THE STIMULATION OF AMINO ACID INCORPORATION IN A MAMMALIAN SYSTEM WITH PHENOBARBITAL, 3-METHYLCHOLANTHRENE AND CYCLOHEXIMIDE

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In a previous paper (Jondorf et al., 1965) we reported on the effect of two different types of carcinostatic compounds on the stimulation of amino acid incorporation brought about by sodium phenobarbital (PB) or 3-methylcholanthrene (3MC) in a rat liver microsomal system. Since the effect of n-pentyl indolyl-3-acetate was confined to the PB-stimulated system, and the terephthalanilide compound affected the 3 MC-stimulated system, we suggested that there might be different mechanisms involved in the two types of stimulation.

We have now been able to further characterize the differences between the mechanism of PB- and 3 MC- stimulation with adrenalectomized and hypophysectomized animals and using the antibiotic cycloheximide (actidione, NSC 185). The latter compound is a potent inhibitor of protein synthesis in vivo (Young et al., 1963) and in vitro (Wettstein et al., 1964; Bennett et al., 1965; Colombo et al., 1965). Nonetheless, rats injected with cycloheximide at dose levels of 0.5 or 1.0 mg/kg yielded liver microsomal fractions that had greatly enhanced amino acid incorporation relative to the controls. The cycloheximide stimulation of amino acid incorporation was not observed in adrenalectomized animals, although hypophysectomized animals were stimulated.

Materials and Methods - 3MC was purchased from Eastman Kodak, Rochester,

N. Y.; PB from the Merck Company, Rahway, N, J. Cycloheximide was generously provided by the Cancer Chemotherapy National Service Center through the courtesy of Dr. J. A. R. Mead. Animals used were female Sprague-Dawley rats of the same age, weighing 160 g. Hypophysectomized and adrenalectomized animals were supplied by Hormone Assay, Inc., Chicago, Ill. The animals were operated two or three days prior to injection.

Rats treated with PB were given 80 mg/kg i.p. for three successive days and were fasted on the third day. Those treated with 3MC were given 50 mg/kg i.p. in corn oil for three successive days and were fasted on the third day. Treatment with cycloheximide was at a dose level of 1 mg/kg i.p. Animals that had undergone endocrine surgery were injected with cycloheximide at a lower dose level (0.5 mg/kg).

Neither adrenalectomized nor hypophysectomized animals were starved on third day of treatment. They were maintained on normal Purina Chow rat cube diet, supplemented with 0.9% saline, and fresh oranges respectively. Control animals received handling and injection volumes of media identical with the experimental ones.

Rats were killed, livers were excized and processed as described by Gelboin (1964). Resuspended microsomal fractions were adjusted to the same protein concentrations on the basis of Lowry et al. (1951) protein analyses. Incubations for non-preincubated (Endogenous RNA-dependent) and preincubated (polyU-dependent) amino acid incorporation at the optimal magnesium chloride concentrations of 5 and 8 μmoles, respectively, and with 105,000 x g supernatant fractions from control intact animals were performed in triplicate as before described (Jondorf et al., 1965).

Results - Fig. 1a shows that the L-(^{14}C) phenylalanine incorporation in the non-preincubated and in the preincubated liver microsomal system from intact 3MC-treated, PB-treated and cycloheximide-treated rats is enhanced relative to the controls. The stimulation of phenylalanine incorporation is greatest in microsomes isolated from rats treated with cycloheximide in vivo and is

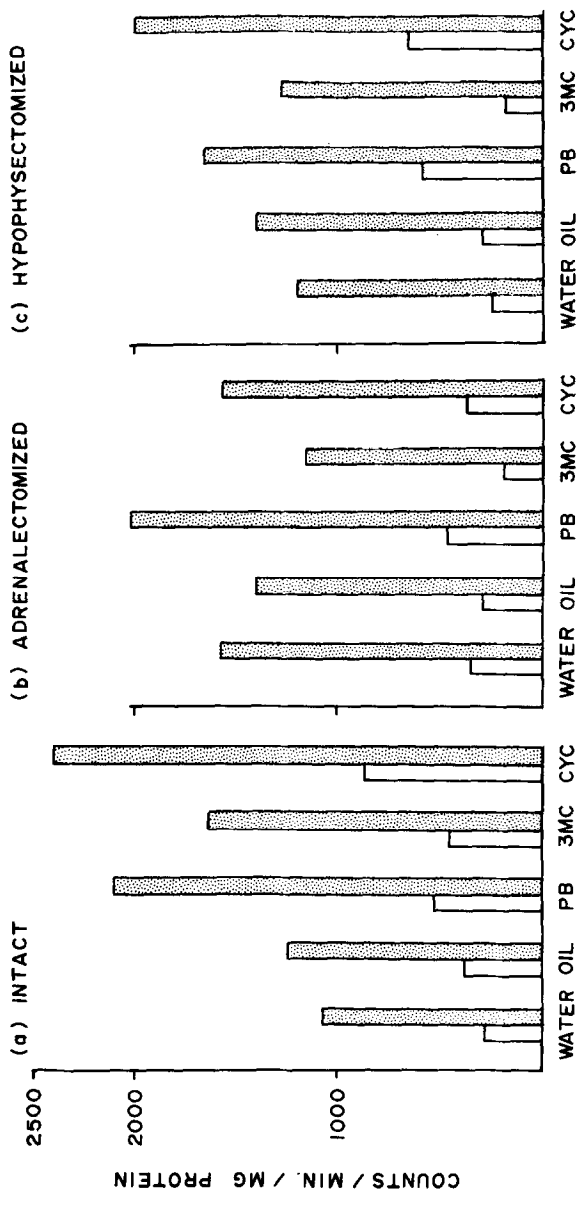

Fig. 1. L-(^{14}C) phenylalanine incorporation in vitro, by microsomal preparations from control, phenobarbital (PB), 3-methylcholanthrene (3MC) and cycloheximide (CYC) treated rats that were (a) intact (b) adrenalectomized and (c) hypophysectomized. Experimental details are as indicated in Methods and Materials.
White and stippled columns represent the (average of three) specific activities from non-preincubated (endogenous-RNA dependent) and preincubated (polyU-dependent) samples respectively.

most pronounced in the endogenous-RNA dependent incubations (over 300% of control values).

The results of the corresponding experiments with adrenalectomized rats are shown in Fig. 1b. It can be seen that the enhancement of phenylalanine incorporation in the liver microsomal system in PB-treated rats is unaffected. Neither 3MC nor cycloheximide treatment of rats *in vivo* evokes a stimulatory response in the *in vitro* incorporation system. 3MC-treated adrenalectomized animals yield microsomes that have a slightly lower phenylalanine incorporation than adrenalectomized controls. This phenomenon was also observed with animals that had been starved on the third day of treatment.

In Fig. 1c the effect of hypophysectomy on the stimulation of amino acid incorporation by the three agents under review can be distinguished. 3MC-treatment of rats that have undergone hypophysectomy does not enhance the phenylalanine incorporation in the subsequently isolated microsomal system. This was observed also in animals starved on the third day. However, the stimulation of the phenylalanine incorporation in the microsomal preparations isolated from PB-treated or cycloheximide-treated rats is not impaired. This suggests that the mechanism of PB and cycloheximide stimulation of amino acid incorporation is independent of pituitary function.

Discussion - The results show that not only phenobarbital (cf. Kato, *et al.*, 1966) and 3MC (cf. Gelboin and Sokoloff, 1964; Gelboin, 1964) but cycloheximide also, stimulate the liver microsomal amino acid incorporating system of the intact rat, in both the endogenous-RNA dependent and in the preincubated polyU-dependent system.

PB stimulation of the system can still be brought about in adrenalectomized (cf. Kato *et al.*, 1966) and in hypophysectomized animals. This suggests that the effect of PB is on the liver directly. Previous work by Orrenius *et al.*, 1965, with thyroidectomized rats showed that thyroid hormones are not involved in phenobarbital-induced enzyme synthesis.

However, since 3MC is ineffective in stimulating the amino acid incorpora-

tion system of adrenalectomized or hypophysectomized animals, the mechanism of 3MC stimulation is dependent on the mediation of the pituitary-adrenal system of the rat. The primary effect of 3MC may be related to the stimulation of ACTH synthesis. Huggins et al., (1959) determined that the induction of mammary carcinomas by agents like 3MC was hormone dependent. The present study illustrates the critical nature of the hormone status of the animals in a short-term response to 3MC.

The mechanism of cycloheximide stimulation of the microsomal system is dependent on the integrity of the adrenal glands, since hypophysectomized animals respond to cycloheximide stimulation but adrenalectomized animals do not. Our findings with adrenalectomized animals are consistent with the observation by Greig and Gibbons (1959) that adrenal cortex extract was effective in protecting rats against fatal doses of cycloheximide. It was surprising to find that cycloheximide stimulated the incorporation of amino acid in intact and hypophysectomized animals, in view of the previous findings by Gorski and Axman (1964) of inhibition of protein synthesis in the rat uterus system, and the studies in vivo with rabbits conducted by Young et al. (1963). However, there is no doubt that cycloheximide administration to rats has a different effect on the subsequently isolated microsomal system than the mere addition of cycloheximide to the microsomal incubation mixtures (Wettstein et al., 1964; Bennett et al., 1965). In their experiments, Fiala and Fiala (1965) also emphasized a hormone dependence of cycloheximide action. The accumulation of RNA in the liver microsomal fraction reported by these workers may in part explain the increased amino acid incorporation in our incubation system in vitro. The stimulation may be related to the formation of artificial messenger RNA for the incorporation of amino acid into trichloroacetic acid insoluble polypeptides which, however, would lack properly co-ordinated biological activity in vivo. Such phenomena were observed by Chantrenne (1964) and Grünberger and Mandel (1965) in bacterial systems grown in media containing purine analogues.

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