Studies on the Induction and Repression of Enzymes in Rat Liver

I. INDUCTION OF THREONINE DEHYDRASE AND ORNITHINE-δ-TRANSAMINASE BY ORAL INTUBATION OF CASEIN HYDROLYSATE*

H. C. PITOT AND CARL PERAINO†

From the McArdle Memorial Laboratory, University of Wisconsin Medical School, Madison 6, Wisconsin

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It has now become firmly established that the environmental control of enzyme synthesis plays a major role in the determination of the phenotype of bacterial cells (1). However, the phenomena of enzyme induction and repression, as well as that of negative feedback at the level of enzyme activity as seen in microorganisms, have not yet been widely studied in mammalian cells. The substrate-induced increases of tryptophan pyrrolase first reported by Knox (2) and studied by many others (3, 4) is perhaps the best studied if not the only example of the control of the synthesis (5) of a single enzyme by its substrate in a multicellular organism. Several mammalian enzymes, e.g. tyrosine (6, 7) and glutamic-alanine (8) transaminases, tryptophan pyrrolase (9), and many others (10), exhibit hormonal control, which appears to be a more common mechanism for enzyme induction in higher organisms. That enzyme induction, i.e. a change in the rate of enzyme synthesis in response to the administration of corticosteroids, has occurred has been shown rigorously in at least two instances in mammalian systems (11, 12).

A major difficulty encountered when studying the control of enzyme synthesis in mammals is the fact that few mammalian enzymes which are responsive to environmental stimuli show increases as large as those seen in bacteria, i.e. 10- to 1000-fold. The purpose of this paper is to report changes in enzyme levels in rat liver under conditions in which the responses of enzymes to environmental stimuli are very great, in one case approaching increases as large as those seen in bacteria, i.e. 10- to 1000-fold.

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EXPERIMENTAL PROCEDURE

Animals and Diets—All animals utilized in these studies were albino male rats weighing 130 to 140 g obtained from the Holtzman Rat Company, Madison, Wisconsin. The animals were placed in individual wire-bottomed cages and were fed a protein-free diet (13). In later experiments, a pellet form of the protein-free diet which was obtained from General Biochemicals, Inc., was used. Animals were maintained on this diet for a period of 5 days before the experiment. At the end of this period, the animals were fasted overnight, and the experiments were begun at 6 a.m., when the body weights of the rats were 100 to 110 g.

In early experiments (see Table I), intact or adrenalectomized rats were fed a 90% casein diet (13) ad libitum for a period of 7 days before their death. Other animals of the same experiment were maintained on Purina laboratory chow diet ad libitum. Cortisone acetate was administered intramuscularly at a dose of 5 mg per day in two doses of 2.5 mg, one in the morning and the other in the late afternoon.

Experimental Conditions—Beginning at zero time (usually 6 a.m.), rats were force-fed by stomach tube 3 ml of a 33% solution of enzymatically hydrolyzed casein (General Biochemicals, Inc.) in distilled water. This regimen was repeated three more times, i.e. at 6, 12, and 18 hours after zero time. At each of these time points and at 24 hours after zero time, animals were killed, and the level of threonine dehydrase and ornithine-δ-transaminase determined. In several experiments, lesser amounts of casein hydrolysate were administered in the same 3-ml volumes. In some experiments, only a single feeding of casein hydrolysate was administered, and in others no hydrolysate was given at all.

Puromycin was injected intraperitoneally at a dosage of 100 mg per kg of body weight at 0, 4, and 8 hours or at 12, 16, and 20 hours during the 24-hour experiment. Actinomycin D was administered by the same route at a level of 1 mg per kg of body weight at 0, 4, 8, and 12, 16, and 18 hours after zero time. At each of these time points and at 24 hours after zero time, animals were killed, and the level of threonine dehydrase and ornithine-δ-transaminase determined. In several experiments, lesser amounts of casein hydrolysate were administered in the same 3-ml volumes. In some experiments, only a single feeding of casein hydrolysate was administered, and in others no hydrolysate was given at all.

Bilateral adrenalectomies were performed by the Endocrine Laboratories, Madison, Wisconsin. The adrenalectomized animals were given a 1% (w/v) NaCl aqueous solution to drink ad libitum and were fed the protein-free diet for 5 days before use in the experiments. These rats were not fasted prior to the experiment. The amount of hydrolysate given was considerably reduced since the 1-g doses were found to be lethal. The amount of casein hydrolysate which permitted observable threonine dehydrase induction without toxicity symptoms in the adrenalectomized rats was found to be about 0.25 g per dose. Also, the experiments with these animals were only carried for 12 hours, since beyond this time, some toxicity (general lethargy and some deaths) became apparent even at these low levels of casein hydrolysate.

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Dietary and hormonal effects on ornithine transaminase and threonine dehydrase activities of rat liver

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Ornithine transaminase unit/kt</th>
<th>Threonine dehydrase unit/kt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purina chow diet</td>
<td>103 ± 9</td>
<td>50 ± 15</td>
</tr>
<tr>
<td>Purina chow diet + cortisone, 5 mg per day for 7 days</td>
<td>75 ± 7</td>
<td>3500 ± 350</td>
</tr>
<tr>
<td>Adrenalectomized Purina chow diet</td>
<td>112 ± 11</td>
<td>50 ± 10</td>
</tr>
<tr>
<td>90% casein diet for 7 days</td>
<td>243 ± 17</td>
<td>5400 ± 640</td>
</tr>
<tr>
<td>90% casein diet for 7 days + cortisone, 5 mg per day for 7 days</td>
<td>252 ± 21</td>
<td>7100 ± 840</td>
</tr>
<tr>
<td>Adrenalectomized + 90% casein diet for 7 days</td>
<td>232 ± 10</td>
<td>3900 ± 400</td>
</tr>
</tbody>
</table>

* See “Experimental Procedure” for details of regimens. Three rats were used for each experiment.

† Micromoles of pyrroline carboxylate formed per g of fresh liver per hour ± standard error of the mean.

‡ Micromoles of α-ketobutyrate formed per g of fresh liver per hour ± standard error of the mean. See text for p values.

Enzyme Assays—At the designated intervals, animals were killed by cervical dislocation, and the livers were rapidly removed and cooled in 0.2 M potassium phosphate buffer, pH 8.0, containing 0.01 M mercaptoethanol. The livers were weighed and homogenized in 4 volumes of the cooling medium with an Ultra Turrax homogenizer (Janke and Kunkel, West Germany). A portion of each homogenate was then centrifuged at 100,000 g for 1 hour in a Spincel model L ultracentrifuge. The whole homogenate was utilized for the assay of ornithine-δ-transaminase by the method of Peraino and Pitot (14). The supernatant fluid (S-3) obtained from the high speed centrifugation was utilized for the threonine dehydrase assays since previous results have shown that the entire activity is in this fraction (15). The method employed was a modification of that used by Goldstein, Knox, and Behrman (16). The reaction was performed in a 1.0 ml volume in small test tubes. The reaction mixture contained 0.2 M K2HPO4 (pH 8.1), 0.25 M l-threonine, and 4 x 10^-4 M pyridoxal phosphate. This mixture minus the enzyme was preincubated with shaking at 37°C for 5 minutes. At the end of this time, 0.1 or 0.2 ml of S-3 was added, and the tubes were incubated for exactly 10 minutes more. At the end of this time, the tubes were removed from the bath and immersed in ice, and 1.0 ml of 10% trichloroacetic acid was added to each tube. By staggering the assays, a large number of tubes could be done in a short time. Control tubes contained the reaction mixture plus enzyme but were stopped with acid at zero time. Under these conditions, the reaction was linear with time and followed zero order kinetics. The α-ketobutyrate formed was determined by the method of Greenberg (17) on an aliquot of the supernatant obtained after centrifugation of the acid-precipitated reaction mixtures. All enzyme units are reported as follows: ornithine transaminase, micromoles of pyrroline carboxylate formed per g of fresh liver per hour; threonine dehydrase, micromoles of α-ketobutyrate formed per g of fresh liver per hour.

Materials—Pyridoxal phosphate was a product of the Sigma Chemical Company. L-Threonine and L-ornithine were obtained from General Biochemicals, Inc. Puromycin hydrochloride was a gift of the Lederle Laboratories while actinomycin D was kindly supplied by Merck and Company. 5-Fluoroorotic acid was a gift of the Cancer Chemotherapy National Service Center through the kind cooperation of Dr. Howard W. Bond. Cortisone acetate was a product of Merck, Sharp, and Dohme.

RESULTS

Previous studies (13, 18, 19) have shown that threonine dehydrase is an enzyme that can be considerably altered in amount by changing the dietary conditions of the animal. Further studies undertaken in this laboratory in conjunction with Dr. Richard Bottomley have shown that both dietary and hormonal factors are involved in the induction of threonine dehydrase whereas only dietary changes have an effect on the level of ornithine transaminase, an enzyme of the mitochondrial sap (14). The results of some of these experiments are seen in Table I. Administration of cortisone, or bilateral adrenalectomy had little or no effect on the level of ornithine transaminase (p > 0.05), whereas the repeated administration of cortisone resulted in a marked rise in threonine dehydrase activity (p < 0.001). However, feeding a high protein diet for 1 week resulted in significant increases in both enzymes whether the animals were adrenalectomized or not (p < 0.001). Administration of cortisone to intact rats on a 90% casein diet gives somewhat higher levels of threonine dehydrase than those on the same diet not given the hormone. The Michaelis constants for threonine and ornithine of the induced and noninduced enzymes were found to be essentially the same. Mixtures of homogenates from animals fed a high protein and a chow regimen gave additive results when assayed for ornithine transaminase and threonine dehydrase.

Inhibition Studies—In view of these data, coupled with the previous findings of a direct dependency of threonine dehydrase levels on the casein content of the diet (18), it became of interest to determine the degree of induction of these enzymes obtainable by acute changes in dietary protein. In a previous communication (20), we have reported on the changes in liver threonine dehydrase and ornithine transaminase obtained when protein-depleted rats were force-fed hydrolyzed casein. In addition, preliminary experiments with inhibitors of protein and RNA synthesis were presented. The work reported here has confirmed and extended these earlier experiments.

In Fig. 1 is shown the effect of early and delayed puromycin administration on the induction of threonine dehydrase and ornithine transaminase by repeated intubations of casein hydrolysate every 6 hours. It is evident that the induction of both ornithine transaminase and threonine dehydrase is markedly inhibited by this compound. If puromycin is administered from 0 to 8 hours, virtually no enzyme induction occurs for 6 hours, and even at 12 hours the induction of threonine dehydrase is 80% inhibited and that of ornithine transaminase more than 50% inhibited by this regimen. When the administration of puromycin is delayed until the 12-hour point, no further significant induction of either enzyme occurs. Interestingly enough, there is only a slight loss of activity of either enzyme after puromycin administration, suggesting that under the conditions reported here, little enzyme turnover is seen.

In Fig. 2 is seen the effect of actinomycin D administration on the dietary induction of threonine dehydrase and ornithine...
FIG. 1. The effect of puromycin administration on the dietary induction of threonine dehydrase (left) and ornithine transaminase (right). All animals were intubated with 1.0 g of casein hydrolysate at 0, 6, 12, and 18 hours. Puromycin-HCl (neutralized to pH 7.0) was administered at a dosage of 100 mg per kg at 0, 4, and 8 hours, or at 12, 16, and 20 hours in the delayed treatment. Enzyme units are explained in the methods. The vertical lines denote ± standard error of the mean. C.H., casein hydrolysate. Each point represents an average ± s.e. of 4 to 10 animals.

transaminase. This antibiotic has been shown effectively to inhibit the DNA-dependent synthesis of RNA (21), presumably by combining with the DNA template, making complimentary RNA synthesis impossible. Evidence to date (21) strongly suggests that this inhibition of RNA synthesis is the only effect of this compound, making it a powerful tool in studies of protein synthesis. In these experiments, this antibiotic proved to be a very effective inhibitor of the dietary induction of threonine dehydrase and ornithine transaminase when given at 0 and 6 hours. This regimen completely suppressed threonine dehydrase induction and inhibited ornithine transaminase synthesis by more than 60% as measured at 12 hours. However, if the administration of the antibiotic were delayed until 12 hours after the initiation of the tube feeding, the inhibitory effect was much less in the case of ornithine transaminase and absent with threonine dehydrase induction. These results indicate that after a 12-hour period of feeding, the induction is no longer dependent, at least for a time, on a concomitant synthesis of complimentary RNA. The significance, if any, of the slight stimulation noted in threonine dehydrase induction by the delayed actinomycin...
A somewhat greater rise in threonine dehydrase, which substantiates a previous report (25).

Further fasting of these animals resulted in a 3-fold increase of threonine dehydrase over the zero time point. Further fasting of these animals resulted in almost complete suppression of the response. Fasting of protein-depleted, adrenalectomized rats resulted in a marked inhibition of further threonine dehydrase induction by the inhibitory effects of puromycin (26), actinomycin D (21, 27), and 5-fluoroorotic acid (23, 24). Furthermore, threonine dehydrase induction by a single dose of casein hydrolysate administered at zero time on threonine dehydrase and ornithine transaminase induction continues for at least 6 more hours. Thus, the sudden cessation of enzyme induction by puromycin could not be accounted for by an inhibition of absorption. Similarly, we have found that the induction of both enzymes becomes insensitive to actinomycin D after only 2 to 3 hours of induction, some time before complete stomach emptying, a finding incompatible with an inhibition of absorption. Furthermore, an 18-hour administration of F-otic acid results in no greater gastric dilution than is seen in controls.

**Single Dose Experiments**—Fig. 5 shows the effect of a single dose of casein hydrolysate administered at zero time on threonine dehydrase and ornithine transaminase. The responses of the two enzymes are noticeably different. Threonine dehydrase shows a peak of induction at 12 hours before returning to a low level at 18 hours and then giving way to a fasting effect as is seen in the adrenalectomized rat. In contrast, ornithine transaminase shows a slow rise over the entire 24-hour period. Fasting results in only a slight rise in ornithine transaminase activity during the 24-hour period.

These results are of importance with reference to the earlier studies with inhibitors, a possible explanation of their action being an inhibition of absorption of the hydrolysate from the gastrointestinal tract. Studies in this laboratory have shown that after a single feeding of 1.0 g of casein hydrolysate, the stomach is essentially empty in 6 hours.4 However, Fig. 5 shows that threonine dehydrase and ornithine transaminase induction continues for at least 6 more hours. Thus, the sudden cessation of enzyme induction by puromycin could not be accounted for by an inhibition of absorption. Similarly, we have found that the induction of both enzymes becomes insensitive to actinomycin D after only 2 to 3 hours of induction, some time before complete stomach emptying, a finding incompatible with an inhibition of absorption. Furthermore, an 18-hour administration of F-otic acid results in no greater gastric dilution than is seen in controls.

**DISCUSSION**

The studies presented here indicate that, under certain circumstances, increases of more than 300-fold (16 ± 5.1 to 5100 ± 300, Figs. 1 to 3) may be obtained within 24 hours with at least one mammalian enzyme, threonine dehydrase. This increase may be equated to a net synthesis of the enzyme as judged by the inhibitory effects of puromycin (26), actinomycin D (21, 27), and 5-fluoroorotic acid (23, 24). Furthermore, threonine dehydrase induction by a single dose of casein hydrolysate exhibits the kinetics of turnover (Fig. 5 (left)), and from the data of the 12- and 18-hour points, the half-life of the enzyme can be calculated as about 3 hours. If inhibition of breakdown or stabilization were responsible for the observed increase in threonine dehydrase, the enzyme level might be expected to at most merely double every 3 hours, whereas it actually increased much more rapidly. Thus, a mammalian system, resting adult liver, may be more capable of rapid changes in the rate and amount of synthesis of certain enzymes, changes which in magnitude are comparable to those seen in microorganisms (1). In fact, when the intermitotic time of bacteria and resting liver are compared, the intermitotic time of bacteria and resting liver are compared, the induction of threonine dehydrase in liver represents considerably more versatility than comparable inductive processes in bacteria, e.g., ß-galactosidase in Escherichia coli: 1000-fold induction, generation time = 20 minutes versus threonine dehydrase in liver; 300-fold induction, generation time = 200 to 400 days in the resting state (25).

**Table II**

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Times of measurement</th>
<th>No. of rats</th>
<th>Threonine dehydrase units†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zero hours control</td>
<td>0</td>
<td>4</td>
<td>34 ± 15</td>
</tr>
<tr>
<td>Plus 0.25 g of casein hydrolysate</td>
<td>0</td>
<td>5</td>
<td>206 ± 52</td>
</tr>
<tr>
<td>Plus 0.25 g of casein hydrolysate + actinomycin D</td>
<td>6</td>
<td>5</td>
<td>41 ± 15</td>
</tr>
<tr>
<td>Fast</td>
<td>6</td>
<td>4</td>
<td>46 ± 10</td>
</tr>
<tr>
<td>Plus 0.25 g of casein hydrolysate</td>
<td>12</td>
<td>6</td>
<td>483 ± 114</td>
</tr>
<tr>
<td>Plus 0.25 g of casein hydrolysate + actinomycin D</td>
<td>12</td>
<td>4</td>
<td>54 ± 29</td>
</tr>
<tr>
<td>Fast</td>
<td>12</td>
<td>5</td>
<td>102 ± 20</td>
</tr>
</tbody>
</table>

* Casein hydrolysate, 0.25 g in 3 ml of H2O, was intubated at 0 and 6 hours. Actinomycin D (1 mg per kg of body weight) was given at 0 and 6 hours.
† Times indicated are measured from initiation of tube feeding.
‡ Micromoles of α-ketobutyrate formed per g of fresh liver per hour, ± standard error of mean.

4 C. Peraino, R. Blake, and H. Pitot, unpublished observations.
FIG. 4. The response of threonine dehydrase (left) and ornithine transaminase (right) to 1.0, 0.5, and 0.1-g levels of casein hydrolysate administered every 6 hours. Conditions are identical with those of Fig. 1 except for the lowered levels of casein hydrolysate. Each point represents an average ± s.e. of 3 to 10 animals.

FIG. 5. The response of threonine dehydrase (left) and ornithine transaminase (right) to a 24-hour fast and to a single dose of 1.0 g of casein hydrolysate given at zero time. Protein-depleted rats were fasted for 6 hours (6 a.m. to 12 noon) before the start of the experiment. In the case of the fasted animals, the fast was continued for 24 hours more. Each point represents an average ± s.e. of 3 to 8 animals.

The absolute requirements for the induction of threonine dehydrase and ornithine transaminase are not at all clear at the present time. In this paper, enzymatically hydrolyzed casein acted as the inducing agent. Since the animals were protein-depleted, it is logical to assume that a complete mixture of amino acids must be fed before hepatic enzyme synthesis could occur. Such is not true with tryptophan pyrrolase induction, since in this case, even protein-deficient rats respond to tryptophan administration by significant short term increases in tryptophan pyrrolase (29). These experiments have also been confirmed in our laboratory in protein-depleted rats. However, threonine or ornithine administered alone to protein-depleted rats produced little or no threonine dehydrase or ornithine transaminase induction. Furthermore, Goldstein et al. (16) were unable to induce threonine dehydrase by administration of threonine alone to chow fed rats presumably on a normal protein intake. A similar effect is seen with hepatic arginase, which has not been induced by arginine (30), but can be induced by a high protein diet (31). Thus, threonine alone appears not to be sufficient for threonine dehydrase induction in the livers of protein-depleted rats or those fed a "normal" diet. In contrast, tryptophan induces tryptophan pyrrolase under both dietary conditions, suggesting a difference in the basic requirements for the induction of the two enzymes in rat liver. If such a difference is assumed to exist, the dietary casein induction of threonine dehydrase and ornithine transaminase may be termed "dietary" enzyme induction to differentiate it from hormonal and pure substrate induction of hepatic enzymes (32).

SUMMARY

Multiple intubations of 1-g aliquots of enzymatically hydrolyzed casein in water to protein-depleted rats resulted in marked increases in hepatic threonine dehydrase (300-fold) and ornithine transaminase (20-fold) over a 24-hour period. Puromycin administration at zero time prevented enzyme induction. When given 12 hours after the first intubation of casein hydrolysate, puromycin inhibited further induction. Actinomycin D and 5-fluoroorotic acid given at zero time also prevented induction; however, these compounds had little or no effect when given from the 12-hour point on. The induction of threonine dehydrase was dependent on the amount of casein hydrolysate given between 0.1 and 1.0 g per dose, while ornithine transaminase showed little induction until 0.5 g of casein per dose was given. Intubation of 0.25-g doses of casein hydrolysate resulted in marked increases in threonine dehydrase in adrenalectomized rats which was completely inhibited by actinomycin D administration. A single dose of 1.0 g of hydrolysate resulted in threonine dehydrase induction up to 12 hours with an almost complete return to the control levels at 18 hours. Under similar conditions, ornithine transaminase induction showed no return to the base-line.
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REFERENCES
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INDUCTION OF THREONINE DEHYDRASE AND ORNITHINE-δ-TRANSAMINASE BY ORAL INTUBATION OF CASEIN HYDROLYSATE
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