

BULLETIN

Of

Haffkine Institute



Haffkine Institute for Training, Research and Testing
Parel, Bombay - 400 012, India

EDITORIAL BOARD

Chairman & Editor

Dr. B. B. GAITONDE, M.D., M.Sc. (Med.), F.A.Sc., F.A.M.S.

Director, Haffkine Institute for Training, Research and Testing, Bombay

Assistant Editor

Mr. R. B. GHOOI, M.Sc.

Scientific Secretary, Haffkine Institute for Training, Research and Testing, Bombay

Members

Dr. C. G. PANDIT, M.B.,B.S., Ph.D., D.P.H., D.T.M.

Ex-Director, Indian Council of Medical Research

Dr. H. I. JHALA, M.D., F.C.P.S., D.T.M.

Superintendent, Sir Harkisondas N. Hospital, Prarthana Samaj, Bombay

Dr. K. G. NAIR, S.E.M., M.D., Ph.D.(Chicago), F.A.C.C. (U.S.A.), F.I.C.A.

(U.S.A.), F.P.C.C. (Hon.)

Director-Professor of Medicine, Seth G. S. Medical College, Bombay

Dr. D. D. BANKER, M.D., Ph.D.

Chief Bacteriologist (Retd.), Glaxo Laboratories, Bombay

Dr. F. FERNANDES, M.Sc., Ph.D.

Head of Microbiology Department (Retd.), Ciba Research Centre, Bombay

Dr. S. S. RAO, M.Sc., Ph.D.

Assistant Director (Retd.), Haffkine Institute, Bombay

Mr. PERCY E. D'SOUZA

Managing Editor, 'Medical Times', Sandoz (India) Ltd., Bombay

Dr. C. V. DELIWALA, M.Sc., Ph.D.

Deputy Director (Retd.), Haffkine Institute, Bombay

Dr. M. V. SANT, M.D., F.C.P.S., D.E. (Prague)

Deputy Director (Retd.), Haffkine Institute, Bombay

Dr. A. N. D. NANAVATI, M.D.

Assistant Director (Retd.), Haffkine Institute, Bombay

Dr. M. B. BHIDE, M.D., F.C.A.I.

Assistant Director, i/c Pharmacology Department, Haffkine Institute for Training, Research and Testing, Bombay

Dr. F. L. SALDANHA, M.Sc., Ph.D., F.I.C. (Cal.)

Deputy Manager (Quality Control), Haffkine Bio-Pharmaceutical Corporation Ltd., Bombay

CONTENTS

	PAGE
<i>Nutritional Requirements of Vibrio and Biotype El Tor Cholerae</i> .. V. P. SHINDE	1
<i>Effects of Drugs on Red Cell Metabolism in vitro Studies</i> H. K. SONI, A. J. BAXI AND A. B. VAIDYA	12
<i>Anaerobic Bacteriology of Septic Wounds</i> .. SWATI R. SALVEKAR, S. MUKERJI AND JAYSHREE K. DAVE	19
<i>Antifilarial Activity of Some Anthelmintic Drugs used in Clinical Practice</i> .. USHA A. SONURLIKAR AND M. B. BHIDE	25
<i>Studies on the Neuromuscular Blocking Activity of Seeds of Solanum khasianum Clarke</i> M. B. BHIDE, P. Y. NAIK AND R. S. JOSHI	31
<i>Synthesis and Study of [2-substituted-4-[4'-Methoxy-3-((2-Hydroxy-5-Halo-or 5-Nitro-benzylidene) Amino) Phenyl] Thiazoles Hydrochlorides for in vitro Antituberculosis Activity-II</i> A. M. PATIL, D. T. CHAUDHARI, MADHUBEN R. PATEL AND S. S. SABNIS	36
<i>Synthesis of N¹-(5-Nitro-2-Furoyl)-N⁴-Substituted Piperazines as Possible Antimicrobial Agents</i> V. S. KHOT, MADHUBEN R. PATEL AND M. H. SHAH	40

Statement about ownership and other particulars about newspaper 'Bulletin of Haffkine Institute' to be published in the first issue every year after last day of February

FORM IV

(See Rule 8)

- | | | |
|--|----|---|
| 1. Place of publication | .. | Haffkine Institute, Acharya Donde Marg, Parel, Bombay 400 012. |
| 2. Periodicity of its publication | .. | Triannually (three times a year—April, August and December). |
| 3. Printer's name | .. | Mr. G. D. Dhond, Manager, Government Central Press, Bombay 400 004. |
| Whether citizen of India? | .. | Yes. |
| If foreigner, state the country of origin | .. | |
| Address | .. | Government Central Press, Netaji Subhash Road, Bombay 400 004. |
| 4. Publisher's name | .. | Dr. B. B. Gaitondé, Director, Haffkine Institute, Bombay 400 012. |
| Whether citizen of India? | .. | Yes. |
| If foreigner, state the country of origin | .. | |
| Address | .. | Haffkine Institute, Acharya Donde Marg, Parel, Bombay 400 012. |
| 5. Editor's name | .. | Dr. B. B. Gaitondé. |
| Whether citizen of India? | .. | Yes. |
| If foreigner, state the country of origin | .. | |
| Address | .. | Haffkine Institute, Acharya Donde Marg, Parel, Bombay 400 012. |
| 6. Names and addresses of individual who own the newspaper and partners or shareholders holding more than one per cent of the total capital. | | Haffkine Institute (Government of Maharashtra Undertaking), Bombay 400 012. |

I, Dr. B. B. Gaitondé, hereby declare that the particulars given above are true to the best of my knowledge and belief.

(Sd.) B. B. GAITONDÉ
(Signature of Publisher)

NUTRITIONAL REQUIREMENTS OF VIBRIO AND BIOTYPE 'EL TOR CHOLERAE'

V. P. SHINDE

Testing Department, Haffkine Institute, Parel, Bombay 400 012

A medium of casein hydrolysate routinely used for production of cholera vaccine is analysed for amino acids and mineral elements. Based on the composition of casein hydrolysate medium, a synthetic medium is prepared and nutritional requirements of *Vibrio* and biotype *El Tor cholerae* are found out by omission of a single nutrient at a time technique. Aspartic acid is found essential for the growth while glutamic acid, glycine and histidine are required to enhance the growth. Magnesium, potassium, phosphate, chloride and sulphate are absolutely essential, whereas, sodium, calcium and iron are required to stimulate the growth of cholera organisms. Requirement of calcium and iron is reduced by increasing the concentration of magnesium in the medium. From the nutritional requirements, a chemically defined medium is elaborated and tested for the growth of two strains of *V. cholerae* (Inaba 569B and Ogawa S/121/58), and two biotype *El Tor cholerae* strains (Inaba, Phil. 6978 and Ogawa, Phil. 1418). Though the growth response is poor (60-65% compared to casein hydrolysate medium), this medium may give controlled cholera vaccine without complicated factors as present in the customarily used medium of protein digests or peptone or meat extracts. This medium may be economical to study the biochemistry of the organisms with respect to their metabolic functions and also to isolate choleraogenic antigens.

Introduction

Growth of cholera vibrios depends on physical conditions and requirement of nutrients. They grow profusely in alkaline medium within pH range 6.5 to 8.5 (1); whereas, pH 7.4 of the medium was recommended for maximum growth of the organisms (2, 3). The reports (4) showed that growth of *V. cholerae* was abundant at temperatures between 30° and 40°C, however, maximum growth was obtained at $37 \pm 0.5^\circ\text{C}$. The essential nutrients present in the medium support for the growth, generation of energy and synthesis of protoplasmic material of microbial cell. The environment in which organism grows must, therefore, be supplied nutrients in a form available to them.

(Received for publication June 28, 1977)

It is reported in the literature (5) that like certain other bacteria, cholera vibrios include two types of strains. Exacting strains which require amino acids for their growth are usually pathogenic and non-exacting strains, which can grow in presence of ammonium salts, instead of amino acids in media, are generally non-pathogenic. When cholera vibrios cultivated in various media containing inorganic salts, glucose and either peptone or casein digest, ammonium sulphate was found to act as a buffer rather than essential nutrient. The results also showed that growth of *V. cholerae* was possible without ammonium salt and failed in absence of either peptone or casein digest in the medium (6). After testing 20 amino acids by adding them singly or in combination

to a basic medium containing inorganic salts and glucose, asparagine, tyrosine and glycine were found essential for growth and multiplication of *V. cholerae* (7). In contrast, others (8, 9) reported that ammonium salt, other inorganic salts and glucose in a medium promoted growth of classical as well as *El Tor cholerae* strains successfully, whereas, they recommended high inoculum in the medium for cultivation. Thind (10) had tried the basic medium (8) and observed that this medium could not give growth of his test strains of cholera-genic vibrios. After a series of trials, further he demonstrated that asparagine, histidine and glutamic acid were the essential amino acids for growth of *V. cholerae*. It is also shown that hypoxanthine is essential to stimulate growth of the organisms. The studies (11) on oxidative metabolism of vibrios indicated that cystine, threonine and asparagine were utilised at faster rate than the other amino acids tested. Basic amino acids showed very poor consumption. Recently, it is reported that arginine in combination with one or more amino acids appeared to be essential for production of cholera-genic antigens (12).

It will be seen from the above that the reports on the requirement of amino acids for growth of cholera-genic organisms are conflicting. As the organisms grow well on a simple medium containing casein hydrolysate or peptone or meat extract and simplicity of the media, not much attention has been paid to study nutritional requirements of cholera-genic organisms for amino acids and mineral elements.

In view of the above, a systematic study on requirement of amino acids and mineral elements in the form of their salts for growth of cholera vibrios was carried out. The results of the experiments are presented and discussed in this paper.

Materials and Methods

Strains of cholera-genic organisms and preparation of inoculum were the same as used previously (13). Casein hydrolysate liquid medium was prepared by the standard procedures (2, 3).

Analytical and Micro-biological Assay Methods—Total nitrogen was determined by micro-keijldahl method. Alpha amino acid nitrogen was determined by the copper method (14, 15). Thirteen amino acids viz., alanine, aspartic acid, cystine, glutamic acid, glycine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, tyrosine and valine were determined by the ion-exchange column chromatography (16). Sulphonated polystyrene Amberlite CG-120, a cation exchange resin (200—400 mesh), was used. Tryptophan and basic amino acids were estimated by the micro-biological assay methods (17). Proline was determined by colorimetric method (18). Calcium, phosphorus and iron were estimated according to A.O.A.C. (19). Magnesium, potassium and sulphate were estimated according to the standard procedures (20). Sodium was determined by the colorimetric method (21) and chloride was estimated by volumetric method (22).

Preparation of Synthetic Medium

Amino Acid Solution—Individual solutions of 18 amino acids as present in casein hydrolysate medium (135 mg N/100 ml) were prepared. Only natural L-isomers of amino acids (N.B.C.) were used (Table I).

Salt Solutions—Inorganic salts of respective elements were used for the preparation of salt solutions. The following quantities of salt solutions : 0.69 ml of 1% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 1.19 ml of 1% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; 2.16 ml of 1% $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$; 3.0 ml of 20% NaCl; 0.47 ml of 10% K_2HPO_4 were used to prepare 100 ml medium. The

values of mineral elements content of casein hydrolysate medium are given in Table II.

Preparation of Medium—One millilitre of each of the amino acid solutions and above mentioned quantities of salt solution excluding dipotassium hydrogen phosphate solution were added in their serial order and diluted to about 75 ml with distilled water. pH 7.5 was then adjusted and phosphate solution was added slowly with thorough mixing and volume was made to 100 ml with water. Thus, the synthetic medium was prepared containing amino acids and mineral elements as present in the medium of casein hydrolysate.

TABLE I

Amino Acid Composition of Casein Hydrolysate Medium (135 mg Nitrogen/100 ml)

Amino Acid (L-isomer)	(mg/100 ml medium)*	
	as Casein Hydrolysate 135 mg N/ 100 ml)	Nitrogen
1. Alanine ..	39.1	6.12
2. Arginine ..	48.3	15.31
3. Aspartic Acid	54.3	5.71
4. Cystine ..	3.5	0.41
5. Glutamic Acid	191.5	18.23
6. Glycine	18.2	3.41
7. Histidine	23.5	6.36
8. Isoleucine	73.7	7.87
9. Leucine ..	82.8	8.84
10. Lysine ..	78.4	15.02
11. Methionine	27.2	2.55
12. Phenylalanine ..	19.9	1.69
13. Proline ..	102.6	12.49
14. Serine ..	46.4	6.18
15. Threonine ..	37.5	4.41
16. Tryptophan ..	0.0	0.00
17. Tyrosine ..	46.9	3.62
18. Valine ..	49.2	3.88
Total, Nitrogen/100 ml. .		120.74

*50 fold individual Amino Acid Solutions prepared and used for preparing Synthetic Media.

TABLE II

Mineral Elements Content of Casein Hydrolysate Medium (135 mg Nitrogen/100 ml)

Element	Casein Hydrolysate (mg/100 ml)	Salts* (mg/100 ml)
Magnesium	0.68	6.9
Calcium	3.50	11.9
Iron	4.25	2.16
Sodium	278.00	600.0
Chloride	364.00	
Potassium	0.08	47.0
Phosphorus	8.41	
Sulphur	2.33	

*Concentration of Salts in the Synthetic Medium.

Experimental

Supplementation of Tryptophan—25 ml of each of plain casein hydrolysate medium (135 mg N/100 ml) and the medium supplemented with tryptophan (12.66 mg of L-tryptophan/100 ml) as present in casein, were taken into a series of Erlenmeyer flasks of 250 ml capacity. The remaining procedure was the same as described earlier (13).

Qualitative Requirement of Amino Acids—Seventeen different media were prepared with the omission of single amino acid. 10 ml of each of synthetic media, consisting of all the amino acids and mineral salts and devoid of single amino acid, were taken into 100 ml Erlenmeyer flasks. The flasks were plugged with cotton and sterilised at 121°C for 15 min. After cooling, 0.2 ml of freshly prepared inoculum was added aseptically to each flask and incubated at 37°C for 48 hr. under stationary condition. Growth response was measured turbidometrically. The growth of the choleraenic organisms was scored as +, ++, +++ and +++++.

Qualitative Requirement of Mineral Elements—It was seen that cholera vibrios required only four amino acids viz., aspartic acid, glutamic acid, glycine and histidine to obtain maximum growth compared with the growth obtained in the synthetic medium containing all amino acids and mineral salts. A synthetic medium having above four essential amino acids and all mineral salts representative of the elements, was prepared. In this, MgCl_2 was used as a source of magnesium with proper concentration. Assay procedure and measurement of growth response of cholera vibrios were the same as for amino acids.

Quantitative Requirement of Amino Acids—It was observed that requirement of calcium and iron was partial. Both the elements were, therefore, omitted from the medium. A double strength synthetic medium consisting of four essential amino acids and mineral salts was prepared after adjusting pH 8.0. As a source of energy, glucose was added at the concentration of 0.1 g/100 ml of the medium. Requirement of amino acid to be assessed was omitted from the medium. In a series of 100 ml flasks, graded amounts (1.0 mg to 10 mg per 10 ml of the medium) of amino acid were taken in 10 levels. The flasks were sterilized at 121°C for 15 min. and then 0.1 ml of sterile 10 % glucose solution was added aseptically. Inoculation, incubation and measurement of growth response were the same as described earlier. Concentration of an amino acid required to support maximum growth, in terms of optical densities, was determined.

Quantitative Requirement of Mineral Elements—A double strength synthetic medium was prepared containing four essential amino acids (aspartic acid, 50 mg; glutamic acid, 30 mg; glycine, 50 mg; and histidine, 20 mg per 100 ml) and mineral salts. Glucose, at a concentration

of 0.1 % was used in the final medium. In addition, ammonium sulphate was used as a source of inorganic nitrogen and sulphur in the medium. The mineral salts to be assessed were omitted in turn from the medium and added in graded amounts. In a series of 100 ml flasks, mineral salts per 10 ml were added: $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.05–0.25 mg); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1–0.8 mg); NaCl (25–200 mg); K_2HPO_4 (1–7 mg); $(\text{NH}_4)_2\text{SO}_4$ (2–14 mg) and hemin as a source of iron (5–25 μg). 5 ml of the double strength synthetic medium devoid of mineral salt to be assessed were taken into the flasks and particular salt solution was added in graded doses and diluted to 10 ml with water. The remaining procedure was the same as described previously.

Interrelationship of Amino Acids—From the quantitative requirement of amino acids and minerals, a primary synthetic medium was elaborated.

Preparation of Double Strength Salt Solution—The essential salts: $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1 g); NaCl (5.0 g); K_2HPO_4 (0.5 g) and $(\text{NH}_4)_2\text{SO}_4$ (1.0 g) were dissolved in distilled water and pH 8.0 was adjusted. The solution was diluted to 500 ml.

Preparation of Amino Acid Solutions—Individual solutions of L-aspartic acid (500 mg %), L-glutamic acid (300 mg %), glycine (500 mg %) and L-histidine $\text{HCl} \cdot \text{H}_2\text{O}$ (200 mg %) were prepared.

Assay Procedure—In a series of 100 ml Erlenmeyer flasks, 5.0 ml of double strength salt solution were taken and 1.0 ml each of the amino acid solutions was added singly or in combination. Volume was made to 10 ml by addition of appropriate amount of distilled water. Remaining procedure was the same as described earlier.

TABLE III
Supplementary Effect of Tryptophan on the Growth of Cholerae Genic Organisms
 (Stationary Condition at 37°C)
 (Mean Values of Five Different Lots)

MEDIUM (135 mg N/100 ml)	Incuba- tion period hrs.	Optical Densities × 100*			
		<i>Vibrio Cholerae</i>		<i>El Tor Cholerae</i>	
		Inaba 569 B	Ogawa S/121/58	Inaba Phil. 6978	Ogawa Phil. 1418
Casein Hydrolysate	24	32	34	40	41
	48	48	44	52	51
	72	52	51	61	59
Casein Hydrolysate Tryptophan	24	31	30	38	39
	48	40	36	44	42
	72	42	42	50	48

*Read on Biochem-absorptiometer (Hilger) at Filter 61 (620 mμ).

TABLE IV
Qualitative Requirement of Amino Acids and Mineral Elements for the Growth of Cholerae Genic Organisms in the Synthetic Medium
 (Stationary Condition)

INGREDIENTS OMITTED	Growth of Organisms, 48 hrs. at 37°C			
	<i>Vibrio Cholerae</i>		<i>El Tor Cholerae</i>	
	Inaba 569 B	Ogawa S/121/58	Inaba Phil. 6978	Ogawa Phil. 1418
<i>Amino Acids—</i>				
All Amino Acids ..	—ve	—ve	—ve	—ve
Aspartic Acid ..	++	+	+	+
Glutamic Acid ..	++	++	++	++
Glycine ..	++	++	+++	+++
Histidine ..	+++	+++	+++	+++
Other Amino Acids ..	++++	++++	++++	++++
<i>Mineral Elements—</i>				
All Minerals ..	—ve	—ve	—ve	—ve
Ca ²⁺ as CaCl ₂ ·2H ₂ O ..	+++	+++	+++	+++
Mg ²⁺ as MgCl ₂ ·6H ₂ O ..	+	+	+	+
Fe ²⁺ as FeSO ₄ ·7H ₂ O ..	+++	+++	+++	+++
NaCl for Na ⁺ and Cl [—] ..	+	+	+	+
K ² K ₂ HPO ₄ for K ⁺ and P [±] ..	+	+	+	+

—ve = No Growth. + = Very Poor Growth. ++ = Visible Growth. +++ = Good Growth.
 ++++ = Growth same as that obtained in Complete Synthetic Medium.

NUTRITIONAL REQUIREMENTS OF CHOLERA ORGANISMS

TABLE V

Quantitative Requirement of Amino Acids and Mineral Elements for the Growth of Choleraenic Organisms

	mg/litre of Medium
<i>Amino Acids—</i>	
Aspartic Acid	500
Glutamic Acid	300
Glycine	500
Histidine	200
<i>Mineral Elements—</i>	
Magnesium ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	9.9
Sodium (NaCl)	1965.0
Chloride (NaCl)	3035.0
Potassium (K_2HPO_4)	112.0
Phosphorus (K_2HPO_4)	89.0
Sulphur (MgSO_4 or $(\text{NH}_4)_2\text{SO}_4$)	...
<i>Carbohydrates—</i>	
D-Glucose	1000.0

N.B.—The need of other elements such as Cu, Co, Fe, Mn, Mo, and Zn remained difficult to demonstrate for the amounts required in traces within the range of usual contamination of glasswares, water and salts used for the preparation of medium.

Results

Supplementary Effect of Tryptophan—Tryptophan when supplemented in the medium of casein hydrolysate, the results of growth response for 24 hr. show that tryptophan does not help to enhance growth (Table III). Moreover, at the end of 48 and 72 hr. under stationary condition, it inhibits growth of all the strains of choleraenic organisms by 15 to 20 % of that growth obtained in the medium of plain casein hydrolysate.

Requirement of Amino Acids—The results on qualitative requirement of amino acids presented in Table IV show that only four amino acids namely, aspartic acid, glutamic acid, glycine and histidine are essential

for growth of *V. cholerae*. In absence of all amino acids from the synthetic medium, even containing ammonium sulphate, growth and multiplication of the organisms are not observed. The requirement of histidine appeared to be limited. The results on quantitative requirement of amino acids to obtain maximum growth of choleraenic organisms are given in Table V.

Requirement of Mineral Salts—When calcium and iron salts are omitted from the medium, growth response of the organisms appeared to be limited, while in absence of magnesium salt or sodium chloride or potassium phosphate, growth response is very poor as compared to the growth obtained in the complete synthetic medium (Table IV). Quantitative requirement of mineral salts in presence of four essential amino acids in the medium showed that requirement of calcium and iron is reduced by increasing amount of magnesium sulphate in the medium (23). The results presented in Table V indicate that magnesium and sulphate (10 mg % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), sodium and chloride (0.5 to 1.0 g % NaCl) and potassium and phosphate (50 to 60 mg % K_2HPO_4) are the most essential inorganic elements for the growth of *V. cholerae*. The requirement of ammonium sulphate as a source of nitrogen could not show need but it may act as a source of sulphate in the medium. It is also seen that growth of cholera vibrios is impaired, if concentration of sodium chloride is increased by more than 1.0 % in the medium.

Interrelationship between Essential Amino Acids—Table VI shows the essential amino acids required for growth of choleraenic vibrios. It is observed from the metabolic interrelationship between the essential amino acids that aspartic acid is the most essential, singly or in combination with

other amino acids, for the growth of all the strains. Incorporation of either glutamic acid or glycine in the medium, *V. cholerae*, Ogawa gives 8% growth, while in presence of glycine alone, growth responses of Inaba 569B and *El Tor cholerae*, Ogawa, are 25 and 14 % respectively as compared to the growth obtained in the medium consisting of all essential amino acids. Amino acid in combination with one, two or three gives better growth of all the strains of choleraogenic organisms.

Growth of Choleraogenic Organisms in Casein Hydrolysate and Synthetic Media—The results presented in Table VII show that the growth of choleraogenic strains of vibrios in the elaborated chemically defined medium is 60 to 65% as compared to that growth obtained in the medium of casein hydrolysate (135 mgN/100 ml). However, it is better than other two synthetic media (Table VIII) of Ranta and McLeod (7) and Thind (10) under stationary and shaking conditions.

TABLE VI

The Interrelationship between the Essential Amino Acids required to support the Growth of Choleraogenic Organisms

(Stationary Condition, 48 hrs. at 37°C)

(Average Values of Three Tests)

Amino Acids in Chemically Defined Medium (pH 8.0)	Optical Densities* × 100*			
	<i>Vibrio cholerae</i>		<i>El Tor Cholerae</i>	
	Inaba 569 B	Ogawa S/121/58	Inaba Phil. 6978	Ogawa Phil. 1418
Asp	18	25	32	35
Asp + Glut	30	27	46	39
Asp + Gly	26	30	38	28
Asp + Hist ..	18	24	34	40
Asp + Glut + Gly ..	32	28	46	40
Asp + Glut + Hist	28	24	42	45
Asp + Gly + Hist	26	31	37	42
Asp + Glut + Gly + Hist	36	35	52	55
Glut	17	03	20	16
Glut + Gly ..	24	18	30	27
Glut + Hist ..	22	18	32	34
Glut + Gly + Hist	25	22	35	28
Gly ..	09	18	24	08
Hist	12	04	18	22
Gly + Hist	23	21	28	29

*Read on Biochem-absorptiometer (Hilger) at Filter 61.

N.B.—Asp=L-Aspartic Acid. Gly=Glycine. Glut=L-Glutamic Acid. Hist=L-Histidine.

TABLE VII

*Growth Response of Choleraenic Organisms in the Formulated Chemically Defined Medium**(Mean Values of Five Different Lots)*

MEDIUM	Optical Densities $\times 100^*$			
	<i>Vibrio Cholerae</i>		<i>El Tor Cholerae</i>	
	Inaba 569 B	Ogawa S/121/58	Inaba Phil. 6978	Ogawa Phil. 1418
<i>Stationary Condition, 48 hrs. at 37°C—</i>				
Casein Hydrolysate ..	52	51	62	67
Ranta & McLeod (1950)	26	24	29	32
Thind (1961)	31	28	36	35
Formulated	33 (65%)	31 (61%)	38 (61%)	40 (60%)
<i>Shaking Condition (100 r.p.m.), 24 hrs. at 37°C—</i>				
Casein Hydrolysate ..	55	54	73	72
Ranta & McLeod (1950)	28	29	38	36
Thind (1961)	30	29	38	40
Formulated	35 (63%)	34 (63%)	48 (65%)	46 (64%)

*Read on Biochem-absorptiometer (Hilger) at Filter 61.

Figures in the paranthesis denote the per cent growth as compared to the growth obtained in casein hydrolysate.

TABLE VIII

Chemical Composition of Synthetic Media

CHEMICAL COMPOUND	g/litre of the Medium (pH 8.0)		
	Thind	Ranta & McLeod	Formulated
Sodium Chloride ..	10.00	5.00	5.00
Ammonium Sulphate ..	1.00		1.00
Magnesium Sulphate (Hydrated)			0.10
Magnesium Sulphate (Anhydr.)		0.10	
Magnesium Chloride (Anhydr.)	0.10		
Potassium Phosphate (Dibasic)	1.00	0.75	0.50
Sodium Thiosulphate ..	0.10		
Asparagine	0.50	0.42	
Aspartic Acid			0.50
Glutamic Acid	0.50		0.30
Glycine		0.51	0.50
Histidine	0.50		0.20
Tyrosine		0.67	
Hypoxanthine	0.50		
D-Glucose	1.00	10.00	1.00

Discussion

The medium of casein hydrolysate is routinely used to grow the virulent strains of *Vibrio* and biotype *El Tor cholerae* for the large scale production of cholera vaccine. The casein hydrolysate medium (135 mg N/100 ml) was, therefore, thought to be the standard medium which satisfies the specific nutrient needs of the organisms. In the first instance, the medium was analysed completely for amino acids and mineral cations and anions to establish the actual nutritional requirements of cholera causing organisms.

It will be seen from the chemical composition of casein hydrolysate medium that tryptophan is totally destroyed by the digestion of casein with strong mineral acids. In order to improve the growth response of cholera organism, the medium was supplemented with tryptophan (24). The results show that tryptophan added in the medium suppresses the growth of all the strains of cholera vibrios. This indicates that supply of tryptophan is not required but it can be synthesised into the cell from the nutrients present in the medium.

The choice of casein hydrolysate or peptone or meat extract for the growth of *V. cholerae* is customary and no systematic studies were carried out so far. Based on the analysis of amino acids and mineral elements, a synthetic medium containing seventeen amino acids and eight mineral elements as present in the medium of casein hydrolysate was prepared. Qualitative and quantitative requirements of amino acids and minerals of choleraogenic organisms are determined by using this medium. The standard procedure of omission of single nutrient at a time was used. It is observed that in absence of all amino acids from the medium, growth of organisms is not possible. This confirms the reported findings

(10) that inorganic nitrogen is not utilized by *V. cholerae* for their multiplication and growth. The requirement of amino acids shows that only aspartic acid, glutamic acid, glycine and histidine are essential to obtain maximum growth of the organisms. The minimum requirement of these essential amino acids was ascertained by the supplementation of each in graded amounts in the synthetic medium. Such procedure of finding out the nutritional requirement of amino acid can not indicate the role of amino acids interrelated to each other in the growth process of the organisms. The determination of such inter-relationship was best done by omitting one, two and three essential amino acids in all possible combinations. The growth in terms of optical densities shows that only aspartic acid is absolutely essential, while glutamic acid, glycine and histidine required to enhance the growth of cholera vibrios. Other amino acids required to build-up the cellular structure appeared to be synthesized from the essential amino acids by the way of oxidative transamination. In support to the absolute requirement of aspartic acid of *V. cholerae*, the authors (11) demonstrated that aspartic acid is oxidised at a much faster rate than asparagine and threonine by the virulent strains of *V. cholerae* during the respiration period of 60 and 120 min. at 38.5°C. Though they summarized in their report that cystine, threonine and asparagine were oxidized at a faster rate, these amino acids could be synthesized from aspartic acid by the organisms.

Though the vibrios are provided with amino acids and glucose, they do not multiply in the medium devoid of all the mineral elements. The results show that magnesium, potassium, phosphate, sulphate and chloride are the most essential cations and anions for the multiplication of

the cells, while sodium, calcium and iron are required to promote growth of cholera vibrios. The requirement of iron is so small that its presence as impurity with other salts is enough to stimulate the growth of the organisms. The requirement of sodium chloride is the function of individual ion supplementation in the medium. It is stated in the report (25) that chloride ion probably plays a role in physiology of the organism; whereas, sodium ion environment is essential to maintain the integrity of the cell.

Apart from the requirement of amino acids and mineral salts, all B-vitamins, nucleic acid precursors, glycerol, Tween 80 etc. were also tried to increase growth response of the organisms. The experiments showed that none of these ingredients are required to promote the growth. It appears that B-vitamins and nucleic acid precursors could be synthesized by the vibrios from the amino acids and anionic parts of the mineral salts supplemented in the medium. Aspartic acid is one of the well-known precursors for the biosynthesis of coenzyme A (26) and aspartic acid,

glutamic acid and glycine are the main precursors required for the biosynthesis of purines and pyrimidines (27).

Thus, after evaluating about 95 inorganic and organic chemical compounds, the nutritional needs of cholera vibrios are assessed and a chemically defined medium is formulated. However, growth response of the organisms in this medium is poor as compared to that of casein hydrolysate medium, but it seems better than other two synthetic media tested (Table VIII). The results indicate that requirement of amino acids may be relative to the amino acid mixture present in the natural protein molecule.

Acknowledgement

Author wishes to express his deep sense of gratitude to Dr. S. D. Ambegaokar, former Assistant Director, Biochemistry Department, for his valuable guidance. He also wishes to record his thanks to Dr. B. B. Gaitonde, Director and Mr. Y. S. Nimbkar, Deputy Director of the Haffkine Institute, Bombay for their interest and encouragement.

REFERENCE

- 1 Read, W.D.B.; Singh, G.; Seal, S. C. and Bose, S. (1939)—Growth and Survival of *V. cholerae* with Special Reference to Growth and Survival in Water. *Ind. Jour. Med. Res.*, **27**, 1–40.
- 2 Sokhey, S. S.; Habbu, M. K. and Bharucha, K. H. (1950)—Hydrolysate of Casein for the Preparation of Plague and Cholera Vaccines. *Bull. W. H. O.*, **3**, 25–31.
- 3 Sokhey, S. S. and Habbu, M. K. (1950)—Casein Hydrolysate Cholera Vaccine. *Bull. W. H. O.*, **3**, 33–42.
- 4 Koch, R. (1884)—*Dtsch. Med. Wschr.*, **10**, Passim.
- 5 Anderson, C. G. (1946)—An Introduction to Bacteriological Chemistry, Livingston Ltd., 2nd Ed., Edinburgh, p. 83–97.
- 6 Jenning, R. K. and Linton, R. W. (1944)—The Biochemistry of *Vibrio cholerae*, II: The Influence of Environmental Factors on Growth. *Arch. Biochem.*, **3**, 429–35.
- 7 Ranta, L. E. and McLeod, M. (1950)—*Vibrio cholerae* in Fluid Media. *Canad. J. Res. (E)*, **28**, 257–62.
- 8 Saxena, K. C.; Bhaskaran, K.; Agarwala, S. C. and Shrivastava, D. L. (1953)—A Simple Medium for the Growth of *Vibrio cholerae*. *J. Sci. Industr. Res.*, **12-B**, 34–35.
- 9 Bhaskaran, K. and Rowley, D. (1956)—Nutritional Studies on *Vibrio cholerae*. *Jour. Gen. Microbiol.*, **15**, 417–22.
- 10 Thind, K. S. (1961)—A Synthetic Medium for *Vibrio cholerae*. *Ind. Jour. Med. Res.*, **49**, 189–93.

- 11 Agarwala, S. C.; Krishna Murti, C. R. and Shrivastava, D. L. (1953)—Studies in Enzyme Make-up of *Vibrio cholerae*, III : Oxidative Metabolism of Vibrios. *J. Sci. Industr. Res.*, **12-B**, 325-29.
- 12 Finkelstein, R. A. and LosPalluto, J. J. (1970)—Session III : Production, Purification and Assay of Cholera Toxin—Production of Highly Purified Cholera toxin and Cholera toxinogen. *J. Infect. Dis.*, **121** (Suppl.), S. 63-72.
- 13 Shinde, V. P.; Ambegaokar, S. D. and Nimbkar, Y. S. (1975)—A Simple Commercial Method of Preparation of the Casein Hydrolysate Medium for the Cultivation of Cholera toxinogenic Vibrios. *Bull. Haff. Inst.*, **3**, 119-27.
- 14 Spies, J. R. and Chamber, D. C. (1951)—Spectrophotometric Analysis of Amino Acids and Peptides with their Copper Salts. *J. Biol. Chem.*, **191**, 787-97.
- 15 Spies, J. R. and Chamber, D. C. (1952)—An Ultraviolet Spectrophotometric Micro Method for Studying Protein Hydrolysis. *J. Biol. Chem.*, **192**, 65-74.
- 16 Moore, S.; Speckman, D. H. and Stein, E. R. (1958)—Chromatography of Amino Acids on Sulfonated Polystyrene Resins : An Improved System. *Anal. Chem.*, **30**, 1185-90.
- 17 Henderson, L. M. and Snell, E. E. (1948)—A Uniform Medium for Determination of Amino Acids with Various Microorganisms. *J. Biol. Chem.*, **172**, 15-29.
- 18 Troil, W. and Lindsley, J. (1955)—A Photometric Method for the Determination of Proline. *J. Biol. Chem.*, **215**, 655-60.
- 19 A. O. A. C. (1970)—Official Methods of Analysis, Association of Official Analytical Chemistry, 11th Ed., Publ., A. O. A. C., Washington, D. C., p. 35-6, 44-5 and 34-5.
- 20 Henry, J. R. (1954)—Clinical Chemistry : Principles and Techniques, Harper and Row Publishers, New York, p. 381-93 & 352-55.
- 21 Venkataraman, A. (1964)—Estimation of Sodium in Serum and Other Biological Fluids. *J. Sci. Industr. Res.*, **13-B**, 713-18.
- 22 Kolmer, J. A.; Spaulding, E. H. and Robinson, W. H. (1951)—Approved Laboratory Technic (Volumetric Determination of Chloride). 5th Ed., Appleton—Century—Croft, Inc., New York, p. 1003-05.
- 23 Shinde, V. P. (1974)—Nutritional Requirements of Cholera Organisms, Ph.D. Thesis entitled ' Studies on Nutritional Requirements of Cholera Organisms ', Bombay University, p. 26-70.
- 24 Block, R. J. and Weiss, K. W. (1956)—Amino Acid Hand Book, Part B, The Amino Acid Composition of Proteins, Publ. Springfield, Illinois, U.S.A., p. 306-27.
- 25 Modak, M. J.; Shinde, V. P. and Bhide, M. B. (1970)—Chloride Dependent Growth Stimulation of *Vibrio cholerae* (569B Inaba). *Indian J. Exp. Biol.*, **8**, 342-43.
- 26 Shive, W. (1950)—The Utilization of Antimetabolites in the Study of Biochemical Processes in Living Organisms, *Annal. N. Y. Acad. Sci.*, **52**, 1212-34.
- 27 Greenberg, D. M. (1961)—Metabolic Pathways, Vol. 2, 2nd Ed., Acad. Press, New York and London, p. 389-457.

■ ■

EFFECTS OF DRUGS ON RED CELL METABOLISM : 'IN VITRO' STUDIES

H. K. SONI*, A. J. BAXI† AND A. B. VAIDYA‡

An attempt has been made to develop an *in vitro* model for screening drugs with possible haemolytic effects using various biochemical, haematological and morphological parameters of normal and G-6-PD deficient red cells.

Two distinct types of mechanisms are seen when potentially haemolytic drugs are used: (i) Oxidative (or peroxidative) changes predominate in the first type. Drugs such as acetylphenylhydrazine (APH) menadione and ascorbic acid produce prominently oxidative changes designated by high levels of methemoglobin and low levels of reduced glutathione. (ii) Drug-membrane interaction in which the changes are offset by increased osmotic fragility and plasma haemoglobin. Oxidative (or peroxidative) changes are conspicuously absent: primaquine is represented in this type.

The *in vitro* model is a simple sensitive and reproducible technique. Adequate information regarding the mode of hemolysis can be obtained in a short period.

Introduction

Adverse effects of drugs on the blood assumed significant proportion in last two decades (1-5). Though several hundred drugs have been suspected to be haematotoxic, the direct causal relationship and identifiable pathogenic mechanisms have not been established except in a small number. The latter include genetically determined red cell enzyme defects predisposing the subjects to drug induced haemolytic anemias (2, 3).

Few reports have appeared recently on *in vitro* effects of drugs on red cell metabolism (4, 5). These investigations are restricted either to a study of a single effect or to a limited number of parameters in the

red cell. We now present comprehensive data on biochemical, haematological and morphological changes in normal and glucose-6-phosphate dehydrogenase (G-6-PD) deficient red cells after incubation with drugs in an attempt to develop an *in vitro* model.

Material and Method

Heparinised blood from normal and G-6-PD deficient human donors was used for the major study. Healthy sheep blood which has been shown to be G-6-PD deficient (6), was also employed for a parallel investigation. Incubation with the drugs was carried out at 37°C for 3 hr. with occasional agitation. Details of the drugs and concentrations used are given in Table I.

Solutions of drugs were prepared and dispensed in 25 ml Erlenmeyer conical flasks. Approximate quantities of the drugs were dried in the oven at 80°—90°C and stored in dark until used.

(Received for publication April 8, 1977)

*Haffkine Institute, Parel, Bombay 400 012.

†Blood Group Reference Centre, Seth G. S. Medical College, Bombay 400 012.

‡Ciba-Geigy Research Centre, Goregaon, Bombay 400 063.

cell G-6-PD levels, there was marked G-6-PD instability, increase in methemoglobin level and decrease in GSH-Px activity at 20 mM APH concentration; catalase activity was slightly reduced. However, marked changes such as increase in methemoglobin level were observed in G-6-PD deficient erythrocytes. Fall in catalase and GSH-Px activity was steep and averaged about 20–30% of the normal activity.

At higher concentration changes were more marked with G-6-PD deficient cells (Table IV).

Menadione—Menadione produced drastic changes in various parameters studied in normal and G-6-PD deficient red cells (Tables V and VI).

Unlike APH, menadione at 4 mM concentration lowered G-6-PD activity to nearly 15 % of the normal. Methemoglobin increase was enormous while catalase and GSH-Px markedly decreased.

Primaquine—Changes produced in normal and G-6-PD deficient red cells after incubation with primaquine are presented in Tables VII and VIII.

Osmotic fragility curves also demonstrated from highly increased fragility to total hemolysis in G-6-PD deficient red cells after primaquine treatment, while no changes were apparent with APH and menadione (*see* Fig 1).

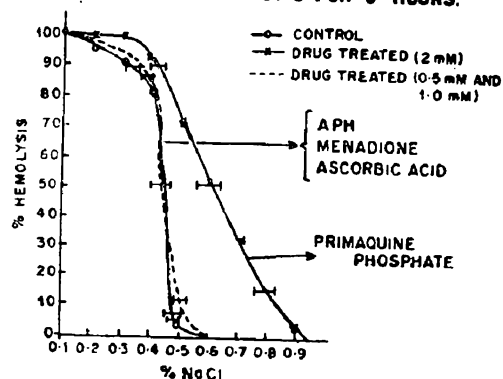
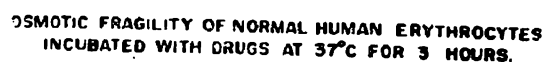
Concentration of Drugs used in the Investigation

Drug	Solvent	Concentration(s) used per ml of blood
Acetylphenylhydrazine (APH).	Ethanol	10 mM to 40 mM.
Menadione	Ethanol	1 mM to 4 mM.
Ascorbic Acid	Water	10 mM to 50 mM.
Primaquine Phosphate.	Water	1 mM to 3 mM.

Effects of various drugs on the biochemical changes in normal and sheep erythrocytes are shown in Table II.

It will be seen that the changes in sheep erythrocytes did not significantly differ from those in normal human erythrocytes. Original thinking that the glucose-6-phosphate dehydrogenase deficient cells may be helpful in the development of the *in vitro* model for screening drugs became no longer tenable.

Acetylphenylhydrazine—Results outlined in Table III indicate that while there were appreciable changes in normal red



Blood smear study for the presence of inclusion bodies revealed that a smaller number of cells contained such bodies after incubation with 1.5 mM primaquine phosphate and 40 mM APH.

Discussion

Despite intensive studies on the probable mechanism of hemolysis, the problem has defied a clear solution. Oxidative processes leading to the formation of hydrogen peroxide have been implicated in red cell aging and destruction (14). Since drug induced haemolytic anemia is intimately associated with G-6-PD deficiency, one of the commonest and widespread of enzymopathies, a clear understanding of associated biochemical events would be of great importance. Thus an *in vitro* system recording simultaneous biochemical, haematological changes has been devised.

Two modes of action of drugs on the red cells were demonstrable. APH and menadione produced marked oxidative changes probably through the formation of hydrogen peroxide. Inhibition of catalase, GSH-Px and to a less extent G-6-PD by these drugs was observed. Increased level of methemoglobin appeared to be one of the terminal changes leading to prehemolytic stage. Absence of increased osmotic fragility and plasma haemoglobin points clearly to the facts that the red cells become extremely vulnerable in this oxidised environment while primaquine appears to interact with membrane directly producing hemolysis on the basis of increased osmotic fragility coupled with absence of oxidative changes in our studies. Our findings are independently supported by those of Bennet and Desforges (5) who also failed to demonstrate any change in the enzymes of pentose phosphate pathways, GSH and methemoglobin in their *in vitro* studies with quinine. These authors proposed that

quinine had no intracellular role to play but the deleterious effects of the drug may be directly on the stroma. Primaquine may have similar effects to quinine.

However, Szeinberg and Marks (17) have shown that variety of compounds such as cysteine, ascorbic acid, primaquine, APH and other stimulate the rate of oxidation in normal red cells, G-6-PD deficient cells did not exhibit similar oxidative changes. On the other hand, Fraser *et al.* (18), who extended the findings of Brodie and Udenfriend (19) were able to demonstrate that 8-amino 5, 6, quinoline-diol (AQD), a metabolite of primaquine, induced hemolytic changes at concentrations much lower than that of primaquine. Changes in osmotic fragility in the present studies are at variance with those of Hopkins and Tudhope (4) who employed mechanical fragility as a physiological maker to observe hemolytic effects. However, changes in GSH-Px inhibition following drug incubation in above study are in agreement with our findings.

Observation on osmotic fragility in the present investigation paralleled those of Miller and Smith (15), indicating primaquine membrane interaction. Whether the hypothesis of Steinbuch and Quentin (16) on the effect of the drugs on the membrane due to cations from the drug forming complexes with the anionic groups of stromal cephalines operates in case of primaquine remains to be elucidated.

In conclusion, we feel that the *in vitro* model devised by us to study the hemolytic effects of drugs has certain advantages for practical application. It is simple, reproducible and clearly defined model. The demonstration of two different mechanism of action of hemolytic process, oxidative as well drug membrane

interaction, gives more credence to the success of such a model.

Acknowledgement

The authors are thankful to Dr. M. B.

Bhide, Assistant Director for advice and interest. Thanks are also due to Dr. B. B. Gaitonde, Director, Haffkine Institute for providing the facilities to carry out this work.

TABLE II

Effect of Drugs on Normal Human and Sheep Erythrocytes incubated at 37°C for 3 hours

Group	Normal Human Erythrocytes		Sheep Erythrocytes	
	GSH mg/100 ml RBCs	%Methemo- globin	GSH mg/100 ml RBCs	%Methemo- globin
Control	80.5 ± 11.0	0.65 ± 0.3	76.0 ± 12.0	0.70 ± 0.30
10 mM APH	60.0 ± 10.0	20.0 ± 3.0	66.0 ± 11.0	8.0 ± 0.30
10 mM Ascorbic Acid	63.5 ± 14.0	12.6 ± 5.0	73.2 ± 16.0	9.5 ± 6.00
4 mM Menadione	10.0 ± 6.0	60.0 ± 8.0	16.0 ± 8.0	52.0 ± 10.00
1 mM Primaquine Phosphate	78.0 ± 11.0	0.65 ± 0.3	77.0 ± 11.0	0.80 ± 0.30

TABLE III

Biochemical Changes in Normal Human Blood incubated at 37°C with APH for 3 hours

Concentration of drug			GSH mg/100 ml RBCs	G-6-PD units/min./ml RBCs	%of Methemo- globin	Catalase K*units/sec.	GSH Peroxidase K*/gm/Hb/ min.
Control	..	..	80.5 ± 8.5	3.4 ± 0.5	0.65 ± 0.35	6.5 ± 0.7	58.8 ± 6.0
10 mM	..	..	60.0 ± 7.0	3.3 ± 0.3	20.4 ± 3.0	6.4 ± 0.6	48.9 ± 7.4
20 mM	..	..	51.6 ± 5.6	3.3 ± 0.3	37.1 ± 3.9	5.9 ± 0.4	26.8 ± 3.8
30 mM	..	..	42.1 ± 6.6	3.5 ± 0.5	54.5 ± 9.5	5.05 ± 0.65	13.6 ± 2.7
40 mM	..	..	19.7 ± 5.3	1.56 ± 0.3	65.2 ± 12.0	4.8 ± 0.7	8.1 ± 2.2

•

TABLE IV

Biochemical Changes in G-6-PD Deficient Blood incubated with APH at 37°C for 3 hours

Concentration of Drug			GSH units/mg/100 ml RBCs	G-6-PD min/ml RBCs	%of Methemo- globin	Catalase K*units/sec.	GSH Peroxidase K*/gm/Hb/ min
Control	..	..	48.6 ± 8.4	0.0	0.72 ± 0.28	4.1 ± 0.3	54.6 ± 4.8
10 mM	..	..	35.2 ± 2.2	0.0	49.9 ± 5.7	2.2 ± 0.3	24.0 ± 5.7
20 mM	..	..	26.5 ± 0.7	0.0	57.3 ± 4.8	1.55 ± 0.2	14.1 ± 3.3
30 mM	..	..	24.6 ± 1.4	0.0	75.0 ± 5.7	1.4 ± 0.13	10.5 ± 0.7
40 mM	..	..	13.3 ± 1.0	0.0	86.5 ± 4.9	1.1 ± 0.11	6.4 ± 2.1

*Activity is expressed as first order reaction constant.

Each mean value is average of six determinations.

TABLE V

Biochemical Changes in Normal Human Blood incubated with Menadione at 37°C for 3 hours

Concentration of Drug	GSH mg/100 ml RBCs	G-6-PD units/min/ml RBCs	% of Methemo- globin	Catalase K*units/sec.	GSH Peroxidase K*gm/Hb/min.
Control ..	76.6 ± 10.5	3.43 ± 0.5	0.71 ± 0.2	6.7 ± 0.4	57.1 ± 4.3
1 mM	36.7 ± 10.0	3.5 ± 0.8	36.0 ± 6.3	5.9 ± 0.65	30.6 ± 3.1
2 mM	28.1 ± 7.1	1.25 ± 0.5	42.9 ± 6.0	4.7 ± 0.7	27.6 ± 2.1
3 mM	20.0 ± 6.2	0.72 ± 0.5	52.1 ± 7.0	4.2 ± 0.4	18.9 ± 4.3
4 mM	9.9 ± 4.6	0.65 ± 0.4	60.0 ± 6.5	3.8 ± 0.4	12.6 ± 3.9

TABLE VI

Biochemical changes in G-6-PD Deficient Blood incubated with Menadione at 37°C for 3 hours

Concentration of Drug	GSH mg/100 ml RBCs	G-6-PD units/min/ml RBCs	% of Methemo- globin	Catalase K*/units/sec.	GSH Peroxidase K*gm/Hb/min.
Control	48.3 ± 7.4	0.0	0.6 ± 0.23	3.9 ± 0.2	48.8 ± 5.0
1 mM	28.4 ± 6.8	0.0	48.4 ± 8.1	2.2 ± 0.2	23.5 ± 3.2
2 mM	18.2 ± 5.8	0.0	57.0 ± 6.8	1.7 ± 0.25	18.8 ± 2.6
3 mM	9.8 ± 3.1	0.0	68.0 ± 7.2	1.1 ± 0.15	12.0 ± 1.6
4 mM	5.6 ± 1.2	0.0	89.4 ± 8.6	0.7 ± 0.1	7.4 ± 0.8

TABLE VII

Biochemical Changes in Normal Human Blood incubated with Primaquine Phosphate at 37°C for 3 hours

Concentration of Drug	GSH mg/100 ml RBCs	G-6-PD- units/min/ml RBCs	% of Methemo- globin	Catalase K*units/sec.	GSH Peroxidase K*gm/Hb/min
Control ..	78.1 ± 9.4	3.45 ± 0.4	0.5 ± 0.3	6.6 ± 0.5	56.6 ± 3.7
0.5 mM ..	76.1 ± 8.0	3.4 ± 0.4	0.8 ± 0.3	6.6 ± 0.5	55.6 ± 3.6
1.0 mM ..	73.7 ± 8.0	3.45 ± 0.4	1.4 ± 0.2	6.5 ± 0.5	53.5 ± 3.6
1.5 mM	70.8 ± 8.5	3.45 ± 0.4	1.6 ± 0.3	6.2 ± 0.04	54.0 ± 3.5
2.0 mM	71.0 ± 9.0	3.5 ± 0.4	1.8 ± 0.3	6.5 ± 0.4	52.0 ± 3.5

*Activity is expressed as first order reaction constant.
Each mean value is average of six determinations.

TABLE VIII

Biochemical Changes in G-6-PD Deficient Blood incubated with Primaquine Phosphate at 37°C for 3 hours

Concentration of Drug	GSH mg/100 ml RBCs	G-6-PD units/min/ml RBCs	% of Methemo- globin	Catalase K*/units/sec.	GSH Peroxidase K*/gm/Hb/min
Control	44.0 ± 7.5	0.0	0.65 ± 0.3	4.2 ± 0.5	56.8 ± 0.9
0.5 mM	40.4 ± 8.5	0.0	0.9 ± 0.3	4.2 ± 0.5	56.0 ± 0.9
1.0 mM	41.6 ± 7.0	0.0	1.1 ± 0.3	4.1 ± 0.4	55.2 ± 1.0
1.5 mM	38.6 ± 8.5	0.0	1.2 ± 0.3	4.1 ± 0.3	54.6 ± 1.0
2.0 mM	38.0 ± 9.0	0.0	1.6 ± 0.2	4.2 ± 0.3	53.4 ± 1.0

*Activity is expressed as first order reaction constant.
Each mean value is average of five determinations.

TABLE IX

Plasma Hemoglobin after Incubation with Drugs at 37°C for 3 hours

Drug	Control mg of Hb/100 ml Plasma		Drug treated blood mg of Hb/100 ml Plasma	
<i>Normal—</i>	<i>Human</i>	<i>Erythrocytes</i>		
40 mM APH ..	0.1 ±	0.02	0.1 ±	0.02
50 mM Ascorbic Acid	0.1 ±	0.02	0.1 ±	0.02
5 mM Menadione	0.1 ±	0.04	0.18 ±	0.04
1 mM Primaquine Phosphate	0.1 ±	0.02	0.1 ±	0.02
2 mM Primaquine Phosphate	0.1 ±	0.03	230.0 ±	50.0
3 mM Primaquine Phosphate	0.1 ±	0.03	800.0 ±	60.0
<i>G-6-PD Deficient Human Erythrocytes—</i>				
40 mM APH ..	0.1 ±	0.02	0.1 ±	0.02
1 mM Primaquine Phosphate	0.12 ±	0.03	0.12 ±	0.02
2 mM Primaquine Phosphate	0.12 ±	0.04	240.0 ±	40.0
3 mM Primaquine Phosphate	0.12 ±	0.04	880.0 ±	60.0

REFERENCES

- 1 Chan, T. K.; Mak, L. W. and Ronald P. Ng. (1971)—Methemoglobinemia, Heinz Bodies and Acute Massive Intravascular Hemolysis in Lysol Poisoning. *Blood*, **38**, 739-44.
- 2 Beutler, E. (1972)—Drug-induced Anemia. *Fed. Proc.*, **31**, 141-46.
- 3 Baxi, A. J. (1972)—Hereditary Red Cell Enzyme Defects and Hemolytic Anemia. *Jour. J. J. Group Hosp.*, **17**, 119-27.
- 4 Hopkins, G. R. and Tudhope, J. (1974)—Lipid Peroxidation in Erythrocytes. *Acta. Haemat.*, **51**, 29-39.
- 5 Bennett, J. M. and Deforges, J. F. (1967)—Quine-induced Hemolysis : Mechanism and Action. *Brit. J. Haemat.*, **13**, 706-14.
- 6 Khanolkar, V. R.; Naik, S. N.; Baxi, A. J. and Bhatia, H. M. (1963)—Studies on Haemoglobin Variants and Glucose-6-Phosphate Dehydrogenase in Indian Sheep and Goat. *Experientia.*, **19**, 472-74.
- 7 World Health Organisation: Technical Report Series (1967)—Standardisation of Procedure for the Study of Glucose-6-Phosphate Dehydrogenase, No. 366.
- 8 Beutler, E.; Duron, O. and Kelly, B. M. (1963)—Improved Method for the Determination of Blood Glutathione. *J. Lab. Clin. Med.*, **61**, 882-88.
- 9 Takahara, S.; Hamilton, H. B.; Neel, J. V.; Kobaras, J. Y.; Orura, Y. and Hishimura, E. (1960)—Hypocatalasemia : A New Genetic Carrier State. *J. Clin. Invest.*, **39**, 610-19.
- 10 Gross, R. T.; Bracci, R.; Rudolph, N.; Schroeder, E. and Cohen, J. A., (1967)—Hydrogen Peroxide Toxicity and Detoxification in Erythrocyte of Newborn Infants. *Blood*, **29**, 481-93.
- 11 Evelyn, K. A. and Malloy, H. T. (1938)—Microdetermination of Oxyhemoglobin, Methemoglobin and Sulfhemoglobin in a Single Sample of Blood. *J. Biol. Chem.*, **126**, 655-62.
- 12 Laboratory Manual of Pediatric Micro and Ultramicro-Biochemical Techniques : Third Edition (1962), Edited by Donough O'brient and Frank, A.; Ibbot, Harper and Row Publishers.
- 13 Parpart, A. K.; Lorenz, P. Z.; Parpart, E. R.; Gregg, J. R. and Chase, A. M. (1974)—The Osmotic Resistance of Human Red Cells. *J. Clin. Invest.*, **26**, 636-40.
- 14 Jandl, J. H.; Engle, I. K. and Allen, D. W. (1970)—Oxidative Hemolysis and Precipitation of Hemoglobin I : Heinz Body Anaemias as an Acceleration of Red Cells Aging. *J. Clin. Invest.*, **39**, 1818-1936.
- 15 Miller, A. and Smith, H.C. (1970)—The Intra-cellular and Membrane Effects of Oxidant Agents on Normal Red Cells. *Brit. J. Haemat.*, **19**, 417-28.
- 16 Steinbuch, M. and Quentin, M. (1957)—A Propos De globul Rough. *Archies Internationale De Pharmacodynamic Et De Therapie*, 110-10.
- 17 Szeinberg, A. and Marks, P. A. (1963)—Substances stimulating Glucose Catabolism by the Oxidative Reactions of the Pantose Phosphate Pathway in Human Erythrocytes. *J. Clin. Invest.*, **40**, 914-24.
- 18 Fraser, I. M.; Tilton, B. E. and Vesell, E. S. (1971)—Effects of Some Metabolite of Haemolytic Drugs on Young and Old Normal and G-6-PD Deficient Human Erythrocytes., *Ann. N. Y. Acad. Sci.*, **179**, 644-53.
- 19 Brodie, B. B. and S. Udenfriend (1950)—Metabolite of Pamaquine in Urine., *Proc. Soc. Exp. Biol. Med.*, **74**, 845-54.

■ ■

ANAEROBIC BACTERIOLOGY OF SEPTIC WOUNDS

SWATI R. SALVEKAR, S. MUKERJI AND JAYSHREE K. DAVE

*Department of Clinical Pathology, Haffkine Institute
Bombay 400 012*

From anaerobic culture of 305 clinical specimens from pre and post-operative wounds, 41 Peptostreptococci, 36 Bacteroides and 18 Clostridia were isolated. For anaerobic culture, classical H₂-replacement method, chrome-alum, chrome-alum and Na₂CO₃ and GasPak System, were evaluated by colony count method. In drug sensitivity tests (by disc-agar diffusion method) Bacteroides, Peptostreptococci and Clostridia were found to be sensitive to metronidazole, chloramphenicol, tetracycline and erythromycin.

Introduction

Human infection due to anaerobic bacteria is often overlooked. However, with increase in the knowledge of the subject and improvement of anaerobic culture techniques (1, 2, 3, 4, 5, 6, 7, 8, 9), identification and isolation of anaerobes have become less complex and more frequent, respectively. Among the anaerobes, infection due to the family of Bacteroidaceae has become increasingly important, as the literature relevant to the subject indicates (10, 11, 12, 13, 14, 15). In India, barring a few reports (16, 17, 18), very little work has been done on this subject. The Clinical Pathology Department of the Haffkine Institute, Bombay, has done some preliminary work on this subject. This is being presented in this article.

Material and Method

Sources of Materials—These are detailed in Table I.

Bacteriological Procedures—The materials collected in swabs were inoculated immediately into Robertson's cooked-meat broth which was boiled for 5 min. and cooled prior to inoculation. The inoculated

tubes were incubated at 37°C for 48 hr. and sub-cultured on two sets of blood (7% sheep) agar containing 100 µg/ml of neomycin; one set was incubated for 48 hr. and the other for 10 days, anaerobically. Colonies of suspected anaerobes were put in Robertson's cooked-meat broth for 24 hr., checked for purity, aerobic and anaerobic growth on blood agar and finally identified on the basis of biochemical characters (13, 19).

Anaerobic Methods—Four methods were tested before use in this study. These were:

- (i) Anaerobic jars working with cold catalyst (Gallenkamp), were evacuated, flushed thrice and finally replaced with hydrogen;
- (ii) As in (i) up to evacuation but H₂ supplied endogenously by chrome-alum method (20);
- (iii) As in (i) up to evacuation but H₂ and CO₂ supplied endogenously by chrome-alum and Na₂CO₃ (20), respectively and
- (iv) GasPak system (BBL).

The comparative efficacy of these methods was tested by noting the number of colonies (21) of *B. fragilis*, Peptostreptococcus and Clostridia on blood agar plates at 37°C for 48 hr. (Table II).

TABLE I
Source of the Clinical Materials and Isolation Results of Anaerobic Culture

Sources	Nos.	Organism isolated			
		Bacteroides	Peptostreptococci	Clostridia	
A. Post-operative Wounds—					
Abdominal ..	15	7	5	5	
Anal-fistula ..	7	4			
Hydrocele ..	20	3	1	1	
Fracture ..	12	2		3	
B. Abscesses—					
Lymph Glands	22	2	6		
T. B. Glands	23		3		
Other Sites	22	8	6		
Appendix	21		1		
Liver	35	2	3		
Empyema	11	2			
Kidney	3	1			
Prostate	11	1	2		
C. Deep Wounds and Burns ..	59	2		9	
D. Cervical and Vaginal Infection ..	44	2	14		
Total ..	305	36	41	18	

TABLE II
Colony Counts of Organisms by Various Anaerobic Methods

Strains tested		Nos. of colonies by following method			
Identity	Count per ml	H ₂ gas	Chrome-alum	Chrome-alum + Na ₂ CO ₃	GasPak
Cl. Tetani (A-471)* ..	10 ⁶	112	90	95	100
Cl. Tetani		40	38	42	35
Mean Count/ml		76 × 10 ⁶	64 × 10 ⁶	68 × 10 ⁶	67 × 10 ⁶
Cl. Welchii (PB-6K)* ..	10 ⁶	208	106	112	201
Cl. Welchii		168	170	170	160
Mean Count/ml		18 × 10 ⁶	17 × 10 ⁶	14 × 10 ⁶	18 × 10 ⁶
B. Fragilis	10 ⁷	76	74	80	85
B. Fragilis		85	90	96	102
Mean Count/ml		81 × 10 ⁶	82 × 10 ⁶	88 × 10 ⁶	93 × 10 ⁶
Peptostreptococcus ..	10 ⁶	460	438	480	502
Peptostreptococcus ..		512	520	525	535
Mean Count/ml		48 × 10 ⁶	47 × 10 ⁶	50 × 10 ⁶	51 × 10 ⁶

*Standard strains obtained from Haffkine Institute, Bombay.
Mean count/ml = mean plate count × count per inoculum.

Sensitivity of Drugs—Minimum inhibitory concentration (MIC) of tetracycline, chloramphenicol, erythromycin and metronidazole against *Clostridia*, *Peptostreptococci* and *Bacteroides* was determined by agar dilution method. This followed by drug sensitivity of the isolates (Table IV) by agar diffusion disc method. For both, techniques recommended by Nastro and

Finegold (22) were used and included *Clostridia* isolated from other sources (Table III & IV). For the tests, the isolates were grown overnight in thioglycollate broth (USP), count adjusted to 10^8 per ml (23) and inoculated on blood agar. Similar procedure was followed in the evaluation of the 4 anaerobic methods (Table II).

TABLE III

MIC of Tetracycline (T) and Metronidazole (M)

Name	Nos.	Drugs	Concentration $\mu\text{g/ml}$								G/M
			0.1	0.2	0.4	0.8	1.6	3.2	6.4	12.8	
<i>Clostridia</i> . .	25	T	4	2	6	3	5	2	2	1	1.43
		M	3	6	3	5	3	2	2	1	1.437
<i>Bacteroides</i>	15	T	4	3	2	1	1	1	1	2	1.38
		M	5	3	2	1	2	2	..	..	1.10
<i>Peptostreptococci</i>	15	T	4	4	2	2	..	1	1	1	1.37
		M	5	4	3	..	1	..	2	..	1.16

G/M = Geometric mean.

TABLE IV

Drug Sensitivity Results of Isolates by Disc Diffusion Method

Drugs	Conc. in $\mu\text{g.}$	Bacteria tested					
		<i>Clostridia</i>		<i>Bacteroides</i>		<i>Peptostreptococci</i>	
		Nos	Sens	Nos.	Sens	Nos.	Sens.
Chloramphenicol	50	58	46	17	10	22	22
Erythromycin	2	58	42	17	9	22	17
Tetracycline . .	10	58	48	17	9	22	18
Metronidazole	5	58	38	17	14	22	20
Colistin	10	58		17		22	
Penicillin	1.5(U)	58	..	17		22	
Neomycin	10	58	1	17		22	
Methicillin ..	.. 10	58	1	17	..	22	..

Results

From 305 clinical materials (Table I), 95 anaerobes (29.93%) were isolated which included 41 *Peptostreptococci*, 36 *Bacteroides* and 18 *Clostridia*. Among the *Bacteroides*, 5 were lost during sub-cultures, the remaining 31 were identified as *B. fragilis* (22), *B. melaninogenicus* (7) and *B. oralis* (2) while *C. tetani* (14), *C. sphenoides* (2), *C. ramosum* (1) and *C. welchii* (1) accounted for the 18 *Clostridia*.

In the MIC (Table III) studies, results of metronidazole and tetracycline are being presented as the same concentration of the drugs were employed for the test. The geometric mean value of the two drugs suggested that a lower concentration of metronidazole was required against *Bacteroides* and *Peptostreptococci*. In drug sensitivity test, metronidazole (Table IV) appeared to be more effective on *Bacteroides* and *Peptostreptococci* as compared to *Clostridia* while the reverse results were noted with chloromycetin, erythromycin and tetracycline.

The results of the colony count (Table II) on plates and the ultimate mean counts of the anaerobes derived thereby indicated that the presence of CO₂ in chrome-alum and GasPak System stimulated the growth of *Bacteroides* and *Peptostreptococci*. The growth of *Clostridia* was not affected by CO₂.

Discussion

For the isolation of anaerobes, techniques such as, use of anaerobically sterilized media or transport media along with anaerobic jars or cabinets, have been recommended by various workers (7, 24, 25, 26). According to Watt, Colles and Brown (27)

results equivalent to anaerobic cabinets are obtainable by using pre-reduced media along with anaerobic jars. This has been supported by others (28) who express doubts regarding the optimum requirement of CO₂ or N₂ for the growth of anaerobes (29).

In this study while evaluating four anaerobic methods, CO₂ (5% to 10%) was observed to stimulate the growth of *Bacteroides* and *Peptostreptococci* but not of *Clostridia*. However, as the growth of the former two anaerobes was not markedly inhibited by H₂, so classical H₂ replacement method, chrome-alum and GasPak System were employed for anaerobic culture.

By using three anaerobic methods mentioned earlier, Robertson's cooked-meat broth which acted as a transport and a primary enrichment medium and neomycin blood agar as selective medium, 95 anaerobes were isolated from 305 clinical materials. This isolation rate of about 30% was low as compared to 81% isolation of *Bacteroides* by Leigh (30), and 50% to 60% anaerobes by Gillespie and Guy (31) from post-operative abdominal septic wounds after major intestinal surgery and intra-abdominal septic wounds. In this study, as the samples from the above areas were less in number; so further study is required before the importance of anaerobic or aerobic infection of that site can be assessed. As regards the drugs, metronidazole appeared to be more effective against *Bacteroides* and *Peptostreptococci* as compared to chloramphenicol, erythromycin and tetracycline (32, 33).

The Institute is continuing further study specially in the field of post-operative wound infections.

Acknowledgement

We are thankful to May and Baker (India) Private Ltd., Bombay, for the necessary financial assistance for this research project, to Mr. N. Nandi and Dr. A. Ganguly of the above concern for taking active interest in the work and to Dr. B. B. Gaitonde, Director of Haffkine Institute, Bombay, for allowing us to publish the

results of this work and also to Shri A. S. Arekar, Statistician of Haffkine Institute for his assistance.

We also express our thanks to Dr. D. S. Pardhanani, Professor of Surgery, G. S. Medical College and K. E. M. Hospital and Dr. P. Biswas, D.M.O., Central Railway Hospital for the generous supply of clinical materials.

REFERENCES

- 1 Hungate, R. E. (1950)—The Anaerobic Mesophilic Cellulolytic Bacteria. *Bact. Rev.*, **14**, 1–49.
- 2 Lowbury, E. J. L. and Lilly, H. A. (1955)—A Selective Medium for *C. Welchii*. *J. Path. Bact.*, **70**, 105–09.
- 3 Finegold, S. M.; Siewert, L. A. and Hewitt, W. L. (1957)—Simple Selective Media for Bacteroides and Other Anaerobes. *Bact. Proc.*, 59.
- Finegold, S. M. (1968)—Infection due to Anaerobes. *Med. Times*, **96**, 174–77.
- 4 Stokes, E. J. (1958)—Anaerobes in Routine Diagnostic Culture. *Lancet*, **i**, 668–70.
- 5 Smith, L. D. S. and Holdman, L. V. (1969)—The Pathogenic Anaerobic Bacteria, Charles C. Thomas Co., Springfield.
- 6 Moore, W.E.C.; Cato, E. P. and Holdman, L. V. (1969)—Anaerobic Bacteria of Gastro-intestinal Flora and Their Occurrence in Clinical Infections. *J. Inf. Dis.*, **119**, 641–49.
- 7 Attebery, H. R. and Finegold, S. M. (1969)—Combined Screw-cap and Rubber-stopper Closure for Hungate Tubes (Pre-reduced Anaerobically Sterilized Roll-tubes and Liquid Media). *Appl. Microbiol.*, **18**, 558–61.
- 8 Suttar, V. L. and Finegold, S. M. (1971)—Antibiotic Susceptibility Tests for Rapid Presumptive Identification of Gram-negative Anaerobic Bacilli. *Appl. Microbiol.*, **21**, 13–20.
- 9 Gorbach, S. L. and Bartlett, J. G. (1974)—Anaerobic Infections. *New Eng. J. Med.*, **May**, **30**, 1237–45.
- 0 Tilloston, J. R. and Lerner, A. M. (1968)—Bacteroides Pneumonia Characteristics of Cases of Empyema. *Arch. Int. Med.*, **68**, 308–17.
- 1 Rothra, E. B. and Schick, S. F. (1969)—Non-clostridial Anaerobic Bacteria in Septic Abortion. *Amer. J. Med.*, **46**, 80–89.
- 2 Okubadejo, O. A.; Green, P. J. and Payne, D.J.H. (1973)—Bacteroides Infections among Hospital Infections. *Br. Med. J.*, **2**, 212–14.
- 3 Mitcheel, A.A.B. (1973)—Incidence and Isolation of Bacteroides Species in Clinical Material and Sensitivity to Antibiotics. *J. Clin. Path.*, **26**, 738–41.
- 4 Lancet (1973)—Bacteroides in Blood. *Jan.*, 27–28.
- 5 Bartlett, J. G. and Finegold, S. M. (1974)—Anaerobic Infection of the Lung and Pleural Fluid. *Amer. Rev. Resp. Dis.*, **110**, 57.
- 6 Dhayagude, R. G. and Purandare, N. M. (1949)—Study on Anaerobic Wound Infections. *Ind. J. Med. Res.*, **37**, 283–92.
- 17 Sen, R.; Saxena, S. N. and Dasgupta, L. R. (1967)—Anaerobic Streptococci in Pathological Processes with Special Reference to Purpural Sepsis. *Ind. J. Med. Res.*, **55**, 795–805.
- 18 Gupta, U.; Talwar, J. R. and Hingorani, V. (1972)—Anaerobic Bacteria isolated from Pyogenic Lesion. *Ind. J. Med. Res.*, **60**, 1557–63.

- 19 Suttar, V. L.; Attebery, H. R.; Rosenblatt, J. E.; Bricknell, K. S. and Finegold, S. M. (1972)—Anaerobic Bacteriology, Wilshire & Sawtelle, Blvds., Los Angeles.
- 20 Marshall, J. H. (1960)—The Production of Anaerobic Condition with Chromous Salts. *J. Gen. Microbiol.*, **22**, 645.
- 21 Collee, J. G.; Watt, B.; Elizabeth, B. and Fowler, R. (1972)—An Evaluation of the GasPak System in the Culture of Anaerobic Bacteria. *J. Appl. Bact.*, **35**, 71–82.
- 22 Nastro, L. J. and Finegold, S. M. (1972)—Bactericidal Activity of Five Antimicrobial Agents against *B. Fragilis*. *J. Inf. Dis.*, **126**, 104–07.
- 23 Baur, A. W.; Kirby, W. M. M.; Sherris, J. C. and Turck, M. (1966)—Antibiotic Susceptibility Testing by Standardized Single Disc Method. *Amer. J. Clin. Path.*, **45**, 493–96.
- 24 Moore, W. E. C. (1966)—Techniques for the Anaerobic Culture of Fastidious Anaerobes. *J. System Bact.*, **16**, 173–98.
- 25 McMinn, H. T. and Crawford, J. J. (1970)—Recovery of Anaerobic Microorganisms from Clinical Specimens in Pre-reduced Media *versus* Recovery by Routine Clinical Laboratory Methods. *Appl. Microbiol.*, **19**, 207–13.
- 26 Peach, S. and Hayek, L. (1974)—The Isolation of Anaerobic Bacteria from Wound Swab. *J. Clin. Path.*, **27**, 578–82.
- 27 Watt, B.; Collee, J. G. and Brown, R. (1974)—The Isolation of Strict Anaerobes: The Use of Anaerobic Cabinet compared with a Conventional Procedure. *J. Med. Microbiol.*, **7**, 319–24.
- 28 Justasin, T. and Nielson, M. L. (1976)—Survival of Anaerobic Bacteria during Transport, *Acta Path. Microbiol. Scand. (B)*, **84**, 51–56.
- 29 Futter, B. V. and Richardson, G. (1971)—Isolation of Anaerobes, Eds., D. A. Shepton and R. G. Board, Academic Press, London & New York.
- 30 Leigh, D. A. (1974)—Clinical Importance of Infections due to *B. Fragilis*. *B. Med. J.*, **3**, 225–28.
- 31 Gillespie, W. A. and Guy, J. (1956)—Bacteroides in Intra-abdominal Sepsis. *Lancet.*, **i**, 1039–42.
- 32 Wheelan, J. P. F. and Hale, J. H. (1973)—Bactericidal Activity of Metronidazole against *B. Fragilis*. *J. Clin. Path.*, **26**, 393–95.
- 33 Seelcor, J. B.; Hale, J. H. and Ingham, H. R. (1975)—Proceedings of the 9th International Congress of Chemotherapy, Page 277–81, Eds. Williams, J. H. and Gaddes, A. M., Plenum Press, New York & London.

■ ■

ANTIFILARIAL ACTIVITY OF SOME ANTHELMINTIC DRUGS USED IN CLINICAL PRACTICE

USHA A. SONURLIKAR AND M. B. BHIDE

Pharmacology Department, Haffkine Institute, Parel, Bombay 400 012

Anthelmintic drugs viz. tetramisole d & l isomers, mebendazole and betoscanate were tested for antifilarial activity *in vitro* against microfilariae of *L. carinii* and *W. bancrofti* and *in vivo* against *L. carinii* in cotton rats. Antifilarial activity was exhibited by L-tetramisole and mebendazole and not by d-tetramisole and betoscanate.

Introduction

Many synthetic organic compounds have exhibited antifilarial activity *in vitro* and in animals but none of them have been proved to be successful in clinical practice (1, 2, 3, 4, 5). Diethylcarbamazine is still the drug of choice against filariasis due to *W. bancrofti*. An introduction of entirely new molecule is an extremely costly and lengthy process. So as an alternative the drugs already in market as anthelmintics were screened for their antifilarial activity.

Material and Method

Drugs Used—(1) Tetramisole (Ethnor), (2) Mebendazole (Ethnor), (3) Betoscanate (Hoechst), (4) Diethylcarbamazine (Lederle).

In Vitro Screening

Against Microfilariae of 'L. carinii'—Microfilariae of *L. carinii* were separated from the blood and were maintained in the medium containing Tyrode with 20% horse serum (6). Small test tube were used in place of capillary tubes for maintaining the microfilariae. The microfilariae were incubated at 28°C. Drugs insoluble in distilled water were used by preparing fine

suspension with 1% Gum Accacia. Microfilariae were observed for 72 hr. at 24 hr. interval. To see the effect of the drug, every time 0.1 ml of the solution was taken on the slide and the number of microfilariae with their motility were noted.

Motility was noted as given below:

- +++ Microfilariae show good motility.
- ++ Microfilariae show reduced motility.
- + Microfilariae show very sluggish movements.
- Microfilariae do not show any movements.

The concentration at which all the microfilariae became non-motile was considered as effective concentration. All work was carried out under sterile condition.

Against Microfilariae of 'W. bancrofti'—Microfilariae of *W. bancrofti* were separated from blood and were maintained on Vero cell line (7). Microfilariae were incubated with the different concentrations of the drug at 37°C. Activity of the compounds was noted for 96 hr., at 24 hr. interval as described for microfilariae of *L. carinii*.

In Vivo Screening

Against 'L. carinii' in Rats—Cotton rats (*Sigmodon hispidus*) infected with *L. carinii* were supplied by department of Zoonosis. White rats were used when cotton rats were not available. Rats were infected with *L. carinii* according to the method described by Hawking *et al.* (8).

Method for counting the Microfilariae—From the tail of the rat 0.01 ml of the blood was collected and a smear was prepared over a circle of 1 cm radius. The smear was dried on the horizontal surface of uniform level to get the equal distribution of microfilariae. The microfilariae along the diameter were then counted and multiplied by the factor to give the microfilariae/10 c mm.

Method of Screening—For drug screening the infected rats were used 15 to 30 days after they become microfilariae positive. Rats showing microfilaria count more than 100 mf/10 mm³, were selected for screening.

Action against Microfilariae—Microfilaria counts were taken on the day of starting the drug treatment and then twice in a week for 4 weeks. Microfilaria count on the day of starting the drug treatment was considered as 100% and the rest of the counts were expressed in percentage. Mean of percentage microfilaria count from all the rats per group was calculated and standard error was calculated.

Action against Adult Worms—Rats were sacrificed to see the action of the compounds on adult worms. At the end of 4 weeks the rats were killed and the worms in the pleural cavity were observed by keeping them in normal saline at 37°C. The number of alive and dead worms and male and female worms were counted. Calcification of the worms was also noted.

The compounds were administered orally in volume of 1 ml/100 gm of body weight for 6 consecutive days. Six rats were used per group. A group of rats was used as a untreated control in each experiment. Diethylcarbamazine was used as a standard drug to compare the results of other drugs at the dose of 6 mg/kg/day for 6 days.

Results

Diethylcarbamazine (DEC)—DEC at the concentration ranging from 12.5 to 500 µg/ml did not exhibit any action on microfilariae of *L. carinii* *in vitro* (Table I). It was found inactive against microfilariae of *W. bancrofti* at 200 µg/ml concentration up to 96 hr. (Table II). DEC reduced the microfilarial count upto 6% during the period of treatment, which was then increased to 96% of the original counts in 4 weeks after the treatment (Figure 1). DEC did not exhibit appreciable action on the adults of *L. carinii* (Table III).

TABLE I

In Vitro Activity against Microfilariae of L. carinii

Drug	Activity	Conc. µg/ml	Period in hr.
Hetrazan	Inactive ..	12.5	72
	Inactive ..	25	72
	Inactive ..	50	72
	Inactive ..	500	72
l-Tetramisole ..	Active	50	48
	Active	25	72
d-Tetramisole ..	Active	500	48
	Inactive ..	250	72
	Inactive ..	50	72
Mebendazole	Inactive ..	50	72
	Inactive ..	25	72

TABLE II

In Vitro Activity of Drugs against Microfilariae of *W. bancrofti*.

Drug	Activity	Conc. $\mu\text{g/ml}$.	Period in hr.
Hetrazan	Inactive ..	200	96
	Inactive ..	100	96
	Inactive ..	50	96
l-Tetramisole	Active	50	72
	Inactive ..	25	96
d-Tetramisole	Inactive ..	50	96
	Inactive ..	25	96
Mebendazole ..	Inactive ..	50	96
	Inactive ..	25	96

l-Tetramisole—*l*-tetramisole was found effective at 48 hr. and 72 hr. with the concentration of 50 $\mu\text{g/ml}$ and 25 $\mu\text{g/ml}$ against microfilariae of *L. carinii* (Table I). Against microfilariae of *W. bancrofti* it showed activity at 72 hr. with 50 $\mu\text{g/ml}$ concentration (Table II). *l*-Tetramisole showed action similar to DEC against microfilariae of *L. carinii* *in vivo*. The microfilaria count was reduced to 4% and 3% during the period of treatment and then increased to 83% and 20% of the

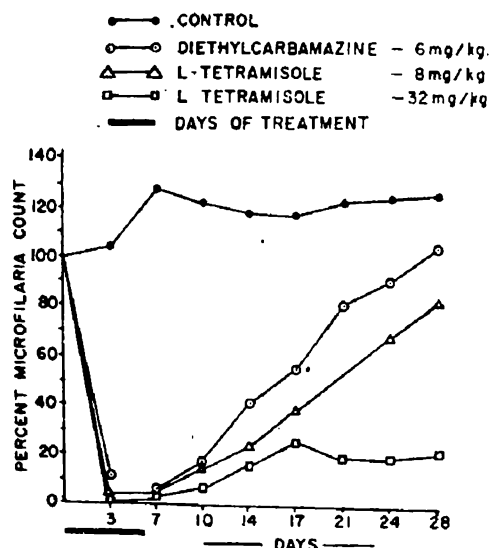


Fig. 1 : Effect of Diethylcarbamazine and *L. Tetramisole* on Microfilaria Count of *L. carinii*.

original value when treated with the dose of 8 mg/kg and 32 mg/kg respectively (Figure 1). With the dose of 8 mg/kg, 30% of the adult worms were dead and 50% of the rats showed calcification of the worms. While with the dose of 32 mg/kg adults from all the rats were calcified and very few worms could be recovered (Table III).

TABLE III

Adult Worms of *L. Carinii* recovered from Treated Rats

Drug	Dose mg/kg $\times 6$	Male		Female		Total	Calci-fication
		Alive	Dead	Alive	Dead		
Control ..		99	8	119	12	228	0/5
Hetrazan	6	39	8	35	5	89	2/6
l-Tetramisole	8	41	25	50	15	131	3/6
	32	4	9	5	7	24	4/4
d-Tetramisole	8	25	7	22	10	65	3/4
	32	19	6	15	2	42	3/4
Mebendazole	8	55	13	44	11	123	6/6
	32	6		4		10	6/6
Betoscanate ..	2	135	16	150	16	318	0/5

d-Tetramisole—*d*-Tetramisole was ineffective against microfilariae of *L. carinii* and *W. bancrofti* in the concentration of 250 µg/ml and 50 µg/ml at 72 hr. and 96 hr. (Tables I and II) respectively. It produced negligible reduction in microfilaria count of *L. carinii*, when rats were treated at the dose of 8 mg/kg. But with 32 mg/kg dose the microfilaria count was reduced to 5% within 3 days of treatment and then increased upto 130% of the original value in 4 weeks. (Partial calcification of the adult worms was observed in the rats treated with *d*-tetramisole, although the number of dead worms was less (Table III).

Diethylcarbamazine and l-Tetramisole—DEC and *l*-tetramisole were administered in the dose of 6 mg/kg and 8 mg/kg for 6 days. The microfilaria count was reduced to 4% during the period of treatment and then increased to 36% of the original count when tested *in vivo*. 35% of the adult worms were dead and all the rats showed partial clarification of the worms (Table III).

Mebendazole—Mebendazole was found ineffective against microfilariae of *L. carinii* and *W. bancrofti* when tested *in vitro* at 50 µg/ml, concentration for 72 hr. and 96 hr. (Tables I and II). Mebendazole when tested *in vivo* at both the dose levels i.e. 8 mg/kg and 32 mg/kg showed consistent reduction in microfilaria count even after discontinuing the treatment. The microfilaria counts were reduced to 9% and 3% of the original value at the end of four weeks (Figure 2). It was found effective at both the dose levels i.e. at 8 mg/kg and 32 mg/kg against adult worms of *L. carinii*. Calcification of the worms was observed in all the treated rats (Table III).

Betoscanate—Betoscanate was tested at the dose of 2 mg/kg/day for 6 days. It exhibited no action against microfilariae and adult worms of *L. carinii*. Microfilaria

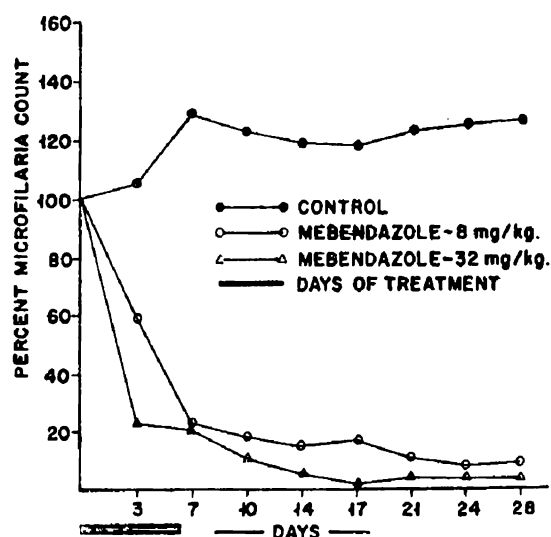


Fig. 2 : Effect of Mebendazole on Microfilaria Count of *L. carinii*.

count was reduced negligibly during the period of treatment (Table III).

Discussion

Tetramisole—Tetramisole is a synthetic anthelmintic drug active against gastrointestinal nematodes at lower dose levels (9). Laevoisomer of tetramisole is twice as active as racemic form and several times active than dextroisomer.

Antifilarial activity of *l*-tetramisole was first reported against *D. immitis* in dogs (10). Action of *l*-tetramisole is also reported against the different filarial parasites such as *B. sergenti* in slow loris, *W. bancrofti* and *B. malayi* in man. (11, 12, 13). *l*-Tetramisole was also found effective *in vitro* against the microfilariae, infective larvae and adults worms of *B. sergenti* (14).

In our experiments *l*-tetramisole was found effective *in vitro*, whereas *d*-tetramisole did not exhibited any activity up to 50 µg/ml. *l*-Tetramisole produced effects similar to DEC on microfilarial count in rats. However adult worms were affected and the calcification of the worms was observed at the end of 28 days with the dose of 32 mg/kg/day for 6 days.

The dextroisomer i.e. d-tetramisole did not much affected microfilaria count during the period of observation. But in all the treated rats, with the dose of 32 mg/kg/day for 6 days partial calcification of the worms was observed.

Diethylcarbamazine l-Tetramisole—l-Tetramisole is an immunostimulant drug, so the combination of the two was tried. But the combination failed to show any additional advantage over the single drug.

Mebendazole—A new broad spectrum anthelmintic was tried against *O. volvulus* in chimpanzees and was found ineffective (15). But combination of l-tetramisole and mebendazole was found effective against *D. perstans* and ineffective against *O. volvulus* (16). Mebendazole alone exhibited good results with prolonged treatment.

We have found mebendazole in active *in vitro* against the microfilariae of *L. carinii* and *W. bancrofti* at the concentration of 50 µg/ml. But *in vivo* it has shown slow reduction in microfilaria count, which was reduced to 9% and 3% at the end of 4 weeks with the dose of 8 mg/kg/day and 32 mg/kg/day for 6 days respectively. Calcification of the worms was observed at both the dose levels.

Acknowledgement

The authors thank Dr. W. N. Gatlewar, Officer in-charge Department of Zoonosis for supplying microfilaria positive blood and infected rats, Dr. U. V. Wagh, Assistant Director, Virus Research Centre, Pune for supplying Vero Cell line and Dr. B. B. Gaitonde, Director, Haffkine Institute, Bombay for providing the facilities to carry out the present work.

REFERENCES

- 1 Hawking, F. and Terry, R. J. (1959)—Chemotherapeutic Properties of Some New Quaternary Ammonium Salts, Part-II : Antifilarial Action against *L. carinii*. *J. Pharmac. Pharmacol.*, **11**, suppl. 94-98.
- 2 Saxena, R.; Iyer, R. N. and Nityanand (1970)—3-ethyl-8-methyl-1, 3, 8, triazabicyclo (4, 4, 0) dean-2-one; A New Antifilarial Agent. *J. Pharmac. Pharmacol.*, **22**, 306-307.
- 3 Lammler, G.; Herzog, H.; Schutz, H. R. and Saupe, E. (1971)—Chemotherapeutic Studies on *Litomosoides carinii* Infection of *Mastomys natalensis* 1 : The Filaricidal Action of 2, 6-bis-Benzimidazoles. *Bull. World Hlth. Org.*, **44**, 751-56.
- 4 Lammler, G.; Herzog, H. and Schutz, H. R. (1971)—Chemotherapeutic Studies on *Litomosoides carinii* Infection of *Mastomys natalensis* 2 : The Activity of Drugs against Adult Parasite. *Bull. World Hlth. Org.*, **44**, 765-770.
- 5 Friedheim, E. A. H. (1974)—Therapeutic Field Trial of a Micro and Microfilaricidal Agent in Caninae Filariasis. *Bull. World Hlth. Org.*, **50**, 572-74.
- 6 Hawking, F.; Sewell, P. and Thurston, J.P. (1950)—The Mode of Action of Hetrazan on Filarial Worms. *Brit. J. Pharmacol. & Chemo.*, **5**, 217-38.
- 7 Sarkar, J. K. (1969)—A Tissue Culture Model for Testing Antimicrofilarial Drugs. *Trans. Roy. Soc. Trop. Med. Hyg.*, **63**, 374-77.
- 8 Hawking, F. and Sewell, P. (1948)—The Maintenance of a Filarial Infection (*Litomosoides carinii*) for Chemotherapeutic Investigation. *Brit. J. Pharmacol. & Chemo.*, **3**, 285-96.
- 9 Theinpont, D.; Brugman, J.; Abadi, K. and Tanamal, S. (1969)—Tetramisole in the Treatment of Nematode Infections in Man. *Am. J. Trop. Med. Hyg.*, **18**, 520-27.
- 10 Tulloch, G. S. and Anderson, R. A (1971)—Acceptability of Daily l-tetramisole by Pound Dogs infected with *D. immitis*. *Nature*, **232**, 644.
- 11 Zaman, V. and Natarajan, P. N. (1973)—Antifilarial Activity of Levamisole on *Brenlia sergenti* and its Comparison with Diethylcarbamazine. *J. Trop. Med. Hyg.*, **76**, 126-30.

- 12 Zaman, V. and Lal, M. (1973)—Treatment of *W. bancrofti* with Levamisole. *Trans. Roy. Soc. Trop. Med. Hyg.*, **57**, 610.
- 13 O'holohan, D. R. and Zaman, V. (1974)—Treatment of *B. malayi* Infection with Levamisole. *J. Trop. Med. Hyg.*, **77**, 113–15.
- 14 Natarajan, P. N. and Zaman, V. (1974)—*In vitro* Action of Levamisole on the Microfilariae, Infective Larvae and Adult Worms of *B. sergenti*. *Int. J. Parasit.*, **4**, 207–210.
- 15 Duke, B. O. L. (1974)—Mebendazole-ineffective against *Onchocerca volvulus*. *Trans. Roy. Soc. Trop. Med. Hyg.*, **68**, 172.
- 16 Maetrens, K. and Wery, M. (1975)—Effect of Mebendazole and Levamisole on *O. volvulus* and *D. perstans*. *Trans. Roy. Soc. Trop. Med. Hyg.*, **96**, 359–60.

■ ■

STUDIES ON THE NEUROMUSCULAR BLOCKING ACTIVITY OF SEEDS OF SOLANUM KHASIANUM, CLARKE

M. B. BHIDE, P. Y. NAIK AND R. S. JOSHI

*Pharmacological Research Unit, CCRIMH, Cirt. No. 2
Department of Pharmacology, Haffkine Institute, Parel
Bombay 400 012*

Aqueous extract of seeds of *Solanum khasianum*, Clarke was found to possess neuromuscular blocking activity both in *in vitro* and *in vivo* preparations, in various species. On isolated phrenic nerve diaphragm preparation of rat and chick biventer cervicis the extract caused sudden increase in muscle tone which gradually returned to normal. The neuromuscular blockade was found to be of mixed type i.e., curare and depolarizing type.

Introduction

Seeds of *Solanum khasianum* are used by local people of Nagaland for getting rid of leeches affecting deep inside the nostrils, ears etc., which could not be reached by other means. The seed powder is also used for controlling pests. It is reported (1) that in leeches exposed to the aqueous extract of seeds produced vigorous looping movement followed by gradual decrease in looping movement, loss of grip and ultimately death. Hence it was of our interest to study the effect of seeds of *Solanum khasianum* on neuromuscular junction.

Material and Method

10 grams of dried seeds of *Solanum khasianum* was powdered and added to 100 ml of distilled water. The mixture was well shaken, allowed to stand for 4 hr. and filtered. The filtrate was used for further pharmacological studies. The content in the filtrate was found to be 20 mg/ml on

evaporation. The neuromuscular blocking activity was evaluated on various *in vivo* and *in vitro* preparations.

1. *Effect on Young Conscious Chicks*—The extract, in a volume of 0.1 ml, was injected intravenously through jugular vein in 3-day old white Leghorn chicks. The animals were observed for the behavioural effects.

2. *Tibialis Anterior Preparations in Cats*—The cats of either sex, weighing between 2.5 to 3.5 kg were anaesthetized with pentobarbitone sodium (40 mg/kg, i.p.). Tibialis anterior nerve muscle preparation was set up following the method described by Brown (2). The lateral popliteal nerve was exposed and placed on the electrodes. It was stimulated through an electronic stimulator using rectangular pulses of 0.5 msec. duration with 4-8 volts at a frequency of 0.1 Hz. The responses were recorded using isometric lever. Drugs were administered close intra-arterially through a cannula inserted into the contralateral femoral artery.

3. *Isolated Phrenic Nerve Diaphragm Preparation of Rat*—Rat phrenic nerve diaphragm preparation was set up in an organ bath containing Kreb's solution maintained at 37°C and bubbled with 95% oxygen and 5% carbon dioxide (3). The phrenic nerve was stimulated by supra maximal rectangular pulses of 0.5 msec. duration with 0.2 Hz through an electronic stimulator. The direct stimulation of the muscle was achieved by using pulses of 5 msec. duration and supra maximal voltage at 0.2 Hz. The tetanic contractions were induced by using a frequency of 50 Hz. The extract was added in the bath to study the effects on the response of the muscle to direct and indirect stimulation.

4. *Frog Rectus Abdominis Muscle Preparation*—Rectus abdominis muscle of the frog *Rana tigrina* was suspended in an organ bath containing frog Ringer solution bubbled with air. The contractions were recorded using a frontal writing lever. The effect of drug, per se, and on acetylcholine induced contractions were recorded.

5. *Chick Biventer Cervicis Preparation*—Chicks weighing between 30 to 50 g were anaesthetized with sodium pentobarbitone (40 mg/kg, i.p.). The biventer muscle was dissected out and set up in an organ bath containing Kreb's Henseleit solution maintained at 37°C and gassed with 95% oxygen and 5% carbon dioxide (4). The muscle was stimulated through the nerve with pulses of 0.5 msec. duration and supra maximal voltage at frequency of 0.2 Hz. The contractions were recorded using a spring lever. Effects of the extract on electrical stimulation and on acetylcholine induced contractions were studied.

Results

1. *Effect on Young Conscious Chicks*—The extract, in a dose range of 50-80 µg/chick produced mixed symptoms like flaccid

paralysis, but no head drop. There was no spasticity but the neck was retracted on one side. Air hunger was also observed. The onset of action was within 1 min. and the effects were completely reversed after 15 min. At doses higher than 80 µg/chick the animals died of convulsions.

2. *Tibialis Anterior Preparation of Cat*—The extract in a dose of 2 mg administered close intra arterially, produced neuromuscular block within 15 min. Neostigmine (100 µg) did not antagonize this effect. The block was partially reversible after about 20 min.

3. *Isolated Phrenic Nerve Diaphragm of Rat*—On this preparation, the extract, in a dose of 200 µg/ml produced sudden contracture of muscle with a simultaneous decrease in the height of contraction induced by indirect stimulation. The contracture of the muscle induced by the extract remained for about 2-3 min. and returned to its normal tone within 10-15 min. and by then response to indirect stimulation was completely abolished (Fig. 1). However, the response to direct muscle stimulation was partially blocked. The blockade was partially reversible but the effect of the second dose was not reproducible on the same tissue.

d-Tubocurarine (2 µg/ml) when added simultaneously with the extract produced the neuromuscular block within 3-4 min. (Fig. 2). At this dose the time required for complete block with d-Tc alone was 8-10 min. Similarly physostigmine (2 µg/ml) when added simultaneously with the extract, partially antagonized the contracture and prolonged the neuromuscular blocking time upto 30-35 min. (Fig. 3).

Unlike d-Tc it failed to produce Wednesky inhibition on tetanic contraction.

TABLE

Overall Effects of the Water Extract of Seeds of 'Solanum khasianum', Clarke

Experiment	Action	Duration	Reversal	Comments
<i>In vivo :</i>				
1. I. V. Injection in Chicks	Paralysis	15 min.	Reversible	Mixed symptoms like flaccid and spastic paralysis.
2. Cat Tibialis Anterior Preparation.	Neuromuscular block. Not antagonized by neostigmine.	15 min.	Partially reversible.	No definite conclusion.
<i>In.vitro :</i>				
1. Rat Phrenic Nerve Diaphragm.	Sudden contracture of the muscle followed by neuromuscular block.	10-15 min.	Reversible	Curare type.
2. Frog Rectus Abdominis Muscle.	Dose dependent contraction, followed by neuromuscular block.			Depolarising type.
3. Chick Biventer Cervicis.	Increase in muscle tone followed by neuromuscular block. Partially antagonizes ACh induced contraction.	5 min.	Partially reversible.	Mixed type.

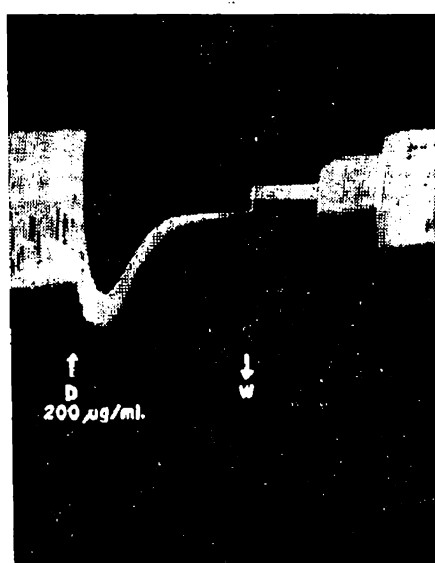


Fig. 1 : Effect of the Extract (D) on Isolated Phrenic Nerve Diaphragm Preparation of Rat.

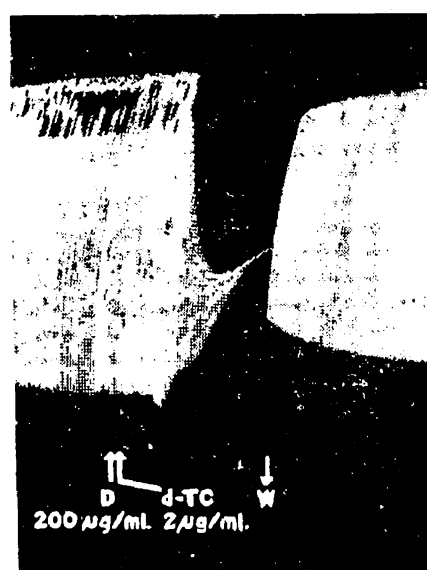


Fig. 2 : Effect of d-tubocurarine (d-Tc) on the Extract Induced Response on Isolated Phrenic Nerve Diaphragm Preparation of Rat.

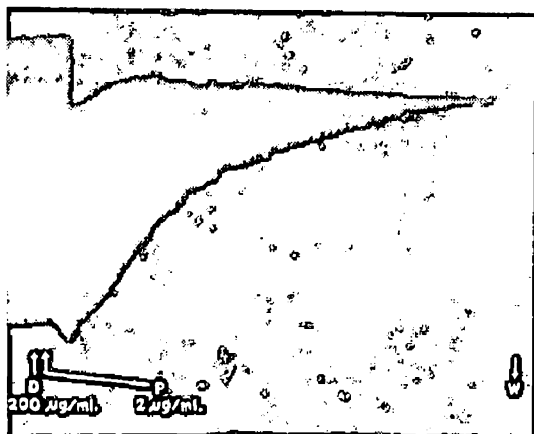


Fig. 3 : Effect of Physostigmine (P) on the Extract (D) Induced Responses on Isolated Phrenic Nerve Diaphragm Preparation of Rat.



Fig. 4 : Effect of Extract (D) on Chick Biventer Cervicis Nerve Muscle Preparation.

4. *Frog Rectus Abdominis Muscle Preparation*—The extract produced a dose dependent contraction of this muscle in a dose range of 100-400 $\mu\text{g/ml}$. This contraction was partially antagonised by d-Tc.

5. *Chick Biventer Cervicis Preparation*—The extract produced a slight increase in the muscle tone followed by a decrease in the height of muscle contraction induced by nerve stimulation. The tone returned to its original level within 5 min. and by then the response to nerve stimulation was completely abolished (Fig. 4). Acetylcholine induced contractions were partially antagonized by the extract at the dose of 200 $\mu\text{g/ml}$.

Discussion

In the present study it is observed that the water extract of *Solanum khasianum* produces neuromuscular block in both *in vivo* and *in vitro* preparations. In chicks it produced flaccid paralysis associated with air hunger and a mild retraction of the neck.

On isolated phrenic nerve diaphragm preparation of rat it produced neuromus-

cular block without affecting much the response to direct muscle stimulation. It is interesting to note that it caused a sudden increase in muscle tone, which gradually reversed to its original level. As d-Tc potentiates and physostigmine partially antagonizes the neuromuscular blocking action of the extract, the mode of its action may be of curare type. But on cat tibialis anterior preparation neostigmine failed to antagonize its action.

The sudden contraction of the diaphragm and to some extent that of chick biventer cervicis muscle and dose dependent contraction of the frog rectus abdominis muscle, suggest a depolarising activity of the extract. This is further supported by the fact that d-Tc partially antagonized the contractions of the rectus muscle induced by the extract.

All the above observations suggest that the water extract of seeds of *Solanum khasianum* possesses a neuromuscular blocking activity which is of a mixed type viz. curare type as well as depolarising type. This needs a further elucidation. Separation of the two fractions having

different modes of action may confirm these observations.

Acknowledgements

The authors are thankful to the Director, Central Council for Research in Indian

Medicine and Homoeopathy, New Delhi for the financial assistance, and to the Director, Haffkine Institute, for the encouragement and the facilities extended.

REFERENCES

- 1 Banerjee, H. D. and Ganguly, D. (1976)—Antileech Property of Seed Extract of *Solanum khasianum*, Clarke. *Indian Drugs*, **13**, 6-7.
- 2 Brown, G. L. (1938)—The Preparation of Tibialis Anterior (Cat) for Close Arterial Injections. *J. Physiol.*, **92**, 22P-23P.
- 3 Bulbring, E. (1946)—Isolated Phrenic Nerve Diaphragm Preparation of Rat. *Brit. J. Pharmacol. Chemother.*, **1**, 38-61.
- 4 Ginsberg, B. L. and Warriner, J. (1960)—The Isolated Chick Biventer Cervicis Preparation. *Brit. J. Pharmacol. Chemother.*, **15**, 410-11.

■ ■

SYNTHESIS AND STUDY OF 2-SUBSTITUTED-4-[4'-METHOXY-3-((2-HYDROXY-5-HALO-OR 5-NITRO-BENZYLIDENE) AMINO) PHENYL] THIAZOLES HYDROCHLORIDES FOR IN VITRO ANTITUBERCULOSIS ACTIVITY-II

A. M. PATIL, D. T. CHAUDHARI, MADHUBEN R. PATEL AND S. S. SABNIS

*Department of Chemotherapy, Haffkine Institute, Parel
Bombay 400 012 (India)*

In view of antituberculosis activity displayed by the azomethine derivatives derived from *para*-N, N-dimethyl-aminobenzaldehyde and *para*-acetamidobenzaldehyde with 2-aryl-4-(3'-amino-4'-methoxyphenyl) thiazoles (I), new azomethine derivatives have been prepared in the present work, from 5-halo- and 5-nitrosalicylaldehydes and above thiazole moiety I. Thirty-two derivatives have been prepared and screened for *in vitro* antituberculosis activity. Twenty-one compounds showed activity and inhibited the growth of *M. tuberculosis* below the concentration of 5 µg/ml. Three compounds had minimum inhibitory concentration at 1 µg/ml.

Introduction

Earlier we have reported the Schiff bases from 4-(3'-amino-4'-methoxyphenyl) thiazoles (I) and *para*-acetamido- and *para*-N, N-dimethylaminobenzaldehydes (1). As an extension of this work, at present, we have synthesised some new azomethines (II) from thiazoles I and 5-chloro-5-bromo- and 5-nitrosalicylaldehydes (III). Muslin *et al.*, had reported number of Schiff bases formed by the above salicylaldehydes (III) and various anilines, and found them active in antituberculosis testing in experimental test systems (2).

Chemistry

All the Schiff bases (II) (Table I) were prepared by previously reported methods (1). 4-(3'-amino-4'-methoxyphenyl) thiazole hydrochloride (I) and appropriate salicylaldehydes III were refluxed in

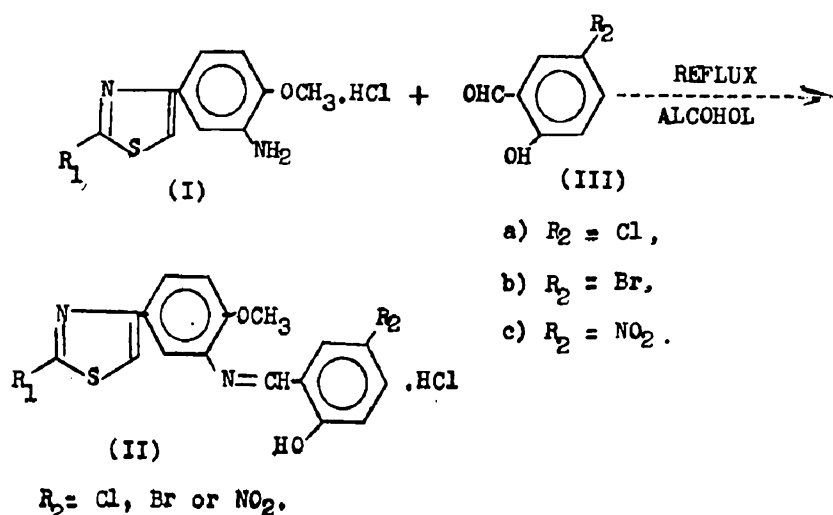
alcohol, and coloured Schiff bases hydrochloride thus formed were isolated in sufficiently pure state.

The requisite 4-(3'-amino-4'-methoxyphenyl) thiazoles (I) were prepared according to the previous method (3) and 5-chloro-5-bromo- and 5-nitrosalicylaldehydes were prepared as reported in literature (4, 5, 6).

Experimental

2-(3-Methoxy-4-benzyloxyphenyl)-4-[3'-((2-hydroxy-5-chloro-benzylidene) amino)-4'-methoxyphenyl] thiazole hydrochloride: (Table-I, 7)—To a solution of 2-(3-methoxy-4-benzyloxyphenyl)-4-(3'-amino-4'-methoxyphenyl) thiazole hydrochloride (0.9 g, 0.002 mole) in alcohol (10 ml) was added alcoholic solution (5 ml) of 5-chlorosalicylaldehyde (0.26 g, 0.002 mole). The mixture was refluxed for 10 min. The resulting clear solution on keeping at room temperature deposited coloured

(Received for publication August 17, 1977)



crystalline solid. This was collected by filtration and washed with dry ethanol and ether to give analytically pure hydrochloride of Schiff base.

Yield: 0.9 g (82%); m.p. 177-80°C.

Similarly, all other compounds reported in Table I were prepared.

Biological Results and Discussion

The *in vitro* tuberculostatic activity was determined on culture of *M. tuberculosis* Var. *Hominis* (strain H₃₇Rv) cultivated on Youmans' medium containing 10 per cent horse serum by a modified method of Doub and Youman (7) and previously reported from this Laboratory (8).

Out of thirty-two compounds twenty-one compounds inhibited the growth of *M. tuberculosis* below 5 µg/ml concentration when tested *in vitro* (Table I). The chlorosalicylaldehyde afforded more active Schiff bases as compared to the Schiff base obtained from 5-bromo- and 5-nitrosalicylaldehydes. Compounds 1, 2 and 6 were the most active compounds among all three series and had inhibitory concentration at

1 µg/ml. All these three compounds are derived from 5-chlorosalicylaldehyde.

4-(3'-amino-4'-methoxyphenyl)-2-(3-methoxy-4-propoxyphenyl)-thiazole and 4-(3'-amino-4'-methoxyphenyl)-2-(3-methoxy-4-benzyloxyphenyl) thiazole proved to be active moieties and gave the active Schiff bases 6, 17 and 28 and 7, 18 and 29 respectively with the three salicylaldehydes (III a, b, c).

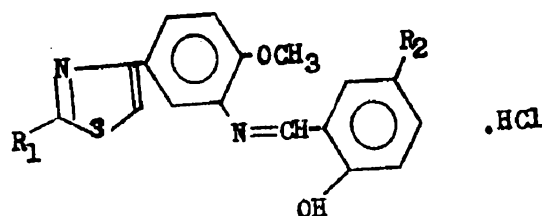
In general, the Schiff bases from 5-chlorosalicylaldehyde are more active than the Schiff bases from 5-bromosalicylaldehyde (III-b) and in turn the Schiff base from III-b are more active than 5-nitrosalicylaldehyde.

Acknowledgement

The authors are thankful to Mr. M. T. Jaokar for elemental microanalyses. They are grateful to Dr. M. H. Shah, Assistant Director, Department of Chemotherapy for his keen interest in present work and to Dr. B. B. Gaitonde, Director, Haffkine Institute, Bombay for providing facilities to carry out the present work.

TABLE

2-Substituted-4-[4'-Methoxy-3'-[2-Hydroxy-5-Halo-or 5-Nitrobenzylidene) Amino) Phenyl] Thiazoles Hydrochlorides.



Serial No.	R ₁	R ₂	M.p.* °C	Molecular formula†	Antituberculosis activity‡ µg/ml.
1	Phenyl	Chloro	213-15	C ₂₃ H ₁₇ ClN ₂ O ₂ S·HCl	1
2	Benzyl	Chloro	138-40	C ₂₄ H ₁₉ ClN ₂ O ₂ S·HCl	1
3	4-Methoxyphenyl ..	Chloro	195-98	C ₂₄ H ₁₉ ClN ₂ O ₃ S·HCl	5
4	3, 4, 5-Trimethoxyphenyl	Chloro	225-29	C ₂₆ H ₂₃ ClN ₂ O ₆ S·HCl	..
5	3-Methoxy-4-ethoxyphenyl	Chloro	166-69	C ₂₆ H ₂₃ ClN ₂ O ₅ S·HCl	2
6	3-Methoxy-4-propoxyphenyl	Chloro	221-23	C ₂₇ H ₂₅ ClN ₂ O ₄ S·HCl	1
7	3-Methoxy-4-Benzoyloxyphenyl	Chloro	177-80	C ₃₁ H ₂₅ ClN ₂ O ₄ S·HCl	2
8	3, 4-Diethoxyphenyl ..	Chloro	228-31	C ₂₇ H ₂₅ ClN ₂ O ₄ S·HCl	2
9	3-Ethoxy-4-Methoxyphenyl	Chloro	243-46	C ₂₆ H ₂₃ ClN ₂ O ₄ S·HCl	..
10	3-Methoxy-4-Hydroxyphenyl	Chloro	165-68	C ₂₄ H ₁₉ ClN ₂ O ₄ S·HCl	2
11	3-Ethoxy-4-Hydroxyphenyl	Chloro	239-41	C ₂₆ H ₂₁ ClN ₂ O ₄ S·HCl	5
12	Phenyl	Bromo	144-48	C ₂₃ H ₁₇ BrN ₂ O ₂ S·HCl	..
13	Benzyl	Bromo	124-27	C ₂₄ H ₁₉ BrN ₂ O ₂ S·HCl	2
14	4-Methoxyphenyl ..	Bromo	192-95	C ₂₄ H ₁₉ BrN ₂ O ₃ S·HCl	5
15	3, 4, 5-Trimethoxyphenyl	Bromo	221-24	C ₂₆ H ₂₃ BrN ₂ O ₆ S·HCl	5
16	3-Methoxy-4-Ethoxyphenyl	Bromo	158-61	C ₂₆ H ₂₃ BrN ₂ O ₄ S·HCl	5
17	3-Methoxy-4-propoxyphenyl	.. Bromo	150-53	C ₂₇ H ₂₅ BrN ₂ O ₄ S·HCl	5
18	3-Methoxy-4-Benzoyloxyphenyl	.. Bromo	187-89	C ₃₁ H ₂₅ BrN ₂ O ₄ S·HCl	5
19	3, 4-Diethoxyphenyl ..	.. Bromo	221-24	C ₂₇ H ₂₅ BrN ₂ O ₄ S·HCl	2
20	3-Ethoxy-4-Methoxyphenyl	Bromo	187-89	C ₂₆ H ₂₃ BrN ₂ O ₄ S·HCl	..
21	3-Methoxy-4-Hydroxyphenyl	.. Bromo	235-38	C ₂₄ H ₁₉ BrN ₂ O ₄ S·HCl	5
22	3-Ethoxy-4-Hydroxyphenyl	.. Bromo	217-20	C ₂₆ H ₂₁ BrN ₂ O ₄ S·HCl	..
23	Phenyl	Nitro	228-31	C ₂₃ H ₁₇ N ₃ O ₄ S·HCl	..
24	Benzyl	Nitro	230-33	C ₂₄ H ₁₉ N ₃ O ₄ S·HCl	..
25	p-Methoxyphenyl ..	Nitro	226-29	C ₂₄ H ₁₉ N ₃ O ₅ S·HCl	..
26	3, 4, 5-Trimethoxyphenyl	Nitro	274-77	C ₂₆ H ₂₃ N ₃ O ₇ S·HCl	..
27	3-Methoxy-4-Ethoxyphenyl	Nitro	231-34	C ₂₆ H ₂₃ N ₃ O ₆ S·HCl	..
28	3-Methoxy-4-Propoxyphenyl	Nitro	209-13	C ₂₇ H ₂₅ N ₃ O ₆ S·HCl	5
29	3-Methoxy-4-Benzoyloxyphenyl	.. Nitro	211-15	C ₃₁ H ₂₅ N ₃ O ₆ S·HCl	2
30	3, 4-Diethoxyphenyl ..	.. Nitro	218-22	C ₂₇ H ₂₅ N ₃ O ₆ S·HCl	..
31	3-Ethoxy-4-Methoxyphenyl	.. Nitro	223-26	C ₂₆ H ₂₃ N ₃ O ₆ S·HCl	2
32	3-Methoxy-4-Hydroxyphenyl	.. Nitro	264-66	C ₂₄ H ₁₉ N ₃ O ₆ S·HCl	5

* Melting points are capillary melting points and are uncorrected.

† Analysed correctly for C, H and N within $\pm 0.4\%$

‡ Minimum inhibitory concentration for *in vitro* antituberculosis action for isonised ± 0.04 µg/ml and for *p*-aminosalicylic acid ± 0.4 µg/ml.

REFERENCES

- 1 Patil, A. M.; Chaudhari, D. T.; Patel, Madhuben and Sabnis, S. S. (1977)—Synthesis and Study of 2-Aryl-4-[3'-(*p*-Substituted Benzylideneamino)-4'-Methoxyphenyl] Thiazoles for *in vitro* antituberculosis Activity-I. *Bull. Haff. Instt.*, **5**, 107-110.
- 2 Muslin, L.; Roth, W. and Erlemeyer, H. (1953)—Metallic Ions and Biological Activity (XIII) : The Tuberculostatic Activity of Several Schiff bases. *Helv. Chim. Acta.*, **36**, 886-90.
- 3 Patil, A. M.; Kirke, P. A. and Chaudhari, D. T. (1976)—Synthesis and Study of 4-(3'-Amino-4'-Methoxyphenyl) Thiazoles for *in vitro* Antituberculosis Activity and as Useful Chemotherapeutic Intermediates. *Bull. Haff. Instt.*, **4**, 51-55.
- 4 Karzunov, N. P. (1963)—5-Chlorosalicylaldehyde, U. S. S. R. Patent, 159497. *Chem. Abstr.* (1964), **60**, 14441a.
- 5 Bougault, J.; Cattelain, E.; Chabrier, P. and Quevauviller, A. (1949)—Derivatives of 5-Bromo-and 3,5-Diiodosalicylaldehyde, *Ann. Pharm. Frac.*, **7**, 163-75 [(*Chem. Abstr.* (1950), **44**, 1452b)].
- 6 Muller, H. S. (1886)—5-Nitrosalicylaldehyde. *Ber.*, **20**, 1929.
- 7 Doub, L. and Youmans, G. P. (1950)—Studies in Tuberculosis Chemotherapy. *Am. Rev. Tuberculosis*, **61**, 407.
- 8 Bundeally, A. E.; Shah, M. H.; Bellare, R. A. and Deliwala, C. V. (1962)—Antitubercular Activity Capriss Fruit. *J. Sci. Industr. Res. India*, **21C**, 305.

■ ■

One compound (4) from this series showed antifungal action at 40 $\mu\text{g/ml}$ against *T. mentagrophytes* and *T. rubrum*. Some of the compounds showed *in vitro* activity against *M. tuberculosis* (H_{37}Rv) in the range of 0.5-10 $\mu\text{g/ml}$. The results are shown in the Table I. The most active compound encountered in the series was N^1 -(5-nitro-2-furoyl)- N^4 -pyrimidyl piperazine (No. 8 of Table I) showing activity at 0.5 $\mu\text{g/ml}$. None of the compounds in this series showed any activity against gram-positive (*S. aureus*) and gram-

negative (*S. typhi*, *E. coli* and *V. comma*) bacteria.

Acknowledgement

The authors wish to thank Dr. C. V. Deliwala Ex-Deputy Director (Research) for his keen interest and Mr. M. T. Jaokar and associates for micro-analyses. We would like to thank Dr. D. T. Chaudhari for his helpful suggestions. Thanks are also due to Dr. B. B. Gaitonde, Director, Haffkine Institute, Bombay for providing the facilities to carry out this work.

REFERENCES

- 1 Zienty, F. B. and Steahly, G. W. (1949)—[1-(Isopropylbenzyl) Piperazines] as Fungicides and Bactericides. *Chem. Abstr.*, **43**, 3558f.
- 2 Wheeler, D. D. and Young, D. C. (1958)—Phthalidylpiperazines. *Chem. Abstr.*, **52**, 17296g.
- 3 Mod, R. M.; Evald, L. S.; Fore, S. P.; Magne, F. C.; Novak, A. K.; Dupay, H. P.; Ortego, J. R. and Fisher, M. T. (1967)—Controlling Microbial Activity. *Chem. Abstr.*, **66**, 28787y.
- 4 Schorr, M.; Mussgaenger, R.; Nesemann, G. and Baner, F. (1962)—Substituted Piperazine : Their Salts and Monoquaternary Compounds. *Chem. Abstr.*, **57**, 2345f.
- 5 Tomcufcik, A. S.; Craig, J. M. and Wilson, S. D. (1967)—4-(2-Aryloxyethyl)-1-Piperazine Carboxylic Acid Esters. *Chem. Abstr.*, **66**, 18722j.
- 6 Rorig, K. J. (1962)—Dialkylaminoethyl Piperazine-propionates. *Chem. Abstr.*, **56**, 8727b.
- 7 Nardi, D.; Massarani, E.; Ross, S.; Tanaja, L. and Degan, L. (1971)—Antibacterial Nitrofurans Derivatives. *J. Med. Chem.*, **14**, 635.
- 8 Boggiano, B. G.; Jackman, G. B.; Petrow, V. and Stephenson, O. (1961)—N-Monosubstituted Piperazines. *Chem. Abstr.*, **55**, 588a.
- 9 Howard, K. L.; Stewart, H. W.; Conroy, E. A. and Denton, J. J. (1953)—1-Heterocyclic Piperazines and 1-Heterocyclic-4-Carboxypiperazines. *J. Org. Chem.*, **48**, 1484.
- 10 Pollard, C. B. and MacDowell, L. G. (1934)—A New Synthesis of N-mono-phenylpiperazine. *J. Amer. Chem. Soc.*, **56**, 2199.
- 11 Schraufstatter, E.; Richter, R. and Ditscheid, W. (1955)—Method III : Fungistatic Method according to Schraufstatter, Richter and Ditscheid (1949), *Acta, Pathol. Microbiol. Scand. suppl.*, **104**, 49.
12. Doub, L. and Youmans, G. P. (1950)—Studies in Tuberculosis Chemotherapy. *Amer. Rev. Tuberc.*, **61**, 407.

■ ■

DIRECTIONS TO CONTRIBUTORS

Bulletin of Haffkine Institute is a triannual publication. It embodies review and research articles in biomedical sciences, mainly devoted to studies on Communicable Diseases.

Contributions should be precise and concise, highlighting original observations and inferences. The type-script (T.S.) should be sent in duplicate, typed in double space on only one side of the sheet with ample margins.

A brief summary not exceeding 200 words, should accompany the article.

The title should be informative and specific, followed by author's name and address.

A short title in not more than forty letters should also be suggested.

The text of the paper should be generally sub-divided into: Introduction, Materials and Methods, or Experimental, Results, Discussion, Acknowledgement and References.

'Short Abstract' of about 100 words suitable for inclusion in 'International Abstracts of Biological Sciences', etc. should be typed on a separate sheet, with author's name, address and title of the paper.

Results should be presented concisely in tables giving only important and relevant data and avoiding incorporation of elaborate observations.

Restraint should be exercised in submitting illustrative matter. Photographs should be well-defined and on glossy paper.

References in the text should be cited by numbers serially as they appear, and to be inserted in parenthesis, e.g. 'Plasmid-mediated resistances to these heavy metals have also been observed in enteric bacilli, especially in P factor carrying organisms (1), and have recently been studied by several workers (2, 3, 4). References at the end should be arranged numberwise serially as they figure in the text with author's name first and abbreviations according to Index Medicus. The following pattern may be followed:

- (i) Reuben, R.; Carey, D. E. and Meyers, R. M. (1968)—Japanese encephalitis studies in Vellore, S. India, *Indian J. Med. Res.*, **56**, 109-18.
- (ii) Cruickshank, R. (1968)—Medical Microbiology, 11th Ed., 180-4, E and S Livingstone Ltd., Edinburgh, p. 28.

Twenty-five reprints will be supplied to the author/s free of cost. Additional reprints may be purchased and should be ordered when galley-proof is returned.

All correspondence should be addressed to the Editor, Bulletin of Haffkine Institute, Haffkine Institute for Training, Research and Testing, Parel, Bombay 400 012 (India).

Annual Subscription : Rs. 20 (Inland) Rs. 25 (Foreign)

Edited and Published by Dr. B. B. Gaitonde, Director, Haffkine Institute for Training, Research and Testing, Bombay 400 012 and printed at the Government Central Press, Bombay 400 004

