There are several lines of evidence which suggest the existence of a causal connection between lower levels of liver haem, as judged from the amount of cytochrome P-450, and porphyria.* A decreased level of cytochrome P-450 has been observed in mice and rats treated with DDC[12] and AIA.[5]† In the case of AIA an increased destruction of liver haem is responsible for the decrease in the level of cytochrome P-450;[5,13] it is possible that DDC may cause the same effect by impairing the synthesis of haem.

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Biochemical Pharmacology, Vol. 21, pp. 272–276. Pergamon Press, 1972. Printed in Great Britain

Plasma levels and urinary excretion of [^{14}C]cyclophosphamide and its radioactive metabolites in rats pretreated with prednisolone*

(*Received* 10 *May* 1971; *accepted* 9 *July* 1971)

THE CYTOTOXIC action of cyclophosphamide (CP) is believed to be related to its metabolic activation by hepatic microsomal enzymes.[1,2] Hayakawa *et al.*[3] reported that simultaneous administration of prednisolone (P) with CP resulted in lower plasma levels of nitrobenzylpyridine-alkylating metabolites of CP in the rat. Since glucocorticoids are often used in conjunction with CP to treat neoplastic diseases, this finding has important therapeutic implications. The availability of [^{14}C]labeled CP permitted the potential drug interaction between P and CP to be investigated in more detail. In this study we have measured the plasma levels and urinary excretion of CP and its radioactive metabolites in rats pretreated with single and repetitive doses of P.

Methods

Male Sprague–Dawley rats (Simonsen Laboratories) weighing 220–300 g were subjected to single or repetitive oral doses of 6·6 or 66 mg/kg of P (CalBiochem) suspended in a 1% carboxymethyl cellulose vehicle. Control rats received the vehicle only. Repetitive P-treatment consisted of 10 daily oral

* Supported by Grant No. GM-12675 from the National Institutes of Health.

doses. On the day of experimentation, cannulation of the femoral artery was performed under ether anesthesia. Rats were allowed to recover for 3 hr prior to receiving an intraperitoneal injection of 10 mg/kg of CP (Mead Johnson), with each animal receiving about 2 μc of ring-labeled [^{14}C]cyclophosphamide* (3·35 mc/m-mole, New England Nuclear). The radioactive CP was chromatographically pure as determined by thin-layer chromatography in the three different solvent systems described below. This dose of CP, which approximates the CD_{50} in rats for the treatment of Yoshida sarcoma,[1] was administered 1 hr after the last oral dose of P, and corresponded in time to the peak plasma levels of P as determined by the method of Peterson *et al.*[4]

Rats were unrestrained and housed in stainless steel metabolism cages to collect 24-hr urine samples which were kept frozen in dry ice. Blood samples of about 0·5 ml were withdrawn at given intervals and replaced with an equivalent volume of heparinized saline (50 units/ml).

[^{14}C]Cyclophosphamide and total metabolites were determined by a modification of the method of Mellett *et al.*[5] The radioactivity in plasma or urine samples was measured by liquid scintillation spectrometry before and after chloroform extraction. The concentration of unchanged drug was calculated from the difference of these two values. A 0·2-ml aliquot of plasma was diluted to 1·0 ml with 0·5 M phosphate buffer, pH 7·0. Total radioactivity in a 0·2-ml aliquot of diluted plasma was obtained by counting in a scintillation medium containing 10% naphthalene, 0·7% 2,5-diphenyloxazole, 0·03% *p*-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene and 5% Cabosil (Cabot Chemical) in dioxane. Another 0·2-ml aliquot of diluted plasma was added to 1·0 ml of the phosphate buffer and 0·1 mg of non-radioactive CP in 0·05 ml of methanol added to facilitate extraction of unchanged CP. Ten ml of chloroform was used to extract plasma and, after separation of the layers, a 0·5-ml aliquot of the aqueous phase was counted in the Cabosil-containing scintillation medium. Quantitative recovery of known amounts of ^{14}C-CP added to rat plasma in the concentrations encountered in these experiments was obtained by this procedure. Undiluted urine samples (0·2–0·5 ml) were analyzed in a similar manner. Quench corrections were made by the twin channels ratio method. Only samples counting twice the rate of an appropriate background sample were included in the data.

The specificity of the extraction method for unchanged CP was assessed by thin-layer chromatography of the chloroform extract of urine from rats dosed with ^{14}C-CP. Repeated experiments showed only unchanged CP in the chloroform extract. Mellet *et al.*[5] and Gibson and Becker[6] reported similar results using this procedure.

Urinary CP metabolites were examined by paper chromatography using the method of Norpoth *et al.*,[7] or by thin-layer chromatography using the following solvent systems: ethanol–ammonia–water (80 : 5 : 5); acetone–methanol–water (4 : 4 : 2); and *n*-butanol–acetic acid–water (4 : 1 : 1). After removal of unchanged CP by chloroform, freeze-dried urine samples (0·8 ml) were quantitatively extracted for radioactivity with methanol; the methanol was evaporated *in vacuo* and spotted on Silica gel G thin-layer plates. The developed chromatograms were scanned for radioactivity using a radiochromatogram scanner (Packard Instruments).

Data were analyzed statistically by a one-way analysis of variance using the least significant difference at $P < 0.05$.

Results and discussion

Repetitive P-treatment. Declines in the plasma levels of unchanged CP and of total CP metabolites (Fig. 1) in the groups pretreated with 10 daily oral doses of P at 6·6 or 66 mg/kg were no different from those of the control group over the 6-hr period observed. Maximum plasma levels of CP were attained within 15–30 min following intraperitoneal injection and declined with an approximate plasma half-life of 1·3 hr. Peak plasma levels of total CP metabolites were reached somewhat later, between 30 min to 1 hr, and declined with a longer approximate plasma half-life of 2·3 hr.

The mean 24-hr urinary excretion of CP (Table 1) in groups pretreated for 10 days with P at dose levels of 6·6 and 66 mg/kg did not differ statistically from the control value. The 24-hr urinary excretion of total CP metabolites (Table 1) for both of the P-treated groups also did not differ from control.

Peak plasma glucocorticoid levels of 440 μg/100 ml of plasma were attained 1 hr after the last of 10 daily oral doses of P given at 66 mg/kg. This value was 17–18 times greater than that cited for endogenous levels of corticosterone in rats by Allan and Kendall,[8] and by Tommasini *et al.*[9] In order to exclude the possibility that a stimulation of CP metabolism might have occurred with repetitive P-treatment, but was perhaps masked by competitive metabolism of the large amounts of glucocorticoid present, rats were pretreated with 10 daily oral doses of P, 66 mg/kg, but did not receive CP until 24 hr after the last P dose, at which time plasma levels of P were undetectable.

* $(ClCH_2CH_2)_2$ N-P (0) O$^{14}CH_2CH_2CH_2NH$.

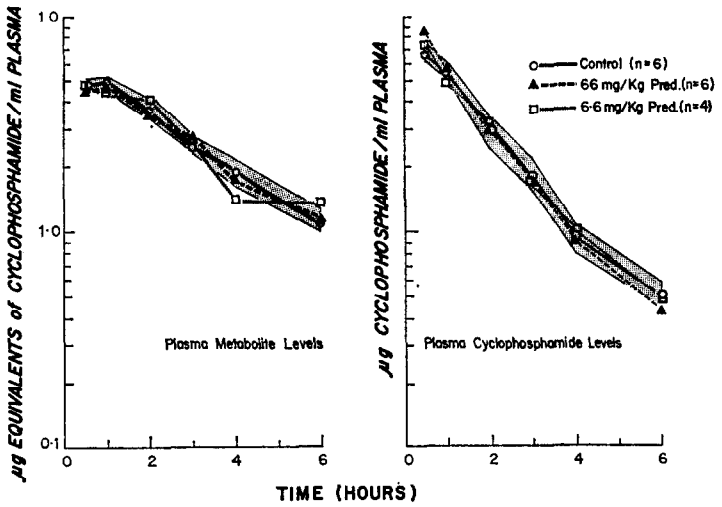

FIG. 1. Plasma levels of [^{14}C]cyclophosphamide and total cyclophosphamide metabolites in rats pretreated with 10 daily oral doses of prednisolone. The values shown are the means of four to six animals and the shaded area represents the standard error of the control group. The variation in the treated groups was not statistically different from that of controls.

As shown in Fig. 2, the changes in plasma levels of CP and of total CP metabolites in the P-treated group were, again, no different from the control group. The 24-hr urinary excretions of CP and total CP metabolites (Table 1) in the P-treated group likewise did not vary significantly from controls.

Single dose treatment with P. To examine the effects of a single dose of P on the plasma levels and excretion of CP, rats were fasted overnight and given a single oral dose of the glucocorticoid, again, at either 6·6 or 66 mg/kg, and CP was administered 1 hr later at peak plasma levels of P.

TABLE 1. CYCLOPHOSPHAMIDE AND TOTAL METABOLITES IN 24-hr URINE OF PREDNISOLONE-TREATED AND CONTROL ANIMALS

Prednisolone treatment		Per cent of ^{14}C dose as:*	
Dose (mg/kg)	No. of days treated	Cyclophosphamide	Total metabolites
6·6	10	12·9 ± 2·8	42·4 ± 8·2
66	10	16·0 ± 1·6	42·9 ± 5·7
Control	10	10·9 ± 1·9	48·4 ± 9·8
66†	10	15·2 ± 1·4	46·9 ± 1·9
Control	10	14·5 ± 5·7	42·4 ± 3·5
6·6	1	11·6 ± 1·7	41·8 ± 3·5
66	1	15·3 ± 1·1	43·1 ± 3·3
Control	1	17·5 ± 2·6	45·5 ± 4·6

* Values are means ± S.E. for four to six animals. No significant differences between prednisolone-treated and control animals were observed.

† Cyclophosphamide was given 24 hr after the last dose of prednisolone instead of at the time of the peak plasma level of prednisolone.

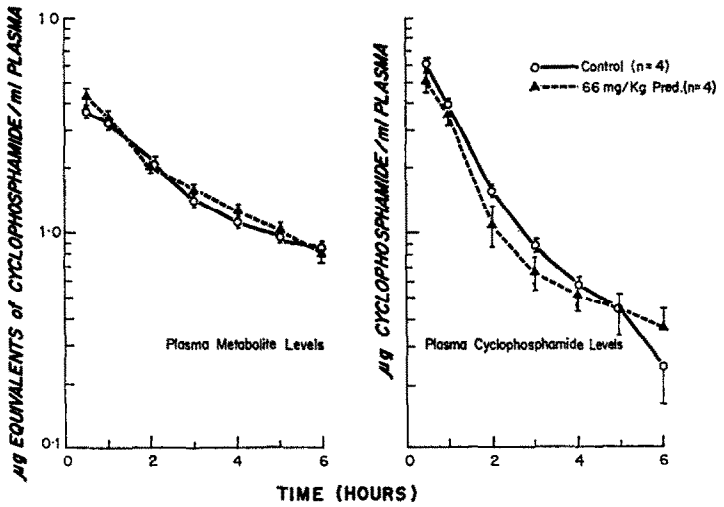

FIG. 2. Plasma levels of [^{14}C]cyclophosphamide and total cyclophosphamide metabolites in rats given cyclophosphamide 24 hr after the tenth daily dose of prednisolone. The values shown are means (± S.E.).

The fall in plasma levels of unchanged CP and total CP metabolites in both of the P-treated groups (Fig. 3) paralleled that of the control group and was not significantly different. The approximate plasma half-lives for the disappearance of CP was about 1 hr, a value close to that calculated from the data of Hayakawa *et al.*[3] for both the controls and P-treated rats in their study. The plasma half-life for total CP metabolites was about 2·8 hr.

The 24-hr urinary excretion (Table 1) of CP and metabolites in both of the P-treated groups did not vary significantly from the controls. The total 24-hr urinary radioactivity, expressed as per cent of injected dose, compares closely to the value reported by Graul *et al.*[2] who used a comparable dose of CP (i.e. 33 mg/kg, i.p.).

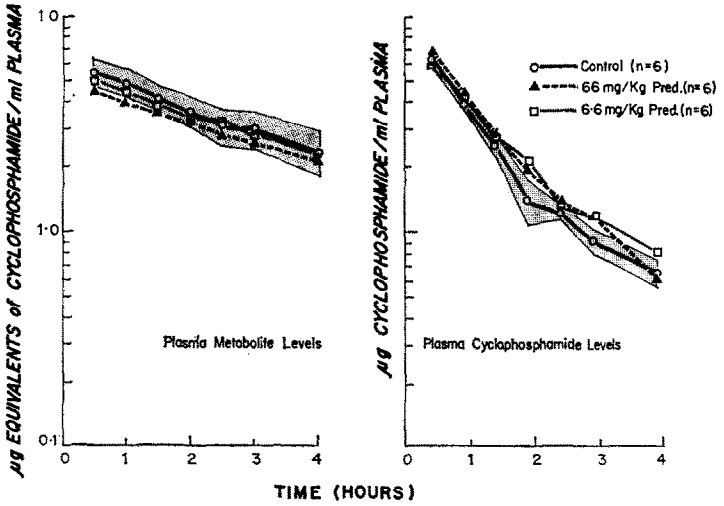

FIG. 3. Plasma levels of [^{14}C]cyclophosphamide and total cyclophosphamide metabolites in rats pretreated with a single dose of prednisolone. Legend is the same as Fig. 1.

Urinary CP metabolite patterns of rats receiving repetitive P-treatment. Radiochromatographic examinations using paper chromatography, and thin-layer chromatography in three separate solvent systems, were made on urine samples of the 10-day-P-treated animals to see if qualitative changes in the metabolite pattern of CP might have occurred. No clear differences were found in the chromatographic patterns of urinary CP metabolites between the P-treated groups and the controls. In general, at least two prominent peaks having R_f values less than CP were characteristic of the thin-layer chromatographic pattern of urinary CP metabolites. Paper chromatography revealed two or three metabolites which were also more polar than the parent compound.

The analytical methods employed in the studies reported here have been shown to detect changes in unchanged ^{14}C-CP and its metabolites in the plasma of mice treated with inhibitors (SKF-525A) and inducers (phenobarbital) of CP metabolism.[6] The same changes were observed in mice using the nitrobenzylpyridine-colorimetric assay[10] suggesting that the colorimetric and radioactive methods are comparable.

Our conclusions based on plasma levels and urinary excretion of CP and metabolites differ from those of Hayakawa *et al.*[3] who administered 100 mg/kg of P and CP simultaneously by the intraperitoneal route. The rather high dose of CP used in their studies was probably dictated by the analytical limits of the colorimetric assay for alkylating metabolites of CP. Our results were obtained with a lower dose of CP and with both single and repetitive doses of P. That our results differ may be due to a difference in the dose of CP or to the route of administration of P. However since no attempt was made to include a measure of variation in the results reported by Hayakawa *et al.*,[3] the importance of the relatively small differences observed in the plasma levels of CP and alkylating metabolites in P-treated and control animals in their study are difficult to assess. The exact correlation between CP metabolism and its antitumor action has yet to be clearly defined; however, if glucocorticoid therapy enhances the antitumor activity of CP by altering its biotransformation, our studies would indicate that the mechanisms involved do not manifest themselves through changes in the over-all plasma disappearance of the unchanged drug or the extent of its urinary elimination in 24 hr.

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