perfused with oxygenated and warmed (35 °C) Tyrode solution. Electrical activity was recovered by means of conventional glass microelectrodes. Each recording microelectrode was also used for intracellular stimulation (current 1–2 μA, duration 3 msec, frequency 0.2–1 Hz) of the cell[10]. After control records, the preparation was perfused with Tyrode solution containing 5×10^{-7} M/l of (±)-verapamil until complete cessation of spontaneous activity ensued. About 5 min after arrest, the preparation was perfused with Tyrode solution containing verapamil and 10^{-5} m/l of noradrenaline bitartarate or 2×10^{-3} M/l of db cAMP.

Results and discussion. The effects of verapamil and db cAMP are shown in the figure. Verapamil reduced the spontaneous rate, decreased the slope of the slow depolarization and diminished the amplitude of the action potential; 12–40 min from the onset of verapamil administration, the action potentials disappears and the records showed only slow depolarization. Within this period, the cells became inexcitable both for depolarizing and for hyperpolarizing pulses (figure, E, F), although in the control conditions, intracellular stimulation during slow depolarization and at the end of repolarization was always effective. In a further 3–8 min, the amplitude and rate of the slow depolarization progressively decreased and finally a stable membrane potential of –45 to –50 mV was recorded.

Addition of noradrenaline was ineffective, whereas db cAMP caused the spontaneous activity to return within 5–20 min. This activity remained as long as the nucleotide was present. Washing out the preparation with pure Tyrode solution, or Tyrode solution containing verapamil, again caused an arrest within 25–75 min. The above results confirm other investigations which indicate that in the cardiac pacemaker slow depolarization as well as the action potential is mainly dependent on the calcium influx[11–13]. Disappearance of the action potential and the complete inexcitability of the cells arrested by verapamil could also support the view that, in the nodal action potential, only the slow current component is present[14].

However, the effect of the db cAMP in the presence of verapamil remains unclear. It is generally accepted that verapamil inhibits calcium influx acting directly on the slow channel[3, 4, 15]. This may explain the lack of action of noradrenaline which, as its actions, increases the inward Ca-current[16, 17]. However, it does not explain why db cAMP reactivates spontaneous activity blocked by verapamil which only slightly, if at all, affects the cAMP content in the heart and does not counteract the adrenergic stimulation of adenylate cyclase[15, 18]. The last finding makes an unspecific action of db cAMP unlikely, because otherwise elevated level of cAMP due to noradrenaline would be also effective.

1 A. Fleckenstein, in: Calcium and the heart. Academic Press, London 1971.
2 A. Fleckenstein, G. Grün, H. Tritthardt and K. Byon, Klin. Wschr. *49*, 32 (1971).
3 A. Fleckenstein, H. Tritthardt, A. Herbst and G. Grün, Pflügers Arch. *307*, 25 (1969).
4 M. Kohlhard, B. Bauer, H. Krause and A. Fleckenstein, Pflügers Arch. *335*, 309 (1972).
5 R. Bayer, R. Kaufmann and R. Mannhold, Naunyn-Schmiedebergs Arch. Pharmak. *290*, 69 (1975).
6 R. Bayer, D. Kalusche, R. Kaufmann and R. Mannhold, Naunyn-Schmiedebergs Arch. Pharmak. *290*, 81 (1975).
7 B. N. Singh and E. M. Vaughan Williams, Cardiovasc. Res. *6*, 109 (1972).
8 R. S. Aronson and P. F. Cranefield, Pflügers Arch. *347*, 101 (1974).
9 M. R. Rosen, J. P. Ilvento, H. Gelband and Ch. Merker, J. Pharmac. exp. Ther. *189*, 414 (1972).
10 W. Tuganowski, J. appl. Physiol. *33*, 130 (1972).
11 H. H. Lu, in: Research in Physiology. Ed. F. F. Kao, K. Koizumi and M. Vassale. Gaggi, Bologna 1971.
12 S. Weidmann, A. Rev. Physiol. *36*, 155 (1974).
13 D. P. Zipes and J. C. Fischer, Circul. Res. *34*, 184 (1974).
14 A. Paes de Carvalho, B. F. Hoffman and M. P. de Carvalho, J. gen. Physiol. *54*, 607 (1969).
15 A. Watanabe and H. R. Beesch, jr, J. Pharmac. exp. Ther. *191*, (1974).
16 H. Reuter, J. Physiol. *242*, 429 (1974).
17 R. W. Tsien, J. gen. Physiol. *67*, 320 (1974).
18 J. Shanefeld, M. E. Hess and N. R. Lewine, J. Pharmac. exp. Ther. *193*, 317 (1975).

Spectral changes resulting from the interaction of some N-alkyl nitrosamines and rat liver microsomes[1]

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Summary. Dimethyl (DMN) and diethyl nitrosamine(DEN) do not give characteristic spectral changes upon interaction with rat liver microsomes, while dipropyl (DPN) and dibutyl (DBN) nitrosamine cause type I spectral changes. The spectral binding constant is 100 mM for DPN and 1.17 mM for DBN. The maximal spectral change is 3.2×10^6 and 1.0×10^6 absorbance units per milligram protein for DPN and DBN respectively.

Many compounds known to be substrates for the hepatic microsomal mixed function oxygenase have been shown to interact with oxidized cytochrome P450 (P450). Remmer et al.[2] using difference spectroscopy showed 2 distinct types of spectral shifts which they later called type I and II. The type I shift was typified by compounds such as aminopyrine, hexobarbital and many other chemicals and was characterized by a trough of about 420 nm and a peak at 385 nm. Compounds such as aniline, pyridine and others gave type II shift, characterized by a peak at about 430 nm and a trough at 395 nm. Type I spectra are generally considered to be caused by nonspecific interactions with a lipophilic site other than the

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2 H. Remmer, J. Schenkman, R. W. Estabrook, H. Sasame and R. Gillette, Mol. Pharmac. *2*, 187 (1966).

heme iron, while type II spectra are known to be result of ferrihemochrome formation with certain nitrogenous bases. Nitrosamines are a group of substances which have been found to be potent carcinogens in a variety of animal species, and it is generally believed that a metabolic activation process is required for their biological actions[3]. Several studies performed with dimethylnitrosamine (DMN) established the need of liver microsomes NADPH and O_2 for its metabolism and activation to an alkylating species, which is believed to be responsible for both toxic and carcinogenic effects[3]. These characteristics of the activation process and its inhibition by CO led to the suggestion that P450 would be involved in DMN activation[3–6]. Notwithstanding, the behavior of the DMN-demethylase enzyme in its response to inducers and inhibitors is not typical of a substrate of the P450 mediated transformations[7–12]. Moreover, DMN does not give spectral changes upon interaction with liver microsomes as expected from substrates of P450 mediated reactions[12,13]. This behavior would be unexpected if nitrosamines were considered as amino derivatives. In this paper we analyzed this fact, and also whether other nitrosamines of higher lipophilic nature than DMN evidence some interaction with hydrophobic regions of P450 as revealed through type I spectral changes.

Methods. Gold label dimethylnitrosamine (DMN) was purchased from Aldrich Chemical Co., diethylnitrosamine (DEN) from T. Schuhardt (Federal Republic of Germany) and dipropylnitrosamine (DPN) and dibutylnitrosamine for E. Kodak. Male Sprague-Dawley rats fasted overnight were used (250–280 g). Liver microsomes were isolated as previously described[13]. The spectral changes were performed in an Amino-Chance Dual Wavelenght-Split beam spectrophotometer, as described by Schenkman et al.[14].

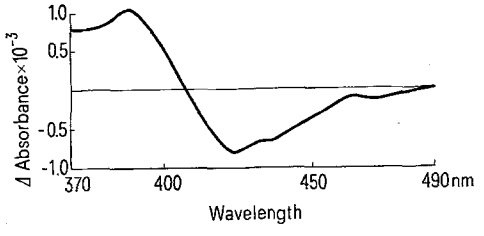

Fig. 1. 6 ml of microsomal suspension diluted to 2.5 mg of protein/ml were divided between 2 cuvettes. DBN (0.2 mM final concentration) was added to the sample cuvette and the spectrum recorded using the Aminco-Chance spectrophotometer in the split beam mode. DPN behaved similarly. There is a peak at 390 nm and a trough at 423 nm.

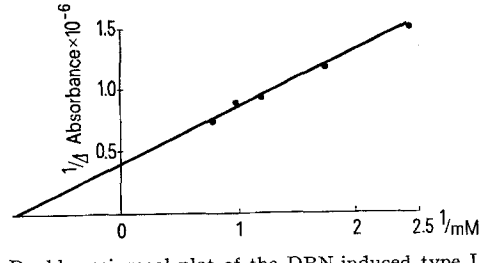

Fig. 2. Double reciprocal plot of the DBN-induced type I spectral changes. Microsomes were suspended to 2.5 mg/ml in 0.025 M Tris 0.15 M KCl buffer pH 7.5 and distributed between 2 cuvettes. DBN (from 0.42 mM to 1.26 mM) was added to sample cuvette and the spectrum was recorded. After triplicate determinations we found that K_s was 1.17 ± 0.23 mM and V_s max was 1.0 ± 0.2×10^6 absorbance units/mg protein. In the case of DPN K_s was 100 ± 15 mM and V_s max 3.2 ± 0.45×10^6 absorbance units/mg protein.

Results. DMN and DEN did not give any spectral change upon interaction on liver microsomal suspensions, inspite of the fact that we used high protein concentrations in the microsomal suspensions or (in the case of DMN) also microsomes derived from phenobarbital preinduced rats. In contrast, DPN and DBN gave a typical type I spectral binding having a peak at 390 nm and a trough at 423 nm (figure 1). The double reciprocal plot of the spectral changes against nitrosamine concentrations is shown in figure 2. In triplicate determinations we found that the spectral binding constant is 100 ± 15 mM for DPN and 1.17 ± 0.23 mM for DBN. The maximal spectral change is 3.2 ± 0.45×10^6 and 1.0 ± 0.2×10^6 absorbance units/mg protein for DPN and DBN respectively.

Discussion. In agreement with previous reports from our and other laboratories, DMN does not give any type I or type II spectral change when added to liver microsomal suspensions derived from either control or Pb-induced rats[12,13]; DEN behaved similarly. Substrate interaction with P450 is assumed to be an essential preliminary step for the oxidation of drugs by the hepatic P450 dependent hepatic microsomal mixed function oxidase[2] and xenobiotics interacting with it alter the spectral properties of microsomal suspensions to give type I or type II spectral changes[14]. In light of these facts, our previous results[13] as well as those reported by others [11,12] would tend to suggest that either P450 is not involved in the metabolism of these nitrosamines, or that it is involved in a manner which is different from that observed for many other substrates of this enzyme system. Both view-points are possible in considering the facts that these enzymes appear to act upon lipid soluble foreign materials converting them into a more polar form which can be secreted through the kidney[15] and that both DMN and DEN are the only water-soluble dialkylnitrosamines[3]. The situation is different for both DPN and DBN. Both gave a type I spectral change. This strongly suggests that P450 participates in their metabolism. DBN would be a better substrate for the mixed function oxygenase system than DPN, as suggest by the values of the spectral binding constans (higher Vs max and smaller K_s for DBN than for DPN) and considering the fact that the kinetics of binding of type I substrates to P450 is frequently

3 P. N. Magee and J. M. Barnes, Adv. Cancer Res. *10*, 163 (1967).
4 P. N. Magee and M. Vandekar, Biochem. J. *70*, 600 (1958).
5 P. Czygan, H. Grein, A. J. Garro, F. Huttever, F. Schaffner, H. Popper, O. Rosenthal and D. Y. Cooper, Cancer Res. *33*, 2983 (1973).
6 A. E. M. McLean and P. A. Day, Biochem. Pharmac. *23*, 1173 (1974).
7 R. Kato, H. Shofi and A. Takanaka, Gann *58*, 467 (1967).
8 N. Venkatesan, J. C. Arcos and M. F. Argus, Life Sci. *7*, 1111 (1968).
9 N. Venkatesan, J. C. Arcos and M. F. Argus, Cancer Res. *30*, 2556 (1970).
10 N. Venkatesan, J. C. Arcos and M. F. Argus, Cancer Res. *30*, 2563 (1970).
11 B. G. Lake, C. E. Heading, J. C. Phillips, S. D. Gangolli and A. G. Lloyd, Biochem. Soc. Trans. *2*, 610 (1974).
12 B. G. Lake, J. C. Phillips, C. E. Heading and S. D. Gangolli, Toxicology *5*, 297 (1976).
13 N. D'Acosta, J. A. Castro, C. R. de Castro, M. I. Díaz Gomez, E. C. de Ferreyra and O. M. de Fenos, Toxic. appl. Pharmac. *32*, 474 (1975).
14 J. B. Schenkman, Biochemistry *9*, 208 (1970).
15 B. B. Brodie, in: Absorption and Distribution of Drugs, p. 199. Ed. T. B. Binus. Williams and Wilkins Co., Baltimore 1964.

similar to the kinetics of their metabolism[16–18]. This would also be expected behaviour if one considers that a relationship has been demonstrated for substrates of these enzymes between their lipid solubility and their ability to be metabolized[19]. The lack of ability to give type II spectral changes by N-nitrosamines is not surprizing inspite of the fact that they are amine derivatives and that it was suggested that type II interactions represent the formation of a ferrihemochrome from the interaction of a basic amine and a ferrihemoprotein[16]. In effect, recent findings support the contention that steric and basic features of nitrogen of amines are of primary importance in type II binding[16]. The strong electron-attracting properties of the nitroso group, as well as its linking to the nitrogen of the amine portion, intensively modify the alkaline nature of the molecule[20] to the extent that it might reduce its ability for interaction with type II binding sites and also could sterically hinder the access to the nitrogen of the ferrihemoprotein as it was previously demonstrated for other molecules[21].

16 J. B. Schenkman, H. Remmer and R. W. Estabrook, Mol. Pharmac. *3*, 113 (1967).
17 J. C. Tophan, Biochem. Pharmac. *19*, 1695 (1970).
18 R. Kato, A. Takanaka and K. Oreada, Biochem. Pharmac. *20*, 447 (1971).
19 L. E. Gaudette and B. B. Brodie, Biochem. Pharmac. *2*, 89 (1959).
20 H. Druckrey, Gann Monograph No. *17*, 107 (1975).
21 R. B. Mailman, A. P. Kulkarni, R. C. Baker and E. Hodgson, Drug Met. Disp. *2*, 301 (1974).

Changes in brain dopamine levels and aggressive behavior with aging in 2 mouse strains

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Summary. The genetic programming of brain monoamine changes with aging show remarkable differences in 2 mouse strains. A marked increase in dopamine occurred in 32-week-old grouped ICR mice and the males showed intense irritability and aggressive behavior. Brain amines changed only slightly in old C57BL6J mice and behavior remained benign. Old females showed similar amine changes but aggressive behavior did not occur in either strain.

We have previously reported[1] the brain monoamine levels of dopamine (DA), norepinephrine (NE) and serotonin (5HT) for 3 mouse strains: C57BL6J, BALBJ and ICR. There are remarkable biochemical and behavioral differences among these strains. Of particular interest is the high brain level of DA in BALB mice, a strain noted for fighting and aggressive behavior among young adult males in contrast to the benign behavior of young adult C57BL6J and ICR males. The turnover rate of dopamine in BALB males also is higher than in the other strains[2]. In the present study the changes of brain monoamines with age and concomitant behavioral changes were investigated in 2 strains of mice.

Methods. The brain amine levels of DA, NE and 5HT in young adults (4 weeks old) and old adults (28–32 weeks old) of C57bL6J and ICR male and female mice were determined as described previously[3]. The mice were kept in groups of 20 or more in large cages. The strains and sexes were separated. General behavior was observed both in the large home cages and further observations were made in small plastic cages holding 4–6 mice. Reactions to handling were also assessed.

Results. The pertinent observations and brain monoamine levels are summarized in the table. In the young adult mice the brain monoamine levels of both males and females of a given strain are similar. It will be noted that the young adult C57BL6J mice have significantly higher brain levels of all 3 monoamines compared to the ICR strain. The high level of NE in this strain is especially

1 G. M. Everett, in: Frontiers of Catecholamine Research, p. 657. Pergamon Press (1973).
2 B. K. Bernard, E. R. Finelstein and G. M. Everett, Physiol. Behavior *15*, 731 (1975).
3 G. M. Everett and J. W. Borcherding, Science *168*, 849 (1970).

Biogenic amines and behavior in young adults and old mice

Strain	Age in weeks	No. of determinations	DA**	Percent change*	NE	Percent change	5HT	Percent change	Aggressive behavior
Males									
ICR	4	10	0.91 ± 0.03		0.53 ± 0.01		0.59 ± 0.01		0 to +
	32	5	1.35 ± 0.05	+48	0.61 ± 0.05	+15	0.76 ± 0.01	+27	+++
C57BL6/J	4	11	1.36 ± 0.02		0.65 ± 0.01		0.69 ± 0.01		0
	28	5	1.45 ± 0.01	+ 7	0.70 ± 0.04	+ 7	0.72 ± 0.04	+ 4	0
Females									
ICR	4	10	0.98 ± 0.02		0.45 ± 0.01		0.61 ± 0.02		0
	32	5	1.60 ± 0.09	+	0.44 ± 0.01	− 4	0.70 ± 0.02	+15	0
C57BL6/J	4	10	1.41 ± 0.01		0.64 ± 0.01		0.80 ± 0.01		0
	32	5	1.76 ± 0.14	+25	0.56 ± 0.01	−13	0.75 ± 0.01	− 6	0

* Percent increase compared to young adults. ** Mean μg/g brain tissue ± SEM.