Biochemical Pharmacology, Vol. 21, pp. 525–533. Pergamon Press, 1972. Printed in Great Britain

ENHANCEMENT BY CARBON TETRACHLORIDE TREATMENT OF HEPATIC TYROSINE AMINOTRANSFERASE INDUCTION IN THE PRESENCE OF NET POLYRIBOSOME BREAKDOWN IN THE RAT*

RAYMOND D. MAGUS,† STEPHEN W. KING, MARY W. PHILLIPS and MATTHEW F. CARLONE

Center for Research in Pharmacology and Toxicology, School of Medicine, University of North Carolina, Chapel Hill, N.C. 27514, U.S.A.

(*Received* 9 *June* 1971; *accepted* 20 *August* 1971)

Abstract—Carbon tetrachloride treatment inhibited the glucocorticoid induction of hepatic tryptophan oxygenase (TPO) while simultaneously enhancing the induction of hepatic tyrosine aminotransferase (TAT) in intact or adrenalectomized rats. In addition, treatment with CCl_4 alone increased the activity of hepatic TAT, but the extent of the CCl_4-induced stimulation of TAT was decreased by adrenalectomy or by hypophysectomy. The CCl_4-induced stimulation of TAT was prevented by high doses of actinomycin D but was enhanced further by 5-fluorouracil. CCl_4 also enhanced the labeling of hepatic polyribosomes with [^{14}C]orotic acid while simultaneously producing breakdown of heavy polysomes. These findings suggest that CCl_4 treatment may enhance the synthesis of certain hepatic enzymes, possibly via synthesis of new polysomes, while simultaneously inhibiting the induction of other enzymes such as TPO.

THE EXPOSURE of animals to CCl_4, either by inhalation or by injection, results in a sequence of widely investigated events leading to hepatic necrosis.[1] In most systems studied, the hepatotoxic actions of CCl_4 are generally catabolic rather than anabolic. The hepatotoxicity is likely due to the degradation of the hepatic endoplasmic reticulum[2] by the proposed free radical-initiated[3] peroxidation and the consequent alteration of protein synthesis.[4]

Animals exposed to CCl_4 have diminished capacity to synthesize tryptophan oxygenase (TPO; EC 1.13.1.12) under conditions which normally implicate the synthesis of new RNA template, whereas enzyme synthesis appears to proceed normally on preexisting RNA template.[5] This is seen in spite of the apparently extensive degradation of endogenous "messenger" RNA and polyribosomes by CCl_4 treatment.[6] Since tryptophan oxygenase represents only one member of a myriad of hepatic inducible enzymes, this enigma prompted an investigation of CCl_4 action on tyrosine aminotransferase (TAT; EC 2.6.1.5), another humoral and cofactor[7,8] inducible enzyme.

* Supported by the U.S. Public Health Service through National Institute of General Medical Sciences Grant GM 13606 (Dr. Thomas C. Butler, principal investigator).

† Deceased May 13, 1971. Reprint requests should be directed to: Dr. David J. Holbrook, Jr., Center for Research in Pharmacology and Toxicology, University of North Carolina, Chapel Hill, N.C. 27514, U.S.A.

MATERIALS AND METHODS

Male Sprague–Dawley rats (from Zivic–Miller Laboratories, Allison Park, Pa.) of 200–250 g weight were used throughout and were maintained over a Sani-Cel (Paxton Processing Co., Paxton, Ill.) bedding with tap water and Purina laboratory chow. Adrenalectomized rats (prepared under ether anesthesia) were used approximately 2–3 days following surgery with supplementation of 0·9 % NaCl in their drinking water. All drug treatments were scheduled to ensure that time of day would not affect enzyme activity values.[9,10]

For enzyme assays, the animals were stunned by a blow on the neck and were then perfused *in situ* via the hepatic portal vein with ice-cold 0·9 % NaCl to remove excess hepatic blood. The livers were quickly excised, blotted free of excess perfusing solution and immediately frozen on dry ice. Livers were homogenized in 3 vol. of an ice-cold medium of 0·15 M KCl–1 mM EDTA–50 mM Tris HCl (pH 7·4 at 4°). TPO was assayed by the Knox-Feigelson method as mentioned in the paper of Magus and Fouts[5] in supernatant fractions obtained by centrifugation at 40,000 g_{max} for 10 min. TAT was assayed in 40,000 g_{max} (20 min) supernatant fractions by the method described by Diamondstone[11] but with incubation for 20 min at 30°. One unit of either enzyme is that amount which catalyzed the formation of one μmole of product during the period of incubation.

DNA analyses were performed on perchloric acid-insoluble material prepared from liver homogenates by the procedure described by Schneider[12] and with the diphenylamine reaction of perchloric acid hydrolysates by the Burton method.[13] Enzyme activities were expressed as units of activity per milligram DNA (i.e. per the cell count)[14] in order to counteract the effect of hepatic weight increase which follows CCl_4 treatment.

Hepatic polyribosome analyses were performed according to a slight modification of the procedure of Henshaw.[15] Livers were perfused *in situ* via the portal vein with ice-cold medium consisting of 0·02 M triethanolamine–HCl, 0·001 M $MgCl_2$, 0·01 M NaCl, 0·25 M sucrose (pH 7·6). Following perfusion, the livers were processed in the cold room. After blotting and weighing, the livers were minced by passage through a Harvard tissue press with a fine mesh disk. The resulting pulp was transferred to homogenization vessels (glass-Teflon, A. H. Thomas & Co.) and homogenized in 4 vol. of the medium described above. After centrifugation at 15,000 *g* for 10 min, a portion of the supernatant material was diluted with an equal volume of freshly prepared 2 per cent deoxycholate in the buffered medium. An amount equivalent to 125 mg liver was layered on a 30-ml convex exponential sucrose gradient (containing the buffered salt medium previously described), isokinetic for a 15 % (w/w) top concentration, prepared as described by McCarty *et al.*,[16] using a mixing reservoir concentration of 32·3 % (w/w) sucrose. Following a 3-hr centrifugation in an SW 25·1 rotor at 25,000 rev/min in a Spinco ultracentrifuge at 5°, the gradients were analyzed (by bottom puncturing and top unloading) in a Gilford spectrophotometer at 260 mμ. Where applicable, fractions of 0·6 ml were collected for radioactivity assay in a Beckman LS 200 scintillation counter in a PPO,* POPOP,† toluene, Triton X-100 counting system,[17] with external standardization. The counting efficiency for ^{14}C was 71 per cent.

* PPO = 2,5-diphenylopazole.
† POPOP = 1,4-bis-2-(4-methyl-5-phenylopazolyl)benzene.

RESULTS

A detailed time course of CCl_4 action was investigated in order to examine the temporal aspects of CCl_4-produced inhibition of TPO induction by hydrocortisone. The data in Fig. 1 indicate that CCl_4 alone in the higher dose administered (1 ml/kg) progressively decreased hepatic TPO activity with time (controls—lower solid line with closed circles). With the lower dose of CCl_4 (0·1 ml/kg), no significant reduction of basal TPO activity was observed within the 24-hr span studied. Induction of TPO by hydrocortisone (TPO assayed 6 hr after administration of hydrocortisone) was inhibited by both doses of CCl_4 although some differences can be observed. That is, the inhibitory effect of CCl_4 was related both to time and to dose, wherein the inhibition by the low dose of CCl_4 was only partial and reversible within the 24-hr period studied. The 1 ml/kg dose completely abolished induction of TPO by hydrocortisone within this same time interval (Fig. 1).

When livers from animals receiving CCl_4 only (0·1 or 1·0 ml/kg, i.p.) were assayed for TAT activity, an unexpected and dose-related long lasting (>24 hr) increase in hepatic enzyme activity was seen (Fig. 2).

An attempt was then made to delineate the possible involvement of the pituitary adrenal system in this unexpected stimulatory effect of CCl_4 on hepatic TAT. CCl_4 was administered to adrenalectomized or hypophysectomized rats. Twelve hr after receiving a 1 ml/kg dose of CCl_4 (i.p.), the animals were killed and assayed for hepatic TAT activity. The data in Table 1 indicate that extirpation of either portion of the pituitary-adrenal axis markedly reduced, but did not completely abolish, the stimulating action of CCl_4 on hepatic TAT at these time intervals. This was not unexpected since previous investigators had shown an increase in circulating corticosteroids following CCl_4 treatment in the rat.[18,19]

Glucocorticoid-inducible enzymes are sensitive to inhibitors or modifiers of RNA or protein synthesis.[20] This information has been contributing evidence for the involvement of new species of RNA template and newly synthesized molecules of enzyme in the induction process.[21] Accordingly, CCl_4 was administered to intact rats which were preexposed to actinomycin D or to 5-fluorouracil.

Animals pretreated with actinomycin D at a 1·25 mg/kg dose retained their ability to respond to CCl_4 in that a four-fold increase in hepatic TAT was obtained in rats treated either with CCl_4 alone (relative to corn oil-treated controls) or with CCl_4 in combination with actinomycin D (relative to actinomycin D-treated controls) (Table 2, expt. 1). Actinomycin D inhibited the TAT response to CCl_4 only if this already toxic dose[22,23] of 1·25 mg/kg of actinomycin D was doubled, although an unexpected appreciable increase in hepatic TAT was produced by the higher dose of actinomycin D alone (Table 2, expt. 2). The administration of actinomycin D alone, at lower doses and with other schedules for enzyme assay, has been reported previously to result in an increased activity of TAT and several other inducible enzymes.[24] The questionable involvement of hepatic RNA synthesis in the action of CCl_4 on TAT was clouded further by our observation that 5-fluorouracil treatment enhanced the action of CCl_4 on hepatic TAT and increased TAT values when administered alone (Table 2, expt. 3). We have since partially characterized the latter phenomenon and have obtained evidence for an alteration by 5-fluorouracil of the apparent turnover of hepatic TAT resulting in potentiation of glucocorticoid induction of the enzyme.[25]

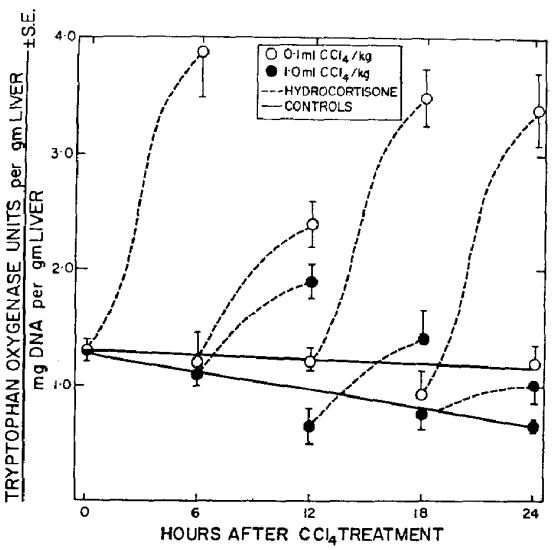

FIG. 1. Time course of inhibition of hepatic TPO induction by hydrocortisone in CCl_4-treated rats. Each point represents data from six male rats. Control animals are those which received only CCl_4 intraperitoneally (i.p.) in corn oil in the dosages indicated. Paired animals, also receiving CCl_4, were given a subcutaneous (s.c.) injection of hydrocortisone sodium succinate (30 mg/kg) at the time points of origin designated by the dashed lines in the figure. Six hr after treatment these animals were killed for enzyme assay.

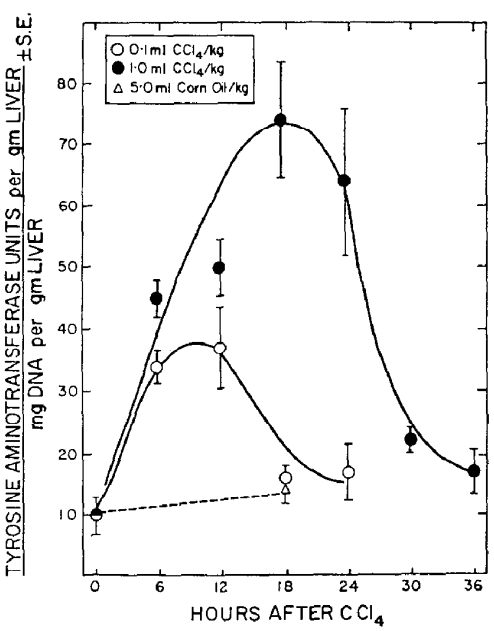

FIG. 2. Effect of CCl_4 treatment on basal hepatic TAT activity in intact rats. This experiment was conducted in a fashion identical with that described under Fig. 1 with the exception that only CCl_4 was administered and the time profile was extended to 36 hr where indicated.

TABLE 1. EFFECT OF ADRENALECTOMY AND HYPOPHYSECTOMY ON TYROSINE AMINOTRANSFERASE ELEVATION BY CCl_4

Treatment	TAT (units/mg DNA ± S. E.)	
	Corn oil*	CCl_4†
Adrenalectomy	6·9 ± 1·0	14 ± 1‡
Hypophysectomy	7·6 ± 0·4	15 ± 2‡

* Five ml/kg., i.p., 12 hr before death.
† One ml/kg (as a 20 per cent solution in corn oil), i.p., 12 hr before death.
‡ Significantly different ($P < 0·05$) from control values. Each mean is composed of six rats.

TABLE 2. EFFECT OF ACTINOMYCIN D AND 5-FLUOROURACIL ON CCl_4-INDUCED INCREASE IN HEPATIC TYROSINE AMINOTRANSFERASE IN INTACT RATS

Treatments	TAT (units/mg DNA ± S. E.)		
	Expt. 1	Expt. 2	Expt. 3
Corn oil (control)*	18 ± 3	11 ± 3	19 ± 2
CCl_4†	75 ± 6‡	65 ± 8‡	62 ± 7‡
Actinomycin D (control)§	14 ± 7	32 ± 4	
Actinomycin D plus CCl_4	56 ± 7‡	30 ± 5	
5-Fluorouracil (control)‖			35 ± 10
5-Fluorouracil plus CCl_4			177 ± 20‡

* Five ml/kg, i.p., 8 hr (expts. 1 and 2) or 18 hr (expt. 3).
† One ml/kg, i.p., 8 hr (expts. 1 and 2) or 18 hr (expt. 3).
‡ Designates significant difference ($P < 0·05$) from the appropriate control in same column. Each mean represents data from six rats.
§ Administered as an aqueous solution, i.p., [1·25 mg/kg (expt. 1) or 2·5 mg/kg (expt. 2)] 30 min prior to CCl_4 (treated rats), or for 8·5 hr (controls).
‖ In aqueous solution (Floricil, Roche), i.p. 250 mg/kg, 16 and 18·5 hr in control rats; 30 min before and 2 hr after CCl_4 administration in treated rats.

While our initial intentions of investigating the effect of CCl_4 on hepatic induction of TAT by hydrocortisone were complicated by the observations described above, it was decided to pursue the original question of CCl_4 action on TAT induction by exogenous glucocorticoid. The data in Fig. 3 show that CCl_4 predictably inhibited the induction of hepatic TPO by hydrocortisone compared with control rats treated with corn oil (right panel), with an appropriate shift in the TPO activity versus hydrocortisone dose curve. The apparent dose–response curve for hydrocortisone on TAT in CCl_4-treated rats, however, was essentially flat at a maximum value compared with control rats treated with corn oil (Fig. 3, left). Since this unusually flat dose–response curve could have resulted from saturation effects with circulating corticosterone, the identical experiment was conducted in 3-day adrenalectomized rats. This experiment, illustrated in Fig. 4, demonstrated that the unusual dose–response relationship between hydrocortisone and TAT in CCl_4-treated rats was retained in the absence of

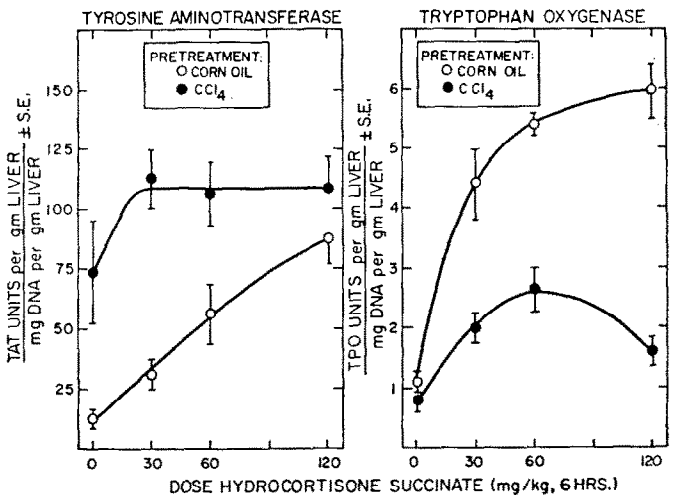

FIG. 3. Divergent effects of CCl_4 pretreatment on induction of hepatic TAT and TPO in intact rats. Male intact rats were pretreated 12 hr with either corn oil (5 ml/kg, i.p.) or CCl_4 (1 ml/kg, i.p. in corn oil). Subsequent to this exposure, hydrocortisone succinate was administered, s.c., in the dosages indicated for a 6-hr period. Following this 6-hr induction period, the animals (six per point) were killed, and their livers were assayed for both TAT and TPO. Zero hydrocortisone dose animals (see figure) were sham-injected, s.c., with saline for a 6-hr period.

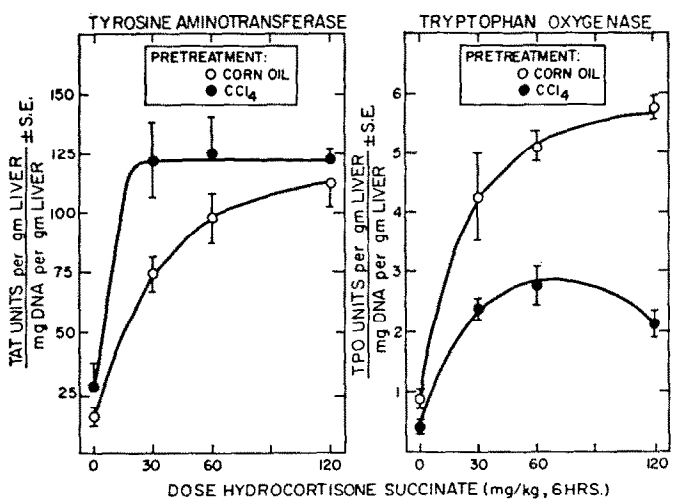

FIG. 4. Divergent effects of CCl_4 pretreatment on induction of hepatic TAT and TPO in adrenalectomized rats. This experiment is identical to that described under Fig. 3, with the exception that the rats in this chart were adrenalectomized.

adrenal glands. Apparently CCl_4 inhibited TPO induction by hydrocortisone while simultaneously sensitizing the regulatory processes for TAT induction by hydrocortisone.

Inasmuch as several reports indicate that CCl_4 inhibits protein synthesis *in vivo*[4,26] and produces polyribosome breakdown *in vivo*,[26,27] it seemed appropriate to examine the effects of CCl_4 on hepatic polyribosome synthesis. Figure 5 shows that treatment

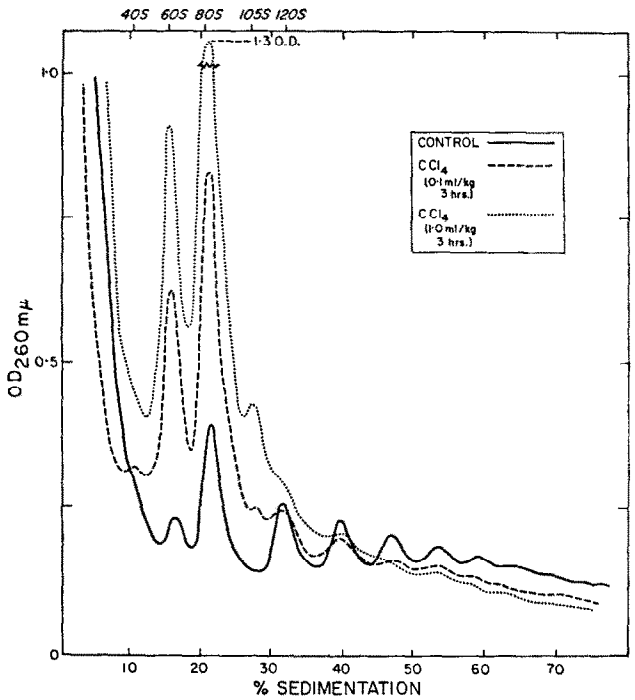

FIG. 5. Dose-related alteration in hepatic polyribosome profiles following CCl_4 treatment. Male rats were treated for a 3-hr period with undiluted CCl_4 in the dosages indicated in the chart; control animals received only a mock injection. Polyribosome preparation was carried out as described in the text. This figure is representative of three rats, and represents typical data obtained from four experiments.

with CCl_4 for 3 hr produces a shift in the polysome profile in that a marked increase in 80 S monomers (and 60 S subunits) is seen concomitantly with a decrease in the heavier sedimenting polysomes. In addition, a distinct peak sedimenting at 105 S is observed (Fig. 5) in CCl_4-treated rats. The disaggregation observed in the ribosomal profile confirmed earlier reports[26,27] and suggested that if TAT enhancement by CCl_4 occurred via increased *de novo* synthesis of the enzyme, such synthesis would necessarily occur in spite of the presence of net polyribosome breakdown and of net inhibition of amino acid incorporation into the total hepatic proteins.[26] The incorporation of [^{14}C]-orotic acid into hepatic polysomes in CCl_4-treated rats was investigated. The data in Fig. 6 indicate that rats treated with CCl_4 had an apparent increased capacity to incorporate [^{14}C]orotic acid into polysomes. The increased incorporation suggests that CCl_4 stimulates polysome synthesis in the presence of net polysome breakdown, i.e. stimulates polysome turnover. This is consistent with the possibility that CCl_4 may stimulate the production of polysomes bearing the messenger for TAT, thereby accounting for the hyperactivity of the enzyme in the livers of CCl_4-treated rats.

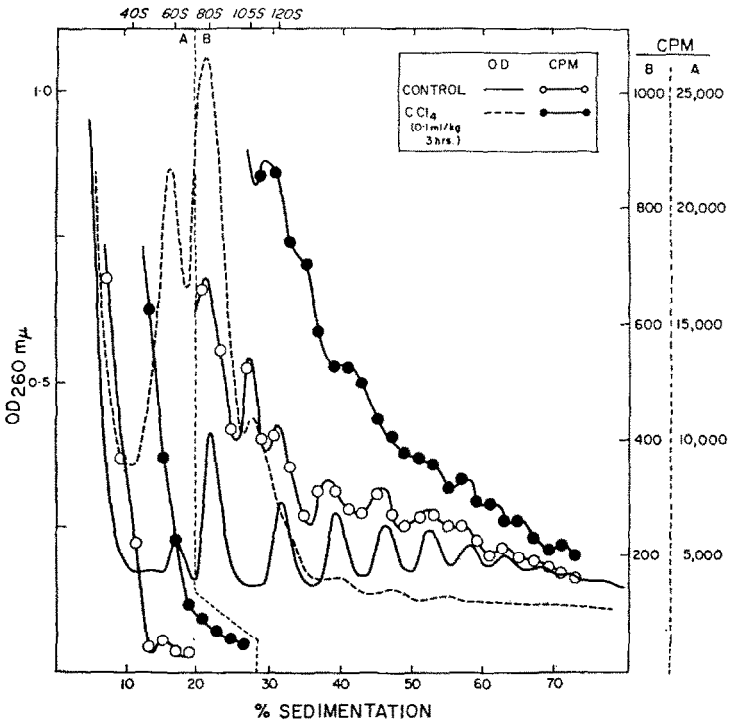

FIG. 6. Stimulation of [6-^{14}C]orotic acid incorporation into polysome RNA by CCl_4 treatment. This preparation is similar to that described under Fig. 5 with the exception that both treated and control rats received 250 μc [6-^{14}C]orotic acid hydrate (New England Nuclear Corp., 40 c/mole) for a 3-hr period of incorporation. The treated animal received both the isotope and low dose of CCl_4, i.p., simultaneously. The panels designated A and B indicate the range of radioactivity relevant to each panel.

DISCUSSION

The experiments described above were undertaken to investigate the possibility that rats suffering from the hepatotoxic effects of CCl_4 treatment would have a reduced capacity to respond to hydrocortisone as an inducer of hepatic TAT. While such treatment indeed inhibited TPO induction, it paradoxically enhanced TAT induction. The over-all action of CCl_4 reported herein appears to indicate that CCl_4 induces (via adrenal corticosterone) or facilitates induction (by exogenous hormone) of TAT, possibly by enhanced synthesis of RNA template or formation of polysomes bearing the TAT messenger RNA. Simultaneously, CCl_4 inhibits similar processes for TPO induction. However, we must question the necessity for the relatively high dose of actinomycin D required to block the action of CCl_4 on basal TAT levels, although this could be due to a CCl_4-actinomycin D interaction. The enhanced labeling of cytoplasmic RNA (polysomes) could be due to stimulation of ribosomal RNA synthesis, or could be explained by CCl_4 inhibition of the *de novo* synthesis of pyrimidine nucleotides thereby allowing the radioactive nucleotides to occupy a greater fraction of the total RNA precursor pool; these questions are currently under investigation.

While the effects of CCl_4 on hepatic TPO regulation are not surprising, those on TAT regulation are paradoxical but not without precedent. Murphy and Malley[19] observed an increase in basal hepatic TAT activity in the CCl_4-treated rat, but failed to observe enhancement of TAT induction under the conditions of their experiments. Treatmcnt with CCl_4 has been found to result in increases in hepatic alkaline phosphatase activities[19,28] and in the activities of other hepatic enzymes.[29]

Paradoxical regulation of hepatic TAT during polysome breakdown by drugs such as 8-azaguanine[30] and 5-azacytidine[31] has been reported by others. This effect of the metabolite analogs has been attributed to stabilization of TAT *in vivo*. However, we were not able to detect a change in the apparent *in vivo* rate of TAT degradation in normal versus CCl_4-treated rats receiving hydrocortisone.

The protein inhibitor cycloheximide, when administered alone to rats, results in a several-fold increase in TAT activity but no effect on basal TPO activity.[32,33] Consistent with other reports of unusual regulatory processes for TAT in rat liver,[34] it is possible that TAT represents a unique class of inducible enzyme(s) and that studies of its regulation must be performed with this consideration.

REFERENCES

1. R. O. Recknagel, *Pharmac. Rev.* **19,** 145 (1967).
2. R. O. Recknagel and A. K. Ghoshal, *Lab. Invest.* **15,** 132 (1966).
3. T. F. Slater, *Nature, Lond.* **209,** 36 (1966).
4. E. A. Smuckler, O. A. Iseri and E. P. Benditt, *J. exp. Med.* **116,** 55 (1962).
5. R. D. Magus and J. R. Fouts, *Molec. Pharmac.* **4,** 465 (1968).
6. M. E. Weksler and H. V. Gelboin, *Biochim. biophys. Acta* **145,** 184 (1967).
7. F. T. Kenney and R. M. Flora, *J. biol. Chem.* **236,** 2699 (1961).
8. O. Greengard and M. Gordon, *J. biol. Chem.* **238,** 3708 (1963).
9. M. I. Rapoport, R. D. Feigin, J. Bruton and W. R. Beisel, *Science* **153,** 1642 (1966).
10. R. J. Wurtman and J. Axelrod, *Proc. natn. Acad. Sci., U.S.A.* **57,** 1594 (1967).
11. T. I. Diamondstone, *Analyt. Biochem.* **16,** 395 (1966).
12. W. C. Schneider, *J. biol. Chem.* **161,** 293 (1945).
13. K. Burton, *Biochem. J.* **62,** 315 (1956).
14. R. Vendrely, in *The Nucleic Acids* (Eds. E. Chargaff and J. N. Davidson) Vol. 2, p. 155. Academic Press, New York (1955).
15. E. C. Henshaw, *J. molec. Biol.* **36,** 401 (1968).
16. K. S. McCarty, D. Stafford and O. Brown, *Analyt. Biochem.* **24,** 314 (1968).
17. M. S. Patterson and R. C. Greene, *Analyt. Chem.* **37,** 854 (1965).
18. T. M. Brody, *Ann. N.Y. Acad. Sci.* **104,** 1065 (1963).
19. S. D. Murphy and S. Malley, *Toxic. appl. Pharmac.* **15,** 117 (1969).
20. R. T. Schimke and D. Doyle, *A. Rev. Biochem.* **39,** 929 (1970).
21. F. T. Kenney, *Adv. Enzyme Regulat.* **1,** 137 (1963).
22. F. S. Philips, H. S. Schwartz, S. S. Sternberg and C. T. C. Tan, *Ann. N.Y. Acad. Sci.* **89,** 348 (1960).
23. S. H. Wilson and M. B. Hoagland, *Biochem. J.* **103,** 556 (1967).
24. F. Rosen, P. N. Raina, R. J. Milholland and C. A. Nichol, *Science* **146,** 661 (1964).
25. R. D. Magus, S. W. King and J. D. Harrison, *Biochem. Pharmac.* **20,** 2239 (1971).
26. E. A. Smuckler and E. P. Benditt, *Biochemistry* **4,** 671 (1965).
27. G. Blobel and V. R. Potter, *J. molec. Biol.* **26,** 293 (1967).
28. D. Koch-Weser, E. Farber and H. Popper, *Archs Path.* **51,** 498 (1951).
29. B. D. Dinman and I. A. Bernstein, *Archs envir. Hlth* **16,** 770 (1968).
30. I. B. Levitan and T. E. Webb, *Biochim. biophys. Acta* **155,** 632 (1968).
31. I. B. Levitan and T. E. Webb, *Biochim. biophys. Acta* **182,** 491 (1969).
32. S. Fiala and E. Fiala, *Nature, Lond.* **210,** 530 (1966).
33. F. Rosen and R. J. Milholland, *Fedn Proc.* **25,** 285 (1966).
34. R. D. Magus, J. D. Harrison and S. W. King, *Biochem. Pharmac.* **20,** 486 (1971).