A Novel Reductive System Involving Flavorprotein in the Rat Intestine

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Biotransformation of foreign compounds (xenobiotics) is of utmost importance to the survival of living organisms against environmental pollutants. The three major primary enzymatic processes involved are oxidative, reductive and hydrolytic systems. Generally speaking in plants and animals, oxidative systems prevail in microorganisms (MATSUMURA, 1975). Recently, several papers have appeared describing reductive reactions on pesticidal chemicals in animal systems indicating the trend for a renewed interest in the role of reductive reactions with respect to detoxication of foreign compounds. (DeBAUN and MENN, 1976; IVIE, 1976; KHALIFA et al., 1976).

Our interest in this subject stems out of the observation of reductive metabolism of DDT to TDE, the first indispensable step for DDT degradation. It has been debated for some time whether the reaction is due to the microbial action in the alimentary system (BARKER et al., 1965; BUNYAN et al., 1966; JEFFRIES and WALKER, 1966; MENDEL and WALTON, 1966; OTTOBONI et al., 1968; PETERSON and ROBISON, 1964). To be sure there have been some evidence that avian and mammalian livers are capable of metabolizing DDT to TDE under anerobic conditions in vitro (BARKER et al., 1965; HASSAL, 1971, 1974; WALKER, 1969). However, the functional meaning of such systems in vivo as well as the enzymatic basis of such activities have not been really clarified.

The purpose of this study is to investigate the possible existance of a reductive system in mammalian tissues. Two insecticide substrates, DDT and ^{14}C-mexacarbate, were used.

MATERIALS AND METHODS

To study the reductive system in mammals, we have chosen the 20,000 g supernatant fraction from the homogenate of the intestinal wall (small intestine) of rats as the source. Anaerobic degradation activites on DDT and mexacarbate were investigated. Mexacarbate was chosen as a convenient substrate since this pesticide degrades much faster than DDT, and thereby facilitated quick assaying of its degradation activities. At the end of the reaction, the products were extracted with diethyl ether, and the degradation activity was mainly monitored by assaying the radioactivity in the aqueous phase.

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Anaerobic degradation in vitro of ^{14}C-mexacarbate by the 20,000 g supernatant: The incubation mixture consisted of 1 ml of the 20,000 g supernatant containing 36 mg fresh weight tissues equivalent in 0.02 M phosphate buffer at pH 6.6 and 10 nanomoles of ^{14}C-mexacarbate in 10 µl ethanol. The cofactors 0.60 µmole FAD or 0.50 µmole NADPH, were added by using 0.2 ml buffer as the vehicle. The volume of incubation mixture in each tube was adjusted to 1.2 ml by using phosphate buffer. Incubation was carried out in Thunberg tubes. After addition of all constituents, the tube was evacuated and nitrogen was flushed; this process was repeated two additional times and finally the nitrogen was evacuated and the incubation carried out under reduced pressure, approximately 10 mm Hg. The system was maintained in a metabolic shaker at 37° for two hours. At the end of incubation, the products were extracted with diethyl ether, and analysis was carried out by using thin-layer chromatography (TLC), along with autoradiography, using benzene-methanol (95:5 v/v) as the mobile phase. The data are expressed in nanomoles and represent the actual degradation activity since blank values were substracted. Blank samples refer to the 20,000 g supernatant to which the substrate was added at the end of incubation. The system was extracted immediately with diethyl ether.

Sephadex gel-filteration: In an attempt to partially purify the protein responsible for such degradation activities the 20,000 g supernatant was first prepared by homogenizing the rat intestinal wall in two parts of buffer, and centrifuging it successively at 8,000 g for 10 minutes and 20,000 g for one hour. Protease hydrolysis was carried out by incubation of the 20,000 g supernatant (containing 34 mg/ml of intestinal wall protein) of enzyme preparation. The resulting hydrolysate was subjected to a gel-filtration column separation by using sephadex G-75. Absorption at 260 or 280 nm was used to monitor the column elution pattern, and the degradation activity was measured individually for each fraction, using water-soluble ^{14}C-metabolites of mexacarbate as the parameter. Two major peaks (I and II) were observed as shown in Fig. 1. Hereafter, peak II will be referred to as flavoprotein preparation.

Anaerobic degradation in vitro of DDT and ^{14}C-mexacarbate by the flavoprotein preparation: Incubation mixtures consisted of 5 ml (1.66 mg protein) of the flavoprotein preparation in case of DDT and 2.5 ml (0.83 mg protein) in case of ^{14}C-mexacarbate; 0.6 µmole of FAD or 0.5 µmole of NADPH; and 140 picomoles of DDT or 10 nanomoles of ^{14}C-mexacarbate in 10 µl ethanol. Incubation conditions, extraction, and expression of data are as mentioned above. With respect to DDT, analysis was carried out by gas chromatography using an electron capture detector and two columns, QF-1 and OV-101 at 185°C. The first column was employed for qualitative identification of the metabolites, while the latter was used for both qualitative and quantitative estimations. As for mexacarbate degradation, ether extracts were analyzed by TLC (mobile phases were: chloroform-methanol 99:1, benzene-methanol

95:5, ether-hexane 4:1, chloroform-acetonitril 4:1) along with autoradiography on X-ray films against authentic reference compounds. The spot corresponding to desmethyl mexacarbate was isolated and characterized by proton magnetic resonance spectroscopy.

RESULTS AND DISCUSSION

By using the 20,000 g supernatant from rat intestine, we could first establish that there was indeed a mexacarbate degradation system which was strongly stimulated by FAD under an anaerobic incubation condition (Table 1). In this system mexacarbate was found to be degraded to relatively polar compounds as judged by the increase in radioactivities in the aqueous phase. In addition, the major ether-soluble product, N-desmethyl mexacarbate as judged by TLC analyses and autoradiography also increased in the presence of FAD.

To study the characteristics of this reductive system, several tests were conducted using the 20,000 g supernatant. The continuous treatment of the system with 1 ppm of streptomycin from the time of dissection to the final incubated (added in the buffer) had no effect on the activity. Neither did the addition of cations Fe^{++}, Co^{++}, Mg^{++} cause the change in the degradation activity. The treatment which caused the activity changes were 10^{-3}M $HgCl_2$ (80% inhibition), CO bubbling (28% inhibition), 10^{-4}M DFP (50% inhibition), 10^{-3}M p-chloromercury benzoate (75% inhibition), 10^{-3}M N-ethyl maliemide (60% stimulation), and mersalyl acid (130% stimulation). The pH optimum was found to be 6.

Preliminary experiments also showed that the protease treatment of the 20,000 g supernatant enhanced the degradation activities on mexacarbate. The protease treated 20,000 g supernatant was then subjected to sephadex gel-filteration.

Degradation activity on mexacarbate was associated with the first 3-5 fraction in each peak. However, further examination of peak I by rechromatography on sephadex G-25 revealed three peaks, all with very low specific degradation activity on mexacarbate. Hence, it was decided that peak II is a better source for the degradation systems on mexacarbates.

Upon examination of these two peaks, it was found that fluorescence spectroscopic characteristics (i.e., fluorescence peak at 520 nm and excitation peak at 450 nm) of peak II and FAD were practically identical. Moreover, both FAD and peak II showed an identical UV-absorption peak at 260 to 270 nm. Other characteristics such as the resistence to protease, heat (up to 90°C for 20 min), etc., agree well with our diagnosis that the system isolated here is a flavoprotein. The molecular weight of the flavoprotein is likely to be in the order of 6,000 to 10,000 as judged by its relative eultion position against standard components (i.e., cytochrome c:13,000) on the same sephadex column.

DDT and mexacarbate were incubated with this flavoprotein preparation (peak II) in the presence and absence of either NADPH or FAD (Table 2). It was found that the addition of FAD, but not NADPH, greatly stimulated the metabolism of both substrates. The most conspicuous increase was observed in the production of TDE in the case of DDT, and desmethylmexacarbate and water soluble metabolites in the case of mexacarbate. The reaction of TDE formation is reductive dechlorination. Moreover, the formation of desmethymexacarbate must involve reductive desmethylation which has never been reported in any biological systems to our knowledge.

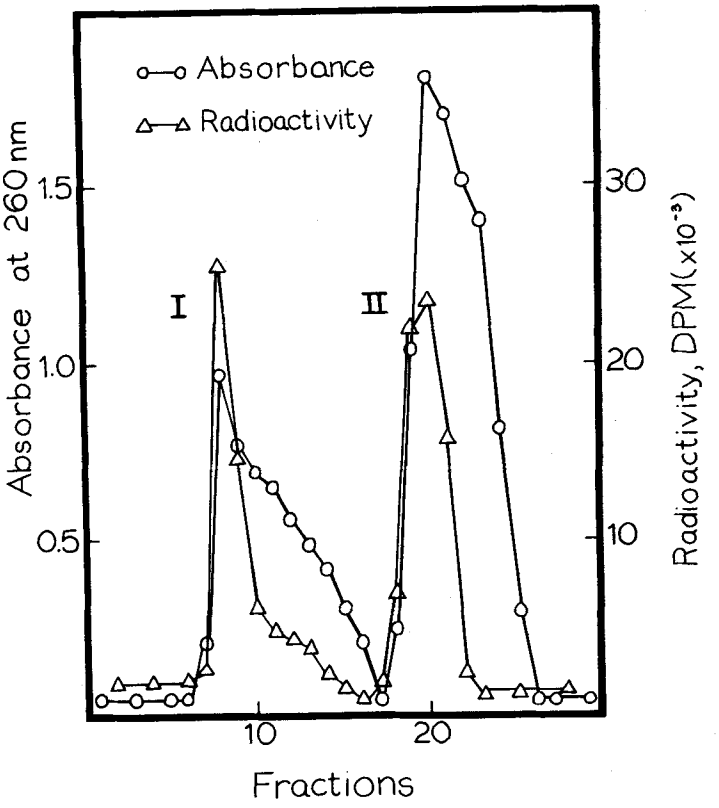

Figure 1. UV absorption and degradation activity (DPM) for the column fractions.

Table 1 Degradation of ^{14}C-Mexacarbate by the 20,000 g Supernatant from Rat Intestine*

Incubation conditions	Mexacarbate Remaining (nanomoles)	Degradation Products (nanomoles)		
		Desmethyl Mexacarbate	Other Ether Soluble	Water Soluble
20,000 g Supernatant	8.80	0.10	0.10	0.05
20,000 Supernatant + FAD	7.40	0.75	0.40	0.50
20,000 g Supernatant + NADPH	8.50	0.20	0.30	0.05

* Averages of 2-3 independent experiments, each experiment consisting of duplicate sets.

Table 2 Degradation of DDT and ^{14}C-Mexacarbate by the Partially Purified Flavoprotein Preparation from Rat Intestine

Substrate Remaining and Degradation Products	Incubation Conditons		
	Flavoprotein only	Flavoprotein + FAD	Flavoprotein + NADPH
	DDT as substrate* (picomoles)		
DDT	124	100	125
TDE	6	27	7
DDE	6	9	4
	^{14}C-Mexacarbate as Substrate (nanomoles)		
Mexacarbate	8.7	5.4	9.0
Desmethyl mexacarbate	0.5	1.8	0.2
Other ether soluble	0.1	0.9	0.1
Water soluble	0.1	1.3	0.1

* The original flavoprotein eluate (peak II, Figure 1) from the sephadex column was found to contain low levels of two compounds which have identical retention time as DDT and TDE (e.g. in one preparation they were 0.05, 0.01 ppm, respectively). It is assumed that these two contaminants are present in a bound form with flavoprotein resolved in peak II. These values were substracted from the experimental value to give the data shown above.

To study the effects of light and oxygen the tubes were incubated in the dark under the same conditions as described in MATERIALS AND METHODS. Two flavin cofactors were used, namely, FAD and riboflavin. The overall degradation activities on mexacarbate, in the presence of FAD, amounted to 4 nanomoles mexacarbate (Table 2). When the reaction was carried out in the dark, the overall degradation activities were decreased to 33% and was further decreased in the presence of oxygen to 10%. When riboflavin was used as the cofactor, degradation activities amounted to 8.9 nanomoles mexacarbate degraded in the presence of light. When the reaction was carried out in dark, the same degradation activity on mexacarbate was experienced. Only in the presence of oxygen in dark did the degradation activities decrease to 13% of the standard value.

The first question one must ask is whether the flavoprotein system discovered here represents a genuine enzymatic system or not. GILLETTE (1971) has previously noted that azo and nitro compounds may be nonenzymatically reduced to amines by reduced cofactors such as NADPH, NADH and reduced flavins such as FADH. KAMM and GILLETTE (1963) also showed that FAD can be reduced by a purified cytochrom c - NADPH system anaerobically, and that this reduced FAD was capable of nonenzymatically converting p-nitrobenzoate to p-aminobenzoate.

The system described herein is not stimulated by NADPH or NADH, and therefore, is different from the above cases. The degradation activity of mexacarbate was, on the other hand, reduced in the absence of light indicating that at least some part of FAD reduction in the standard reaction scheme is carried out nonenzymatically by some electron donors (they could be amines or amino acids, but not NADH or NADPH) (PENZER and RADD, 1968; PYROM, 1968). The rest of the FAD reducing reaction which takes place in the dark could be carried out by enzymatic systems or by flavoprotein itself. It has been known that flavoproteins are capable of reducing flavin cofactors. Thus, in view of the essential role of flavoproteins, it is most logical to assume that the sole function of flavoprotein here is to convert FAD to FADH which actually reacts with DDT or mexacarbate to produce respective degradation products. It must be stressed here that FAD, as low as 10 μg per reaction tube (10^{-5}M), resulted in maximum stimulation of reductive activities, and furthermore, this FAD concentration is roughly in the same magnitude of its level in various animal tissues. Thus, even though the actual reductive reaction itself could be carried out nonenzymatic, the phenomenon itself should not be regarded as irrelevant phenomenon in vivo. Also, flavoproteins are known to be omnipresent in various biological systems. In animals, they are present in the liver as well as in the alimentary canal. Thus, along with the evidence that favorable anaerobic conditions exist in at least the lower alimentary canal in vivo, the chance is that such a flavoprotein-flavin cofactor catalyzed reaction do play significant roles for degradation of certain xenobiotics.

The discovery that flavoproteins-flavin cofactor combinations, whether they are microbially derived or mammalian in origin, are capable of degrading various xenobiotic substrates in the presence of FAD or other flavin confactors under anaerobic conditions helps clarify many phenomena hitherto considered unexplainable. For instance, it has been shown by FRENCH and HOOPINGARNER (1971) that cell membrane fractions from Escherichia coli actively convert DDT to TDE, and that the reaction is strongly stimulated by FAD. Similarly WEDEMEYER (1966) observed earlier that the reduction activity on DDT in the cell-free extract from Aerobacter aerogenes was stimulated by the addition of flavine mononucleotide (FMN) with light illumination. Such reductive reaction was strongest under an anaerobic condition at an acidic pH. As for mammalian systems, it is interesting to note that basically similar biochemical characteristics are found in the DDT reducing enzyme in the liver. According to HASSALL's (1971) description, the system is heat-stable, is optimal at an acidic pH, and is stimulated by exogenously added riboflavin. Thus, it is likely that the underlying basic mechanism common throughout these phenomena is anaerobic reduction by involving flavoprotein-flavin cofactor systems.

The meaning and significance of such flavoprotein involved system in vivo in elimination of toxic foreign compounds must be further examined, particularly in relation to other documented reductive systems such as popphyrin -Fe^{++} involved systems (CASTRO, 1964; KHALIFA et al., 1976; MISKUS et al., 1965) and specific NADPH requiring nitro reductase systems (GILLETTE, 1971; HITCHCOCK and MURPHY, 1967; ROSE and YOUNG, 1973; SYMMS and JUCHAU, 1974). Nevertheless, this system can be easily distinguished from others by the stimulated effect of FAD (and other flavins), characteristic heat stability, and acidic pH requirements, and therefore, we feel certain that its contribution will be properly assessed in the near future.

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