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Lipid Solubility and Affinity for N-Demethylation of Dansylamides in Isolated Rat Hepatocytes

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Isolated hepatocytes carry out the N-demethylation of dansylamide at near linear rates for up to 8 h. This reaction was measured by following the release of tritium into water on hydroxylation of ^{3}H-labeled methyl groups. The competitive inhibition of dansylamide by dansylated amino acids was studied in this system as an example of competing drug metabolism in a series of compounds which are identical around the site of metabolism and different remote to that site. A correlation between lipid solubility and the K_i was not found over the entire range of substrate analogues. While most of the high K_m inhibitors seem to correlate with lipid solubility, the highly lipophilic derivatives of the leucines and phenylalanine are in a separate group. Lipid solubilities of the dansylated amino acids were little affected by changes in pH and thus behaved as "essential nonelectrolytes".

Liver parenchymal cells have been recognized as the main locus of drug metabolism via microsomal hydroxylation pathways. Teleologically this process is necessary to excrete foreign materials in the kidney. Excretion is facilitated after introduction or uncovering of polar groups in an otherwise lipid-soluble xenobiotic, which would be reabsorbed in the latter part of the nephron. The degree of lipid solubility, as a rule, determines to a large extent the susceptibility of a xenobiotic to P-450-mediated reactions[1] in a system which otherwise shows very little substrate specificity. Binding of substrates precedes the metabolic transformation. Jefcoate et al.[2] have shown that the binding energy for the interaction of *n*-alkylamines with P-450 is linearly related with alkyl chain length, providing evidence for a hydrophobic cavity in the heme region of P-450 sufficient to accommodate large, hydrophobic substrates.

In this communication we are trying to investigate the utility of isolated hepatocytes for a study of the potential correlation between lipid solubility and the K_i of competitive substrates (inhibitors) of the metabolism of a radioactive parent molecule. Similar studies have been done before; however, the variations on the molecule to be metabolized were on the actual site of hydroxylation. An example of this approach was reported in 1963 by McMahon et al.[3] These authors reported that oxidative O-dealkylation of a series of alkyl and arylalkyl *p*-nitrophenyl esters in rat liver microsomes was dependent on the size of the alkyl groups. For saturated alkyl groups, the K_m and V_{max} values decreased with increasing length of the groups, which resulted in an overall decrease of the reaction rate for a given concentration. In vivo dealkylation rates correlated well with in vitro results. Creaven et al.[4] and Davies and Creaven,[5] studying a series of substituted hydroxybiphenyl derivatives, showed progressively decreasing rates of microsomal O-dealkylation as the alkyl substituent was increased from methyl through *n*-butyl but did not determine K_m and V_{max}.

We were particularly interested in whether lipid solubility would be the only variable in determining the effectiveness of structurally similar inhibitors in cases where the variation in the molecule was far distant from the site of hydroxylation and consisted of more than a chain-length alteration. For these experiments a series of dansylated amino acids was employed. The inhibition of [*N-methyl*-^{3}H]dansylamide N-demethylation by different dansylamino acids was measured and compared with the relative lipid solubility of the various derivatized amino acids. From the measured effectiveness of dansylamide N-demethylation, we have calculated the K_i values. Theoretically, if classical Michaelis–Menten enzyme kinetics are obeyed, one would predict the K_m of an inhibitor's own metabolism to be identical with its K_i in the competitively inhibited reaction. To study the relevance of lipid solubility as a singular determinant of drug metabolism in a series of similar compounds under conditions close to the in vivo situation, but in the absence of biological variation, a minimal requirement is an intact cellular system.

Despite the advantages of liver cells as compared to microsomes, there have been relatively few attempts to utilize this system to study drug metabolism. Berry[6] has successfully used isolated hepatocytes to study the action of pyruvate on ethanol metabolism. Holtzman et al.[7] have

found that these cells will metabolize aniline, ethylmorphine, and 3,4-benzo[a]pyrene, while Corona et al.[8] and Zimmerman et al.[9] have studied hepatotoxicity by drugs in isolated liver cells. Moldéus et al.[10,11] have studied drug–cytochrome P-450 interactions and drug metabolism linked to cytochrome P-450 in isolated hepatocytes. Von Bahr et al.[12] have also studied the binding of drugs to cytochrome P-450 in this system. Recently, Vadi et al.[13] have studied the metabolism of 3,4-benzopyrene in isolated rat liver cells and have shown that the rate of the primary, oxidative step was similar to that catalyzed by isolated rat liver microsomes. Moldéus et al.[14] have investigated conjugation reactions in this system.

In a recent communication[15] we have described drug metabolism experiments in isolated liver cells prepared by perfusion of isolated rat livers with collagenase. We have shown that these isolated hepatocytes metabolize drugs for extended periods of time (8 h). There are many advantages of such a drug-metabolizing system for experiments such as ours. While maintaining the organizational level of the isolated perfused liver, one can do many parallel experiments with aliquots of cells from one liver. This system lends itself to multiple sampling and ready manipulation of experimental parameters and eliminates biological variations. The results of our experiments indicate that lipid solubility is not the singular parameter controlling drug metabolism in a limited series of analogues when an integrated system such as hepatocytes is employed. We have found that isolated hepatocytes rapidly metabolize drugs and we believe that this is a promising system for future drug metabolism studies.

Experimental Section

Abbreviations used: EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; dansyl chloride, 5-dimethylamine-1-naphthalenesulfonyl chloride; SKF-525-A, 2-diethylaminoethyl-2,2-diphenyl valerate hydrochloride.

Materials and Methods. Animals. Male Sprague–Dawley rats, 200–250 g, were maintained on a standard Purina rat chow diet at 22 °C in temperature controlled rooms on a cycle of 12 h of light and 12 h of darkness.

Materials. Collagenase (CLS-111, 132 U/mg) was obtained from Worthington Biochemical Corp. EDTA, HEPES, and bovine albumin, fraction V, were obtained from Sigma Chemical Co. Amino acids (100X), L-glutamine (200 mM), and "antibiotic–antimycotic" (10000 units of penicillin, 10000 μg of streptomycin, and 25 μg of Fungizone per ml) were purchased from Grand Island Biological Co. Dextran T-70 was from Pharmacia and dextrose from Mallinckrodt. Lithium lactate was obtained from Nutritional Biochemicals Corp. and dansyl chloride from Pierce Chemical Co. Dansyl chloride [*N*-methyl-^{3}H], 1–5 Ci/mmol, was purchased from New England Nuclear. Dansylated amino acids were purchased from Seikagaku Fine Biochemicals. SKF-525-A was obtained from Smith Kline and French Labs.

Radioactive Compounds. Radioactive dansylamide was synthesized by reacting dansyl chloride in acetone with excess gaseous ammonia. After standing overnight, the reaction mixture was taken to dryness and an aliquot of the residue repeatedly recrystallized from alcohol–water with nonlabeled dansylamide. The specific radioactivity did not change after three recrystallizations and the compound migrated as a single radioactive spot on TLC.

Radioactive dansyl acid was synthesized by hydrolysis of the labeled dansyl chloride with 0.1 N NaOH. Its purity was checked by TLC in benzene–pyridine–acetic acid (16:4:1) in which it migrated as a single spot cochromatographing with standard dansyl acid. This spot was scraped off the plate, counted, and found to contain 97% of the radioactivity of the original dansyl chloride aliquot.

Perfusion System and Preparation of Hepatocytes. The techniques for cell preparation have been previously described.[15] The perfusion system incorporated the following features: isolated in vitro organ; a circulating perfusate volume of 100 ml; enclosed silastic membrane oxygenating system, constant volume nonpulsatile pump; perfusion pressure monitoring; the canulated organ submerged in buffer inside the thermostated perfusion vessel.[16] The ischemic period was no longer than 15 s.

Incubation Conditions. Each incubation reaction contained 5-ml cells which were pipetted into 25-ml Erlenmeyer flasks, the bottoms of which had been heated and pushed inward to form a conically raised center which kept the cells from settling in the flask when incubated in a gyratory shaker. The opening of the pipet tip used for the dispension of the cells was enlarged to 3 mm and fire polished. The 100-ml stock-cell suspension was swirled continuously while pipetting to ensure equal aliquots of cells in each reaction. The concentration was 45–60 mg wet weight cells/ml or about 4.4–5.9 × 10^6 cells/ml. All incubations were carried out at 30 °C. We have found that liver cells retain their viability at 30 °C for longer periods than at 37 °C while carrying out all measured metabolic functions. The reaction flasks were swirled in a gyrating incubator at 80 revolutions per minute. The appropriate concentrations of drug in 50 μl of redistilled dimethyl sulfoxide plus 0.5 μCi of very high specific activity (5 Ci/mmol) labeled drug were added to each reaction.

Cell Viability. The trypan blue exclusion test (0.25% trypan blue in perfusion buffer) indicates that about 95% of the cells have intact cellular membranes preventing the stain to reach the nucleus. In a previous communication,[15] we have reported that these cells utilize oxygen at a linear rate, synthesize glucose from alanine, pyruvate, fructose, and lactate at rates comparable to the isolated perfused liver, and metabolize citrate in a linear fashion for 8 h. In the meantime, we have established that these cells respond to various physiological stimuli, such as glucagon, insulin, epinephrine, and cAMP, with respect to rates of glycogenolysis and gluconeogenesis.[17]

Radioactive Assay. The measurement of N-dealkylation of [*N*-methyl-^{3}H]dansylamide was based on the exchange of tritium into water following the formal interjection of an oxygen atom into a C–H bond, a technique similar to that used by Thompson et al.[18] to study ethylmorphine *N*-demethylase. One-third of the ^{3}H becomes exchangeable with H_2O and the remaining two-thirds are released on successive metabolism of formaldehyde to formic acid and CO_2. In this series of reactions the primary P-450 catalyzed step is the slowest reaction and controls the rate of tritium release into the aqueous media as we have shown in earlier experiments by the addition of methanol, formaldehyde, and formate.[15] At various time intervals, 500 μl of the incubation medium containing cells was sampled and centrifuged and 200 μl of the supernatant placed on a short Dowex-50 cation-exchange column in the H^+ form. The column was eluted with 2 ml of water, and the effluent was mixed with 14 ml of a solution containing 333 ml of Triton X-100 (Rohm and Haas) and 667 ml of toluene and 3.8 g of Omnifluor. The vials were then counted in a Beckman LS 250 liquid scintillation counter. Since the substrate (dansylamide) and its metabolites are held by the ion-exchange column and the tritiated water passes through, it is possible to quantitatively separate the two.

Fluorescence Measurements. Fluorescence of the dansylated amino acids was measured in an Aminco-Bowman spectrophotofluorometer at an excitation wavelength of 350 nm and an emission wavelength of 494 nm, as described by Seiler.[19]

Lipid Solubility. Relative lipid solubilities of dansylated amino acids were determined by two different methods. The first method involved equilibrating the compounds between equal volumes of chloroform and phosphate buffer. To 5 ml of chloroform, 0.5 μmol of each compound was added and the fluorescence determined. The chloroform solutions were then extracted by shaking for 5 h with 5 ml of 0.1 N phosphate buffer, pH 7.4, and the fluorescence remaining in the chloroform layer was redetermined. The difference between the first and second fluorescence determinations reflects the amount of drug which entered the buffer phase. From this, the chloroform–buffer ratios were calculated. The second method of determining relative lipid solubilities employed TLC. This experiment was designed to show the effects of pH on lipid solubility. 1-Butanol was saturated with 1 N acetic acid at pH 2.5 or 1 N pyridine at pH 8.0. The dansylated compounds were spotted on silica gel plates and developed in either the butanol–acetic acid or butanol–pyridine

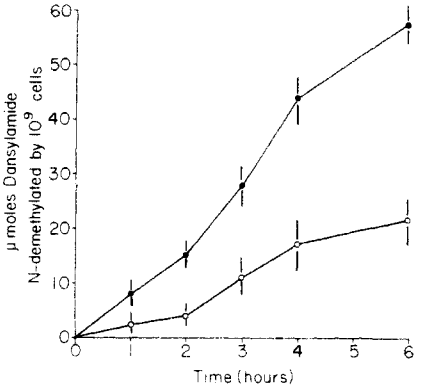

Figure 1. N-Demethylation of 0.5 mM dansylamide by isolated rat hepatocytes (•); N-demethylation of 0.5 mM dansylamide + 50 μg/ml (0.13 mM) of SKF-525-A (○). Incubations were carried out at 30 °C with liver cell concentrations of 50 mg/ml wet weight. Points are the average of duplicate samples from duplicate reactions. A representative experiment is shown. Confidence levels are indicated as ± standard error of the mean.

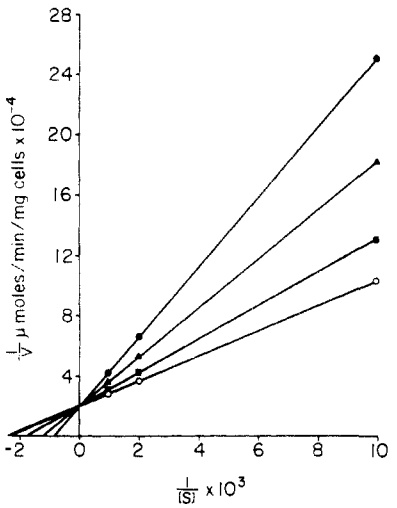

Figure 2. Lineweaver-Burk plot of dansylamide N-demethylation (○); dansylamide + 0.1 mM dansyl-L-isoleucine (▪); dansylamide + 0.5 mM dansyl-L-isoleucine (▲); dansylamide + 1.0 mM dansyl-L-isoleucine (•). A cell concentration of 50 mg/ml wet weight was incubated at 30 °C as described in the text. Points are the average of duplicate samples from duplicate reactions taken at 90 min.

solutions. The plates were then dried and the compounds identified with a fluorescent light. The relative R_f values should parallel the relative solubilities of the compounds in butanol, if no other effects such as adsorption on silica gel are involved.

Presentation of Data. Experiments were repeated at least three times. Reactions were run in duplicate, and duplicate samples were taken from each reaction for assays. In each case data from a representative experiment are given, and values are the average of the four assays per point (duplicate reactions, duplicate samples). Even though the absolute values vary from experiment to experiment (10–15%) the relative relationships between experimental conditions remain within close limits (see Figure 1).

Results and Discussion

Isolated liver cells linearly N-demethylated dansylamide for as long as 8 h. This metabolism was inhibited by 50 μg/ml (0.13 mM) of SKF-525-A (Figure 1). The rate of ^3H_2O release was concentration dependent and a Lineweaver–Burk plot shows the Michaelis constant for dansylamide to be 0.435 mM (Figure 2).

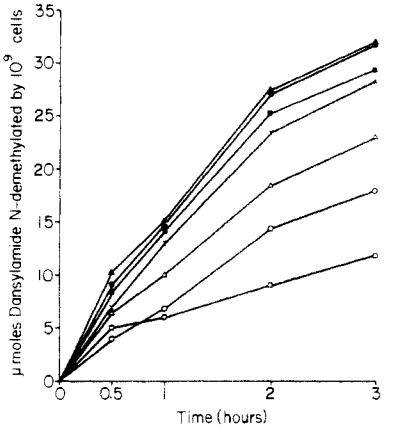

Figure 3. Inhibition of 0.5 mM dansylamide N-demethylation by 0.5 mM dansyl acid (•); 1.0 mM dansyl acid (▪); 2.0 mM dansyl acid (×); 0.5 mM dansyl-L-isoleucine (△); 1.0 mM dansyl-L-isoleucine (○); 2.0 mM dansyl-L-isoleucine (□); no inhibitor (▲). Incubations were carried out at 30 °C with liver cell concentrations of 50 mg/ml wet weight. Points are the average of duplicate samples from duplicate reactions not differing by more than 10%.

Table I.[a] Percent Inhibition by 1 mM Dansylated Amino Acids on N-Demethylation of 0.5 mM Dansylamide

Dansyl-L-glutamic acid	7% activation
Dansyl-L-proline	6% activation
Dansyl-L-arginine	3.5% inhibition
1-Dimethylaminonaphthalene-5-sulfonic acid	5% inhibition
Dansyl-L-aspartic acid	5.25% inhibition
Dansyl-L-valine	5.4% inhibition
Dansyl-L-alanine	16.4% inhibition
Dansyl-L-tryptophan	21.5% inhibition
Dansyl-L-glycine	29% inhibition
Dansyl-L-methionine	42.75% inhibition
Dansyl-L-isoleucine	44.5% inhibition
Dansyl-L-leucine	46.5% inhibition
Dansyl-L-phenylalanine	51.7% inhibition

[a] Relative inhibitions of 1.0 mM dansylamino acids on the N-demethylation of 0.5 mM dansylamide. The inhibitors were added at time 0, and the precent inhibition was determined after 90 min of incubation. A cell concentration of 50 mg/ml wet weight was incubated at 30 °C as described in the text. Values are the average of duplicate samples from duplicate reactions.

Inhibition of dansylamide N-demethylation was measured in the presence of 12 different dansylated amino acids, as well as dansyl acid. A representative experiment in which dansyl-L-isoleucine and dansyl acid are the inhibitors is shown in Figure 3. The reactions proceed at near linear rates in all cases. At a concentration of 0.5 mM dansylamide, 0.5 mM, 1.0 mM, and 2.0 mM dansyl acid, one of the weakest inhibitors, had little effect on dansylamide N-demethylation, while dansyl-L-isoleucine, one of the most potent inhibitors, showed an inhibition of 60% at 2mM as determined at 90 min. Similar results have been found for dansylglycine, an inhibitor of intermediate strength. When these data are plotted according to the method of Lineweaver and Burk, the typical picture of competitive inhibition is seen (Figure 2). The relative inhibitions of 1 mM dansylamino acids on N-demethylation of 0.5 mM dansylamide are shown in Table I. The degree of inhibition was determined at 90 min as the metabolism was linear between 0.5 and 2 h (see Figure 3). In all cases, the relative inhibitions are independent of the time of incubation. The N-demethylation of [*N-methyl-*^3H]dansyl acid was also competitively inhibited by dansylamide (data not shown). The K_i for dansylamide

Table II.[a] Chloroform-Phosphate Buffer Partition Coefficient

Dansyl-	Coeff	Dansyl-	Coeff
Glu	0.011	Trp	0.064
Gly	0.014	Val	0.074
Acid	0.015	Pro	0.158
Arg	0.030	Phe	0.169
Asp	0.045	Leu	0.221
Met	0.059	Ile	0.265
Ala	0.061	Amide	7.330

[a] Table II shows the relative chloroform-phosphate buffer partition coefficients of the tested dansylamino acids. The partition coefficients are listed in order of increasing chloroform solubility. To 5 ml of chloroform, 0.5 μmol of each dansylamino acid was added, and the fluorescence determined. The chloroform solutions were then extracted by shaking for 5 h with 5 ml of 0.1 N phosphate buffer, pH 7.4, and the fluorescence in the chloroform was redetermined. The difference between the first and second fluorescence determinations reflects the amount of drug that enters the buffer phase. From this the chloroform-buffer ratios were calculated.

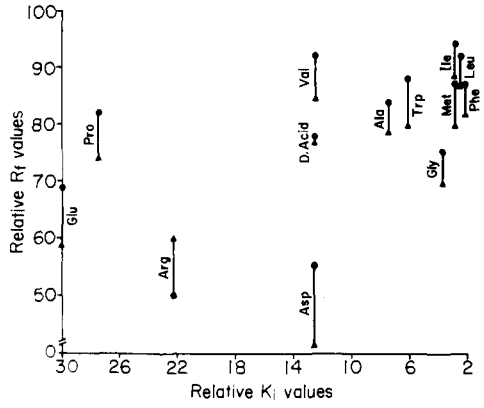

Figure 4. Relationship between relative R_f values of dansylamino acids and their potencies as inhibitors of N-demethylation. Relative R_f values of dansylamino acids were determined in basic (▲) and acidic (●) solvent systems by thin-layer chromatography. The K_i values were calculated from the potencies of the compounds as inhibitors of N-demethylation. The two extreme relative R_f values are connected by a line and plotted over the relative K_i values.

was found to be 0.637 mM in this competition experiment. We feel this is in reasonable agreement with the K_m of 0.435 mM found in an earlier experiment with dansylamide by itself.

The relative chloroform solubilities of the tested dansylated amino acids are shown in Table II. In most cases the derivatives with the higher relative chloroform solubilities are the more potent inhibitors of dansylamide N-demethylation. However, there are several analogues that are either relatively soluble in chloroform but poor inhibitors (dansyl-L-proline) or relatively insoluble but effective inhibitors (dansyl-L-glycine). The best inhibitors, the dansyl derivatives of L-leucine, ammonia, L-isoleucine, and L-phenylalanine, had K_i values which did not fit a linear correlation with lipid solubility as extrapolated from the remaining dansylamino acids.

Since Collander has reported a linear correlation between the permeability of cells and the lipid solubility of organic nonelectrolytes,[20] we were interested to determine whether the dansylamino acids would behave as "essential nonelectrolytes" and have lipid solubilities little effected by pH changes. When the dansylated amino acids were developed on TLC plates in either acidic or basic solvents, the compounds migrated in a similar fashion with relatively

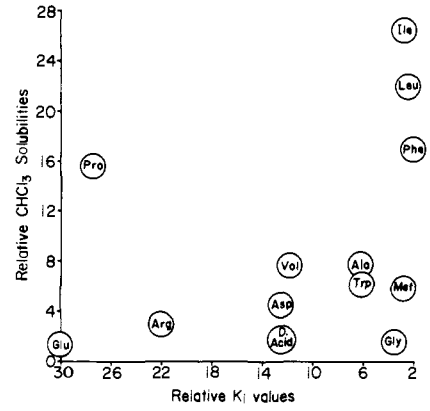

Figure 5. Relationship between relative chloroform solubilities of dansylamino acids and their potencies as inhibitors of N-demethylation. Relative chloroform solubilities of a series of dansylamino acids are plotted over their relative K_i values. Chloroform solubilities were determined by partitioning the compounds between chloroform and phosphate buffer. The K_i values were calculated from the potencies of the compounds as inhibitors of dansylamide N-demethylation.

Table III[a]

Dansyl-	K_i values × 10^{-3} M	Dansyl-	K_i values × 10^{-3} M
Glu	0.304	Trp	0.060
Pro	0.275	Gly	0.037
Arg	0.222	Met	0.029
Acid	0.127	Ile	0.027
Asp	0.125	Leu	0.025
Val	0.124	Phe	0.021
Ala	0.074		

[a] Table III contains the relative K_i values of the tested dansylamino acids as determined from their potencies as inhibitors of dansylamide N-demethylation. The K_i values were calculated from the percent inhibition of 1.0 mM dansylamino acids on the N-demethylation of 0.5 mM dansylamide. The formula used for the calculations was that of Michaelis-Menten for a simple inhibitor.

small changes of R_f value (lipid solubility) as compared to the large pH differential (pH 3–8) (Figure 4). This indicates that differences in the pH of the microenvironment around the P-450 complex and the various transport barriers cannot alone account for the discrepancies between lipid solubility and K_i wherever those were observed.

In Figure 5 the relative chloroform solubilities are plotted against the relative K_i values of the dansyl derivatives. This plot illustrates that there is no definite correlation between K_i and lipid solubility under these experimental conditions. The K_i values were determined from the percent inhibition using the Michaelis-Menton equation for a simple inhibitor

$$K_i = \frac{K_m[I]v_i}{V_{max}[S] - V_i(K_m t[S])}$$

and are shown in Table III. Comparing the TLC data (Figure 4) with the partition experiment (Figure 5), it seems that better correlation is seen in the former, an indication that the R_f values might be influenced by adsorption phenomena.

Collander[20] had also shown that molecular volumes play a role in determining cell permeability to organic nonelectrolytes. That is, smaller molecules with similar olive oil–water partition coefficients more readily pass the cellular membrane than larger ones. In those instances where discrepancies in dansylamino acid lipid solubilities

and their effectiveness as inhibitors are observed, a case could be made for smaller molecular volumes due to hydrophobic interaction between the naphthyl ring and the amino acid side chain. A similar consideration might apply to dansyl-L-proline, an amino acid derivative of more rigid structure.

If one exempts the derivatives of phenylalanine and the leucines and the dansyl derivative of proline from the rest of the compounds, a correlation coefficient for K_i and lipid solubility is found to be 0.739.

One might consider the tritium isotope effect from the [*N-methyl*-^{3}H]dansylamide and dansyl acid and to what degree this could potentially effect the observed rates of N-demethylation. The rate of N-demethylation of the nonlabeled material is not expected to be significantly higher than that measured since, first, we do not measure small, initial, but rather significant overall conversions; second, Thompson et al.[18] have found that colorimetric and radioactive assays of [*N-methyl*-^{3}H]ethylmorphine N-demethylation yield identical quantitative values. In addition, Elison et al.[21,22] have compared the rates of demethylation of morphine (R–NH–CH_3) and trideuterio-*N*-methylmorphine (R–NH–CD_3) by rat liver microsomes. The rate of demethylation of the deuterated substrate was only slightly less than that of the hydrogenated compound. Another parameter which might affect the overall drug metabolic rates is that amino acids are actively transported, and it is possible that their dansylated derivatives are also substrates for these transport systems. These agents could also affect the drug-metabolizing system at a number of other points such as NADPH synthesis or respiration; however, the competitive kinetics argue against such alternative mechanisms.

The isolated liver cells are definitely a complicated system and to study the dependence of drug metabolism on lipid solubility, similar experiments will have to be carried out with liver microsomes. The comparison of these two sets of data will then allow the evaluation of the question of whether the anomalies which we have observed are inherent to the P-450 system or are introduced by the multicompartmentation of the intact cell. It should be pointed out that liver cells constitute a higher level of biological organization, so that information collected in liver cell experiments should be closer to the in vivo situation than experiments performed with microsomes.

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Notes

Hashish.[1] Unsaturated Side-Chain Analogues of Δ^8-Tetrahydrocannabinol with Potent Biological Activity

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Two Δ^8-THC derivatives, **4a** and **4b**, with functionalized side chains were synthesized. Treatment of (+)-*trans*-*p*-mentha-2,8-dien-1-ol with the resorcinal **2b** followed by removal of the dithiol group with HgO–BF_3·Et_2O gave the aldehyde **3b**. A Wittig reaction of dimethyl (2-oxoheptyl)phosphate with **3b** furnished **4a**, which was reduced to **4b**. Compounds **4a** and **4b** showed potent cannabinoid-like activity in mice.

The main active constituent of marihuana is Δ^9-tetrahydrocannabinol (THC) which causes CNS depression and ataxia in laboratory animals. The most prominent effects are decreased locomotor activity, increased sensitivity to stimuli, such as sound and touch, general depression, and, at high doses, static and dynamic ataxia.[2]

In natural THC's, i.e., Δ^8- and Δ^9-THC's, the effect of side-chain modification in the aromatic ring has not been studied as extensively as in the synthetic $\Delta^{6a,10a}$-THC's. However, the limited examples which have been reported[3]