Biochemical Pharmacology, Vol. 17, pp. 839–842. Pergamon Press. 1968. Printed in Great Britain

Further studies with glutarimide antibiotics—II. Some effects of cycloheximide pretreatment on hepatic protein synthesis and release of nascent polypeptides in intact and adrenalectomized rats

(*Received* 25 *September* 1967; *accepted* 27 *November* 1967)

IT HAS BEEN shown previously that pretreatment with cycloheximide inhibits the incorporation of L-(^{14}C)-amino acids into the protein of various organs in intact animals.[1–4] Evidence is here presented that the administration of cycloheximide to rats inhibits the amino acid incorporation into liver microsomal protein *in vivo*, except in adrenalectomized animals 24 hr after treatment with the antibiotic. The degree of inhibition of amino acid uptake into protein *in vivo* is dose-dependent, time-dependent, and apparently hormone-dependent. The rate of loss of radioactive nascent polypeptide from liver microsomal preparations labeled *in vivo*, during subsequent incubation *in vitro*, is related neither to adrenalectomy nor to effects of cycloheximide *in vitro*.

METHODS

The animals used in this study were adrenalectomized (5 days post-operative) and matching intact female Sprague–Dawley rats weighing 160–170 g, supplied by Hormone Assay Inc. (Chicago, Ill). They were maintained on Purina Chow rat cubes and 0·9% saline (adrenalectomized) or water (intact) *ad libitum* until 24 hr before sacrifice. During that period all animals were starved, but their fluid intake was not restricted. Starvation ensured a lowering of liver glycogen and eliminated any possible differential effect on glycogen content resulting from treatment with cycloheximide.[5]

The rats were pretreated with water or with various dose levels of cycloheximide intraperitoneally (0·8 ml injection volume per 160 g rat) for 1, 2 or 24 hr. They were then injected with uniformly labeled L-(^{14}C)-leucine (20 μc/2 μmole/0·8 ml) i.p. Twenty minutes later the rats were killed by stunning and decapitation. Their livers were then immediately excised and individually processed according to Jondorf *et al.*[6] for the preparation of microsomal fractions. After the assay of protein,[7] sp. act. were determined by precipitation of triplicate samples of the microsomal material with 10% (w/v) TCA containing unlabeled L-leucine (1 mg/ml), and subsequent purification required for scintillation counting.[6]

For the measurement of the release of radioactive protein from liver microsomes labeled *in vivo*, aliquots of microsomal protein (3·0 mg) were incubated for 15 min (in triplicate) at 37° in reaction vessels containing the following other components (*cf.* Jondorf *et al.*[6]): potassium phosphate (pH 7·4), 10 μmole; magnesium chloride, 5 μmole; sucrose, 120 μmole; ATP (disodium salt), 1·25 μmole; GTP (sodium salt), 0·25 μmole; phosphocreatine (disodium salt), 40 μmole; creatine phosphokinase (EC 2.7.3.2.), 0·25 mg; reduced glutathione (adjusted to pH 7·4 with 1ON potassium hydroxide), 50 μmole; unlabeled 105,000 *g* supernatant fraction from intact control liver, 1·0 mg; water to adjust volume of reaction mixtures to 0·85 ml. In some experiments incubations were carried out in the presence of 0·10 mg cycloheximide in the reaction mixture. After the incubation, the samples were chilled, diluted to 12·5 ml with 0·25 M sucrose, and were then subjected to centrifugation at 105,000 *g* for 1 hr. The re-pelleted microsomes were redispersed after careful rinsing and were then prepared for scintillation counting as already described.[6] The ratio of specific activities before and after incubation is a measure of the release of labeled protein (see Ennis and Lubin[8]).

RESULTS

The effects of various dose levels of cycloheximide and variations in the time of treatment with the antibiotic, on the incorporation of L-(^{14}C)-leucine into liver microsomal protein *in vivo* in intact and adrenalectomized rats, are summarized in Table 1. It is clear that at 1 and 2 hr after treatment of rats with cycloheximide at 0·5, 1·0 and 5·0 mg/kg dose levels there is a dose-dependent inhibition of amino acid incorporation into protein *in vivo* in intact rats, which is in agreement with the findings of

TABLE 1. EFFECT OF CYCLOHEXIMIDE PRETREATMENT AT THREE DIFFERENT DOSE LEVELS ON THE INCORPORATION OF L-(^{14}C)-LEUCINE INTO RAT LIVER MICROSOMAL PROTEIN *in vivo*, OF INTACT AND ADRENALECTOMIZED RATS AT 1, 2 AND 24 HR

Time (hr)	Cycloheximide treatment (mg/kg)	Animals	Microsomal sp. act. (S_1)*	Microsomal sp. act. after incubation (S_2)†	Ratio of sp. act. (S_2/S_1)
1 hr‡	0	Intact	5812	4766	0·82
	0·5	Intact	1862 (32%)§	1486	0·80
	1	Intact	1006 (17%)	815	0·81
	5	Intact	378 (6%)	316	0·83
	0	Adrenalect.	6603	5283	0·80
	0·5	Adrenalect.	1684 (25%)	1363	0·81
	1	Adrenalect.	1087 (16%)	909	0·84
	5	Adrenalect.	401 (6%)	318	0·79
2 hr‡	0	Intact	5742	4384‖	0·76‖
	0·5	Intact	1561 (27%)	1183	0·75
	1	Intact	1231 (21%)	935	0·76
	5	Intact	267 (5%)	217	0·81
	0	Adrenalect.	5215	3874‖	0·76‖
	0·5	Adrenalect.	1387 (26%)	1065	0·77
	1	Adrenalect.	531 (10%)	3018	0·75
	5	Adrenalect.	215 (4%)	166	0·77
24 hr‡	0	Intact	5347	4027‖	0·75‖
	0·5	Intact	4581 (86%)¶	3366	0·74
	1	Intact	4103 (77%)	3051	0·74
	0	Adrenalect.	5817	4374‖	0·75‖
	0·5	Adrenalect.	6788 (117%)**	5077	0·75
	1	Adrenalect.	(lethal)		

* Figures quoted for S_1 are the average sp. act. (expressed as cpm/mg microsomal protein) of 3 simultaneous determinations after labeling of animals *in vivo* with L-(^{14}C)-leucine for 20 min as described under "Methods".

† Figures quoted for S_2 refer to average values of residual activity (also expressed as cpm/mg microsomal protein) after incubation of labeled microsomal samples (in triplicate) in the complete system *in vitro* for 15 min as indicated in the text. The ratio S_2/S_1 is a measure of the release of radioactive protein from the microsomal fractions labeled *in vivo*.

‡ One of 3 similar experiments.

§ Figures in parentheses are percentages of control values which are arbitrarily set at 100 per cent.

‖ Identical results were obtained when incubation *in vitro* was carried out in the presence of cycloheximide (0·10 mg/incubation).

¶ With 6 pairs of animals, the treated values (±S.D.) were 82 ± 5 per cent of the controls.

** With 6 pairs of animals, the treated values (±S.D.) were 116 ± 4 per cent of the controls.

Trakatellis *et al.*[3] with mice. At 1 and 2 hr after treatment and with similar dose levels of cycloheximide, the response in adrenalectomized rats resembles that observed in intact rats. However, at 24 hr there is a definite but smaller inhibition of protein synthesis in intact animals at dose levels of 0·5 and 1·0 mg/kg cycloheximide, a response which differs from that in adrenalectomized animals (which can survive well at 0·5 mg/kg but not at greater dose levels for the 24-hr period of the treatment), where no inhibition of protein synthesis is noted. Instead, in successive experiments, there was an increased synthesis of protein at the hepatic microsomal level of the order of 15 per cent. It was also noted that the 25 per cent increase in liver weight induced in intact rats by treatment with cycloheximide (0·5 mg/kg, 24 hr) does not occur when adrenalectomized rats are similarly treated.

It can be said therefore that the administration of cycloheximide to adrenalectomized rats evokes a different response both *in vivo* and *in vitro*[9] than the administration of the drug to intact rats after 24 hr of pretreatment.

Table 1 also shows the results of experiments on the release of radioactive protein from microsomal fractions labeled *in vivo* and then incubated *in vitro* as described under Methods. The ratios of the radioactivity remaining after incubation to the radioactivity incorporated during labeling *in vivo* in all the experimental animals, intact and adrenalectomized, control and cycloheximide-treated for 1, 2 or 24 hr, are very closely related.

The loss of labeled polypeptide, according to the theory of a tape mechanism of protein synthesis,[3,10] is connected with the movement of individual ribosomes along strands of messenger RNA. This does not appear to be affected either by cycloheximide pretreatment or by adrenalectomy in the rat liver micromosal system under the experimental conditions described here.

Furthermore, when liver microsomal fractions obtained from intact or adrenalectomized rats labeled with L-(^{14}C)-leucine *in vivo* are incubated *in vitro* in the presence not only of the usual cofactors but also of cycloheximide (0·10 mg per incubation), the release of labeled nascent polypeptide is not affected by the physical addition of the antibiotic to the incubation mixture.

DISCUSSION

Cycloheximide has different effects on protein synthesis *in vivo* and *in vitro*. Pretreatment of animals with cycloheximide *in vivo* inhibits protein synthesis *in vivo*,[1–4] but stimulates amino acid incorporation into protein in a system using liver microsomal preparations from pretreated sources.[4, 6, 9] The addition of cycloheximide to various incorporation systems *in vitro* inhibits protein synthesis.[4, 8, 11–19]

As a possible explanation for the inhibitory effect of cycloheximide on protein synthesis at the microsomal level, it has been suggested that the antibiotic affects the dynamic equilibrium between free ribosomes and polysomes under conditions of protein synthesis.[12, 15] This would imply that the antibiotic interfered in the translational read-out of the messenger-RNA molecules by the ribosomal particles. Such an explanation is inconsistent with previous findings in mouse[3] and rat liver[4] systems where cycloheximide had no direct effect on polysome and free ribosome populations in liver microsomal preparations.

The explanation that the mode of action of cycloheximide might involve some interference with the ribosomal read-out process of the messenger-RNA molecules cannot easily be reconciled with the present studies on the release of radioactive protein from microsomal preparations prelabeled with L-(^{14}C)-leucine. For, if there were interference, one would expect the release of radioactive nascent polypeptide to be inhibited by the presence of cycloheximide in the incubation system *in vitro* under conditions that were found to favor inhibition of amino acid incorporation into protein *in vitro*.[4, 20] However, the release of radioactive protein from the prelabeled microsomes under the experimental conditions here described, is not in any way affected by the presence of cycloheximide in the system (see Ennis and Lubin[8]).

It has also been suggested that cycloheximide interfered in the transfer reaction of amino acids from soluble RNA to protein.[3, 8, 17, 19] However, previous investigations in this laboratory[6, 9, 20] have established that changes in protein synthesis in rat liver, brought about by cycloheximide, are associated predominantly with the microsomal fraction and not with factors present in the soluble, 105,000 *g* supernatant fraction.

The present study reveals that there are differences in the response to cycloheximide in intact and adrenalectomized rats at 24 hr after treatment with the antibiotic. There is no inhibition of amino acid incorporation *in vivo* in the adrenalectomized rats, although there is a definite inhibition in intact rats. This recalls the earlier work of Fiala and Fiala[21] on the comparison of hepatic glycogen depletion in rats receiving cycloheximide alone and in combination with hydrocortisone (an agent known to protect against cycloheximide poisoning[22, 23]), and the observation that adrenalectomized rats are more sensitive than intact rats to the toxic effects of cycloheximide.[9, 21] These findings help to establish that in the mammalian organism the mode of action of cycloheximide is influenced by adrenal secretion. It remains to be determined whether the inhibitory action of cycloheximide pretreatment (0·5 mg/ g for 24 hr) on the amino acid incorporation *in vivo* and the stimulation of amino acid incorporation *in vitro*[9] can be restored in adrenalectomized rats by injecting them with hydrocortisone or corticosterone.[24]

Acknowledgement—This investigation was supported financially by Grant GM-13749 from the United States Public Health Service.

Department of Pharmacology,
The George Washington University,
School of Medicine,
Washington, D.C., U.S.A.

W. R. JONDORF

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Biochemical Pharmacology, Vol. 17, pp. 842–845. Pergamon Press. 1968. Printed in Great Britain

Inhibition of hexobarbital metabolism by diethylnitrosamine

(*Received* 11 *September* 1967; *accepted* 7 *December* 1967)

THE ACTIVITIES of various drug metabolising enzyme systems found in the microsomal fraction of liver homogenates can be greatly influenced by the pretreatment of animals with any of a considerable range of foreign chemicals.[1, 2] Inhibition may result from administration of compounds such as the diethylamino-ethanol ester of diphenylpropylacetic acid (SKF 525-A) while stimulatory agents include drugs like phenobarbital, insecticides of the chlorinated hydrocarbon type and certain polycyclic carcinogenic hydrocarbons.

Previously, it has been shown[3] that treatment of rats with the hepatocarcinogen diethylnitrosamine (DEN) increases the activity of liver UDP-glucuronyltransferase—the microsomal drug metabolising enzyme involved in the formation of glucuronides.[4] The present communication reports the effects of this treatment on the metabolism and duration of action of hexobarbital in rats.