Biochemical Pharmacology, 1962, Vol. 11, pp. 211–220. Pergamon Press Ltd., Printed in Great Britain.

FACTORS INFLUENCING INDUCTION OF HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYMES*

R. KATO, E. CHIESARA and P. VASSANELLI

Institute of Pharmacology, University of Milan, Milan, Italy

(*Received* 2 *October* 1961; *accepted* 14 *November* 1961)

Abstract—The influence of some factors on the induction of the microsomal drug-metabolizing enzymes by phenobarbital was studied to obtain further information on the mechanism of induction. Only the administration of ethionine 30 min before the administration of phenobarbital completely inhibits the effect of phenobarbital. On the contrary, norleucine, *p*-fluorphenylalanine and methioninesulphoxide did not modify the effect of phenobarbital. An activator or an inhibitor were not found in the liver of the phenobarbital or the phenobarbital + ethionine pretreated rats.

Treatment with phenobarbital can increase the enzyme activity also in rats which have low enzyme activities after administration of carbon tetrachloride and low dietary protein.

IT IS well known that pretreatment with some drugs can induce an increased capacity to metabolize the same or other drugs and at the same time the pharmacological effects of the drugs are diminished.[1–10] There are many compounds which can effect induction (inducing drugs) or of which the metabolism undergoes induction (induced drugs) have been reported.[2–6, 8]

An increasing in activity of microsomal enzymes, concerned in some common metabolic steps of the induced drugs, was held to be involved in the enzymatic induction.[8] The mechanism by which the inducing drugs can produce an increase in activity of the microsomal enzymes is not yet known. Several workers showed that the phenomenon of the induction was not apparent after treatment with the inducers together with ethionine,[1, 3, 5, 7, 11–13] and it is generally recognized that the inhibitory action of ethionine on the enzyme induction is due to its inhibitory action on protein biosynthesis.

The work reported here was carried out to obtain further information on the mechanism of the induction and particularly on the relationship between protein metabolism and the induction phenomenon.

MATERIALS AND METHODS

Male Sprague–Dawley rats weighing from 60 to 70 g were used for all experiments unless otherwise specified. Sodium phenobarbital (60 mg/kg) and phenaglycodol (90 mg/kg) were generally used as the inducing drugs by intraperitoneal injection.

Meprobamate, carisoprodol, pentobarbital, hexobarbital and strychnine were used as the induced drugs (substrates). The determinations of meprobamate and

* Presented at First International Pharmacological Meeting, Stockholm, 1961.

pentobarbital and hexobarbital were carried out according to the methods of Hoffman and Ludwig[14] and Brodie *et al.*,[15] respectively. The determinations of carisoprodol and strychnine were carried out according to the methods reported previously.[16, 17] Liver enzyme activities were determined by measuring the metabolized drugs in liver slices or in the microsomal preparation after an incubation of 2 hr.

The rat was killed by decapitation and the liver immediately removed and sliced with a microtome. Liver slices (500 mg) were suspended in a Warburg flask which contained 6 ml of Krebs phosphate buffered Ringer (pH 7·4 for hexobarbital and meprobamate metabolisms and pH 8·2 for strychnine metabolism) and 0·2 ml of the substrates (final concentrations of strychnine, pentobarbital, hexobarbital and meprobamate 2×10^{-4} M, 2×10^{-4} M, 8×10^{-4} M, 3×10^{-4} M, respectively), and incubated in an atmosphere of oxygen at 37 °C and shaken. At the end of the incubation period, the reaction mixture was homogenized and 2 ml of the homogenate were used for the determination of the substrates.

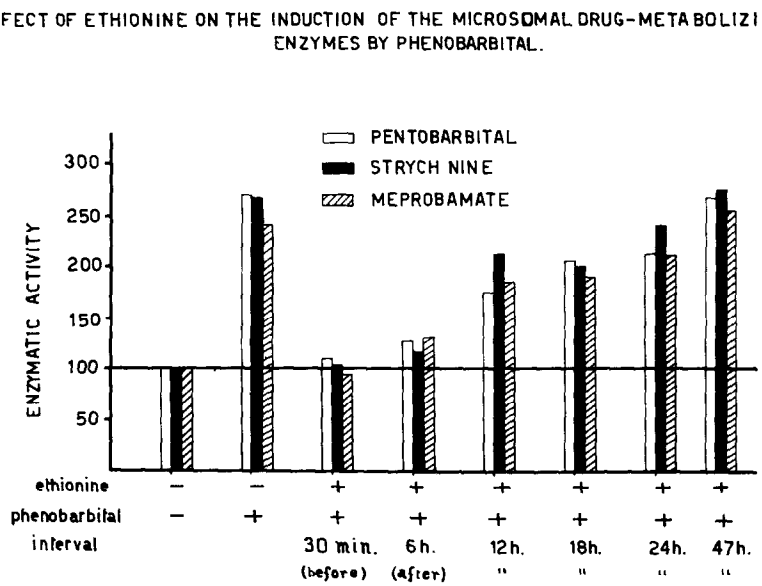

FIG. 1. Effect of delayed administration of ethionine after the administration of phenobarbital on the induction of the microsomal drug-metabolizing enzymes by phenobarbital. Ethionine (200 mg/kg) was injected intraperitoneally 30 min before or 6 hr, 12 hr, 18 hr, 24 hr and 47 hr after the phenobarbital administration (60 mg/kg, i.p.). The enzyme activities were determined on the microsomal-containing supernatant. The values given represent averages obtained from at least six rats, and expressed as per cent of the control rats (pentobarbital 78 μg/g per 2 hr, strychnine 208 μg/g per 2 hr, meprobamate 85 μg/g per 2 hr).

In the experiments with microsomal preparations the liver was homogenized in 2 parts of isotonic KCl (1·15 per cent) with a Potter–Elvehjem type homogenizer. The nuclei and mitocondria were sedimented by centrifugation of the homogenate at 8500 g for 15 min.

The incubation mixture (5·0 ml) contained 2 ml of the microsome-containing supernatant, 0·1 ml of 20 μmole glucose-6-phosphate, 0·4 μmole TPN, 100 μmole nicotinamide and 75 μmole $MgCl_2$ and 1 M KCl and 2·3 ml of 0·1 M sodium phosphate buffer pH 7·4 or 8·2 and 0·2 ml of the substrate. In some experiments the microsomes were separated by centrifugation of the microsome-containing supernatant at 10,500 g for 60 min.

Sleeping time after hexobarbital was taken as the duration of loss of the righting reflex.

Relation of time-interval between administration of phenobarbital and ethionine on the intensity of induction of the microsomal drug-metabolizing enzymes by phenobarbital

The effect of ethionine on the induction of the microsomal drug-metabolizing enzymes by phenobarbital was examined at various time-intervals between administration of phenobarbital and ethionine. Ethionine when administered 30 min before the injection of phenobarbital can completely inhibit the induction of the microsomal enzymes by phenobarbital.

If ethionine was administered after phenobarbital, the inhibitory effects of ethionine was progressively diminished with increasing time intervals between the administration of the two drugs (Fig. 1, the experiment with the microsome-containing supernatant).

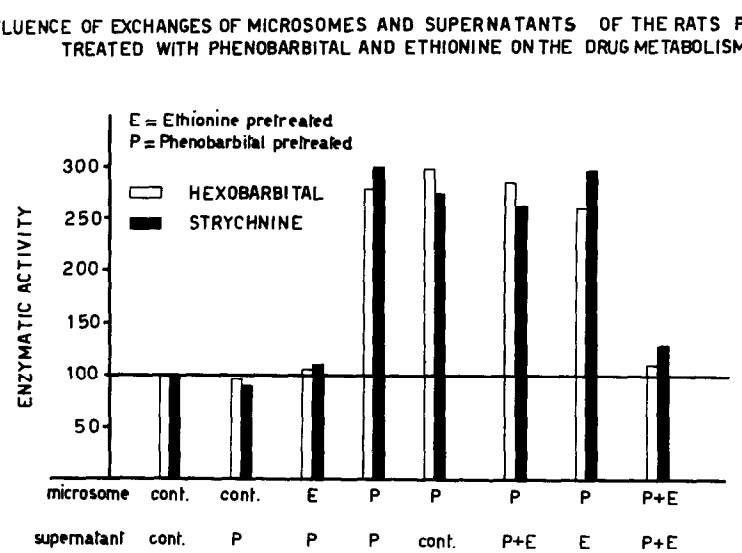

FIG. 2. Influence on the enzyme activities of exchange of the microsome and supernatant of the phenobarbital — or the pentobarbital + ethionine-pretreated rats. Male rats weighing 60 g, were injected intraperitoneally with phenobarbital (60 mg/kg) or ethionine and, 30 min later phenobarbital. The rats were sacrificed 48 hr after the injection of phenobarbital. After the separation of the microsomes and the supernatants they were interchanged and the microsomes were resuspensed. The values given represent averages obtained at least from four rats and expressed as per cent of the control rats (hexobarbital 269 μg/g per 2 hr, strychnine 168 μg/g per 2 hr).

Possible presence of an inhibitor or an activator of the enzymes in the liver of the pretreated rats

Exchanges of the microsomes and the supernatant obtained from control, phenobarbital, ethionine, or phenobarbital + ethionine pretreated rats were carried out to detect any modification of the enzyme activities in the microsomal fraction preparations (Fig. 2).

The results indicate that only the microsomes were responsible for the high enzyme activities in the phenobarbital pretreated rats, and that there is no activator or inhibitor in supernatant fractions obtained from phenobarbital or phenobarbital – ethionine pretreated rat.

Similar results were also obtained with phenaglycodol instead of phenobarbital.

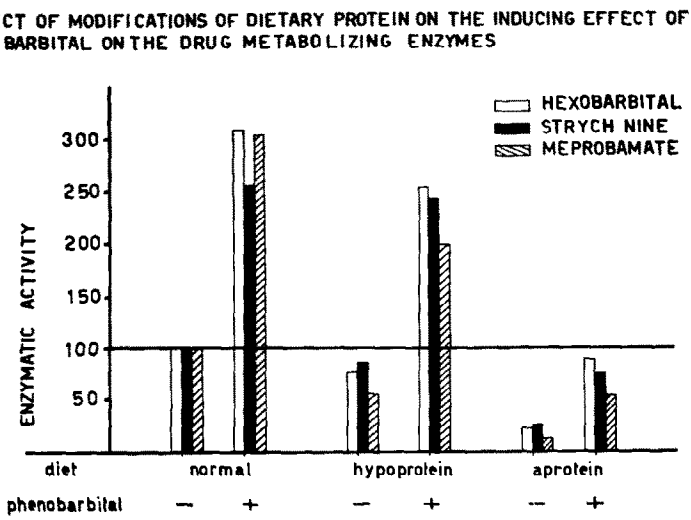

FIG. 3. Effect of dietary protein on the induction of the microsomal drug-metabolizing enzyme by phenobarbital. The experiments were on female rats, weighing 100 g. The rats were divided into three groups, and the first group fed on normal diet and the second group on hypoprotein diet of Razente and the third group on protein-free diet of Knowlton for 1 week. Phenobarbital (60 mg/kg, i.p.) was injected into half the rats in each group and all the rats were sacrificed 48 hr after. The determination of the enzyme activities was carried out with the microsome-containing supernatant. The values given represent averages obtained from at least six rats and expressed as per cent of the control rats (hexobarbital 233 μg/g per 2 hr, strychnine 165 μg/g per 2 hr, meprobamate 77 μg/g per 2 hr).

Effect of dietary protein on the induction of the enzyme by phenobarbital

The fact that ethionine can completely inhibit the induction of the enzyme by phenobarbital suggests the importance of protein biosynthesis in the induction of the enzymes. Experiments were carried out on three groups of rats which were maintained for 1 week on a normal diet (18 per cent protein) a hypoprotein diet of Razente (6 per cent protein) or protein-free (aprotein) diet of Knowlton.

As shown in Fig. 3 (the experiments with the microsome-containing supernatant)

the results obtained in this experiment were very different from the results obtained with ethionine pretreatment.

Ethionine could completely inhibit the induction of the enzymes when given at a dose which did not itself modify the enzyme activities, on the contrary, the rats maintained with the hypoprotein or the aprotein diet, have low activities of the hepatic enzymes, but in their liver the phenomena of induction of the enzymes is still clearly observed as in normal rats.

Similar results were obtained in the experiment with liver slices.

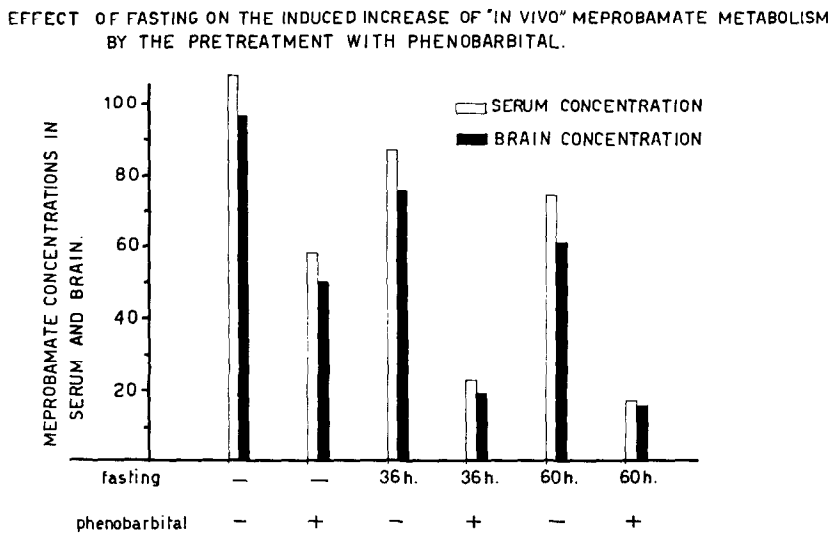

FIG. 4. Effect of fasting on the induction of the microsomal drug-metabolizing enzymes by phenobarbital. The experiments on rats weighing 160 g. The rats were divided into three groups and the first group was fasted for 60 hr and the second group was fasted for 36 hr. Phenobarbital (60 mg/kg, i.p.) was injected into half of each group and all the rats were injected with meprobamate (200 mg/kg, i.p.) 36 hr after phenobarbital. The determination of meprobamate concentrations in serum and in brain was carried out 3 hr after the meprobamate administration. The values given represent averages obtained from at least eight rats. Meprobamate concentrations were expressed in μg/ml for serum and μg/g for brain.

Effect of fasting on the induction of the enzyme by phenobarbital

It is well known that in the fasting rats a marked decrease in liver weight and variations of some enzyme activities occur in 24 hr.[19–21]

The results reported here were obtained from *in vivo* metabolism of meprobamate (Fig. 4). It is of interest that meprobamate metabolism increases in fasting rats and the effect of phenobarbital is more marked in fasting rats.

Similar results were obtained for meprobamate metabolism in liver slices.

Effect of ethionine, norleucine, p-fluorphenylalanine and methionine sulphoxide on the induction of the microsomal drug-metabolizing enzymes by phenobarbital

Previous work suggested that the inhibitory action of ethionine on the induction of the enzyme may be due to its inhibitory action on biosynthesis of the enzyme protein.

Inhibitions by some amino acid analogue on induction of some bacterial enzymes have recently been reported by several authors.[23] It is of interest to know whether the induction of the hepatic microsomal drug-metabolizing enzymes is inhibited not only by ethionine, but also by other amino acid analogues.

Ethionine, as shown in Fig. 5 (the experiments with the liver slices), can inhibit completely the induction of the enzymes by phenobarbital with a dose which by itself does not modify the normal enzyme activities.

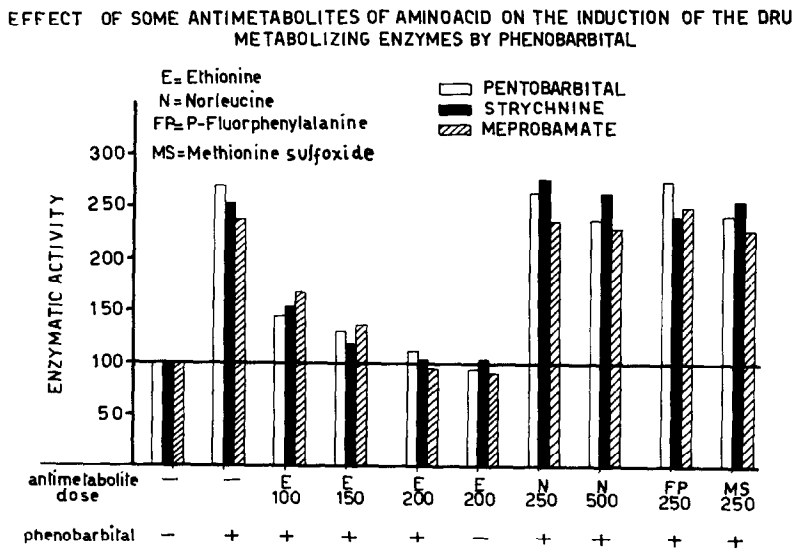

FIG. 5. Effect of ethionine, norleucine and *p*-fluorphenylalanine and methioninesulphoxide on the induction of the microsomal drug-metabolizing enzymes by phenobarbital. The experiments were on male rats weighing 60 g. Ethionine (200 mg/kg, 150 mg/kg, 200 mg/kg), norleucine (200 mg/kg, 500 mg/kg), *p*-fluorphenylalanine (250 mg/kg) and methioninesulphoxide (250 mg/kg) were intraperitoneally injected 30 min before the injection of the phenobarbital (60 mg/kg, i.p.). The enzyme activities were determined on the liver slices 48 hr after the injection of phenobarbital. The values given represent averages obtained from at least six rats and expressed as per cent of the control rats. (Pentobarbital 185 μg/g per 2 hr, strychnine 404 μg/g per 2 hr, meprobamate 187 μg/g per 2 hr)

Ethionine has shown an inhibitory action even with small doses. On the contrary, norleucine, *p*-fluorophenylalanine and methionine sulphoxide were not capable of inhibiting the induction by phenobarbital.

Similar results were obtained with phenaglycodol instead of phenobarbital or with the liver microsome-containing supernatant.

Effect of carbon tetrachloride on the induction of the microsomal drug-metabolizing enzymes by phenobarbital

The administration of carbon tetrachloride induces a decrease in the activities of the hepatic microsomal drug-metabolizing enzymes. Experiments were carried out to

ascertain the efficiency of phenobarbital in the rat intoxicated with carbon tetrachloride. The results show that the induction of the drug metabolizing enzymes by phenobarbital takes place also in the liver intoxicated with carbon tetrachloride, and that the induction cannot be modified by CCl_4 in doses that do not change the enzyme levels of normal rats (Fig. 6, the experiments with the microsome-containing supernatant). The similar results were also obtained for the duration of sleeping-time after hexobarbital (Fig. 7).

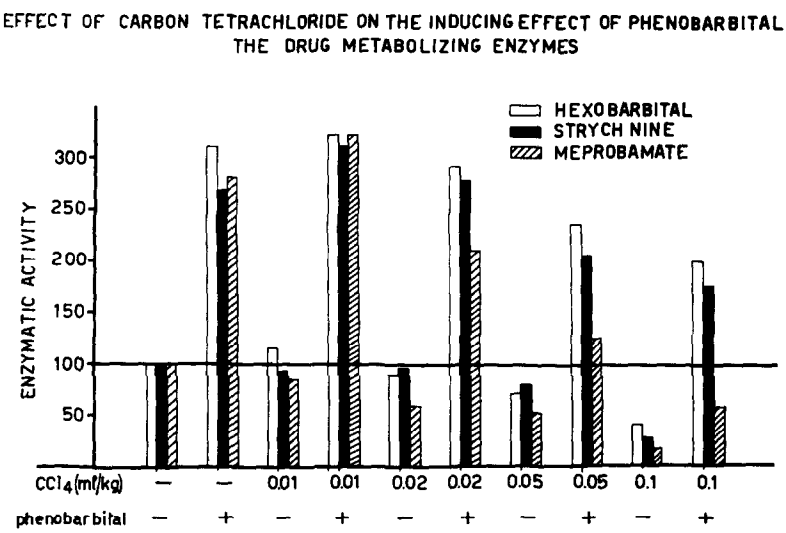

FIG. 6. Effect of carbon tetrachloride on the induction of the microsomal drug-metabolizing enzyme by phenobarbital. The experiments were on female rats weighing 100 g, carbon tetrachloride was diluted with arachic oil and was given intraperitoneally in doses of 0·01 ml, 0·02 ml, 0·05 ml and 0·1 ml/kg in total volume of 1 ml/kg. Phenobarbital (60 mg/kg, i.p.) was injected 30 min after the injections of CCl_4. The rats were sacrificed 36 hr after phenobarbital. The enzyme activities were determined with the microsome-containing supernatant. The values given represent averages obtained from at least six rats and expressed as per cent of the control rats. (Hexobarbital 208 μg/g per 2 hr, strychnine 167 μg/g per 2 hr, meprobamate 74 μg/g per 2 hr).

Possible interference of the effect of phenobarbital by simultaneous injection with chemically related compounds, another type of the inducing drugs, or pharmacological antagonists

Most of barbiturated and other related compounds, such as glutethimide, primidone, diphenylhydantoin, urethane have similar effect to phenobarbital.[8] It was reported by Conney *et al.* that administration of 3:4-benzypyrene or 20-methylcholanthrene increases the demethylase activities towards azo dye and the metabolism of zoxazolamine and other compounds, but, according to our results, it did not increase the metabolism of hexobarbital, pentobarbital, strychnine, meprobamate and carisoprodol.[5, 24, 25]

The possible interference with the effect of phenobarbital by simultaneous injection with some pirimidine derivatives, 3:4-benzpyrene, 20-methylcholanthrene and some analeptic drugs was examined.

2:4-Dichlorpirimidine (150 mg/kg) and barbituric acid (200 mg/kg i.p.), 3:4-benzpyrene (50 mg/kg, i.p.), 20-methylcholanthrene (50 mg/kg, i.p.), amphetamine (20 mg/kg, o.p.), bemegride (15 mg/kg, i.p.), did not modify effect of phenobarbital and also did not show any modification of the enzyme activities of control rats.

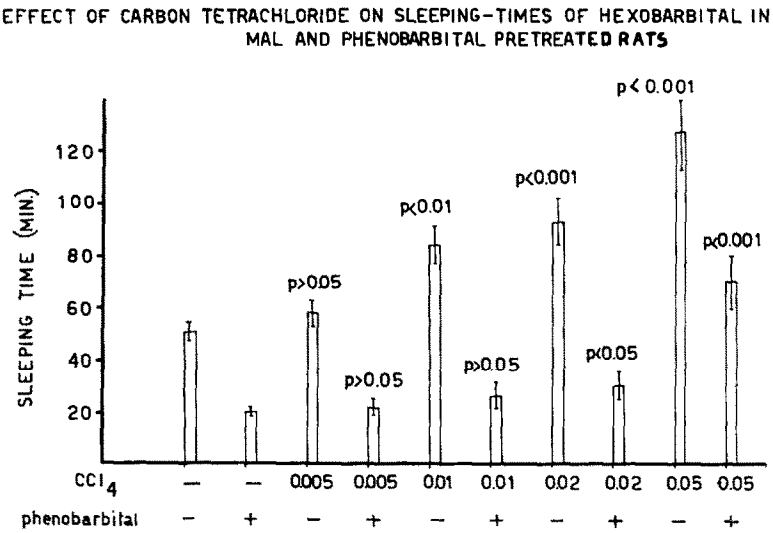

FIG. 7. Effect of carbon tetrachloride on the decrease of sleeping time induced by phenobarbital. The experiments were on female rats of 140 g. Carbon tetrachloride was given intraperitoneally diluted with arachic oil and given in doses of 0·005 ml, 0·01 ml, 0·02 ml and 0·05 ml/kg in total volume of 1 ml/kg. Phenobarbital (60 mg/kg, i.p.) was injected 30 min after the injection of CCl_4. Hexobarbital (100 mg/kg) was injected intraperitoneally 36 hr after phenobarbital for determination of sleeping-time. The values given present average obtained from eight animals. Probabilities were calculated in the differences between normal rats and carbon tetrachloride-pretreated rats or phenobarbital-pretreated rats and phenobarbital + carbon tetrachloride-pretreated rats.

DISCUSSION

In this work it was demonstrated that ethionine is a specific agent for inhibiting the induction of the microsomal drug-metabolizing enzymes by phenobarbital. The inhibitory action of ethionine may be due to its action on protein biosynthesis. But it is not clear why only ethionine has the inhibitory action on the induction of the microsomal drug-metabolizing enzymes. The accumulation of lipid in liver by ethionine could not be considered responsible.[22] Norleucine, *p*-fluorphenylalanine and methioninesulfoxide have potent inhibitory actions, like ethionine, on protein biosynthesis and on induction of some bacterial enzymes,[23] but it is conceivable that the amino acid analogues have an inhibitory effect on enzymes induction only in bacteria and they have no action on the enzyme induction in rats. This observation

might offer another possible interpretation of the induction of hepatic drug-metabolizing enzymes, that the induction is not due to increase of enzyme protein.

Many agents which modify the hepatic enzyme activity of rats, i.e. administration of alloxan, deoxycholic acid or inhibitors of the reticulo-endothelial system (powdered carbon, iron oxide or trypan-blue) and ligation of common bile duct, show modification of the effect of phenobarbital, such as administration of carbon tetrachloride, or feeding on aprotein diet. But the all above-mentioned agents could not completely inhibit the effect of phenobarbital which was demonstrated by ethionine, even in conditions of marked decrease of the enzyme activities of non phenobarbital-pretreated rats.[22]

The modifications produced by low protein and protein-free diet or CCl_4 suggest the disturbance of the enzyme biosynthesis by low protein pool or lowing of cell function after CCl_4. Under such conditions phenobarbital, in contrast to ethionine, can stimulate enzyme biosynthesis.

The ineffectiveness of the pyrimidine derivatives, 3:4-benzpyrene or 20-methylcholanthrene and the analeptic drugs on modification of phenobarbital effect suggest that: (1) the process of the induction by two types of compound was a completely independent process, and that there is no competition in the enzyme biosynthesis in the hepatic microsomes of pretreated rats; (2) there is no competition in the stimulation of the enzyme biosynthesis with a substance of related chemical structure, and the stimulation is not connected with the pyrimidine ring of phenobarbital; (3) the depressant effect of phenobarbital on the central nervous system is not of importance for the induction.

Concerning the mechanism of the induction, it is possible to suppose that the method by which the administered drug is metabolized may be one of the important factors.[8] Indeed, most of the drugs which have an inducing effect, are metabolized by microsomal liver enzymes which require TPNH and oxygen and which are inhibited by SKF 525 A (6) (22) (26).

REFERENCES

1. A. H. Conney, E. C. Miller and J. A. Miller, *Cancer Res.* **16**, 450 (1956).
2. A. H. Conney, C. Davidson, R. Gastel and J. J. Burns, *J. Pharmacol.* **130**, 1 (1960).
3. A. H. Conney, I. A. Michaelson and J. J. Burns, *J. Pharmacol.* **132**, 202 (1961).
4. H. Remmer, *Arch. exp. Path. Pharmak.* **237**, 296 (1959).
5. R. Kato, *Atti Soc. lombarda. Sci. Med. Biol.* **14**, 777 (1959).
6. R. Kato, *Atti Soc. lombarda. Sci. Med. Biol.* **14**, 783 (1959).
7. R. Kato, *Med. Exp.* **3**, 95 (1960).
8. R. Kato and E. Chiesara, *Brit. J. Pharmacol.* In press.
9. R. Kato. To be published.
10. R. Kato, E. Chiesara and G. Frontino, *Jap. J. Pharmacol.* **11**, 31, (1961).
11. R. Kato and E. Chiesara, *Atti Accad. Med. Lombarda.* **15**, 392 (1960).
12. R. Kato, G. Frontino and P. Vassanelli, *Jap. J. Pharmacol.* **11**, 25, (1961).
13. J. M. Fujmoto and G. L. Plea, *J. Pharmacol.* **131**, 283 (1961).
14. A. J. Hoffman and B. J. Ludwig, *J. Amer. Pharm. Ass.* **48**, 740 (1959).
15. B. B. Brodie, J. J. Burns, L. C. Mark, P. A. Lief, E. Bernstein and E. M. Popper, *J. Pharmacol.* **109**, 26 (1953).
16. R. Kato, P. Vassanelli and G. Frontino, *Med. exp.* In press.
17. R. Kato, E. Chiesara and P. Vassanelli, *Jap. J. Pharmacol.* In press.
18. R. H. Adamson and J. R. Fouts, *J. Pharmacol.* **127**, 87 (1959).

19. R. L. DIXON, R. W. SHULTICE and J. R. FOUTS, *Proc. Soc. exp. Biol. Med.* **103**, 333 (1960).
20. G. WEBER and A. CANTERO, *Amer. J. Physiol.* **190**, 229 (1957).
21. G. N. CATRAVAS and H. S. ANKER, *J. Biol. Chem.* **232**, 669 (1958).
22. R. KATO, E. CHIESARA and P. VASSANELLI. Unpublished data.
23. M. VAUGHAN and D. STEIBERG, *Advanc. Protein Chem.* **14**, 115 (1959).
24. A. H. CONNEY, J. R. GILLETTE, J. K. INSCOE, E. R. TRAMS and H. S. POSNER, *Science* **130**, 1478 (1959).
25. R. KATO, E. CHIESARA and P. VASSANELLI, *Atti Accad. Med. Lombarda.* **15**, 443 (1960).
26. R. KATO, *Proceeding 1st Europ. Symp. Med. Enzymol., Milano,* 1960, p. 363. Karger, Basel.