Liver poly(A)-containing RNA isolated from rats in different dietary states was translated in a cell free protein synthesizing system employing reticulocyte lysates. Immunoprecipitation of the cell-free reaction products with goat anti-6-phosphogluconate dehydrogenase followed by sodium dodecyl sulfate-urea-gel electrophoresis showed that the induction of this lipogenic enzyme was accompanied by a corresponding increase in the concentration of its specific translatable mRNA.

Currently we have been using rat liver 6-phosphogluconate (6-phospho-n-gluconate:NADP+ oxidoreductase (decarboxylating EC 1.1.1.44)) as a model to study the nutritional regulation of enzyme levels in mammals. If rats are fasted and refed a high carbohydrate non-fat diet, the level of this enzyme, as well as other lipogenic enzymes, has been shown to increase (1, 2) due to an increased rate of synthesis (3).

At least two distinct mechanisms may regulate the induction of lipogenic enzymes. Fatty acid synthetase (4, 5), glucose-6-phosphate dehydrogenase (6, 7), and malic enzyme (8) synthesis are repressed by cAMP while 6-phosphogluconate dehydrogenase synthesis is not influenced by cAMP (3). However, the induction of both glucose-6-P dehydrogenase and 6-phosphogluconate dehydrogenase is proportional to the amount of carbohydrate eaten during fasting-refeeding experiments (9, 10). While some lipogenic enzymes appear to be related by both nutritional and hormonal factors, 6-phosphogluconate dehydrogenase may be regulated by the nutritional state without an involvement of cAMP (3). Therefore, 6-phosphogluconate dehydrogenase should serve as a less complicated system with which to investigate mechanisms regulating the nutritional induction of enzymes.

In this paper, we describe the isolation of an active poly(A)-containing RNA fraction, the translation of this RNA in a reticulocyte lysate translation system, and the subsequent characterization of the 6-phosphogluconate dehydrogenase synthesized in vitro. These results provide the first evidence that the induction of a lipogenic enzyme is associated with an increase in the concentration of the specific translatable mRNA coding for its synthesis.
was retained and made 20 min with respect to magnesium acetate. The solution was centrifuged again for 10 min at 25,000 x g. Seven milliliters of the supernatant fraction were run through (medium velocity 58 x g) and equilibrated with 1 mM Tris/acetate, pH 7.6, 50 mM KCl, 1 mM magnesium acetate, 4 mM 2-mercaptoethanol. The most turbid 7 ml of the elute were pooled and centrifuged again at 23,000 x g for 10 min. The supernatant fraction was removed, the S23 extract, or wheat germ extract, and was stored in small aliquots at -70°C.

For protein synthesis, reactants were present in the assay mixture in the following concentrations: 20 mM Hepes (pH 7.6), 20 mM KCl, 1 mM magnesium acetate, 0.3 mM spermidine, 110 mM potassium acetate, 1 mM ATP, 0.02 mM GTP, 8 mM creatine phosphate, 2.3 mM dithiothreitol, 40 μg/ml of creatine phosphokinase, 60 μM concentration of each of 19 amino acids except leucine, and 35 μCi of L-[4,5-3H]leucine (58 Ci/mmol). Each assay contained 20 μl of wheat germ extract plus varying amounts of RNA for a total assay volume of 0.1 ml. Reactions were incubated at 30°C and terminated after 90 min by pipetting 50-μl aliquots onto Whatman No. 3MM paper discs and washing as previously described (3).

**Translation of mRNA in Reticulocyte Lysates** — Reticulocyte lysate assays were prepared and carried out essentially as outlined by Palmiter (16). A standard reaction mixture of 500 μl contained 15 μl of poly(A)-containing RNA (1 mg/ml), 100 μl of reagent mixture, 100 μl of lysate, and 50 μl of [3H]leucine (1 μCi/ml, 58 Ci/mmole) or [3H]tyrosine (1 μCi/ml, 45 Ci/mmole). The reagent mixture was prepared fresh for each assay from frozen stock solutions. The concentrations of reactants were those outlined in the complete reaction mixture. The reagent mixture provided 19 unlabeled amino acids (minus leucine) each at 100 μM (in reactions in which [3H]tyrosine was substituted for [3H]leucine, nonradioactive leucine was included and nonradioactive tyrosine omitted), 80 mM KCl, 2 mM MgAc, 1 mM dithiothreitol, 16 mM creatine phosphate, 115 μM of creatine phosphokinase, 30 μM hemin (depending on the lysate used), and 20 mM Hepes, pH 7.4.

After protein synthesis had proceeded for 90 min at 25°C, Triton X-100 and sodium deoxycholate were added to give a concentration of 1% Triton X-100 and 1% sodium deoxycholate. The reaction mixture was then subjected to limited proteolysis (22) as follows. The immunoprecipitates were increased to limited proteolysis (22) as follows. The immunoprecipitates were dissolved in 0.1 ml of sample buffer and boiling for 5 min prior to disc gel electrophoresis on 15% gels as outlined by Laemmli (20).

**Messenger RNA Levels for 6-Phosphogluconate Dehydrogenase** — Various methods are available for the isolation of active mRNA fractions from whole tissue or tissue homogenates. In determining an approach to quantitating mRNA levels for 6-phosphogluconate dehydrogenase, which represents at the most only 0.4% of the cytoplasmic protein, we have utilized methods which allowed both a rapid and gentle isolation. The methods which best suited these criteria were the rapid isolation of total rat liver polysomes and mRNPs followed by extraction of poly(A)-containing RNA by dissociation in SDS and affinity chromatography on oligo(dT)-cellulose. Utilizing these methods, an enriched poly(A)-containing RNA fraction could be obtained in a relatively short period of time (on the order of 4-12 h) after killing of the rat.

In order to ensure uniform quality for each poly(A)-containing RNA preparation, the wheat germ translation system was used to determine the activity of each RNA fraction isolated from rats in different dietary states. Messenger RNA activity was determined by adding increasing amounts of poly(A)-containing RNA to the wheat germ translation system (Fig. 1). Good preparations of mRNA routinely gave about 7.5 to 8.5 x 10^6 efp.m of [3H]leucine incorporated into protein per μg of poly(A)-containing RNA when assayed at the optimal concentration of added RNA using the conditions described.
Messenger RNA Levels for 6-Phosphogluconate Dehydrogenase

FIG. 1. Activity of poly(A)-containing RNA isolated from rats in different nutritional states. Poly(A)-containing RNA was isolated from magnesium-precipitated polysomes and assayed in the wheat germ cell-free system as outlined under "Materials and Methods." Poly(A)-containing RNA was isolated from induced (○), pellet-fed (□), and starved (△) rats.

FIG. 2. Synthesis of 6-phosphogluconate dehydrogenase in reticulocyte lysates. Immunoprecipitates from the reticulocyte lysate incubation mixtures were reduced, alkylated, and run on 10% SDS-urea gels as previously described (3). Reticulocyte cell-free assays were incubated with poly(A)-containing RNA from induced (A), pellet-fed (B), and starved rats (C) or with no exogenous RNA (D) added (background). The gels were sliced into 2-mm sections, solubilized in Protosol, and counted in a Toluene scintillation solution (3). A separate gel with purified 6-phosphogluconate dehydrogenase was run as a marker for the enzyme.

under "Materials and Methods." In our hands however, the wheat germ system was not very satisfactory for quantitating mRNA levels of 6-phosphogluconate dehydrogenase. Small molecular weight fragments which were immunoprecipitated by anti-6-phosphogluconate dehydrogenase serum made it difficult to quantitate the amount of radioactive 6-phosphogluconate dehydrogenase which was synthesized.

When poly(A)-containing RNA was translated in reticulocyte lysates, however, the 6-phosphogluconate dehydrogenase which was synthesized could be isolated by immunoprecipitation with the specific antiserum. After the immunoprecipitate was reduced, alkylated, and subjected to electrophoresis on SDS-gels, a single major peak of radioactivity was observed which co-migrated with purified 6-phosphogluconate dehydrogenase (Fig. 2). The minor peaks observed are most likely due to nonspecific globin contamination or nascent chain products. The absence of radioactivity in the 6-phosphogluconate dehydrogenase peak when no poly(A)-containing RNA was added illustrates the very low background observed with this system. As evidence that the translation product which co-migrates with purified 6-phosphogluconate dehydrogenase is indeed that enzyme, reticulocyte lysate assays incubated with and without poly(A)-containing RNA were applied to small DEAE-cellulose columns under conditions where 6-phosphogluconate dehydrogenase binds to the column. Fig. 3 illustrates that the radioactive 6-phosphogluconate dehydrogenase synthesized in the reticulocyte lysate binds to the column under the same conditions as the nonradioactive pure protein. In addition, Fig. 3 demonstrates that if the reticulocyte lysate assay previously incubated with mRNA is applied...
Messenger RNA Levels for 6-Phosphogluconate Dehydrogenase

Fig. 4. Cyanogen bromide fragments from $^{125}$I-labeled 6-phosphogluconate dehydrogenase (6-PGDH) standard and the immunoprecipitated translation product. The iodinated standard and the translation product were prepared and subjected to cleavage with cyanogen bromide as described under "Materials and Methods." The fragments were subjected to electrophoresis on 15% SDS-gels which were then sliced into 1-mm sections, solubilized in 150 µl of Protosol, and counted in Enconfluor for $^3$H (○). The iodinated protein was counted directly in a γ counter ( ● ). The arrow indicates the $R_m$ of intact 6-phosphogluconate dehydrogenase.

Fig. 5. Peptides produced by a limited tryptic digestion of $^{125}$I-labeled 6-phosphogluconate dehydrogenase (6-PGDH) standard and the $^3$H-labeled translation product. Both proteins were reacted with tosylphenylalanyl chloromethyl ketone-treated trypsin as described under "Materials and Methods." Peptides from the $^{125}$I-labeled 6-phosphogluconate dehydrogenase standard ( ● ) and the $^3$H-tyrosine-labeled immunoprecipitated protein ( ○ ) were separated by SDS-disc gel electrophoresis on 15% gels, sliced and counted as in Fig. 4. The arrow indicates the $R_m$ of intact 6-phosphogluconate dehydrogenase.

Using this approach, it was possible to resolve peptides with relatively low levels of radioactivity.

Fig. 4 shows a comparison of the cyanogen bromide fragments from pure $^{125}$I-labeled 6-phosphogluconate dehydrogenase with those from the $^3$H-tyrosine-labeled translation product. In Fig. 5 we show a similar comparison of the peptides produced after limited digestion with trypsin as recently described by Cleveland et al. (22). Cyanogen bromide cleavage and limited digestion with trypsin both produce a number of peptides which are partially resolved by SDS-disc gel electrophoresis. With both procedures there is good coincidence of peaks from the immunoprecipitated translation product with those from pure 6-phosphogluconate dehydrogenase. This demonstrates that the translation product which comigrates with the 6-phosphogluconate dehydrogenase subunit is indeed 6-phosphogluconate dehydrogenase.

Fig. 6 illustrates that the amount of 6-phosphogluconate dehydrogenase synthesized is a linear function of the amount of rat liver poly(A)-containing RNA added to the reticulocyte lysate translation system. Fifteen micrograms of poly(A)-containing RNA per assay falls within the linear range for both induced and pellet-fed rats. Since the RNA translated in Fig. 6 was isolated from equivalent amounts of liver in each of the dietary states, and since the RNA from pellet-fed rats coded for the synthesis of less 6-phosphogluconate dehydrogenase, these data suggest that there are reduced amounts of 6-phosphogluconate dehydrogenase mRNA in noninduced rats.

In Table 1, we report the amount of 6-phosphogluconate dehydrogenase mRNA present in livers of rats in three different dietary states. In making these measurements it was essential that the quality of mRNA isolated from each dietary state be comparable so the results would not be misinterpreted due to low recovery or degradation of mRNA. Consequently, each RNA preparation was translated in a wheat germ system which has low endogenous mRNA levels. Only those preparations showing high activity (see Fig. 1) were used for subsequent translation and quantitation in reticulocyte lysates. Equal amounts of poly(A)-containing RNA, isolated from

to a DEAE-cellulose column prior to immunoprecipitation, the minor peaks noted in Fig. 2 can be eliminated.

The immunoprecipitated translation product was also subjected to cyanogen bromide cleavage or to limited digestion with trypsin in order to compare the protein to authentic 6-phosphogluconate dehydrogenase. To ensure that the translation product and the standard 6-phosphogluconate dehydrogenase were comparably labeled, the pure enzyme was iodinated on the tyrosine with $^{125}$I and the translation product was synthesized using $^3$H-tyrosine. The peptides produced were resolved by SDS-die gel electrophoresis on 15% gels.

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This approach involved the assumption that increased levels of messenger RNA for 6-phosphogluconate dehydrogenase would result in higher levels of specific protein being made when this RNA was translated in a cell-free system. The rationale of this requirement was satisfied. Great care was taken in the preparation of total rat liver poly(A)-containing RNA in a wheat germ cell-free system in order to ensure uniform messenger activity. Fifteen micrograms of each of the poly(A)-containing RNA's was translated and the 6-phosphogluconate dehydrogenase synthesized was immunoprecipitated and run on gels as in Fig. 2. The counts per min in each 6-phosphogluconate dehydrogenase peak is reported below along with the specific activity of the enzyme in the liver from which the RNA was isolated. The numbers have been normalized to take into account small differences in the activities of the reticulocyte lysate preparations used in the assays. Addition of exogenous mRNA resulted in an 18%, on the average, inhibition of total incorporation, regardless of the dietary origin of the poly(A)-containing RNA used in the reticulocyte lysate assays.

### DISCUSSION

In quantitating messenger RNA levels for 6-phosphogluconate dehydrogenase, we have used an indirect assay employing translation of total rat liver poly(A)-containing RNA in a heterologous protein-synthesizing system. The rationale of this approach involved the assumption that increased levels of messenger RNA for 6-phosphogluconate dehydrogenase would result in higher levels of specific protein being made when this RNA was translated in a cell-free system. The linear relationship between the amount of poly(A)-containing RNA and the amount of 6-phosphogluconate dehydrogenase synthesized (Fig. 6) for both induced and non-induced rats confirms the validity of this assumption.

The second requirement is assurance that the radioactivity we are measuring is in 6-phosphogluconate dehydrogenase. The third control which we ran was to translate every preparation of poly(A)-containing RNA in reticulocyte lysates, the only protein precipitated has an R, identical to that of the authentic 6-phosphogluconate dehydrogenase subunit. In a separate experiment we treated the antiserum with enough pure 6-phosphogluconate dehydrogenase to remove any antiserum specific for that enzyme and centrifuged to remove the immunoprecipitate (data not shown). The remaining supernatant fraction contained no anti-6-phosphogluconate dehydrogenase but would still contain antiseraums to proteins other than 6-phosphogluconate dehydrogenase if they had been present in the original antiserum. This treated antiserum was no longer able to immunoprecipitate any radioactive protein synthesized from poly(A)-containing RNA translated in a reticulocyte lysate. Thus, the antiserum which immunoprecipitates the radioactive peaks appearing in Fig. 2 is specific for 6-phosphogluconate dehydrogenase.

In addition, the translation product which reacts with the anti-6-phosphogluconate dehydrogenase serum is adsorbed to and eluted from DEAE-cellulose by the same conditions which bind and elute authentic 6-phosphogluconate dehydrogenase (Fig. 3). Finally, if the immunoprecipitated translation product is subjected to disc gel electrophoresis and the radioactive peak is subjected to cleavage with cyanogen bromide or to a limited digestion with trypsin, the peptides produced co-migrate with peptides produced under identical conditions from pure 6-phosphogluconate dehydrogenase (Figs. 4 and 5). Taken together all of these experiments provide very strong evidence that the radioactivity we are measuring is in 6-phosphogluconate dehydrogenase.

The third control which we ran was to translate every preparation of poly(A)-containing RNA in a wheat germ translation system in order to ensure that each preparation was of uniform quality. This eliminates errors which might have occurred if some RNA preparations had been exposed to ribonuclease or if the poly(A)-containing RNA recovery from the oligo(dT)-cellulose was variable. If care was taken to ensure uniform messenger activity, Fifteen micrograms of each of the poly(A)-containing RNA's was translated and the 6-phosphogluconate dehydrogenase synthesized was immunoprecipitated and run on gels as in Fig. 2. The counts per min in each 6-phosphogluconate dehydrogenase peak is reported below along with the specific activity of the enzyme in the liver from which the RNA was isolated. The numbers have been normalized to take into account small differences in the activities of the reticulocyte lysate preparations used in the assays. Addition of exogenous mRNA resulted in an 18%, on the average, inhibition of total incorporation, regardless of the dietary origin of the poly(A)-containing RNA used in the reticulocyte lysate assays.

### TABLE 1

**Induction of mRNA levels for 6-phosphogluconate dehydrogenase**

<table>
<thead>
<tr>
<th>Nutritional state</th>
<th>6-Phosphogluconate dehydrogenase specific activity</th>
<th>Radioactive 6-phosphogluconate dehydrogenase synthesized in the reticulocyte lysate system (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/mg protein</td>
<td>cpm/µg RNA x 10^4</td>
</tr>
<tr>
<td>Induced</td>
<td>0.37</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>0.23</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>0.23</td>
<td>7.7</td>
</tr>
<tr>
<td>Mean = 0.28 ± 0.047*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pollent. fed</td>
<td>0.071</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>0.083</td>
<td>7.5</td>
</tr>
<tr>
<td></td>
<td>0.064</td>
<td>8.6</td>
</tr>
<tr>
<td>Mean = 0.070 ± 0.0055</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasted</td>
<td>0.059</td>
<td>7.7</td>
</tr>
<tr>
<td></td>
<td>0.055</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>0.030</td>
<td>8.0</td>
</tr>
<tr>
<td>Mean = 0.055 ± 0.0026</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Standard Error.
ensure that every poly(A)-containing RNA preparation was prepared using identical procedures; the translational activity (column A in Table I) was quite reproducible and independent of the nutritional state of the animal. It was important, however, that exactly the same amount of RNA was applied to each oligo(dT)-cellulose column.

Our wheat germ preparations were unsatisfactory for the quantitation of 6-phosphogluconate dehydrogenase, possibly because they appear to be contaminated with ribonuclease. Although the reticulocyte lysate system has a very large background due to endogenous globin mRNA, radioactive globin is removed during the immunoprecipitation and subsequent washes and does not interfere with the quantitation of the 6-phosphogluconate dehydrogenase which is synthesized. It is absolutely necessary, however, to run SDS-disc gel electrophoresis on the immunoprecipitate in order to ensure that the radioactivity is all due to that enzyme. Even if the reticulocyte lysate supernatant fraction remaining after the poly(A)-containing RNA is translated is subjected to chromatography on DEAE-cellulose to purify the 6-phosphogluconate dehydrogenase prior to immunoprecipitation there are enough nonspecific counts in the washed immunoprecipitate to require SDS-disc gel electrophoresis. 6-Phosphogluconate dehydrogenase represents such a small amount of the total cytoplasmic protein (0.4 to 0.07%) that complete separation of the enzyme from all other proteins is difficult even with a specific antiserum. However, with proper care and electrophoresis of the immunoprecipitates on SDS-gels, one can quantitate relative levels of mRNA which represent less than 0.1% of the total protein synthesis.

Table I shows a good correlation between the level of 6-phosphogluconate dehydrogenase and the amount of specific mRNA coding for its synthesis. We had previously established that the induction of this enzyme is entirely due to an increase in its rate of synthesis (3). Thus, the dietary induction of rat liver 6-phosphogluconate dehydrogenase is accompanied by an increase in the rate of enzyme synthesis and an increase in the level of mRNA coding for the synthesis of the enzyme.

The most likely mechanism for an increase in the concentration of 6-phosphogluconate dehydrogenase mRNA would be an increase in the rate at which the gene coding for the enzyme is transcribed. However, present data does not exclude several other possibilities such as changes in the processing or transport of the mRNA from the nucleus to the cytoplasm, changes in the rate of degradation of the mRNA, or changes in the rate of initiation of 6-phosphogluconate dehydrogenase synthesis which results in a protection of the mRNA if it is on larger polysomes in the induced animal. Experiments are currently in progress which will attempt to differentiate among these possibilities. In any event, any postulated mechanism for the regulation of lipogenic enzyme induction must include the probability that the rate of enzyme synthesis is in part regulated by changing the levels of mRNA coding for the synthesis of these enzymes.

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