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ON THE INHIBITION OF MICROSOMAL DRUG METABOLISM BY SKF 525-A*

S. JENNER and K. J. NETTER

Department of Pharmacology, Section on Toxicology, University of Mainz, D 65 Mainz, Germany

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Abstract—Kinetic experiments have been carried out on the inhibition of the O-demethylation of p-nitroanisole and the N-demethylation of N-monomethyl-p-nitroaniline by β-diethylaminoethyl-diphenyl-n-propylacetate-HCl (SKF 525–A). The source of the enzyme were liver microsomes of male mice pretreated with phenobarbital. Addition of original SKF 525–A to the reaction mixture resulted in an apparently non-competitive inhibition of both demethylation reactions. When the substance was recrystallized from benzene the non-competitive type of inhibition was converted to an apparent competitive type of inhibition. Use of other solvents for recrystallization such as water, methanol, cyclohexane and chlorobenzene did not lead to a change of the type of inhibition. Therefore recrystallization from benzene seemed to be unique in causing this peculiar change of kinetic behaviour. Benzene produces no chemical alteration of the SKF 525–A molecule that could be detected by a number of physicochemical methods employed.

MANY compounds of different chemical nature inhibit the oxidative metabolism of foreign compounds by hepatic microsomal mixed-function oxidase. Among these SKF 525–A (β-diethylaminoethyl-diphenyl-n-propylacetate-HCl) is the most frequently used inhibitor. For the mechanism of action of this inhibitor several explanations have been offered. They are based on: (a) a combination of SKF 525–A (as an alternative substrate) with the substrate-binding site of cytochrome P 450 resulting in a competitive inhibition,[1] (b) a nonspecific alteration of the enzyme protein resulting in a formally non-competitive inhibition, (c) a specific action in deviating the electron flow thus dissociating NADPH consumption and drug oxidation (uncoupling)[2] and (d) an interference with the penetration of substrate through the microsomal membrane. Since kinetic experiments could possibly decide between these different explanations, several authors have tried to classify the inhibition as competitive or non-competive. Depending on the drug substrates used, however, either type of inhibition has been found as summarized in Table 1. Possible reasons for these discrepancies have recently been discussed by Mannering.[15]

In view of the conflicting results of these kinetic experiments the finding[11] was particularly interesting that SKF 525–A, recrystallized from benzene, appeared as a competitive inhibitor in a system that the original compound normally inhibits non-competitively. This observation suggests that the variable results may be due to differences in the chemical pretreatment of SKF 525–A and not to differences in microsomes. Therefore the effect of recrystallization of SKF 525–A from benzene and other solvents on the type of inhibition was investigated. It is shown that benzene recrystallization *per se* changes the type of O-demethylation of p-nitroanisole and N-demethylation of N-monomethyl-p-nitroaniline from non-competitive to competitive.

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TABLE 1. INHIBITION OF DRUG OXIDATIONS BY SKF 525-A

Substrate	K_m of substrates (mM)	K_i of SKF 525-A (mM)	Authors	Ref.
Non-competitive type of inhibition				
Monomethyl-4-aminoantipyrine			La Du *et al.*	1955 (3)
o-Nitroanisole	0·8	0·08	Netter	1960 (4)
p-Nitroanisole	0·2		Netter *et al.*	1964 (5)
Trichloroethylene	100	11	Byington *et al.*	1965 (6)
Aniline	1·7	0·16	Ikeda *et al.*	1968 (7)
Diphenylhydantoin	0·037		Kutt *et al.*	1970 (8)
Aminopyrine	6		Ackermann	1970 (9)
Competitive type of inhibition				
N-Methyl-butynamine			McMahon	1962 (10)
o-Nitroanisole	0·7		Gilbert	1965 (11)
Ethylmorphine	0·58	0·06	Anders *et al.*	1966 (1)
N,N-Dimethylaniline		0·015	Abou-Donia *et al.*	1968 (12)
Aminopyrine			Graham *et al.*	1970 (13)
Meperidine	0·39		Eade *et al.*	1970 (14)

MATERIALS AND METHODS

Animals and preparation of microsomes. Male mice of the strain NMRI/Han weighing about 20 g were kept on a standard laboratory diet (Altromin®) and water *ad libitum*. For induction of microsomal demethylating activity the animals received 60 mg/kg of sodium phenobarbital in 0·1 ml per 10 g of body weight three to four times every 8–12 hr intraperitoneally. Twelve hr after the last injection the animals were sacrificed by decapitation and exsanguination. There was no previous restriction of food and water. Pooled livers of several mice were homogenized in a Potter–Elvejhem type homogenizer in five volumes of ice-cold sucrose-EDTA-tris buffer solution of pH 7·4 (0·25 M, $5{\cdot}4.10^{-3}$ M, 2.10^{-2} M, resp). Microsomes were prepared and washed by differential centrifugation in a Spinco Model L ultracentrifuge as described previously.[4] Protein was determined by the biuret reaction using bovine serum albumin as a standard.

Determination of enzymatic activity. The velocity of p-nitrophenol formation due to oxidative O-demethylation of p-nitroanisole was measured by direct spectrophotometry at 420 nm in a suspension of microsomes (0·5 or 1 mg protein/ml) and in the presence of 20 mM nicotinamide in 70 mM phosphate buffer pH 7·85.[5] In order to achieve a continuous formation of NADPH from NADP the assay mixture (total volume 2 ml) contained 2·2 mM di–sodium glucose-6-phosphate and 0·7 Racker units of glucose-6-phosphate dehydrogenase (0·05 ml of a commercial suspension). Both the sample and the reference cuvettes contained the same solution. After allowing 5 min for adjustment of the temperature to 37° the reaction was started by the addition of 0·05 μmole of NADP (0·04 ml) to the sample cuvette. N-demethylation was measured similarly by following the disappearance of N-monomethyl-p-nitroaniline at 420 nm[16] where the resulting p-nitroaniline absorbs less than the parent compound. The results obtained from these kinetic experiments were evaluated graphically in a $1/v$ vs. $1/[S]$ diagram according to Lineweaver and Burk.[21] Substrate concentrations were in the 10^{-4} M range, inhibition experiments were carried out in the presence of 0·67–$2{\cdot}0.10^{-4}$ M SKF 525-A.

Recrystallization of SKF 525–A. For recrystallization from benzene the original SKF 525–A, which according to the manufacturers was crystallized from a mixture of acetone and ethyl ether, was dissolved in benzene (reagent grade, E. Merck AG, Darmstadt) in a quantity of about 3 mg in 1 ml by slightly warming the solution under hot running tap water. From this solution SKF 525–A was precipitated by slowly adding an equal volume of n-heptane (reagent grade, E. Merck AG, Darmstadt). The supernatant solvent was removed and the crystals were dried in a desiccator *in vacuo* for several days over silica gel. They showed a silky appearance. Microscopically they were rectangular flat crystals of varying size. Recrystallization from cyclohexane and chlorobenzene under the same conditions led to the same result. Recrystallization from methanol or water was carried out in a rotating vacuum desiccator yielding different crystals in the case of water. The melting points were not changed in all the compounds obtained and corresponded to that of the original substance (121–124°, uncorrected).

To prove the complete removal of benzene from the recrystallized SKF 525–A 2 mg of the material were placed in a glass-stoppered cuvette and heated to 90° in a drying oven. No photometrical absorption due to benzene vapors could be detected at 260 nm by this sensitive method.

The purity of the recrystallized product was further shown by thin layer chromatography on silica HF 254 (E. Merck AG, Darmstadt) using a developing system of ethanol, glacial acetic acid, and water (60:30:10, by vol.).[17] The developing time was 1 hr. The substance was localized by inspection in ultraviolet light as well as by spraying with Dragendorff's reagent.[18] The amounts applied to the silica plates were 20 and 50 μg, respectively. UV spectra were taken in 70 mM phosphate buffer at pH 7·85.

Nuclear magnetic resonance spectra of crude and recrystallized SKF 525–A were measured at the Biochemistry Laboratory of the Veterans Administration Hospital, Dallas, Texas by Dr. P. A. Srere, and at the Department of Inorganic Chemistry, University of Mainz, by Dr. B. Mathiasch.

RESULTS

Inhibition by SKF 525–A *recrystallized from benzene.* As shown in Fig. 1a the inhibition of the demethylation of p-nitroanisole by crude, non-recrystallized SKF 525–A (charge Nos. TEC 921–122, HR-1–238) proves to be non-competitive. This applies to microsomes of untreated as well as phenobarbital pretreated mice. The figure represents one out of a series of three identical and independent experiments. The Michaelis constants for the uninhibited control reactions were found to be 0·1 and 0·08 mM. When SKF 525–A was recrystallized from benzene as described in the Method section, it exhibits a competitive type of inhibition as presented in Fig. 1b. Again one out of five identical experiments is shown.

Since it could be argued that this striking change might for specific reasons be confined only to p-nitroanisole demethylation, we also studied the N-demethylation of N-monomethyl-p-nitroaniline. As can be seen from Figs. 2a and b, crude SKF 525–A inhibits also this reaction in a non-competitive manner, whereas the benzene recrystallized compound produces a competitive inhibition. The inhibitor constants in both cases do not undergo marked changes, although there is a tendency towards lower values for the recrystallized SKF 525–A.

Inhibition by SKF 525–A *recrystallized from other solvents.* Since the conversion of

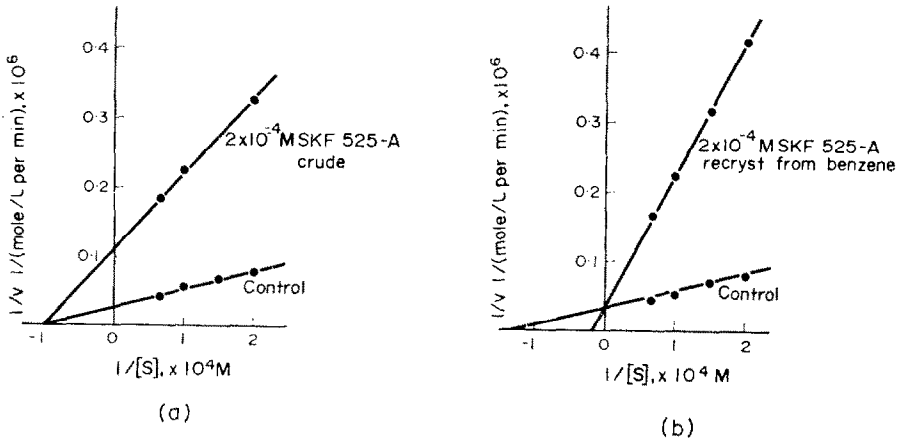

FIG. 1. Inhibition of the O-demethylation of p-nitroanisole by SKF 525-A in phenobarbital stimulated mouse liver microsomes (1 mg protein/ml). Michaelis constants (K_m) were taken graphically from the diagram.[22] Inhibition constants (K_i) were determined from a Dixon plot[23] based on the values from the $1/v$ vs. $1/[S]$ diagram. In cases of non-competitive inhibition they were also calculated according to.[24] a: crude SKF 525-A: K_i: 0·06 mM, K_m for substrate: 0·1 mM. b: benzene recrystallized SKF 525-A: K_i: 0·04 mM. K_m for substrate: 0·08 mM.

the type of inhibition could be demonstrated after recrystallization from benzene, it was to be asked if recrystallization from other solvents would also produce this effect. The diagrams of Fig. 3 show that recrystallization from water, methanol, cyclohexane or chlorobenzene does not lead to competitive inhibition of p-nitroanisole O-demethylation. Also stepwise addition of benzene to chlorobenzene [from 10–80% (v/v)] to form a solvent mixture for recrystallization, from which SKF 525-A then was precipitated by addition of n-heptane, did not change the type of inhibition to become

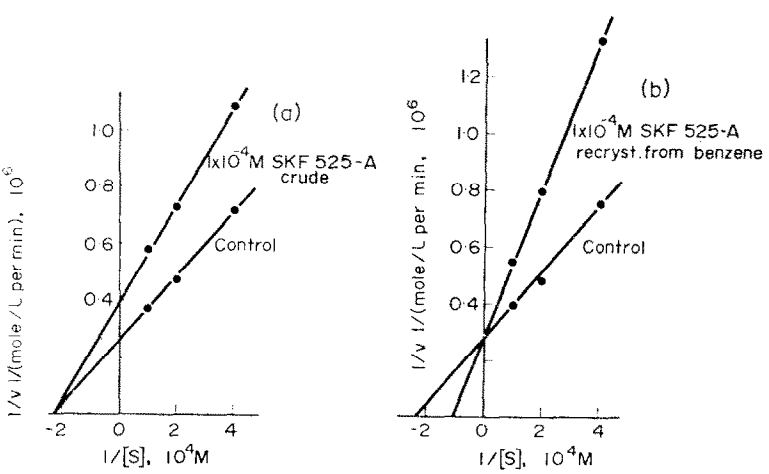

FIG. 2. Inhibition of N-demethylation of N-monomethyl-p-nitroaniline by SKF 525-A, in phenobarbital stimulated mouse liver microsomes (0·5 mg protein/ml). Procedure as in Fig. 1. a: crude SKF 525-A: K_i: 0·19 mM. K_m for substrate 0·04 mM. b: benzene recrystallized SKF 525-A: K_i: 0·08 mM. K_m for substrate: 0·045 mM.

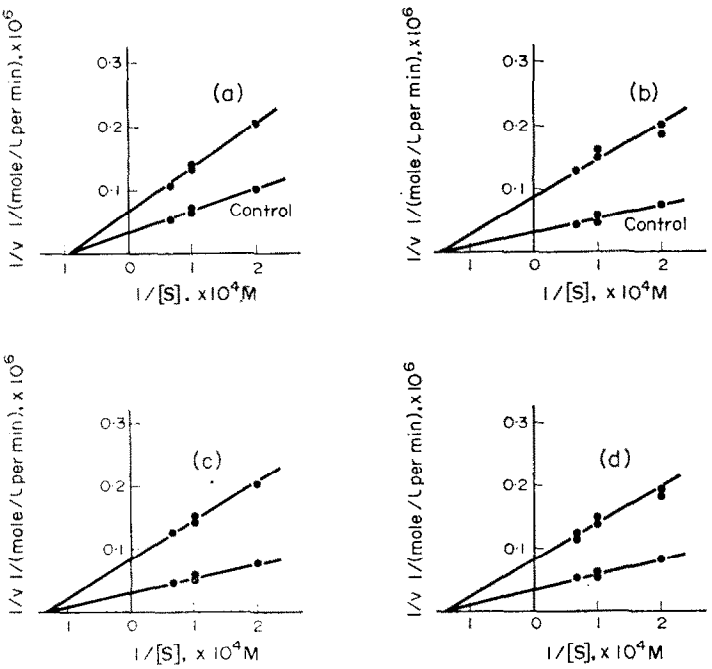

FIG. 3. Inhibition of p-nitroanisole O-demethylation by SKF 525–A recrystallized from different solvents. Phenobarbital stimulated mouse liver microsomes (1 mg protein/ml). SKF 525–A concentration 1.10^{-4} M. Recrystallizing solvents: water (a), methanol (b), cyclohexane (c), chlorobenzene (d).

competitive. It rather produces mixed types of inhibition, which are difficult to interpret. Competitive inhibition was obtained only after recrystallization from pure benzene. The effect of behaving as a competitive inhibitor remained with the benzene recrystallized product for about two years, during which time it was stored in a desiccator at — 4°.

Criteria for identity of crude and recrystallized SKF 525–A. From the foregoing it would seem important to establish a chemical or physiochemical difference between the two forms of SKF 525–A. Thin layer chromatography revealed a single spot for both crude and recrystallized SKF 525–A, with identical R_f values at 0·54. There was also no detectable difference when the mother liquor was evaporated to dryness and the resulting crystals subjected to thin layer chromatography. In the ultraviolet region identical spectra were obtained with a peak value at 203 nm. Nuclear magnetic resonance spectra showed no difference between crude and benzene recrystallized SKF 525–A. Considerations based on molecular models of SKF 525–A suggested that the free rotation of one of the benzene rings could be hindered by the diethylaminoethyl moiety of the molecule.[19] Also it was suspected that a third benzene ring could be intercalated between the two existing ones and be retained there firmly. Examination of the spectrum of Fig. 4 excludes these possibilities, since the protons of both benzene rings (7·24 ppm) appear homogeneous and thus indicate free rotation. Furthermore, there is no sign for the presence of a third benzene ring within the molecule.

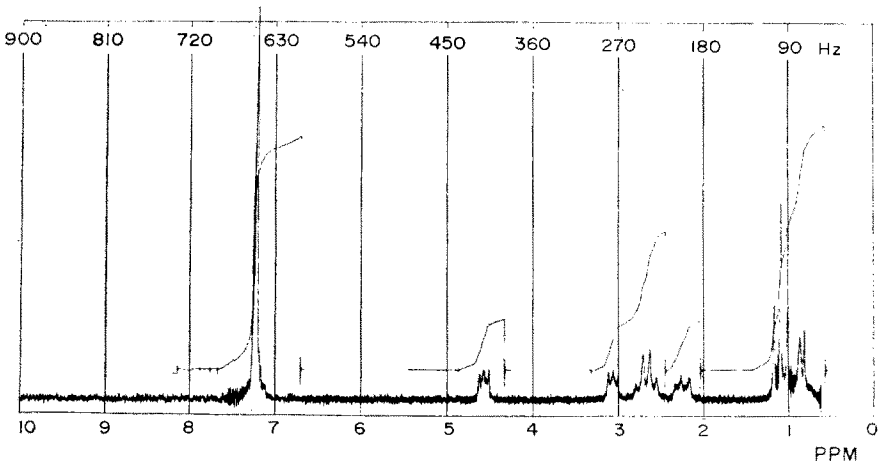

FIG. 4. Nuclear magnetic resonance spectrum of benzene recrystallized SKF 525–A. SKF 525–A (Lot No. TEC 921–122) was dissolved in C_2HCl_3 (30 mg/0·4 ml) and a spectrum was recorded at 90 MC in a 5 mm sample tube at 27° in an NMR spectrograph KIS–11 of Bruker Physik AG, Karlsruhe, with automatic internal stabilization by the tetramethylsilane (TMS) signal. The upper scale shows the increment in frequency, the lower the corresponding ppm values. Spectra of benzene recrystallized and crude SKF 525–A were identical.

DISCUSSION

The reported experiments on the inhibition of O- and N-demethylation reactions in mouse liver microsomes show that recrystallization of SKF 525–A, which was originally crystallized from a mixture of acetone and ether, from benzene results in a change of the kinetic type of inhibition. It is modified from non-competitive to competitive by this procedure. Apparently this effect can be achieved only with benzene, since recrystallization from other solvents produces no change.

Alterations of the apparent mechanism of inhibition of drug oxidation reactions have been described previously. Thus the kinetic type of inhibition of microsomal degradation of aniline, aminopyrine, and ethylmorphine by nicotinamide, 2,4-dichloro-6-phenylphenoxyethylamine (DPEA), and amino-ethyldiphenylpropylacetate (SKF 26754–A) changes in various ways with animal species or concentration of the inhibitors.[20] Moreover, nicotinamide is able to convert the inhibition of the N-demethylation of ethylmorphine by SKF 26754–A from a predominantly non-competitive to an apparent competitive type. This effect casts doubts on the validity of inhibition experiments carried out in the presence of nicotinamide, which is usually added in order to prevent the hydrolysis of NADP. More recently the effect of low (4 mM) and high (50 mM) concentrations of nicotinamide on various N-dealkylation reactions has been studied with the result that 50 mM nicotinamide inhibits dealkylation of morphine and ethylmorphine competitively while that of SKF 525–A as a substrate shows an uncompetitive inhibition.[21] In the experiments presented here, however, the experimental design was such that the only variation was the recrystallization of SKF 525–A from benzene or other solvents. This eliminates the above described effects of nicotinamide.

At present no explanation for the observed change in inhibition type can be offered. Instead a number of arguments seem to have been ruled out. Thus the suggestion

seems to be eliminated that benzene might be bound firmly to the SKF 525–A crystals and be transferred into the microsomal suspension. Furthermore, with none of the methods applied a chemical difference could be detected that would explain the observed behaviour; melting point, mixed melting point, thin layer chromatography, nuclear magnetic resonance spectroscopy and ultraviolet spectroscopy. So we are left at present with the possibility of an attachment of a benzene ring, that does not show up in the above tests, or of the loss of the hydrochloride moiety. The latter should not interfere, however, as soon as the compound is dissolved in buffer.

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