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Amino Acid Induction and Carbohydrate Repression of Dimethylnitrosamine Demethylase in Rat Liver[1]

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SUMMARY

Starvation of rats for 24 hr considerably enhances hepatic dimethylnitrosamine demethylase activity, and 3-methylcholanthrene pretreatment inhibits the enzyme in starved animals to the same extent as in fed animals. Determination of the kinetic constants following starvation revealed significant increase of the apparent V_{max} indicating increase in the amount of demethylase. There was no significant change in the K_m. Studies with actinomycin D provide strong support that starvation-induced increase is due to *de novo* protein synthesis, consistent with the observed increase in maximal velocity.

Ingestion of glucose markedly inhibits demethylase activity while ingestion of casein alone stimulates it appreciably, in a manner analogous to such phenomena with a few other hepatic enzymes. These results and previous data suggest that the level of dimethylnitrosamine demethylase in liver is under the control of multiple regulatory factors.

INTRODUCTION

The activity of microsomal drug-metabolizing enzymes varies with species, sex, genetic background, and the physiological and nutritional state of the animal (reviewed in Ref. 3). Information available on the dietary control of microsomal drug-metabolizing enzymes is scarce in contrast to extensive studies on the dietary regulation of enzymes such as tryptophan pyrrolase (22, 24), tyrosine transaminase (4, 5, 24), and arginase (18). For example, starvation of male rats (11) decreases the metabolism of a variety of drugs, including aminopyrine and hexobarbital, and enhances aniline hydroxylation without altering the metabolism of oxazolamine. DAB[2] reductase activity in rats has been shown to be enhanced by fasting (7).

Our previous studies (20, 21) describing the inhibitory effect of MC on liver microsomal DMN demethylase were performed on rats fed *ad libitum*. Because of the known diverse effects of fasting on enzyme activities, we proceeded to determine the effects of starvation on control DMN demethylase activity as well as the action of MC on DMN demethylase in starved animals. The present study shows considerable enhancement in demethylase activity due to fasting, and also demonstrates decreased enzyme activity in MC-treated rats under conditions of starvation. We have also observed, with the DMN demethylase system, the phenomena of amino acid induction and carbohydrate repression, analogous to the mechanisms of regulation of DAB reductase (7), threonine dehydrase and ornithine δ-transaminase (16, 17), and serine dehydratase (8) in mammalian tissues.

MATERIALS AND METHODS

Chemicals and Solutions, and Treatment of Animals. The sources of biochemicals and the preparation of solutions have been described previously (21). Immature, male Sprague-Dawley rats (Holtzman Co., Madison, Wis.) were used. These were fed basal diet (2) (containing here 4 mg riboflavin/kg) *ad libitum* for 3 to 4 days before the beginning of the treatment (at which time they weighed 55 to 70 g). Special diets of finely ground cellulose (Alphacel) or "vitamin-free" casein (both from Nutritional Biochemicals Corp., Cleveland, Ohio) or glucose (Cerelose; Corn Products Co., Englewood Cliffs, N. J.) were fed *ad libitum* for 24 hr before sacrifice.

DMN Demethylase Assay. The general methods of microsome isolation, demethylation reaction and assay of HCHO, and microsomal protein determination were as previously described (20). For all experiments except the kinetic studies, livers from 2 to 4 rats in each group were pooled to give sufficient microsomes. In kinetic studies, 10 to 12 rats in each group were pooled to obtain the necessary quantity of microsomes. Each reaction flask contained microsomes equivalent to 1.5 g liver, wet weight, as in other experiments; however, the incubation period was here 30 min compared to 40 min in other experiments. Solutions of DMN, freshly prepared, were added to the reaction flasks to yield concentrations of 1, 2, 5, 10, 20, 40, and 80 μmoles/10 ml reaction mixture.

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[2]The abbreviations used are: DAB, 4-dimethylaminoazobenzene; MC, 3-methylcholanthrene; DMN, dimethylnitrosamine (also known as *N*-nitrosodimethylamine).

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RESULTS

Effect of MC on DMN Demethylation in Starved Rats. Chart 1 shows that starvation more than doubles the activity of the demethylase. Pretreatment by MC inhibits the enzyme in starved animals to the same extent as in fed animals. As the compounded result of MC-induced inhibition and starvation-induced enhancement, the activity of MC-treated starved animals lies between the activities of fed and starved rats. Under similar experimental conditions, starvation has been shown to elevate and MC administration to enhance further the liver DAB reductase level (7).

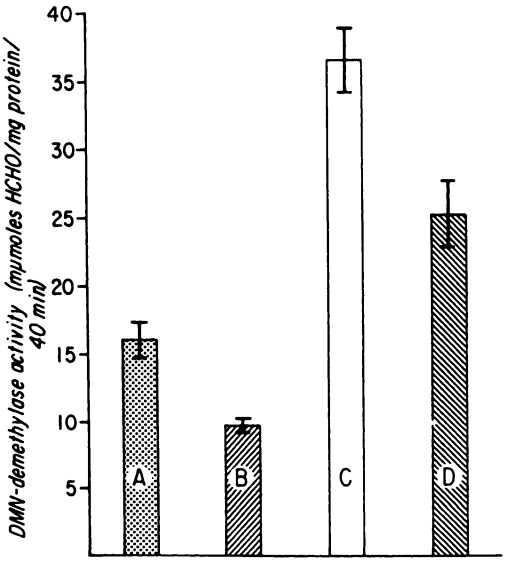

Chart 1. Effect of MC on DMN demethylase in fed and starved rats. *Bars* correspond to the following groups: *A*, control-fed; *B*, MC-fed; *C*, control-starved; *D*, MC-starved. Animals were fed *ad libitum* or starved for 24 hr before sacrifice and were given i.p. injections of corn oil or MC (40 mg/kg) at the beginning of starvation. Values represent the mean ± S.E. of 10 determinations. Probabilities for the significance of the differences between the means are: $p < 0.001$ for control-fed *vs.* control-starved (126.3% increase), control-fed *vs.* MC-fed (39.6% inhibition), and MC-fed *vs.* MC-starved (158.2% increase); $p < 0.01$ for control-starved *vs.* MC-starved (31.1% inhibition).

Kinetic Parameters of DMN Demethylase during Starvation. To test whether the observed increase in demethylase activity due to starvation arises from an increased amount of enzyme and/or enhanced affinity of enzyme for substrate, the kinetic parameters of DMN demethylase in starved rats were determined. Starvation did not bring about significant change in the K_m of the enzyme: fed, $[35.2 \pm 4.4] \times 10^{-5}$ M; starved, $[52.9 \pm 8.0] \times 10^{-5}$ M ($p \cong 0.10$). On the other hand, the V_{max} was appreciably elevated in starved animals: fed, 20.7 ± 1.4 mμmoles HCHO/mg protein/30 min; starved, 31.4 ± 1.4 mμmoles HCHO/mg protein/30 min. This represents a 51.7% increase of the V_{max} ($p < 0.001$; each value is the average of 5 experiments).

Effect of Actinomycin D on Starvation-induced Enhancement of Demethylase. Actinomycin D, a well-known inhibitor of DNA-dependent RNA synthesis, has been used in the present study to elucidate whether the starvation-induced increase in demethylase activity reflects an enhanced output of mRNA molecules and/or stabilization of already existing template. As is evident from Chart 2, when the antibiotic is administered throughout the period of fasting (at 0, 6, 12, and 18 hr), there is essentially complete blocking of the starvation-induced increase. The enzyme activities of actinomycin D-treated rats, both fed and fasted (*bars C* and *D*), are not significantly different, and the values are considerably lower than that of control animals fed *ad libitum* (*bar A*).

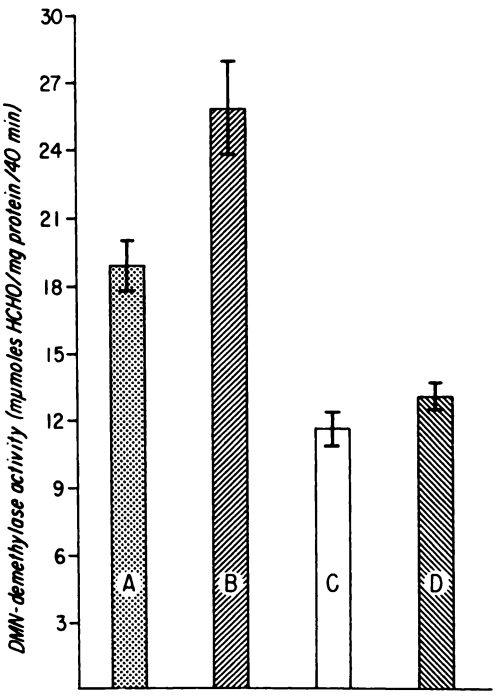

Chart 2. Effect of actinomycin D administration on starvation-induced increase of DMN demethylase activity in rat liver. *Bars* correspond to the following groups: *A*, control-fed; *B*, control-starved; *C*, actinomycin D-fed; *D*, actinomycin D-starved. Actinomycin D (20 μg) or 0.154 M sodium chloride–0.04 M sodium phosphate (pH 7.4) solution was given i.p. at 0, 6, 12, and 18 hr. Thus, each rat receiving the antibiotic received a total of 80 μg actinomycin D during this period. Starvation was begun at 0 hr, and animals were sacrificed at 24 hr. Values represent the mean ± S.E. of 6 determinations. Probabilities for the significance of the differences between the means are: $p < 0.02$ for control-fed *vs.* control-starved (36.9% increase); $p < 0.001$ for control-fed *vs.* actinomycin-fed (38.9% inhibition) and control-starved *vs.* actinomycin-starved (49.6% inhibition); $0.10 < p < 0.20$ for actinomycin-fed *vs.* actinomycin-starved (13.0% increase).

Effect of "Delayed Actinomycin D" on Starvation-induced Increase. In these experiments (Chart 3), the administration of actinomycin D was delayed until 6 hr after fasting began, to give sufficient time for production of mRNA and/or

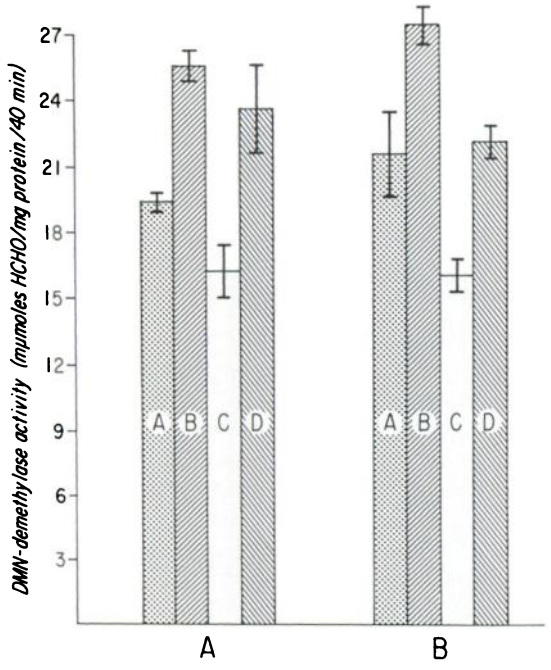

Chart 3. Effect of delayed administration of actinomycin D on starvation-induced increase of DMN demethylase activity in rat liver. *Bars* correspond to the following groups: *A*, control-fed; *B*, control-starved; *C*, actinomycin D-fed; *D*, actinomycin D-starved. Starvation was begun at 0 hr, and animals were sacrificed at 24 hr. Actinomycin D (40 μg for experiments in Chart 3A and 56 μg for experiments in Chart 3B) or 0.154 M sodium chloride–0.04 M sodium phosphate (pH 7.4) solution was given i.p. at 6 and 13 hr. Hence, each antibiotic-treated rat received in the experiments in Chart 3A a total of 80 μg actinomycin D and in Chart 3B experiments a total of 112 μg. Values represent the mean ± S.E. of 5 determinations. Probabilities for the significance of the differences between the means (for Chart 3B) are: $p < 0.05$ for control-fed *vs.* control-starved (27.0% increase) and control-fed *vs.* actinomycin-fed (25.6% inhibition); $p < 0.01$ for control-starved *vs.* actinomycin-starved (19.3% inhibition); $p < 0.001$ for actinomycin-fed *vs.* actinomycin-starved (37.8% increase).

stabilization of template to occur, if such a mechanism were operative in the present case. The animals in the 1st series received a total dose of 80 μg actinomycin D (Chart 3*A*), while the animals in the 2nd series received 112 μg (Chart 3*B*).

The data presented in Chart 3 show that the demethylase activity of animals starved for 6 hr and then given actinomycin D is appreciably higher than that of fed animals also receiving "delayed actinomycin D," but still notably lower than that of starved rats given only 0.154 M NaCl–0.04 M sodium phosphate solution. This is in contrast to the DAB reductase system (7), where the inducing effect of starvation is completely resistant to treatment with actinomycin D under similar dose and time schedules of administration.

Starvation induces the DMN demethylase activity to a much lesser extent in the experiments in Charts 2 and 3 than in those in Chart 1, even when the animals were not treated with actinomycin D. This lowering of enzyme activity is probably due to stress produced by multiple injections (*cf.* Ref. 21).

Regulation of the Liver DMN Demethylase Level by Total Amino Acids and by Glucose. The nature of the dietary constituent, the absence of which is responsible for the elevation of demethylase activity during starvation, was next elucidated. In the experiments presented in Table 1, the rats were either starved or fed only glucose, casein, or cellulose for 24 hr before sacrifice. The group receiving nonnutritive cellulose was included in these experiments in order to establish whether the starvation-induced increase of enzyme activity is due to the withdrawal of specific dietary constituent(s) or to stress arising from hunger and accompanying hormonal changes. At sacrifice, it was verified that these animals did indeed eat the nonnutritive cellulose. Table 1 shows that feeding of only glucose to rats brings about a dramatic decrease in demethylase activity in comparison to the starved animals; this activity is quite close to that in rats fed the basal diet *ad libitum* (20). Glucose added *in vitro* to the reaction flask elicited no change in the demethylase activity of microsomes from fed or fasted animals. In contrast, casein feeding further enhances the enzyme activity, in comparison to the starved rats; feeding nonnutritive cellulose results in no change. It appears, therefore, that while glucose represses the enzyme, the total amino acids released from casein induce the demethylase activity. Further experiments will be necessary to establish whether the protein-induced increase is due, as is probable, to the replenishment of the total protein anabolic pool in these rapidly metabolizing, starved weanling animals or to the specific inducer action of certain amino acid(s).

Table 1

Amino acid induction and carbohydrate repression of rat liver DMN demethylase

Animals were either fasted or fed a dietary regimen consisting solely of casein, glucose, or cellulose for 24 hr before assay. Values represent averages of 5 to 6 experiments, and the standard errors are given.

Dietary regimen	Demethylase activity (mμmoles HCHO/mg protein/40 min)
I. Starved	36.2 ± 2.0
Fed casein	59.7 ± 4.3[a]
II. Starved	44.6 ± 5.2
Fed glucose	17.4 ± 3.1[a]
III. Starved	43.2 ± 6.0
Fed cellulose	41.6 ± 4.2[b]

[a]Highly significant difference ($p < 0.01$) from the respective control group.
[b]Not different ($p > 0.80$) from the control group.

DISCUSSION

Increase of DMN Demethylase Synthesis during Starvation and Its Inhibition by MC. In this study with DMN demethylase, starvation enhances enzyme activity and MC displays its inhibitory effect in fasted as well as in fed rats. Several other instances (6, 7, 10) concur that, in general, microsomal enzyme inducers exert either a stimulatory or an inhibitory effect on drug metabolism, irrespective of whether

the animals have been fed or fasted for a short time (24 to 48 hr) before assay.

The increase of the V_{max} of DMN demethylation as a result of fasting for 24 hr is suggestive of either an accelerated synthesis or a lowered rate of degradation of the enzyme. When actinomycin D is administered at the beginning of fasting and thereafter until sacrifice, starvation-induced elevation of demethylase activity is completely blocked (Chart 2), indicating that enhanced synthesis of enzyme takes place during starvation. This is further substantiated in the experiments with "delayed actinomycin D," *i.e.*, when the antibiotic administration was not begun until 6 hr after the initiation of starvation (Chart 3*A* and 3*B*). The activity in starved, delayed actinomycin D-treated rats is higher than the activity in fed, delayed actinomycin D-treated animals, but the former is, nonetheless, considerably lower than the activity in starved animals receiving only the NaCl-phosphate solution (most clearly seen in Chart 3*B*). It is possible, therefore, that during the 6-hr starvation (preceding the first administration of the NaCl-phosphate solution or actinomycin D), intense transcription of DNA into mRNA or stabilization of already existing mRNA molecules has occurred. In case of either of the two events, administration of actinomycin D (but not the NaCl-phosphate solution) at 6 and 13 hr after starvation began could lead to less overall mRNA production and, hence, decreased enzyme synthesis provided that some decay of mRNA takes place and is not replenished in the 18-hr period (*i.e.*, between the first treatment with actinomycin D at 6 hr and sacrifice at 24 hr). That such decay of mRNA does indeed occur within the 18-hr period is supported by the consistent and very substantial inhibition of the demethylase by actinomycin D given 18 and 11 hr before sacrifice in rats fed *ad libitum* (Chart 3*A* and 3*B*). The starvation-induced greater DMN demethylase synthesis is, therefore, the consequence of enhanced transcription or greater stabilization of messenger template. Experiments with other inhibitors may permit distinguishing between the two possibilities.

Carbohydrate Repression of DMN Demethylase. While repression of enzymes by catabolites or glucose in microorganisms (1, 13, 15) is a well-known and extensively studied phenomenon, the existence of such a phenomenon in mammalian tissues has been brought to attention only recently (7, 12, 16, 19, 23). In the present study, glucose was found to inhibit DMN demethylase considerably, and it is this effect which is released by starvation. In animal tissues, glucose appears to bring about primarily a cessation of serine dehydratase synthesis and an increase of degradation of the enzyme, suggesting glucose action at the template level (8). A similar action of glucose at the level of translation has been proposed in the regulation of DAB reductase (7). By analogy, it is possible that glucose affects the regulation of DMN demethylase level at the translation step.

Amino Acid Induction of DMN Demethylase. Feeding casein alone for 24 hr causes a marked stimulation of DMN demethylase activity beyond that of starved rats. This is reminiscent of the induction of liver threonin dehydrase (16), ornithine δ-transaminase (16), and serine dehydratase (8) by the feeding of casein hydrolysate. It appears, therefore, that the observed increase in demethylase activity due to starvation actually represents the net effect of interplay between inhibition by carbohydrates and stimulation by amino acids. Such a situation is possible during starvation, when glycogen and protein stores of the animal are known to be broken down. It is possible that similar interactions between carbohydrates and amino acids occur with other drug-metabolizing enzymes. Kato (9) has reported decreased activities of several drug-metabolizing enzymes in rats receiving protein-free diet for 10 days before assay compared to activities in animals receiving low-protein ration (5% protein); the latter activity is, in turn, less than that found in animals receiving standard diet (18% protein) for the same period before sacrifice.

The observed effects of glucose and amino acids on DMN demethylase activity do not appear to be caused by hormonal changes arising from hunger. Rats fed nonnutritive cellulose *ad libitum* showed the same enzyme activity as starved animals. The inhibitory effect of glucose on DMN demethylase in this study is also in agreement with a recent finding (14) that feeding a carbohydrate diet free of protein protects rats against the lethal and hepatotoxic effects of DMN. While the inhibitory effect of glucose on liver DAB reductase reported by Jervell *et al.* (7) is the first example of glucose effect on microsomal drug-metabolizing enzymes, the present report is the first to show both the amino acid induction and the glucose repression of any such enzyme system. Thus, it seems that also the "atypical" microsomal drug-metabolizing enzymes are under the control of multiple regulatory factors, encountered thus far with other enzymes, and further research might bring more such instances to light.

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REFERENCES

1. Adhya, S., and Echols, H. Glucose Effect and the Galactose Enzymes of *Escherichia coli:* Correlation between Glucose Inhibition of Induction and Inducer Transport. J. Bacteriol., *92:* 601–608, 1966.
2. Arcos, J. C., Gosch, H. H., and Zickafoose, D. Fine Structural Alterations in Cell Particles during Chemical Carcinogenesis. III. Selective Action of Hepatic Carcinogens Other Than 3'-Methyl-4-dimethylaminoazobenzene on Different Types of Mitochondrial Swelling. Effect of Stimulated Liver Growth. J. Biophys. Biochem. Cytol., *10:* 23–36, 1961.
3. Conney, A. H., and Burns, J. J. Factors Influencing Drug Metabolism. Advan. Pharmacol., *1:* 31–58, 1962.
4. Goswami, M. N. D., and Chataigner, F. Starvation-induced Adaptation of Rat Liver Tyrosine Transaminase and Serine Dehydrase. Experientia, *22:* 370–371, 1966.
5. Greengard, O., Baker, G. T., Horowitz, M. L., and Knox, W. E. Effect of Irradiation and Starvation on the Regulation of Rat Liver Enzymes. Proc. Natl. Acad. Sci. U. S., *56:* 1303–1309, 1966.

6. Hospador, M. A. and Manthei, R. W. Influence of Age and Diet on the Induction of Hexobarbital-metabolizing Enzymes in the Mouse. Proc. Soc. Explt. Biol. Med., *128:* 130–132, 1968.
7. Jervell, K. F., Christoffersen, T., and Morland, J. Studies on the 3-Methylcholanthrene Induction and Carbohydrate Repression of Rat Liver Dimethylaminoazobenzene Reductase. Arch. Biochem. Biophys., *111:* 15–22, 1965.
8. Jost, J. P., Khairallah, E. A., and Pitot, H. C. Studies on the Induction and Repression of Enzymes in Rat Liver. V. Regulation of the Rate of Synthesis and Degradation of Serine Dehydratase by Dietary Amino Acids and Glucose. J. Biol. Chem., *243:* 3057–3066, 1968.
9. Kato, R. Sex Difference in the Activities of Microsomal Drug-metabolizing Enzyme Systems in Relation to Dietary Protein, Japan. J. Pharmacol., *16:* 221–223, 1966.
10. Kato, R., Chiesara, E., and Vassanelli, P. Factors Influencing Induction of Hepatic Microsomal Drug-metabolizing Enzymes. Biochem. Pharmacol., *11:* 211–220, 1962.
11. Kato, R., and Gillette, J. R. Effect of Starvation on NADPH-dependent Enzymes in Liver Microsomes of Male and Female Rats. J. Pharmacol. Exptl. Therap., *150:* 279–284, 1965.
12. Lardy, H. A., Foster, D. O., Young, J. W., Shrago, E., and Ray, P. D. Hormonal Control of Enzymes Participating in Gluconeogenesis and Lipogenesis. J. Cell. Comp. Physiol., *66* (Suppl. 1): 39–54, 1965.
13. Magasanik, B. Catabolite Repression. Cold Spring Harbor Symp. Quant. Biol., *26:* 249–256, 1961.
14. McLean, A. E. M., and Verschuuren, H. G. Effects of Diet and Microsomal Enzyme Induction on the Toxicity of Dimethyl Nitrosamine. Brit. J. Exptl. Pathol., *50:* 22–25, 1969.
15. Moses, V., and Prevost, C. Catabolite Repression of β-Galactosidase Synthesis in *Escherichia coli.* Biochem. J., *100:* 336–353, 1966.
16. Peraino, C., and Pitot, H. C. Studies on the Induction and Repression of Enzymes in Rat Liver. II. Carbohydrate Repression of Dietary and Hormonal Induction of Threonine Dehydrase and Ornithine δ-Transaminase. J. Biol. Chem., *239:* 4308–4313, 1964.
17. Pitot, H. C., and Peraino, C. 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. J. Biol. Chem., *239:* 1783–1788, 1964.
18. Schimke, R. T. The Importance of Both Synthesis and Degradation in the Control of Arginase Levels in Rat Liver. J. Biol. Chem., *239:* 3808–3817, 1964.
19. Tschudy, D. P., Welland, F. H., Collins, A., and Hunter, G. The Effect of Carbohydrate Feeding on the Induction of δ-Aminolevulinic Acid Synthetase. Metabolism, *13:* 396–406, 1964.
20. Venkatesan, N., Arcos, J. C., and Argus, M. F. Differential Effect of Polycyclic Hydrocarbons on the Demethylation of the Carcinogen Dimethylnitrosamine by Rat Tissues. Life Sci., *7:* 1111–1119, 1968.
21. Venkatesan, N., Argus, M. F., and Arcos, J. C. Mechanism of 3-Methylcholanthrene-induced Inhibition of Dimethylnitrosamine Demethylase in Rat Liver. Cancer Res., *30:* 2556–2562, 1970.
22. Wu, S. Y., and Rosenthal, H. L. Induction of Tryptophan Pyrrolase Activity in Starving Rabbits of Different Ages. Proc. Soc. Exptl. Biol. Med., *122:* 414–417, 1966.
23. Young, J. W., Shrago, E., and Lardy, H. A. Metabolic Control of Enzymes Involved in Lipogenesis and Gluconeogenesis. Biochemistry, *3:* 1687–1692, 1964.
24. Yuwiler, A., Geller, E., and Schapiro, S. Response Differences in Some Steroid-sensitive Hepatic Enzymes in the Fasting Rat. Can. J. Physiol. Pharmacol., *47:* 317–328, 1969.