REFERENCES

1. D. S. ZAHARKO and R. L. DEDRICK, *Proc. Fifth Int. Congr. Pharmac.*, Vol. 3, p. 316. Karger, Basel (1973).
2. J. FOLKMAN and V. H. MARK, *Trans. N.Y. Acad. Sci.* **30,** 1187 (1968).
3. V. SCHMIDT, W. ZAPOL, W. PRENSKY, T. WONDERS, I. WODINSKY and R. KITZ, *Trans. Am. Soc. artif. internal Organs* **18,** 45 (1972).
4. E. R. GARRETT and P. B. CHEMBURKAR, *J. pharm. Sci.* **57,** 944 (1968).
5. E. R. GARRETT and P. B. CHEMBURKAR, *J. pharm. Sci.* **57,** 949 (1968).
6. E. R. GARRETT and P. B. CHEMBURKAR, *J. pharm. Sci.* **57,** 1401 (1968).
7. A. C. TANQUARY and R. E. LACEY (Eds.), *Controlled Release of Biologically Active Agents*, Adv. Exp. Med. Biol. Ser., Plenum Press, New York (in press).
8. G. BENAGIANO, M. ERMINI, C. C. CHANG, K. SUNDARAM and F. A. KINCL, *Acta endocr. Copenh.* **63,** 29 (1970).
9. D. G. LIEGLER, E. S. HENDERSON, M. A. HAHN and V. T. OLIVERIO, *Clin. Pharmac. Ther.* **10,** 849 (1969).
10. W. S. HOFFMAN, *The Biochemistry of Clinical Medicine*, 3rd Edn., p. 8. Year Book Medical Publishers, Chicago (1964).
11. D. S. DITTMER (Ed.) *Blood and Other Body Fluids*, pp. 35–36. Federation of American Societies for Experimental Biology, Washington, D.C. (1961).
12. T. FUJII and H. C. THOMAS, *J. phys. Chem., Ithaca* **62,** 1566 (1958).
13. J. R. BERTINO and G. A. FISCHER, *Meth. med. Res.* **10,** 297 (1964).
14. K. B. BISCHOFF, R. L. DEDRICK, D. S. ZAHARKO and J. A. LONGSTRETH, *J. pharm. Sci.* **60,** 1128 (1971).
15. D. S. ZAHARKO, H. BRUCKNER and V. T. OLIVERIO, *Science, N.Y.* **166,** 887 (1969).
16. D. S. ZAHARKO and V. T. OLIVERIO, *Biochem. Pharmac.* **19,** 2923 (1970).

Biochemical Pharmacology, Vol. 23, pp. 2461–2464. Pergamon Press, 1974. Printed in Great Britain.

Effects of reserpine pretreatment on microsomal enzyme activity*

(*Received* 4 *December* 1973; *accepted* 15 *February* 1974)

THE METABOLISM of reserpine by the liver has long been thought to be largely due to a hydrolytic cleavage involving a non-specific esterase.[1,2] Recently, however, an NADPH, oxygen-dependent mixed function oxidase also has been implicated as an intermediate step in reserpine's biotransformation.[3,4] Since reserpine appears to be a substrate for the microsomal system, it seems reasonable to assume that it may also have the ability to induce microsomal enzymes. This supposition is supported by the observation[5] that, after both large and small doses of reserpine, there is a prominent increase in the amount of hepatic smooth surfaced endoplasmic reticulum. It is the purpose of the present study to describe some of the effects produced by short-term reserpine administration on hepatic microsomal drug metabolism.

Male Wistar rats (175–200 g), albino rabbits (1 to 1·5 kg) and guinea pigs (350–500 g) were used in this study. All animals were maintained on and had free access to food and water. For metabolism studies *in vitro*, animals were sacrificed by stunning followed by exsanguination. Their livers were removed, weighed and homogenized in ice-cold 1·15% KCl. Microsomes were prepared by differential centrifugation according to methods already described.[6] Reaction mixtures of 5-ml vol. were incubated in a Dubnoff metabolic shaker at 37° with air as the gaseous phase. Each 5-ml reaction mixture contained the following constituents: nicotinamide adenine dinucleotide phosphate, 2·0 μmoles; glucose 6-phosphate, 25 μmoles; glucose 6-phosphate dehydrogenase, 2·0 units; nicotinamide, 20 μmoles; magnesium chloride, 25 μmoles; and the microsomes derived from 333 mg liver. The pH of the incubation mixtures was adjusted to 7·4 with 0·1 M phosphate buffer. The pathways studied, the method of assay and the amount of substrate added were: side-chain oxidation of hexobarbital,[7] 3·0 μmoles; and the aromatic hydroxylation of aniline,[8] 10 μmoles. Microsomal protein was determined by the method of Lowry *et al.*[9] using a Technicon auto-analyzer.

* This work was supported by funds from the U.S. Public Health Service (GM 16433 and Research Career Development Award 1 K04 GM 12522).

Single injections of reserpine (0·5–5·0 mg/kg, i.p.) were administered to rats, and 24 hr later changes in several hepatic parameters were measured. Doses of reserpine above 0·5 mg/kg resulted in a 25 per cent reduction of body weight within 24 hr; however, no changes were observed either in liver/body weight ratios or in the concentration of microsomal protein/g of liver (Table 1). Hexobarbital metabolism was increased 24 hr after reserpine only at the 2·5 mg/kg dose, while the rate of aniline *p*-hydroxylation was elevated at 1·0, 2·5 and 5·0 mg/kg. Metabolic rates were elevated irrespective of whether the data were expressed on the basis of mg protein or total liver. The effect of reserpine on hexobarbital oxidation may be merely a random observation, since it could not be reproduced later in this study.

When daily doses of reserpine (2·5 mg/kg, i.p.) were given for up to 4 days, much more profound effects were seen (Table 2). Body weight again decreased as early as 1 day after drug administration and continued to fall until after 4 days of treatment the average animal weight was only 62 per cent of control. Further

TABLE 1. EFFECTS OF SINGLE DOSES OF RESERPINE ON MICROSOMAL DRUG METABOLISM IN THE RAT

Dose (mg/kg)	N	Body wt (g)	LW/BW*	Microsomal protein (mg/g)	Hexobarbital (nmoles/mg protein/15 min)	Aniline
Control	11	190 ± 6	4·4 ± 0·2	23·6 ± 0·8	221 ± 28	33 ± 2
0·5	11	186 ± 7	4·5 ± 0·1	21·3 ± 0·9	226 ± 31	37 ± 3
1·0	7	166 ± 5†	4·2 ± 0·2	22·1 ± 1·2	215 ± 6	46 ± 2†
2·5	11	174 ± 7†	4·0 ± 0·2	24·3 ± 0·7	300 ± 19†	61 ± 4†
5·0	9	155 ± 6†	4·4 ± 0·1	23·2 ± 1·6	192 ± 40	49 ± 4†

* Liver wt/100 g body wt. All values in the table are mean ± S. E. M.
† $P < 0·05$.

TABLE 2. EFFECTS OF DAILY DOSES OF RESERPINE (2·5 mg/kg) ON MICROSOMAL DRUG METABOLISM IN THE RAT

Days of treatment	N	Body wt (g)	LW/BW*	Microsomal protein (mg/g)	Hexobarbital (nmoles/mg protein/15 min)	Aniline
0	14	190 ± 7	4·6 ± 0·1	22·1 ± 0·9	221 ± 23	31 ± 2
1	13	159 ± 5†	4·4 ± 0·2	22·5 ± 0·8	197 ± 22	43 ± 3†
2	12	147 ± 7†	4·4 ± 0·2	21·3 ± 1·0	187 ± 20	46 ± 3†
3	15	151 ± 7†	3·8 ± 0·2†	19·3 ± 1·2†	230 ± 20	51 ± 3†
4	3	118 ± 6†	2·9 ± 0·1†	10·1 ± 1·0†	273 ± 95	29 ± 1

* Liver wt/100 g body wt. All values in the table are mean ± S. E. M.
† $P < 0·05$.

treatment resulted in unacceptable increases in animal mortality. Both liver to body weight ratios and concentration of microsomal protein were reduced after 3 days of reserpine injections. Hexobarbital oxidation was unchanged during drug treatment, while aniline metabolism increased on days 1–3 and then dropped to slightly below control values 24 hr after the fourth daily dose of reserpine.

Daily reserpine injections (2·5 mg/kg) were also given to rabbits and guinea-pigs in order to see if the previously observed increase in aniline metabolism produced by reserpine was species specific. Both species were much more sensitive to reserpine, and therefore no more than two successive days of treatment at 2·5 mg/kg was used. Rabbits and guinea-pigs increased their rates of aniline *p*-hydroxylation after reserpine (Table 3). Rabbits required 2 days of treatment, while guinea-pigs showed enhanced aniline metabolism after a single reserpine injection. The peak percentage increases in aniline metabolism observed were 23 and 57 per cent for rabbits and guinea-pigs, respectively (Table 3), while the maximum activity increase in rats was 65 per cent (Table 2).

When appropriate dosage schedules are used, both single and short-term daily injections of reserpine can result in increases in aniline metabolism in rats. Under the same conditions, hexobarbital metabolism is only minimally affected. This increase does not appear to be species specific, since aniline metabolism in rabbits and guinea pigs was also augmented after reserpine administration. Rats showed the greatest increase in aniline hydroxylation, followed by guinea-pigs and rabbits.

Reserpine treatment produced losses in body weight, liver weight and/or microsomal protein, which are particularly marked when larger doses are given for several days. The diarrhea and fluid loss which usually

TABLE 3. EFFECT OF DAILY DOSES OF RESERPINE (2·5 mg/kg) ON ANILINE METABOLISM IN THE RABBIT AND GUINEA-PIG

Species	Days of treatment	N	Aniline*
Rabbit			
	0	4	23·0 ± 1·7
	1	4	27·1 ± 3·4
	2	3	28·6 ± 1·8†
Guinea-pig			
	0	6	29·1 ± 1·9
	1	4	45·7 ± 5·3†
	2	4	39·7 ± 4·0†

* Nmoles metabolized/mg of protein/15 min.
† $P < 0.05$.

accompanied reserpine treatment, and the observed decrease in food consumption by these animals, suggest that the altered metabolism may be due to some form of physiologic stress. This stress could result either from starvation or from a specific activation of the pituitary–adrenal axis. The latter explanation seems the less likely, since Furner and Stitzel[10] have demonstrated that stress can bring about changes in drug metabolism independent of the presence of an intact adrenal gland, and Cushman and Hilton[11] reported no direct effect of reserpine on the adrenal cortex.

Increased aniline metabolism secondary to a reserpine-induced starvation seems to be a more likely possibility. Both reserpine administration and starvation result in similar histologic changes in hepatocytes.[5] These include the appearance of two types of cytoplasmic lipid droplets, alterations in mitochondrial structure and, perhaps most importantly, prominent increases in smooth surfaced endoplasmic reticulum. The latter structure has, of course, been shown to be the subcellular component most directly involved in microsomal drug oxidation. The finding that pair-fed (i.e. similarly starved) and reserpine-treated animals showed similar alterations in smooth endoplasmic reticulum[5] suggests that most of reserpine's effects are due to an enforced starvation. Further support for maintaining that the effects of reserpine are mediated secondarily to starvation is found in the studies of Kato and Gillette,[8] who showed that, when male rats were starved for 72 hr, aniline but not hexobarbital metabolism was enhanced.

The increased microsomal metabolism produced by reserpine appears to resemble more closely that caused by polycyclic hydrocarbons than it does that of the phenobarbital-type of inducing agent. Both polycyclic hydrocarbons and reserpine, but not phenobarbital, are able to increase microsomal enzyme activity after a single injection *in vivo*. Furthermore, both reserpine and the polycyclic hydrocarbons appear to stimulate aniline more than hexobarbital biotransformation. Whether or not reserpine interacts with microsomal P-450 in a similar manner to that of the polycyclic compounds is yet to be determined.

Perhaps it should not have been totally unexpected to find that reserpine administration can alter the activity of drug-metabolizing enzymes, since reserpine's interactions with microsomal enzymes have been reported recently. Reserpine can both be metabolized[3] by and serve as a competitive inhibitor[4] for hepatic drug-metabolizing enzymes. Although the mechanism by which reserpine causes these changes is still unclear, the present study provides evidence that this drug, which is used clinically both chronically and in combination with other agents, has the potential for altering the duration and intensity of action of a co-administered drug.

Acknowledgement—The excellent technical assistance of Ms. Jane Dean was greatly appreciated.

ROBERT E. STITZEL

Department of Pharmacology,
West Virginia University Medical Center,
Morgantown, W. Va. 26506, *U.S.A.*

REFERENCES

1. H. SHEPPARD and W. H. TSIEN, *Proc. Soc. exp. Biol. Med.* **90,** 437 (1955).
2. H. SHEPPARD, W. H. TSIEN, E. B. SIGG and A. J. PLUMMER, *Archs int. Pharmacodyn. Thér.* **113,** 160 (1957).
3. R. E. STITZEL, L. A. WAGNER and R. J. STAWARZ, *J. Pharmac. exp. Ther.* **182,** 500 (1972).

4. R. J. STAWARZ and R. E. STITZEL, *Pharmacology*, in press.
5. W. B. WINBORN and L. L. SEELIG, *Lab. Invest.* **23**, 216 (1970).
6. J. S. MCCARTHY and R. E. STITZEL, *J. Pharmac. exp. Ther.* **176**, 772 (1971).
7. J. R. COOPER and B. B. BRODIE, *J. Pharmac. exp. Ther.* **114**, 408 (1955).
8. R. KATO and J. R. GILLETTE, *J. Pharmac. exp. Ther.* **150**, 279 (1965)
9. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
10. R. L. FURNER and R. E. STITZEL, *Biochem. Pharmac.* **17**, 121 (1968).
11. P. CUSHMAN and J. G. HILTON, *J. Endocr.* **31**, 181 (1965).

Biochemical Pharmacology, Vol. 23, pp. 2464–2466. Pergamon Press, 1974. Printed in Great Britain.

Hormone and adenosine 3′, 5′ cyclic monophosphate stimulated phosphorylation of human erythrocyte membranes

(*Received* 8 *October* 1973; *accepted* 6 *March* 1974)

ADENOSINE 3′, 5′ cyclic monophosphate (cyclic AMP) has now been implicated in a large number of control mechanisms in the cell.[1] The widespread distribution of cyclic AMP-dependent protein kinases led Kuo and Greengard[2] to postulate that the primary function of cyclic AMP was to stimulate phosphorylation of specific proteins. *In vitro* a variety of proteins serve as substrate for cyclic AMP-dependent protein kinases: histones,[3] phosphorylase kinase,[4] ribosomal proteins[5] and troponin.[6] However with the exception of phosphorylase kinase[4] the cyclic AMP stimulated phosphorylation has not been associated with any specific modulation of biological activity.

We therefore decided to study the cyclic AMP-stimulated phosphorylation of the erythrocyte membrane and its possible role in the regulation of cation transport in the intact cell.

MATERIALS AND METHODS

ATP and cyclic AMP were purchased from Sigma (London) Chemicals Co. Ltd, London, S.W.6, U.K. and N^6, O^2-dibutyryl cyclic AMP from Boehringer Corp., London Ltd, London, W.5, U.K. Acetyl choline and noradrenaline were supplied by Koch-Light Laboratories Ltd, Colnbrook, Bucks, U.K. and theophylline by BDH (Chemicals) Ltd, Poole, Dorset, U.K. The sodium salt of (γ-^{32}P) ATP (2200–3000 mCi/m-mole) and [^{32}P] Pi orthophosphate (30 Ci/g phosphorus) were purchased from the Radiochemical Centre, Amersham, Bucks., U.K. Prostaglandin E_1 and E_2 were kindly supplied by Dr. J. E. Pike, The Upjohn Co., Kalamazoo, Michigan 49001, U.S.A. and persantin, RA 233 and RA 433 were a generous gift from Boehringer Ingelheim Ltd, Isleworth House, Great West Road, Isleworth, Middlesex, U.K.

The erythrocytes were prepared from recently expired acid–citrate–dextrose blood supplied by the Clinical Pathology Department, Manchester Royal Infirmary. Fresh human blood was obtained from local volunteers by venepuncture and fresh rat blood by cardiac puncture. Citrate was the anti-coagulant when fresh blood was taken.

Erythrocyte membranes were prepared by washing the ghosts six times with hypothonic sodium phosphate buffer as previously described by Duffy and Schwarz[7] except no EDTA was included in the washing buffers. Phosphorylation of the isolated membranes was carried out by the method of Duffy and Schwarz.[8] For the phosphorylation of intact erythrocyte membranes, red blood cells were allowed to synthesize their own [γ-^{32}P] ATP from [^{32}P] Pi (1 μCl/ml whole blood) added to fresh whole blood. The incubation was carried out at 37° for 45 min in a shaking water bath. The erythrocytes were freed of extracellular [^{32}P] Pi, plasma and white cells by washing four times with ice cold isotonic choline chloride. They were then suspended in an isotonic solution of the following composition: NaCl 135 mM, KCl 5·0 mM, $MgCl_2$ 1·0 mM, $CaCl_2$ 1mM, Na_2HPO_4 2·5 mM and glucose 11 mM. The dibutyryl derivative of cyclic AMP (1 mM) was added and incubation was carried out at 37° for 45 min. The reaction was terminated by the addition of 60 mOsm ice cold sodium phosphate buffer (pH 7·2). The membranes were then prepared as described[7] except that ice cold washing buffers were used and 1 mM carrier ATP was added to the last two washes. Protein was measured by the method of Lowry *et al.*,[9] using crystalline bovine serum albumin as standard.