The Spectrum of Rhinovirus Inhibition by 2-(α-Hydroxybenzyl)-benzimidazole and D-(—)-2-(α-Hydroxybenzyl)-benzimidazole HCl* (33393)

JACK M. GWALTNEY, JR. (Introduced by C. M. Kunin)

Departments of Preventive Medicine and Internal Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22901

2-(α-Hydroxybenzyl)-benzimidazole (HBB) has been shown to be a specific inhibitor of picornavirus multiplication (1, 2–4). A concentration of 493 μ*M* or less of HBB inhibits the cytopathic effect of many of the enteroviruses; inhibited strains exhibit quantitative differences in susceptibility (1, 5). HBB has also shown activity against some strains of the more recently discovered rhinovirus subgroup (6) of the picornavirus group (7–11).

The current investigation was initiated to extend observations on the spectrum of inhibitory action of HBB for the numbered rhinovirus serotypes. HBB sensitivity of varying degree was noted for half of the virus strains tested. During the course of the investigation the more active hydrochloride of the D-(—) isomer of HBB (D-HBB·HCl) became available for investigation (12). Results of testing with this compound suggest that D-HBB·HCl susceptibility of varying degree may be a common characteristic of all rhinoviruses.

Materials and Methods. Viruses. Rhinovirus strains representative of the 55 numbered serotypes and one subtype (6) were isolated during an epidemiological study of acute respiratory disease (13) or were obtained from other investigators.[1] Strains isolated from ill persons were usually in early passage when tested while strains obtained from other sources were mainly prototypes at higher passage levels.

Compounds. The HBB[2] and D-HBB·HCl[3] were suspended in medium containing equal volumes of Eagle's MEM and medium 199. Suspensions were mixed overnight at room temperature by an electromagnetic stirring device. Two percent fetal calf serum was incorporated into the medium either prior to stirring or at the time of testing. Solutions were prepared at weekly or 2-week intervals and stored at room temperature.

Cell cultures. Diploid human embryonic lung (WI-38)[4] and HeLa (Rhino)[5] cultures in screw-cap tubes obtained from commercial sources were maintained on 49% Eagle's MEM, 49% medium 199, and 2% fetal calf serum prior to testing.

Experimental design. Concentrations of 223 and 447 μ*M* HBB and of 77, 115, 192, 383, and 574 μ*M* D-HBB·HCl were tested against rhinovirus inocula ranging from 3 to 100 $TCID_{50}$ per ml. Testing was done in triplicate or quadruplicate cell culture tubes with virus titrations maintained simultaneously on medium without compound. Control tubes for each concentration of compound and tests of a sensitive rhinovirus were included in each series of experiments. A known resistant rhinovirus was also tested in medium containing compound concentrations under test in early experiments. Tubes were incubated at 35 or 37° in a roller drum and

* This work was done under the sponsorship of the Commission on Acute Respiratory Diseases of the Armed Forces Epidemiological Board and was supported in part by Research Contract DA-49-007-MD-1000 from the US Army Medical Research and Development Command, Department of the Army, and by Public Health Service Research Contract PH-43-63-1128 from the Institute of Allergy and Infectious Diseases.

1 Kindly supplied by Drs. M. A. Mufson, D. Hamre, and V. V. Hamparian.

2 Kindly supplied by Dr. Lewis H. Sarett, Merck Sharp and Dohme, Rahway, New Jersey.

3 Kindly supplied by Dr. Arthur F. Wagner, Merck Sharp and Dohme, Rahway, New Jersey.

4 Flow Laboratories, Rockville, Md.; Grand Island Biological Company, Grand Island, New York; Microbiological Associates, Bethesda, Md.; HEM Research, Rockville, Md.

5 Grand Island Biological Company, Grand Island, New York.

TABLE I. Rhinoviruses Tested vs HBB and D-HBB • HCl in WI-38 Cell Culture Tubes.

Rhinovirus		Drug concentration (μM)						
		HBB		D-HBB • HCl				
Type	Strain	223	447	77	115	192	383	574
1A	proto.	—(300)[a]						
	SF1382	—(300)						
	SF1382					—(100)		
	SF1382					—(10)		
	SF1382					—(10)	—(10)	—(10)
1B	SF704	—(300)						
	SF704					—(100)		
	proto.		—(10)					
	proto.					—(30)	—(30)	tr(30)
2	proto.	—(300)						
	proto.					—(100)		
	proto.					—(100)		
	proto.					—(30)	±(30)	+(30)
3	SF1399	+(100)						
	proto.	+(30)			+(30)	+(30)		
4	SF748	—(100)						
	SF748					+(100)		
	proto.				tr(100)			
	proto.				+(100)			
5	proto.		+(30)					
	proto.				+(30)			
6	SF1349	—(10)						
	SF1349					±(10)		
7	proto.		—(100)					
	proto.					tr(10)	±(10)	+(10)
8	proto.		—(100)					
	proto.		tr(100)			—(100)	—(100)	±(100)
	proto.		—(100)			—(100)	±(100)	+(100)
9	proto.		tr(30)					
	proto.					tr(30)	±(30)	+(30)
10	proto.		+(30)					
	proto.					±(30)	+(30)	+(30)
11	SF747	±(300)						
	SF747					+(30)		
	proto.				+(3)			
	proto.				±(100)			
12	proto.						+(100)	+(100)
13	SF1384	±(300)						
	SF1384					—(30)		
	SF1384			tr(10)		±(10)	+(10)	
	SF1384					—(30)	±(30)	+(30)
14	SF725	±(30)						
	proto.		+(30)					
	proto.		+(100)			+(100)	+(100)	+(100)
	proto.				+(100)			

TABLE I (continued)

Rhinovirus		Drug concentration (μM)						
		HBB		D-HBB • HCl				
Type	Strain	223	447	77	115	192	383	574
15	SF525	−(10)						
	proto.					±(30)		
	proto.					+(100)		
	proto.				−(30)			
16	proto.		−(30)			−(30)	−(30)	
	proto.	+(10)				±(10)	+(10)	+(10)
	proto.						+(100)	
17	SF460	−(10)						
	SF460					+(100)		
	proto.				tr(30)			
18	proto.	+(100)				±(100)	+(100)	+(100)
19	proto.		tr(10)			tr(10)	±(10)	+(10)
20	proto.				−(100)			
	proto.				−(30)	−(30)		
	SF1582					±(30)		
	proto.					±(30)		
21	CH51	±(300)						
	CH51					+(10)		
22	proto.		±(30)			±(30)	+(30)	+(30)
23	SF1322	−(10)						
	proto.						+(100)	+(100)
24	proto.				−(100)			
	proto.					tr(30)	±(30)	+(30)
	proto.					−(100)	tr(100)	+(100)
25	proto.		tr(30)			tr(30)	±(30)	+(30)
26	proto.	+(3)						
	proto.					+(100)		
	Chi 127-1				+(100)			
27	SF274	+(300)						
	SF274			−(30)		−(30)	+(30)	
	SF274				−(30)			
	proto.		+(10)			+(10)	+(10)	+(10)
28	proto.		tr(100)			tr(100)	+(100)	+(100)
29	SF127	−(10)						
	SF127					tr(100)		
	SF133	−(30)						
	proto.		−(100)			−(100)	tr(100)	+(100)
30	CH91	tr(300)						
	CH91	±(100)						
	proto.		−(100)			−(100)	−(100)	+(100)
31	SF1240	−(300)						
	SF1240					−(100)		
	SF1240					−(30)		
	SF1240			+(30)		±(30)	+(30)	
	Sheff 30/60				−(100)			

TABLE I (continued)

Rhinovirus		Drug concentration (μM)						
		HBB		D-HBB · HCl				
Type	Strain	223	447	77	115	192	383	574
32	SF578	tr(100)						
	SF578					±(100)		
	proto.		±(100)			±(100)	+(100)	+(100)
33	SF692	−(100)						
	SF692					+(100)		
	SF692				−(100)			
	SF692					±(30)		
	SF692					−(30)		
34	SF1540	+(100)						
	SF1540					+(3)		
	SF1540				tr(10)	tr(10)		
	SF1540					±(30)		
	proto.				−(100)			
35	SF795	−(30)						
	SF1616					+(10)		
	proto.				tr(10)			
36	proto.		−(100)			−(100)	−(100)	±(100)
	proto.		+(100)			±(100)	±(100)	+(100)
37	proto.		+(30)			+(30)	+(30)	+(30)
	proto.				−(100)			
38	proto.		tr(30)			−(30)	±(30)	±(30)
39	SF299	±(100)						
	SF299	−(100)						
	proto.		−(30)			−(30)	±(30)	+(30)
40	proto.				−(100)			
	proto.					−(100)	±(100)	+(100)
41	SF220	±(100)						
	SF220					+(100)		
	proto.				−(100)			
42	proto.		+(3)			+(3)	+(3)	+(3)
	proto.				±(100)			
43	proto.		+(100)			±(100)	+(100)	+(100)
44	proto.		tr(30)			tr(30)	−(30)	±(30)
45	proto.				−(100)			
	proto.				−(30)	−(30)		
	proto.					−(100)	±(100)	+(100)
	proto.					tr(100)	±(100)	+(100)
46	CH202					±(100)		
	proto.		+(30)			+(30)	+(30)	+(30)
	proto.				−(30)			
	proto.				tr(100)	tr(100)		
	proto.					tr(100)	±(100)	±(100)
47	SF42			(100)		±(100)	+(100)	
48	proto.		+(100)			+(100)	+(100)	+(100)
	proto.				−(100)			

TABLE I (continued)

Rhinovirus		Drug concentration (μM)						
		HBB		D-HBB · HCl				
Type	Strain	223	447	77	115	192	383	574
49	SF414			—(30)		—(30)	+(30)	
50	proto.		+(100)			±(100)	+(100)	+(100)
51	SF357			—(100)		—(100)	±(100)	
	SF357		±(100)	—(100)		±(100)	+(100)	
52	proto.		+(30)			+(30)	+(30)	+(30)
53	proto.		+(100)			±(100)	+(100)	+(100)
54	proto.		±(100)			tr(100)	±(100)	+(100)
55	proto.		+(100)			±(100)	±(100)	+(100)

[a] Virus $TCID_{50}$/ml.

read daily for cytopathic effect (CPE).

Determination of virus inhibitory activity of compounds. Tests were judged complete when virus control tubes showed 75% or greater destruction of cell sheets. Comparisons were made at that time with the percentage of cell sheet destruction in tubes containing virus-compound mixtures. Observed differences of 75% or more were graded as "+," 75–50% as "±," 50–25% as trace, and less than 25% as "—" inhibition.

Observations of compound cytotoxicity. WI-38 cell culture tubes exposed for 2 weeks to test concentrations of D-HBB · HCl were washed with Hanks' balanced salt solution, trypsinized, suspended in growth medium (10% fetal calf serum in Eagle's BME), and transferred to 60 × 15-mm plastic petri dishes.[6] Rates of cell growth during incubation at 35° in an atmosphere of 2% CO_2 in air were compared to growth rates of control cell culture tubes which had been on medium without compound.

Results. Rhinovirus inhibition. Results of testing rhinovirus strains of the numbered types against HBB and D-HBB · HCl in human embryonic lung cell (WI-38) culture tubes are shown in Table I. Experiments performed simultaneously with a particular virus strain are recorded on the same line. A strain or strains of all 55 serotypes except type 1A and subtype 1B showed + or ± amounts of inhibition on exposure to one or more concentrations of the compounds. The amount of inhibition of a particular virus strain was repeatedly observed to increase as the concentration of the compounds was increased. Also, succeedingly greater concentrations of HBB and D-HBB · HCl inhibited a progressively larger proportion of strains (Table II). Calculation of the relative activities of the compounds is based on the work of Kadin *et al.* (12) which showed D-HBB · HCl to be approximately 1.5 times as effective as DL-HBB.

Eight strains of rhinovirus type 7 and nine of rhinovirus type 24 were tested against a 192 μM concentration of D-HBB · HCl in WI-38 cell culture tubes (Table III). The strains of the same serotype showed similar but not completely uniform responses to compound exposure.

Strains of 36 types were tested in HeLa cell culture tubes, and the result of these experiments was compared to similar testing in WI-38 cell culture tubes done either simultaneously or at different times (Table IV). Agreement was found in results obtained with the two cell lines with approximately three-quarters of the strains regardless of whether or not testing was done simultaneously.

The above results have been confirmed by experiments now in progress using a more sensitive and quantitative gradient plate plaque reduction method which allows testing

[6] Falcon Plastics, Los Angeles, Calif.

TABLE II. Inhibitory Effect of HBB and D-HBB · HCl on Rhinovirus Types in WI-38 Cell Culture Tubes.

	Concentration			Rhinovirus types		
					Inhibited (+ or ±)	
	(μg/ml)	μM	Activity[a]	No. tested	(no.)	(%)
D-HBB · HCl	20	77	0.6	6[b]	1	17
	30	115	0.9	23[b]	7	30
HBB	50	223	1.0	26	11	42
D-HBB · HCl	50	192	1.5	53	30	57
HBB	100	447	2.0	31	18	58
D-HBB · HCl	100	383	3.0	42	33	79
	150	574	4.5	38	36	95

[a] Based on the assumption of a relative activity of 2 and 3 for HBB and D-HBB · HCl, respectively (11).

[b] Most strains tested had previously shown sensitivity to a 192 μM concentration of D-HBB · HCl.

at compound levels which are not toxic to cells (14). With this technique a strain of type 1A showed inhibition at a 184 μM concentration of D-HBB · HCl. This method also demonstrated a relatively high proportion of compound-resistant virions of this strain which explains the failure of the cell culture tube method to show virus inhibition. Strains of a highly sensitive rhinovirus (type 14) and of a moderately sensitive one (type 22) were inhibited by 57 and 103 μM concentrations, respectively, of D-HBB · HCl using the plaque reduction method.

TABLE III. D-HBB · HCl Testing vs Multiple Strains of Rhinovirus Types 7 and 24.

Rhinovirus		Inhibition by D-HBB · HCl
Type	Strain	(192 μM)
7	SF 1346	+(100)[a]
	SF 1348	±(100)
	SF 1379	tr(100)
	SF 1380	tr(100)
	SF 1396	±(30)
	SF 1397	±(30)
	SF 1476	tr(30)
	Prototype	tr(100)
24	SF 680	—(30)
	SF 860	—(100)
	SF 1448	—(100)
	SF 1561	—(100)
	SF 1562	±(30)
	SF 1563	—(30)
	SF 1615	—(100)
	SF 1750	—(100)
	Prototype	tr(100)

[a] Virus $TCID_{50}$.

Compound cytotoxicity. The 574 μM concentration of D-HBB · HCl was the highest which allowed successful testing without cell culture toxicity evidenced by nonspecific degeneration. At this concentration WI-38 cell cultures frequently showed morphologic evidence of toxicity in the form of cell shrinkage. Similar changes were noted less frequently at the 383 μM concentration. These changes were reversible, and disappeared in cells refed medium without compound. Rates of cell growth determined by the time required for development of confluent cell sheets in petri dishes were reduced for cells with prior exposure to 574 and 383 μM but not to 192 μM compound concentrations.

Discussion. Testing for inhibition by HBB has been reported by others for strains of 28 rhinovirus types (Table V). Most showed no sensitivity to this compound at the concentrations used which ranged from 200 to 440 μM. Some strains showed partial or inconsistent inhibition. The results of the current experiments with HBB are in general agreement with these earlier reports, although par-

TABLE IV. Comparison of Testing 192 μM D-HBB • HCl vs Rhinovirus Strains in WI-38 and HeLa Cell Culture Tubes.

Rhinovirus		Cell culture		Rhinovirus		Cell culture	
Type	Strain	WI-38	HeLa	Type	Strain	WI-38	HeLa
1B	SF 704	—(100)[a]	—(30)	27	SF 274	—(30)	±(100)
2	prototype	—(30)	—(10)	28	prototype	tr(100)	—(100)
3[b]	prototype	+(100)	+(100)	29	SF 127	tr(100)	NT
4	SF 748	+(100)	+(30)		prototype	—(100)	+(100)
6	SF 1349	±(10)	+(100)	29[b]	prototype	tr(30)	—(100)
7[b]	prototype	tr(100)	+(3)	31[b]	SF 1240	—(30)	tr(3)
8	prototype	—(100)	tr(3)	33	SF 692	+(100)	tr(100)
9	prototype	tr(30)	—(100)	33[b]	SF 692	±(30)	—(30)
11	SF 747	+(30)	NT	35	SF 1616	+(10)	+(100)
	prototype	NT	+(30)	37	prototype	+(30)	+(100)
12[b]	prototype	tr(100)	tr(100)	39	prototype	—(30)	NT
14[b]	prototype	+(10)	+(100)		SF 299	NT	—(100)
15	SF 525	±(30)	NT	40	prototype	+(100)	—(100)
	prototype	+(100)	+(100)	40[b]	prototype	—(100)	±(30)
16	prototype	—(30)	NT	40[b]	prototype	—(100)	tr(100)
	prototype	±(10)	NT	42	prototype	+(3)	+(30)
	prototype	tr(100)	±(100)	45	prototype	—(30)	±(100)
17	SF 460	+(100)	+(100)		prototype	—(100)	tr(100)
18	prototype	±(100)	+(100)	46	prototype	—(100)	tr(100)
19	prototype	tr(10)	—(100)	48	prototype	+(100)	+(100)
20[b]	prototype	±(30)	+(100)	51	prototype	±(100)	—(100)
20[b]	prototype	tr(30)	±(100)	51[b]	prototype	tr(100)	tr(30)
	SF 1582	±(30)	—(30)	52	prototype	+(30)	+(100)
21	CH 51	+(10)	±(100)				
24	prototype	+(100)	+(100)				
25	prototype	tr(30)	—(30)				
25[b]	prototype	—(3)	—(30)				
26	prototype	+(100)	+(100)				

[a] Virus $TCID_{50}$/ml.

[b] Tests performed simultaneously.

tial or inconsistent HBB sensitivity was observed for strains of a few types which previously showed no inhibition. On the other hand only type 1A and subtype 1B failed to show some degree of inhibition at a 574 μM concentration or less of D-HBB • HCl. This suggests that inhibition by this compound is a common characteristic of the classified rhinovirus types.

Separation of the possible compound effects of direct virus inhibition vs virus inhibition secondary to cell changes cannot be made in the cell culture tube experiments involving the higher compound concentrations. The current findings of cell toxicity at compound concentrations of 383 and 574 μM confirm the earlier work of Kadin *et al.* (15) who observed morphological changes in cells exposed to 493 μM D-HBB • HCl. Tamm *et al.* (16) reported morphological changes in cells exposed to 329 μM HBB.

Two possible explanations have to be considered for virus strains showing inhibition at cytotoxic concentrations of compound: (i) Inhibition at the higher concentration is not virus-specific, and results secondarily from toxicity of the compound for cells. (ii) Inhibition even at the high concentration is virus-specific, and the effects of the compound on cells are of no consequence for the replication of virus.

The linear increase in the proportion of strains inhibited by increasing compound concentrations suggested that inhibition at

TABLE V. Rhinovirus Testing vs HBB Reported by Other Authors.

Virus type	Cell culture	HBB conc (μM)	Inhibition	Ref.
1A	HEL	440	No	2
	WI-26	220	Inconsistent	7
	HeLa	220	No	7
	HEL	200	No	8
1B	HEL	440	No	2
2	HEL	440	No	2
	WI-26	220	Inconsistent	7
	HeLa	220	No	7
3	WI-26	220	Inconsistent	7
	HeLa	220	No	7
7	WI-26	220	Inconsistent	7
	HeLa	220	No	7
13	WI-26	200	No	9
14	WI-26	200	Partial	9
15	WI-26	200	No	9
16	WI-26	200	No	9
17	WI-26	200	No	9
19	WI-26	220	Inconsistent	7
22	HEL	200	No	11
24	HEL	200	No	11
26	HEL	200	Yes	11
28	HEL	200	No	11
29	HEL	200	No	8
30	HEL	200	No	8
31	HEL	200	No	8
32	WI-26	200	No	10
33	WI-26	200	No	10
34	HEL	200	No	11
35	HEL	200	No	11
36	HEL	200	No	11
37	HEL	200	No	11
38	HEL	200	No	11
40	HEL	200	No	11
41	HEL	200	No	11
51	HEL	200	No	11

the higher concentrations was also due in part to direct effect on the virus. No sharp break was observed in compound effect with increasing concentrations to suggest that a new mode of action such as cell poisoning had become predominant. Direct effect on the virus was also suggested by the fact that WI-38 cells exposed to 383 μM concentrations of D-HBB · HCl supported the growth of a number of rhinovirus strains and that cells exposed to 574 μM concentrations still supported growth of strains of types 1A and 1B. Finally the preliminary results of plaque reduction experiments now in progress which allow testing at noncytotoxic compound concentrations provide direct evidence that the observed effect is virus-specific.

The HBB antiviral activity for poliovirus has been attributed to interference with the synthesis of viral-induced RNA polymerase (4). If true for rhinoviruses, the present findings suggest that the viral-directed RNA polymerases of classified rhinoviruses may be homogenous in regard to D-HBB · HCl susceptibility but that wide quantitative differences in this characteristic exist.

Summary. Rhinovirus strains representative of the 55 numbered serotypes and one subtype were tested in HeLa and WI-38 cell culture tubes for inhibition by 223 and 447 μM concentrations of 2-(α-hydroxybenzyl)-benzimidazole (HBB) and 77, 115, 192, 383, and 574 μM concentrations of D-(—)-2-(α-hydroxybenzyl)-benzimidazole · HCl (D-HBB · HCl). Half of strains tested showed some inhibition at a 447 μM concentration of HBB and strains of all types except 1A and subtype 1B showed some degree of inhibition at a D-HBB · HCl concentration of 574 μM or less. An increasing proportion of strains were inhibited as test concentrations of the compounds were increased. Multiple strains of rhinovirus types 7 and 24 showed similar degrees of sensitivity to a 192 μM concentration of D-HBB · HCl. Comparative testing of strains of 36 types vs a 192 μM concentration of D-HBB · HCl in WI-38 and HeLa cell culture tubes gave similar but not identical results. D-HBB · HCl concentrations of 574 and 383 μM were toxic for cells. The behavior of viruses which are insensitive to HBB or D-HBB · HCl at nontoxic concentrations, but show some sensitivity at higher concentrations, is difficult to interpret because of the problem in separating virus-specific from toxic actions of the compounds. Indirect evidence for a virus-specific inhibitory effect is provided by the linear increase in the proportion of sensitive strains with increasing compound concentrations and by the growth of some rhinovirus strains in cells exposed to cytotoxic concentrations. Experiments now in progress with a more sensitive gradient plate plaque reduction

technique, using D-HBB · HCl concentrations below cytotoxic levels, support this hypothesis. These results suggest that rhinoviruses as a group show a wide range of sensitivity to these compounds but that most types share to some degree the characteristic of D-HBB · HCl sensitivity.

1. Eggers, H. J. and Tamm, I., Virology **13**, 545 (1961).

2. Tamm, I. and Eggers, H. J., Virology **18**, 439 (1962).

3. Eggers, H. J. and Tamm, I., Nature **197**, 1327 (1963).

4. Baltimore, D., Eggers, H. J., Franklin, R. M., and Tamm, I., Proc. Natl. Acad. Sci. U.S. **49**, 843 (1963).

5. Eggers, H. J. and Tamm, I., J. Exptl. Med. **113**, 657 (1961).

6. Kapikian, A. Z., Nature **213**, 761 (1967).

7. Hamparian, V. V., Hilleman, M. R., and Ketler, A., Proc. Soc. Exptl. Biol. Med. **112**, 1040 (1963).

8. Connelly, A. P. and Hamre, D., J. Lab. Clin. Med. **63**, 30 (1964).

9. Kisch, A. L., Webb, P. A., and Johnson, K. M., Am. J. Hyg. **79**, 125 (1964).

10. Webb, P. A., Johnson, K. M., and Mufson, M. A., Proc. Soc. Exptl. Biol. Med. **116**, 845 (1964).

11. Hamre, D., Connelly, A. P., and Procknow, J., J. Lab. Clin. Med. **64**, 450 (1964).

12. Kadin, S. B., Eggers, H. J., and Tamm, I., Nature **213**, 761 (1964).

13. Gwaltney, J. M., Jr., Hendley, J. O., Simon, G., and Jordan, W. S., Jr., Am. J. Epideminol. **87**, 158 (1968).

14. Kucera, L. S. and Herrmann, E. C., Jr., Proc. Soc. Exptl. Biol. Med. **122**, 258 (1966).

15. Kadin, S. B., Eggers, H. J., and Tamm, I., Nature **201**, 639 (1964).

16. Tamm, I., Bablanian, R., Nemes, M. M., Shunk, C. H., Robinson, F. M., and Folkers, K. J. Exptl. Med. **113**, 625 (1961).

Received May 15, 1968. P.S.E.B.M., 1968, Vol. 129.