[CONTRIBUTION FROM THE CHEMICAL DIVISION, DEPARTMENT OF MEDICINE, UNIVERSITY OF CHICAGO]

Studies on Pheohemin a and Pheohemin b[1]

BY GORO KIKUCHI[2] AND E. S. GUZMAN BARRON[3]

RECEIVED JANUARY 27, 1959

Studies were made on the properties of pheohemin a and pheohemin b and their derivatives, in order to obtain some information on the structural relationship between the pheohemins and the hemin moieties of the cytochromes of the "a" group. The absorption maxima and the extinction coefficients of pheohemin a, pheohemin b and their various nitrogen base complexes were determined. The oxidation–reduction potentials of pheohemin a, pheohemin b and their nitrogen base complexes were found to be more positive than those of protohemin, spirographishemin and their corresponding derivatives. E_0' of pheohemin b was more positive than that of pheohemin a. Pheohemin b was found to be a more active catalyst than pheohemin a for the oxidation of methyl linolenate and of ascorbic acid by molecular oxygen. The catalytic activities of various nitrogen base complexes of pheohemin a were also studied. The role of the formyl side chain of the iron porphyrins was discussed.

Introduction

The oxidation–reduction potentials of protohemin and several of its nitrogen base complexes were studied extensively by Barron.[4] He also reported[5] that the oxidation–reduction potentials of spirographishemin derivatives were more positive than those of the corresponding protohemin derivatives, and the absorption maxima of spirographishemin derivatives were located at longer wave lengths than the corresponding bands of protohemin derivatives. These facts suggested a possible correlation between the displacement of the absorption bands of the iron porphyrin toward longer wave length and the shift of the oxidation-reduction potential toward that of oxygen. In this connection, it is of interest to note that the cytochromes of "a" group absorb light at longer wave lengths and have higher oxidation-reduction potentials than the "b" and "c" cytochromes.[6,7]

Pheohemin a[8] and pheohemin b,[9] which are iron complexes of pheophorbide a and pheophorbide b, respectively, have absorption bands at longer wave lengths[8,10] than spirographishemin.[5,10] It has been reported that pheohemin b and hemin a[11–15] have a formyl side chain, whereas pheohemin a and hemin a_2[16] have no formyl group. In this paper, studies were made on the properties of pheohemin a and pheohemin b and their derivatives, in order to obtain some information on the structural relationship between the pheohemins and the hemin moieties of the cytochromes of the "a" group. This question has been discussed by many workers.[10–15,17]

(1) This work was aided by a contract, No. AT(11-1) 233, between the Atomic Energy Commission and the University of Chicago, and supported by the Douglas Smith Foundation for Medical Research of the University of Chicago.

(2) Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York. Address after May, 1959: Department of Biochemistry, Nippon Medical School, Tokyo, Japan.

(3) Deceased 1957.

(4) E. S. G. Barron, *J. Biol. Chem.*, **121**, 285 (1937).

(5) E. S. G. Barron, *ibid.*, **133**, 51 (1940).

(6) E. G. Ball, *Biochem. Z.*, **295**, 262 (1938).

(7) W. W. Wainio, *J. Biol. Chem.*, **216**, 593 (1955).

(8) K. Kunz, W. Morneweg and H. Müller, *Z. physiol. Chem.*, **199**, 93 (1931).

(9) O. Warburg, *Ber.* **64**, 682 (1931).

(10) O. Warburg and E. Negelein, *Biochem. Z.*, **244**, 9 (1932).

(11) O. Warburg and H. S. Gewitz, *Z. phyiol. Chem.*, **288**, 1 (1951).

(12) M. Kiese and H. Dannenberg, *Biochem. Z.*, **322**, 395 (1952).

(13) J. E. Falk and C. Rimington, *Biochem. J.*, **51**, 36 (1952).

(14) R. Lemberg, *Nature*, **172**, 619 (1953).

(15) M. Kiese and H. Kurz, *Biochem. Z.*, **325**, 299 (1954).

(16) J. Barrett, *Biochem. J.*, **64**, 626 (1956).

(17) E. Negelein and W. Gerischen, *Biochem. Z.*, **268**, 1 (1934).

Experimental

A mixture of pheophytin a and pheophytin b was supplied by the American Chlorophyll Division, Strong, Cobb and Co. Pheophytins were hydrolyzed and the resultant pheophorbides were fractionated to pheophorbide a and pheophorbide b according to the method of Willstätter.[18] Pheohemin a and pheohemin b were prepared by a slight modification of the methods of Warburg[9] and Kunz[8] and crystallized as the propionic acid salts. The iron content of the crystalline pheohemins was determined by the method of Scott.[19] The purity of pheohemin a was found to be 100% and that of pheohemin b, 93%, assuming the molecular weight of pheohemin a propionate and pheohemin b propionate as 740 and 754,[9] respectively.

The oxidation–reduction potentials were measured at 30° with a type K Leeds and Northrup potentiometer, using the Michaelis standard acetate (*p*H 4.62) as the reference electrode. Fifty-ml. aliquots of 10^{-4} *M* ferric pheohemin samples were titrated with sodium dithionite. The total volume of $Na_2S_2O_4$ added did not exceed 1.5 ml.

Oxygen uptake was measured by the conventional Warburg's manometric technique. Absorption spectra were measured with the Beckman DU or DK-2 spectrophotometer.

Results

I. Oxidation–Reduction Potentials of Pheohemin a, Pheohemin b, and their Base Complexes.—As a preliminary experiment, the complex formation for the pheohemins with nitrogen bases was studied spectrophotometrically. The absorption maxima and the extinction coefficients of pheohemin a, pheohemin b and their completed base complexes are given in Table I.

The titration curves of pheohemin samples (Fig. 1) were in all cases steeper than the theoretical titration curve where $n = 1$ in the equation of

$$E_h = E_0' - \frac{RT}{nF} \ln \frac{(Fe^{++}\ \text{hemin})}{(Fe^{+++}\ \text{hemin})}$$

This is in contrast to the control experiments with protohemin and with ferricyanide which gave curves in good agreement with the theoretical curves where $n = 1$. The slope of the titration curve for pheohemin–pilocarpine complex was found to be particularly steeper than the other complexes. A similar discrepancy between the observed and the theoretical E_h values was also reported for the protohemin–pilocarpine complex.[4] Therefore, in this paper, E_0' values were calculated from E_h values at the inflection points on the titration curves.

1. Pheohemin a.—Pheohemin a was titrated with $Na_2S_2O_4$ at varying *p*H values and in the

(18) R. Willstätter and A. Stoll, "Investigations on Chlorophyll" (English translation), The Science Press Printing Co., Lancaster, Pa., 1928.

(19) E. M. Scott, *Arch. Biochem.*, **6**, 27 (1945).

TABLE I

PROPERTIES OF ABSORPTION SPECTRA OF PHEOHEMIN a AND PHEOHEMIN b

Solution in	Ferric mμ	Ferric εmM	Ferrous mμ	Ferrous εmM
Pheohemin a				
NaOH (0.1 *N*)	391	58.1	415	86
	585	8.2		
	690	9.6	640	20.6
Pyridine (1.0 *M*)	417	60	415	54.8
	638	15	585	16.2
			645	24.2
Cyanide (0.1 *M*)	423	60	430	56.3
	630	15	(Shoulder at approx. 595)	
			633	18.3
Pilocarpine (0.1 *M*)	414	62	420	45
	640	13.8	(Shoulder at approx. 595)	
			642	20.5
α-Picoline (1.0 *M*)	412	56.4	420	55.2
	625	13	590	16.4
			645	22.3
Pheohemin b				
NaOH (0.1 *N*)	402	40.3	425	55
	665	6.3	440	57
			575	10
			623	11.9
Pyridine (1.0 *M*)	425	44	435–440	50
	600	11.2	(Shoulder at approx. 550)	
			558–610	16.4
Cyanide (0.1 *M*)	435	52	438	43
	605	12	462	44
			603	14.8
			(Shoulder at approx. 623)	

presence of a variety of complexing agents; the results are shown graphically in Fig. 2 and in Table II. We were unable to determine an accurate E_0' value for the pyridine complex below *p*H 9.0. The complex formation of pheohemin a with pyridine was incomplete at this *p*H as shown by the marked increase in the E_h value toward the negative after about half of the pheohemin a was reduced with $Na_2S_2O_4$. $\Delta E_0'/\Delta p$H value for free pheohemin a, pheohemin a–pyridine complex, and pheohemin a–α-picoline complex was 0.06, while a value of 0.03 was obtained for the pheohemin a–pilocarpine complex. The E_0' of the cyanide complex was independent of *p*H. The oxidation–reduction potential of the free pheohemin a was more negative than the potential of any of its base complexes at the same *p*H. E_0' values of the base complexes of pheohemin a were more positive in the following order: cyanide < pilocarpine < α-picoline < pyridine complex. The general pattern of the oxidation–reduction potentials of pheohemin a under varying conditions as shown in Fig. 2 is very similar to that of protohemin.[4]

2. Pheohemin b.—Similar titration experiments were carried out with pheohemin b and results are shown in Table II and Fig. 2. The oxidation–reduction potentials of the pheohemin b and its base complexes are more positive, in general, than those of the corresponding pheohemin a.

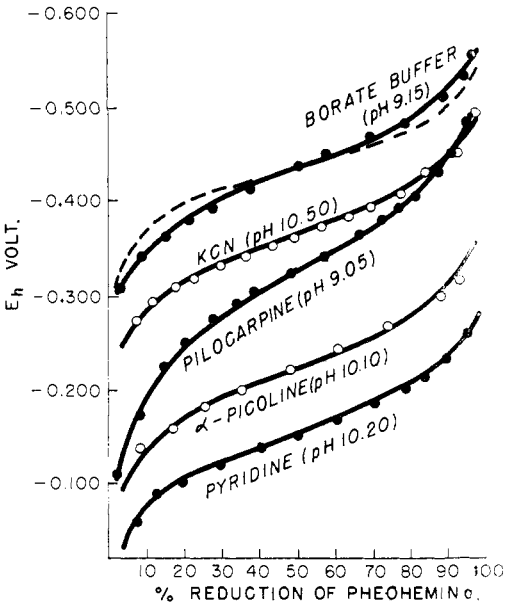


Fig. 1.—Titration curves of pheohemin a and its base complexes. The dotted line represents the theoretical curve when $n = 1$.

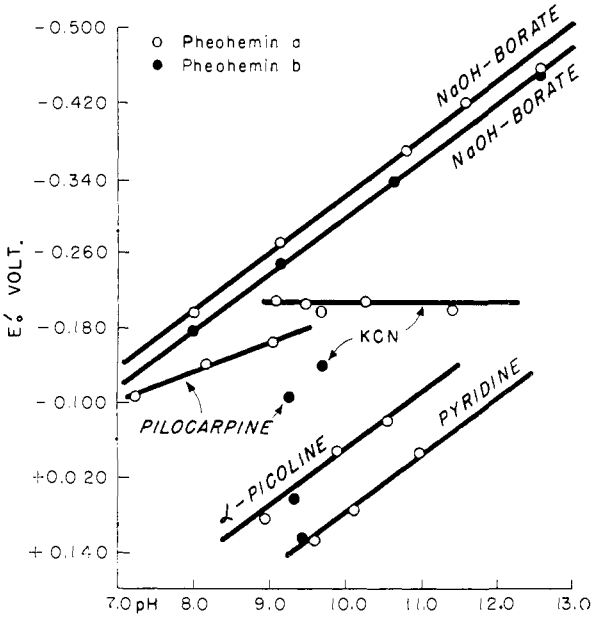


Fig. 2.—Variation with *p*H of the oxidation–reduction potentials of pheohemins and their base complexes.

II. Activities of Pheohemin a and Pheohemin b as Oxidative Catalysts.—The activities of pheohemin a and pheohemin b in catalyzing the oxidation of methyl linolenate and ascorbic acid were compared with that of protohemin. With methyl linolenate as the substrate (Table III), the catalytic activity of protohemin was almost identical to that of the pheohemins a and b within the first 5 minutes, after which the rate of reaction catalyzed by protohemin decreased gradually, indicating the rapid decomposition of the protohemin mole-

TABLE II

OXIDATION-REDUCTION POTENTIALS OF PHEOHEMIN a, PHEOHEMIN b AND ITS BASE COMPLEXES

Medium	*p*H	E_0'
Pheohemin a		
0.1 *N* NaOH	12.56	−0.374
.01 *N* NaOH, 0.1 *M* NaCl	11.60	− .338
.1 *M* borate buffer	10.82	− .290
.1 *M* borate buffer	9.15	− .190
.1 *M* borate buffer	8.00	− .116
.1 *M* KCN, 0.1 *M* borate buffer	10.28	− .127
.1 *M* KCN, 0.1 *M* borate buffer	9.70	− .116
.1 *M* KCN, 0.1 *M* borate buffer	9.50	− .124
.1 *M* KCN, 0.1 *M* borate buffer	9.10	− .124
1.0 *M* pyridine, 0.1 *M* borate buffer	11.00	+ .035
1.0 *M* pyridine, 0.05 *M* borate buffer	10.10	+ .094
1.0 *M* pyridine, 0.05 *M* borate buffer	9.60	+ .125
0.2 *M* pilocarpine, 0.1 *M* borate buffer	9.05	− .084
0.2 *M* pilocarpine, 0.1 *M* borate buffer	8.19	− .061
0.2 *M* pilocarpine, 0.1 *M* borate buffer	7.23	− .026
1.0 *M* α-picoline, 0.025 *M* borate buffer	10.55	− .003
1.0 *M* α-picoline, 0.025 *M* borate buffer	9.90	+ .033
1.0 *M* α-picoline, 0.025 *M* borate buffer	8.92	+ .103
Pheohemin b		
0.1 *N* NaOH	12.61	−0.363
.1 *M* borate buffer	10.64	− .257
.1 *M* borate buffer	9.16	− .166
.1 *M* borate buffer	8.00	− .097
.1 *M* KCN, 0.1 *M* borate buffer	9.67	− .058
1.0 *M* pyridine, 0.1 *M* borate buffer	9.37	+ .124
0.2 *M* pilocarpine, 0.1 *M* borate buffer	9.25	− .025
1.0 *M* α-picoline	9.42	+ .074

cule.[20,21] Apparently the pheohemins were more resistant to such autocatalytic oxidative decomposition. When ascorbic acid was used as the substrate (Table IV), the catalytic activity of protohemin was lower than that of the pheohemins. In all cases, the catalytic activity of pheohemin b was higher than that of pheohemin a.

TABLE III

COMPARISON OF THE CATALYTIC ACTIVITIES OF PROTOHEMIN, PHEOHEMIN a AND PHEOHEMIN b IN THE OXIDATION OF METHYL LINOLENATE

Each reaction mixture contained 0.03 μmole of hemin, 30 μmoles of methyl linolenate, 0.2 ml. of 20% Tween 20 and 2.2 ml. of 0.1 *M* borate buffer, *p*H 9.28, in a final volume of 3.0 ml. 0.15 ml. of 15% KOH was in the center cup. Temperature was 28°. Blank value obtained without addition of hemin was 20 μl. oxygen uptake after 40 minutes of reaction. The data in the table are the values minus the blank values.

Time, min.	Oxygen uptake in μl. Protohemin	Pheohemin a	Pheohemin b
2	49	39	42
5	78	74	81
10	113	121	137
20	149	182	205
30	173	222	245
40	193	252	272

In Table V, the catalytic activities of the free pheohemin a and of pheohemin a–base complexes are compared. The oxidation of methyl linolenate

(20) R. Lemberg and J. W. Legge, "Haematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949, p. 453.

(21) K. Kaziro and G. Kikuchi, *J. Biochem.* (*Japan*), **39**, 1 (1952).

TABLE IV

COMPARISON OF THE CATALYTIC ACTIVITIES OF PROTOHEMIN, PHEOHEMIN a AND PHEOHEMIN b IN THE OXIDATION OF ASCORBIC ACID

Each reaction mixture contained 0.06 μmole of hemin, 20 μmoles of ascorbic acid (neutralized) and 2.5 ml. of *M*/15 phosphate buffer, *p*H 7.4, in a final volume of 3.0 ml. 0.15 ml. of 15% KOH was in the center cup. Temperature was 28°. Blank value without hemin amounted to 12 μl. after 60 minutes of reaction. The data in the table are the values minus the blank values.

Time, min.	Oxygen uptake in μl. Protohemin	Pheohemin a	Pheohemin b
5	7	19	32
10	14	32	50
20	25	54	69
30	37	74	85
40	45	91	99
50	56	107	110
60	63	120	120

catalyzed by pheohemin a was inhibited markedly by the addition of cyanide. However, the activity of pheohemin a–cyanide complex in catalyzing the oxidation of ascorbic acid was found to be higher than that of free pheohemin a. The pilocarpine complex showed the highest activity for the oxidation of either substrate.

TABLE V

COMPARISON OF THE CATALYTIC ACTIVITIES OF PHEOHEMIN a AND ITS BASE COMPLEXES

With methyl linolenate as the substrate, reaction mixtures contained 0.03 μmole of pheohemin a, 30 μmoles of substrate, 0.2 ml. of 20% Tween 20 and 2.2 ml. of 0.1 *M* borate buffer or 0.1 *M* pilocarpine or 1.0 *M* pyridine or 0.1 *M* KCN. *p*H value was adjusted to 9.28 in all cases. Final volume was 3.0 ml. With ascorbic acid as substrate, reaction mixtures contained 0.06 μmole of pheohemin a, 20 μmoles of ascorbic acid (neutralized) and 2.5 ml. of *M*/15 phosphate buffer or 0.2 *M* pilocarpine or 1.0 *M* pyridine or 0.1 *M* KCN at *p*H 7.4. Final volume was 3.0 ml. 0.15 ml. of 15% KOH was in the center cup. Temperature was 28°.

	Oxygen uptake in μl. Methyl linolenate 10 min.	30 min.	Ascorbic acid 10 min.	30 min.
Borate buffer	110	210	28	69
KCN	6	15	40	96
Pyridine	96	173	47	106
Pilocarpine	122	223	57	133

Discussion

As shown in Table II and Fig. 2, the E_0' of the free pheohemin b is more positive than that of free pheohemin a. This relation is similar to Barron's finding that the E_0 of spirographishemin (−0.230 volt at *p*H 9.63) was more positive than that of protohemin (−0.316 volt at *p*H 9.63). The replacement of the vinyl or methyl side chain of the pyrrole ring of the porphyrin by a formyl group would probably cause a shift of E_0' toward more positive values.

In contrast to the relationship observed for the spectra of protohemin and spirographishemin, it was found that the absorption spectrum of pheohemin a is more rhodofied than that of pheohemin b. It would therefore appear that the formyl group is not necessarily the rhodofying group. There also seems to be no direct correlation between the increase in oxidation–reduction potential and the shift of the absorption spectrum toward longer

wave lengths. However, it is noteworthy that the oxidation–reduction potential of pheohemin a which has no formyl side chain was found to be more positive than that of spirographishemin.

Pheohemin b has been found to have a more positive oxidation–reduction potential than pheohemin a and also to be more effective as an oxidative catalyst. These results suggest that this difference in catalytic activity may be due to the presence of the formyl group which may also account for the high oxidative activity of cytochrome oxidase.

CHICAGO, ILLINOIS

[CONTRIBUTION FROM THE RESEARCH LABORATORIES OF THE U. S. VITAMIN AND PHARMACEUTICAL CORPORATION]

Aminocyclohexyl Esters

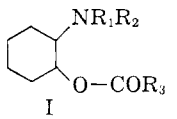
BY SEYMOUR L. SHAPIRO, HAROLD SOLOWAY, HARRIS J. SHAPIRO AND LOUIS FREEDMAN

RECEIVED JANUARY 27, 1959

A series of *trans*-2-(substituted amino)-cyclohexyl benzoates of the type I have been synthesized and examined for pharmacological activity. Significant responses have been noted with these compounds as local anesthetics, hypotensives and central nervous system depressants.

In the course of developments leading to the elucidation of the structure[1] and total synthesis[2] of reserpine, many workers have undertaken studies[3] in the search for similar or modified pharmacological activity in simple analogs.

In our laboratories a similar objective was approached from the viewpoint that in one simple sense, reserpine in terms of its nitrogen at position 4 of ring D and its oxygen function at position 18 of ring E, might be regarded as an aromatic ester of a dialkylamino substituted cyclohexanol. In this paper, the synthesis and examination for pharmacological activity of a number of *trans*-aminocyclohexyl esters[4] of the type I are reported and the scope of the compounds is described in Table I.



I

An inspection of the literature indicates previous exploration[5,6] of analogs of I although the reported work reflects a period when careful examination for depression of central nervous system response was not being conducted.

The reaction of the secondary amine R_1R_2NH with cyclohexene oxide afforded the *trans*-substituted aminocyclohexanol[6,7] which in turn was esterified using the acid chloride R_3COCl to afford the cyclohexyl esters I in moderate to good yield.

Pharmacology.—In view of the initial stimulus of this work as an investigation of simple congeners of reserpine, it was of interest to evaluate the compounds as hypotensive agents,[8a] inhibitors of central nervous system response[8b] and potentiators of Evipal sleeping time.[8b] In addition, the majority of compounds were screened for local anesthetic[8c] and anti-Parkinson effects.[8c] The data have been compiled in Table II.

Many of the compounds evaluated show local anesthetic activity of a far greater intensity than procaine. In the assessment of the structural effects two particular group variations showed the best activity. The use of $-NR_1R_2$ = N-methyldiethylaminoethylamino is associated generally with the highest absolute activities as well as over-all effectiveness (compounds 27, 23, 25), while the use of the pyrrolidino group is the next most effective (compounds 8, 5, 7, 6).

The hypotensive effect was confined largely to the N-methylpiperazine derivatives. Significant depression in central nervous system activity was noted with relatively few compounds and, interestingly, while the only structure which afforded this effect coupled with hypotension was a 3,4,5-trimethoxybenzoate (compound 36), this compound failed to prolong Evipal sleeping time.[9] The fail-

(1) (a) C. F. Huebner, H. B. MacPhillamy, E. Schlittler and A. F. St. Andre, *Experientia*, **11**, 303 (1955); (b) C. F. Huebner and E. Wenkert, THIS JOURNAL, **77**, 4180 (1955); (c) P. A. Diassi, F. L. Weisenborn, C. M. Dylion and O. Wintersteiner, *ibid.*, **77**, 4687 (1955).

(2) R. B. Woodward, F. E. Bader, H. Bickel, A. J. Frey and R. W. Kierstead, *Tetrahedron*, **2**, 1 (1958).

(3) (a) B. V. Rama Sastry and A. Lasslo, *J. Org. Chem.*, **23**, 1577 (1958); (b) G. Di Paco and C. S. Tauro, *Farmaco (Pavia), Ed. sci.*, **64**, 429 (1958); (c) R. A. Lucas, M. E. Kuehne, M. J. Ceglowski, R. L. Dziemian and H. B. MacPhillamy, American Chemical Society Meeting, Chicago, Ill., September, 1958, p. 6-O; (d) F. A. Turner and J. E. Gearien, *ibid.*, p. 6-O; (e) F. M. Miller and M. S. Weinberg, American Chemical Society Meeting, Atlantic City, N. J., September, 1956, p. 11-N.

(4) Important pharmacological activity has been noted by Friess and co-workers with *cis*-2-dimethylaminocyclohexanol and related structures; (a) P. A. French, W. C. Alford and S. L. Friess, *J. Org. Chem.*, **23**, 24 (1958); (b) S. L. Friess, THIS JOURNAL, **79**, 3269 (1957).

(5) T. S. Kusner, *Ukrain. Khem. Zhur.*, **7**, Wiss. Abt., 179 (1932) [*C. A.*, **27**, 3476 (1933)]; (b) H. Heckel and R. Adams, THIS JOURNAL, **49**, 1303 (1927); (c) F. E. King and D. Holmes, *J. Chem. Soc.*, 164 (1947); (d) R. Granger and J. Fraux, *Trav. soc. pharm. Montpellier*, **7**, 22 (1947–1948) [*C. A.*, **48**, 10804*h* (1954)].

(6) More recent work has been reported during the course of our investigations; (a) L. Dúbravková, I. Ježo, P. Šefčovič and Z. Votický, *Chem. Zvesti*, **11**, 150 (1957) [*C. A.*, **51**, 15455*d* (1957)]; (b) J. Kovář and K. Bláha, *Chem. Listy*, **52**, 283 (1958) [*C. A.*, **52**, 11005*f* (1958)].

(7) An extensive literature indicates that the reaction of amines with cyclohexene oxide yields the *trans*-2-substituted aminocyclohexanol; (a) F. N. Hayes, L. C. King and D. E. Peterson, THIS JOURNAL, **78**, 2527 (1956); (b) T. Taguchi and M. Nakayama, *ibid.*, **73**, 5679 (1951); (c) K. Bláha and J. Kovář, *Chem. Listy*, **52**, 77 (1958) [*C. A.*, **52**, 12864*f* (1958)]; (d) L. R. Hawkins and R. A. B. Bannard, *Can. J. Chem.*, **36**, 220 (1958); (e) F. G. Bordwell and R. J. Kern, THIS JOURNAL, **77**, 1141 (1955).

(8) The methods used for evaluation of the noted pharmacological responses have been detailed previously; (a) S. L. Shapiro, H. Soloway and L. Freedman, *ibid.*, **80**, 2743 (1958); (b) S. L. Shapiro, I. M. Rose, E. Roskin and L. Freedman, *ibid.*, **80**, 1648 (1958); (c) S. L. Shapiro, H. Soloway, E. Chodos and L. Freedman, *ibid.*, **81**, 203 (1959).

(9) For the mechanism of the sedative action of reserpine, see S. Garattini and L. Valzelli, *Science*, **128**, 1278 (1958).