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Estrogen-Induced Changes in Translation, and Specific Messenger RNA Levels during Oviduct Differentiation

(ovalbumin/morphological changes/protein synthesis/ribosomes/chick)

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ABSTRACT Estrogen-induced morphologic differentiation of chick oviduct is accompanied by increases in the total endogenous mRNA activity of oviduct polysomes. Concomitant increases are also noted in ribosome translational capacity and activity of peptide chain initiation factors. Once the differentiation process nears completion (about 7 days of estrogen administration), total ribosome-bound mRNA activity decreases, but the translational machinery remains very active. In addition, estrogen induces the accumulation of ovalbumin mRNA before ovalbumin is demonstrable in the oviduct. The data suggest that the rate-limiting event in the hormonal induction of cell-specific proteins, such as ovalbumin, is the synthesis and intracellular accumulation of specific mRNA for such proteins.

Morphologic differentiation, growth, and synthesis of cell-specific proteins occur in the oviduct when estrogen is given daily to immature chicks (1–3). One of these proteins, ovalbumin, has become widely used as a chemical marker of hormone-mediated differentiation.

Studies in our laboratories have shown that qualitative and quantitative changes in nuclear gene transcription precede the appearance of cell-specific oviduct proteins (3–6). We have also reported an increase in the formation and biosynthetic activity of oviduct polyribosomes during estrogen differentiation (5–7). Based on the above indirect experiments, we have tentatively concluded that the assembly of oviduct polyribosomes and increased protein synthesis reflected earlier changes in the functional messenger RNA activity of the tissue (5–7).

Subsequently, techniques have been developed in our laboratory that allow us to assess more accurately oviduct-protein synthesis in systems capable of peptide chain initiation (8–9). Using these systems, we have now examined both estrogen-induced changes in endogenous mRNA activity on oviduct ribosomes and the ability of single ribosomes to translate exogenous synthetic mRNA [poly(U)]. We have further demonstrated an estrogen-induced increase in the translational activity of oviduct initiation factors measured with heterologous ribosomes. Finally, our recent ability to translate specific ovalbumin mRNA in a rabbit reticulocyte lysate (10, 11) has enabled us to quantitate simultaneously ovalbumin mRNA activity and tissue levels of ovalbumin.

MATERIALS AND METHODS

Animal Materials. 7-day-old female Rhode Island Red chicks received daily subcutaneous injections of 5 mg of diethylstilbestrol in sesame oil, and were killed by cervical dislocation according to protocols described in the text and figure legends. Rabbit reticulocyte lysate, prepared according to the method of Gilbert and Anderson (12), was the generous gift of Drs. P. M. Prichard and W. French Anderson of the NIH.

Preparation of Chick Oviduct and Rabbit Reticulocyte Ribosomes and Initiation Factors. The magnum portions of the oviducts were removed and polyribosomes were prepared (5). Crude initiation factors and 0.5 M KCl-washed ribosomes (S-ribosomes) were prepared from the polyribosomes (9). AS_{70} enzyme fraction was prepared from the 150,000 $\times$ g supernatant fraction of chick oviduct (9). All materials were stored at $-196°$. Rabbit reticulocyte lysate prepared by diluting the packed cell volume 1:4 was used for the preparation (12) of S-ribosomes and crude initiation factors (fraction I).

Cell-Free Protein Synthesis with Endogenous Message. The incubation mixture contained these components, in a total volume of 100 μl: Tris·HCl (pH 7.2 at 23°), 30 mM; ATP (neutralized to pH 7.0), 1.0 mM; GTP (neutralized to pH 7.0), 0.5 mM; phosphoenolpyruvate (neutralized to pH 7.0), 7.5 mM; pyruvate kinase, 0.3 IU; dithiothreitol, 1.0 mM; [^{14}C]L-valine, 1.0 μM (572 dpm/pmol); and 19 [^{12}C]L-amino acids, 10.0 μM (each). Optimal $MgCl_2$ concentrations were 3.0 and 4.0 mM for the reticulocyte and oviduct systems, respectively. The reaction mixtures also contained ribosomes (in limiting amounts), initiation factors, and the AS_{70} enzyme fraction (saturating amounts of the latter two components), as described in the text or figure legends. Incubation was for 20 min at 37° (at which time protein synthesis was linear), and reactions were stopped by addition of 1.0 ml of 10% trichloroacetic acid. Samples were prepared and counted by liquid scintillation spectrometry (5, 13).

Poly(U)-Directed Polymerization of [^{14}C]Phe-tRNA. The incubation mixture contained the following materials, in a total volume of 50 μl: Tris·HCl (pH 7.6 at 23°), 60 mM; GTP (pH 7.0), 1.0 mM; phosphoenolpyruvate (pH 7.0), 4.5 mM; pyruvic kinase, 0.3 IU; 2-mercaptoethanol, 3 mM; $MgCl_2$, 5.0 mM; 5.5 pmol of chick oviduct [^{14}C]L-Phe-tRNA (0.6% charged, 840 dpm/pmol), prepared by the method described by Anderson (14); 0.4 A_{260} unit of poly(U) (where indicated), 90 μg of reticulocyte fraction I protein; and 0.5 A_{260} (about 85 μg of ribosomal RNA) of oviduct S-ribosomes from chicks treated with estrogen, as noted in the text and figure. Incubation was for 3 min at 37°. The reaction was

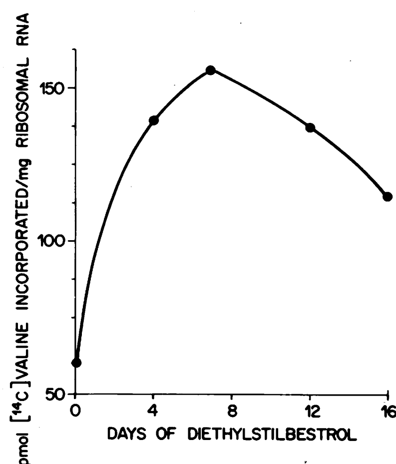

FIG. 1. The effect of estrogen on S-ribosome protein synthesis. Immature chicks received daily injections of diethylstilbestrol. S-ribosomes were prepared from the oviduct magnum at the times indicated (5, 9). Each reaction mixture contained about 85 μg of rRNA (0.5 A_{260} units), 12 μl of fraction I protein (80 μg of protein) and 180 μg of AS_{70} protein. The concentration of each component of the reaction mixture is given in *Methods*. Incubation was for 20 min at 37°. A blank of 0.7 pmol was obtained in the absence of S-ribosomes, and was subtracted from each point. Triplicate assays varied by less than 0.5 pmol.

stopped with 1.0 ml of 10% trichloroacetic acid, and the samples were processed as described (5, 13).

Isolation of Estrogen-Stimulated Total RNA. Total oviduct nucleic acid was prepared (10, 11) from diethylstilbestrol-treated chicks. The nucleic-acid preparations were then filtered on nitrocellulose membrane filters (Millipore) to partially purify mRNA activity (15, 16). The filter-bound RNA was removed from the filters with sodium dodecyl sulfate, precipitated with ethanol, dissolved in water, and stored at −196° before assay.

Ovalbumin Synthesis in Rabbit Reticulocyte Lysate. The assay contained, in a final volume of 0.5 ml: rabbit reticulocyte lysate (1:4, v/v), 0.2 ml; ATP (pH 7.0), 1.0 mM; GTP (pH 7.0), 0.2 mM; phosphoenolpyruvate (pH 7.0), 7.5 mM; pyruvate kinase, 0.3 IU; $MgCl_2$, 2.0 mM; Tris·HCl (pH 7.4 at 23°), 20 mM; KCl, 100 mM; [^{14}C]L-valine, 10 μM (572 dpm/pmol); 19 [^{12}C]L-amino acids, 20 μM (each); reticulocyte FI (90 μg of protein); and 10 μg of the mRNA preparation. Incubation was for 30 min at 37°. In order to determine total incorporated radioactivity, 25-μl aliquots were precipitated in trichloroacetic acid and counted by the standard procedure (5, 13). The remainder of the reaction mixtures were used to measure ovalbumin synthesis. Radioactive material precipitated by ovalbumin antiserum was assayed by the method described in detail by Means et al. (11).

Ovalbumin Determination in the Oviduct. Oviducts were removed from the same groups of chicks used to isolate total nucleic acid. The tissue was homogenized in 0.15 M NaCl–20 mM $NaPO_4$ buffer (pH 6.8), and the homogenate was centrifuged at 150,000 × g for 1 hr. The supernatant was removed, and ovalbumin was measured by an immunochemical procedure (17).

Materials and Chemicals. Diethylstilbestrol was purchased from Merck and Co. Sucrose (ribonuclease free), Tris (ultra-pure), ammonium sulfate (ultra-pure), and nonradioactive amino acids were obtained from Schwarz–Mann Research Laboratories. ATP, GTP, phosphoenolpyruvate, pyruvate kinase, and dithiothreitol were purchased from Calbiochem. Polyuridylic acid and rabbit antiserum to chicken egg albumin [Pentex (R) lot 50123] were obtained from Miles Laboratories.

RESULTS

Oviduct S-ribosomes isolated from chicks at various stages of estrogen-mediated tissue differentiation were assayed for endogenous mRNA activity in a heterologous protein-synthesizing system (Fig. 1). The S-ribosomes were prepared by washing oviduct polyribosomes in 0.5 M KCl in the absence of Mg^{+2}. These conditions result in formation of monosomes that contain mRNA and are highly active in protein synthesis when supplied with an exogenous source of initiation factors (7–9). In order to use these experiments to assess accurately ribosome-bound mRNA activity, it was necessary to establish conditions that were optimal for peptide chain initiation. Therefore, these assays were performed in the presence of saturating amounts of rabbit reticulocyte Fraction I (80 μg of protein saturates 0.5 A_{260} of ribosomes at 3 mM Mg^{+2}).

It can be seen from Fig. 1 that under optimal conditions (and measured as rate of protein synthesis, rather than extent) total endogenous mRNA activity of oviduct ribosomes from chicks treated with estrogen for 4 days is markedly increased over particles from unstimulated animals. Endogenous mRNA activity reaches a peak between 4 and 7 days of treatment. The total activity decreases, however, with continued hormone stimulation.

It was of interest to determine whether the eventual decline in protein synthesis noted in Fig. 1 is due to an overall decline in biosynthetic capacity of the ribosomes or to decreased total mRNA activity. We had observed that oviduct

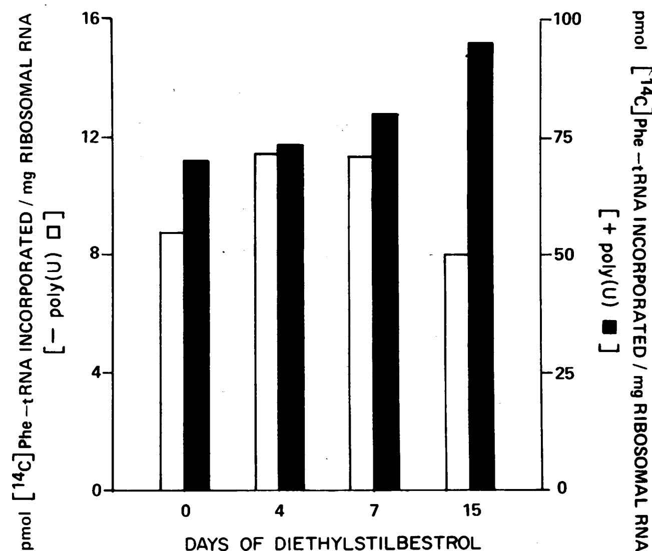

FIG. 2. The effect of estrogen on poly(U)-dependent polymerization of Phe-tRNA. Each reaction mixture contained about 85 μg of rRNA (0.5 A_{260}), 15 μl of reticulocyte fraction I (90 μg of protein), and 5.5 pmol of chick oviduct [^{14}C]L-Phe-tRNA (840 dpm/mol). Incubation was for 3 min at 37°. This reaction is linear up to 5 min with 1.0 A_{260} of S-ribosomes. A requirement for additional transfer factors could not be demonstrated in the presence of reticulocyte fraction I.

polyribosomes are not stimulated by poly(U) to synthesize polyphenylalanine, thus precluding a measurement of total ribosomal activity (7). However, ribosomes do show a dependency on poly(U) for polyphenylalanine synthesis after they have been washed in 0.5 M KCl, thus allowing a measurement of total ribosomal activity independent of endogenous mRNA. Therefore, using a constant number of ribosomes (measured as ribosomal RNA), we have measured poly(U)-dependent [^{14}C]Phe-tRNA polymerization throughout the course of estrogen administration to immature chicks. Again, saturating amounts of rabbit reticulocyte Fraction I were used in all reaction mixtures. It can be seen from the data in Fig. 2 that in the absence of poly(U) (left ordinate), incorporation of phenylalanine into peptide at different times is similar to that seen in Fig. 1 for [^{14}C]valine incorporation in the endogenous mRNA-directed system. When poly(U) is added, there is a significant stimulation (6- to 12-fold) of the relative capacity for polyphenylalanine synthesis in all the ribosome preparations (right ordinate; note 6-fold increase in scale). Moreover, in the presence of poly(U) the capacity for peptide synthesis continues to increase throughout estrogen treatment, whereas, when synthesis is dependent upon endogenous message activity, decreases are noted by 15 days. The difference between the total ribosomal synthetic capacity [plus poly(U)] and the endogenous message-dependent activity (minus [poly(U)] is a determination of the capacity of ribosomes to support protein synthesis. In an unstimulated chick there is a lower endogenous mRNA activity, but an increased ribosomal synthetic capacity. These results would be compatible with a relative messenger RNA deficiency. With continued estrogen treatment, the total ribosomal synthetic capacity appears to remain high in spite of a decrease in endogenous ribosome-bound mRNA.

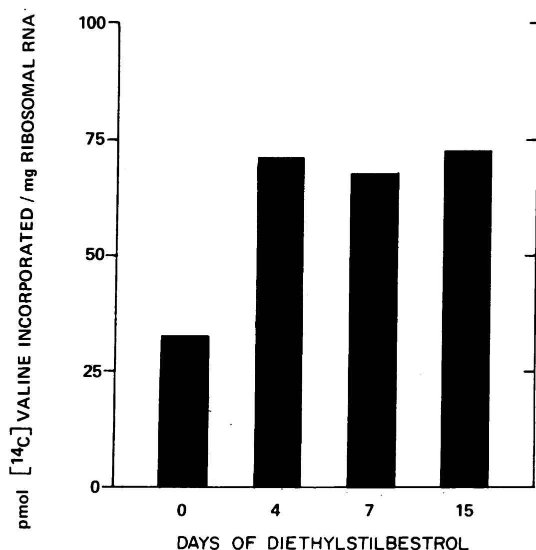

FIG. 3. Effect of estrogen on the activity of oviduct initiation factors. Immature chicks received daily injections of diethylstilbestrol. Polyribosomes were prepared from the oviduct magnum and washed with 0.5 M KCl (5, 9). Saturating amounts of the crude initiation factors for each preparation were determined in separate experiments. Each reaction mixture contained 0.2 A_{260} of rabbit reticulocyte S-ribosomes (50 μg of rRNA), 180 μg of AS_{70} protein, and saturating amounts of the initiation factor fractions. The $MgCl_2$ concentration was 4.0 mM. Incubation was for 20 min at 37°. Blanks of about 0.2 pmol were obtained in the absence of S-ribosomes.

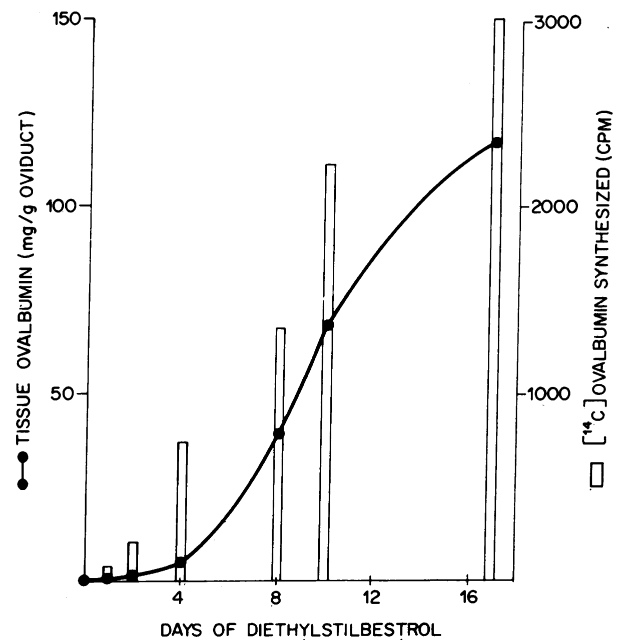

FIG. 4. Effect of estrogen on ovalbumin mRNA activity, and tissue concentrations of ovalbumin. Immature chicks received daily injections of diethylstilbestrol. The magnum portion of the oviducts was removed, and the total nucleic acid was extracted (10, 11). The tissue levels of ovalbumin were determined from the same groups of animals by an immunochemical method (17). Messenger RNA was partially purified by the Millipore filter technique (15, 16), and was assayed in the reticulocyte lysate. Each reaction tube contained 10 μg of exogenous mRNA and the components described in *Methods* ([^{14}C]valine was used as a label). Incubation was for 30 min at 37°. The synthesis of ovalbumin is proportional to the amount of added RNA up to 16 μg of RNA, and is linear for 45 min.

The activity of a crude preparation of chick oviduct initiation factors was assayed at various times during estrogen stimulation. Activity was measured as rate of protein synthesis with a saturating amount of each avian-factor fraction in a system containing 0.2 A_{260} of rabbit reticulocyte S-ribosomes at 4.0 mM Mg^{+2}. AS_{70} enzyme fraction prepared from chick oviduct was also added to the reaction mixture, since a 15% dependency was found for this material. The results of these studies were shown in Fig. 3. Administration of estrogen for 4 days results in increased initiation-factor activity in the heterologous protein-synthesizing system. However, the stimulatory activity of the factors remains constant during the remainder of the study. Therefore, estrogen appears to induce a significant increase in the activity of initiation factors, presumably by the synthesis of new enzyme proteins. Once this enhanced activity is obtained, there is no further increase despite further changes in the rate of protein synthesis.

We have recently shown that the activity of ovalbumin mRNA in the oviduct is dependent upon the hormonal state of the animal, as are the oviduct levels of ovalbumin (3, 11). In the present study, we attempted to correlate messenger activity with tissue levels of ovalbumin by determining whether there was detectable ovalbumin messenger activity that preceded the appearance of ovalbumin in the tissue, and

whether the time-dependent activity of this specific messenger was different from the total messenger activity. The results of this study are shown in Fig. 4. A significant level of specific ovalbumin mRNA activity, assayed in a rabbit reticulocyte lysate (10, 11), is detected at least 24 hr before the first demonstrable appearance of ovalbumin in the oviduct. Moreover, ovalbumin mRNA activity continues to increase with estrogen administration, and coincides with further increases in oviduct ovalbumin synthesis. At the later time points, the tissue levels of ovalbumin probably reflect both synthesis and accumulation of this protein. Both ovalbumin mRNA activity and oviduct ovalbumin levels have reached 60–70% of the values obtained in a laying hen (5260 cpm of [^{14}C]ovalbumin/10 μg of oviduct RNA and 173 mg of ovalbumin per g of oviduct) after 16 days of estrogen treatment. Thus, ovalbumin synthesis continues to increase at a time when total oviduct protein synthesis is decreasing.

DISCUSSION

The first detectable signs of oviduct morphologic differentiation, which appear by 12 hr after a single injection of estrogen to immature chicks, include increased mitotic activity and increased nuclear volume. Appearance of immature tubular gland cells can be seen at 36 hr, and the differentiation process is nearly completed by 6–8 days (1–3). Thus, as would be expected, morphologic differentiation precedes functional differentiation, such as the appearance of cell-specific proteins. It is not surprising then that major changes in hybridizable species of RNA (3, 18), chromatin-template activity and composition (3, 19), and RNA polymerase activity (3, 4) are primarily associated with morphologic differentiation. This is also true for ribosome synthesis and polyribosome activity (5–7). All of these events have been demonstrated to reach a maximum between 4–7 days of estrogen administration and subsequently decline.

Our studies show that endogenous messenger activity of oviduct ribosomes follows the general trend of the differentiation process, peaking at about 7 days of estrogen treatment. Moreover, estrogen appears to increase either the synthesis, the activity, or the subcellular distribution of peptide chain initiation factors during the initial stages of morphologic differentiation. Regardless of the precise level of control, it is interesting to note that, whereas continued estrogen treatment results in a decreased total mRNA activity after completion of the differentiation process, the activity of the ribosome-bound initiation factors and the translational capacity of the ribosomes appear to remain elevated. This finding would suggest that the oviduct translational apparatus remains "primed" to respond rapidly to intracellular changes in mRNA concentration.

Indeed ovalbumin mRNA activity first appears between 1 and 2 days, and corresponds to the development of primitive gland cells. This is before detectable levels of the cell-specific protein ovalbumin can be found in the oviduct. However, ovalbumin mRNA and *in vivo* ovalbumin synthesis increase in concert throughout 16 days of estrogen treatment. Once the differentiation process has occurred and total mRNA activity begins to decline, one can still detect marked increases in accumulation and translation of specific mRNA molecules, such as the one that codes for ovalbumin. Moreover, we have previously demonstrated that withdrawal of estrogen results in disappearance of ovalbumin mRNA activity. The activity is again demonstrable within 24 hr after readministration of estrogen (10, 11).

Growth and differentiation, therefore, require a multiplicity of complex changes in the transcriptional and translational mechanisms operable in the whole organ. Not only do increases in endogenous mRNA activity occur, but also increases in tRNA, ribosomal RNA, ribosome synthesis, and synthesis of protein factors required for translation are noted. On the other hand, independent of the tissue-differentiation process, the data of Fig. 4 indicate that the rate-limiting factor for the synthesis of specific hormone-inducible proteins, such as ovalbumin, is the intracellular concentration of the specific mRNA molecules for these proteins.

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1. Kohler, P. O., Grimley, P. M. & O'Malley, B. W. (1969) *Science* **160**, 86–87.
2. Kohler, P. O., Grimley, P. M. & O'Malley, B. W. (1969) *J. Cell Biol.* **40**, 8–27.
3. O'Malley, B. W., McGuire, W. L., Kohler, P. O. & Korenman, S. G. (1969) *Recent Progr. Horm. Res.* **25**, 105–160.
4. McGuire, W. L. & O'Malley, B. W. (1968) *Biochim. Biophys. Acta* **157**, 187–194.
5. Means, A. R., Abrass, I. B. & O'Malley, B. W. (1971) *Biochemistry* **10**, 1561–1570.
6. Means, A. R. & O'Malley, B. W. (1972) *Metabolism* **21**, 357–370.
7. Means, A. R. & O'Malley, B. W. (1971) *Acta Endocrinol. (Copenhagen) Suppl.* **153**, 318–336.
8. Means, A. R., Comstock, J. P. & O'Malley, B. W. (1971) *Biochem. Biophys. Res. Commun.* **45**, 759–766.
9. Comstock, J. P., O'Malley, B. W. & Means, A. R. (1972) *Biochemistry* **11**, 646–652.
10. Rosenfeld, G. C., Comstock, J. P., Means, A. R. & O'Malley, B. W. (1972) *Biochem. Biophys. Res. Commun.*, **46**, 1695–1703.
11. Means, A. R., Comstock, J. P., Rosenfeld, G. C. & O'Malley, B. W. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 1146–1150.
12. Gilbert, J. M. & Anderson, W. F. (1970) *J. Biol. Chem.* **245**, 2342–2349.
13. Means, A. R., Hall, P. F., Nicol, L. W., Sawyer, W. H. & Baker, C. A. (1969) *Biochemistry* **8**, 1488–1495.
14. Anderson, W. F. (1969) *Biochemistry* **8**, 3687–3691.
15. Rosenfeld, G. C., Comstock, J. P., Means, A. R. & O'Malley, B. W. (1972) *Biochem. Biophys. Res. Commun.*, **47**, 387–392.
16. Brawerman, G., Mendecki, J. & Lee, S. Y (1972) *Biochemistry* **11**, 637–641.
17. Korenman, S. G. & O'Malley, B. W. (1967) *Biochim. Biophys. Acta* **140**, 174–176.
18. O'Malley, B. W. & McGuire, W. L. (1968) *Proc. Nat. Acad. Sci. USA* **60**, 1527–1534.
19. Spelsberg, T. C., Steggles, A. W. & O'Malley, B. W. (1971) *Biochim. Biophys. Acta* **254**, 129–134.