for the rapid accumulation of DA within the nerve terminals of the striatum observed when the drug is administered *in vivo*.

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REFERENCES

1. R. H. ROTH and N. J. GIARMAN, *Biochem. Pharmac.* **19**, 1087 (1970).
2. G. L. GESSA, L. VARGIU, F. CRABAI, G. C. BOERO, F. CUBONI and R. CAMBA, *Life Sci.* **5**, 1921 (1966).
3. R. H. ROTH and Y. SURH, *Biochem. Pharmac.* **19**, 2001 (1970).
4. N. J. GIARMAN and K. F. SCHMIDT, *Br. J. Pharmac. Chemother.* **20**, 563 (1963).
5. R. H. ROTH, *Int. encyclopedia of pharmacology and therapeutics*, Chap. 18, Pergamon Press, Oxford (1972).
6. N.-E. ANDÉN, A. DAHLSTRÖM, K. FUXE, K. LARSSON, L. OLSEN and U. UNGERSTEDT, *Acta physiol. Scand.* **67**, 313 (1966).
7. P. F. SPANO, A. TAGLIAMONTE, P. TAGLIAMONTE and G. L. GESSA, *J. Neurochem.* **18**, 1831 (1971).
8. J. E. HARRIS and R. H. ROTH, *Molec. Pharmac.* **7**, 543 (1971).
9. M. J. BESSON, A. CHERAMY and J. GLOWINSKI, *J. Pharmac. exp. Ther.* **177**, 196 (1971).
10. R. H. ROTH and E. A. STONE, *Biochem. Pharmac.* **17**, 1581 (1968).
11. S. SPECTOR, A. SJOERDSMA and S. UDENFRIEND, *J. Pharmac. exp. Ther.* **147**, 86 (1965).
12. S. UDENFRIEND, in *Fluorescence assay in biology and medicine* (Eds. N. O. KAPLAN and H. A. SCHERAGA) p. 129. Academic Press, New York (1962).
13. P. W. K. WONG, M. E. O'FLYNN and T. INOUYE, *Clin. Chem.* **10**, 263 (1961).
14. J. T. COYLE and S. H. SNYDER, *J. Pharmac. exp. Ther.* **170**, 221 (1969).
15. M. J. KUHAR, R. H. ROTH and G. K. AGHAJANIAN, *J. Pharmac. exp. Ther.* **181**, 36 (1972).
16. G. BUSTOS and R. H. ROTH, *Br. J. Pharmac.* **44**, 817 (1972).
17. J. R. WALTERS and R. H. ROTH, *Biochem. Pharmac.*, **21**, 2111–2121 (1972).
18. R. H. ROTH, *Eur. J. Pharmac.* **15**, 52 (1971).
19. J. R. WALTERS, G. AGHAJANIAN and R. H. ROTH, *Proc. Int. Pharmacological Meetings*, in press.

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Effect of puromycin on binding and on metabolism *in vitro* of substrates by rat liver microsomes

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THE ADMINISTRATION of puromycin, an inhibitor of protein synthesis, prevents drug-induced increases in microsomal enzyme activity.[1–3] The mechanism of this effect is thought to be due to puromycin interaction with the ribosome-messenger RNA complex leading to the release of incomplete protein chains from the ribosomes. Since puromycin is demethylated by rat liver microsomes,[4] it also could be expected to act as an alternate substrate affecting the metabolism of other drugs.[5] Gelboin and Blackburn[2] reported that treatment of rats with puromycin did not alter the basal level of benzypyrene hydroxylase in several tissues. However, data presented by Alvares *et al.*[6] indicate that puromycin administered hourly by i.p. injections in a dose of 20 mg/kg, caused a slight decrease in V_{max} of benzypyrene hydroxylase of rat liver microsomes.

The purpose of the research presented in this communication was to characterize the reaction of puromycin with rat liver microsomes, to investigate the effects of puromycin on the binding of sub-

strates to microsomal cytochrome P-450 and to relate these findings with its effects on the metabolism *in vitro* of microsomal enzyme substrates.

Puromycin dihydrochloride was purchased from Nutritional Biochemicals Company (Cleveland, Ohio). β-Diethylaminoethyldiphenylpropylacetate (SKF 525-A) was obtained through the courtesy of Smith, Kline & French Laboratories.

Preparation of microsomes. Male Sprague–Dawley rats weighing 215–300 g were decapitated without anesthesia, their livers removed, chilled, blotted, weighed and homogenized in 3 vol. of ice cold 0·15 M potassium chloride (KCl) using a motor driven Teflon pestle glass homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min at 0–4° in a Sorvall model RC-2B centrifuge. After carefully aspirating the floating fat layer, the underlying supernatant fraction was decanted and centrifuged at 105,000 *g* for 1 hr at 0–4° in a Beckman model L2-65 ultracentrifuge. The resulting microsomal pellet was resuspended in fresh 0·15 M KCl, recentrifuged at 105,000 *g* for 1 hr and stored at −15° overnight. Thawed microsomes were resuspended in 0·15 M KCl and the protein content was determined by the method of Gornall *et al.*[7]

Binding studies. The binding of puromycin, aniline, SKF 525-A and hexobarbital to washed microsomes was determined by a modification of the method of Remmer *et al.*[8] using microsomes diluted to 1·75–2 mg protein/ml with 0·3 M phosphate buffer, pH 7·4. Five to seven serial 1-μl additions of substrate to the sample cuvette containing 3·0 ml of microsomes were made using a Hamilton syringe and repeating dispenser. Equal volumes of 0·3 M phosphate buffer were added to the reference cuvette. After each addition, the difference spectrum was recorded between 340 and 490 nm using the Aminco–Chance spectrophotometer in the split beam mode. When the effect of puromycin (modifier) upon the binding of other drugs (substrates) was to be determined, the modifier was added to both reference and sample cuvettes and after recording a base line, the substrate was added to the sample cuvette and the difference spectrum determined as described above. The apparent dissociation constant (K_s) and maximal absorbance (ΔA_{max}) of the various drugs were calculated by the method of Wilkinson[9] using an Olivetti–Underwood programma 101 computer. ΔA was determined by measuring the amplitudes of the peak to trough of the absorption spectrum.[10]

Enzyme assays. The incubation mixture contained the following: microsomal suspension, 1 ml; nicotinamide adenine dinucleotide phosphate (NADP), 5 μmoles; glucose 6-phosphate (G6P), 25 μmoles; glucose 6-phosphate dehydrogenase (G6PD), 2 units; magnesium sulfate, 25 μmoles; drug substrate, 0·5 ml; enough 0·1 M phosphate buffer, pH 7·4, to make a final volume of 5·0 ml. To determine the effect of puromycin on the metabolism *in vitro* of substrates, 0·5 ml of puromycin solution was substituted for 0·5 ml of phosphate buffer. Four or five concentrations of each substrate were used as follows: aniline (0·5, 0·2, 0·1 and 0·05 mM); hexobarbital (0·5, 0·4, 0·3, 0·2 and 0·1 mM). Mixtures were incubated for 30 min at 37° under air in a Dubnoff metabolic shaker at 120 oscillations/min. The aromatic hydroxylation of aniline was assayed by measuring *p*-aminophenol formed[11] and the side chain oxidation of hexobarbital was determined by measuring disappearance of substrate[12]. Appropriate recoveries and blanks were used in each determination. In the case of hexobarbital metabolism, incubation flasks containing puromycin (1 and 2 mM) were included in order to detect absorption at 245 and 280 nm which might be due to either puromycin or one of its metabolites. Apparent Michaelis–Menten constants (K_m) and V_{max} were calculated as described for K_s and ΔA_{max}.

Cytochrome P-450 content. The cytochrome P-450 content of hepatic microsomes (2·5 mg protein/ml suspended in 0·1 M phosphate buffer, pH 7·4) was determined by the method of Omura and Sato[13] using the Aminco–Chance recording spectrophotometer in the split beam mode.

Binding of puromycin to washed microsomes. Puromycin produces a type II difference spectrum when added to liver microsomes. The positions of the trough and peak were 392 and 428 nm respectively. Table 1 summarizes the spectral constants of puromycin binding to washed microsomes

TABLE 1. PUROMYCIN SPECTRAL CONSTANTS

Concentration of puromycin (mM)	K_s*† (mM)	ΔA_{max}/mg protein*†‡ ($\times 10^{-3}$)	ΔA_{max}/μmole P-450*†‡
0·024–0·168	0·060 ± 0·007	38·24 ± 1·79	22·45 ± 1·05
0·120–0·600	0·076 ± 0·013	42·60 ± 1·52	25·02 ± 0·89

* Values are mean ±S.E.

† Values obtained from 5–7 point double reciprocal plot.

‡ ΔA is the sum of amplitudes of the peak (428 nm) and trough (392 nm) of the difference spectra.

at two different concentrations. There was no significant difference between the K_s and ΔA_{max} calculated at these two levels.

Effect of puromycin on binding of other substrates. Puromycin at concentrations of 0·024 and 0·048 mM significantly decreased the ΔA_{max} of aniline (type II compound) without significantly affecting its K_s (Table 2). This effect of puromycin is analogous to that of a non-competitive inhibitor of an enzyme-substrate reaction. Puromycin at a concentration of 0·120 mM also non-competitively inhibited the binding of SKF 525-A, a type I compound (Table 2). However, 0·096 mM puromycin significantly increased the K_s of hexobarbital (type I compound) without affecting its ΔA_{max} (Table 2). This effect is analogous to that of a competitive inhibitor of an enzyme-substrate reaction.

TABLE 2. EFFECT OF PUROMYCIN ON SPECTRAL CONSTANTS OF ANILINE, SKF 525-A AND HEXOBARBITAL

Substrate	Modifier	K_s*† (mM)	A_{max}/mg protein*†‡ ($\times 10^{-3}$)
Aniline	None (control)	0·287 ± 0·043	31·85 ± 1·96
	Puromycin (0·024 mM)	0·300 ± 0·026	24·29 ± 0·93§
	Puromycin (0·048 mM)	0·397 ± 0·057	23·61 ± 1·73§
SKF 525-A	None (control)	0·0057 ± 0·0006	38·53 ± 0·89
	Puromycin (0·024 mM)	0·0063 ± 0·0010	35·17 ± 1·25
	Puromycin (0·120 mM)	0·0055 ± 0·0008	33·77 ± 1·00§
Hexobarbital	None (control)	0·112 ± 0·005	26·87 ± 0·31
	Puromycin (0·048 mM)	0·135 ± 0·013	26·61 ± 0·76
	Puromycin (0·096 mM)	0·143 ± 0·012‖	26·83 ± 0·58

* Values are mean ±S.E.
† Values obtained from 5–7 point double reciprocal plot.
‡ ΔA is the sum of amplitudes of the peak and trough of the difference spectra.
§ Value significantly different from control value ($P < 0·01$).
‖ Value significantly different from control value ($P < 0·05$).

Effect of puromycin on in vitro *drug metabolism.* Puromycin in a concentration of 1 mM significantly decreased the V_{max} of aniline metabolism but did not affect the apparent K_m for this reaction (Table 3).

TABLE 3. EFFECT OF PUROMYCIN ON KINETICS OF ANILINE AND HEXOBARBITAL METABOLISM

Substrate	Modifier	K_m*† (mM)	V_{max}*†
			(nmoles *p*-amino-phenol formed/mg protein/hr)
Aniline	None (control)	0·086 ± 0·009	35·80 ± 1·30
	Puromycin (0·1 mM)	0·089 ± 0·005	33·33 ± 0·61
	Puromycin (1 mM)	0·073 ± 0·008	28·0 ± 0·9‡
			(nmoles/metab/mg protein/hr)
Hexobarbital	None (control)	0·348 ± 0·050	303·23 ± 23·35
	Puromycin (1 mM)	0·412 ± 0·075	209·91 ± 21·24§
	Puromycin (2 mM)	1·044 ± 0·171‡	357·27 ± 43·81

* Values are mean ±S.E.
† Values obtained from a 4 or 5 point double reciprocal plot.
‡ Value significantly different from control value ($P < 0·01$).
§ Value significantly different from control value ($P < 0·05$).

The inhibitory effect appears to be non-competitive. The V_{max} of hexobarbital metabolism was significantly reduced by 1 mM puromycin. On the other hand, a higher concentration of puromycin (2 mM) significantly increased the K_m of hexobarbital without affecting the V_{max} (Table 3).

In the present study, puromycin has been shown to induce a type II difference spectrum when added to liver microsomes. The spectral dissociation constant (K_s) which is regarded as a measure of the affinity of a substrate for cytochrome P-450[14] was found to be 0·06–0·08 mM for puromycin. Therefore, puromycin can be considered as a microsomal binder of moderate affinity, falling in between the high affinity binders such as SKF 525-A (K_s = 0·005 mM) and the weak binders such as aniline (K_s = 0·286 mM). Supporting this classification of puromycin as a moderate binder is the observation that the difference spectrum of aniline was easily affected by low concentrations of puromycin while higher concentrations of puromycin were needed to alter the difference spectra of hexobarbital and SKF 525-A.

Schenkman *et al.*[15] proposed that the binding of substrates to microsomal hemoprotein precedes enzymatic conversion of the substrate and that the appearance of a spectral change therefore may be considered as evidence for the formation of an enzyme–substrate complex. These authors showed that the Michaelis constants of hexobarbital and aminopyrine were nearly the same as their spectral dissociation constants. A similar correlation between difference spectra and drug metabolism was shown for a series of barbiturates by Topham.[16] In the present study, puromycin affected the binding and the metabolism *in vitro* of aniline in the same manner. Puromycin decreased the ΔA_{max} and the apparent V_{max} of aniline hydroxylase without affecting the K_s or K_m. However, such correlation was not present in the case of hexobarbital. Puromycin increased the K_s of hexobarbital without affecting ΔA_{max} but exerted a dual action on hexobarbital metabolism. Low concentrations of puromycin acted as a non-competitive inhibitor of hexobarbital metabolism while higher concentrations showed competitive inhibition. In addition, the spectral dissociation constant of puromycin (0·06–0·08 mM) is one-fifth the apparent Michaelis constant for the demethylation of puromycin (K_m = 0·355 mM) as reported by Mazel *et al.*[4]

Since aniline, a type II compound, displaces CO from the CO-cytochrome P-450 complex,[15] it has been suggested that substrates which produce type II spectral change interact with the iron at the CO-binding site of the heme. The interaction of type I compounds is supposed to take place at a different site since addition of hexobarbital does not displace CO. The observation by Chaplin and Mannering[17] that the digestion of microsomes with phospholipase C destroys type I binding without decreasing type II binding provides further evidence that the two binding sites differ and support the concept that type I binding site is closely associated with membrane phospholipids. Since puromycin was shown to induce a type II spectrum, it is expected that puromycin binds to the CO-binding site of cytochrome P-450 in a way similar to that of aniline. It would also be expected that aniline and puromycin will compete for the same binding site. However, results obtained in this study show that puromycin inhibits the binding of aniline in a non-competitive fashion. On the other hand, puromycin affected the binding of two type I compounds namely hexobarbital and SKF 525-A in completely different ways. These results associated with the results obtained in the metabolism studies *in vitro* suggest that puromycin might be binding to another site in addition to the CO-binding site of cytochrome P-450. This site could be the ribonucleic acid associated with microsomal membranes (Membron)[18] since puromycin is known to bind to ribosomes.[19]

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REFERENCES

1. A. H. CONNEY and A. G. GILMAN, *J. biol. Chem.* **238,** 3682 (1963).
2. H. V. GELBOIN and N. R. BLACKBURN, *Cancer Res.* **24,** 355 (1964).
3. S. ORRENIUS, J. E. ERICSSON and L. ERNSTER, *J. Cell Biol.* **25,** 627 (1964).
4. P. MAZEL, A. KERZA-KWIATECKI and J. SIMANIS, *Biochim. biophys. Acta* **114,** 72 (1966).
5. M. W. ANDERS, *A. Rev. Pharmac.* **11,** 37 (1971).
6. A. P. ALVARES, G. SCHILLING and R. KUNTZMAN, *Life Sci.* **10,** 129 (1971).
7. A. G. GORNALL, C. J. BARDAWILL and M. M. DAVID, *J. biol. Chem.* **177,** 751 (1949).
8. H. REMMER, J. SCHENKMAN, R. W. ESTABROOK, H. SASAME, J. R. GILLETTE, S. NARISIMHULU, D. Y. COOPER and O. ROSENTHAL, *Molec. Pharmac.* **2,** 187 (1966).

9. G. N. WILKINSON, *Biochem. J.* **80,** 324 (1961).
10. K. C. LEIBMAN and R. W. ESTABROOK, *Molec. Pharmac.* **7,** 26 (1971).
11. R. KATO and J. R. GILLETTE, *J. Pharmac. exp. Ther.* **150,** 279 (1965).
12. J. R. COOPER and B. B. BRODIE, *J. Pharmac. exp. Ther.* **114,** 409 (1955).
13. T. OMURA and R. SATO, *J. biol. Chem.* **239,** 2370 (1964).
14. H. REMMER, R. W. ESTABROOK, J. B. SCHENKMAN and H. GREIM, *Naunyn-Schmiedebergs Arch, exp. Path. Pharmak.* **259,** 98 (1968).
15. J. B. SCHENKMAN, H. REMMER and R. W. ESTABROOK, *Molec. Pharmac.* **3,** 113 (1967).
16. J. C. TOPHAM, *Biochem. Pharmac.* **19,** 1695 (1970).
17. M. D. CHAPLIN and G. J. MANNERING, *Molec. Pharmac.* **6,** 631 (1970).
18. H. C. PITOT, N. SLADEK, W. RAGLAND, R. K. MURRAY, G. MOYER, H. D. SOLING and J. P. JOST in *Microsomes and drug oxidations* (Eds. J. R. GILLETTE, A. H. CONNEY, G. J. COSMIDES, R. W, ESTABROOK, J. R. FOUTS and G. J. MANNERING), p. 59. Academic Press, New York (1969).
19. S. PESTKA, *Cold Spring Harb. Symp. Quant. Biol.* **34,** 395 (1969)

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Salicylate poisoning—Effect on 2, 3-diphosphoglycerate levels in the rat

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SALICYLATE is known to inhibit enzymes at several steps in erythrocyte glycolysis.[1–3] 2,3-Diphosphoglycerate (2,3-DPG) is a product of erythrocyte glycolysis found in many mammalian species, and its concentration in the erythrocyte has been found to have an important effect on the affinity of hemoglobin for oxygen.[4,5] Decreased levels of 2,3-DPG have been shown to increase this affinity, and this in turn may impede delivery of oxygen to tissues.[6] For this reason, the present study was designed to study the effect of large doses of salicylate on levels of erythrocyte 2,3-DPG.

Male Sprague–Dawley rats (mean body wt, 270 g) were given sodium salicylate (100 mg/ml) intraperitoneally in doses of either 400, 500 or 600 mg/kg. A fourth group run concurrently with the others was given an equivalent volume of isotonic saline. No anesthesia was used, and the animals were not disturbed between procedures. They had access to food and water *ad lib.* Blood was obtained by amputation of the tail tip just before and approximately 24 hr after the administration of the sodium salicylate. In three of the animals given 600 mg/kg of sodium salicylate, blood was also obtained at 4 hr. Analyses were performed for 2,3-DPG by a chromotropic acid method[7] as follows. Heparinized packed red blood cells were washed four times in iced isotonic saline and then mixed with 2 vol. of 6·7% trichloroacetic acid per volume of packed red cells. The samples were kept frozen for up to 7 days and after defrosting, mixing and centrifugation, 0·1 ml of the supernatant was mixed with 4 ml of a solution containing 10 mg of chromotropic acid (Eastman Organic Chemicals) per 100 ml of concentrated sulfuric acid and incubated in a boiling water bath for 135 min. A reagent blank of 0·1 ml H_2O and standards of 0·1 ml with a concentration of 1, 2 and 3 μmoles/ml of 2,3-diphosphoglyceric acid (Sigma Chemical Company) were run simultaneously with the unknown samples. Standards and the unknown samples were analyzed in duplicate. The optical densities at 695 nm were determined in a Coleman model 6D spectrophotometer, the standards graphed, and the unknown values were read from the resulting curve. The sample of whole blood was also analyzed for hemoglobin concentration and hematocrit, and the mean corpuscular hemoglobin concentration (MCHC) was calculated as previously described.[8] The concentration of 2,3-DPG in the unknown sample was multiplied by three to correct for dilution with the trichloroacetic acid and then divided by the MCHC and multiplied by 100 to give the concentration of DPG in micromoles per gram of hemoglobin. Salicylate concentration in serum was determined by Trinder's technique.[9] Two of the nine rats given 500 mg/kg and two of fourteen given 600 mg/kg of sodium salicylate died before the second blood sample was obtained.

The results are presented in Table 1. At 24 hr after the administration of sodium salicylate, there