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EFFECT OF SULPHONYLUREAS (TOLAZAMIDE, TOLBUTAMIDE AND CHLORPROPAMIDE) ON THE METABOLISM OF DIPHENYLHYDANTOIN IN THE RAT

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Abstract—The blood sugar lowering sulphonylureas chlorpropamide, tolazamide and tolbutamide showed competitive inhibition of diphenylhydantoin hydroxylation by the 9000 *g*-supernatant of rat liver homogenates. Tolbutamide was the strongest inhibitor and chlorpropamide the weakest. The enzyme kinetics could not be presented satisfactory by Lineweaver–Burk plots; therefore log–dose response curves were used and the potency of the inhibitors was calculated according to the method of Arunlakshana and Schild. The relative inhibitory potency of the three sulphonylureas chlorpropamide–tolazamide–tolbutamide was 1:2·5:4·3.

SEVERAL species, including man[1] and rat[2] metabolize diphenylhydantoin (DPH) mainly by ring hydroxylation in the para-position to 5-(*p*-phenyl)-5 phenylhydantoin (5-HPPH). This metabolism can be influenced by several drugs. Phenobarbital (in man[3]), hydroxyzine (in mice[4]) and lynestrol (in mice[5]) are known to stimulate DPH metabolism, while phenothiazine derivatives, isoniazid and many others are reported to inhibit it.[6] Though DPH itself stimulates the metabolism of dicoumarol,[7] DPH intoxication, probably due to inhibition of metabolism, was observed in dicoumarol-treated patients.[8]

Pannekoek[9] observed a patient who developed signs of DPH intoxication after concurrent administration of tolazamide; the high level of DPH in blood decreased after tolazamide was replaced by insulin, while the same DPH dose was maintained.

Tolazamide, a sulphonylurea is a blood sugar lowering agent; it is partly ring hydroxylated in the rat liver to *p*-hydroxyltolazamide.[10] It seemed possible that these drugs might interfere with each others metabolic inactivation and this led us to study the metabolism of DPH in rats in the presence of tolazamide. We have also investigated the influence of two other sulphonylureas—tolbutamide, which is hydroxylated at the phenyl-ring,[10,11] and chlorpropamide, which is not ring hydroxylated.[12,13]

MATERIALS AND METHODS

In preliminary experiments the influence of tolazamide on the rate of DPH metabolism *in vivo* was examined in rats. Male Wistar rats (TNO, Zeist, Holland, mean

weight 230 g, having free access to water and food) were used. The half-life of DPH in plasma after i.v. injection was estimated (at least four rats contributed to each point of the curve).

Other groups of animals were pretreated with different doses of tolazamide (solvent: 0·05 M Na_2HPO_4 and 0·02 N NaOH in water, pH 11·5). After either the last dose of tolazamide or solvent, DPH was injected into a tail vein. The animals were anaesthetized with ether, and blood samples were taken by aortic puncture. The DPH content of the samples was assayed by the method of Thurkow *et al.*[14]

In some experiments the animals were decapitated, the brains were removed and DPH content was estimated by the same method. Differences between groups were tested with Wilcoxon's two-sample test; they were considered to be significant when $P < 0{\cdot}05$.

Enzyme preparation and incubation. The effect of tolazamide and other sulphonylureas on the enzymatic conversion of DPH was further studied in liver homogenates from rats, using [^{14}C]-labelled DPH. Again male Wistar rats, weighing 200–250 g, were used. The animals were decapitated and the livers were quickly put in ice-cold 0·15 M KCl. A 20 per cent homogenate was made using a Potter–Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 9000 *g*. The postmitochondrial supernatant was diluted 1:1 with 0·15 M KCl.

DPH metabolism *in vitro* was studied according to the method described by Kutt *et al.*[6] ATP, NAD and NADP (Boehringer, Mannheim) were dissolved in 0·8 M sodium phosphate buffer (pH 7·4) so that final concentrations of $3{\cdot}9 \times 10^{-3}$ M, $8{\cdot}2 \times 10^{-4}$ M and $9{\cdot}3 \times 10^{-4}$ M respectively, were obtained in the incubate.

[^{14}C]DPH (New England Nuclear) was dissolved in 0·05 per cent (w/v) NaOH and supplemented with non-radioactive DPH (N.V. Chemische Industrie, Katwijk, Holland) until the required concentrations were obtained (radioactivity approx. 90,000 dpm/ml of incubation medium). When tolazamide, (Upjohn), tolbutamide (Boehringer) or chlorpropamide (Boehringer) were added to the incubation medium, these substances were dissolved together with DPH in 0·05 per cent (w/v) NaOH. In some experiments 5×10^{-4} M SKF 525-A was added. The incubations were performed at 37° in a Dubnoff shaker under constant aeration with a 5 per cent CO_2 95 per cent O_2-mixture (flow-rate 2 l./min).

The reaction was started by adding 0·6 ml of the enzyme preparation to the incubation medium consisting of 0·15 ml DPH solution, 0·25 ml 0·05 per cent (w/v) NaOH (eventually containing one of the sulphonylureas) and 0·5 ml of the coenzymes solution in phosphate buffer. The reaction was stopped by adding 1 ml of the incubate to 5·5 ml chloroform + 1·5 ml 0·3 M NaH_2PO_4-solution, this being the first step of the extraction procedure.[14] Metabolite formation was measured as radioactivity that remained in the first aqueous phase.

Thin layer chromatography and liquid scintillation counting of [^{14}C]DPH *and metabolites.* For purity control of [^{14}C]-labelled DPH and for more detailed study of metabolite formation thin layer chromatography (t.l.c.) and liquid scintillation counting (l.s.c.) were used.

Thin layer chromatography was performed on Merck Kieselgel F 254-plates, and developed with chloroform–methanol (95:5). After drying, the plates were visualized under u.v. light (DPH and metabolites were resolved as yellowish spots); they were then either scanned with a Berthold scanner or various zones were scraped off into

tubes containing 0·5 ml NCS (Amersham, Searle) and 10 ml toluene with 50 mg PPO and 0·5 mg POPOP. The samples were counted for 10 min in a Nuclear-Chicago Mark I 300-scanner. Known amounts of 5-HPPH (Aldrich Chemical Co., Milwaukee), [^{14}C]-labelled-DPH and non-radioactive DPH served as control. One large peak of radioactivity was shown by direct scanning after 287·415 dis/min had been applied to the plate. This peak had the same R_f value (0·52) as non-radioactive reference DPH. No impurities were observed, though impurities of less than 1 per cent of the total radioactivity could have been detected with this method.

The fate of DPH during the subsequent steps of the procedure was monitored using LSC purity control. To the aqueous phases 1 ml water and—just before counting—10 ml Instagel (Packard) was added. The chloroform phases (evaporated to dryness) were solubilized with NCS-toluene as described. The radioactivity in the samples was expressed as a percentage of the initial amount of DPH. After extraction of 2×10^{-4} M [^{14}C]DPH (activity 90·000 dpm/ml) only 2·4 per cent of the total radioactivity remained in the first aqueous phase (see Thurkow *et al.*[14]), while 101·4 per cent was recovered in the basic phosphate (l.s.c. of the dried chloroform-phase did not give reliable results, so this part of the procedure was abandoned). After t.l.c. of the last chloroform phase and scraping off the plates in various zones only 1·7 per cent of the total radioactivity was found outside the active spot.

These results indicate that nearly all the radioactive material was [^{14}C]DPH and it was almost completely removed from aqueous media at pH 7·3 by our extraction procedure.

RESULTS

In vivo *experiments.* The plasma half-life ($t_{\frac{1}{2}}$) of DPH after a single intravenous injection of 25 mg/kg in rat proved to be 45 min and that of tolazamide (10 mg/kg) 85 min.

Blood levels of DPH after administration to rats that also received either tolazamide or its solvent are given in Table 1. Rats that had been pretreated for 5 days with tolazamide showed slightly (though significantly) higher DPH-blood levels than animals that had been given the solvent. Estimation of brain levels of DPH in rats using different doses and time schedules also showed minor though consistent increases of DPH in those animals that had been treated with tolazamide. This justified a more detailed study of DPH metabolism by rat-liver homogenates.

TABLE 1. DPH BLOOD LEVELS IN RATS TREATED WITH TOLAZAMIDE

Dose (mg/kg)			Sample collected after	
DPH	Tolazamide	*n*	(min)	μg DPH/ml
25	0*	10	30	10·4 ± 0·5
	10*	10	30	10·8 ± 0·5
14	0†	14	45	4·8 ± 0·5
	7†	14	45	6·7 ± 0·7‡

Controls received the corresponding volume of tolazamide-solvent.
* One single tolazamide injection, at the same time as DPH.
† Five days pretreatment with tolazamide.
‡ $P < 0·05$ (according to Wilcoxon's test).

Incubation experiments. Incubation of rat liver postmitochondrial supernatant with 2×10^{-4} M [^{14}C]DPH for various times (0, 10, 20, 30, 60 and 120 min) followed by extraction showed an increase in radioactive material in the aqueous phase due to the formation of 5-HPPH, which was confirmed by TLC using non-radioactive 5-HPPH as reference substance ($R = 0{\cdot}20$). After incubation for 120 min a second spot, R_f 0·80, was detected, probably another metabolite. Blank values (0-min incubated at 37°) showed that approx. 2·5 per cent of unmetabolized DPH remained in this aqueous phase; only traces of metabolite appeared in the chloroform after 60 min of incubation. So in further experiments l.s.c. of the aqueous phase could be used to measure the rate of DPH-metabolism.

The rate of metabolism in time at various DPH concentrations is presented in Fig. 1; there is no difference between (5×10^{-4}) and 10×10^{-4} M) DPH concentrations. The initial velocity of conversion decreases after 10 min of incubation; a uniform incubation time of 5 min was therefore maintained in our experiments. Figure 2 shows a Lineweaver–Burk plot of the mean result of four experiments; the relationship between $1/v$ and $1/s$ is not linear. When the best fitting line through the points representing the lower substrate concentration is drawn, an apparent K_m of $0{\cdot}4 \times 10^{-5}$ M is found, whereas this value becomes $1{\cdot}5 \times 10^{-5}$ when only the highest concentrations are considered.

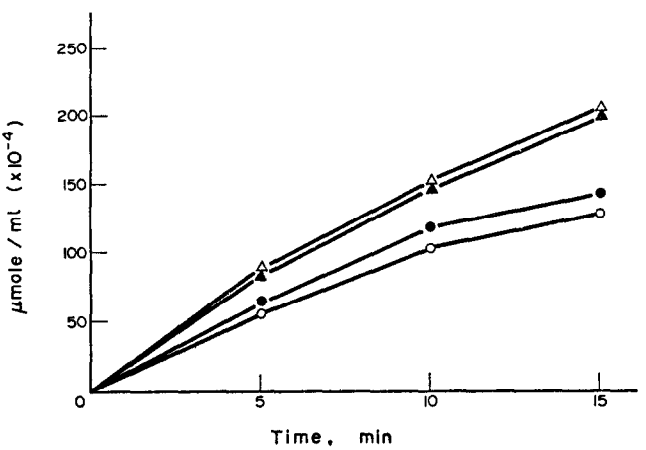

FIG. 1. Hydroxylation of DPH in time by rat liver 9000 *g* supernatant. (○) DPH concn. 1×10^{-4} M; (●) DPH concn. 2×10^{-4} M; (△) DPH concn. 5×10^{-4} M; (▲) DPH concn. 10×10^{-4} M; *v* is expressed as µmoles DPH converted/ml of undiluted 9000 *g* supernatant.

Because of these unsatisfactory results we also plotted log [*s*] vs *v* (Fig. 3). This gave a linear dose–response curve for DPH metabolism. In the presence of sulphonylurea this curve shifted parallel to the right, the greatest shift being achieved with the highest concentrations of inhibitor. This indicates that the inhibition of DPH metabolism by sulphonylurea has—at least partly—a competitive character (Ariens[15]). When the three drugs were present at the same concentration, tolbutamide had the greatest effect, followed by tolazamide, while chlorpropamide was the weakest inhibitor. (This order was found at all three concentrations of the inhibitors that were used, Fig. 3a, b and c). In the presence of 5×10^{-4} M SKF 525-A no metabolite formation was observed.

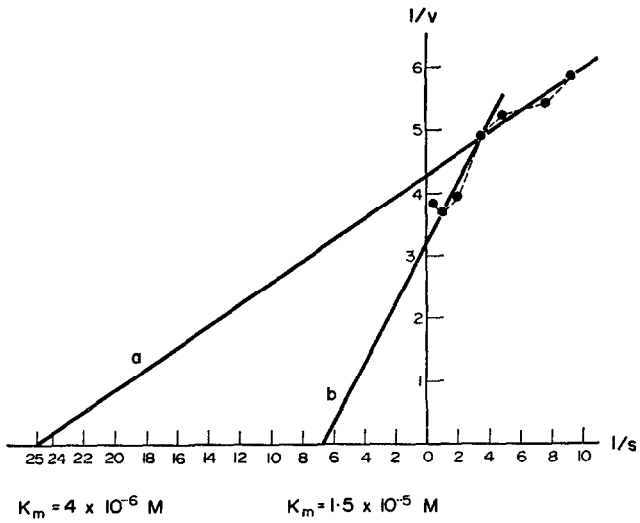

FIG. 2. Lineweaver–Burk plots of the rate of hydroxylation of DPH by rat liver 9000 *g* supernatant (mean of 4 experiments, i.e. each point is based on 4 separate values). The best fitting lines through the points representing: (a) the lower substrate concentrations; (b) the higher substrate concentrations are shown; v is expressed as μmoles DPH converted/min/ml of undiluted 9000 *g* supernatant.

The potency of inhibitors can be expressed by their pA_2-value; this value is defined (Arunlakshana and Schild[16]) as the negative logarithm of that concentration of antagonist (i.e. inhibitor) at which the agonist (i.e. substrate) concentration has to be doubled in order to get the same effect (i.e. velocity) as in the absence of antagonist. By plotting log $(A/a - 1)$ vs pA (where A is the effect in the presence and a in the absence of antagonist) a straight line is found that intersects the X-axis at the pA_2-value. This is shown in Fig. 4; the pA_2-values for chlorpropamide, tolazamide and tolbutamide were found to be 3·0, 3·4 and 3·63 respectively. This means that in equal concentrations chlorpropamide is 2·5 times a weaker inhibitor of DPH-metabolism than tolazamide and approx. 4·3 times weaker than tolbutamide.

DISCUSSION

The plasma half-life of DPH in our experiments was shorter (0·75 hr), though only slightly, than that found by Gerber *et al.*[17] (1·2 hr). A preferential increase in DPH-brain levels after tolazamide could not be demonstrated, in contrast with DPH-brain levels after pretreatment with sulthiame.[18] This indicates that an eventual influence of tolazamide on the mode of action of DPH cannot be attributed to enhancement of the cerebral concentration of DPH.

In our *in vitro* experiments the only metabolite that could be determined after the short incubation time that we used was 5-HPPH. Chang *et al.*[19] isolated another non-conjugated metabolite of DPH from 24 hr rat urine; this was 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5 phenylhydantoin. The second spot on the t.l.c. plate ($R_f = 0{\cdot}08$) that we observed in some experiments after 120 min of incubation may have been due to the formation of this metabolite.

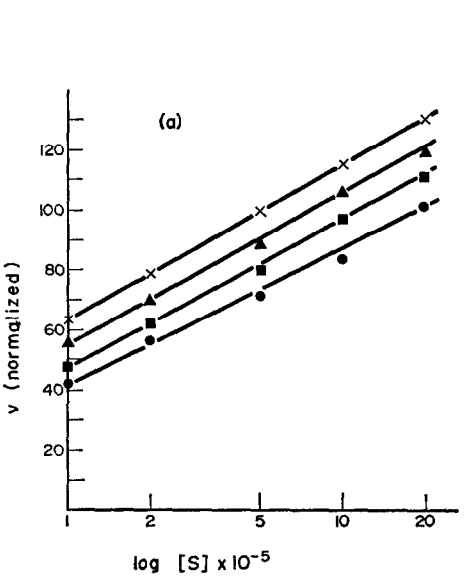

Fig. 3a

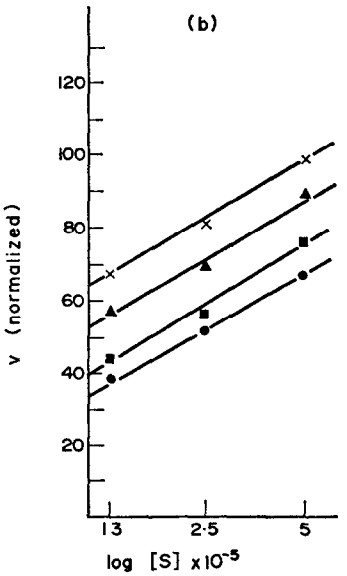

Fig. 3b

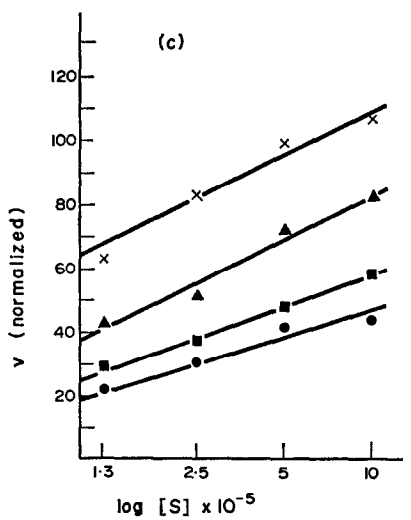

Fig. 3c

FIG. 3. Log-dose effect curves for DPH metabolism. (×) Control; (▲) with chlorpropamide; (■) with tolazamide; (●) with tolbutamide. Concentration of inhibitor: (a) $7{\cdot}5 \times 10^{-4}$ M; (b) 10×10^{-4} M; (c) 25×10^{-4} M. The curves are normalized, the 5×10^{-5} M concentration of DPH being the reference point (100).

The inhibition of DPH-metabolism by 5×10^{-4} M SKF 525 A, the well-known inhibitor of mixed function oxidase, was almost complete. This confirms that also in our experiments DPH is hydroxylated in rat-liver by the mixed function oxidase system.

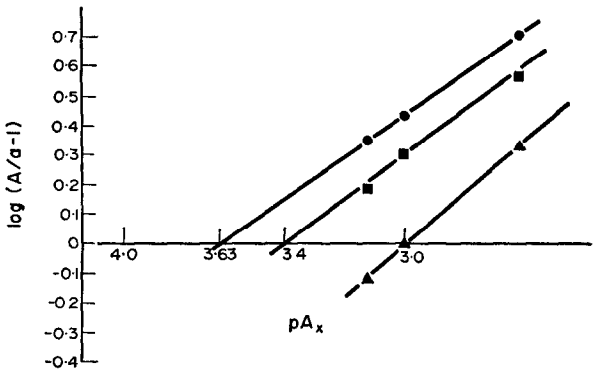

FIG. 4. Arunlakshana–Schild plots for the inhibition of DPH-metabolism by (▲) chlorpropamide; (■) tolazamide and (●) tolbutamide. The data are taken from Fig. 3.

The rate of 5-HPPH formation did not seem to increase with substrate concentrations higher than 5×10^{-4} M. Kutt *et al.*[6] found no increase in the velocity of DPH metabolism in 9000 *g*-supertanant fractions of rat-liver homogenates with substrate concentrations above 1×10^{-4} M; the decrease in velocity of DPH-conversion after 10 min of incubation (Kutt *et al.*[20]) could be confirmed in our experiments. Kutt *et al.*[6] calculated a mean K_m of $3{\cdot}73 \pm 0{\cdot}18 \times 10^{-5}$ M from sixteen different Lineweaver–Burk plots; however, none of these is shown in their figures. Conversion of the Hofstee plot that is presented by these authors results in a Lineweaver–Burk plot that is also non-linear, just as in our experiments. This non-linearity may cause difficulties in drawing the best fitting straight line and thus differences in K_m ($3{\cdot}73 \times 10^{-5}$ M against 6×10^{-6} M (resp. $1{\cdot}5 \times 10^{-5}$) in our results) may be explained.

Possibly the irregularities of the kinetics with DPH as a substrate are related to the low water solubility of this compound; Hansen and Fouts[21] have discussed the consequences of such a situation. The inhibition by the sulfonylureas was much weaker than by SKF 525-A. The non-linearity of the Lineweaver–Burk plots prevented us from drawing conclusions as to the nature of the inhibition. The log dose–response curves that we finally used for the characterization of DPH metabolism are much less sensitive to variations, especially when low substrate concentrations are used. In our experiments the inhibition of DPH hydroxylation caused by sulphonylurea proved to be (at least partly) competitive, as was shown by the parallel shift to the right of the dose–response curves. The potency of the inhibitors, expressed in pA_2-values (according to Arunlakshana and Schild[16]) was relatively low. It reflected the degree of oxidative metabolism to which the three inhibitors in our study are subjected. The clinical implications of these findings are the subject of further investigation in our laboratory.

REFERENCES

1. T. C. BUTLER, *J. Pharmac. exp. Ther.* **119**, 1 (1957).
2. E. W. MAYNERT, *J. Pharmac. exp. Ther.* **130**, 275 (1960).
3. S. A. CUCINELL, A. H. CONNEY, M. S. SANSUR and J. J. BURNS, *Clin. Pharmac. Ther.* **6**, 420 (1965).

4. N. GERBER, W. L. WELLER, R. LYNN, R. E. RANGO, B. J. SWEETMAN and M. T. BUSH, *J. Pharmac exp. Toxicol.* **178,** 567 (1971).
5. C. L. RÜMKE and J. NOORDHOEK, *Eur. J. Pharmac.* **6,** 163 (1969).
6. H. KUTT and K. VEREBELY, *Biochem. Pharmac.* **19,** 675 (1970).
7. J. MØLHOLM HANSEN, K. SIERSBAEK-NIELSEN, M. KRISTENSEN, L. SKOVSTED and L. KORSGAARD CHRISTENSEN, *Acta med. scand.* **189,** 15 (1971)
8. J. MØLHOLM HANSEN, M. KRISTENSEN, L. SKOVSTED and L. KORSGAARD CHRISTENSEN, *Lancet* **2,** 265 (1966).
9. J. H. PANNEKOEK, personal communication (1969).
10. J. TAGG, D. M. YASUDA, M. TANABE and CH. MITOMA, *Biochem. Pharmac.* **16,** 143 (1967).
11. R. C. THOMAS and G. J. IKEDA, *J. med. Chem.* **9,** 507 (1966).
12. J. S. WELLES, M. A. ROOT and R. C. ANDERSON, *Proc. Soc. exp. Biol. Med.* **101,** 668 (1959).
13. J. A. TAYLOR, *Clin. Pharmac. Ther.* **13,** 710 (1972).
14. I. THURKOW, H. WESSELING and D. K. F. MEIJER, *Clin. Chim. Acta* **37,** 509 (1972).
15. E. J. ARIENS, A. M. SIMONIS and J. M. VAN ROSSUM, in *Molecular Pharmacology* (Ed. E. J. ARIENS), Vol. 1, p. 251. Academic Press, New York (1964).
16. O. ARUNLAKSHANA and H. O. SCHILD, *Br. J. Pharmac.* **14,** 48 (1959).
17. N. GERBER and K. ARNOLD, *J. Pharmac. exp. Ther.* **164,** 232 (1968).
18. P. L. MORSELLI, M. RIZZO and S. GARATTINI, *Biochem. Pharmac.* **19,** 1846 (1970).
19. T. CHANG, A. SAVORY and A. J. GLAZKO, *Biochem. biophys. Res. Commun.* **38,** 444 (1970).
20. H. KUTT and J. R. FOUTS, *J. Pharmac. exp. Ther.* **176,** 11 (1971).
21. A. R. HANSEN and J. R. FOUTS, *Chem. Biol. Interactions* **5,** 167 (1972).