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EFFECTS OF ENANTIOMERIC HOMOGENEITY ON THE *IN VITRO* METABOLISM AND *IN VIVO* ANTICANCER ACTIVITY OF (+)- AND (−)-CYCLOPHOSPHAMIDE

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Abstract—Separate incubation kinetic measurements for the metabolic "activation" of enantiomerically pure (+)- and (−)-cyclophosphamide (CP) and the racemate of CP by identical phenobarbital (PB)-induced mouse liver microsomal mixed-function oxidase preparations gave, respectively, V_{max} 13.8 ± 1.0, 20.0 ± 1.5 and 16.3 ± 1.1 μmol bis-2-chloroethylamine (nor-HN2) equiv./g wet liver/hr, and K_m 0.37 ± 0.02, 0.56 ± 0.04 and 0.45 ± 0.02 mM. The absolute magnitude of the apparent V_{max} kinetic parameter increased by *ca.* 50 per cent in a subsequent comparative run between (+)- and (−)-CP using a second preparation of the hepatic microsomal ozidase; however, the relative behaviour of CP enantiomers toward enzymatic "activation" was constant, within experimental error, and revealed that $V^-_{max}/V^+_{max} = 1.34 \pm 0.17$ and $K^-_m/K^+_m = 1.35 \pm 0.14$. Removal and quantitative measurement, as a function of time, of free acrolein that is produced by incubation of CP with PB-induced microsomes repeatedly gave a roughly congruous family of "skewed bell-shaped" curves having maxima in the order (±)-CP > (+)-CP > (−)-CP; however, the differences between these acrolein time-course profiles were relatively small (*ca.* 10–20 per cent). Repitition of such measurements with freshly prepared mouse liver cytosol and NAD^+ added to the incubation mixtures of CP and liver microsomes led to comparable suppression of the maxima. Isolation of CP from separate (+)- and (−)-CP incubation mixtures, followed by determination of enantiomeric homogeneity by nuclear magnetic resonance methods, demonstrated that CP is not racemized during *in vitro* liver microsomal metabolism. Aminal screening data (test/control precentages) for (+)-, (−)-, and (±)-CP activity against mouse L-1210 lymphoid leukemia showed no outstanding differences in therapeutic value. Collectively, these various experimental probes suggest that there is an unusually low degree of biological stereoselectivity associated with the metabolism of CP enantiomers.

Cyclophosphamide* (CP) exists in stereochemically nonidentical enantiomeric forms, due to the presence of an asymmetric phosphorus center, and the racemic mixture of (+)- and (−)-CP ("Cytoxan") is administered currently to cancer patients either alone or in combination therapy [1]. The relatively broad spectrum of human cancers found responsive to chemotherapy with (±)-CP [2] has naturally prompted an extensive interest in the metabolism of this drug, and a substantial amount of chemical and pharmacological data [2] that has been obtained with (±)-CP supports the basic set of transformations shown in Fig. 1. Enzymatic oxidation of the C-4 position in CP by hepatic microsomal mixed-function oxidase produces 4-hydroxycyclophosphamide (4-HO-CP), which may then equilibrate with aldophosphamide (AP) [3], the aldehyde hydrate of AP [4], and/or thioether conjugates of 4-HO-CP/AP [5, 6]. Fragmentation of AP subsequently affords acrolein and phosphoramide mustard, which has been suggested [7, 8] as being required for cytotoxicity. Enzyme-mediated conversions of 4-HO-CP and AP into the relatively nontoxic urinary metabolites 4-ketocyclophosphamide (4-keto-CP) and carboxyphosphamide are, on the other hand, associated with drug detoxification. Hence, the possibility for differential partitioning of 4-HO-CP/AP between these toxification and detoxification pathways in cancerous vs normal cells has been used to rationalize the unique oncostatic selectivity of CP, as compared to other phosphoramidic mustards [7, 9, 10].

Assuming that CP and all chiral pre-fragmentation metabolites of CP are configurationally stable about phosphorus, *in vivo*, and given that biological phenomena usually exhibit marked selectivity toward enantiomers, we began a systematic study of CP which initially focused on fundamental stereochemistry [11–13] and dynamical properties [14, 15]. More recent work has been concerned with the elucidation of biochemical selectivity and relative anticancer efficacy for the enantiomers of CP. Such data are reported herein and are compared with results that have been published by other investigators [16, 17] during the course of our own studies.

MATERIALS AND METHODS

(+)- and (−)-CP-enantiomers. Diastereomeric 3-α(-methylbenzyl)cyclophosphamides were synthesized from optically pure (−)-(*S*)-α-methylbenzylamine and were chromatographically separated according to earlier reported procedures [11]. Thin-layer chro-

*2-[Bis(2-chloroethyl)amino]-2H-1,3,2-oxazaphosphorinane 2-oxide.

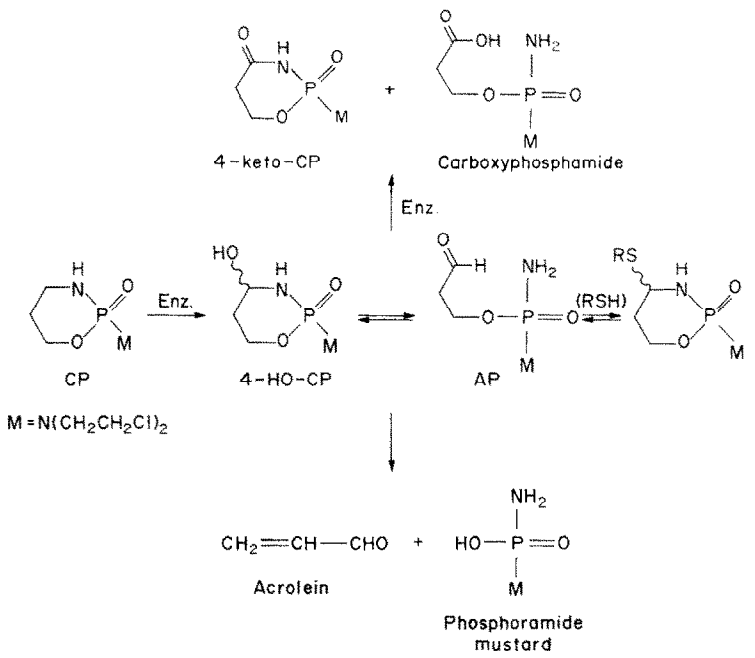

Fig. 1. Metabolic reactions of cyclophosphamide (CP).

matography (T.L.C.) and high-resolution 220 MHz ^{1}H nuclear magnetic resonance (N.M.R.) analyses of these precursors to the CP enantiomers demonstrated that diasteromeric purity was in excess of 99 per cent. Each diastereomer was then subjected to hydrogenolysis in aqueous ethanol using 10% Pd/C catalyst and 50 psi H_2 (25°, 2 days) [18], and the resultant samples of (+)-(*R*)- and (−)-(*S*)-CP [13, 19] were isolated chromatographically [12] as anhydrous crystals in *ca.* 30 per cent yield. Fourier transform 220 MHz ^{1}H N.M.R. measurements utilizing accumulated free induction decay signals from (+)- and (−)-CP solutions containing the chiral shift reagent tris-[3-(trifluoromethyl hydroxymethylene)-*d*-camphorato] europium (III), $Eu(tfc)_3$ [12], unambiguously established that the enantiomeric homogeneity of the CP samples exceeded 99 per cent, and thereby demonstrated that the Pd/C-catalyzed reduction route to CP is highly stereospecific.* Assessment of CP enantiomeric homogeneity by optical rotation has been reported [12, 18]; however, the relatively low optical rotatory strength of CP(absolute value of $[\alpha]_D^{20} = 2.3 \pm 0.2°$ methanol) may lead to complications that result from possible contamination by small amounts of strongly rotating optically active components. This situation, for example, has been encountered during studies with CP isolated from the urine of patients receiving CP treatment.†

(±)-*CP*. Anhydrous racemic CP was synthesized by the straightforward extension of the general procedures of Friedman and Seligman [21] and was isolated from cold water as a crystalline monohydrate, following initial purification by column chromatography [silica gel; chloroform–methanol (9:1)].

Isolation of liver microsomes and liver cytosol. Male Dub ICR mice (19–25 g, Flow Research Animals) were used throughout; mice receiving pretreatment were given i.p. injections of phenobarbital (PB): 30 mg/kg in the first morning and evening, with 60 mg/kg in the morning for 2 consective days. The animals were fed a standard chow diet *ad lib.* and were starved for 8–10 hr before cervical dislocation on the fourth morning. Isolation of liver microsomes as pellets for resuspension in phosphate buffer was carried out according to literature methodology [22], which also afforded liver cytosol in the supernatant fluid during centrifugation at 9000 *g*. Cytochrome P450 content in liver microsomes from PB-treated mice was typically *ca.* 1.5 nmol P450/mg of protein, and was determined by a combination of standard cytochrome P450 [23] and protein [24] assay methods.

Kinetics for liver microsomal conversion of CP into alkylating metabolites. The methodology for measuring the kinetics of enzymatic CP "activation" was essentially identical to that reported by Sladek [22]. Separate samples of (+)-, (−)-, and (±)-CP (1.0 to 0.2 mM) were incubated in duplicate runs at 37° using phosphate buffer (pH 7.4, 0.1 M) which contained freshly prepared liver microsomes and cofactors. The reaction mixtures were quenched by protein precipitation [$ZnSO_4$, $Ba(OH)_2$] and were assayed colorimetrically for soluble metabolites capable of alkylating 4-(*p*-nitrobenzyl)pyridine (NBP) [25]. The total concentration of this metabolite mixture was

*A new synthetic route to (+)- and (−)-CP has been recently published by Kawashima *et al.* [20]. These investigators established enantiomeric purity of CP samples by ^{1}H N.M.R. using $Eu(hfc)_3$, which is the heptafluoropropyl analog of $Eu(tfc)_3$, and it was reported that $Eu(hfc)_3$ is the chiral shift reagent of choice for CP.

†P. B. Farmer *et al.*, personal communication.

determined on a relative basis using a Beer's Law correlation that had been obtained previously with known amounts of bis-2-chloroethylamine (nor-HN2), and metabolite concentrations are thus expressed in terms of μmol nor-HN2 equivalents. Pertinent details regarding the kinetic measurements are discussed below; however, it should be noted that the recognized [22] vulnerability of the NBP assay procedure to extraneous error factors required strict systematization, and that the reliability of individual data points was insured by triplicate colorimetric measurements.

Time-course for liver microsomal-induced conversion of CP into acrolein. The procedure of Alarcon *et al.* [26, 27] for monitoring the production of acrolein was utilized. Samples of (+)-, (−)-, and (±)-CP (20 μmol) were incubated separately at 37° with an amount of microsomes equivalent to 0.75 g wet liver in phosphate buffer (pH 7.4, 0.1 M) containing all necessary cofactors. The final volume of each incubation mixture was 10 ml, and a constant flow rate of 120 ml/min was used for the oxygen purging gas. Acrolein concentrations in cold water (60 ml) traps, which were replaced in tandem every 15 min, were determined by reaction [27] with recrystallized *m*-aminophenol and quantitative assay for the 7-hydroxyquinoline so produced, using an Aminco–Bowman fluorometer.

The influence of liver cytosol upon the time-course of acrolein collection was measured by conducting a set of CP incubations identical to those described, with the addition of both freshly isolated cytosol equivalent to 0.20 g wet liver and NAD^+ (5.4 μmol). Use of cytosol equivalent to 0.75 g wet liver led to lowered acrolein concentrations that were near the limits of detection for the fluorometric method employed.

To gauge the efficiency and time-profile of acrolein removal from these incubation mixtures, a control solution of acrolein (50 μmol) in phosphate buffer without microsomes was subjected to the standard purging conditions and acrolein assay method.

Isolation of unmetabolized CP samples from liver microsomal incubations. Separate (+)- and (−)-CP samples were incubated with liver microsomes in the usual manner and, after CP activation had ceased (30 min), the incubation mixtures were either directly extracted with chloroform or were first treated with $ZnSO_4$ and $Ba(OH)_2$ to precipitate protein and then subjected to extraction. Solvent removal *in vacuo* followed by chromatography [silica gel; chloroform–methanol (9:1)] of residual material gave purified CP for high-resolution ^1H N.M.R. measurement of enantiomeric composition [12].

Anticancer screening of CP samples. Enantiomerically pure anhydrous (+)-CP (NSC No. 273034) and (−)-CP (NSC No. 273033) were evaluated (National Cancer Institute Developmental Therapeutics Program) against mouse L-1210 lymphoid leukemia according to standard protocol [28], which was applied simultaneously to (±)-CP·H_2O for comparative purposes. Test samples were administered i.p. (distilled water vehicle) on day 1 only at doses between 500 and 50 mg/kg. Groups of female (five) and male (six) mice were used and results were evaluated on day 30. Mean survival time was the evaluation parameter, and compounds exhibiting a test/control precentage (T/C %) greater than 125 are considered to be active in this screening procedure.

RESULTS AND DISCUSSION

Stereoselectivity factors associated with liver microsomal conversion of CP into soluble alkylating metabolites. In contradistinction to the extensive amount of information available [29–31] concerning relationships between hepatic microsomal mixed-function oxidase activity and substrate chemical constitution, there have been relatively few reports [32–34] on enzymatic stereoselectivity with regard to the absolute configuration of a given chiral substrate. The net or overall stereoselectivity for CP enantiomers which is exhibited by this multiple-form enzyme system [35, 36] was evaluated *in vitro* with non-fractionated (pelleted) mouse liver microsomes. To date there have been no basic qualitative differences between CP metabolism results obtained with mouse liver as opposed to human liver [37]. The enzymatic activation of CP refers specifically to the transformation of CP into 4-HO-CP; hence, the incubation kinetic methodology which has been applied in the past [22] and adopted by us for monitoring this mono-oxidation process is rather indirect. The technique involves measurement of the total concentration (relative to nor-HN2) of the soluble metabolites that alkylate NBP and, therefore, cannot provide true turnover numbers for the enzyme reaction of interest. At a heuristic level, however, it was assumed that such pseudo or apparent activation rate data for microsomal activation of (+)- and (−)-CP might nevertheless provide a starting point for attempts to correlate stereoselective biological processes with the relative therapeutic efficiences of CP enantiomers.

The appearance of soluble NBP-alkylating metabolites from (±)-CP incubation mixtures was linear over a standard reaction period of 8.0 min, during which time approx. 7 per cent conversion of (±)-CP could be affected. Rate decelerations were found after this time period and may be due to the incursion of lipid peroxidation [38]; however, other reports indicate linearity over 20 [22] and 40 min [39]. Lineweaver–Burk plots shown in Fig. 2 represent the average of duplicate runs for the separate activation of (+)- and (−)-CP by the same liver microsomal preparation used for similar kinetic measurements with (±)-CP (data not shown). Comparison of the derived apparent V_{max} and K_m values listed in Table 1 (run 1) with corresponding values obtained from a second liver microsome preparation (run 2) shows clearly that there is substantial variation in the absolute magnitude of these pseudo kinetic parameters, while the averaged ratios indicate a small but real difference in behaviour between (+)- and (−)-CP: $V^-_{max}/V^+_{max} = 1.34 \pm 0.17$ and $K^-_m/K^+_m = 1.35 \pm 0.14$.

While the interpretation of these relative V_{max} and K_m values for (+)- and (−)-CP is complicated by the absence of direct information concerning (a) the relative abundance of soluble CP-metabolites, which provide colorimetric NBP-derivates having different extinction coefficients, and (b) the fractionation of chiral metabolites by insolubilization reactions, we note that other investigations of CP metabolism are

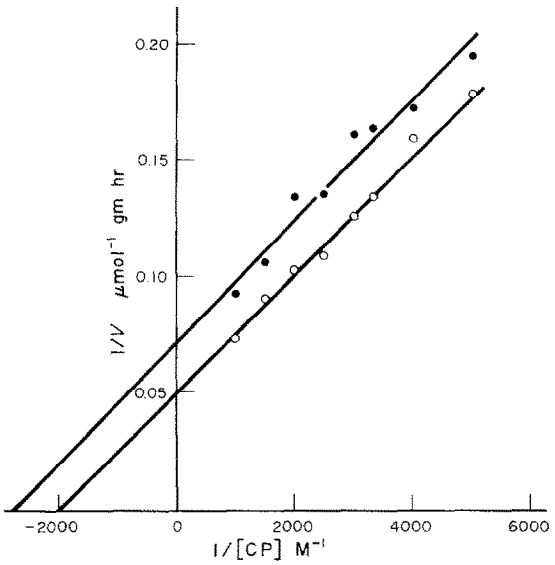

Fig. 2. Lineweaver–Burk plots for the production of soluble alkylating metabolites during incubation [2] of (+)-cyclophosphamide (●) and (−)-cyclophosphamide (○) in phosphate buffer (pH 7.4, 0.1 M) at 37° for 8.0 min with necessary cofactors and liver microsomal mixed-function oxidase obtained from phenobarbital-treated male Dub ICR mice. The apparent values of V refer to μmol nor-HN2 equiv./g wet liver/hr, and [CP] refers to mM cyclophosphamide. The derived apparent values of V_{max} and K_m are given in Table 1 (Run 1), together with the parameters obtained from a similar plot for (±)-CP which was omitted for the sake of clarity. Each line represents a least-square fit of averaged data points from duplicate experiments involving triplicate colorimetric determinations [21] of the metabolite mixture capable of alkylating 4-(*p*-nitrobenzyl)pyridine (NBP).

also consistent with the tentative conclusion that liver enzyme turnover rates for CP enantiomers are not appreciably different. Thus, mass spectroscopic studies* of CP-pseudoracemate metabolism by mouse liver microsomes also indicate little difference in the rates of metabolism of the enantiomers, with (+)-CP being metabolized very slightly faster than (−)-CP. Furthermore, similar spectroscopic techniques have shown that the initial disappearance rates for (+)- and (−)-CP during metabolism are virtually identical [16] and that excreted CP in mouse urine was not enriched markedly in either enantiomer [40].

The V_{max} value of 16.3 ± 1.1 μmol nor-HN2 equiv./g wet liver/hr obtained for (±)-CP (Table1, run 1) is approximately four times greater than that reported by Sladek [22], who used liver microsomes from untreated male rats, and is consistent with the observation [39, 41] that PB inductions akin to those utilized herein afford higher levels of enzyme activity toward CP. Such inducing agents have been reported [33] to influence enzymatic stereoselectivity toward enantiomers; unfortunately, however, our untreated mice yielded microsomes which result in unacceptably large errors in attempted comparative kinetic measurements with (−)- and (+)-CP.† The value of V_{max} for (±)-CP in Table 1, run 1 is, within experimental error, equal to the average of V^{+}_{max} and V^{-}_{max} for run 1 (16.3 ± 1.1 vs 16.9 ± 1.2 nor-HN2 equiv./g wet liver/hr), as are the K_m values (0.45 ± 0.02 vs 0.46 ± ± 0.03), which suggests that the NBP-alkylating metabolites are formed in a non-inhibitory fashion.

A final and important aspect related to stereoselectivity of microsomal activation of CP concerns CP racemization that could, in principle, be competitive with the enzymatic C-4 oxidation. In the extreme case, relatively rapid stereomutation at the asymmetric phosphorus center would lead to conversion of pure (+)- and (−)-CP into either racemic or partially racemized CP prior to 4-HO-CP formation and would thereby obscure the interpretation of kinetic data and screening results. There are at least three reasonable CP racemization pathways: (a) addition of a fifth ligand (L) to phosphorus giving either a symmetrical pentaccordinate dihydroxyphosphorane (L = OH) or an unsymmetrical phosphorane (L ≠ OH) capable of intramolecular ligand exchange [43–45], (b) reversible electron-transfer from the Fe(II) heme-enzyme [31] center to bound CP substrate, which produces a stereochemically labile phosphoranyl radical anion ($R_1R_2R_3\dot{P}O^-$) [46], and (c) oxygen-transfer from Fe(II) to CP to afford a stereochemically non-rigid phosphorane ($R_1R_2R_3PO_3$). As a test for such racemization pathways, separate incubations of (+)- and (−)-CP with liver microsomes under standard conditions were followed by recovery of unmetabolized CP and Fourier transform ^{1}H N.M.R. assay for enantiomeric purity. No racemization was detected (< 1per cent), which implies that if enzyme-mediated CP stereomutation is occuring the stereomutated substrate does not re-enter the "pool" of available enantiomerically pure CP. These data do not exclude the possibility of CP racemization followed by 4-HO-CP formation; however, comparative rate data which are discussed in the next section allow for the definite conclusion that NBP-alkylating products from (+)- and (−)-CP cannot be formed in completely racemic form.

Stereoselectivity factors associated with metabolic conversion of CP into acrolein and phosphoramide mustard. In vivo fragmentation of 4-HO-CP/AP into acrolein and phosphoramide mustard is reportedly [3] a first-order kinetic process, while oxidation of 4-HO-CP/AP into the relatively nontoxic 4-keto-CP and carboxyphosphamide urinary metabolites is presumably enzymatic and is believed to involve soluble enzymes that are particularly effective in liver cytosol as compared to other tissue. For example, a bioassay system has been utilized by Cox *et al.* [10] to demonstrate that marked detoxification of (±)-CP incubation mixtures is affected by treatment with

*P. B. Farmer *et al.*, personal communication.

†While it is thought [22, 42] that cytochrome P450 (or an analogous heme-enzyme system) is responsible for conversion of CP into 4-HO-CP, control experiments with PB-induced microsomal preparations revealed that heating at 37° before substrate addition and assay for cytochrome P450 markedly reduced the enzymatic activity toward (±)-CP without a proportionate reduction in cytochrome P450. For example, increasing the preincubation heating period from 4 to 8 min led to a 50 per cent decrease in the concentration of (±)-CP metabolites that alkylate NBP; however, this decrease in activity was accompanied by only a 14 per cent decrease in the value of P450/mg of protein.

Table 1. Apparent kinetic parameters for incubation (37°) of cyclophosphamide (CP) with liver microsomal mixed-function oxidase obtained from phenobarbital treated male Dub ICR mice.

Run*	Substrate	V_{max}† (μmol/g/hr)	V^-_{max}/V^+_{max}	K_m (mM)	K^-_m/K^+_m
1	(−)-CP	20.0 ± 1.5	1.45 ± 0.22	0.56 ± 0.04	1.51 ± 0.19
	(+)-CP	13.8 ± 1.0		0.37 ± 0.02	
	Avg.	16.9 ± 1.2		0.46 ± 0.03	
	(±)-CP	16.3 ± 1.1		0.45 ± 0.02	
2	(−)-CP	27.4 ± 1.6	1.23 ± 0.13	0.57 ± 0.02	1.19 ± 0.09
	(+)-CP	22.2 ± 1.0		0.48 ± 0.02	
	Avg.		1.34 ± 0.17		1.35 ± 0.14

*Runs 1 and 2 refer to "identical" incubations (see Fig. 2 caption for details) which utilized liver microsomes that were freshly prepared on two separate occasions; the Lineweaver–Burk plots for (+)- and (−)-CP in run 1 are shown in Fig. 2. Each V_{max} and K_m value within a run represents an average of duplictate kinetic experiments.

†V_{max} values refer to the production of soluble metabolites capable of alkylating 4-(*p*-nitrobenzyl)pyridine and are measured in terms of μmol bis-2-chloroethylamine equiv. [22]/g wet liver/hr.

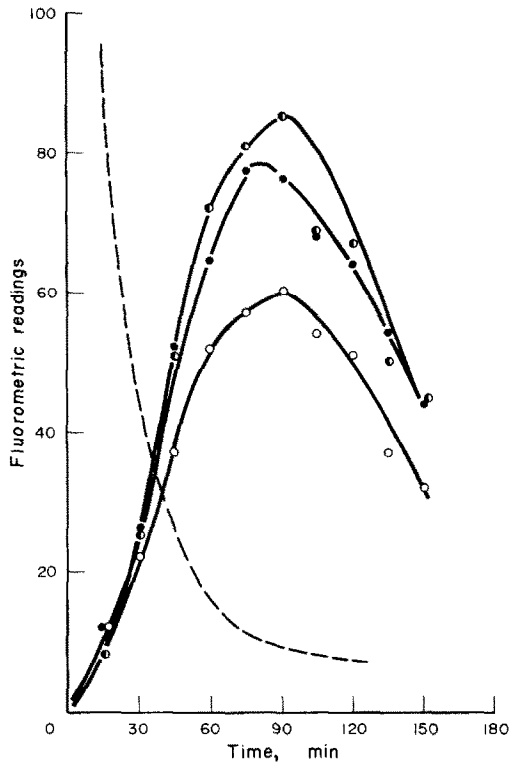

Fig. 3. Time-course studies, according to the method of Alarcon *et al.* [26, 27], of acrolein removal from separate incubation (37°) mixtures in phosphate buffer (pH 7.4, 0.1 M) containing 20 μmol (±)-cyclophosphamide (CP) (◐), (+)-CP (●) or (−)-CP (○) and microsomes equivalent to 0.75 g wet liver from phenobarbital-treated male Dub ICR mice, together with necessary cofactors. Final volumes were 10 ml and the flow rate for oxygen carrier gas was 120 ml/min. Concentrations of acrolein collected in cold water traps (60 ml, replaced every 15 min) were determined by a fluorometric method [26, 27]. The dashed curve represents the time-course for acrolein removal under typical conditions, minus liver microsomes.

liver-cell cytosol and that the extent of such deactivation correlated with NAD^+-dependent aldehyde dehydrogenase. Figure 3 shows the relative amounts of acrolein that were removed as a function of time from the gas phase of separate incubation mixtures of (+)-, (−)-, and (±)-CP with cofactors and equal portions of the same liver microsomal preparation. Based on our control data, it may be assumed that CP activation in these incubations had ceased after approx. 15 min and that the chronological profiles for the subsequently collected acrolein indirectly monitor the fate of 4-HO-CP/AP, for which the published [3] half-life is 80 min at 37° in phosphate buffer (pH 7, 0.07 M). While the absolute magnitude of fluorometric readings for a given 15-min collection period with each CP sample varied from run to run, as did the precise profile shape (presumably due to subtle differences between liver microsomal preparations), the qualitative relative relationships between these three profiles were reproducible. The observation (see control curve in Fig. 3) that acrolein is exponentially removed from phosphate buffer at a rate which is roughly comparable to the reported [3] 80 min half-life of 4-HO-CP/AP militates against a rigorous quantitative treatment of the acrolein metabolite profiles in Fig. 3. Qualitatively, however, it may be seen that (+)-CP leads to approx. 10–20 per cent more acrolein than (−)-CP during the period of "maximal collection" (approx. 60–120 min), and that the quantity of acrolein from (±)-CP exceeds that from (+)-CP by *ca.* 5–10 per cent. Comparison of the set of curves shown in Fig. 3 with a set obtained in an analogous series of incubations wherein liver-cell cytosol was added (Fig. 4) indicates that the expected diminution in acrolein collection is not accompanied by an experimentally significant degree of enantiomer selectivity, as the profile maxima are attenuated to approximately the same extent. The conclusion that neither the cytosol enzyme oxidations nor binding/conjugation processes involving 4-HO-CP/AP exhibit pronounced stereoselectivity may be

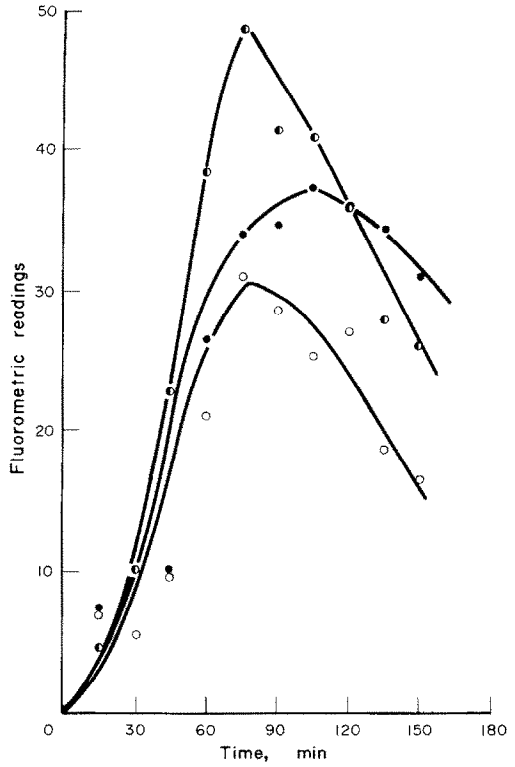

Fig. 4. Time-course studies of acrolein removal from separate cyclophosphamide incubations, as described in Fig. 3, with added NAD^+ (5.4 μmol) and cytosol equivalent to 0.20 g wet liver.

countered by arguing, for example, that the observed overall nonstereoselective influence of multi-component liver-cell cytosol could result from enantiomer-selective enzymatic transformations and enantiomer selective binding/conjugation which happen to operate in opposing stereochemical senses. This circumstance may be justifiable in that 4-keto-CP and carboxyphosphamide recovered from the urine of mice treated with CP-pseudoracemates undergo approx. 60–70 and 10–50 per cent enantiomeric enrichment [40], respectively; however, this reasoning is weakened by the fact that specific causative factors for such enrichment are unknown at this time.

A final point to consider regarding the acrolein profiles in Fig. 3 is the somewhat greater amount of acrolein which is collected during metabolism of (±)-CP as opposed to that starting from either pure enantiomer. This phenomenon contrasts with the behaviour of (±)-CP as seen in the soluble NBP-alkylating data [Table 1, $V^{\pm}_{max} = (V^{+}_{max} + V^{-}_{max})/2$] and may be rationalized by assuming competitive inhibition between 4-HO-CP/AP enantiomers in processes which control the rate of acrolein production. The amount of collectable (free) acrolein actually measured is influenced by subsequent conjugation reactions with glutathione [47] and other thiol functionalities [5, 6]; however, the achiral nature of acrolein allows one to neglect these factors because they operate in an identical fashion regardless of the stereochemical nature of the initial CP substrate.

Comparative anticancer screening data for CP enantiomers. The relationship between enantiomeric purity of CP and anticancer activity was evaluated by National Cancer Institute standard screening tests [28] against mouse L-1210 lymphoid leukemia. From the results summarized in Table 2, it can be seen that (+)- and (−)-CP exhibit differences between T/C values for males vs females at a given dosage, with such differences being most noticeable for 500 and 250 mg/kg injections. Male mice responded best when treated with (−)-CP [maximum T/C = 261% at 250 mg/kg vs maximum T/C = 223% at 500 mg/kg with (+)-CP], while females showed the best response with (+)-CP [maximum T/C = 280% at 250 mg/kg vs maximum T/C = 253% at 500 mg/kg with (−)-CP]. However, in both sex groups the differences between maximum T/C percentages for (+)- and (−)-CP are obviously small. The data for male mice further indicate that the highest T/C value obtained for (±)-CP·H_2O (297% at 250 mg/kg) is somewhat greater than the best T/C percentage shown by enantiomerically pure (−)-CP (261% at 250 mg/kg).

The salient feature of the above L-1210 test results, viz. that CP enantiomeric purity has little effect on therapeutic value, parallels the findings of Cox *et al.* [16], which were published during the course of our studies and indicate that therapeutic indices ($TI = LD_{50}/ID_{90}$) for CP enantiomers against ADJ/PC6 plasma cell tumor in mice cover only a 2-fold activity range: TI = 69, 93, and 128 for (+)-, (±)-, and (−)-CP respectively. Inasmuch as the initial metabolism of 4-methylcyclophosphamide (4-MeCP) appears to be somewhat analogous to that of CP [9], it is not suprising that recently published [48] *in vivo* screening data for *cis*-4-MeCP enantiomers [49] against ADJ/PC6 plasma cell tumor also show small activity differences: TI = 31, 51, and 58 for (+)-, (±)-, and (−)-*cis*-4-MeCP respectively. The corresponding *trans*-4-MeCP [49] sample series gave TI values of 32, 57, and 25 [48], and thereby revealed, curiously, a greater activity for the racemate as opposed to either pure enantiomer.

Anticancer activity of enantiomeric 5-membered ring CP analogs synthesized from amino acids has been measured [50]; however, gross structural (metabolic) differences between such molecules and CP preclude a legitimate comparison of results.

In conclusion, the relationship between biological stereoselectivity and absolute seterochemistry at the asymmetric phosphorus center in CP has been evaluated by use of enantiomerically pure and racemic CP samples to measure, in a relative manner, (a) the rates of liver microsomal activation of CP to give soluble NBP-alkylating metabolites, (b) the chronological profiles for release of acrolein from microsomally activated CP, (c) the influence of liver-cell cytosol components on such acrolein profiles, and (d) the therapeutic effectiveness of CP in a L-1210 lymphoid leukemia test system in mice. While experimentally significant differences between (+)- and (−)-, and (±)-CP obtain for (a)–(c), these inequities are small by comparison to those normally anticipated for biological systems. Small differences in relative effectiveness against mouse L-1210 leukemia also hold for the pure enantiomers and racemate of

Table 2. Anticancer screening data for enantiomerically homogeneous and racemic samples of cyclophosphamide (CP) against mouse L-1210 lymphoid leukemia in mice*

Sample	Dose (mg/kg)	Sex	Cures†	T/C (%)‡
(+)-(*R*)-CP [NSC No. 273034]	500	M	2/6	223
		F	2/5	271
	250	M	0/6	188
		F	3/5	280
	125	M	0/6	143
		F	0/5	173
	62.5	M	0/6	122
		F	0/5	121
(−)-(*S*)-CP [NSC No. 273033]	500	M	0/6	138
		F	0/5	253
	250	M	2/6	261
		F	0/5	188
	125	M	0/6	156
		F	0/5	140
	62.5	M	0/6	127
		F	0/5	142
(±)-(*R,S*)-CP·H_2O [NSC No. 26271]	500	M	0/6	§
	400	F	1/6	228
	250	M	3/6	297
	200	F	0/6	208
	125	M	0/6	161
	100	F	0/6	133
	62.5	M	0/6	138
	50	F	0/6	118

* Test samples were administered (water vehicle, i.p. injection) 1 day after mouse inoculation with 10^5 cells. The overall evaluation period was 30 days, with survival on day 5 used to determine toxicity. Unless noted otherwise, the listed doses were found to be 100 per cent nontoxic.

†First number refers to survivors; second number refers to size of the test group.

‡See Ref. 28; values > 125 indicate activity in this test system.

§Fifty per cent toxicity

CP; however, the ultimate significance that can be attached to these and related [16] screening results must await further *in vivo* tests. At the present time it appears as if there is a lack of appreciable stereochemical discrimination between (+)- and (−)-CP during intitial hepatic microsomal activation; however, details regarding subsequent stereochemical differentiation between the enantiomers of chiral CP metabolites are not adequately understood. *In vitro* and *in vivo* studies aimed at more direct evaluations of synthetically obtained enantiomerically pure 4-HO-CP/AP, 4-keto-CP, and carboxyphosphamide are in progress and will be reported in the future.

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