"pockets" that do not exist in a single-bond environment. Conceivably, PLA bears sites that prefer such pockets and others which prefer highly apolar regions. For this reason, perhaps, the protein is more soluble in chloroethanol or chloroform–methanol than in more apolar or polar solvents. One might consider the possibility, therefore, that unsaturated domains in a membrane lipid core provide islands of lesser hydrophobicity and that these are attractive to PLA.

Acknowledgments

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Changes in the Hepatic Levels of Messenger Ribonucleic Acid for Malic Enzyme during Induction by Thyroid Hormone or Diet†

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ABSTRACT: Levels of hepatic messenger ribonucleic acid (mRNA) for malic enzyme [L-malate:NADP oxidoreductase (decarboxylating), EC 1.1.1.40] were quantitated in different dietary and hormonal states of the rat. Polysomal or total cellular poly(A)-containing RNA was translated in the rabbit reticulocyte lysate system, which had been treated to reduce endogenous mRNA activity. The relative level of incorporation of radiolabeled amino acid into malic enzyme was determined by immunoprecipitation with antibody to malic enzyme and formaldehyde-fixed *Staphylococcus aureus* (Cowens I strain) as an immunoadsorbent. The immunoprecipitated product comigrated with purified malic enzyme on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. No malic enzyme was detected when nonspecific antisera or an excess of unlabeled malic enzyme was added during immunoprecipitation. The level of malic enzyme mRNA was found to markedly increase relative to euthyroid, chow-fed rats when the animal was either fed a high carbohydrate, fat-free diet or made hyperthyroid. Animals receiving both treatments had a further increase in mRNA activity to a level which was ~0.2% of the total incorporation of [^{3}H]leucine. Levels of malic enzyme activity and the relative rate of synthesis were found to increase roughly in proportion to mRNA levels in these three states. Thus, the induction of malic enzyme by thyroid hormone or high carbohydrate, fat-free diet is due largely to an increase in the mRNA coding for this enzyme.

The activity of rat hepatic malic enzyme [L-malate:NADP oxidoreductase (decarboxylating), EC 1.1.1.40] can be modulated by both hormonal and dietary factors. The thyroidal status of the rat has profound effects on the levels of this cytosolic enzyme (Tepperman & Tepperman, 1964; Wise & Ball, 1964; Ruegamer et al., 1965; Tarentino et al., 1966). Hypothyroid rats have reduced levels of hepatic malic enzyme activity, whereas hyperthyroid rats have greatly increased levels compared to euthyroid animals. Consequently, malic enzyme activity has become a commonly employed marker of tissue effects of thyroid hormone for the rat liver (Oppenheimer et al., 1977). The level of hepatic malic enzyme activity is also known to be influenced by the diet of the animal (Tepperman & Tepperman, 1964; Wise & Ball, 1964; Tarentino et al., 1966; Fitch & Chaikoff, 1960; Pande et al., 1964). Starvation causes a dramatic decrease in enzyme activity, whereas diets

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high in carbohydrate and low in fats lead to increased activity relative to normal chow-fed animals. In this regard, malic enzyme behaves similarly to a group of enzymes, including the NADP-linked hexosemonophosphate dehydrogenases and fatty acid synthetase, which are involved in lipogenesis. Recent studies in our laboratory have demonstrated that the effects of thyroid hormone and diet on malic enzyme are interrelated (Mariash et al., 1980). For instance, in hypothyroid rats, little effect of the high carbohydrate, fat-free diet on malic enzyme is observed. Furthermore, the dose–response relationship of thyroid hormone and malic enzyme activity with or without the high carbohydrate, fat-free diet suggests that the two factors do not act in an additive fashion but are related in a more complex manner.

These changes in malic enzyme activity with thyroidal or dietary states have been shown by immunochemical techniques to be due to changes in the amount of enzyme present, rather than to alteration in the activity of a constant amount of enzyme (Isohashi et al., 1971). Measurements of the rate of incorporation of amino acid into malic enzyme during induction have also suggested that changes in the enzyme mass are primarily due to corresponding variation in the rate of enzyme synthesis (Gibson et al., 1972; Murphy & Walker, 1974; Li et al., 1975; Silpananta & Goodridge, 1971; Goodridge & Adelman, 1976). Since the rate of enzyme synthesis clearly is influenced by multiple intracellular events, we have attempted in our initial studies to determine whether levels of translationally active mRNA for malic enzyme are changed by thyroidal or dietary states which alter enzyme activity. The assay for malic enzyme mRNA utilizes the nuclease-treated reticulocyte lysate protein synthesizing system of Pelham & Jackson (1976) for translation of poly(A)-containing RNA and specific immunoprecipitation with antibody to malic enzyme and heat-killed, formaldehyde-fixed *Staphylococcus aureus* (Cowens I strain) as an immunoabsorbent (Kessler, 1975; Jonsson & Kronvall, 1974). This assay has allowed us to measure specific mRNA species which constitute as little as 0.01% of the total mRNA activity present.

Materials and Methods

Animals. Male Sprague–Dawley rats weighing ~200 g were used in all experiments. Control euthyroid rats were maintained on lab chow (Purina). Dietary induction of malic enzyme was performed by feeding rats "Fat Free Test Diet" (ICN Pharmaceuticals) ad libitum for 7 days. This diet differs from normal lab chow primarily in the absence of any fat (as opposed to 22% in chow diet) and the simpler nature of its carbohydrate source (sucrose, 58% by weight). Both of these factors have been shown to be important for dietary induction of the lipogenic enzymes (Fitch & Chaikoff, 1960; Tepperman & Tepperman, 1964). Rats were made hyperthyroid by intraperitoneal injection of 15 μg of L-triiodothyronine (Sigma) per 100-g body weight daily for 7 days.

Assay and Purification of Malic Enzyme. Malic enzyme activity was assayed by the method of Hsu & Lardy (1967). One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 nmol of $NADP^+$ in 1 min.

Malic enzyme was purified from the livers of hyperthyroid rats fed the high carbohydrate, fat-free diet by a modification of the method of Yeung & Carrico (1976). A 20% (w/v) homogenate of tissue was centrifuged at 12000*g* for 15 min and then at 100000*g* for 1.5 h. The supernatant fraction was then subjected to a 60–80% ammonium sulfate fractionation, prior to affinity purification on immobilized N^6-(6-aminohexyl)adenosine 2′,5′-diphosphate (P-L Biochemicals). Malic enzyme was eluted with 0.5 mM $NADP^+$, concentrated by ultrafiltration, and chromatographed on a Bio-Gel A-0.5m (Bio-Rad Laboratories) column. Malic enzyme was concentrated and stored at 4 °C, where its activity was stable for at least several months. Analysis of the final preparation on sodium dodecyl sulfate ($NaDodSO_4$)[1]–polyacrylamide gel electrophoresis revealed a single peak of protein with an apparent subunit molecular weight of 63 000, in reasonable agreement with a previously reported estimate for the rat liver enzyme (Li et al., 1975). From the amount of protein loaded and the sensitivity of the stain, we approximate the purity of this preparation to be greater than 98%. The final specific activity of the malic enzyme was 49 600 units/mg.

Antibody to Purified Malic Enzyme. Antisera to purified malic enzyme were obtained by subcutaneous injection of 500 μg of enzyme in Freund's complete adjuvant into 5-lb New Zealand white rabbits. Injections were repeated 3 times at 1-week intervals. One week after the last injection animals were bled and antisera prepared. The IgG fraction was obtained by DEAE-Sephadex chromatography (Fahey & Terry, 1973). Double immunodiffusion by the method of Ouchterlony (Ouchterlony & Nilsson, 1973) revealed only one precipitin band of identity between purified malic enzyme and total rat liver homogenate.

Relative Rate of Malic Enzyme Synthesis. Animals were injected intraperitoneally with 0.5 mCi of [^{3}H]leucine (ICN, 40–50 mCi/mmol) 45 min before sacrifice. Livers were removed and homogenized in 3 volumes of 0.32 M sucrose and 3 mM $MgCl_2$. Homogenates were centrifuged at 100000*g* for 1 h. The incorporation of [^{3}H]leucine into total cytosolic protein was determined by precipitation in 20% (w/v) trichloroacetic acid. Incorporation into malic enzyme was determined by adding enough antisera to malic enzyme to quantitatively precipitate all enzyme activity in a 1–2-mL sample of the 100000*g* supernatant fraction. Purified unlabeled malic enzyme was added to samples containing less than 6.5 units so that all samples had this total amount of enzyme. After incubation for 2 h at 30 °C, the samples were layered over cushions of 1 M sucrose containing 1% Triton X-100 and centrifuged at 10000*g* for 10 min. The completion of immunoprecipitation was routinely checked by assaying the enzyme activity in the original supernatant fraction. Pellets were washed 3 times with 0.15 M NaCl and dissolved in 0.3 mL of 88% formic acid for counting in Aquasol (New England Nuclear). Backgrounds were determined by reprecipitating the original supernatant fraction with an equal amount of antisera and unlabeled purified malic enzyme (Schimke et al., 1965).

Isolation of Poly(A)-Containing RNA. Polysomal RNA was isolated by a modification of the method suggested to us by Dr. A. Goodridge (Goodridge et al., 1979). Fifteen minutes prior to killing, rats were injected intraperitoneally with 1 mg of cyclohexamide per 100-g body weight to block ribosomes attached to mRNA and, thus, reduce potential ribonuclease degradation. Livers were removed and homogenized in 25 mM Tris-HCl, pH 7.6, 25 mM NaCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.25 mM EDTA, and 5 μg/mL cyclohexamide. The homogenate was centrifuged at 750*g* for 10 min and then at 15000*g* for 15 min. The supernatant fraction was brought to 0.5 mg/mL heparin, 1% Triton, and 0.1 M $MgCl_2$ and incubated for 1 h at 4 °C (Palmiter, 1974). Precipitated material was collected by centrifugation through 1 M sucrose cushions and resuspended in 0.5 M KCl, 10 mM Tris-HCl,

[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; SaC, heat-killed, formaldehyde-fixed *Staphylococcus aureus* (Cowens I strain).

pH 7.6, 30 mM EDTA, 0.5% $NaDodSO_4$, and 0.2% sodium deoxycholate. Poly(A)-containing RNA was isolated by oligo(dT)–cellulose (Collaborative Research) chromatography (Krystosek et al., 1975). The final RNA was precipitated with ethanol, collected and redissolved in H_2O, and stored at −80 °C. The concentration of RNA was determined based on an absorbance of 25 at 260 nm for a 1 mg/mL solution.

Total cellular poly(A)-containing RNA was isolated by extraction of whole liver with phenol–chloroform at pH 9. Frozen (−80 °C) liver samples were homogenized in a Virtis homogenizer in 10 volumes of 0.1 M Tris-HCl, pH 9, 0.1 M NaCl, 5 mM EDTA, 0.5% (w/v) sodium *N*-lauroylsarcosinate, and 0.5 mg/mL heparin and 10 volumes of buffer-saturated phenol, followed by the addition of 5 volumes of chloroform and extraction for 10 min at 25 °C. Following centrifugation to separate layers, the aqueous phase was reextracted 3 additional times with an equal volume of phenol–chloroform (1:1). The final aqueous layer was precipitated with 2 volumes of ethanol at −20 °C, washed 3 times with 3 M sodium acetate, pH 5.5, and 5 mM EDTA (Palmiter, 1974), and reprecipitated with ethanol. Total cellular RNA samples were resuspended in H_2O and heated to 68 °C for 2 min prior to oligo(dT)–cellulose chromatography for isolation of poly(A)-containing RNA.

In Vitro Translation of RNA. Rabbit reticulocyte lysate was prepared by the method of Evans & Lingrel (1969). The lysate was treated with 30 μg/mL micrococcal nuclease (P-L Biochemicals) as described by Pelham & Jackson (1976). In vitro translational assays contained the following in a volume of 120 μL: 20 mM Hepes, pH 7.6, 80 mM KCl, 2 mM magnesium acetate, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 40 μg/mL creatine phosphokinase (Worthington), 80 μM of 19 unlabeled amino acids, 20 μg/mL poly(A)-containing RNA, and either 20 μCi of [^{3}H]leucine (New England Nuclear, 120 Ci/mmol) or 67 μCi of [^{35}S]-methionine (New England Nuclear, 700 Ci/mmol). Reactions were incubated for 45 min at 30 °C and then centrifuged at 100000*g* for 1 h to remove ribosomes. Total incorporation into released proteins was determined by spotting a 1–2-μL aliquot of the supernatant fraction on Whatman 3MM paper, followed by washing through the hot trichloroacetic acid procedure of Mans & Novelli (1961).

Immunoprecipitation of in Vitro Translational Products. The ribosomal supernatant fraction was first adjusted to 0.25% NP-40 and then precleared by adding 50 μL of a 10% suspension of heat-killed, formaldehyde-fixed *S. aureus*, Cowens I strain (Kessler, 1975; Jonsson & Kronvall, 1974) (SaC, Calbiochem). After 15 min at 4 °C, the tubes were centrifuged in a Beckman microfuge for 2 min and the supernatant was transferred to a clean tube. Appropriate antibody was added for 30 min at 4 °C, followed by 10 μL of 10% SaC for 15 min at 4 °C. Tubes were centrifuged, and the supernatant fraction was discarded. SaC pellets were washed 4 times by resuspension in 0.5 mL of 0.0125 M potassium phosphate (pH 7.6), 0.2 M NaCl, and 0.25% NP-40. The final pellet was eluted with 75 μL of 2% $NaDodSO_4$, 2% 2-mercaptoethanol, and 0.5 M sodium phosphate, pH 7.1, heated to 95 °C for 3 min, and subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis on 7.5% gels (0.5 × 9.4 cm). The gels were sliced into 0.217-cm sections, dissolved in 0.5 mL of 30% H_2O_2 at 60 °C for 5 h, and counted in Aquasol (New England Nuclear).

Results

Detection and Quantitation of mRNA for Malic Enzyme. Polysomal poly(A)-containing RNA isolated from livers of hyperthyroid rats which were fed the high carbohydrate, fat-free diet was used for initial characterization of the in vitro translational system. With this RNA, it was possible to incorporate ~2.2 × 10^6 cpm of [^{35}S]methionine into hot trichloroacetic acid precipitable material in a 45-min period. In the absence of exogenous RNA, incorporation in the nuclease-treated reticulocyte lysate system was roughly 5% as high. Immunoprecipitation of the translational products with specific antibody to purified malic enzyme, followed by adsorption onto heat-killed, formaldehyde-fixed *S. aureus* (Cowens I strain), was carried out. This procedure provides an extremely quick method for collecting antibody–antigen complexes with low background levels of nonspecific adsorption. An $NaDodSO_4$–polyacrylamide gel electrophoresis profile of the immunoprecipitated products is shown in Figure 1. A single peak of radioactivity was observed. The migration of this peak was identical with that of purified malic enzyme. No specific peak was observed when antibody to malic enzyme was replaced by nonimmune rabbit serum or antibody to purified chick albumin. Addition of excess unlabeled malic enzyme to the immunoprecipitation effectively eliminated the peak of ^{35}S-labeled malic enzyme (data not shown). Finally, if rat liver RNA was omitted, no observable peak of radioactivity could be detected, indicating that malic enzyme is not a product of endogenous RNA in the reticulocyte lysate. Thus, we conclude that the peak of ^{35}S-labeled material present in the immunoprecipitated material is due to malic enzyme synthesized in vitro from the rat liver polysomal RNA.

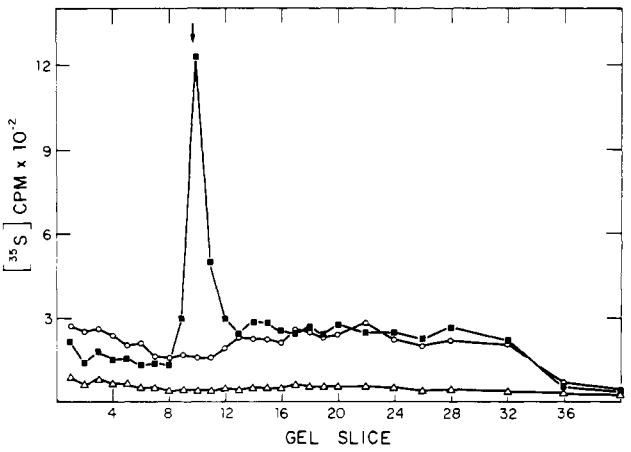

FIGURE 1: Translation of malic enzyme from rat liver polysomal RNA. Translation in nuclease-treated reticulocyte lysate system using either polysomal poly(A)-containing RNA from hyperthyroid rat fed high carbohydrate diet (■, ○) or no exogenous RNA (△). [^{35}S]-Methionine-labeled products were immune precipitated with either antibody to malic enzyme (■, △) or nonimmunized rabbit sera (○). Gels were run for 18 h at 2.5 mA/gel, at which time the tracking dye was ~2 cm from the end of the gel. The position of the purified malic enzyme run in a parallel gel is shown by the arrow.

Levels of mRNA for malic enzyme were quantitated by comparing the radioactivity which comigrated with authentic malic enzyme in the $NaDodSO_4$ gel of immune-precipitated products to the total radioactivity incorporated into the protein. For quantitation of the recovery of malic enzyme during immune precipitation, ^{125}I-labeled malic enzyme, iodinated by the method of Bolton & Hunter (1973), was added to a normal translational assay. This assay was then processed in the standard fashion. The final immunoprecipitated products were analyzed by $NaDodSO_4$ gel electrophoresis. A single peak of ^{125}I was observed which comigrated with marker malic enzyme, detected by staining the purified protein run in a parallel gel. Thus, there was no evidence for breakdown of malic enzyme under translational or processing conditions. The recovery of

Table I: Induction of Malic Enzyme and mRNA Coding for Malic Enzyme[a]

group	malic enzyme activity (units/mg of protein)	relative rate of synthesis (%)	polysomal mRNA for malic enzyme (% ^{35}S cpm)	polysomal mRNA for malic enzyme (% ^{3}H cpm)	mRNA for malic enzyme in total cellular mRNA (% ^{35}S cpm)	poly(A)-containing RNA content (μg/mg of DNA)
euthyroid	17.6 (±1.8)	0.051 (±0.008)	0.007	0.008	*b*	38.2 (±1.70)
euthyroid and high carbohydrate diet	163 (±16)	0.316 (±0.066)	0.083	0.122	0.0364	35.4 (± 0.28)
hyperthyroid	141 (±7)	0.203 (±0.018)	0.042	0.100	0.0378	37.2 (±2.66)
hyperthyroid and high carbohydrate diet	308 (±27)	0.830 (±0.306)	0.13	0.198	0.1022	34.7 (±2.93)

[a] For each experimental group, enzyme activities, relative rates of synthesis, and poly(A)-containing RNA contents were measured on four individual rats, and the values represent the mean plus or minus standard error. For malic enzyme mRNA determinations, RNA for each group was extracted from a single pooled sample containing equal portions of the individual livers. [b] Below levels detectable in the assay.

^{125}I-labeled malic enzyme was found to vary from 45 to 54% in four separate experiments. Levels of malic enzyme mRNA were corrected for the average recovery of 50%. It must be pointed out, however, that the ^{125}I-labeled malic enzyme presumably represents the native tetrameric form of enzyme isolated from liver (Li et al., 1975). The translated product is initially present as the subunit; it is not known whether this subunit wiill associate to form native enzyme under in vitro conditions. The effect of the iodination on the immunoprecipitability of malic enzyme also has not been closely examined. Thus, it is conceivable that the recovery of ^{125}I-labeled malic enzyme may be an overestimate or underestimate of that of the in vitro synthesized product.

When comparing RNA preparations from animals under different treatment regimens, it is important to establish that the percentage of malic enzyme synthesized does not vary with RNA concentration. Such behavior has been reported for certain mRNA species during in vitro translation in the wheat germ system (Sonenshein & Brawerman, 1976, 1977). Accordingly, polysomal poly(A)-containing RNA from livers of hyperthyroid rats fed the high carbohydrate, fat-free diet was translated over a range of RNA concentration from 3 to 36 μg/mL. Both incorporations of [^{35}S]methionine into total protein and into malic enzyme increased proportionally with RNA concentration. No systematic deviation in the percentage of malic enzyme was seen at any RNA concentration. The percentage of malic enzyme varied from 0.087 to 0.14 with a mean of 0.11 and a standard deviation of 0.017.

Malic Enzyme Activity and Relative Rates of Synthesis in Various States. For the purpose of comparing mRNA for malic enzyme with enzyme activity and relative rates of synthesis, four experimental groups were used: (1) euthyroid rats fed normal chow diet, (2) euthyroid rats fed high carbohydrate, fat-free diet, (3) rats made hyperthyroid by daily injection of 15 μg of L-triiodothyronine per 100-g body weight and fed normal chow diet, and (4) rats made hyperthyroid as above, but fed the high carbohydrate, fat-free diet. Treatments were continued for 7 days. For a test of the effectiveness of these various regimens, malic enzyme activity was determined in hepatic cytosolic samples of these rats (Table I, column 2). As expected, both thyroid hormone and high carbohydrate diet caused a marked induction of enzyme activity. Animals receiving thyroid hormone had enzyme levels ninefold higher than normal controls, whereas the dietary induction accounted for an eightfold increase in activity. The animals receiving both thyroid hormone and the high carbohydrate diet showed a further increase in malic enzyme activity to a level approximately 17 times higher than normal controls.

Previous reports have indicated that the induction of malic enzyme activity by either thyroid hormone or diet is due to an increased rate of enzyme synthesis (Isohashi et al., 1971; Gibson et al., 1972; Murphy & Walker, 1974; Li et al., 1975). For confirmation of this conclusion, studies were performed to measure the in vivo rate of incorporation of [^{3}H]leucine into malic enzyme in the four experimental groups. The rate of incorporation into malic enzyme was expressed as the incorporation of leucine into immunoprecipitable malic enzyme relative to incorporation into total cytosolic proteins. If a common leucine precursor pool is assumed, potential changes in the specific activity of leucine in the various states will not affect the results. In normal animals, 0.05% of the leucine incorporation into total cytosolic protein was precipitated by antibody to malic enzyme (Table I, column 3). This value was increased 6.2-fold in animals fed a high carbohydrate diet, 4-fold in hyperthyroid animals, and 16.3-fold in the combined treatment group. Thus, it appears that the changes in enzyme activity induced by hormone and diet can be accounted for largely by changes in the rates of synthesis of malic enzyme.

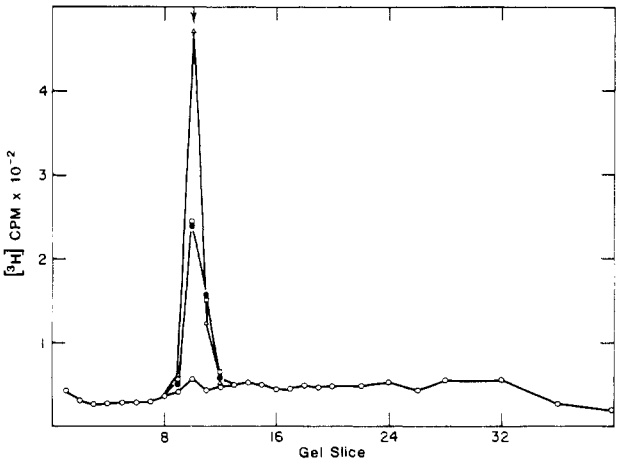

FIGURE 2: Levels of mRNA for malic enzyme in various dietary and thyroidal states. Polysomal poly(A)-containing RNA from euthyroid chow-fed rats (○), euthyroid rats fed high carbohydrate diet (●), hyperthyroid, chow-fed rats (□), or hyperthyroid rat fed high carbohydrate diet (△) was translated at a concentration of 20 μg/mL. Reactions were subjected to immunoprecipitation for malic enzyme as described under Materials and Methods and in the legend to Figure 1. Background counts per minute in all gels were essentially coincident with that of euthyroid, chow-fed rats and were omitted for clarity. The position of purified malic enzyme run in a parallel gel is shown by the arrow.

Levels of mRNA for Malic Enzyme in Various States. We next sought to determine the levels of mRNA for malic enzyme present in the polysomal poly(A)-containing RNA fraction for the four experimental groups. RNA samples were translated in the nuclease-treated reticulocyte lysate, and the final immunoprecipitates were then analyzed by NaDod-

SO_4–polyacrylamide gel electrophoresis (Figure 2). The amount of total incorporation into protein was roughly the same for the four RNA samples. The level of mRNA for malic enzyme in euthyroid rats fed normal diets is barely detectable with this assay technique. The extremely small peak of radioactivity which comigrates with malic enzyme, however, does occur reproducibly with euthyroid RNA. Attempts to quantitate this peak obviously provide only a gross estimate of the mRNA level. On the other hand, the highest levels of malic enzyme mRNA were observed in the hyperthyroid rats fed the high carbohydrate, fat-free diet. The quantitation of these data is shown in Table I. Two separate translational assays using either [^{35}S]methionine (column 4) or [^{3}H]leucine (column 5) as the labeled amino acid were performed. The approximate increase in mRNA for malic enzyme over chow-fed, euthyroid control was 14-fold for rats fed high carbohydrate diet, 9-fold for hyperthyroid rats, and 22-fold for rats receiving both treatments. Thus, a reasonably good correlation between relative levels of polysomal mRNA for malic enzyme and enzyme activity is observed for the four experimental groups.

Since the liver contains a substantial proportion of poly-(A)-containing RNA not bound to polyribosomes (Atryzek & Fausto, 1979), it was of interest to quantitate malic enzyme mRNA activity in total cellular extracts. Accordingly, a second portion of the livers used to isolate polysomes was extracted with phenol–chloroform. This RNA was then translated and the quantity of malic enzyme mRNA activity determined (Table I, column 6). As observed with the polysomal poly(A)-containing RNA, levels of malic enzyme mRNA increase roughly in proportion after treatment with either thyroid hormone or high carbohydrate, fat-free diet and are further enhanced by administration of both treatments. Thus, it does not appear that there is any major shift in the proportion of polyribosomal-bound mRNA for malic enzyme during dietary or hormonal induction.

Concomitant measurements of the levels of total cytoplasmic poly(A)-containing RNA were made by a method we recently described (Towle et al., 1979). No significant differences were detected between the four experimental groups (Table I, column 7). The higher relative levels of malic enzyme mRNA thus correlate with increased absolute quantities of specific mRNA. Therefore, the control of malic enzyme activity in the rat liver appears to occur largely through an increased rate of enzyme synthesis mediated via higher levels of mRNA coding for the protein.

Discussion

Malic enzyme in the rat liver provides an excellent model for studying regulation by both thyroid hormone and nutritional factors as well as the potential interaction between these two control mechanisms. Administration of either thyroid hormone or high carbohydrate, fat-free diet caused an induction of hepatic malic enzyme activity which could be largely accounted for by changes in the rate of enzyme synthesis, as has been previously reported (Tepperman & Tepperman, 1964; Wise & Ball, 1964; Ruegamer et al., 1965; Tarentino et al., 1966; Oppenheimer et al., 1977; Fitch & Chaikoff, 1960; Pande et al., 1964; Isohashi et al., 1971; Gibson et al., 1972; Murphy & Walker, 1974; Li et al., 1975; Silpananta & Goodridge, 1971; Goodridge & Adelman, 1976). Levels of mRNA for malic enzyme, measured in the polysomal fraction or in total cellular extracts, increased roughly in proportion to the increases in enzyme activity. Thus, the induction by both hormonal and dietary factors involves some step in the production of mRNA for malic enzyme. There are, of course, many possible sites at which the level of mRNA could be controlled, including transcription, processing, and degradation of the mRNA. The present study does not allow us to differentiate between these possibilities.

There are several areas of this study which deserve further attention. The method used for isolation of polysomes was designed to maintain polysomal integrity by avoiding conditions which may lyse lysosomes until their removal by differential centrifugation. The absence of detergents during the initial centrifugation steps, however, will lead to some loss of membrane-bound polysomes which become entrapped in the nuclear or mitochondrial–lysosomal fractions. Since malic enzyme would be expected to be found in free polysomes, the slight enhancement of malic enzyme mRNA in the polysomal fraction relative to the total cellular extract is not unexpected. This partial enrichment in the polysomal fraction may also explain why we did not detect malic enzyme mRNA in the total cellular extracts of normal livers. In preliminary experiments we have been able to increase the sensitivity of the in vitro translational assay by affinity chromatography of the in vitro products prior to immunoprecipitation. By utilization of this technique, mRNA for malic enzyme was detected in total cellular poly(A)-containing RNA of euthyroid liver ($\sim$0.004% of total products).

The use of a translational assay for quantitating specific mRNA levels is subject to certain reservations. Different mRNA species may be translated with somewhat varying efficiencies in the in vitro system. Thus, the absolute percentage of a specific product synthesized relative to the total protein might not accurately reflect the percentage of mRNA for that specific product in the total RNA preparation. A difference between in vitro and in vivo translational efficiency of malic enzyme mRNA could account for part of the difference observed between the percentage of malic enzyme mRNA detected in the RNA preparations and the relative rate of malic enzyme synthesis. Another factor in this difference, however, is probably due to the fact that the rate of synthesis is quantitated relative to incorporation into cytosolic (100000*g* supernatant fraction) protein and mRNA relative to incorporation into total protein. For the purposes of this study, we are most interested in comparing levels of specific product between RNA isolated from differing conditions of animal maintenance. It would seem reasonable to assume that differing efficiencies of translation should not vary greatly between RNA isolated from different states and, thus, the relative proportions of specific mRNA will vary roughly in proportion to the actual levels of the mRNA species present.

In addition to malic enzyme (Goodridge et al., 1979), the response of certain other lipogenic enzymes to dietary influences has been examined with respect to specific mRNA levels. Measurement of rat liver fatty acid synthetase revealed coordinate increases in enzyme activity and polysomal mRNA after feeding with high carbohydrate, fat-free diet (Flick et al., 1978; Nepokroeff & Porter, 1978). Similarly, polysomal mRNA for 6-phosphogluconate dehydrogenase was found to increase in proportion to enzyme activity during dietary induction (Hutchinson & Holton, 1978). For glucose-6-phosphate dehydrogenase, however, increased mRNA levels could only account for a portion of the increase in enzyme activity induced by the high carbohydrate, fat-free diet (Sun & Holton, 1978). This finding suggests that for glucose-6-phosphate dehydrogenase regulation of the efficiency of translation of mRNA may also occur. Since all previous studies on lipogenic enzymes which have been performed have utilized polysomal RNA as a source of mRNA, it has not been possible to rule

out the existence of a population of nontranslated mRNA for specific enzyme present in states of reduced synthesis. In the present study, however, no major difference was observed in the level of malic enzyme mRNA between polysomal and total cellular poly(A)-containing RNA. Thus, the existence of such an inactive mRNA pool for malic enzyme is unlikely. The mechanism by which nutritional factors alter mRNA production remains to be elucidated. The relative levels of fat and simple carbohydrate, such as glucose or sucrose, are important factors in determining the extent of induction (Tepperman & Tepperman, 1964; Michaelis & Szepesi, 1973; Reed & Tarver, 1975). The induction could conceivably occur by a direct interaction of some dietary factor with a regulatory component of mRNA production. Alternatively, the signal could occur through secondary stimulation of some undefined intracellular or hormonal process.

For thyroid hormone, there are two other examples of specific mRNA species whose level is modulated by hormones. Growth hormone mRNA has been shown to be induced by thyroid hormone in rat pituitary derived cell cultures (Martial et al., 1977; Seo et al., 1977). In rat liver, mRNA for α_{2U}-globulin, a protein of unknown function found in the urine of male rats, was found to increase upon administration of thyroid hormone to hypothyroid rats (Roy et al., 1976; Kurtz et al., 1976). As far as we know, malic enzyme is the first enzyme which has been shown to have increased mRNA levels in response to thyroid hormone.

In the context of evidence suggesting a nuclear site of thyroid hormone initiation (Oppenheimer, 1979; Samuels, 1978), it is tempting to speculate that the proximate signal for the induction of malic enzyme mRNA by thyroid hormone is the binding of T_3 to specific nuclear receptors. Support for this hypothesis has recently been obtained in preliminary experiments in which we have measured the relative rate of malic enzyme synthesis after the injection of hypothyroid rats with T_3 (Mariash et al., 1980). Extrapolation of the time course to base line values provided an estimate of 1 to 2 h for the time lapse between the cellular penetration of T_3 which occurs within minutes after the injection of hormone and the onset of malic enzyme induction. This time course is entirely consistent with those of other well established hormone response systems in which the time of appearance of specific translational products has been ascertained after receptor occupancy (Samuels & Shapiro, 1976; Kurtz et al., 1978; Tsai et al., 1975; Palmiter et al., 1976; Jost et al., 1978).

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Steroid–Protein Interactions. Influence of Steroid Structure and Temperature on the Binding of Steroids to Guinea Pig Corticosteroid-Binding Globulin†

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ABSTRACT: To better understand the nature of the interactions between steroids and proteins, we have analyzed the thermodynamic and structural requirements of steroid binding by the corticosteroid-binding globulin purified from pregnant guinea pig serum. The affinity constant of the protein–cortisol complex is inversely related to temperature. At 4 °C the association of cortisol is enthalpy and entropy driven, whereas at 37 °C enthalpy is the driving force, $\Delta S°$ being negative. By comparison of the K_a values determined by equilibrium dialysis for individual steroids that differ in only one structural change, the effect of this change on the binding affinity can be assessed. The steroid-binding site appears to be best adapted to bind cortisol; alteration of this structure invariably decreases the affinity. The free energy contributions by individual substituents are approximately additive in determining the total free energy of binding. For example, the low affinity of progesterone relative to cortisol for guinea pig corticosteroid-binding globulin results from the absence of the three hydroxyls at C-11, C-17, and C-21. The total free energy contribution of the three hydroxyls, calculated as the sum of their individual contributions, is −1.6 kcal/mol, a value which is also found experimentally as the difference in the binding energy between the cortisol and progesterone complexes. Important structures for optimal binding are the 3- and 20-oxo groups, a double bond at the 4 position, a 19-methyl group, and an 11β-hydroxy group. In contrast to the steroid-binding site of the guinea pig progesterone-binding globulin, which is predominantly hydrophobic in nature, the binding site of guinea pig corticosteroid-binding globulin apparently contains several hydrophilic groups capable of forming hydrogen bonds. Half of the free energy of binding at 4 °C can be attributed to the three hydroxy and the two oxo groups of cortisol. Based on the evaluation of the binding constants of 45 steroids, a model for the steroid binding site of guinea pig CBG is proposed.

The corticosteroid-binding globulin (CBG)[1] of the guinea pig exists at a relatively high concentration during late pregnancy and can be isolated from serum in pure form with a high yield (Mickelson & Westphal, 1979a). Guinea pig CBG has an affinity constant for cortisol that is ~20 times larger than that for progesterone. Human CBG, in contrast, binds both cortisol and progesterone with similar affinity (Stroupe et al., 1978). In the present study, the affinity constants of guinea pig CBG complexes with a number of steroids have been determined in order to define the conditions producing strong or weak interaction at various locations in the steroid molecule. As a result, a complementary image of the binding site may be deduced. The influence of temperature on the binding activity was also investigated. The information derived from this study will serve as a prelude to a systematic investigation of the nature of the steroid binding site using chemical modification techniques. Recently, the guinea pig CBG–cortisol complex prepared in our laboratory has been crystallized and X-ray crystallographic analysis has been initiated.[2]

Materials and Methods

Pooled serum from guinea pigs in the last 7–14 days of pregnancy was obtained from Dutchland Laboratory Animals,

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[1] Abbreviations used: CBG, corticosteroid-binding globulin or transcortin; K_a, equilibrium association constant; PBG, progesterone-binding globulin.

[2] Personal communication from Dr. Alex McPherson, Jr., University of California, Riverside.

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