Biochemical Pharmacology, Vol. 22, pp. 2247–2252. Pergamon Press, 1973. Printed in Great Britain.

ON THE CONVULSIVE ACTION OF CASTRIX

YASUKO MURAKAMI and KATASHI MAKINO

Department of Biochemistry, Jikei University, School of Medicine, Tokyo, Japan

(*Received* 13 *November* 1972; *accepted* 2 *March* 1973)

Abstract—Castrix, a vitamin B_6 antagonist, shifted the visible absorption spectrum of methylene blue towards the longer wavelength with increase of absorbancy. This spectral alteration was similar to that observed by dilution of the dye. Several castrix derivatives also had some ability to alter the spectrum of methylene blue, and an approximate correlation was found between their ability to alter the spectrum and their convulsant activity. This correlation was observed also with other metachromatic dyes; toluidine blue and thionine. A similar shift of spectrum of methylene blue was observed also after the addition of substances known to have good electron donor or good electron acceptor properties, pyridoxal Schiff bases or some vitamin B_6 antagonists. The electron donor property of castrix was shown by studying its complex with iodine monochloride and it was suggested that the convulsive action of castrix may be due to the electron donor property.

CASTRIX (2-chloro-4-dimethylamino-6-methylpyrimidine) produces severe convulsions in mice which are protected by vitamin B_6.[1] The mechanism whereby castrix competes with vitamin B_6 is uncertain and cannot be explained by the suggested mechanism for other vitamin B_6 antagonists.[2] Castrix does not combine with pyridoxal phosphate, and does not inhibit glutamic decarboxylase, pyridoxine phosphate oxidase, or pyridoxamine phosphate oxidase. Castrix does not have the hydroxymethyl group and its inhibitory action on pyridoxal kinase is very weak. Furthermore, it is interesting that convulsions produced by castrix were protected by subconvulsive doses of several vitamin B_6 antagonists.[2]

The experiments described in this paper suggested that the convulsive action of castrix may be due to its electron donating property.

MATERIALS AND METHODS

Pyridoxal derivatives were gifts from Dr. T. Kuroda of Wakamoto Pharmaceutical Co. Ltd. Castrix was kindly supplied by Mr. Umeda of Nihon Tokushu Noyaku Co. Ltd.

Absorption spectra were determined with a Hitachi 124-002 spectrophotometer at room temperature.

The synthesis of the complex of castrix and iodine monochloride: castrix, 34 mg and iodine monochloride, 34 mg were dissolved separately in each 1 ml of carbon tetrachloride. The iodine monochloride solution was then added dropwise to the castrix solution. The yellow crystalline precipitates which formed immediately were washed thoroughly with carbon tetrachloride and dried. The complex was analysed

by dissolving it in hot glacial acetic acid and titrating the resulting solution iodometrically. Elementary analysis were kindly performed in the R. and D. Centre of the Toshiba Electric Co.

The convulsive action of castrix derivatives was determined as described in previous paper[2] and CD_{60} was obtained from a dose response curve.

RESULTS AND DISCUSSION

The effect of castrix derivatives on spectra of metachromatic dyes. When castrix was added to a methylene blue solution at room temperature, a slight change of the tone was noticed. The effect of castrix was clearly shown by freezing the solution of methylene blue which alone changed its colour from blue to purple-pink but in the presence of castrix remained blue. Methylene blue is a well-known metachromatic dye. The interaction of castrix with metachromatic dyes, methylene blue, toluidine blue or thionine, were examined spectrophotometrically. The spectra of these dyes had two peaks or one peak and one shoulder under the experimental conditions. As shown in Fig. 1 these peaks or shoulders were shifted towards the longer wavelength by an addition of castrix, and the peak at the longer wavelength became higher. Several castrix derivatives also altered similarly the spectra of the dyes. The ability of castrix derivatives to shift the spectra of the dyes could be determined by the extent of shifts of absorption peaks towards the longer wavelength or by the ratio of the height of the

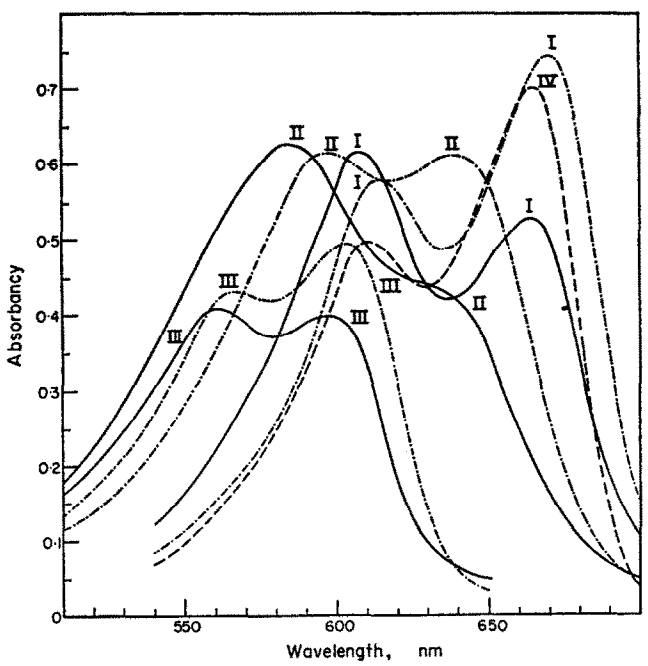

FIG. 1. Effect of castrix (5×10^{-3} M) on the spectra of metachromatic dyes was examined in 0·1 M carbonate buffer, pH 9·2. After standing at room temperature for 10 min spectra of the solutions were read in a cuvette with a 1·0 mm lt path length. Final concn of dye: methylene blue (I), $1{\cdot}2 \times 10^{-4}$ M; toluidine blue (II), $1{\cdot}5 \times 10^{-4}$ M; thionine (III), 3×10^{-4} M. (———). In the absence of castrix; (—·—) in the presence of castrix. Curve IV was the spectrum of methylene blue, $2{\cdot}4 \times 10^{-5}$ M which was recorded on a 5-fold-expanded recorder scale.

two absorption peaks. The ratio of height of the two absorption peaks of dyes in the presence of castrix derivatives was plotted against the CD_{60} of castrix derivatives (Fig. 2). Castrix, 2-chloro-4-dimethylamino-5-methylpyrimidine, 2-chloro-4,6-dimethylpyrimidine and 2-chloro-4-amino-6-methylpyrimidine fitted into a straight line. The last derivative did not cause convulsions in mice and scarcely shifted the spectra of the dyes. The other three compounds produced convulsions in mice as reported previously.[2] 4-Dimethylamino-6-methylpyrimidine which had a different convulsive action from castrix did not fit into the straight line. 2-Chloro-4-dimethylamino-pyrimidine had a strong depressive action and the proportion of mice with convulsions decreased in higher dosage.[2] This derivative also did not fit into the straight line, but the point falling under it may indicate that its convulsive action was weakened by the depressive action.

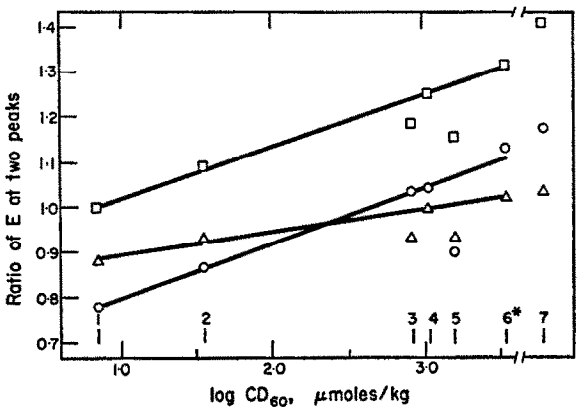

FIG. 2. The effect of castrix derivatives on spectra of metachromatic dyes. Final concentration of each castrix derivative was 5×10^{-3} M. Other conditions as in Fig. 1. (□) Toluidine blue, (△) thionine, (○) methylene blue, (1) castrix, (2) 2-chloro-4-dimethylamino-5-methylpyrimidine, (3) 4-dimethylamino-6-methylpyrimidine, (4) 2-chloro-4,6-dimethylpyrimidine, (5) 2-chloro-4-dimethylaminopyrimidine, (6) 2-chloro-4-amino-6-methylpyrimidine, (7) none.

* This derivative did not cause convulsions in mice at a dosage of 3800 μmoles (500 mg/kg).

The effect of several substances known to have good electron donor or acceptor properties or of vitamin B_6-related substances on the spectrum of methylene blue. The mechanism whereby castrix altered the spectra of metachromatic dyes is obscure. Since the spectral shift by castrix was somewhat similar to that observed by dilution of the dye (Fig. 1), it may be related to the mechanism of metachromasia. Szent-Györgyi has pointed out that in metachromatic dyes the k values for both the highest filled and lowest empty orbitals are very small, which makes the dyes both good electron donors and good acceptors and metachromasia is therefore connected with charge transfer.[3] Therefore, the fact that castrix altered the spectra of metachromatic dyes may indicate that it has good electron acceptor or donor property. Several substances known to have good electron acceptor or donor properties were found to alter the spectrum of methylene blue similarly to castrix (Table 1). Vitamin B_6 and its antagonists were also tested for their ability to shift the spectrum of methylene blue. As shown in Table 1 pyridoxine, pyridoxamine, pyridoxal, pyridoxal phosphate and

TABLE 1. THE EFFECT OF SEVERAL SUBSTANCES KNOWN TO HAVE GOOD ELECTRON DONOR OR ACCEPTOR PROPERTIES OR OF VITAMIN B_6 RELATED SUBSTANCES ON THE SPECTRUM OF METHYLENE BLUE

Substances	Ratio of height of the two absorption peaks of methylene blue (E of absorption peak at the shorter wavelength/E of absorption peak at the longer wavelength).*	
	5×10^{-3} M	5×10^{-2} M
FMN	0·420†	
Riboflavin	0·450†	
Isonicotinyl hydrazone of PL‡	0·485†	
Isonicotinyl hydrazone of PLP‡	0·500†	
Caffeine	0·700	
NADH	0·718	
Thiosemicarbazone of PLP	0·767	
Castrix	0·765	
Adenosine	0·792	
Semicarbazone of PLP	0·802	
NAD	0·805	
5-Deoxy PL + alanine§	0·903	
PL + alanine§	0·920	
Hydrazone of PL	0·946	
PLP + alanine§	1·00	
Toxopyrimidine	1·05	0·675
Isonicotinyl hydrazide	1·09	0·843
PLP	1·12	
Pyridoxine + alanine§	1·15	
Alanine§	1·15	
Pyridoxine	1·15	0·907
PL	1·16	
5-Deoxy PL	1·15	
Aminooxyacetic acid	1·17	
4-Deoxypyridoxine		0·785
Pyridoxamine		1·04
Gamma-aminobutyric acid		1·20
Thiosemicarbazide		1·22
Penicillamine		1·23
Semicarbazide		1·23
None	1·20 ± 0·03	

* Each reaction mixture contained methylene blue, $1{\cdot}2 \times 10^{-4}$ M; carbonate buffer, 0·1 M and an appropriate substance. Final pH was 9·2. After standing at room temperature for 10 min the spectrum of the solution was read in a cuvette with 1·0 mm light path length against an appropriate blank.

† The absorption peak of methylene blue at the shorter wavelength disappeared and became the shoulder.

‡ PL: Pyridoxal, PLP: Pyridoxal phosphate.

§ Concentration of alanine was 0·1 M.

5-deoxypyridoxal had only a weak effect. The effect of pyridoxal, pyridoxal phosphate and 5-deoxypyridoxal was increased by forming Schiff bases with amino acids. Several vitamin B_6 antagonists were also found to shift the spectrum of methylene blue alone or by forming pyridoxal Schiff base. For these vitamin B_6 antagonists the order of the convulsant activity was castrix > thiosemecarbazide > toxopyrimidine > isonicotinylhydrazide > 4-deoxypyridoxine > semicarbazide (estimated with mouse convulsion provoking activity $CD_{99\cdot9}$ i.p., moles/kg of body wt[2,6–10]). An approximate

correlation between the convulsant activity and the ability to shift the spectrum of methylene blue seems to exist.

Complex of castrix with iodine monochloride. If castrix is a good electron donor, it will form a complex with halogens or polyhalides.[4] The yellow complex was actually prepared by mixing equimolar solutions of iodine monochloride and castrix. The analysis of the complex agreed reasonably with theoretical values for 1:1 complex (Table 2). The interaction of castrix and iodine monochloride was examined spectrophotometrically in carbon tetrachloride. When castrix was added to iodine monochloride, an absorption maximum at 460 nm of iodine monochloride was shifted

TABLE 2. COMPLEX OF CASTRIX WITH IODINE MONOCHLORIDE

	Analyses		
	Carbon (%)	Hydrogen (%)	Iodometric equiv. (%)
Calcd. for castrix·ICl	25·09	3·00	167·0
Found	25·04	2·88	168·8

towards the shorter wavelength. With increasing castrix concentration (final castrix concn: 5×10^{-4} M to $1{\cdot}0 \times 10^{-2}$ M in iodine monochloride, $2{\cdot}5 \times 10^{-3}$ M) a sharp isobestic point was observed at 400 nm and 1:1 complex formation was indicated by a mole ratio method.[5] The average dissociation constant of the castrix–iodine complex in carbon tetrachloride at 25° was $8{\cdot}7 \times 10^{-5}$ (solution $2{\cdot}5 \times 10^{-3}$ M for both castrix and iodine monochloride). This result indicated that castrix had good electron donor property. It has been noted by Popov *et al.* that other convulsants, such as strychnine, metathamide, hydrazides and pentamethylenetetrazole have electron donor properties.[4] Pyridoxal in its Schiff base with amino acid altered the spectrum of methylene blue as described above, suggesting that pyridoxal Schiff base should be a good electron donor or acceptor. Molecular orbital calculations indicated Schiff bases of pyridoxal phosphate are good electron acceptors or good electron donors.[11] It seems to be likely that castrix, a good electron donor, interacts with pyridoxal phosphate Schiff base which is a good electron acceptor or donor, causing convulsions in mice.

Acknowledgements—The authors are grateful to Dr. M. Matsuda for his helpful discussion. This work was supported by a research grant from the Japanese Ministry of Education.

REFERENCES

1. O. KARLOG and E. KNUDSEN, *Nature* **200,** 790 (1963).
2. Y. MURAKAMI, K. MURAKAMI and K. MAKINO, *Biochem. Pharmac.* **21,** 277 (1972).
3. A. SZENT-GYÖRGYI, in *Introduction to a Submolecular Biology*, p. 101. Adaemic Press, New York (1960).
4. A. I. POPOV, C. C. BISI and M. CRAFT, *J. Am. chem. Soc.* **80,** 6513 (1958).
5. A. I. POPOV and R. H. RYGG, *J. Am. chem. Soc.* **79,** 4622 (1957).
6. K. MAKINO and J. YAMASHITA, *Vitamin* (in Japanese) **41,** 440 (1970).
7. K. MAKINO, T. KINOSHITA, T. SASAKI and T. SHIOI, *Nature* **173,** 34 (1954).
8. J. NOZAKI, *Vitamin* (in Japanese) **16,** 516 (1959).

9. J. NOZAKI, *Vitamin* (in Japanese) **14,** 310 (1958).
10. C. C. PFEIFFER, in *Inhibition in the Nervous System and Gamma-aminobutyric Acid* (Ed. E. ROBERTS), p. 324. Pergamon Press, Oxford (1960).
11. A. PERAULT, B. PULLMAN and C. VALDEMORO, *Biochem. biophys. Acta* **46,** 555 (1961).