cm^{-1}; mass spectrum: m/e 343 ($M^+ - HCl$) (1.8%), 345 (0.9), and 58 (100); ^1H-NMR: δ 7.67–6.07 (m, 5, C_6H_3, C_1H and C_2H), 4.9 (ragged t, 1, C_3H), 4.27–2.50 [m, 8, $CH_2N(CH_3)_2$], 2.50–1.77 (broad s, 1, C_4H), 1.28 [s, 8, $(CH_2)_4$], and 0.83 (t, 3, CH_3) ppm.

Anal.—Calc. for $C_{18}H_{28}Cl_3NO$: C, 56.76; H, 7.36; N, 3.68. Found: C, 56.82; H, 7.42; N, 3.67.

The 2,4-dichloro analog VI*b*, prepared in a similar manner in 38% yield, was recrystallized from dry acetone as colorless needles, mp 142.5°; IR: 3320 (br) (OH), 2680 (br) [$NH^+(CH_3)_2$], 1580 (w) (CH=CH), and 955 (m) (CH=CH); mass spectrum: m/e 343 ($M^+ - HCl$) (0.9%), 345 (0.6), and 58 (100); ^1H-NMR ($CDCl_3$): δ 7.56–6.70 (m, 4, C_6H_3 and C_1H), 6.50–5.96 (dd, 1, C_2H), 4.83 (ragged t, 1, C_3H), 4.08–2.53 [m, 8, $CH_2N(CH_3)_2$], 2.53–1.90 (broad s, 1, C_4H), 1.30 [s, 8, $(CH_2)_4$], and 0.87 (t, 3, CH_3) ppm.

Anal.—Calc. for $C_{18}H_{28}Cl_3NO$: C, 56.76; H, 7.36; N, 3.68. Found: C, 57.03; H, 7.14; N, 3.69.

1-(*p*-Chlorophenyl) - 4-dimethylaminomethyl - 1,3-nonadiene Hydrochloride—*threo*-(*E*)-1-(*p*-Chlorophenyl) - 4-dimethylaminomethyl-1-nonen-3-ol (10 g, 0.029 mole), mp 157° [lit. (13) mp 151–152°], prepared by the published procedure (13), was added to phosphoric acid (85% v/v). The solution was stirred at room temperature for 24 hr. The reaction mixture, on extraction with ether, gave a yellow oil (3.2 g). This oil was treated with ethanolic hydrochloric acid (10% w/v) to give a colorless solid.

Recrystallization of the precipitate from ether–ethanol gave 1-(*p*-chlorophenyl)-4-dimethylaminomethyl-1,3-nonadiene hydrochloride (1.4 g, 15%), mp 203°, as colorless plates; IR (free base, neat): 3020 (w) (CH=CH), 2760, 2800 (m) (NCH_3), 1625 (w) (CH=CH), and 945 (s) (CH=CH); mass spectrum: m/e: 291 ($M^+ - HCl$) (21%), 220 (35), and 58 (100); ^1H-NMR ($CDCl_3$): δ 7.30 (s, 4, C_6H_4), 7.27–6.17 (m, 3, C_1H, C_2H, C_3H), 4.10–2.30 [m, 8, $CH_2N(CH_3)_2$], 1.40 [s, 8, $(CH_2)_4$], and 0.83 (t, 3, CH_3) ppm.

Anal.—Calc. for $C_{18}H_{27}Cl_2N$: C, 74.10; H, 8.92; N, 4.80. Found: C, 74.73; H, 8.56; N, 4.80.

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Effect of Antineoplastic and Cytotoxic Mannich Bases Derived from Conjugated Styryl Ketones on Mitochondrial Respiration in Rat Liver Cells

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Received November 28, 1977, from the **College of Pharmacy and the ‡Department of Chemistry and Chemical Engineering, University of Saskatchewan, Saskatoon, Saskatchewan, S7N 0W0, Canada.* Accepted for publication March 7, 1978.

Abstract □ Five cytotoxic Mannich bases (5-dimethylamino-1-substituted phenyl-1-penten-3-ones), three having antineoplastic activity, were evaluated for respiratory-inhibiting properties in rat liver mitochondria in the presence of four substrates: succinate, glutamate, 3-hydroxybutyrate, and palmitylcarnitine. Four compounds (I*b*–I*e*) showed significant inhibiting properties which, on occasion, were reversed partially by coenzyme Q_{10}. Evaluation of the spectra of the mitochondrial cytochromes indicated that I*b*–I*e* blocked the electron transport chain prior to the sequence of cytochromes. Since inhibition occurred when different substrates were used, a common site of action for I*b*–I*e* is likely; competition of I*b*–I*e* with coenzyme Q_{10} probably occurs. Compounds I*a*–I*e* inhibited RNA polymerase from Swiss mouse kidney cells but were virtually bereft of activity *versus* RNA polymerase from L-1210 leukemia cells. Polarography of the Mannich bases and the related styryl ketones showed that antineoplastic activity was associated with higher half-wave potentials.

Keyphrases □ 1-Phenyl-1-penten-3-one derivatives—effect on enzyme activity in rat liver mitochondria, various substrates □ Enzyme activity—effect of 1-phenyl-1-penten-3-one derivatives in rat liver mitochondria □ Structure–activity relationships—1-phenyl-1-penten-3-one derivatives evaluated for effect on enzyme activity in rat liver mitochondria

Mannich bases have a wide range of biological activities including antimicrobial effects (1–3), analgesic activity (4), local anesthetic properties (5, 6), and psychotropic effects (7). Recently, a number of Mannich bases and related compounds including 5-dimethylamino-1-substituted phenyl-1-penten-3-ones I*a*–I*e* were shown to have cyto-

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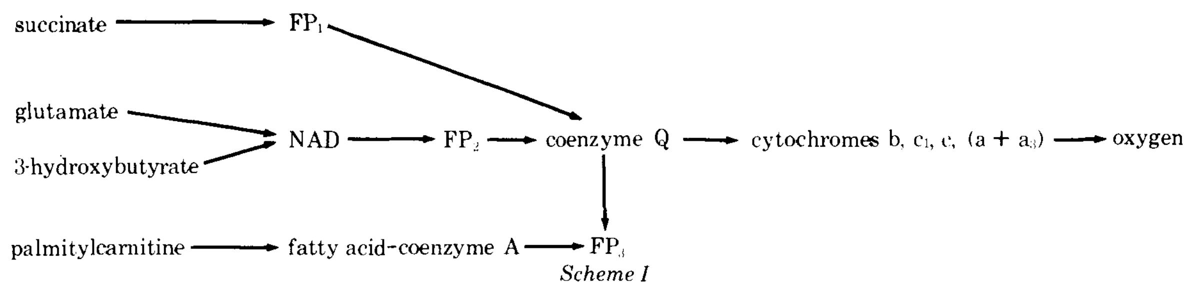


Scheme I

toxic activities in the KB *in vitro* screen (Table I) (8), which may be an indication of tumor-inhibiting properties (9). While I*a*–I*e* showed no activity in the L-1210 lymphoid leukemia screen in mice, three of the derivatives were assessed in the murine P-388 lymphocytic leukemia test system and showed antineoplastic activity at low dose levels (8).

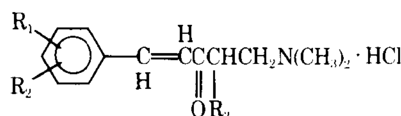
Since certain compounds containing a benzylidene group attached to electron-withdrawing functions, such as cyano and nitro moieties, interfere with mitochondrial functions (10) and some anticancer agents interfere with mitochondria (11), solutions of I*a*–I*e* were added to disks on complex ethanol and complex glucose media inoculated with a strain of *Saccharomyces cerevisiae* (12). In the absence of mitochondrial function, the yeast will grow on complex glucose medium because energy is provided by glucose fermentation. However, the complex ethanol medium contains a nonfermentable energy source and interference with mitochondrial function prevents yeast growth. Thus, a comparison of the differential in growth on these two media in the presence of inhibitors gives a quantitative estimation of the efficacy in blocking mitochondrial function.

Table I shows that the biological activities of I*a*–I*e* may be attributed, in part at least, to interference with mitochondrial function in *S. cerevisiae*. The questions arise whether rat liver mitochondria are also inhibited by I*a*–I*e* and what processes occurring in the mitochondria are affected by these Mannich bases.

RESULTS AND DISCUSSION

A number of important biochemical events occur in the mitochondria, including oxidative phosphorylation, the fatty acid and citric acid cycles, and ion transport activities (13). In this study, the effect of I*a*–I*e* on respiration was investigated. In normal cells, respiration is coupled to phosphorylation in a process referred to as oxidative phosphorylation. This process occurs when a number of substrates such as succinate, pyruvate, and malate are reduced by dehydrogenases and the pairs of electrons formed are transferred to coenzyme Q *via* either flavoproteins or an NAD–flavoprotein route. The electrons are then transferred by a series of cytochromes from coenzyme Q_{10} to molecular oxygen, which is then reduced to water (14).

The various substrates added to isolated rat liver mitochondria were



I*a*: $R_1 = R_2 = R_3 = H$
I*b*: $R_1 = R_2 = H$, $R_3 = (CH_2)_4CH_3$
I*c*: $R_1 = 2\text{-Cl}$, $R_2 = 4\text{-Cl}$, $R_3 = (CH_2)_4CH_3$
I*d*: $R_1 = 2\text{-Cl}$, $R_2 = 6\text{-Cl}$, $R_3 = (CH_2)_4CH_3$
I*e*: $R_1 = 3\text{-Cl}$, $R_2 = 4\text{-Cl}$, $R_3 = (CH_2)_4CH_3$

succinate, glutamate, 3-hydroxybutyrate, and palmitylcarnitine; electrons are transferred from these four substrates to coenzyme Q_{10} by three mechanisms involving separate flavoproteins designated FP_1, FP_2, and FP_3 (Scheme I). Table II shows the effects of various concentrations of the Mannich bases I*b*–I*e* on mitochondrial respiration in the presence of the four substrates. At the dose levels examined, all compounds except I*a* had inhibiting properties; I*a* showed the weakest effect on interference with mitochondrial function with *S. cerevisiae* and also had the lowest cytotoxicity in the KB screen.

The inhibition of respiration that occurred on addition of the drug showed that only uncoupling of oxidative phosphorylation did not occur since well-known uncouplers such as 2,4-dinitrophenol added to the mitochondria gave a rapid increase in oxygen consumption. The question arises as to the site of action of I*a*–I*e* in the electron transport chain. An examination of the normal difference spectra of the mitochondria under both aerobic and anaerobic conditions revealed the presence of cytochromes c and a_3 in both the oxidized and reduced forms (15), respectively, after the addition of succinate to the anaerobic sample. However, in the presence of I*b*–I*e*, cytochromes c and a_3 remained in the oxidized state in the presence of succinate under anaerobic conditions. This result indicates that the point of blockage of these compounds likely occurs before cytochrome c.

When antimycin A was added to the mitochondria, cytochromes c and a were blocked, but, in addition, a difference spectrum also was obtained for cytochrome b. This finding confirms that antimycin A blocks the transfer of electrons to cytochrome b (16). The failure of the Mannich bases I*b*–I*e* to act in a similar manner to antimycin A suggests that they have a different point of inhibition than antimycin A or that blockade of the electron transport chain by these compounds occurred between cytochromes b and c. The possibility also exists that cytochrome b is not on the direct respiratory chain but may represent a side or shunt pathway.

The fact that I*b*–I*e* inhibited each substrate suggested a common site of action, namely, coenzyme Q_{10}. Table II shows that the inhibitory effect of I*b*–I*e* on respiration may be reversed by the addition of coenzyme Q_{10}. This finding strengthens the hypothesis that the Mannich bases, which

Table I—Effect of the Mannich Bases against the KB Tumor, P-388 Lymphocytic Leukemia, and Yeast Mitochondria [a]

			Mean Area of Inhibition of *S. cerevisiae*[d] Growth in Presence of I[e]	
Compound	KB[b]	P-388 Lymphocytic Leukemia[c]	Complex Ethanol Medium, mm^2	Complex Glucose Medium, mm^2
I*a*	11	N.A.	32.4	0.0
I*b*	1.5	N.A.	307.7	15.2
I*c*	1.0	130 (18)	123.2	41.5
I*d*	2.8	115 (28)	41.5	0.0
I*e*	1.2	142 (6.25)	128.7	27.9

[a] With the exception of the KB result for I*a*, the data are taken from Refs. 8 and 12 (reproduced with permission of the copyright owner). [b] The figures, in micrograms per milliliter, in the KB cell culture screen indicate the dose inhibiting 50% growth of human epidermoid carcinoma of the nasopharynx in Eagle's medium. [c] Compounds I*c* and I*e* were dissolved in saline, and I*d* was dissolved in hydroxypropylcellulose. The solutions of I*d* and I*e* were injected intraperitoneally into BDF_1 mice; with I*c*, the CDF_1 strain was used. Nine daily injections were made with I*c* and I*e*, and one injection every 4 days was made with I*d*. The figures quoted are the maximum activities obtained and are ratios of the survival time of treated animals to control animals expressed as a percentage. The figures in parentheses are the optimum doses in milligrams per kilogram. N.A. = result not available. [d] Strain GR 13 constructed in the Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. [e] The concentration of solutions added to the medium was 5 mg/ml for I*a* and I*b* and 1 mg/ml for I*c*–I*e*.

Table II—Inhibition of Respiration in Rat Liver Mitochondria by I*b*–I*e* Using Different Substrates

		Succinate				Glutamate				3-Hydroxybutyrate				Palmitylcarnitine			
Compound	Concentration, μmoles	Inhibition, %	*SE*	Reversal with Ubiquinone, %	*SE*	Inhibition, %	*SE*	Reversal with Ubiquinone, %	*SE*	Inhibition, %	*SE*	Reversal with Ubiquinone, %	*SE*	Inhibition, %	*SE*	Reversal with Ubiquinone, %	*SE*
I*b*	5	84.34	2.24	7.69	2.06	85.56	2.99	6.43	2.07	94.69	0.94	−0.07	0.10	79.81	1.69	0.24	3.50
	2.5	49.55	3.84	−3.51	10.61	80.98	2.69	7.91	4.52	91.83	1.85	0.31	2.94	69.29	4.29	−1.83	7.40
	0.5	0	—	—	—	2.88	7.66	−73.32	31.08	65.27	9.02	−41.34	36.81	2.48	9.12	11.38	184.46
I*c*	5	97.58	0.56	0.67	0.25	86.36	2.86	11.61	4.56	95.50	1.83	3.04	1.14	88.59	3.57	5.10	6.35
	2.5	96.83	1.13	1.89	1.21	78.01	1.72	3.45	2.27	94.74	1.62	−0.43	3.68	84.32	3.70	14.14	5.77
	0.5	84.77	0.78	31.04	25.66	79.57	1.63	−0.37	2.54	95.00	1.21	1.88	0.95	66.53	7.69	−0.21	1.54
	0.1	10.88	16.17	18.17	9.90	80.12	2.61	0.17	5.30	89.66	1.64	−1.22	2.30	0	—	—	—
I*d*	5	95.13	0.95	2.32	1.74	83.67	2.97	8.40	2.88	93.34	1.76	2.89	0.83	78.92	2.49	−0.06	3.34
	2.5	95.52	0.79	−0.11	0.72	82.10	3.88	3.21	2.12	95.00	0.90	2.49	1.64	83.42	2.29	−4.70	3.50
	0.5	84.43	0.90	10.31	3.26	81.56	2.26	−0.01	5.73	93.82	1.18	4.67	0.56	41.38	4.66	0.66	7.71
	0.1	7.96	8.85	142.39	111.44	70.41	3.89	13.16	16.88	89.74	0.90	−2.69	1.72	0	—	—	—
I*e*	5	96.76	1.21	−0.28	0.57	86.14	2.53	−1.34	3.65	102.30	4.52	3.56	1.63	80.12	4.18	7.80	8.05
	2.5	93.61	1.47	−1.23	0.93	89.99	1.68	5.44	1.44	98.32	0.92	1.71	1.08	87.92	3.24	−1.06	1.74
	0.5	79.46	2.72	15.55	5.45	82.63	4.08	0.73	2.13	90.92	1.89	−1.47	3.09	79.80	2.92	−1.03	3.50
	0.1	80.13	2.61	23.80	22.87	74.59	4.11	5.10	6.60	92.56	1.75	−123.53	120.64	0	—	—	—

contain an α,β-unsaturated keto group like coenzyme Q_{10}, may compete with coenzyme Q_{10} for the electrons produced from the various substrates.

With succinate as the substrate, increasing concentrations of coenzyme Q_{10} increased the degree of reversal of the inhibition produced by I*b*–I*e* (Table III). Coenzyme Q_{10} alone did not stimulate respiration when added to uninhibited mitochondrial preparations. The degree of reversal of inhibition by coenzyme Q_{10} caused by I*b*–I*e* varied between substrates, being highest with succinate. This variation may possibly be caused by I*b*–I*e* acting at a second site, prior to coenzyme Q_{10}, with glutamate, 3-hydroxybutyrate, and palmitylcarnitine. This possibility is under investigation.

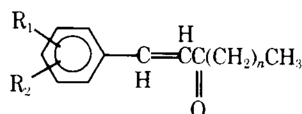
Correlations between antitumor and antimicrobial activities with redox potentials were established previously (17–19). It was considered that if the Mannich bases act as receptors for electrons in place of ubiquinone, then a relationship between the half-wave potentials of I and respiratory-inhibiting properties may be found. Table IV indicates that the half-wave potentials ($E_{1/2}$) of I*a*–I*e*, although varying over a narrow range, parallel the effect on respiration; *i.e.*, the dichlorinated compounds have higher $E_{1/2}$ values than the unsubstituted nonenone I*b*, which, in turn, has a half-wave potential higher than I*a*, which was bereft of effect on respiration under the conditions employed. The α,β-unsaturated ketones II*a*–II*e*, which were inactive in the L-1210 lymphoid leukemia screen (8), had lower half-wave potentials than the corresponding Mannich bases, suggesting that higher $E_{1/2}$ values favor antineoplastic activity.

Efforts are underway to examine whether the antineoplastic and cytotoxic Mannich bases act at other sites in the mammalian cell than the mitochondria. Since a number of antitumor agents interfere with RNA polymerase (20), I*a*–I*e* were tested for inhibitory activity toward solubilized nuclear DNA-dependent RNA polymerases from L-1210 leukemia and Swiss mouse kidney cells. Table IV indicates that I*b* was the most active compound in both tests.

EXPERIMENTAL

Syntheses—The compounds were prepared by the literature procedure (21).

Inhibition of Respiration in Rat Liver Mitochondria—Adult, male, Wistar rats, which had been starved for 18 hr, were sacrificed by decapitation. The livers were removed, and 10 g of the liver was placed in 10 ml of ice-cold 3.4 m*M* tromethamine[1] (III) hydrochloride buffer (pH 7.40 at room temperature). The 3.4 m*M* III hydrochloride buffer contained 0.25 *M* sucrose and 1 m*M* ethylene glycol bis(aminoethyl) tetraacetate. All following procedures were carried out at 0°.



II*a*: $R_1 = R_2 = H$, $n = 1$
II*b*: $R_1 = R_2 = H$, $n = 5$
II*c*: $R_1 = 2\text{-Cl}$, $R_2 = 4\text{-Cl}$, $n = 5$
II*d*: $R_1 = 2\text{-Cl}$, $R_2 = 6\text{-Cl}$, $n = 5$
II*e*: $R_1 = 3\text{-Cl}$, $R_2 = 4\text{-Cl}$, $n = 5$

The liver was homogenized[2], and the homogenate was then diluted with buffer so that it contained 10% liver and 90% buffer. The homogenate was then centrifuged at 2000 rpm (500×*g*) for 10 min. The supernate was removed carefully and centrifuged at 9500 rpm (10,000×*g*) for 7 min. The supernate was discarded, and the mitochondrial pellet was resuspended in approximately 30 ml of buffer and recentrifuged at 9500 rpm (10,000×*g*) for 7 min. Resuspension of the pellet and recentrifugation was carried out once more. Finally, the pellet was resuspended in 10 ml of buffer. In the respiratory experiments (*vide infra*), 1 ml of this suspension was diluted with 5 ml of buffer to give a protein concentration of approximately 3.5 mg/ml.

The medium for the determination of I*a*–I*e* on mitochondrial respiration contained potassium chloride (0.1790 g), magnesium chloride (0.1708 g), III monophosphate (0.1315 g), and sucrose (9.24 g) dissolved in 100 ml of 10 m*M* III hydrochloride. The 10 m*M* III hydrochloride had a pH of 7.40 at 25°. The pH of the respiratory medium was adjusted to 7.40 at 37° using a 0.5 *M* III solution. The Mannich bases were dissolved in 10 m*M* III hydrochloride buffer in such concentrations that 10 μl of solution contained 5 μmoles of compound. Disodium succinate, monosodium glutamate, and β-hydroxybutyric acid (sodium salt) were dissolved in 10 m*M* III hydrochloride, and the palmitylcarnitine was dissolved in absolute ethanol.

The cells of an oxygen monitor[3] were surrounded by circulating water from a constant-temperature bath at 37°, and to a cell were added the medium (2.5 ml) and mitochondrial suspension (0.5 ml). After a baseline had been recorded, the substrate (15.0 μmoles of succinate, glutamate, or β-hydroxybutyrate or 1.0 μmole of palmitylcarnitine) was added, and the increase in respiration was recorded for approximately 2 min. With palmitylcarnitine, 0.5 ml of bovine serum albumin solution (60 mg/ml) also was added to protect the mitochondria from the detergent action of the substrate. The requisite volume of the solution of the drug was added, and the effect on mitochondrial respiration was noted.

In experiments attempting reversal of the effect of the Mannich bases on mitochondrial respiration with ubiquinone, 5 μl of a solution of ubiquinone in ethanol containing 2.25 nmoles/5 μl of ubiquinone was added to the cell approximately 2 min after the addition of the Mannich base, and the effect on respiration was noted. Preliminary experiments established that the ubiquinone and ethanol in these concentrations had no effect on respiration; in addition, solutions of the Mannich bases and ubiquinone were stable during the experiment (UV evidence). The literature method (15) was followed in examining the spectra of the cytochromes in rat liver mitochondria.

[1] Sigma Chemical Co., St. Louis, MO 63178.
[2] With a Potter-Elvehjem glass–Teflon (du Pont) homogenizer, Kontes Glass Co., Vineland, NJ 08360.
[3] YSI Model 53, Yellow Springs Instrument Co., Yellow Springs, OH 45387.

Table III—Effect of Increasing Concentrations of Coenzyme Q_{10} on Respiration Blocked by *Ib*–*Ie*

Compound	Concentration, µmoles	Reversal with 4.5 nmoles of Ubiquinone, %		Reversal with 9.0 nmoles of Ubiquinone, %	
		Trial 1	Trial 2	Trial 1	Trial 2
I*b*	2.5	31.4	25.0	37.5	34.5
I*c*	0.5	36.4	32.0	53.6	55.6
I*d*	0.5	16.7	17.0	28.6	32.4
I*e*	0.5	32.5	30.4	46.1	35.0

Table IV—Measurement of Half-Wave Potentials of I*a*–I*e* and II*a*–II*e* and Assay of I*a*–I*e* against RNA Polymerases

Compound	$E_{1/2}$	Inhibition of RNA Polymerase, %	
		L-1210	Mouse Kidney
I*a*	−1.25	0	20.3
I*b*	−1.19	5.9	27.9
I*c*	−1.06	0	12.5
I*d*	−1.09	0	7.6
I*e*	−1.11	0	18.2
II*a*	−1.40		
II*b*	−1.38		
II*c*	−1.25		
II*d*	−1.28		
II*e*	−1.30		

The effect of increased concentration of ubiquinone on succinate-induced respiration after the addition of Mannich bases was shown using I*b*–I*e*. The concentrations of drugs used were: 2.5 µmoles of I*b* and 0.5 µmole of I*c*–I*e*. The concentrations of ubiquinone were 4.5 and 9.0 nmoles. Each trial was repeated twice.

In every case that rat liver mitochondria were used, the protein content was determined by a modification (22) of the previously described procedure (23).

Effect of I*a*–I*e* on RNA Polymerases—The solubilized nuclear DNA-dependent RNA polymerases from L-1210 leukemia and Swiss mouse kidney cells were prepared according to the literature procedure (24), and each assay was carried out in duplicate using a concentration of 10 m*M*.

Measurement of Half-Wave Potentials—A polarograph[4] was calibrated in the following manner. A solution of 0.01 *N* cadmium chloride (5.0 ml) was diluted to 50.0 ml with 0.1 *N* potassium chloride. The mixture was purged with nitrogen, and five polarograms were obtained. The average half-wave potential ($E_{1/2}$) was compared to the literature value of −0.600 v (25). The half-wave potentials found in this experiment differed by 0.011 v from the value obtained when the solutions contained 40% (v/v) ethyl alcohol (*vide infra*).

Approximately 1 mg of I*a*–I*e* was dissolved in 1.0 ml of ethyl alcohol and placed in a 25-ml volumetric flask. The flask was filled to volume with a solution of 0.1 *N* potassium chloride containing 40% (v/v) ethyl alcohol. The solution was shaken and emptied into an electrolytic cell, and nitrogen was bubbled through the solution for 10 min. The temperature of the solutions varied from 21.7 to 22.5°; five determinations were undertaken (<2% variation), and the average result was obtained.

[4] Metrohm Polarecord E 261 model R, Fisher Scientific Co., Edmonton, Alberta, Canada.

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