Biochemical Pharmacology, 1965, Vol. 14, pp. 515–524. Pergamon Press Ltd., Printed in Great Britain.

ENHANCED SULFOBROMOPHTHALEIN DISAPPEARANCE IN MICE PRETREATED WITH VARIOUS DRUGS*

JAMES M. FUJIMOTO, W. FOSTER EICH and HUDSON R. NICHOLS

Department of Pharmacology,
Tulane University School of Medicine, New Orleans, L., U.S.A.

(*Received* 3 *March* 1964; *accepted* 4 *May* 1964)

Abstract—Pretreatment of mice with certain drugs caused changes in liver function subsequently measured by concentrations of sulfobromophthalein in plasma. Drugs such as phenobarbital, nikethamide, urethan, and β-phenylisopropylhydrazine produced significantly elevated concentration of sulfobromophthalein in plasma soon after pretreatment. Findings of greatest interest were the enhanced disappearance of sulfobromophthalein from plasma with many of the above agents as well as with SKF 525A, N-methyl-3-piperidyl-N′N′-diphenylcarbamate, and tolbutamide. Countercurrent analyses of acetone extracts of plasma indicated the enhanced disappearance of the parent compound, sulfobromophthalein. Countercurrent analyses of liver extracts indicated a hepatic mechanism which appears to follow an adaptive type of response.

ADMINISTRATION of a variety of drugs to animals leads to an accelerated metabolism of certain drugs by enzymes in the microsomal fraction of the liver.[1, 2] The purpose of this study is to demonstrate that these drugs also increase the function of the liver in yet another way. To this end, the effect upon liver function in mice of phenobarbital, tolbutamide, SKF 525A, urethan, nikethamide, β-phenylisopropylhydrazine (PIH), N-methyl-3-piperidyl-N[1]N[1]-diphenylcarbamate (MPDC), and 3-methylcholanthrene-(3MC) has been measured by sulfobromophthalein (BSP) disappearance. Because the major part of the metabolism of BSP is carried out by enzymes in the soluble rather than the microsomal fraction,[3] changes in the disposition of BSP caused by these drugs suggest the occurrence of effects beyond that seen on the microsomal fraction. Since the removal of BSP from blood plasma is dependent not only on metabolism but on uptake and secretion by the liver, an attempt has been made to assess the contribution of these factors to the described effect.

METHODS

Male albino mice weighing 15 to 35 g were fasted overnight or at least 2 hr before treatment with the drugs under study. The main drugs used were phenobarbital sodium (100 mg/kg body weight), tolbutamide or tolbutamide sodium (1,000 mg/kg), and DL-ethionine (200 mg/kg). Other drugs used in more limited fashion are mentioned in Results. The drugs were administered orally in concentrations, 0·1 ml of solution

* This work was supported by Grant GM-05514 from the Division of General Medical Sciences, National Institute of Health.

/10 g body weight, and the fasting was continued to the end of the experiment. At the end of the specified interval after treatment with the drug (hereafter called the pretreatment time), the BSP test was done according to the method of Kutob and Plaa.[1] This test involved intravenous administration of BSP sodium, 100 mg/kg, followed in 15 min by anesthetizing the mouse rapidly with ether (for about 1 min) and obtaining a blood plasma sample on which the BSP determination was done. In earlier experiments the cardiac puncture technique of Kutob and Plaa was used to obtain blood; in the later experiments another technique, satisfactory and more easily performed, was employed. The skin in the axillary region was incised and followed by a deep incision of the muscle mass to sever the large blood vessels to the foreleg. Blood collected rapidly in the pocket made by the flap of skin and the axilla and was aspirated into a heparinized tuberculin syringe. The blood was placed in test tubes containing 0·01 ml of 2% sodium oxalate (in earlier experiments) or 0·01 ml of heparin (in later experiments). For the BSP determination, the tubes were centrifuged and two 100-μl aliquots of plasma were transferred to small test tubes containing 1 ml of isotonic saline. To one tube, 0·05 ml of 10% sodium hydroxide and to the other, 0·05 of 10% hydrochloric acid were added. The optical densities (absorbances) of these two samples were estimated at 580 mμ on a Coleman Jr. spectrophotometer. The difference in optical densities between the two samples was directly proportional to the BSP concentration as described by Kutob and Plaa, and a difference of 1 unit of optical density equaled 23·1 mg/100 ml BSP sodium in plasma. For convenience this test was referred to as the "15-minute BSP test". In subsequent experiments, blood samples were obtained not only at 15 min, but also at 7 and 30 min after BSP administration. Thus the variables tested were the drug, the pretreatment time, and the blood-sampling time.

In order to characterize the nature of the material being measured in the BSP test, countercurrent analyses were performed on plasma extracts. For this purpose, blood samples from 9 to 10 mice of a group were pooled, and a 3-ml aliquot of the plasma was transferred to a glass stoppered tube containing 9 ml of acetone. The tube was shaken thoroughly and centrifuged. Several measured aliquots (to make a total of 8 ml, which is equivalent to 2·25 ml of plasma) of the supernatant phase were dried in a plastic weighing dish placed in the hood (several aliquots were necessary because of the limited size of the dish). The air-dried residue was taken up in the countercurrent solvent system. In other experiments, not only the plasma but the livers were taken for countercurrent analyses. A sample of 1 g ± 10 mg (range) of liver was taken from each mouse immediately after blood sampling and homogenized in 3 ml of distilled water; then a 2-ml aliquot of homogenate was added to 6 ml of acetone and the mixture shaken and centrifuged. Three-ml aliquots of each of the acetone extract were pooled for the 9 to 10 animals in each group. A 15-ml aliquot (corresponding to 15/16 g of liver, assumed that 1 g of liver is 1 ml) of the pooled extract was air dried, and the residue was taken up in the countercurrent system.

The countercurrent solvent system consisted of 10 ml of each phase of a mixture of 600 ml of ethyl ether, 200 ml of *n*-butanol, 800 ml of 1 N HCl. The bottom layer was moved manually and an 8-plate transfer performed. After the distribution procedure, 2 ml of 10 N NaOH was added to the tubes, and the tubes were shaken and the phases allowed to separate. The bottom phase of each tube was read at 580 mμ on the Coleman Jr. spectrophotometer, and 0·10 O.D. corresponded to 1 μg BSP sodium/ml of final lower phase. The total recovery for the procedure was checked by adding known

quantities of BSP to liver homogenates, preparing the acetone extracts, and running the countercurrent procedure. The recoveries ranged from 95 to 101 per cent when compared to the procedure using BSP in water in place of the homogenate.

RESULTS

The effect of pretreatment with phenobarbital on the plasma concentration of BSP was diphasic (Fig. 1). For the 6-hr pretreatment time the plasma BSP concentration

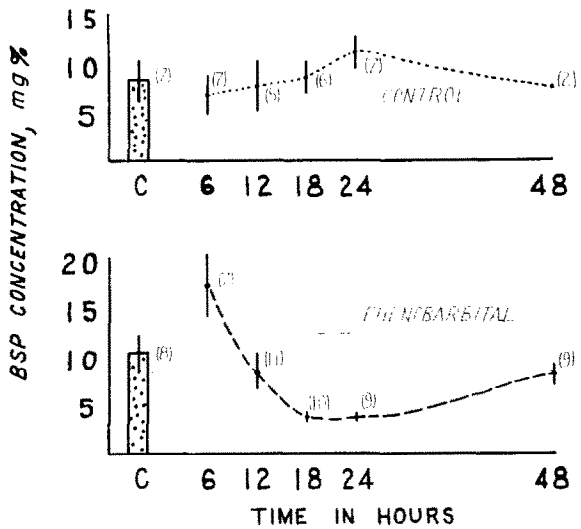

FIG. 1. The effect of phenobarbital sodium (100 mg/kg, p.o.) on plasma concentrations of BSP sodium (100 mg/kg, i.v.). Upper panel: control, C, was given no pretreatment; other groups were pretreated p.o. with water at the indicated times before the 15-minute BSP test was done. Lower panel: C as above; others were given phenobarbital; ( ) = number of animals; vertical lines = standard error.

was increased, and for the 18- and 24-hr pretreatment times the concentration was decreased, compared to control values. Control values were unaffected by administering water (solvent for the drug solution) to the mice at the specified times, compared to an untreated control group. The retention of BSP found at 6 hr was not particularly surprising in that previous work with another barbiturate[5] had given similar results. However, the decreased concentration of BSP at 18 and 24 hr was a new finding, which was taken to indicate an increase in function of the liver because the liver is the major organ involved in the removal and disposition of BSP.

Tolbutamide (Fig. 2) also caused significant lowering of BSP concentration. The values at 18, 24, and 48 hr were significantly different from the control; but at 6 hr, tolbutamide did not produce an increase as phenobarbital had. Thus the response to tolbutamide was not diphasic.

The finding that these two drugs lower the BSP concentration in plasma suggested the occurrence of an adaptive type of phenomenon in that both phenobarbital[1, 6] and tolbutamide[7] produce adaptive increases in activity of enzymes in the microsomal

fraction of the liver after a latent period similar to that seen in the present experiments. Therefore, antagonism studies were performed with ethionine along the lines of a previous study.[8] In Table 1, 24-hr pretreatment with phenobarbital or tolbutamide gave the expected lowering of BSP concentration, whereas ethionine given with either one of these agents at 24 hr blocked the effect. The blocking effect was obtained with

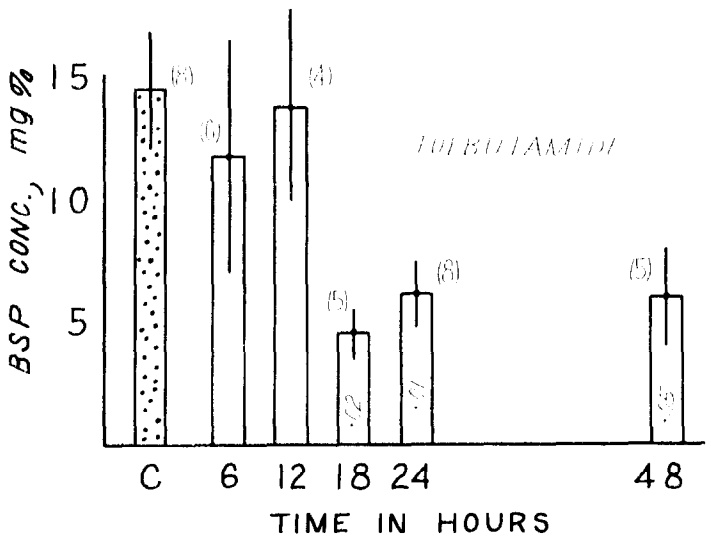

FIG. 2. The effect of tolbutamide sodium (1,000 mg/kg, p.o.) on plasma concentrations of BSP sodium (100 mg/kg, i.v.). Legend as in Fig. 1. Numbers within bars are P values.

TABLE 1. EFFECT OF PRETREATMENT WITH ETHIONINE, PHENOBARBITAL, AND TOLBUTAMIDE ON PLASMA BSP CONCENTRATION (15-MINUTE BSP TEST)

Drug	Pretreatment time (hr)	No. of mice	Plasma BSP (mg/100 ml)*
Control		7	11·3 ± 5·1
Ethionine	24	8	11·1 ± 5·3
Phenobarbital	24	9	3·0† ± 0·5
Phenobarbital + ethionine	24 + 24	5	10·3 ± 3·7
Phenobarbital	48	7	3·2† ± 0·3
Phenobarbital + ethionine	48 + 24	6	4·8 ± 2·9
Control		4	14·0 ± 1·5
Ethionine	24	7	13·7 ± 1·7
Tolbutamide	24	8	5·8† ± 1·4
Tolbutamide + ethionine	24 + 24	9	11·9 ± 1·2
Tolbutamide	48	9	12·9 ± 1·7
Tolbutamide + ethionine	48 + 24	8	11·1 ± 1·2

* ± S.E.

† $P < 0.05$ compared to control value. Doses of drugs are given in Methods.

a dose of ethionine which in itself had no effect on the BSP concentration. Thus these data suggest an adaptive type of effect caused by the phenobarbital and tolbutamide. The results for the 48-hr pretreatment with phenobarbital were partially supportive. When phenobarbital was given 24 hr prior to the ethionine, the ethionine was less effective, apparently because the adaptive changes had already taken place when the ethionine was given so that it had less blocking effect.[8] With the tolbutamide, the results at 48 hr were not particularly informative since the primary effect of tolbutamide, that of lowering the BSP concentration, was not obtained in this experiment. Since it was felt that reproducible responses to 48-hr pretreatment with both phenobarbital and tolbutamide were not easily obtained, no further studies with 48-hr pretreatment were done.

Because many drugs are known to produce adaptive changes in liver microsomal enzyme activity,[1] a few selected drugs[2] which were immediately available were tested for the diphasic effect on plasma concentration of BSP. A number of drugs produced a diphasic effect (Table 2) and it is evident that no correlation existed between the

TABLE 2. EFFECT OF DRUG PRETREATMENT ON BSP PLASMA CONCENTRATION (15-MINUTE BSP TEST)

	Drug*	Dose (mg/kg)	No. of mice	Pretreatment time (hr)	Plasma BSP (mg/100 ml)
A	Control		11		7·2 ± 0·8
	Phenobarbital	100	9	24	3·0† ± 0·5
	MPDC	150	8	24	3·4† ± 0·8
B	Control		10		9·8 ± 1·0
	Phenobarbital	100	5	24	5·0† ± 0·7
	SKF 525A	100	9	24	4·4† ± 0·7
	3 MC	45	8	24	6·8 ± 1·5
C	Control		8		7·3 ± 1·0
	Nikethamide	200	5	24	5·4 ± 1·1
	PIH	50	4	24	3·3† ± 1·5
	Urethan	1,200	10	24	4·4† ± 0·6
	Phenobarbital	100	4	24	3·1† ± 0·6
D	Control		9		8·4 ± 0·7
	Urethan	1,200	9	6	12·0† ± 0·9
	MPDC	150	7	1	9·3 ± 0·6
	PIH	50	7	1	10·0† ± 0·4
E	Control		8		7·3 ± 1·0
	SKF 525A	100	9	1	8·0 ± 0·5
	Nikethamide	200	8	1	17·3† ± 2·9

* MPDC = N-methyl-3-piperidyl-N′,N′-diphenylcarbamate HCl; SKF 525A = β-diethylaminoethyl diphenylpropylacetate HCl; 3MC = 3-methylcholanthrene administered in oil; PIH = β-phenylisopropylhydrazine HCl.

† $P<0.05$ compared to control.

initial degree of inhibition and the subsequent extent of enhancement of BSP disappearance from plasma. For instance, nikethamide pretreatment resulted in good inhibition but only small enhancement, whereas SKF 525A caused no inhibition but good enhancement.

In order to characterize the material measured in the BSP test, countercurrent analyses were performed on acetone extracts of the plasma and liver. In Fig. 3, countercurrent distribution of a known sample of BSP mixed with a metabolite of BSP

indicated that good separation of BSP and the metabolite was attainable with the ether–butanol–HCl solvent system. To prepare the metabolite used here, the bile of anesthetized rats given BSP was collected (by cannulating the bile duct) and streaked on to a paper chromatogram. After development, the major metabolic product, which presumably corresponded to the glutathione conjugate of BSP, was eluted from the

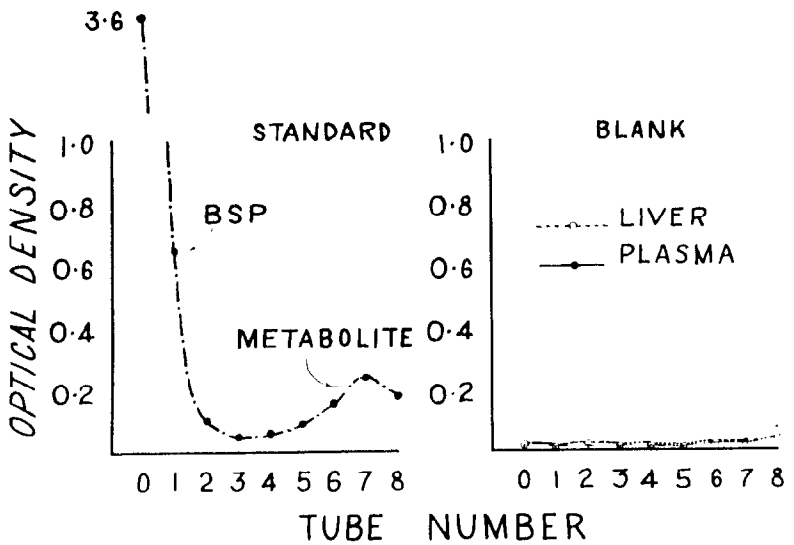

FIG. 3. Countercurrent distribution analysis of BSP and metabolite (left) and liver and plasma acetone extracts of untreated mice (right). Lower phase was mobile phase.

paper and isolated in partially purified form. In the chromatograms other minor metabolites of BSP traveled in intermediate positions between BSP and this metabolite. Since the solvent systems used in the chromatographic and countercurrent procedures were similar, this relationship could be carried over to the countercurrent system. On the right side of Fig. 3, countercurrent analyses of acetone extracts of control liver and plasma show the extent of blank interference.

In Fig. 4, the results for plasma indicate that the major component present was BSP with little if any other component detectable. Also, the amount of BSP in the plasma from the control group was greater than that in the plasma from mice treated with phenobarbital or tolbutamide.

With the optical density/BSP concentration factor given in the section on *Methods*, totals in tubes 0 through 2 were calculated in terms of plasma concentration of BSP. For control, phenobarbital, and tolbutamide groups, the values were 6·6, 3·3, and 3·6 mg/100 ml respectively. It was therefore concluded that the differences demonstrated for plasma in the earlier experiments were indeed due to the BSP and not to metabolites. The results on the right side of the figure show that the livers contained appreciable levels of metabolite and large amounts of BSP at this time. The livers of the control mice appear to contain more BSP and metabolite than those of phenobarbital- or tolbutamide-pretreated groups. Calculation of the total amounts of BSP (tubes 0 through 2) for control, phenobarbital, and tolbutamide groups gave 427, 344, and 345 μg BSP/g liver, respectively. These values were approximations in that 1 g of

liver was assumed to be 1 ml. It appeared that the more rapid removal of BSP from the plasma of mice treated with the drugs could be due to a more rapid hepatic turnover; however, it was not clear whether the effect was caused by an increase in uptake, metabolism, secretion, or a combination of all these factors. To assess some of them,

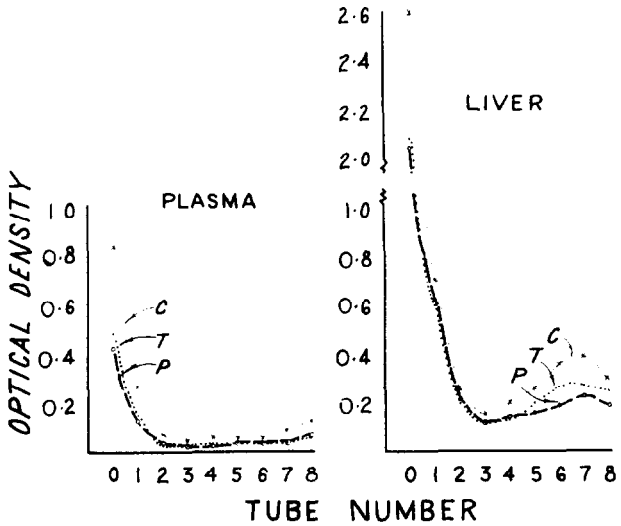

FIG. 4. Countercurrent distribution analyses of plasma and liver acetone extracts of control, C; phenobarbital sodium, P, 100 mg/kg, p.o.; and tolbutamide sodium, T, 1,000 mg/kg, p.o.; in 24-hr pretreated mice 15 min after administration of BSP sodium (100 mg/kg, i.v.).

a temporal study was done in an earlier (7-min) and a later (30-min) time of analysis after administration of BSP.

Again, differences were demonstrated at 7 min between the control and the phenobarbital and tolbutamide groups with calculations of totals in the first 3 tubes giving values of 29, 16, and 14 mg/100 ml BSP in plasma. In the liver, a somewhat different picture was seen at 7 min than was seen at 15 min (Fig. 5). Here the drug-treated groups appeared to have more BSP and metabolite than the control group, with approximate values of 527, 532, and 469 μg BSP/g liver for phenobarbital, tolbutamide, and control groups respectively. The high values for the groups treated with drugs indicated enhanced uptake of BSP by the liver. Metabolism and secretion may also have been enhanced because the change in concentration of BSP in the liver from 7 to 15 min was much steeper for the drug-treated than for the control group (from about 530 to 345 vs. 469 to 427 μg/g respectively). However, the same steep change could have arisen solely from the enhanced uptake with rapid fall in plasma concentration of BSP. It was hoped that by 30 min all the changes would be magnified; however, Fig. 6 indicated that the differences were no longer present by this time.

The possibility that the drugs had enhanced secretion of BSP and metabolite into the bile was further considered. Since it was not technically feasible to collect bile in these mice, segments of the gastrointestinal tract were homogenized, extracted with acetone, and analyzed. Comparison was made of such segments of the gastrointestinal tract as the stomach plus the small intestine, small intestine alone, and 6 inches of the

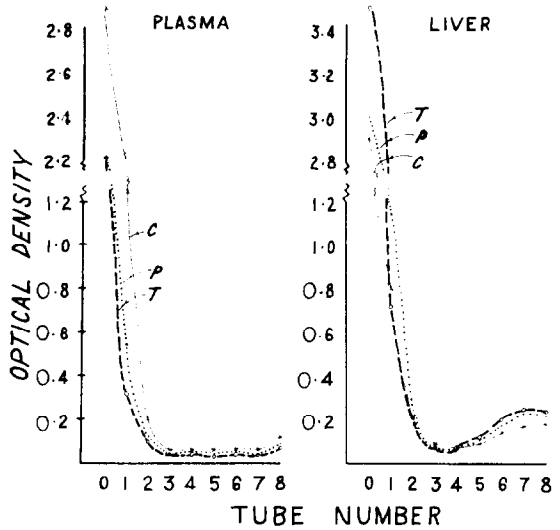

FIG. 5. Countercurrent distribution analyses of plasma and liver acetone extracts as in previous figure except 7 min after BSP administration.

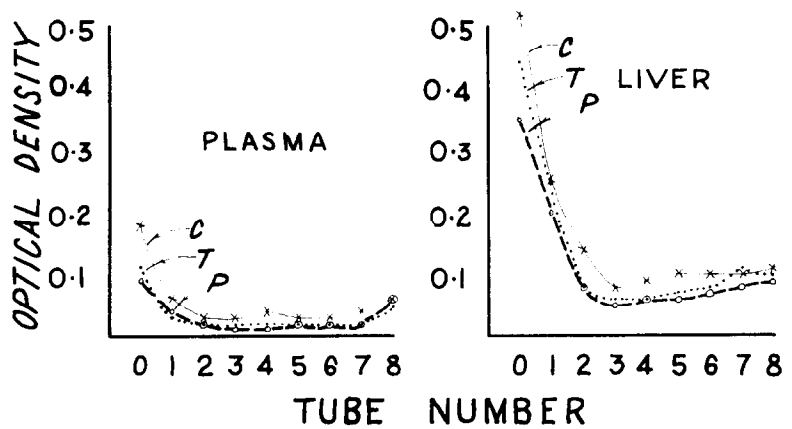

FIG. 6. Countercurrent distribution analyses of plasma and liver acetone extracts as in previous two figures except 30 min after BSP administration.

small intestine. The countercurrent analyses indicated no demonstrable differences between the groups for the 7-, 15-, and 30-min periods. The tissue blanks were high, and inability to find differences meant either that secretion into bile was not particularly enhanced or that the technique was not sufficiently sensitive for detecting differences that might have existed.

DISCUSSION

Various drugs have been shown to enhance the disappearance of BSP from the plasma. This phase was preceded in some cases by an inhibitory phase; in other cases no initial inhibitory phase was seen. The phase of enhanced disappearance occurred about 24 hr after pretreatment with the agents and was detected by the significantly lower concentrations of BSP-like material (at 15 min) in the plasma from pretreated

mice. Since the method of Kutob and Plaa[4] measured total BSP content (BSP plus metabolites), it was necessary to assess more specifically the nature of the material being measured. Countercurrent analyses performed in the solvent system described by Thomas and Plaa[9] for their differential extraction procedure, demonstrated that the pretreatment had enhanced the disappearance of the parent compound, BSP. Analyses of the liver and plasma at 7, 15, and 30 min indicated that the effect was obtained through a hepatic mechanism. At 7 min the concentration of BSP and metabolite appeared to be higher in the livers of the drug-treated group; at 15 min the BSP content of livers of the control group appeared higher, and at 30 min no difference existed between the groups. These results suggest that an increase in liver function had been produced by the drug pretreatment. Since the procedures used in the present study to measure liver function did not sharply delineate factors of uptake, storage, biotransformation, and secretion, it was not clear how the enhanced disappearance of BSP was brought about, even though the one factor, uptake, was increased. Thus these data are of value primarily in establishing that an increased removal of BSP had been produced by a mechanism involving the liver. Other techniques will have to be utilized to study the hepatic mechanisms.

The time course of the effect and the finding that ethionine blocked the effect suggest an adaptive type of response similar to that produced by these agents on the microsomal fraction of the liver. However, the results cannot be explained by the effect on the microsomal enzyme system because BSP is not metabolized in major part by the microsomal fraction.[3] Although glucuronide conjugation may be demonstrated *in vitro* with liver microsomes,[10] no such metabolic product of BSP has been identified in quantity *in vivo*.

Many of these agents were shown to produce a diphasic effect on hexobarbital action corresponding to an initial inhibition and subsequent stimulation of hexobarbital metabolism.[2] The probability was high that an inhibitor of hexobarbital metabolism would also be a stimulator of the same system. These compounds in the present study that enhanced BSP removal stimulated hexobarbital metabolism (but not vice versa). However, the parallelism is poor when applied to the initial or inhibitory phases of the diphasic effect. For instance, SKF 525A and MPDC inhibit hexobarbital metabolism[2] but have no inhibitory effect on BSP clearance. Others such as nikethamide and PIH[2] inhibit both.

Hasselblatt and Hukuhara[11] reported the interesting finding that administration of tolbutamide to rabbits that were being infused intravenously with BSP led to a decrease in the blood levels of BSP. The tolbutamide by virtue of decreasing the binding of BSP to plasma proteins was thought to augment the removal of BSP by the liver. This direct effect of tolbutamide was not observed in our study, since, with the 1- and 6-hr pretreatment times when blood levels of tolbutamide should have been substantially high, no effect was seen. Also, such a mechanism could not explain the enhanced disappearance of BSP seen later in our study, since 24 hr after the administration of tolbutamide only a very small amount, if any, of tolbutamide would be present in the blood to affect binding of BSP to plasma proteins. Our results are therefore, better explained by the adaptive type of change discussed earlier.

Acknowledgement—The authors wish to acknowledge the technical assistance of Mr. Louis E. Estingoy and drugs from the following firms: Hynson, Westcott and Dunning; Lakeside Laboratories; Smith Kline and French; Upjohn; CIBA.

REFERENCES

1. A. H. CONNEY and J. J. BURNS, *Advanc. Pharmacol.*, **1**, 31 (1962).
2. D. M. SERRONE and J. M. FUJIMOTO, *Biochem. Pharmacol.* **11**, 609 (1962).
3. B. COMBES and G. S. STAKELUM, *J. clin. Invest.* **40**, 981 (1961).
4. S. D. KUTOB and G. L. PLAA, *J. appl. Physiol.* **17**, 123 (1962).
5. J. M. FUJIMOTO, K. B. PEARCE and G. L. PLAA, *J. Pharmacol. exp. Ther.* **129**, 139 (1960).
6. H. REMMER, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **235**, 279 (1959).
7. H. REMMER, in *Enzymes and Drug Action*, J. L. MONGAR and A. V. S. DE REUCK, Eds., Little, Brown, Boston (1962).
8. J. M. FUJIMOTO and G. L. PLAA, *J. Pharmacol. exp. Ther.* **131**, 282 (1961).
9. M. C. THOMAS and G. L. PLAA, *Amer. J. clin. Path.* **34**, 488 (1960).
10. A. K. BROWN and H. BURNETT, *Amer. J. Dis. Child.* **94**, 510 (1957).
11. A. HASSELBLATT and T. HUKUHARA, *Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak.* **243**, 307 (1962).