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STUDY OF ANAESTHETIC AGENTS FOR THEIR ABILITY TO ELICIT PORPHYRIN BIOSYNTHESIS IN CHICK EMBRYO LIVER

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Abstract—Effects of some anaesthetic drugs on the activity of δ-aminolevulinate synthetase and on the formation of porphyrins and cytochrome P-450 were studied in 18-day-old chick embryo livers *in ovo*. The drugs were either tested alone or with a small dose of 1,4-dihydro-3,5-dicarbethoxycollidine, which reproduces in the embryo liver a partial block in the heme biosynthesis pathway similar to that found in cells of human patients with porphyrias. Two series of local anaesthetics were tested: procaine and its derivatives (proxymetacaine, oxybuprocaine, butacaine and tetracaine) had no (or very slight) porphyrogenic effects. In contrast, lidocaine and its derivatives (bupivacaine, mepivacaine, etidocaine, pyrrocaine and prilocaine) were found to induce δ-aminolevulinate synthetase and to cause accumulation of porphyrins and cytochrome P-450. Some other drugs used in anaesthesiology were tested: fentanyl, morphine, sodium oxybate, pancuronium, pethidine and phenoperidine were found to be non-porphyrogenic; alcuronium was a slight inducer. It is suggested that the inducing drugs should be avoided in patients with hepatic porphyrias.

Acute hereditary hepatic porphyrias (acute intermittent porphyria, hereditary coproporphyria and variegata porphyria) are disorders of heme synthesis in which overproduction of heme precursors is often accompanied by severe clinical manifestations. Each of the inherited porphyrias is characterized by a specific defect in the activity of one of the enzymes of heme biosynthesis [1]. Most of the time the diseases remain clinically latent and only occasionally result in acute abdominal and/or neuropsychiatric symptoms. Occurrence of the symptoms often follows exposure to drugs such as barbiturates, sulfonamides, oestrogens etc. [1]. As a result of their deficiency of a heme pathway enzyme, patients with acute porphyrias are known to have a limited capacity for heme synthesis in the liver: however, exposure to "porphyrogenic" drugs could lead to a marked induction of the rate-controlling enzyme δ-aminolevulinate synthetase (ALA-S)† (EC 2.3.1.37) thereby resulting in overproduction of porphyrins and their precursors ALA and PBG. Barbiturates and many other lipophilic compounds induce ALA-S and cytochrome P-450 in intact mammalian liver, but only a very few compounds produce an experimental porphyria with massive overproduction of porphyrins in normal liver [2–7]. However, the lipophilic compounds become very effective (i.e. porphyrogenic) if a partial block in liver heme biosynthesis exists either as a result of a genetic defect (patients with acute porphyrias) or in some experimental animals (rat, mouse and chick embryo) following a pretreatment with a specific inhibitor of heme biosynthesis. De Matteis and Stonard [8] were the first to propose the use of a small dose of DDC for producing an experimental animal model for drug sensitivity in acute hepatic porphyrias. DDC treatment leads to the formation in the liver of *N*-methyl protoporphyrin which is the inhibitor of ferrochelatase (EC 4.99.1.1), the last enzyme in heme biosynthesis [9]. Anderson [5] has adapted this method to the chick embryo *in ovo* (for testing antihypertensive drugs). He underlined the advantage of using a small dose of DDC in combination with drugs being tested.

The primary aim of our study was to test some general and/or local anaesthetics (especially those used by dentists), because porphyric patients are quite likely to be exposed at some time to one of these agents. We used the chick embryo liver *in ovo* as a model system because its advantages over other experimental animal models have already been demonstrated [7, 10]. "Inducing" effects of drugs were studied after injection of (a) the drug *alone*, by measuring increases in hepatic ALA-S activity, porphyrin accumulation and cytochrome P-450; and (b) after injection of the drug *with an additional small dose of DDC*, by measuring ALA-S activity and porphyrin accumulation in the embryo livers.

MATERIALS AND METHODS

[1-4^{14}C]Succinic acid was obtained from Amersham (U.K.). AIA was a gift from Hoffman-La Roche (Switzerland). DDC was obtained from Eastman Organic Chemicals (Rochester, NY).

Succinyl-CoA synthetase (partially purified from

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† AIA, allylisopropylacetamide; ALA, δ-aminolevulinic acid; ALA-S, δ-aminolevulinate synthetase; DDC, 1,4-dihydro-3,5-dicarbethoxycollidine; DMSO, dimethylsulfoxide; PBG, porphobilinogen.

R. Spheroides) was a gift from Drs P. and R. Labbe (University Paris VII, Colombes, France).

The following drugs were provided as gifts from laboratories in France: procaine from Merain, Paris; lidocaine, etidocaine and prilocaine from R. Bellon, Neuilly; bupivacaine from Winthrop, Clichy; oxybuprocaine and proxymetacaine from Merck-Sharp & Dohme, Paris; butacaine from Opacalcium, Paris; pyrrocaine from A.T.O. Zizine, Paris; tetracaine and morphine from Assistance Publique, Paris; alcuronium chloride from Roche, Neuilly; pancuronium bromide from Organon Teknika, Saint Denis; pethidine from Specia, Paris; sodium γ-hydroxybutyrate from Egic, Montargis.

Fertilized 16-day-old chick embryos (Warren Strain, CNRZ Jouy en Josas, France) were kept in an incubator at 37° with 70% humidity. Drugs were dissolved in either a small vol. (0.1–0.3 ml) of 0.15 M NaCl or DMSO (for water-insoluble drugs). Following the usual methods, sterile injections of drugs were made when the eggs were 18 days old. Normally, after 24 hr, the embryos were killed by decapitation, the livers were removed, separated from the gall-bladder and rinsed with saline prior to homogenization with 3 vols of 0.25 M sucrose/0.02 M Tris buffer (pH 7.4). As previously noted by Rifkind *et al.* [3, 10] and Anderson [5], responses to a given dose of an inducing drug vary considerably when measurements are carried out in individual embryos, regardless of the route of administration. Therefore, for this study, at least six embryo livers were pooled for each determination.

In assays with the drug alone, two types of controls were included: one control with the 0.15 M NaCl vehicle (that was used for water-soluble drugs) and one control with the DMSO vehicle (that was used for water-insoluble drugs); in assays with drugs combined with DDC, DDC was injected with DMSO (0.1–0.2 ml) as solvent and the companion drug was either in NaCl (0.15 M) or DMSO: controls were composed of eggs injected with the small dose of DDC alone.

Porphyrins. These were extracted from the whole homogenate with 1 N perchloric acid:methanol (1:1 v/v); the type and the concns of porhyrins were studied with a spectrofluorimeter [Hitachi Perkin–Elmer (Model 204)] using a technique previously described [11]. The type of accumulated porphyrins was confirmed by high-pressure liquid chromatography [12].

Enzyme assays. The homogenates were centrifuged at 800 *g* for 15 min and the supernatant was sonicated 3 times for 15 sec. Enzyme assays were performed as follows.

(a) ALA-S activity was measured following the radiochemical method of Strand *et al.* [13] with [^{14}C]succinic acid and a succinyl-CoA-generating system; recovery of ALA was 75–85% as determined spectrophotometrically with the Ehrlich reagent. Enzyme activity was expressed as pmoles ALA per 30 min per mg of protein.

(b) Ferrochelatase activity was measured using the radiochemical method of Bonkowsky *et al.* [14] with some modifications previously described [15]. Enzyme activity was expressed as nmoles heme per hour per mg of protein.

Cytochrome P-450. This was measured as follows: 10 ml of the 800 *g* supernatant were centrifuged at 12,000 *g* for 15 min; 5 ml of the supernatant were then centrifuged at 105,000 *g* for 30 min; after washing twice, microsomes (pellet) were resuspended in potassium phosphate buffer [100 mM (pH 7.4)] and the protein concn adjusted to 2 mg/ml. The level of cytochrome P-450 was measured in the microsomal fraction using the method of Omura and Sato [16] with a dual beam spectrophotometer (Beckman 35).

Proteins. These were measured by the method of Lowry *et al.* [17].

RESULTS

Preliminary studies

A time-course for induction by DDC at porphyrogenic dose (4 mg/egg), is shown in Fig. 1. It was found that liver ALA-S activity was highest 15–18 hr after injection of the drug and ALA-S levels were still elevated at 24 hr. The porphyrin content of the liver strictly followed the rise of ALA-S, but the highest level still persisted at 24 hr. In contrast, a significant increase of cytochrome P-450 was only observed at 24 hr.

Various doses of DDC were then tested to fix the optimal dose to eventually potentiate the inducing effects of a drug. Table 1 shows the results obtained with DDC combined with AIA as inducer (2 mg/egg); porphyrin, ALA-S and ferrochelatase in the liver were measured 22–24 hr after drug injection. It was found that 0.25 mg of DDC was the proper dose, because: (a) when injected alone it gave only a relatively small increase in porphyrin level with a minor change in ALA-S activity, and (b) when injected with the inducer it gave the *maximal-fold* increase of both parameters. Moreover a significant inhibition of ferrochelatase still occurred at 24 hr.

Tables 1 and 2 summarize the effects at 24 hr of two known inducers (AIA and phenobarbital) injected either alone or combined with a small dose of DDC (0.25 mg/egg). AIA and/or phenobarbital injected alone caused the expected increase in ALA-S activity and in the cytochrome P-450 level,

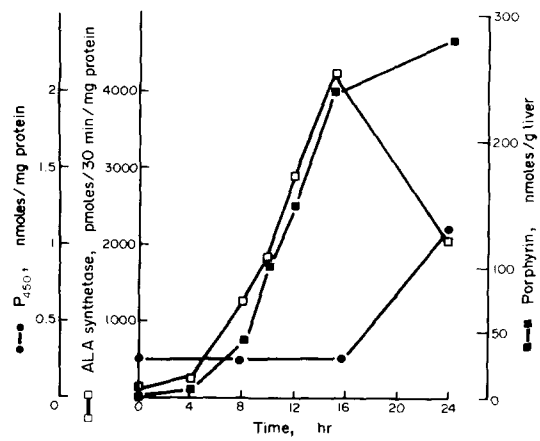

Fig. 1. Time-course of effects on hepatic heme metabolism. DDC (4 mg/egg) was administered in a single injection to 42 eggs. Values represent the average of results from two independent determinations made on six pooled livers from 18-day-old chick embryos, □, ALA synthetase activity; ■, total porphyrin concn; ●, cytochrome P-450 content.

but with a low porphyrin accumulation. When a small dose of DDC was injected simultaneously with the inducers, a more marked increase in both ALA-S activity and porphyrin content was observed as compared to the minor change found in controls treated with DDC alone (Tables 1 and 2).

Fig. 2 shows a time-course for induction by phenobarbital (4 mg) injected with a small dose of DDC (0.25 mg/egg). Liver ALA-S and porphyrins were both found to be highest at 24 hr.

Following these preliminary results it was decided to study the effects of the drugs usually at 22–24 hr for both series of assays (drugs alone and drugs with DDC) (see Discussion).

Tested drugs

Table 2 shows the effects of some loco-regional anaesthetics: procaine, lidocaine and their respective derivatives. Procainamide (which is not an anaesthetic agent) was tested as well because of its structural similarity to the other compounds (see Discussion). For each series of assays a drug was considered to be inducing when the following data were obtained: (a) without DDC: ALA-S activity, porphyrin and cytochrome P-450 concn at least higher than the mean control level plus 3 S.D.; and (b) with DDC (0.25 mg): ALA-S activity and porphyrin concn at least higher than the mean control level plus 3 S.D.

According to the results, loco-regional anaesthetic agents have been classified as follows: (a) the inducing drugs were found to be lidocaine and other drugs with structures derived from aniline (bupivacaine, mepivacaine, etidocaine, prilocaine and pyrrocaine); (b) the non-inducing drugs were found to be oxybuprocaine and proxymetacaine; and (c) procaine, tetracaine and butacaine were found to slightly increase either ALA-S activity or porphyrin level and were therefore classified as doubtful.

Some other drugs used in anaesthesiology were tested: phenoperidine, sodium oxybate, pancuronium and fentanyl were found to be non-inducing drugs; alcuronium was a slight inducer (see Table 3).

DISCUSSION

Several animal species have already been tested for their sensitivity to drugs known as inducers of porphyric attacks [7]. In contrast to rodents, 18-day-old chick embryos *in ovo* appear to be one of the best test systems, because they combine a marked sensitivity to inducing drugs (well correlated with their effects in porphyric patients [7]) and a considerable ease of handling.

We used this system to examine the effects of loco-regional anaesthetics on the hepatic heme biosynthesis pathway. Our preliminary studies (Table 1) confirmed the previous report of Anderson [5]: (a) the use of a small "priming" dose (0.25 mg/egg) of DDC in combination with the drug being tested, and (b) a fluorimetric measurement of liver porphyrins at 24 hr after injection are a sensitive and rapid method to test drugs for their porphyrogenic effect.

As already noted [5] differential effects of inducing drugs injected alone were observed in avian liver.

Table 1. *Effects of increasing doses of DDC on hepatic ALA synthetase, porphyrin and ferrochelatase*

DDC (mg) (in 0.2 ml DMSO)	ALA synthetase activity (pmoles/30 min/mg protein)			Porphyrin (nmoles/g liver)			Ferrochelatase activity with or without AIA* (nmoles/hr/mg protein)	% inhibition
	Without AIA	With AIA (2 mg)	Fold increase	Without AIA	With AIA (2 mg)	Fold increase		
0	80	1800	22.5	0.5	2	4	3	0
0.15	128	2700	21	11	170	15.5	1.6	50
0.25	150	4640	31	10	250	25	1.2	60
0.30	287	5470	19	27	300	11	1.2	60
0.50	390	7300	19	28	440	16	0.9	70

DDC with or without allylisopropylacetamide (AIA) was administered in a single injection into eggs. ALA synthetase, porphyrin and ferrochelatase were measured 22–24 hr after injections. Values represent the average of results from two independent determinations made on six pooled livers from 18-day-old chick embryos.

* AIA has no effect on ferrochelatase activity.

Table 2. Effects of local anaesthetics (with or without DDC) on hepatic ALA synthetase, porphyrin and cytochrome P-450

	Dose (mg)	Ratio man/egg*	Drug alone			Drug and DDC (0.25 mg)	
			ALA synthetase (pmoles/30 min/mg protein)	Porphyrin (nmoles/g liver)	Cytochrome P-450† (nmoles/mg protein)	ALA synthetase (pmoles/30 min/ mg protein)	Porphyrin (nmoles/g liver)
Controls (in 0.2 ml DMSO)			80 ± 30	0.50 ± 0.25	0.30 ± 0.05	—	—
Controls (0.25 mg DDC)			—	—	—	150 ± 90	10 ± 3
Known inducer							
Phenobarbital	4	50	1180	2.2	1.22	4900 ± 870	125 ± 30
AIA	2	—	1770	1.8	0.74	5932 ± 1248	231 ± 53
Lidocaine‡	2	50	400	8.2	0.69	5070	224
Mepivacaine‡	2	50	1360	3.4	1.19	9200	264
Bupivacaine‡	2	50	260	1.9	0.87	3160	210
Prilocaine‡	10	10	350	0.5	0.87	1830	108
Etidocaine‡	2	50	310	0.9	1.10	3330	256
Pyrrocaine‡	2	50	390	1.6	0.69	1800	216
Procaine	2	50	280	0.5	0.29	690	18
Oxybuprocaine	1	—	150	0.5	0.30	400	11
Proxymetacaine	2	—	110	0.4	0.23	270	11
Tetracaine	2	50	170	0.4	0.38	360	54
Butacaine	2	50	70	0.6	0.31	170	28
Procainamide	2	—	90	0.5	—	300	7

Assays were performed at 24 hr after drug injection (with or without DDC) to eggs. Values represent the average of results from two independent determinations made on six pooled livers from 18-day-old chick embryos. Values of controls are means ± S.D. for at least two groups of six pooled embryo livers.

* Ratio man/egg is the ratio of the average human dose and the dose injected into eggs.

† Cytochrome P-450 was not measured when DDC was injected with studied drug because at low dose DDC prevents cytochrome P-450 increase (see Refs 5 and 10).

‡ Inducer.

Table 3. Effects of miscellaneous drugs (with or without DDC) on hepatic ALA synthetase, porphyrin and cytochrome P-450

	Dose (mg)	Ratio man/egg*	Drug alone			Drug and DDC (0.25 mg)	
			ALA synthetase (pmoles/30 min/ mg protein)	Porphyrin (nmoles/g liver)	Cytochrome P-450† (nmoles/mg protein) (microsomes)	ALA synthetase (pmoles/30 min/ mg protein)	Porphyrin (nmoles/g liver)
Controls (0.2 ml DMSO)			80 ± 30	0.50 ± 0.25	0.30 ± 0.05	—	—
Controls (0.25 mg DDC)			—	—	—	150 ± 90	10 ± 3
Known inducer							
Phenobarbital	4	50	1180	2.2	1.22	4900 ± 870	125 ± 30
AIA	2	—	1770	1.8	0.74	5932 ± 1248	231 ± 53
Alcuronium‡	1	20	125	0.40	nd	520	68
Fentanyl	0.01	50	80	0.35	0.32	75	8.5
Pancuronium	0.5	10	60	0.40	0.34	95	8
Pethidine	5	80	70	0.35	0.28	50	1
Phenoperidine	0.2	30	90	0.45	0.34	90	10
Sodium oxybate	50	60	80	0.40	0.27	70	7
Morphine	1	40	20	0.40	0.36	50	12

Assays were performed at 24 hr after drug injection (with or without DDC) to eggs. Values represent the average of results from two independent determinations made on six pooled livers from 18-day-old chick embryos. Values of controls are means ± S.D. for at least two groups of six pooled embryo livers.

* Ratio man/egg is the ratio of the average human dose and the dose injected into eggs.

† Cytochrome P-450 was not measured when DDC was injected with studied drug because at low dose DDC prevents cytochrome P-450 increase (see Refs. 5 and 10).

‡ Inducer.

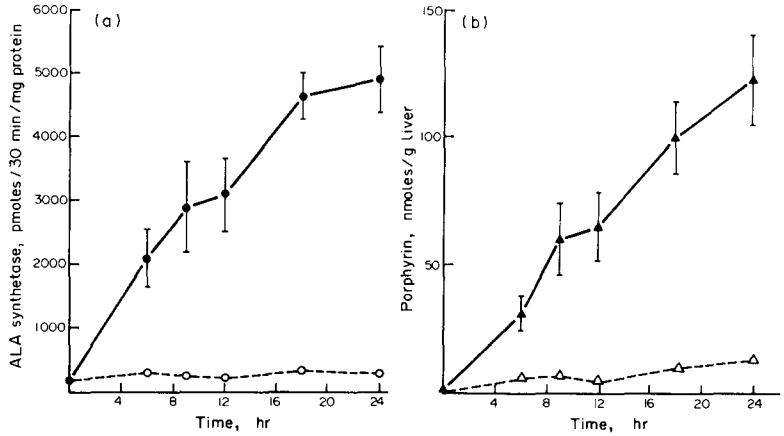

Fig. 2. Time-course of phenobarbital combined with DDC: effects on hepatic ALA synthetase (a) and porphyrin accumulation (b). Phenobarbital (4 mg/egg) combined with a priming dose of DDC (0.25 mg/egg) were administered in a single injection to 36 eggs. For DDC + phenobarbital, values represent means ± S.D. of results from four independent determinations made on six pooled livers from 18-day-old chick embryos. For DDC alone, values represent the average of results from two independent determinations made on six pooled livers from 18-day-old chick embryos. ● and ▲, phenobarbital + DDC; ○ and △, DDC alone.

(a) If induction of hepatic heme biosynthesis usually results in significant increases in liver ALA-S activity, maximal induction of the enzyme occurred at time intervals which could be very different from one drug to another [5]. In our study on the effects of drugs injected alone, ALA-S activities were significantly increased at 24 hr (Fig. 1 and Table 2) for all inducing drugs being tested.

(b) If induction of ALA-S is generally associated with an increase in hepatic cytochrome P-450 levels, porphyrin accumulations are often very slight (Table 2). In contrast, giving a small "priming" dose (0.25 mg/egg) of DDC (inhibitor of ferrochelatase) in combination with an inducing drug, resulted in chicken embryo porphyrin accumulation and increased ALA-S activities, data which are similar to that found during crises in patients with hepatic porphyria (Table 2), and very different from the data obtained with DDC alone (at the small dose used, DDC caused little increase in ALA-S activity and porphyrin content in the liver). Moreover, a good correlation ($r = 0.89$) was found to exist between ALA-S activities and the accumulation of porphyrin, both measured at 24 hr (Fig. 2 and Table 2).

Some local anaesthetic agents have already been tested by Parikh and Moore [4] by measuring in rats the changes induced in the activity of hepatic ALA-S. A significant increase was found only with lidocaine, no increase in activity was obtained with prilocaine, and only a mild increase (20–25%) with bupivacaine. This discrepancy with our results (we found a high increase with each of these products—see Table 2) can probably be explained by the remarkable sensitivity of the chick embryo liver; possible species differences in the metabolic response to drugs need to be considered when comparing experimental results and also when extrapolating from animal to man. However, at this time, all drugs which have been clearly involved in precipitating acute attacks in humans and which have then been tested in chick embryo (*in ovo*) were found to be inducers (with an increase in ALA-S activity and/or porphyrin accumulation in the liver in this system) [7].

The porphyrogenic effect of drugs is a dose-related phenomenon. It is however very difficult to be sure that the dose injected in chicken embryos is equivalent to the dose administered to man; the criterion of the ratio dose/wt (w/w) cannot be used, because the amount of drug injected would be too small for it to induce. Comparing the dose (100–200 mg/day) used in human therapeutics for a well-known inducer (phenobarbital) and the dose (2–5 mg) of this drug injected in chicken embryo to obtain a significant increase in ALA-S activity and liver porphyrin concn, it was decided that the dose used to test any drug should be between 1/20 and 1/100 of the human dose; in other words, the dose of a drug administered to chick embryos was 20–80 times (w/w) the average dose encountered by patients. When the tested drug seemed to be slightly porphyrogenic (mild increase in ALA-S activity and/or porphyrin accumulation), we confirmed the data by using a higher concn (i.e. alcuronium).

Our studies on the porphyrogenic effects of the two series of loco-regional anaesthetic agents are evocative of the relationship between the structure and the porphyrogenic effects of these molecules. Several features have already been described regarding the porphyrogenic activity of drugs [7]. At least three of them (lipophilicity, bulky hydrophobic groups containing a carbonyl group and steric factors inhibiting hydrolysis in esters and amides) are found in the porphyrogenic drugs described in our work. The degree of hydrophilicity and the steric obstruction from hydrolysis are the two structural conditions which could explain the marked difference between the porphyrin-inducing activity of the two series. Drugs of the lidocaine series (which have been found to be inducers) are much less hydrophilic than procaine and its derivatives, which are not (or slight) inducers. The second condition (steric resistance to

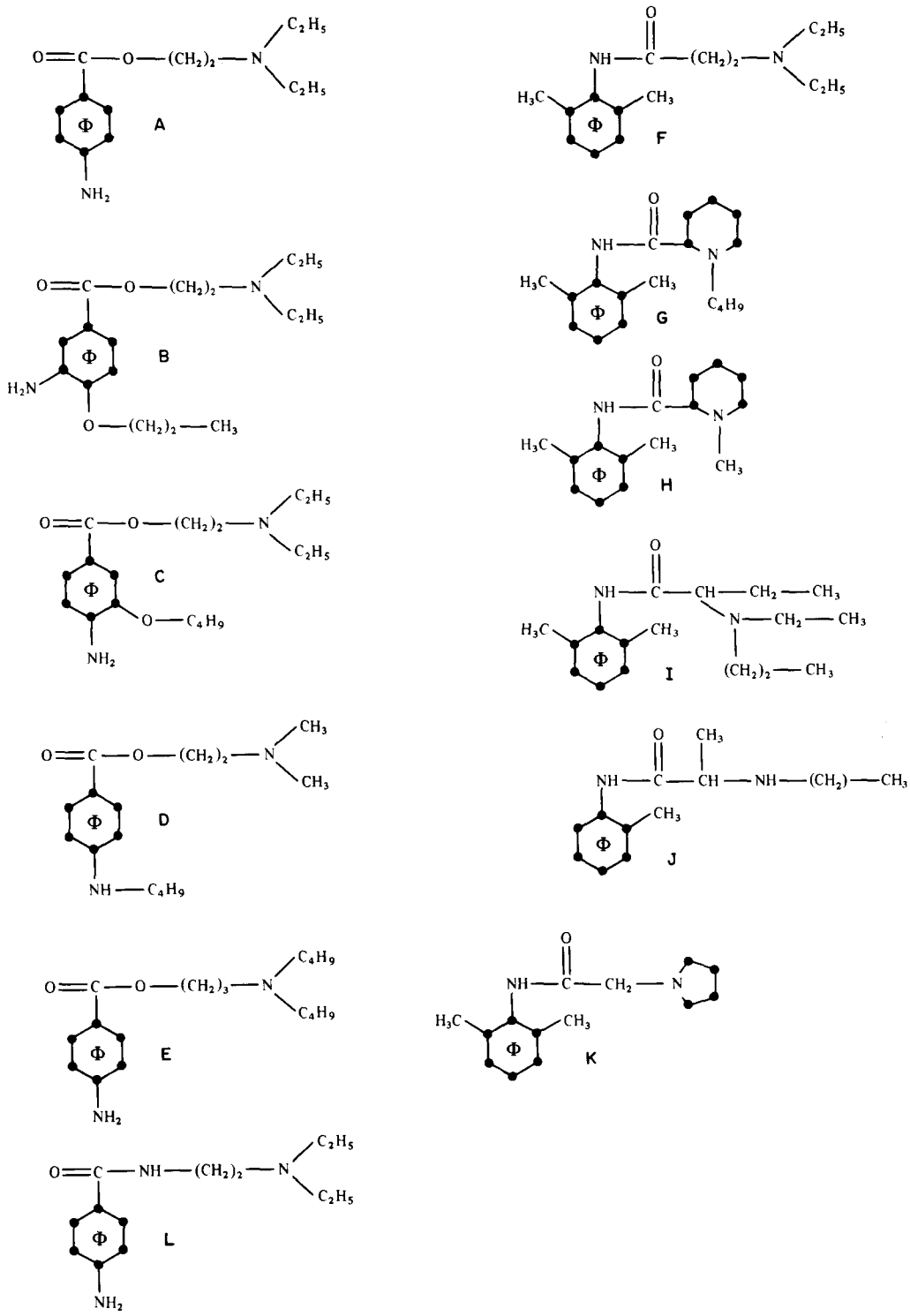

Fig. 3. Some tested drugs. Left side: procaine and its derivatives. Right side: lidocaine and its derivatives. (**A**) Procaine; (**B**) proxymetacaine; (**C**) oxybuprocaine; (**D**) tetracaine; (**E**) butacaine; (**F**) lidocaine; (**G**) bupivacaine; (**H**) mepivacaine; (**I**) etidocaine; (**J**) prilocaine; (**K**) pyrrocaine; (**L**) procainamide.

hydrolysis), has been demonstrated by Racz and Marks [18] in their studies of a series of esters and amides: the essential feature for ALA-S-inducing capacity was an ester or amide group *resistant* to hydrolysis. Procaine and its derivatives contain an ester group which sterically appears to be readily hydrolysable; they are not (or slight) inducers. The substitution of the ester group of procaine by an amide group (procainamide) without changing the steric features does not change the inducing capacity of the compound (Table 2). By contrast, lidocaine and its derivatives possess an amide whose hydrolysis seems sterically hindered by two methyl groups in positions 2 and 6 of the benzene ring. Moreover prilocaine, which has only one methyl group, is a less potent inducer than the others (Table 1).

Among the other anaesthetic drugs tested, it would be useful to remember that pancuronium, fentanyl, phenoperidine and sodium oxybate seem to be safe; alcuronium is a slight inducer, but at a relatively high dose.

These studies on an experimental animal model allowed us to identify new porphyrogenic drugs whose use (particularly in anaesthesia) should probably be avoided in porphyric patients.

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