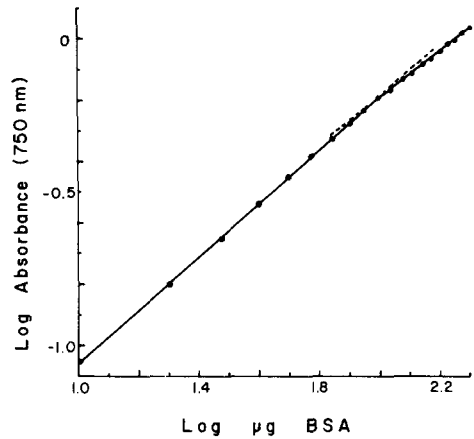

FIG. 2. Log–log plot of the standard curve for 10–200 µg of BSA using the standard assay volume.

a yellow precipitate, which also occurs in samples previously precipitated with TCA. After centrifugation of the yellow Lubrol precipitate, the absorbance values are greater than those of controls by an average of 107.1%. No precipitate forms with the method described in this paper and the absorbance values are 101.2% of controls. Sucrose and Tris interfere with both assay methods by producing color in the reagent blanks and by reducing the extinction for the proteins. For the various proteins, the DOC–TCA method produced an average recovery of 98.4 ± 3.9% ($\bar{X}$ ± SD, $n = 6$), whereas the TCA method gave a recovery of 85.8 ± 5.7% of the protein. In the presence of the interfering substances for both BSA and membrane protein, the recovery was 97.4 ± 4.4% ($\bar{X}$ ± SD, $n = 6$) for the DOC–TCA method and 89.7 ± 5.2% for the TCA method.

DISCUSSION

The stock reagents and working solutions prepared as described in Methods are stable at room temperature (20°C) for at least 1 year, except for the CTC solution which is stable for 2 months and Reagent A which is stable for 2–3 weeks. The working solutions permit the addition of only two reagents to the protein samples, using volumes that are widely available with automatic pipets. The analysis can be carried out in 10 × 75-mm disposable culture tubes for both the standard and microassay volumes. These modifications greatly simplify the assay for either small or large number of samples handled routinely or sporadically.

The presence of SDS further simplifies the assay by alleviating possible interferences by lipids (6) and nonionic and cationic detergents (4,5). SDS also promotes rapid solubilization of protein precipitates and denaturation of membrane and proteolipid proteins in the presence of sodium hydroxide

TABLE 2

COMPARISON OF THE ORIGINAL METHOD OF LOWRY *ET AL.* TO THIS REVISED PROTEIN ASSAY METHOD WITH RESPECT TO DIFFERENT PROTEINS AND INTERFERING SUBSTANCES[a]

Protein + interfering substance	Absorbance (750 nm)[b]			
	Lowry *et al.*		Peterson	
	Direct	TCA ppt.	Direct	DOC–TCA ppt.
Hemoglobin	0.467	0.372	0.427	0.429
Catalase	0.443	0.347	0.437	0.401
BSA (fraction V)	0.389	0.352	0.381	0.382
BSA (fatty acid free)	0.434	0.398	0.408	0.402
BSA (crystalline)	0.456	0.408	0.432	0.444
+ Lubrol	0.486[c]	0.447[c]	0.436	0.450
+ Sucrose	0.362	0.399	0.250	0.422
+ Tris	0.316	0.419	0.226	0.432
Membrane protein	0.469	0.399	0.442	0.427
+ Lubrol	0.495[c]	0.426[c]	0.456	0.424
+ Sucrose	0.318	0.388	0.242	0.404
+ Tris	0.293	0.409	0.266	0.420

[a] See Methods for details.

[b] Values represent the average of triplicates read against appropriate reagent blanks.

[c] Samples centrifuged before reading.

(6). This assures a rapid and complete reaction with the Reagent A constituents under almost all situations likely to be encountered, thus adding to the simplicity and general applicability of the technique.

Quantitative protein precipitation by the DOC–TCA method of Bensadoun and Weinstein (3) has been confirmed using the modified procedure outlined in Methods. Furthermore, as shown in Fig. 1, the procedure is equally effective with membrane proteins where quantitative recovery is observed at less than 1 μg/ml of membrane protein. The membrane proteins can be quantitatively recovered even in the presence of 0.58% Lubrol WX and 80 mM sucrose (final concentrations in the assay; see Table 1). Since these are considered quite challenging conditions for precipitation, it is assumed that membrane proteins can also be recovered from the numerous other interfering substances as investigated by Bensadoun and Weinstein (3) for BSA. The DOC–TCA precipitation thus allows for a single, rapid, and quantitative protein precipitation method for removal of interfering substances, which is applicable to most all commonly occurring situations. It should be pointed out that Lubrol WX, a nonionic detergent, can be tolerated directly in the assay due to the presence of SDS (see Table 2), but is usually accompanied by other substances such as sucrose and buffers, which are interfering and must be removed.

The standard curve, as shown in Fig. 2, is linear when plotted on a log–log scale, with a break to a second linear slope occurring at 40 μg of BSA/ml. Stauffer (12) showed the break to occur at a final concentration of 127 μg of BSA/ml. The final concentrations of Stauffer's reagents are slightly different from ours, but little else can be offered to explain the discrepancy. In our hands the curve is not affected by a prior DOC–TCA precipitation step, the use of membrane proteins, the use of a 4× concentrated copper sulfate–potassium tartrate solution, or the presence of SDS. Our results show that the break occurs consistently at a final protein concentration of 40 μg/ml when using either the standard or microassay procedure. The first linear portion of the log–log standard curve is within the most accurate range of the absorbance scale, and is quite adequate for routine analysis. Most important, the linear transformation permits samples to be analyzed in an entirely objective fashion and quite rapidly with programmable calculators. Moreover, the two-sample point standard method permits the convenient inclusion of standards with each assay, which removes any problems with inadvertent storage of the samples before their absorbances can be read. Samples requiring excessive storage can be kept with little loss of sensitivity if stored at 0°C.

The results in Table 2 reveal that the relative extinction for various protein standards is similar between the original method of Lowry *et al.* (2) and the revised procedure outlined in Methods. The absolute extinction, however, was slightly higher for each protein standard assayed by the method of Lowry *et al.* (average 105%). There is no reason to assume that proteins behave differently with the modified procedures suggested in this paper. Furthermore, with the revised procedure, nonionic and cationic detergents, as previously noted, can be tolerated directly in the assay. With the method of Lowry *et al.*, Lubrol is found to interfere even after removal of the yellow precipitate by centrifugation. The DOC–TCA precipitation method is effective in the recovery of the various types of proteins and in the removal of sucrose and Tris interferences (Table 2). Standard TCA precipitation procedures give recoveries of less than 90% at 50 μg of protein, and drastically less at low protein concentrations (3). Since the sample size can be up to 1.0 ml with the 2.5 ml standard assay volume of the revised method, as opposed to 0.2 ml for the original method of Lowry *et al.*, it is possible to analyze substantially more dilute solutions of proteins by the new method, without special preparation of more concentrated reagents. The effectiveness of the DOC–TCA precipitation step permits even more dilute solutions to be analyzed irrespective of the presence of any interfering substances.

In summary, this paper offers a consolidation of recent modifications (3–6,11) of the method of Lowry *et al.* (2) for protein estimation. Changes in these modifications have been made to facilitate convenience in rapid determination of routine protein samples. The method is applicable to sol-

uble, membrane, and proteolipid proteins in dilute solutions containing interfering substances. Utilization of the linear transformation provides a rapid method for an objective estimate of protein concentration in multiple samples.

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. Lowell E. Hokin for his helpful suggestions during this study and in the preparation of this manuscript. This work was supported by grants from the National Institutes of Health (HL-16318) and the National Science Foundation (GB-40368X).

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