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POLYAMINES AS MODULATORS OF DRUG OXIDATION REACTIONS CATALYZED BY CYTOCHROME P-450 FROM LIVER MICROSOMES

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Abstract—The effect of polyamines on the activity of the mixed-function oxidase (MFO) system from human, rat and rabbit liver microsomes was investigated in detail. It was shown that polyamine (spermine) stimulates NADPH-dependent activity of the MFO system several-fold whatever the substrate (foreign drug or natural), not only with microsomes but also with the reconstituted system consisting of highly purifed cytochrome P-450 (LM_2 isozyme), cytochrome P-450 NADPH reductase and dilauroylphosphorylcholine. Stimulation (extent and concentration dependence) appeared to be dependent on a number of parameters such as ionic strength, pH, animal species and treatment, nature of the substrate, and was stereospecific (different effect on 6β-and 16α-testosterone hydroxylation). Further, the spermine effect was evaluated on some elementary steps of the cytochrome P-450 reaction cycle, like substrate binding, P-450 reduction and second electron transfer. Finally, it was shown that the organic peroxide dependent activity was not stimulated by spermine with microsomes nor with the purified P-450 LM_2 isozyme.

On the basis of this study, it was concluded that the locus of polyamine action is cytochrome P-450 and that stimulation could result either from increased stability of the oxyferrous intermediate of P-450 or from an increased rate of second electron transfer from reductase to P-450.

Liver microsomes from man and animal species harbor a multienzyme mixed-function oxidase (MFO) system whose terminal oxidase is cytochrome P-450 [1]. The MFO system is involved in the metabolism of a number of natural substrates (steroid hormones, biogenic amines, cholesterol, bile salts, prostaglandins etc.) and the disposition of an extremely wide variety of drugs and foreign chemicals [2]. In particular, it is well-established that this system is involved either in the elimination or in the generation of carcinogenic chemicals [3–5]. Any information on the control and regulation of the liver microsomal MFO system is therefore of interest, in view of the many important biochemical processes in which it is involved. In the past 15 yr a large amount of work has been devoted to the study of the mechanisms controlling this system. It is now clearly established that MFO activity can be strongly modulated by inducers like phenobarbital, methylcholanthrene, β-naphthoflavone and other chemicals [3, 6]. This modulation, which is accompanied by enzyme induction in the liver, was ascribed to *de novo* synthesis of specific forms of cytochrome P-450 in response to specific inducers [3]. Some of these forms (for instance P-450 LM_2 and LM_4 specifically induced in the rabbit liver by phenobarbital and β-naphthoflavone) have now been isolated, highly purified and fully characterized [7].

Besides this 'genetic modulation', it is reasonable to suspect other physiological means of regulation leading to the expression of a 'physicochemical modulation'. If we except such trivial parameters as pH and ionic content, as well as the use of non-physiological conditions such as high concentrations of organic solvents like acetone [8], ethylisocyanide [9], detergents like emulgen and Triton X-100 [10] and flavonoids [11], very few investigations indeed have been devoted to the study of a physiological physico-chemical modulation of the MFO system from liver microsomes. Among the possible candidates to such a modulation are phospholipids [12, 13] and polyamines [14–16].

Polyamines (putrescine, spermidine and spermine) are ubiquitous organic polycations. In the past few years these have been shown to be involved in a variety of physiological processes including cell growth and differentiation, nucleic acid and protein synthesis and membrane metabolism [17–19]. In this respect, previous investigations [14–16] emphasized the stimulating effect of polyamines on the rat liver microsomal MFO system. However, these reports did not provide sufficiently extensive and systematic data so that the locus of polyamine action and the molecular mechanism of their effect could not be thoroughly documented. Moreover, the observations were confined to rat liver microsomes so that extrapolation to other animal species and man liver was not possible.

Because of our interest in both polyamines effect in enzyme reactions [20, 21] and cytochrome P-450 from liver microsomes [22, 23], we recently decided to undertake a thorough study of polyamines as modulators of the liver microsomal MFO system.

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The first part of this investigation, mainly carried out on microsomes, is presented in this paper (a further extension will be made on a highly purified preparation of cytochrome P-450 from rabbit liver). Our results show that: (1) polyamines stimulate the MFO system of liver microsomes from the rat, rabbit and man, both on xenobiotics and physiological substrates; (2) the locus of the polyamine effect is cytochrome P-450; and (3) polyamines stimulate the system, either by stabilizing the oxyferrous intermediate of cytochrome P-450, or by favouring the second electron transfer in the P-450 reaction cycle. A preliminary report on this work appeared recently [24].

MATERIALS AND METHODS

Microsomes and enzymes preparations

Human. Human liver autopsy samples were obtained from the Department of Pathology, Hôpital Guy de Chauliac (Montpellier, France) Two preparations were used in this work: subject A (59-yr-old female), and subject B (60-yr-old female). In both cases death was due to carbon monoxide asphyxia. Liver samples were sliced into 1-cm sections and washed several times with cold 0.9% NaCl. Samples were then homogenized in 4 vols of 0.1 M Tris–HCl (pH 7.4), 0.1 M KCl, 0.1 mM EDTA and 50 μM butylated hydroxytoluene. The homogenate was potterised and centrifuged for 30 min at 10,000 *g*. Supernatant was collected and pellets were resuspended in the same buffer and centrifuged again at 10,000 *g*. Supernatants were then centrifuged for 90 min at 105,000 *g*. Microsomes were resuspended and homogenized at 30 mg protein/ml in 0.1 M Tris–HCl (pH 7.4), 0.1 M KCl, 0.1 mM EDTA, 20% glycerol and stored at −20°. The cytochrome P-450 content of the microsomes, determined according to Omura and Sato [25] was 0.18 and 0.15 nmoles/mg protein for samples A and B respectively.

Rat. Male Wistar untreated rats (weighing 200–250 g) were used in this work. Microsomes were prepared according to the procedure used for human liver, except that livers were perfused *in situ* with cold 0.9% NaCl.

Rabbit. Male New Zealand rabbits (weighing 2.0–2.5 kg) were used in this work. For phenobarbital treatment, the animals were allowed to drink a 0.1% solution of phenobarbital (pH 7.0) for 5 days. For TAO treatment, TAO (triacetyloleandomycin) was incorporated into the food (1 mmole/kg) for 7 days. Liver microsome highly purified P-450 LM_2 and NADPH cytochrome P-450 reductase were prepared according to previously published procedures [7, 26]. The specific content of LM_2 was 16–18 nmoles/mg, while the sp. act. of the reductase was 5922 nmoles/min/nmole with cytochrome *c* as the substrate.

Assays

Incubation mixtures contained 50 mM Tris–HCl (pH 7.4) (at 37°), microsomes (the concentration was 1 mg/ml, except with benzo[*a*]pyrene activity where the concentration was 0.1 mg/ml), regenerating system consisting of 2 mM glucose-6-phosphate and 1 u/ml glucose-6-phosphate dehydrogenase, an appropriate dilution of a spermine solution [50 or 5 mM in water (pH 7.0)], and an appropriate dilution of substrate solution. Benzphetamine, chlorcyclizine and ethoxycoumarin were dissolved in pure water while testosterone and benzo[*a*]pyrene solutions were made in acetone. In this last case, the substrate concentration was high so as to keep the final concentration of the organic solvent in the incubation mixture as low as possible (0.5% v/v). When the reconstituted system was used, microsomes were replaced by a mixture of 0.5 μM cytochrome P-450 LM_2, 0.5 μM NADPH cytochrome P-450 reductase, and 45 mg/ml dilauroylphosphatidylcholine preincubated for 5 min at room temperature, and the NADPH-regenerating system was omitted. In all cases, reaction was initiated by the addition of NADPH, 0.2 mM with microsomes, 1 mM with the reconstituted system, after 3 min incubation at 37°. When NADH synergism was studied, the reaction was initiated by the simultaneous addition of 0.5 mM NADPH and 0.5 mM NADH.

Benzphetamine and chlorcyclizine demethylation were followed by measuring the amount of formaldehyde released in the medium against time using the method of Nash [27]. Ethoxycoumarin deethylation was fluorometrically monitored according to a procedure published earlier [28], hydroxycoumarin being used as the standard. Testosterone hydroxylation in the 6β and 16α positions (hydroxylation in the 2α and 7α positions is negligible with rabbit liver microsomes) was measured by high-performance liquid chromatography (HPLC). The procedure developed in this laboratory included: quenching of the 1-ml reaction mixture in ice, followed by addition of 50 nmoles of 6α-OH-estradiol used as the extraction label and of an equal volume of dichloromethane, agitation for 10 min followed by centrifugation at 5000 *g* for 5 min and collection of the organic phase. The extraction of testosterone and hydroxylated metabolites was repeated once, the solvent was evaporated under a stream of nitrogen at room temperature and the tubes were stopped and stored in the dark at −20° until HPLC analysis. The solid residues were then dissolved in 20 μl of acetone and immediately analysed by HPLC. The HPLC system (Gradient Gilson) consisted of two high-pressure pumps (model 302), a Holochrome HD detector, a Waters Bondapack C18-10 μm column protected by a guard column, a sample injection valve and pressure gauge. Absorbance of the effluent was recorded at 240 nm on a Tarkan W + W recorder 600. Elution programmed with an APPLE II microcomputer was carried out at ambient temperature (18–19°) at a flow rate of 1 ml/min and 1400 psi with a mobile phase of water/methanol [58/42 (v/v) from time 0 to 11 min and 27/63 (v/v) from 11.1 to 18 min]. The rate of production of hydroxymetabolites of testosterone was calculated from the peaks area, the extraction ratio determined with 6α-OH-estradiol as the standard being taken into account. Benzo[*a*]pyrene hydroxylation was fluorometrically monitored, according to a method published earlier [29]. 3-OH-Benzo[*a*]pyrene was used as standard. For each substrate kinetics were determined over various time intervals within which the product formation against time was linear. Turnovers are expressed as nmoles product per minute per mg microsomal protein with

microsomes, or per nmole P-450 with the reconstituted system; in the case of benzo[*a*]pyrene the turnover is given as pmoles 3-OH metabolite formed per min per mg. Cumene hydroperoxide dependent deethylation of ethoxycoumarin was carried out under the conditions already described except that NADPH and the regenerating system were omitted, the reaction being initiated by addition of 1 mM cumene hydroperoxide dissolved in acetone.

NADPH-dependent cytochrome P-450 reduction

The incubation mixture was the same as described in the previous section, excepted that the NADPH-regenerating system was omitted. One millilitre of incubation mixture (containing 1 mg of microsomal proteins) was placed in a glass cuvette of the Aminco DW2 spectrophotometer. The cuvette was carefully stoppered and carbon monoxide entered by gentle bubbling for 20 min at 0°. The cuvette was then placed into the sample compartment of the spectrophotometer and incubated for 5 min at 20°; this temperature was chosen because it allowed the convenient recording of the reduction kinetics. When the O.D. reached a stable level (dual-wavelength mode was used between 450 and 490 nm), a small amount of NADPH solution (final concentration 0.5 mM) saturated with carbon monoxide was anaerobically added with a Hamilton syringe and, after quick agitation, the O.D. increase was immediately recorded. When a stable level was reached, generally after 10–30 min (depending on the microsomes and treatment of animals), a few crystals of sodium dithionite were added to the mixture in order to ascertain whether total reduction had occurred. In most cases, the NADPH-dependent reduction represented more than 90% of the total. Kinetics of the reduction of P-450 were generally multiphasic and could be mathematically described by the sum of two expontial functions. The rate constant of the various phases as well as their relative contribution to the overall process were conventionally obtained from semilogarithmic plots of final absorbance minus absorbance at a given time against time after mixing.

Apparatus

Optical spectrophotometric measurements were made with an Aminco DW2 spectrophotometer. For ethoxycoumarin and benzo[*a*]pyrene assays determination of product formed was made with an Aminco–Bowman spectrofluorimeter. Both apparatuses were equipped with a thermostated cell holder compartment. HPLC analyses were made with a gradient Gilson apparatus equipped with two pumps (model 302) and a holochrome HD detector.

Materials

Polyamines (putrescine, spermidine and spermine) were from Sigma Chemical Co. (St. Louis, MO); stock solutions in pure water were prepared daily; pH was adjusted to 7.0. Ethoxycoumarin was prepared according to a published procedure [28]. Benzo[*a*]pyrene was purchased from Aldrich Chemical Co. (Milwaukee, WI), chlorcyclizine-HCl from Wellcome Co. (Research Triangle Park, CN) and testosterone and hydroxymetabolites from steraloids (Wilton, NH). Benzphetamine, the hydroxymetabolite of benzo[*a*]pyrene and 2β-OH-testosterone were kindly provided by Dr Glady (Upjohn Laboratories), Drs Longfellow and Keith (NCI Chemical

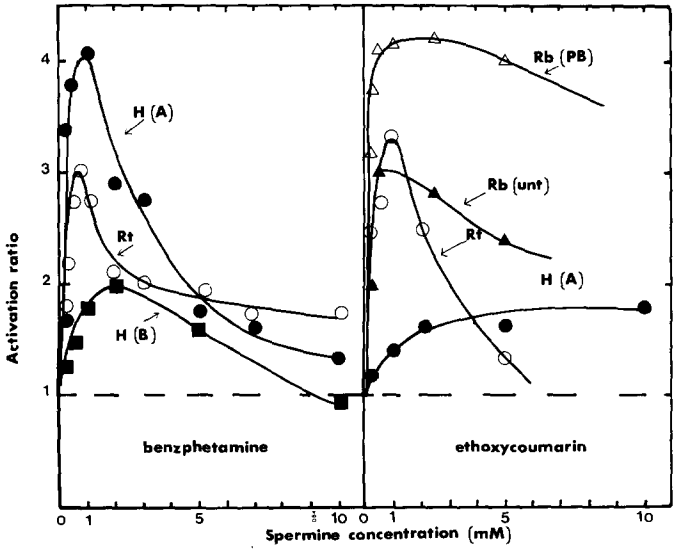

Fig. 1. Activation ratio against spermine concentration. Activation ratio = turnover in the presence of spermine/control turnover (in absence of spermine). H (A): human liver microsomes from subject A; control turnover: 0.043 nmoles/min/mg with benzphetamine and 0.104 nmoles/min/mg with ethoxycoumarin. H (B): human liver microsomes from subject B; control turnover: 0.103 nmoles/min/mg. Rt: rat liver microsomes; control turnover: 2.93 nmoles/min/mg with benzphetamine and 0.88 nmoles/min/mg with ethoxycoumarin. Rb (PB) and Rb (unt): rabbit liver microsomes from phenobarbital and untreated animals respectively; control turnover: 2.50 and 0.83 nmoles/min/mg respectively. Putative relative uncertainty ±10%. For further details see Materials and Methods.

Table 1. Effect of spermine on 6β- and 16α-testosterone hydroxylase of untreated rabbit liver microsomes

Spm (mM)	0	0.1	0.5	1	2	5
6β-OH-Testosterone	2.3 ± 0.2	3.1 ± 0.3	4.5 ± 0.4	4.3 ± 0.4	5.2 ± 0.4	3.8 ± 0.4
16α-OH-Testosterone	0.5 ± 0.1	0.7 ± 0.2	0.6 ± 0.1	0.8 ± 0.2	0.8 ± 0.2	0.7 ± 0.2

Activity is given in nmoles product per minute per nmole P-450 (see Materials and Methods for detail).

Repository, Chicago, IL) and Dr Kirk (MRC, London, U.K.) respectively. Other chemicals were of analytical or the best available grade, especially those used in HPLC measurement.

RESULTS

In our preliminary report [24] we showed that the effect of polyamines was dependent on the number of charges, i.e. the largest effect was observed with spermine at the lowest concentration. Clearly, spermine appeared as the most efficient of the series and the investigation was continued with this compound. From Fig. 1, and Tables 1 and 2, it is clear that spermine stimulates not only the MFO system from rat liver microsomes as shown by other investigators [14–16], but also the MFO system from man and rabbit liver microsomes. Accordingly, generalization of the effect of polyamines as physiological modulators of the drug-metabolizing liver enzyme system is not unreasonable. Both maximal amplitude and concentration dependence of the stimulation depend on the species, the nature of the substrate and the treatment of the animals. Also evident from Fig. 1 is the finding that in some cases (human subject A with benzphetamine and phenobarbital-treated rabbit with ethoxycoumarin, for instance) very low spermine concentrations (50–100 μM) significantly activates the MFO system. Results concerning the effect of spermine on 6β and 16α hydroxylation of testosterone by rabbit liver microsomes are presented in Table 1. These data show that in addition to being species-, substrate- and treatment-dependent, the activation level is also stereospecific, i.e. a larger effect is observed on the 6β position. Similar findings were made with liver microsomes from treated rabbits.

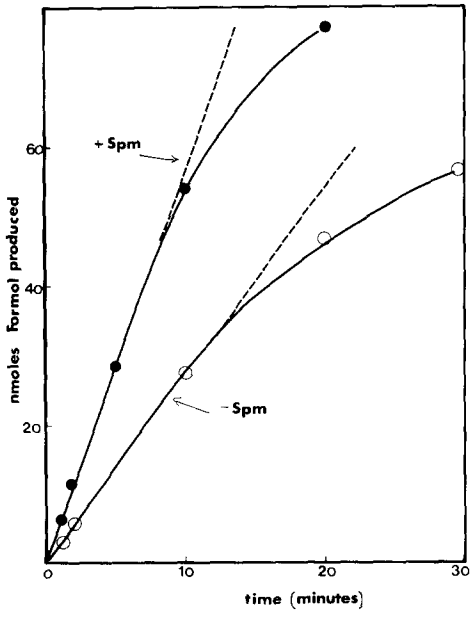

Fig. 2. Time course of formol released during metabolism of benzphetamine by the reconstituted system (P-450 LM_2 isozyme–reductase–dilauroylphosphorylcholine) in the absence and in the presence of 2 mM spermine. Putative relative uncertainty ±10%. For further detail see Materials and Methods.

In contrast to a previous report [16], but in agreement with our preliminary observations [24], spermine (and the other polyamines) is also a potent activator of the reconstituted MFO system (Fig. 2). This system consists of highly purified cytochrome P-450 isozyme LM_2, NADPH cytochrome P-450 reductase and dilauroylphosphatidylcholine. Here again stimulation can be observed with low polyamine concentrations as in microsomes. These results with the reconstituted system are important since they allow us to rule out the possibility suggested elsewhere [14, 15], namely that polyamines stimulate the MFO system by stabilizing the microsomal membranes. Further, it is clearly apparent from Fig. 2 that activation does occur on the linear part of the kinetics in contrast to Kitada *et al.* [16] who only observed that the polyamine effect was to increase the interval of time within which the kinetics remained linear.

In order to tentatively identify the locus of polyamine action and to elucidate the mechanism of activation, a series of experiments was designed and will now be described in detail.

Effect of ionic strength on spermine activation

Fig. 3 shows the effect of ionic strength on the stimulation by spermine of the MFO system from liver microsomes of phenobarbital-treated rabbits, using ethoxycoumarin as the substrate. In these experiments, the ionic strength was varied by modifying the potassium phosphate or magnesium chloride concentration, the pH of the medium being kept constant. It appears that at low spermine concentrations (<1 mM), stimulation decreases as ionic strength increases. At higher concentrations of spermine (>1 mM), stimulation first increases, then decreases as ionic strength increases. On the other hand, as the ionic strength increases, a larger concentration of spermine is required to produce maximal activation. Finally, magnesium inhibits more efficiently than potassium the effect of spermine. It

Table 2. Effect of spermine on rate of reduction of P-450 and on activity in different preparations of rabbit liver microsomes

Microsomes from		Spm (mM)			
		0	0.5	2	10
Untreated rabbit	k_f (min^{-1})	1.3 ± 0.2	2.7 ± 0.5	2.1 ± 0.3	1.5 ± 0.2
	k_s (min^{-1})	0.18 ± 0.05	0.37 ± 0.08	0.22 ± 0.05	0.13 ± 0.02
	AHH (pmoles/min/mg)	277 ± 25	510 ± 50	477 ± 50	280 ± 25
	Chlorcyclizine demethylation (nmoles/min/mg)	0.5 ± 0.1	1.2 ± 0.1	1.5 ± 0.1	1.6 ± 0.1
TAO-treated rabbit	k_f (min^{-1})	12 ± 2	12 ± 2	12 ± 2	nd
	k_s (min^{-1})	2.5 ± 0.3	2.6 ± 0.3	2.5 ± 0.3	nd
	AHH (pmoles/min/mg)	193 ± 20	373 ± 35	240 ± 25	100 ± 10
	Chlorcyclizine demethylation (nmoles/min/mg)	1.6 ± 0.2	2.2 ± 0.2	3.1 ± 0.3	2.7 ± 0.3

k_f and k_s, rate constants of fast and slow phases, respectively. AHH, aryl hydrocarbon hydroxylase activity (see Materials and Methods for details).

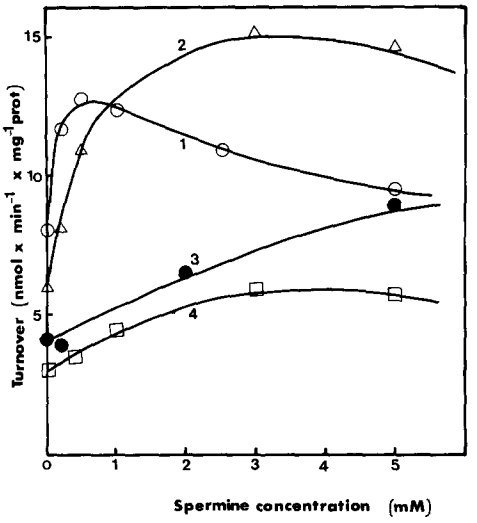

Fig. 3. Turnover of ethoxycoumarin deethylase activity of microsomes from phenobarbital-treated rabbits as a function of spermine concentration. Effect of ionic strength. (1) 0.01 M potassium phosphate (pH 7.4); (2) 0.05 M phosphate; (3) 0.1 M phosphate; (4) 0.01 M phosphate plus 10 mM magnesium chloride. Putative relative uncertainty ±10%. For further details see Materials and Methods.

is clear from these data that the interaction of spermine with its target in the MFO system is predominantly electrostatic, as expected from its polycationic nature.

Effect of pH on spermine activation

Since spermine appears to tightly bind one of the components of the MFO system, it is likely from the polyelectrolyte theory that a pH profile shift of the activity of the system should be observed [20, 21]. This effect is illustrated in Fig. 4 where ethoxycoumarin deethylase activity of liver microsomes from phenobarbital-induced rabbit is plotted against pH, in the absence and in the presence of 1 and 5 mM spermine. It is clearly observed that, as spermine concentration increases, the pH optimum of the reaction is displaced towards lower values, while, in the meantime, activation occurs. The interpretation of this effect is as follows: as spermine binds to its target, there results a local, rather strong positive electrostatic potential which repels the protons. Accordingly, the local pH is higher than the bulk pH and the pH-profile (activity against bulk pH) appears therefore to be displaced towards lower values [20, 21]. These results strongly suggest that spermine binding occurs in close proximity (a few Ångströms) of the molecular structure involved in the limiting step of the overall reaction. It is therefore likely that spermine binds near the active site either on the P-450 molecule or on the active complex between P-450 and the reductase. It should be mentioned here that, owing to the pH-profile shift, the activation ratio depends on the pH: it increases from 2 at pH 8.8 to nearly 13 at 6.9. In most of our measurements this effect has been taken into account.

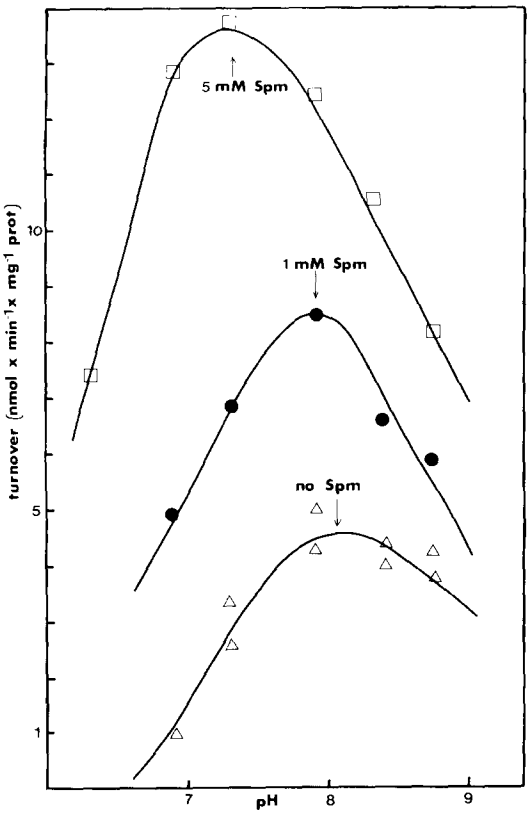

Fig. 4. Effect of spermine on the pH-profile of ethoxycoumarin deethylase activity of microsomes from phenobarbital-treated rabbits. Buffers used: from pH 6.0 to 7.3, 50 mM Bistris–HCl; from 7.3 to 9.0, 50 mM Tris–HCl. Putative relative uncertainty ±10%. For further detail see Materials and Methods.

Effect of spermine on substrate binding

Although substrate binding is not likely to represent the limiting step in the cytochrome P-450 reaction cycle [30], it is of interest to know whether K_m values of the substrates are modified by the presence of spermine. A series of experiments on phenobarbital-treated rabbit liver microsomes using ethoxycoumarin and benzphetamine as substrates, was carried out to document this point. An example of such experiments is shown in Fig. 5 where Line weaver–Burk plots of ethoxycoumarin deethylase activity are recorded. These plots present usually two linear parts, which could reflect the occurrence in microsomes of at least two populations of different P-450 isozymes, actively metabolizing ethoxycoumarin. Clearly, both low- and high-affinity K_ms of ethoxycoumarin are not modified significantly, whereas the V_{max}s are increased in the presence of 5 mM spermine. In particular, the V_{max} associated with the high-affinity component is increased to a larger extent than that associated with the low-affinity component. If high- and low-affinity components represent two different cytochrome P-450 isozymes, such a difference is to be expected. In the case of benzphetamine (only one component is apparently present here) K_m = 46.8 and 29.8 μM, in the absence and in the presence of 2 mM spermine respectively; the small difference is either not significant or indicates that binding of the substrate is but slightly increased. However, it is clear from these data that binding of the substrate is not the critical step in the polyamine action.

Effect of spermine on NADPH-mediated reduction of cytochrome P-450

In the reaction cycle of cytochrome P-450, after substrate binding, the second step consists in the reduction of the cytochrome which receives a first electron from NADPH via the reductase. A detailed study of polyamine effect was undertaken on this process which can be spectrophotometrically monitored. The data reported in Table 2 were obtained with two different preparations of microsomes, benzpyrene and chlorcyclizine being used as the sub-

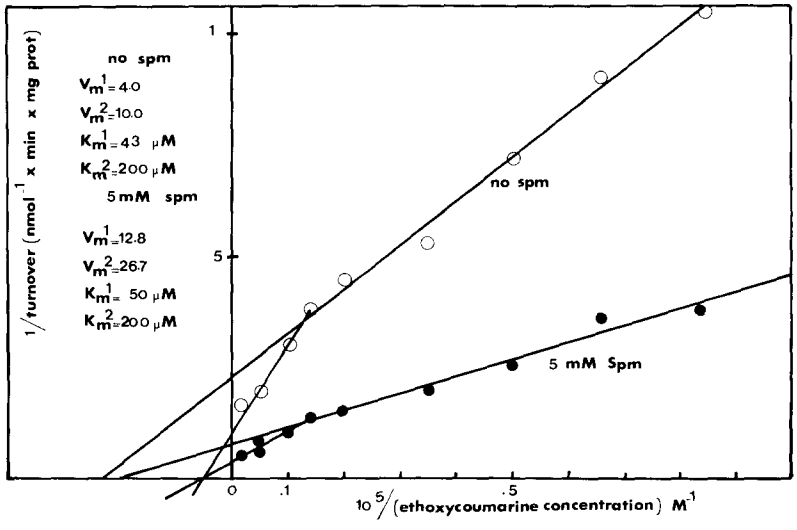

Fig. 5. Lineweaver–Burk plots of ethoxycoumarin deethylase activity of liver microsomes from phenobarbital-treated rabbit, in the absence and in the presence of 5 mM spermine. Putative relative uncertainty ±10%. For further detail see Materials and Methods.

strates, and illustrate two different types of response. It must be first mentioned that reduction of cytochrome P-450 within microsomes is generally a biphasic process [30]. As is the case for substrate binding, such a behaviour could result from the presence of at least two different populations of isozyme with different rates of reduction. Although likely, this hypothesis has not yet been clearly established. Concerning the rate constants of reduction of P-450 in liver microsomes from untreated rabbits, spermine being absent, our data are in good agreement with a recent report [11]. On the other hand, they appear to be an order of magnitude lower than the corresponding values obtained from rat liver microsomes [31]. It can be seen that the effect of spermine on reduction of cytochrome P-450 roughly parallels that observed on both benzpyrene hydroxylate activity (aryl hydrocarbon hydroxylase, AHH, activity) and chlorcyclizine demethylase activity with the same microsomes. While reduction of cytochrome P-450 remains biphasic (the relative amplitude of each phase is not significantly affected by spermine: fast phase 70%, slow phase 30%), the rate constant of both phases is increased at low polyamine concentrations. At higher spermine levels the effect is lower and disappears at 10 mM. However, such an observation could not be made with liver microsomes from TAO-treated rabbits, although spermine stimulates AHH and chlorcyclizine demethylase activities with these microsomes to the same extent as it does with untreated microsomes (see Table 2). TAO-treated rabbits were used in this work because it was recently demonstrated in our laboratory that this antibiotic behaves as a new type of inducer, stimulating the synthesis of form LM_{3b} of cytochrome P-450, known up to now as non-inducible by classical inducers [23]. On the basis of our observations that spermine effect depends not only on the species and substrate but also on the treatment of animals, the conflicting results reported in Table 2 are not unexpected. However, it has to be realized that reduction of cytochrome P-450, in these two preparations of microsomes, cannot be the rate-limiting step. These observations suggest therefore that the locus of spermine action could be cytochrome P-450 or the active complex cytochrome P-450 reductase, but that first electron transfer cannot be the critical step in spermine action.

Effect of spermine on the NADH synergism

After reduction of cytochrome P-450, the next step in the reaction cycle is the binding of an oxygen molecule. This process is extremely fast (50–100 msec) as observed in this and in other laboratories [32], and cannot be seriously considered as critically affected by polyamines. This step results in the formation of the so-called oxyferrous intermediate. At this stage two possibilities might occur. In the normal situation, the oxyferrous intermediate receives from NADPH via the reductase (as for first electron reduction) a second electron and then becomes competent for the hydroxylation reaction. However, if the second electron is not provided sufficiently fast, and because of its intrinsic instability [30, 33], the oxyferrous intermediate autoxidizes unproductively, restoring the oxidized enzyme–substrate complex. It is not yet clearly established whether this second electron transfer is the actual limiting step in the P-450 reaction cycle. Furthermore, direct observation of this elementary step is not possible. In order to tentatively gain information on the polyamine effect on this step we have undertaken a study of the effect of spermine on the NADH synergism. It is known since the work of Hildebrandt and Estabrook [34] that the NADH synergism results from the fact that another microsomal constituent, cytochrome b_5 can, like the NADPH cytochrome P-450 reductase, efficiently transfer the second electron on the oxyferrous intermediate of P-450. A

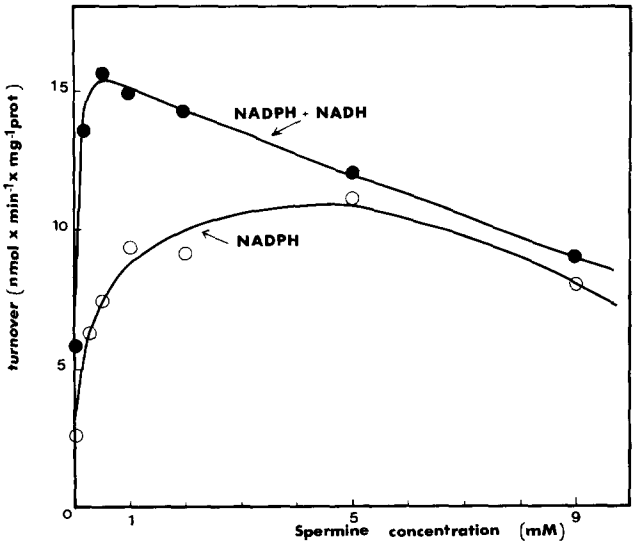

Fig. 6. Effect of spermine on NADH synergism of NADPH-dependent ethoxycoumarin deethylation by rabbit liver microsomes from phenobarbital-treated animals. Putative relative uncertainty ±10%. For further detail see Materials and Methods.

recent work from our laboratory has directly confirmed this point [22]. It appeared therefore of interest to see whether or not spermine activation was modified in the presence of NADH. Fig. 6 illustrates a typical series of experiments carried out on the same preparation of liver microsomes from phenobarbital-treated rabbits, using ethoxycoumarin as substrate. Clearly the concentration dependence of spermine activation is strongly modified in the presence of NADH: maximal activation is observed with a much lower concentration of spermine. Since it is known that cytochrome b_5 has no effect on those elementary steps, preceding the second electron transfer, these results suggest that the locus of spermine action is the oxyferrous intermediate of P-450 or another intermediate or step beyond transfer of the second electron on the way to the release of hydroxylated substrate. On the other hand, spermine could stimulate this electron transfer by modifying the redox potential of the oxyferrous intermediate. Preliminary experiments (not shown here) by stopped-flow spectrophotometry clearly showed that spermine does not stabilize the oxyferrous intermediate of P-450 LM_2.

Effect of spermine on the hydroperoxide-dependent hydroxylation in microsomes

In addition to the normal mechanism involving NADPH, NADPH cytochrome P-450 reductase, and molecular oxygen, it was shown a few years ago that cytochrome P-450 could catalyze hydroxylation reactions by utilizing organic hydroperoxides as oxygen suppliers [35, 36]. It was therefore of interest to see whether polyamines also stimulated the system under these conditions. Experiments were carried out with rabbit liver microsomes from control and phenobarbital-treated animals, ethoxycoumarin as substrate and cumene hydroperoxide. Ethoxycoumarin was hydroxylated under these conditions although to a smaller rate than under normal conditions; however, we did not observe any stimulation by spermine in the concentration interval from 0 to 10 mM. Experiments carried out with the reconstituted system (using P-450 LM_2) were also negative in this respect. It is worth emphasizing here that neither NADPH- nor NADH-mediated cytochrome *c* reduction by microsomes was activated by polyamines.

DISCUSSION

Although our results on the stimulation of the liver microsomal MFO system by polyamines (Fig. 1) are in good agreement with previous reports [14–16], the interpretation that we propose in this paper is different. Chapman [14] and Jellinck and Perry [15] reasoned from their data that polyamines stimulate the liver microsomal MFO system by stabilizing the microsomal membranes which actually harbor the system. This interpretation must be ruled out on the basis of the results presented in this paper and in preliminary work [24] on the reconstituted system for which, given the relative amount of protein and phospholipid, any membrane structure cannot seriously be suspected to be reconstituted. Yet polyamines stimulate it to the same extent as microsomes and roughly the same concentration is required to produce maximal activation. On the other hand, Kitada *et al.* [16] carried out their experiments in high ionic strength conditions (0.1 M sodium–potassium phosphate, 10 mM magnesium chloride) which according to our results strongly inhibit the polyamine effect. Although their observation that polyamines inhibit lipid peroxidation is interesting and could be ascribed to the ability of spermine to scavenge chemical radicals as recently reported [37], it is not likely to explain the activating effect observed on the hydroxylation reaction. Results presented in Fig. 2 rule out this connection between these effects.

In contrast to these previous reports, the detailed investigation presented in this paper allows us to emphasize the following points: (1) polyamines stimulate P-450-dependent hydroxylation reactions in both microsomes and a reconstituted system (2) treatment of animals with different P-450 inducers modifies both the level and concentration dependence of the stimulation, (3) polyamine stimulation is substrate-dependent and stereospecific, (4) polyamines modify the pH-profile of the hydroxylation reactions, (5) in some cases, polyamines stimulate the reduction of P-450, (6) polyamine stimulation is strongly modified when NADH is present in addition to NADPH, and (7) polyamines do not stimulate the cumene hydroperoxide dependent hydroxylation reaction.

Collectively these different results strongly suggest that the locus of polyamine actions is cytochrome P-450. The findings that the hydroperoxide-supported hydroxylation is not activated and that NADH modifies the level and concentration dependence of activation suggest that the second electron transfer between reductase or cytochrome b_5 and the oxyferrous intermediate of P-450 is favoured. This would account for a different concentration dependence of spermine stimulation in the absence and in the presence of NADH, since electron transfer from reductase and cytochrome b_5 is very likely to occur at a different rate. On the other hand, this allows us to rule out the possibility that spermine stimulation originates from an elementary step *before* activation by the second electron of the oxyferrous intermediate, although reduction of cytochrome P-450 is indeed stimulated in some occasions (see Table 2). However, considering the second electron possibility, it is not clear whether spermine favours the interaction between P-450 and reductase or b_5 (decrease of the K_d for instance) or modifies the redox potential of the oxyferrous intermediate, so that the electron transfer is made easier. Further experiments on the elementary steps of the reaction cycle, using a reconstituted system with different isozymes, are necessary to reach a better understanding of this process. In particular, studies on the effect of polyamines on such parameters as hydrogen peroxide production during turnover of the system, kinetics of P-450 isozymes autoxidation, and P-450 reductase binding, appear promising in this respect.

Whatever the actual mechanism of polyamine-mediated activation, it is clear from the present work that this effect occurs in different animal species and in particular in man. Since polyamines are essential

cell components whose level in liver may be high and is subjected to large fluctuations [38] (particularly interesting in this respect is the finding by Russel [39] that those compounds known as inducers of cytochrome P-450 in liver microsomes stimulate the synthesis of polyamines) it is not unreasonable to suspect them as modulators of the MFO system *in vivo*. A comparative study of drug metabolism in the liver cell (in primary culture or freshly isolated) in the presence and absence of the newly synthesized specific inhibitor of polyamine synthesis (DL-α-difluoromethylornithine) [40] could allow us to know whether or not polyamines actually modulate the liver microsomal MFO system *in vivo*.

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