

BULLETIN

Of

Haffkine Institute



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Parel, Bombay - 400 012, India

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STUDIES ON THE HYPOGLYCEMIC EFFECT OF FRACTIONS FROM $MgSO_4$ PRECIPITATE OF *BORDETELLA PERTUSSIS*' EXTRACTS

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The hypoglycemic effect observed with the $MgSO_4$ precipitate of *B. pertussis* extracts, was found to be almost equal on reducing the dose ten times after purification by DEAE-cellulose column chromatography. There was no significant change in liver glycogen and serum free fatty acids (FFA), accompanying the hypoglycemic effect in mice.

Introduction

One of the observed physiological changes in experimental animals receiving whole cells or impure extracts of *B. pertussis* is hypoglycemia (1-3). The maximum hypoglycemic effect is observed between days 4 and 16 after injection of *B. pertussis* and the results are shown to be similar in all the species studied (3). In addition a clinical trial with *B. pertussis* vaccine for the treatment of insulin resistant diabetes has been reported (4).

The studies conducted so far have been with *B. pertussis* cells or vaccine without attempting to purify the hypoglycemic factor, although it is known that along with the primary toxic manifestations, various other physiological changes are observed in mice receiving whole cells or impure extracts (5). The present communication reports the results of a preliminary study on the puri-

fication of a $MgSO_4$ precipitate from *B. pertussis* extracts and comparison of its hypoglycemic action with the purified fractions.

Experimental

Bordetella pertussis cells (Strain 25525) were supplied by the Bacteriology Department of Haffkine Institute. Alkaline saline extract (SE) was prepared from the cells as described (6). The SE was adjusted to pH 5.6 and treated with 0.45M solution of $MgSO_4$ to a final concentration of 0.03 M. The suspension was dialysed thoroughly against distilled water and centrifuged at $35,000 \times g$ for 45 minutes in a Spinco Model L refrigerated ultracentrifuge. The residue was lyophilized (Fr.A)

The fraction Fr. A was tested for its hypoglycemic effect by administering intraperitoneally, 1 mg. in 0.5 ml. sterilized saline in mice and measuring the fasting (16 hours after) blood sugar level

Received for publication 3rd May 1979.

(7) on different days. The controls received 0.5 ml. saline. The mice (Haffkine strain, selectively bred) all weighed between 18-20 g. Seven mice were used in each control or test experiment. The Fr. A was further purified by extraction with phosphate buffer pH 7.1 (0.15 M) and subjecting extracts to DEAE cellulose column chromatography using a gradient, 0 to 1M in NaCl. The volume under the peaks were pooled and lyophilized. For determining the minimum lethal

dose (MLD), 1 mg. Fr. A. (50 mg/kg body weight) and 0.1 mg. Fr. 3 (5 mg/kg body weight) were administered intraperitoneally in saline to mice. After a period of 4 days the animals were fasted and challenged with standard histamine hydrochloride. The controls received saline only. Liver glycogen was estimated by the phenol sulphuric acid procedure (8) while the serum free fatty acids (FFA) after chloroform-n-heptane extraction, were estimated colorimetrically (9).

OUTLINE OF PROCEDURE

B. PERTUSSIS CELLS

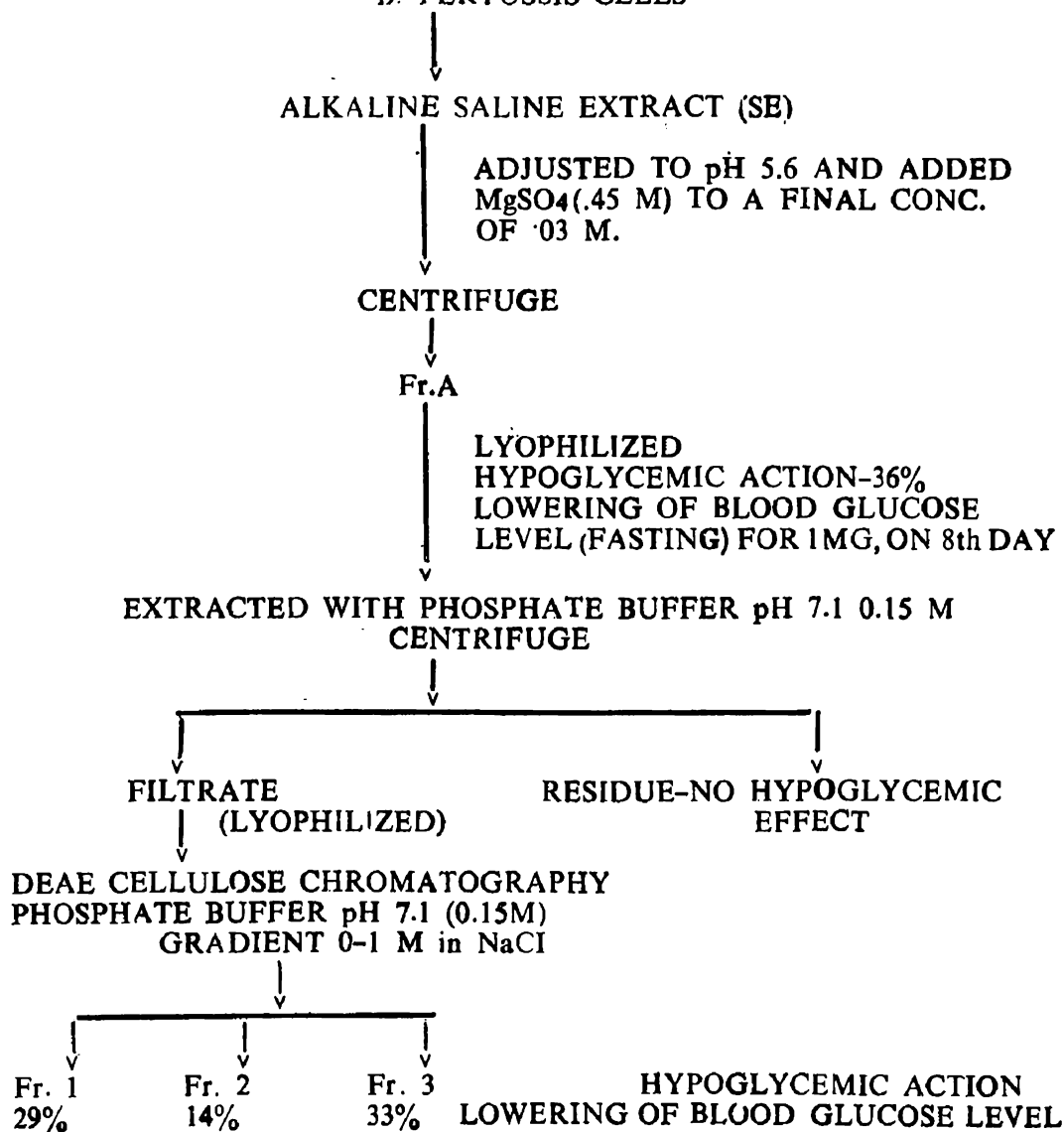


Fig. 1

Results and Discussion

Based on the reports that the hypoglycemic and the histamine sensitizing factors are two coexistent but distinct constituents found in ammonium sulphate fractions (upto 75%) (10), the fraction Fr. A reported to contain nearly all HSF (11) was initially separated from SE with a view to study its hypoglycemic effect. When intraperitoneally administered to mice at 1 mg. concentration, the fasting blood sugar level was lowered by 36% (blood sugar level 63.6 ± 5.6 mg. % against 100 mg. % for control) on the 8th day (Fig. 1), thus proving the presence of the hypoglycemic effect in Fr. A. Following DEAE cellulose column chromatography three fractions were isolated (Fig. 2). At a dose concentration of 0.1 mg, Fr. 1 showed 29% (71 ± 3 mg.%), Fr. 2, 14% (86 ± 5 mg.%), and Fr. 3, 33% (67 ± 3 mg. %) lowering respectively of fasting blood sugar level in mice. Thus, Fr. 3 in parti-

cular shows almost the same hypoglycemic effect as Fr. A but at one-tenth the concentration (Fig. 1 and Table I). The above procedure also appears to have achieved separation of the HSF from the hypoglycemic factor to some extent. The MLD for Fr. A was 3 mg. of histamine standard while that for the purified Fr. 3 was > 3 mg. It may, however, be noticed that for the same two concentrations Fr. 3 showed improvement in blood sugar lowering action as compared to Fr. A. In addition, the absorption patterns (Fig. 2) suggests that the nucleoproteins precipitated from *B. pertussis* could be investigated for their hypoglycemic effect. Such studies could be of benefit particularly in view of the report that in a clinical trial in patients with Diabetes mellitus, *B. pertussis* vaccine was found to temporarily overcome insulin resistance and reduce insulin requirements (4).

TABLE I

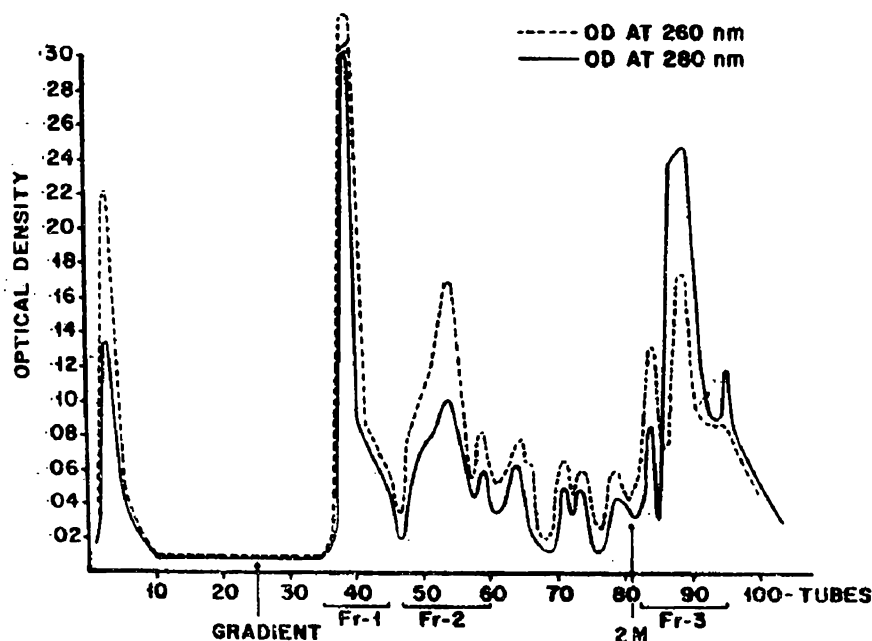
*Blood Sugar Lowering Effect of Fractions from $MgSO_4$ Precipitate of *B. Pertussis* Extracts*

(Fasting Blood Sugar Level of Control is 100 mg%)

No.	Fraction	Dose mg	Blood Sugar Level mg%	$\pm$ S.E.	%Lowering
1	Fr. A	1.0	63.6	± 5.6	36.4
2	Fr. 1	0.1	71.0	± 3.0	29.0
3	Fr. 2	0.1	86.0	± 5.0	14.0
4	Fr. 3	0.1	67.0	± 3.0	33.0

Fig. II

DEAE-CELLULOSE CHROMATOGRAPHY OF Fr-A
ELUTION-PHOSPHATE BUFFER pH 7.1, 0.15 M WITH GRADIENT 0 TO 2 M IN
SODIUM CHLORIDE. COLUMN-1 X 16 CM, RATE-10 ML/HR.



In an effort to understand the mechanism of hypoglycemic action, the effect of Fr. 3 on liver glycogen content and serum free fatty acids (FFA) was examined in mice. At a concentration of 0.1 mg. there was no significant change in the levels of liver glycogen (Table II) and serum FFA (Table III).

The hypoglycemic effect may find explanation in the observation that increased glucose uptake and utilization due to oxidation to CO_2 was noticed in spleen slices from mice given *B. pertussis* (12). A similar phenomenon was also demonstrated in peripheral lymphocytes sensitized to *B. Pertussis*. It would hence be informa-

tive to investigate the mechanism underlying the control of glucose transport which is known to be regulated by glucose-6-phosphate in the skeletal muscle (13). Further, since in a variety of cellular functions the effect produced by exogenous cyclic GMP is known to be opposite to that of cyclic AMP (14), it may also prove worthwhile investigating cyclic GMP levels in *B. pertussis* sensitized animals.

Acknowledgement

We acknowledge the help of Mr. R. S. Karbhari and his staff from the Bacteriology Department of Haffkine Institute for making *B. Pertussis* cells available.

TABLE II

Liver Glycogen Levels in Mice Sensitized with Fr. 3 (0.1 mg)

Groups		*Liver weight g	†Glycogen content mg	Glycogen mg/g liver	Mean $\pm$ SB
Control	1	1.7965	2.640	1.47	
	2	1.9270	6.560	3.40	
	3	2.1535	3.880	1.80	1.6
	4	1.5735	1.920	1.22	± 0.62
	5	1.6955	0.520	0.31	
Test	1	1.7970	0.760	0.43	
	2	1.8610	1.200	0.64	
	3	1.6831	4.200	2.52	1.26
	4	1.9355	2.800	1.44	± 0.42

*Each liver weight value represents that pooled from 2 animals.

†Each value is the mean of 3 independent estimations. The difference between the control and test values are not significant (N.S.) $p > 0.05$ (unpaired 't' test.)

TABLE III

Free Fatty Acid (FFA) Levels in Serum from Mice Sensitized with Fr. 3 (0.1 mg)

Control Group		Test Group	
No.	FFA levels mg/100 ml	No.	FFA levels mg/100 ml
1	245.0	1	122.5
2	197.5	2	190.0
3	245.0	3	230.0
Mean $\pm$ SE	229.2 $\pm$ 15.83	Mean $\pm$ SE	180 $\pm$ 35.83

Blood from 3 animals was pooled together for each group (n=9). Each FFA value represents the mean of 3 independent estimations. The difference between control and test value is not significant (NS) $p > 0.05$ (unpaired 't' test).

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EFFECTS OF ASCORBIC ACID ON OXYGEN UPTAKE OF HAMMERSCHMIDTIELLA DIESINGI, LEIDY, 1850.

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The oxygen uptake studies were carried out on *Hammerschmidtella diesingi*, a nematode parasite, of American Cockroach to investigate the possible effects of ascorbic acid. Oxygen uptake was found to be elevated due to the addition of ascorbic acid in the incubation medium. It is concluded that ascorbic acid *per se* has a stimulatory effect on oxygen uptake of *H. diesingi*.

Introduction

In our earlier studies on a host-parasite model of *Periplaneta americana* L., an American cockroach and *Hammerschmidtella diesingi*, an Oxyurid pinworm, it was observed that the parasite exhibits interspecific competition (1). The biochemical studies on *H. diesingi* showed that the parasite was able to utilize glucose, galactose and mannose in a preferential manner (2). Further studies using glucose and TCA cycle intermediates revealed that *H. diesingi* utilized these substrates on equimolar basis suggesting that the oxidation takes place via conventional TCA cycle

(3). Recently, we have reported that oxygen uptake patterns of *H. diesingi* in these substrates underline a basic mammalian pattern in relation to its substrate utilization (4).

The role of ascorbic acid in insect biochemistry is well known (5). Saraf *et al* (6) have made a very interesting observation showing that hepatic caeca of *P. americana* contain appreciable amount of ascorbic acid. *H. diesingi* invariably is found to habitate in this region.. Ascorbic acid has been also reported to have an accelerating effect on the hatching of eggs of *H. diesingi* (7), *Ascaris lumbricoides* and *A. gaili* (8).

These observations encouraged us to study the effect of ascorbic acid on the oxygen-uptake pattern of *H. diesingi*. The preliminary findings are reported here.

MATERIALS AND METHODS

Adult cockroaches, *P. americana*, were collected, dissected and *H. diesingi* pinworms were identified and removed (7). After several washings with normal saline (0.9% NaCl), the worms were transferred in batches of 20 worms to each Warburg manometer flask.

Since oxygen uptake was found to be consistent in normal saline (4), the same has been used as a basic incubation medium. The glucose and DL-ascorbic acid solutions (1% w/v each) were separately prepared in saline. The volumes of these solutions in appropriate flasks were exactly 1.0 ml. along with the worms.

In other experiments, 1% glucose saline containing 0.5% (w/v) ascorbic acid was used, and the pH was adjusted either to 2.5 or 7.5.

Manometric oxygen uptake studies were carried out by conventional manometric techniques (4).

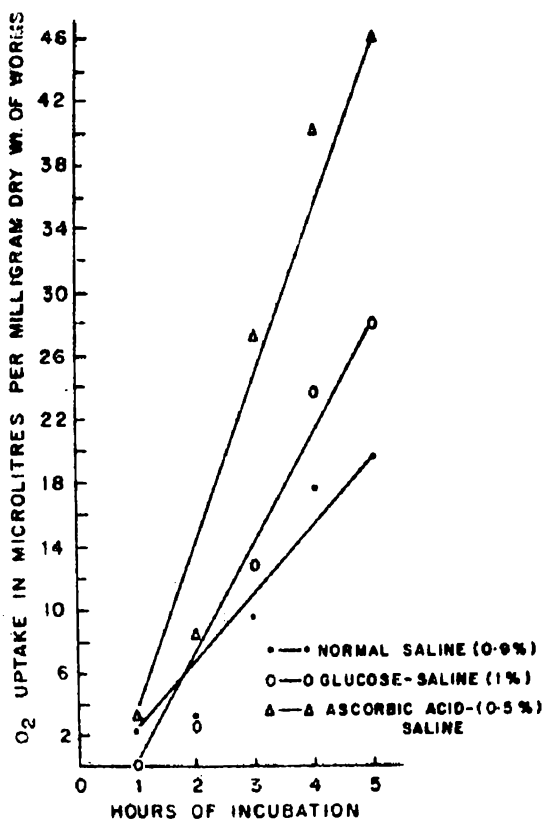


Fig. 1

RESULTS

Figure 1 shows total oxygen uptake pattern of *H. diesingi* in glucose and ascorbic acid saline as compared to that in normal saline. Ascorbic acid is seen to have stimulated oxygen uptake to a significantly high level.

In the next experiment, pH adjustments were made to 7.5 in all solutions so as to rule out the possible artifact due to change in H^+ ion concentration. Figure 2 shows that despite the pH adjustment to 7.5 (which is also the pH of glucose saline solution) there is higher oxygen uptake in ascorbic acid-glucose-saline as compared with glucose saline. The oxygen uptake due to auto-oxidation

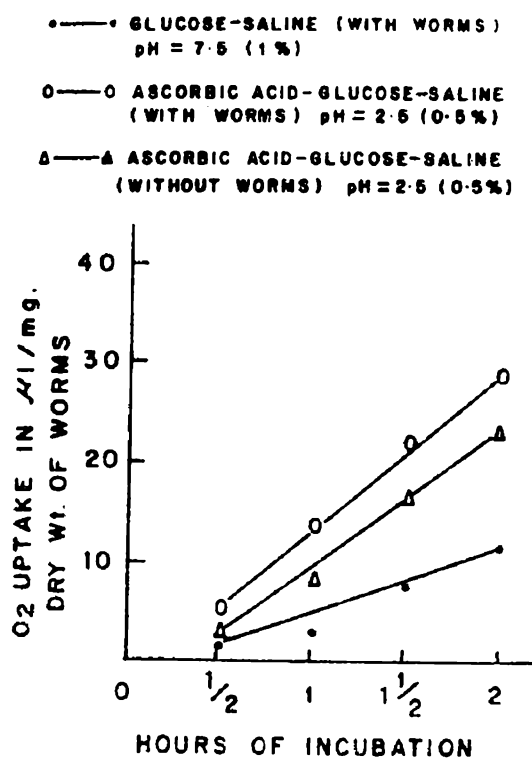


Fig. 2

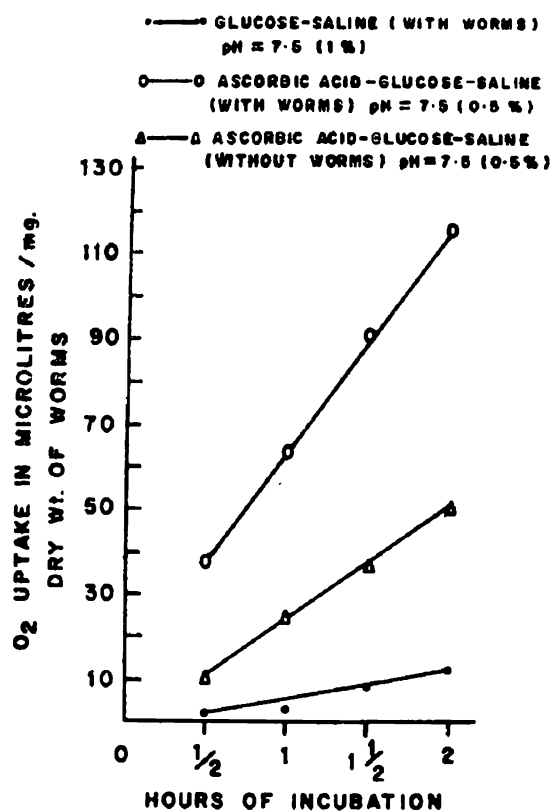


Fig. 3

tion of ascorbic acid at pH 7.5 is appreciable but its contribution, as seen in Figure 2, to total oxygen uptake in the same medium by the worms is not significant.

The pH of glucose-saline was adjusted to 2.5 so as to study the pattern in acidic medium. The oxygen uptake, as seen in Figure 3, is reduced in both the categories as compared to earlier values at pH 7.5. Despite of this reduction, ascorbic acid seems to have a significant stimulatory effect on oxygen uptake of the worms.

DISCUSSION

The increase in oxygen uptake of the worm at pH 7.5 was due to a stimulatory effect of ascorbic acid. The auto-oxidation of ascorbic acid at pH 7.5 was fairly high but insignificant as compared to the total uptake of the worms. Since ascorbic acid is unstable at pH 7.5 (9), the oxygen uptake was also studied at pH 2.5. At this pH also ascorbic acid showed a stimulatory effect. Thus, it is clear that ascorbic acid *per se* has stimulatory effect on oxygen uptake of *H. diesingi*.

The role played by ascorbic acid in elevating oxygen uptake of *H. diesingi* is not yet fully understood. Some of the factors involved in elevating oxygen uptake due to ascorbic acid may be subscribed to its reported involvement in redox potential (10), competitive transport across the cell membrane (11) and stimulatory

action on oxidation of lipids and amino acids (12). It is interesting to note that Patnaik and Kanungo (13) observed that the decrease in oxygen uptake of the skin of aged rats was associated with the lowering of ascorbic acid levels. This observation is significant as *H. diesingi* utilizes carbohydrates in a manner similar to mammalian system (4).

It is, however, necessary to study the mechanism by which ascorbic acid exerts the stimulatory effect on the parasite's respiration.

SUMMARY

The earlier studies from this laboratory had revealed that *H. diesingi*, a nematode parasite found in *P. americana*, utilizes different monosaccharides in preferential manner. The oxygen uptake studies revealed that it

utilizes the substrate through conventional TCA cycle. Presently, we report the studies on the effects of ascorbic acid on the pattern of oxygen uptake by these worms. Ascorbic acid was found to have significant stimulatory effect on oxygen uptake at 2.5 and 7.5 pH. The contribution due to autoxidation of ascorbic acid was insignificant. It is concluded that ascorbic acid *per se* stimulates the oxygen uptake. Further studies are in progress to understand the mode of action of ascorbic acid.

ACKNOWLEDGEMENTS

We are grateful to our Director, Dr. B. B. Gaitonde, for the encouragement given during the course of this investigation. We are also thankful to Dr. P. N. Akolkar for his valuable comments in the preparation of this manuscript.

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SYNTHESIS AND STUDY OF 2-SUBSTITUTED ALKYL OR ARYL AMINO BENZOTHAZOLES AS ANTITUBERCULOSIS AGENTS

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2-Aminothiophenol condensed with substituted aryl or alkyl isothiocyanates in a non-polar solvent at higher temperature to obtain 2-alkyl or substituted aryl aminobenzoles. These were screened for *in vitro* anti-tuberculosis activity. Eight compounds inhibited the growth of H 37 Rv strain of *Mycobacterium tuberculosis* at concentration 3.12 ug/ml.

A variety of benzothiazoles are known for their prodigious range of activity in the field of medicinal chemistry. Especially, they were very useful as antibacterial (1), antituberculosis (2), anthelmintic (3) or fungicides (4). However, 2-alkyl or substituted aryl amino benzothiazoles seem to be less explored for these activities and this fact initiated us to synthesize and test them for anti-tuberculosis and antibacterial activities.

CHEMISTRY

The 2-substituted aminobenzo-thiazoles were synthesised by the known method i.e., 2-aminothiophenol (I) was treated with appropriate alkyl or substituted aryl isothiocyanate (II) in boiling xylene (1) or toluene which gave sufficiently pure compound with the elimination of H_2S gas.

The alkyl or substituted aryl isothiocyanates required as intermediates were prepared by known methods (5).

BIOLOGICAL RESULTS

The *in vitro* tuberculostatic activity was determined on culture of *M. tuberculosis* var *Hominis* (strain H₃₇Rv) cultivated in Youman's medium containing 10% horse serum by the modified method of Doub and Youmans (6).

Out of twenty-four compounds, compound Nos. 2, 3, 7, 10, 11, 15, 17 and 19 inhibited the growth of *M. tuberculosis* at 3.12 ug./ml. concentration. Compound Nos. 5, 6, and 9 were active at 6.25 ug./ml. concentration and other compounds have the antituberculosis activity at concentrations ranging from 12.5 ug./ml. to 100 ug./ml.

It is observed in the 2-substituted arylaminobenzothiazole series (Table 1) that the unsubstituted phenyl amino benzothiazole (No. 11) has activity upto 3.12 ug./ml.; however, when the substituent in the phenyl ring is electronegative in nature like chloro or nitro group, the activity is considerably reduced. When substituents like methoxy, ethoxy, propoxy or butoxy is present, the activity goes on increasing with length of carbon chain or is retained.

All the compounds were found to be inactive against *S. aureus* and *S. typhi*.

EXPERIMENTAL PROCEDURE

The general method for the preparation of 2-alkyl amino or 2-substituted aryl amino benzothiazole (Nos. 1 to 24) is illustrated by the following typical experiment.

2-Benzylaminobenzothiazole :

A mixture of 2.5 g of *o*-aminothiophenol (0.02 mole) and 3.0 g benzyl isothiocyanate (0.02 mole) in *o*-xylene was refluxed. After complete evolution of H₂S gas, the reaction mixture was cooled to room temperature. Crystalline compound separated out which was filtered and recrystallized from hexane-toluene (4:6) mixture to give 2-benzyl aminobenzothiazole (No. 10). Yield: 4.32 g (90%), m.p. 156°C.

Similarly, all other compounds reported in Table 1 were prepared.

ACKNOWLEDGEMENT

The authors are thankful to Dr. M. H. Shah, Assistant Director, Department of Chemotherapy for his keen interest in the work. They are also grateful to Mr. M. T. Jaokar and associates for elemental microanalysis. The authors are indebted to Dr. K. D. Sharma, Director, Haffkine Institute, for providing excellent facilities to carry out the present work.

TABLE I

2-Alkylamino benzothiazoles

Where, R=Alkyl or substituted aryl.

Sr. No.	R	Molecular formula	M.P. °C*	Elemental analysis						Antituber- culosis activity (ug/ml) **
				C		H		N		
				Cal.	Found	Cal.	Found	Cal.	Found	
1.	Methyl	C ₈ H ₈ N ₂ S	Oil	58.53	58.87	4.87	4.62	17.07	17.37	—
2.	Ethyl	C ₉ H ₁₀ N ₂ S	Oil	60.67	60.32	5.61	5.83	15.73	15.42	3.12
3.	n-Propyl	C ₁₀ H ₁₂ N ₂ S	84	62.50	62.67	6.25	6.42	14.58	14.80	3.12
4.	iso-Propyl	C ₁₀ H ₁₂ N ₂ S	127	62.50	62.80	6.25	6.45	15.45	14.78	50.00
5.	n-Butyl	C ₁₁ H ₁₄ N ₂ S	96	64.07	64.15	6.79	6.95	13.72	13.92	6.25
6.	iso-Butyl	C ₁₁ H ₁₄ N ₂ S	112	64.07	63.90	6.79	7.18	13.72	13.51	6.25
7.	n-Hexyl	C ₁₃ H ₁₈ N ₂ S	68	66.66	67.03	7.69	7.24	11.96	12.16	3.12
8.	Cyclohexyl	C ₁₃ H ₁₆ N ₂ S	177	67.24	67.37	6.89	6.74	12.06	11.81	100
9.	Allyl	C ₁₀ H ₁₀ N ₂ S	75	63.15	63.28	5.26	5.47	14.73	14.62	6.25
10.	Benzyl	C ₁₄ H ₁₂ N ₂ S	156	70.00	69.55	5.00	5.63	11.66	11.04	3.12
11.	Phenyl	C ₁₃ H ₁₀ N ₂ S	159	69.02	69.32	4.42	4.72	12.38	12.64	3.12
12.	4-Chloro-phenyl	C ₁₃ H ₉ Cl N ₂ S	205	60.00	60.60	3.46	3.70	10.76	10.48	100
13.	4-Bromo-phenyl	C ₁₃ H ₉ Br N ₂ S	215	51.14	51.24	2.95	3.07	9.18	9.08	200
14.	2-Nitro-phenyl	C ₁₃ H ₉ N ₃ O ₂ S	120	57.56	57.86	3.32	3.67	15.49	15.82	100
15.	4-Methyl-phenyl	C ₁₄ H ₁₂ N ₂ S	175	70.00	69.67	5.00	4.92	11.66	11.48	3.12

Contd.

Contd. From TABLE I

Sr. No.	R	Molecular formula	M.P. °C*	Elemental analysis						Antituber- culosis activity (ug/ml) **
				C		H		N		
				Cal.	Found	Cal.	Found	Cal.	Found	
16.	4-Methox- phenyl	C ₁₄ H ₁₂ N ₂ OS	161	65.62	65.47	4.68	4.48	10.93	10.87	100
17.	4-Ethoxy- phenyl	C ₁₅ H ₁₄ N ₂ OS	166	66.66	66.82	5.18	5.28	10.37	10.49	3.12
18.	4-Propoxy- phenyl	C ₁₆ H ₁₆ N ₂ OS	156	67.60	67.47	5.63	5.82	9.95	9.73	—
19.	4-Butoxy- phenyl	C ₁₇ H ₁₈ N ₂ OS	156	68.45	68.91	6.04	5.85	9.39	9.54	3.12
20.	2-Methoxy-4-nitro- phenyl	C ₁₄ H ₁₁ N ₂ O ₃ S	177	58.53	58.70	3.83	3.54	9.75	9.86	100
21.	4-N. N-Dimethyl-amino- phenyl	C ₁₅ H ₁₅ N ₃ S	199	66.91	66.68	5.57	5.83	15.61	15.34	12.5
22.	4-Acetyl- phenyl	C ₁₅ H ₁₂ N ₂ OS	188	67.16	67.37	4.47	4.69	10.44	10.67	12.5
23.	4-Benzoyl- phenyl	C ₂₀ H ₁₄ N ₂ OS	171	72.72	72.58	4.24	4.37	8.48	8.73	50
24.	4-(p-Chlorobenzoyl)- phenyl	C ₂₀ H ₁₃ ClN ₂ OS	215	65.84	65.57	3.56	3.73	7.68	7.72	100

* Melting points are capillary melting points and are uncorrected.

** Minimum inhibitory concentration *in vitro* antituberculosis action for isoniazide =0.04 ug/ml and for p-aminosalicylic acid=0.4 ug/ml.

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**2- [5',-SUBSTITUTED-1',3',4',-OXADIAZOL-2'-YL THIO-
METHYL]-5 (or 6)-SUBSTITUTED-BENZIMIDAZOLES
AS ANTHELMINTIC AGENTS.**

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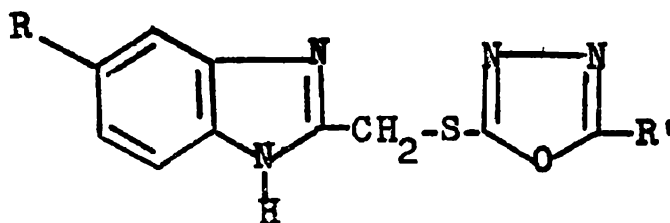
ABSTRACT

A few 2-[(5'-substituted-1',3',4'-oxadiazol-2-yl)thiomethyl]-5(or 6)-substituted benzimidazoles were synthesised and screened against *H. nana*. Only two compounds exhibited activity.

Benzimidazole derivatives possessing high order of antibacterial and antiviral^{2a, b} activity and broad spectrum antiparasitic activity³⁻⁵ have been reported. 1, 2, 4-Oxadiazole ring system has also received considerable attention⁶. Some of these derivatives for example, 3-substituted-1,2,4-oxadiazoles have shown significant nematocidal activity. Si-

milarly, 1,3,4-oxadiadoles also are active nematocides^{8a, b} 9a, b.

We report in this paper the synthesis and screening of a series of compounds (I) which possess benzimidazole as well as 1,3,4-oxadiazole moieties linked together through -CH₂S- as antiparasitic agents.



(I)

Where

R = H, NO₂;
R' = C₆H₅; 2,4-Cl₂C₆H₃; 4-Cl-C₆H₄; 4-CH₃C H₄;
3,4,5, -(CH₃O)₃-C₆H₂; 4-pyridyl.

Received for Publication 5th Feb. 1979

CHEMISTRY :

The proposed oxadiazolyl thiomethyl benzimidazoles were obtained by reacting 2-chloromethyl-5(or6)-substituted benzimidazole with appropriate 2-substituted 5-mercapto-1,3,4-oxadiazole. The required 2-chloromethyl-5(or6)-substituted benzimidazoles were prepared as reported^{10,11} and 2-substituted-5-mercapto-1,3,4-oxadiazoles were obtained from the corresponding hydrazide by the procedure described by Hoggarth¹².

EXPERIMENTAL

2-Chloromethylbenzimidazole was prepared from o-phenylenediamine and chloroacetic acid in 4N hydrochloric acid with 80% yield¹⁰. The 2-chloromethyl-5 (or 6)-nitrobenzimidazole was prepared from 4-nitro-1, 2-phenylenediamine and chloroacetic acid in 4N hydrochloric acid with 78% yield.

2-Substituted 5-mercapto 1, 3, 4-oxadiazoles were prepared by Hoggarth's method¹². Appropriate hydrazides were refluxed with carbon disulphide and alcoholic potassium hydroxide and finally cyclized using hydrochloric acid to give 5-substituted-2-mercapto 1, 3, 4-oxadiazole with 70-90% yield. (Table 1).

2-(5'-p-Chlorophenyl-1',3',4'-oxadiazol-2'-yl) thiomethyl-5 (or 6)-nitrobenzimidazole :

A solution of 2-mercapto-5-p-chlorophenyl-1,3,4 oxadiazole

(1.06 g; 0.005 mol) in minimum quantity of 10% sodium hydroxide was added, into a stirred alcoholic solution of 2-chloromethyl-5 (or 6)-nitrobenzimidazole (1.05 g; 0.005 mol). This mixture was slightly warmed and kept overnight at room temperature. The solid that separated next day was filtered and recrystallised twice from ethanol. The Yield was 59% (1.14 g), and m.p. 228-230°.

Analysis calculated for

$C_{16}H_{10}ClN_5O_3S$: C,49.55 ; H,2.58 ;
N,18.06%

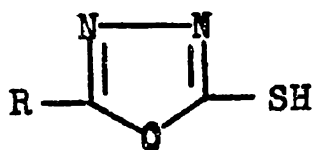
Found : C,49.64 ; H,2.70 ;
N,17.60%

All other compounds (Table 2) were prepared as described above.

BIOLOGICAL RESULTS AND DISCUSSION :

The oxadiazolyl benzimidazole derivatives synthesised were tested in vivo¹³ against *Hymenolepis mana* at 500 mg./kg. dose. The detailed screening results are shown in the last column in Table 2.

Only two compounds out of nine showed some activity. Remaining seven were inactive. 2-(5'-Tolyl-1', 3', 4'-oxadiazol-2'-yl)-5-nitrothiomethylbenzimidazole was the most active showing 97% efficacy as compared to 100% shown by Yomesan at 500 mg./kg. dose.

TABLE I**2-Substituted-5-mercapto-1,3,4-oxadiazoles**

No.	R	Yield	M.p. °C (found)	Reported m.p. °C
1.	Phenyl	83	219-20	220
2.	<i>p</i> -Chlorophenyl	90	175-76	176
3.	2,4-Dichlorophenyl	71	177-78 ^a	—
4.	<i>p</i> -Tolyl	89	215-17 ^b	—
5.	3,4,5-Trimethoxyphenyl	60	189-90 ^c	—
6.	4-Pyridyl	65	264-65	264

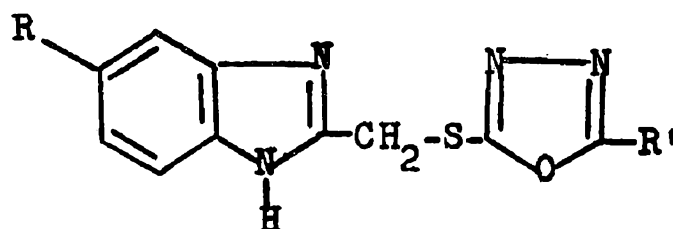
a) Anal. found : N, 11.38; C₈ H₄ Cl₂ N₂ OS requires N, 11.32%

b) Anal. found : N, 14.98; C₉ H₈ N₂ OS requires N, 14.58%

c) Anal. found : N, 10.95; C₁₁H₁₂N₂O₄S requires N, 10.44%.

TABLE II

2-(5'-Substituted-1',3',4',-oxadiazol-2'-yl) thiomethyl-5 (or 6)-substituted benzimidazoles.



No.	R	R'	M. p. °C _a (Uncorr.)	Yield %	ANALYSIS (%)						Percentage efficacy at 500 mg/kg dose.
					Calculated			Found			
					C	H	N	C	H	N	
1	H	Phenyl	206-207	56	62.33	3.89	18.18	62.68	4.34	18.19	83
2	H	p-Chloro- phenyl	183-184	61	56.06	3.21	16.35	56.04	3.04	16.55	not
3	H	3,4,5-Trime- thoxy phenyl	157-159	58	57.15	4.52	14.07	56.68	4.58	14.42	effective
4	H	4-Pyridyl	188-189	66	58.25	3.56	22.85	58.44	3.56	22.79	"
5	NO ₂	p-Chloro- phenyl	228-230	60	49.55	2.58	18.06	49.64	2.70	17.60	"
6	NO ₂	2,4-Dichloro- phenyl	234-235	60	45.50	2.18	16.59	46.00	2.25	16.46	"
7	NO ₂	p-Tolyl	230-231	62	55.38	3.54	19.07	55.03	3.59	19.20	97
8	NO ₂	3,4,5-Trime- thoxy phenyl	233-234	56	51.46	3.83	15.80	50.99	3.66	15.27	not
9	NO ₂	4-Pyridyl	248-249	63	50.85	2.82	23.73	51.36	2.64	23.20	effective

Crystallization solvent : For all compounds ethanol was used for crystallization.

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INVESTIGATION OF INDIGENOUS PLANTS FOR LARVICIDAL ACTIVITY

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ABSTRACT :

The increasing cost of chemical insecticides and resurgence menace of insect borne diseases, particularly *Malaria* and *Leishmaniasis*, has stimulated for alternative and/or supplementary insecticides.

The present study is an attempt to evaluate larvicidal properties of three indigenous plants, viz. *Acorus calamus*, *Azadirachta indica* and *Ocimum sanctum*, IV instar larvae of *Culex p fatigans* reared in laboratory were used for evaluation.

Introduction

The discovery and use of insecticides has proved to be major breakthrough in the improvement of agricultural yields and in public health. Insecticides have been estimated to save more lives than all the antibiotics combined. About 15 million people were saved from dying of malaria alone, during the years 1955-1965 (1). Unfortunately with the exposure of insect populations to wide spread mass chemical treatments the phenomenon of insecticide resistance has cropped up. Over 250 agricultural pest species and 100 species of disease-carrying arthropods have now been reported as highly tolerant to one or more of the main groups of insecticides. (2).

Besides creating problems of resistance, insecticides have posed several environmental hazards. Wide scale application of DDT has produced two important effects (3) :

- I. A persistence in soil and water, with consequent spreading to areas where DDT was never sprayed.
- II. A gradual accumulation in the fatty tissues of some organisms, including man.

The other types of insecticides have been shown to present different environmental problems. Though they are less stable than DDT and therefore less likely to accumulate they are comparatively more toxic to vertebrates, and thus to man.

Apart from the resistance problem and environmental hazards, the rising cost of petroleum products have made it difficult for developing countries to resort to the use of conventional insecticides. This factor makes it necessary for them to look for alternative insecticides.

It has been reported that about two thousands plants possess insecticidal properties (4). They are scattered over 170 families of plants. Among these plants, insecticidal properties of "Neem" (*A. Indica*) are well documented (5,6,7) This paper reports evaluation of Neem and other plants for mosquito larvicidal property.

MATERIALS AND METHODS

Several fractions of Neem (*Azadirachta indica*), Vekhand (*Acorus calamus*) and Tulsi (*Ocimum sanctum*) were assessed for mosquito larvicidal activity. Larvicidal property of these fractions was evaluated against IV stage of *Culex fatigans* larvae

In the case of *Azadirachta indica* (Neem) and *Ocimum sanctum* (tulsi) plant leaves were used for obtaining fractions. In the case of *Acorus calamus* (vekhand) several fractions of rhizome were exploited

Petroleum ether, ether, chloroform and alcohol extracts of finely ground material were obtained by using soxhlet and percolator. Purification of petroleum ether extract of *Acorus calamus* was

performed according to the method described by Chavan et al. (8).

Mosquito larvicidal activity was evaluated as per methods recommended by World Health Organization (9, 10).

All experimental exposures were done using 500 ml. glass beakers. Twenty five larval mosquitces of the speces *Culex pipens fatigans* were placed in 250 ml. tap water containing various concentrations of serially diluted extracts and held for 24 hours mortality count. Larvae were then decanted and transferred to fresh water and fed with 1 ml. of 1.0% yeast extract suspension in water. One or two days after treatment, dead larvae were removed and counted for percentage mortality.

Three replications were employed for each dilution of the extract. Various solvents employed for extractions from these indigenous plants like petroleum ether, ether, acetone, alcohol were also evaluated for their toxicity to mosquito larvae. Twenty five larvae were used as control. They were not exposed to any toxicant and were maintained in a 500 ml. beaker containing 250 ml. water.

Larvae incapable of rising to or submerging from the surface upon probing were considered as dead. Total number of larvae pupating and pupae emerging into adults were also recorded.

TABLE I

**Larvicidal activity of extracts of *Acorus calamus* Linn
(*Araceae*)**

Extract	% Concentration	% Mortality
1. Petroleum ether extract	0.1	100
	0.01	100
2. Ether extract	0.1	20
	0.01	10
3. Chloroform extract	0.1	40
	0.0	20
4. Alcohol extract	0.1	35
	0.01	20
5. Water extract	0.1	5
	0.01	5

TABLE II

**Larvicidal activity of purified oil fraction of *Acorus calamus* Linn
(*Araceae*)**

% Concentration	% Mortality	
	Pyrethum extract	<i>Acorum calamus</i> oil C-118-124°C/6mm
1.0	100	100
0.1	100	100
0.01	90	100
0.001	80	90

RESULTS

Table I shows larvicidal activity of various fractions of *Acorus calamus* (Vekhand). It will be seen from the Table that only petroleum ether extract exhibits larvicidal activity.

In Table II larvicidal activity of purified fraction of *A. calamus* is shown in different concentrations. Percent mortality is compared with pyrethrum extract, known plant based larvicide. The activity is seen to be comparable to pyrethrum extract in all the concentrations. Table III presents details about larvicidal activity of different fractions of *Azadiracta indica* (Neem). It will be seen that two fractions i. e. Petroleum ether extract (NPZI) and etherextract (NPZ II) showed larvicidal activity.

The activity was found to persist for 120 hours in case of NPZ I and NPZ II as shown in Table IV, as was the case with pyrethrum extract used for comparison. Further fractionation of NPZ-I using neutral alumina column and elution with petroleum ether, benzene, methanol showed that only fraction I possessed larvicidal property (Table V).

Larvicidal properties of various fractions of *O. sanctum* (Tulsi) are shown in Table VI. Only two fractions i.e. Petroleum ether extract hot (TPZ-I) and petroleum ether extract cold (TPZ-II) were

found to exhibit larvicidal activity when exposed. The residual activity was found to last for only 72 hours.

DISCUSSION

Present studies show that in the case of *Acorus calamus* 0.1% and 0.01% of petroleum ether extracts produce 100% mortality in *Culex p fatigans* IV stage larvae. On the other hand, in ether, chloroform, alcohol and water extracts mortality was very low, ranging from 5 to 40% only.

In case of 'purified oil extract' of *Acorus calamus*, larvicidal activity at 0.01 and 0.001% dose levels was found to be comparable to pyrethrum extract.

Results obtained support the findings of various other investigators. Mirnov, (11) was the first to observe that *Anopheles maculipennis* and *Musca domestica* adults are killed within 40 minutes when sprayed with decoction of *A. calamus* rhizome. Subramanian, (12 & 13) worked on the insecticidal properties of *A. calamus*. Studies were confined to the direct use of the powdered rhizome. Dixit et al. (14) studied the insecticidal activity of solvent extracts and steam volatile principle of the rhizome of *A. calamus*. They found that petroleum ether extract was more toxic than kerosene to mosquitoes and houseflies. Ether, petroleum ether, and alcoholic extracts of *A. calamus* rhizomes were

TABLE IIILarvicidal activity of various extracts of *A. indica*

Sr. No.	Extract	% concn.	24 hrs kill %	% Dead Larvae
1.	Petroleum ether (NPZ-I)	1.0	100	100
2.	Ether (NPZ-II)	1.0	75	100
3.	Chloroform (NPZ-III)	1.0	65	100
4.	Alcohol (NPZ-IV)	0.5	60	95
5.	Control	—	—	—

TABLE IVResidual larvicidal activity of various extracts of *A. indica*

No.	Extract	% conc. extract	% Mortality after hrs					
			24	48	72	96	120	144
1.	Pet. ether (NPZ-I)	1.0	100	100	80	80	75	60
		0.2	100	100	70	70	55	50
2.	Ether (NPZ-II)	0.5	100	100	80	80	75	60
3.	Pyrethrum	0.2	100	100	84	80	70	70
4.	Solvent control	—	—	—	—	—	—	—
5.	Water control	—	—	—	—	—	—	—

TABLE V

Larvicidal activity of fractions of extract NPZ-I of *A. indica*

Sr. No.	Fraction	% of concentration	24 hrs. kill	% of Dead Larvae	% of Dead Pupae	% of Dead Adult	% of Live adult
1.	Fraction I	1.0	100	100	—	—	—
2.	Fraction II	1.0	80	80	10	—	—
3.	Fraction III	1.0	40	50	10	10	20
4.	Fraction IV	1.0	55	55	—	10	35
5.	Fraction V	1.0	25	40	—	5	55
6.	Control	—	—	—	—	—	—

examined by Mukherjee and Govind, (15) for their contact and residual toxicity against *M. nebulosus* adults and ovicidal and stomach poison effect against *B. mori*. The ether extract has been found to be more toxic than any other extracts.

Effects of the oil vapours of *A. calamus* were investigated by Sexena and Srivastava, on the eggs and first instar nymphs of *D. koenigii* (16). They found that vapours effect the hatching of eggs. Loss of fecundity in *D. koenigii* due to vapours of *A. calamus* oil was also observed by Saxena and Mathur (17). Different extracts of *A. calamus* were evaluated for their mosquito larvicidal activity against 4th instar larvae of *Culex P. fatigans* by

Chavan et al., (8). They found that petroleum ether extract is highly toxic.

In the case of *Azadirachta indica* it was noticed that two fractions of 1% of petroleum ether extract and ether extract i. e., NPZ-I and NPZ-II produced 100% and 75% mortality in 24 hours. Other extracts did not show any significant mortality.

While studying the residual activity of the extract NPZ-I and NPZ-II it was noticed that at concentrations 1, 0.2 and 0.5% produced 100% mortality in 48 hours respectively. The activity was low after 48 hours, and was found to be 60% after 144 hours. Similar result were obtained in the case of pyrethrum extract.

TABLE VI

Larvicidal activity of extracts of *Ocinum sanctum* (Tulsi) (Labiatae)

Sr. No.	Extract with Code No.	% Concn. of Extract	%24 hrs. kill	% Dead Larvae	%Dead P pae	% Dead adults	%Live Adults
1.	Pet. Ether (Cold) TPZ-I	1.0	100	100	-	-	-
2.	Pet. Ether (Hot) TPZ-II	1.0	100	100	-	-	-
3.	Ether (Cold) TPZ-III	1.0	85	85	-	-	15
4.	Ether (Hot) TPZ-IV	1.0	-	60	10	-	30
5.	Chloroform (Cold) TPZ-V	1.0	80	80	10	-	10
6.	Chloroform (Hot) TPZ-VI	01.	30	80	-	-	20
7.	Acetone (Hot) TPZ-VII	01.	-	50	20	15	15
8.	Acetone (Cold) TPZ-VIII	1.0	-	60	15	-	25
9.	Water, TPZ-IX	1.0	35	95	-	-	5
10.	Pyrethrum Extract	0.2	100	100	-	-	-
11.	Petroleum Ether	—	-	-	-	15	85
12.	Water	—	-	-	-	-	100

TABLE VII
Residual Larvicidal activity of Extract of *Ocimum sanctum* (Tulsi)

Sr. No.	Extract	% Concn. of Extract	Percentage Mortality after hours				
			24	48	72	168	336
1.	Pet. ether TPZ-II	1.0	100	100	100	65	60
2.	Pet. ether TPZ-II	0.6	100	100	100	65	60
3.	Pet. ether TPZ-II	0.4	85	85	85	50	50
4.	Pet ether TPZ-II	0.2	75	75	70	50	40
5.	Control (PE)	—	10	—	—	—	—
6.	Control Water	—	—	—	—	—	—

NPZ—I was subjected to further fractionation on column chromatography using neutral alumina column and eluting with petroleum ether, benzene, methanol and collecting several fractions. Screening of these fractions showed that only fraction I and II possessed larvicidal activity.

Extracts from leaves of *Azadirachta indica* were used for protecting plants from attack of locust. Holman, (18) found that extracts of Neem leaves checked the attack of locusts and he claimed that extract can be successfully used for protecting fruits, vegetables and cereals against insects (18) Heal et al., (19) reported that Neem exhibited toxicity against cockroaches in

America. Pruthi et al. observed the repellent action of Neem to stored grain pests (20). Roonwal (21) stated that locust do not eat Neem leaves. Jotwani et al., (22) reported the Neem seeds acted as a protectant against important storage pest of wheat. McMillan et al showed that extract of *Azadirachta indica* could serve as an active feeding deterrent and growth retardant (23).

Gill et al. (24) reported for the first time systemic activity of Neem derivatives. Neem extracts possess growth retarding properties. The larvae or eggs of several insect species failed to develop normally when exposed to Neem treatment. Similar results

were observed and depicted in Table-V, which reveals that Fractions III, IV & V give considerable low kill after 24 hours of exposure. At the same time emergence of adult is also very low.

Studies with *Ocimum sanctum* showed that two fractions to possess larvicidal activity. The action was found to persist for 72 hours.

The leaves of *O. Sanctum* yield a bright yellow volatile oil which is reported to possess antibacterial and insecticidal proper-

ties. It has marked insecticidal activity against mosquitoes though it is not comparable to that of pyrethrum. It has also been reported to possess mosquito repellent action. It will be seen from studies carried out that some fractions from *Acorus calamus* and *Aradirachta indica* possess mosquito larvicidal activity.

Insecticides of plant origin have been extensively used in agriculture but to a very limited extent in public health. Yet in the development of new insecticides they deserve careful consideration.

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PATHOGENECITY STUDIES ON *SALMONELLA ROAN*

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ABSTRACT :

In this study we report on the animal pathogenicity test carried out in swiss-NIH mice for *Salmonella roan* isolate. The *Salmonella roan* was isolated from food samples. In pathogenicity studies on mice *S. roan* behaved like *S. enteritidis* which is a known pathogen. LD₅₀ value for *S. roan* was 2.8×10^4 organisms and produced histopathological changes characteristic of *Salmonella* infection.

Introduction

With the ever increasing use and export of processed food products, the problem of bacterial food poisoning has thrown a new challenge to public health authorities. Of the outbreaks of bacterial food poisoning, Salmonellosis remains one of the major ones. The genus *Salmonella* encompasses over 1000 strains based on the serotypes (1). In India alone 109 species of *Salmonella* were described till 1970 (2). The aberrant biochemical characters of occasional strains and the genetic

mutability of the serotypes add to the complexity of diverse antigenic types of *Salmonella*. On rare occasions antigenic similarities among different species also complicate the identification. In addition food technologists are always confronted with the problem of having to decide on the pathogenicity to man of an isolate hitherto not known to be associated with human disease. Since human experiments are impossible, it is logical to test and compare the pathogenicity to laboratory animals of such *Salmonella* isolates with *Salmonella*

known to be pathogenic to man. We report a recently conducted animal pathogenecity study on *Salmonella roan* (o:38 H:1 v:e. n, x) isolated from food samples. This study was carried out on behalf of Central Institute of Fisheries Technology, Cochin, India.

Materials & Methods :

Bacterial Cultures: 1) *Salmonella roan* was referred by Central Institute of Fisheries Technology, Cochin, India. Biochemical characters were confirmed as per the identification scheme of Edwards and Ewing (4). Serotyping for O and H antigens was carried out at Salmonella Reference Laboratory, Kasauli, India.

***S. enteritidis*:** The isolate in our laboratory from the infected laboratory animals was checked for the characteristic biochemical reactions and was confirmed sero-

logically by the Salmonella Reference Laboratory, Kasauli, India.

Animals: Albino mice of NIH strain weighing 18-20 gm. were supplied by Haffkine Biopharmaceutical Corp. Ltd. Bombay.

LD₅₀ values: In order to determine LD₅₀ values, mice were injected by intraperitoneal route 0.5 ml. of the different dilutions of the test organism suspended in 2.5% mucin. Nutrient broth culture of the test organism having viable count of 1.4×10^9 ml. was diluted from 10^{-1} to 10^{-6} . These dilutions were mixed with equal volume of 5% mucin and 0.5 ml. were injected intraperitoneally. Mice were observed for a period of 14 days. LD₅₀ values were calculated by the method of Reed & Muench (5). Briefly it is as follows :

Proportionate distance from LD₅₀ value of the minimum dose causing greater than 50% mortality (x)

$$= \frac{\left(\begin{array}{l} \% \text{ mortality greater} \\ \text{than 50\%} \end{array} \right)}{\left(\begin{array}{l} \% \text{ mortality greater} \\ \text{than 50\%} \end{array} \right)} - \frac{\left(\begin{array}{l} \% \text{ maximum mortality} \\ \text{observed at the next} \\ \text{dose causing less than} \\ \text{50\% mortality} \end{array} \right)}{\left(\begin{array}{l} \% \text{ maximum mortality} \\ \text{observed at the next} \\ \text{dose causing less than} \\ \text{50\% mortality} \end{array} \right)}$$

Negative Log. of LD₅₀ Titre

= Negative Log of minimum dilution causing greater than 50% mortality (d) + Proportionate distance (x)

$$\text{LD}_{50} \text{ value} = 10^{(d+x)} \times \frac{\text{Count of undiluted bacterial suspension}}{\text{ml}} \times 0.5$$

Cumulative Mortality Records :

Daily cumulative mortality records were maintained for each dose separately. Time points of reaching 50% & 100% mortality were noted down.

Autopsy Studies : 15 mice were injected by interaperitoneal route 2.2×10^4 organisms (1 LD₅₀ dose), suspended in 2.5% mucin. Three animals were sacrificed on each of the days 1, 3, & 6 respectively. Two animals were sacrificed on day 14. An autopsy was performed on each animal. A fragment of spleen & a fragment of liver were separately collected with aseptic technique. These were ground in a sterile mortar and pestle. The material was inoculated into 10 ml. of Selenite F broth. After overnight incubation at 37°C samples were plated on MacConkey's Agar. Non lactose fermenter colonies were picked up and their biochemical characters were studied.

Results :

LD₅₀ value for *Salmonella roan* was 2.8×10^7 , while that for *Salmonella enteritidis* was 5.7×10^4 organisms.

Minimum time required to achieve 50% mortality (Table I) for *S. roan* was 4 days when one LD₅₀ dose of the organisms was injected. The mortality time point of achieving 50% mortality for *S. enteritidis* was approximately

5 days. With increasing doses 50% mortality could be achieved in a much shorter time. 100% mortality for *S. roan* could be achieved only with 3.5×10^4 (approximately 1000 LD₅₀ doses) and that too within 1 day. 100% mortality for *S. enteritidis* could be achieved with 2.25×10^6 organisms (50 LD₅₀ doses) or more. The 50LD₅₀ dose could achieve 100% mortality in five days. Higher doses required much shorter period to achieve 100% mortality.

Autopsy Studies :**Morbid Anatomical Changes**

Gross Changes : Almost all the organs were congested at autopsy. No other changes were observable in animals that were sacrificed 24 hours after challenge. The other three groups of animals i.e. those that were sacrificed on days 3, 6 & 14 showed enlargement of spleen, Liver, Mesenteric lymph nodes and Peyer's patches. The enlargement of Spleen, Mesenteric lymph nodes and Peyer's patches were particularly marked in the animals that were sacrificed on days 6 & 14.

Histological Changes :

(1) **Liver :** In general there were no architectural alterations. Twenty four hours after challenge the liver showed intense sinusoidal congestion and cloudy change. No other pathology could be identified. On days 3 & 6 the

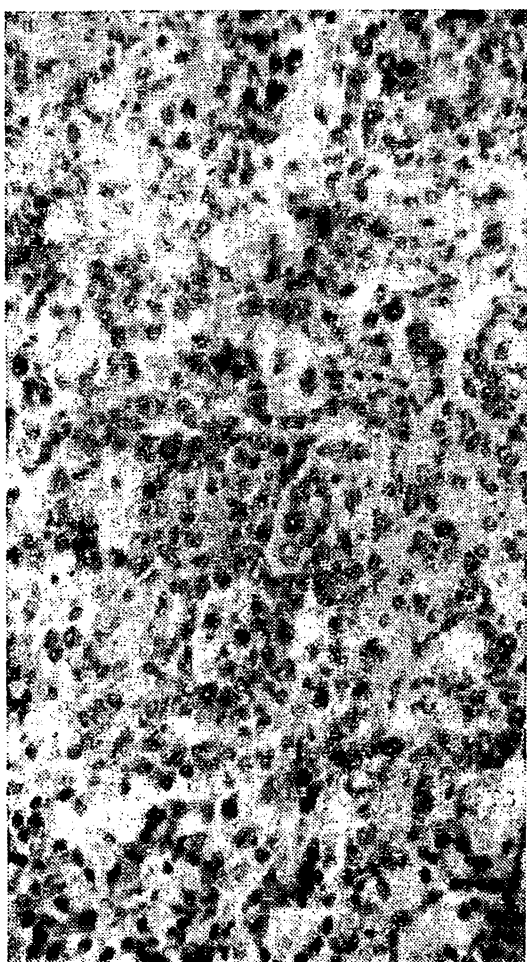


Fig. 1

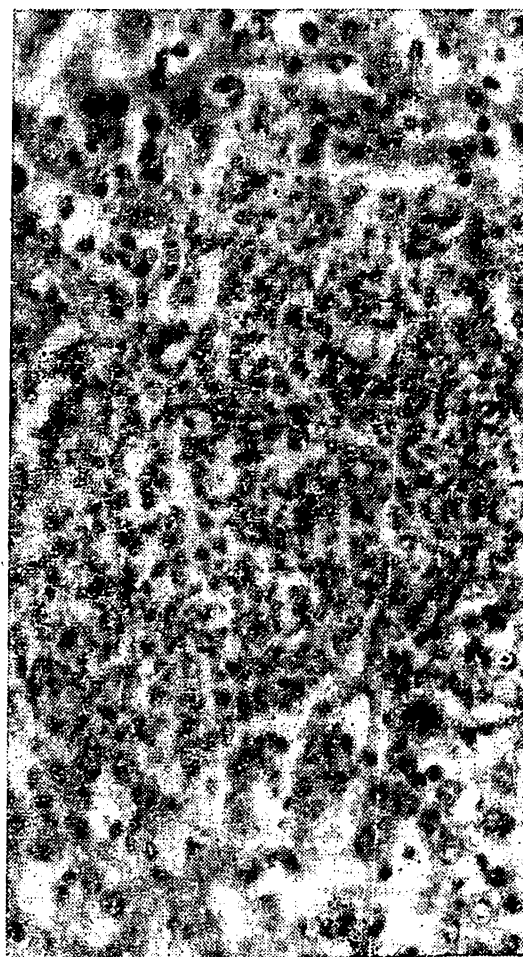


Fig. 2

TABLE I

Mortality time Points for *S. roan*

No. of organisms	Time in days for 50% mortality	Time in days for 100% mortality
3.5×10^2	Not achieved	Not achieved
3.5×10^3	7	„
3.5×10^4	4	„
3.5×10^5	1	„
3.5×10^6	1	„
3.5×10^7	less than 1	„

TABLE II

Mortality time points for *S. entritidis*

No. of organisms	Time in days for 50% mortality	Time in days for 100% mortality
2.25×10^3	Not achieved	Not achieved
2.25×10^4	„	„
2.25×10^5	5	„
2.25×10^6	3	5
2.25×10^7	less than 1	less than 1
2.25×10^8	less than 1	less than 1

liver showed changes that were quite similar. The portal tracts revealed mild mononuclear infiltration at places. The parenchymatous cells showed a general cloudy change. There were focal areas of necrosis of the hepatic parenchyma. The necrotic foci were infiltrated by macrophages and lymphocytes. The sinusoids showed numerous prominent Kupfer cells. The day 14, liver showed an occasional small focus of necrosis. Kupfer cells were numerous. Many more portal triads revealed fair amount of mononuclear infiltration. (Fig. 1).

Spleen: At 24 hours splenic red pulp was markedly congested. No other changes could be seen at this time. Spleens of animals sacrificed on day 3, 6 & 14 showed similar changes. The red pulp was congested. The perivascular lymphoid follicles appeared enlarged. The enlargement was maximum in day 14 spleens. There were no prominent germinal centres. The marginal zones of lymphoid follicles revealed mixed cellularity consisting of macrophages. These were particularly numerous on days 3 & 6. (Fig. 2).

Mesenteric Lymph nodes :

At 24 hours the nodes revealed congestion and mild macrophage infiltration of paracortical areas. The day 3, 6 & 14 nodes revealed essentially similar changes. The cortical lymphoid follicles appeared smaller. The paracortical areas

were enlarged and they had encroached upon the adjoining follicles. The enlargement was particularly marked in day 6 & 14 samples. The paracortical areas showed mixed cellularity consisting of lymphocytes and macrophages. The sinusoids were dilated and contained numerous macrophages. No necrotic foci could be seen.

Peyer's Patches :

At 24 hours there were no changes. The day 3, 6 & 14 Peyer's patches revealed lymphoid hyperplasia. No other pathology could be observed.

The other organs showed congestion and cloudy change.

Bacteriological Studies :

Non-lactose fermenting gram-ve bacilli could be isolated from Livers and Spleens of all the 11 mice sacrificed at various time points. The isolates showed biochemical characters identical with those of the strain submitted for investigation.

Discussion :

The results indicate that the strain of *Salmonella roan* is pathogenic to mice. The LD₅₀ value of *S. roan* is comparable to that of *S. enteritidis* (Table I & II). *S. enteritidis* is a known pathogen for mice & hence used in this study for comparison. Time points for achieving 50% mortality are also comparable.

The morbid anatomical changes seen in Liver, Spleen and mesenteric lymph nodes are very much similar to the effects caused by Salmonella in general (6) i.e.

1) Focal necrosis of liver with macrophage infiltration of the necrotic foci, Kupfer cell hyperplasia.

2) Acute congestion of spleen with lymphoid hyperplasia and macrophage infiltration in the marginal zone of periarterial lymphoid sheath.

3) Paracortical hyperplasia with predominant macrophage infiltration of Lymphnode.

These data indicate that the

strain behaves very much similar to *S. enteritidis* when tested in mice. Although pathogenicity to mice does not necessarily indicate pathogenicity to man, the possibility of *S. roan* being pathogenic for man as well, is very high. This is because, the host specificity such as that of *S. typhi* for man forms one of the exceptions and as a general rule Salmonella behave as pathogens to most of the mammals.

Acknowledgements

The authors wish to express their gratitude to the Director, Haffkine Institute for granting permission to publish the material.

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COMPARISON OF DISC SENSITIVITY OF CLINICAL ISOLATES FOR GENTAMICIN, NEOMYCIN & FRAMYCETIN.

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ABSTRACT :

Antibiotic sensitivity studies employing disc diffusion technique were carried out on 500 clinical isolates against three aminoglycosides namely Gentamicin, Framycetin and Neomycin. 86.6% of the isolates were sensitive to Gentamicin (10 mcg. disc) while only 49.8% and 49.4% of the isolates were sensitive to Framycetin (30 mcg. disc.) and Neomycin (30 mcg. disc.). Even when higher strength viz. 100 mcg. discs. of Framycetin were employed rise in sensitivity was marginal (61%). Multiple drug resistance was found to be much more common than resistance to a single drug and there appears to be cross resistance between Framycetin and Neomycin. Significance of the data is discussed in the text.

Introduction

Aminoglycosides form one of the most useful group of antibiotics in combating infections of a fairly wide spectrum. Even with increased incidence of multiple drug resistance mediated by R-factors, resistance for the majority of aminoglycosides was remarkably low. However, in the past few years plasmid mediated drug resistance to these antibiotics is being increasingly reported. Till 1967, plasmid conferring resistance to Gentamicin was not known (1). But this did not remain true for very long (2). Hence, it becomes necessary to

update information on the emergence of resistance in clinical isolates to such useful drugs. We have investigated 500 clinical isolates from various sources for sensitivity against three aminoglycosidic antibiotics namely Neomycin, Framycetin & Gentamicin.

Material & Methods

1) **Bacterial Isolates:** The bacterial strains subjected to antibiotic sensitivity test were isolated and identified at Haffkine Institute. Table I shows the source of the isolates.

2) **Antibiotic Sensitivity Test:** Disc diffusion method of Bauer

Received for publication on 3rd Sept, 1979.

TABLE I

Source of isolates

Urinary tract infections	79
Isolates from cases of diarrhoea	82
Pus samples	85
Conjunctival swabs	21
Ear, Nose, Throat swabs	32
Swabs from Cases of burns	119
Miscellaneous infections	82
Total	500

et al (3) was employed. The concentrations of antibiotics employed per disc were as follows.

Gentamicin	— 10 mcg.
Framycetin	— 30 mcg.
Framycetin	— 100 mcg.
Neomycin	— 30 mcg.

Results :

Staph aureus, *E. coli*, *Klebsiella*, *Proteus* and *Pseudomonas* formed the predominant isolates tested.

Staph aureus strains were isolated from conjunctiva, nose, throat & miscellaneous sources including pus from abscesses of varied sites. *E. coli* *Klebsiella*, *Proteus* & *Pseudomonas* tested in

this study were isolated from all the above mentioned sites and also from urine. Fifty eight of the *E. coli* strains were Enteropathogenic (EPEC), isolated from cases of diarrhoea. Eighteen strains of *Salmonella* (*S. enteritidis* and *S. typhimurium*) were also isolated from cases of diarrhoea. 119 strains of *Pseudomonas* were isolated from cases of burns.

Table II reveals the Antibiotic Disc Sensitivity results of Gentamicin, Framycetin (30 mcg. & 100 mcg. discs.) and Neomycin. Out of 500 different isolates studied 86.6% were sensitive to Gentamicin, 49.8% were sensitive to Framycetin (30 mcg. disc.), and 49.4% were sensitive to Neomycin. The number of sensitive

TABLE II

Organisms	Total No of strains studied	G 10			F 30			F 100			N30		
		S	R	PR	S	R	PR	S	R	PR	S	R	PR
<i>Beta haemolytic</i>													
<i>Streptococci</i>	9	8	1	0	5	3	1	5	2	2	5	3	1
<i>Streptococcus faecalis</i>	2	1	1	0	0	2	0	0	2	0	0	2	0
<i>Staph. aureus</i>	94	90	4	0	72	19	3	78	12	4	70	19	5
		(95.7%)			(76.6%)			(83%)			(74.5%)		
<i>E. Coli</i>	102	99	3	0	74	17	11	85	12	5	74	18	10
		(97 %)			(72.5%)			(83%)			(72.5%)		
<i>Klebsiella</i>	64	55	9	0	44	16	4	48	16	0	42	18	4
		(85.9%)			(68.7%)			(75%)			(65.6%)		
<i>Enterobacter</i>	1	1	0	0	1	0	0	1	0	0	1	0	0
<i>Citrobacter</i>	1	1	0	0	0	1	0	0	0	1	0	1	0
<i>Proteus</i>	37	28	9	0	18	16	3	20	14	3	19	17	1
		(75.6%)			(48.6%)			(54%)			(51.3%)		
<i>Salmonella</i>	18	16	2	0	10	6	2	12	5	1	4	13	1
		(88.8%)			(55.5%)			(66.6%)			(22.2%)		
<i>Shigella</i>	1	1	0	0	1	0	0	1	0	0	1	0	0
<i>Pseudomonas</i>	171	133	37	1	24	130	17	55	63	53	31	130	10
		(77.7%)			(14 %)			(32%)			(18 %)		
Total	500	433	66	1	249	210	41	305	126	69	247	221	32
		(86.6 %)			(49.8%)			(61%)			(49.4%)		

TABLE III

Patterns of drug resistance

Antibiotic markers	No. of resistant isolates	% Resistant isolates
G alone	1	0.2
F alone	13	2.6
N alone	16	3.2
G and N	1	0.2
G and F	0	0
N and F	160	32
G, N and F	64	12.8

G = Gentamicin

F = Frymycetin

N = Neomycin

strains increased marginally to 61% with Framycetin 100 mcg. discs.

Discussion :

Plasmid borne resistance to aminoglycosides has been attributed to enzymatic inactivation (4). These are acetylating and phosphorylating enzymes. Often cross-resistance to related antibiotics is shown by many organisms. For example Kanamycin phosphotransferase can inactivate Neomycin as well (4). Davies (5) has recently suggested that the mechanism of resistance to aminoglycosides consists of a block in the transport of the drug and the modification of the antibiotic by the enzyme is not the primary

mechanism. If this is true then the phenomenon of cross resistance by virtue of affinity for inactivating enzyme may not hold true. Moreover, plasmids coding for Gentamicin resistance were not known till 1967 (1). Smith reported for the first time R-factor mediated resistance for Gentamicin in 1967 (2). All this data necessitates continuous vigilance through in-vitro testing of useful drugs such as aminoglycosides.

Studies on the genetics of drug resistance were not carried out by us. However, the results have been analysed for the presence or otherwise of multiple drug resistance (Table III). Multiple drug resistance was found to be

much more common than resistance to a single drug. Maximum number of isolates were found to be resistant to Neomycin and Framycetin (32% of the strains). It was also very significant that resistance to Gentamicin was always associated with resistance to Neomycin and Framycetin. Only 0.2% strains were resistant to Gentamicin alone where as 12.8% of the strains were resistant to all the three antibiotics. There appears to be cross resistance between Neomycin and Framycetin. Such cross resistance among closely related aminoglycosides may be due to R plasmids.

We have carried out antibiotic sensitivity studies with Framycetin discs of two strengths namely 30 mcg. & 100 mcg. antibiotic per disc. This was done in view of certain recent reports employing 100 mcg. Framycetin discs. (7, 8). Since Framycetin is an antibiotic closely related to Neomycin (some authorities even consider it to be the same as Neomycin B (6)) it was felt that proper concentration to be employed in the disc should be the same as recommended for Neomycin by Baur et al (3) viz 30 mcg. and it would be incorrect and misleading to use 100 mcg. Framycetin discs in actual practice. However, we carried out the antibiotic sensitivity study with Framycetin discs of both the strengths viz. 30 mcg. and 100 mcg. antibiotic per disc. Our results indicate that employment of

30 mcg. discs for Framycetin is appropriate and realistic because the sensitivity test results with 30 mcg. discs were comparable with Neomycin 30 mcg. discs. Out of 500 strains studied 49.4% were sensitive to Neomycin (30 mcg.) and 49.8% were sensitive to Framycetin (30 mcg.). Even when 100 mcg. discs of Framycetin were employed the overall sensitivity response increased only marginally (61% strains) which was still considerably less than the response obtained with Gentamicin (86.6% strains) used in standard strength (10 mcg. per disc). This difference was particularly noticeable in *Pseudomonas* sensitivity results. 77.7% of the strains were sensitive to Gentamicin where as only 14% were sensitive to Framycetin (30 mcg. discs.) and 18% were sensitive to Neomycin. Even with 100 mcg. Framycetin discs only 32% of the *Pseudomonas* strains were found sensitive. These results directly contradict reports of Subramaniam et al (7) and Mahajan & Angra (8).

Drug sensitivity studies on Enteropathogenic *E. coli* (EPEC) and *Salmonella* were carried out because majority of those isolated were found to be resistant to Chloramphenicol, (58% of *E. coli* and 63% of *Salmonella*) a commonly employed drug in the treatment of diarrhoea. It was also observed that 78% of *Salmonella* and 63% of EPEC were resistant

to Neomycin, another commonly employed drug in the treatment of diarrhoeas. Eventhough, high cost excludes the use of Gentamicin in the treatment of diarrhoeas, it is significant that 98.4% of EPEC and 88.8% of *Salmonella* were sensitive to Gentamicin.

Acknowledgements

The authors wish to express their gratitude to the Director, Haffkine Institute for granting permission to publish the material.

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BOOK REVIEW

TITLE LYMPHOCYTE HYBRIDOMAS Current topics in Microbiology and Immunology — Vol. 81 Editors — F. Melchers, M. Potter, N. L. Warner. Springer — Verlag — Berlin — Heidelberg — New York, 1978

Review

The monograph under review is the 81st Volume of Current topics in Microbiology and Immunology. Edited by F. Melchers, M. Potter, N. L. Warner. It is devoted to the papers presented at the 2nd Workshop on 'Functional Properties of Tumours of T. and B. Lymphocytes'. Sponsored by the National Cancer Institute (NIH) April 3-5, 1978.

The workshop was initiated to focus the wide interest created on this subject following the discovery by Kohler and Milstein. By successful fusion of myeloma cells with spleen cells from animals previously immunised, Kohler and Milstein demonstrated the production of continuous cultures of hybridomas secreting antibodies of predefined specificity.

The monograph consists of 39 papers presented at the meeting. It includes two tables which show at a glance the various cell systems that have been hybridised to date. So far only mouse plasmacytoma lines are available. To help investigators to start work with standard cell lines, the Salk

Institute, Tumour Distribution Centre has undertaken to supply the standard sublines.

Several of the papers deal with technological problems encountered in attempts to obtain highest concentration of functional hybrids.

Attempts are directed to the study of the various factors involved in improving the frequency of cell fusion. Basically this is dependent on the clonal type present in the normal cell population capable of fusion with the neoplastic partner. The problem therefore highlights the importance of biochemical or biophysical characterisation of the component in the tumour cell population.

Some of the reports deal with hybridisation of mouse myeloma cells with normal Ig secreting cells of other species. These include attempts to fuse mouse myeloma cells with human CLL cells and human lymphoblastoid cell lines.

Few of the studies pertain to the extension of this approach to produce hybrids of T. lymphocytes.

Successful culture of lymphocyte hybridomas offers an effective practical solution to the production of monospecific antibodies. So it has stimulated wide interest among cell biologists extending to a variety of problems ranging from details of chemical, structural changes in the antibody molecule; genetic expression and biomedical studies which may have relevance to clinical medicine. This volume presents a comprehensive picture of the status of the field to date and

gives glimpses of the future application of this approach to biomedical problems. It forms a very useful base for those interested in using this approach.

Each of the papers was followed by a discussion. Although some of the points brought up in the discussion have been alluded to, in the preface I feel inclusion of the discussion at the end of each article would have further enhanced the interest in the subject.

Dr. (Miss) Beatriz M. Braganca,
Haffkine Institute.

PROCEEDINGS OF THE CRITICS' CLUB

(January to April, 1979)

The Critics' Club of the Haffkine Institute began the year's activities with an eminent speaker on 2nd January — Prof. George Wald, American Biologist who won international acclaim for his work on Vitamin A and vision. Prof. Wald spoke on "Vision and other important matters". Addressing the scientists of the Institute, Prof. Wald covered a wide range of subjects ranging from science to religion and philosophy. He held the audience spell-bound for over an hour. He concluded his talk by saying that inspite of the immense amount of knowledge gathered on the subject of vision, scientists cannot claim to have understood the value of vision. Prof. Wald is a receipient of the Nobel award for Medicine and Physiology, which was conferred on him in 1967, in recognition of his work on the biochemistry of vision.

Miss. Sushila Menon of the Department of Zoonosis addressed the Critics' Club, on 19th January and discussed her work on the establishment of Hookworm infection in laboratory animals. For the first time the human strain of this parasite (*Necator americanus*) was established in infant rabbits without the concomitant use of immunosuppressants. The workers suggested that this model may

prove to be an important advance in the study of host-parasne interactions.

Dr. Shah, of the Chemotherapy Department discussed before the club the use of plastic containers for transfusion fluid. He enumerated the advantages these containers have over the conventional glass bottles. Reports of leaching of the plasticisers in the fluid had however, checked the indiscriminate use of plastics for this purpose. Work done in his department showed that the P.V.C. containers of Indian origin were of sufficiently high quality and did not present any problems when used to store aqueous fluids. He concluded with a note of caution against the use of such containers for blood, as the problems in this were many and careful study was necessary.

Miss Theresa Joseph, of the Department of Clinical Pathology, spoke before the Critics' Club on the Bacteriology of Infant Diarrhoeas. A study involving 150 cases of infant diarrhoeas was carried out in the institute. The predominant organism to be isolated was the Enteropathogenic *Escherichia Coli* (EPEC), surprisingly 18% of the samples showed the presence of *Salmonella*. The workers extended the study to test the sensitivity of the isolates to commonly used antibacterial agents. The results of these tests

revealed that a high percentage of isolates of EPEC and Salmonella were resistant to Chloramphenicol and Neomycin, the commonly used agents. Septran (a combination of Trimethoprim and Sulphamethoxazole in the ratio 1:5) was found to be effective against all EPEC and 96% of Salmonella isolates. The value of Chloramphenicol and Neomycin in combat infantile diarrhoea is therefore questioned by the workers.

A visiting scientist from England Dr. Dawn Owen gave two talks to the Critics' Club. Dr. Owen, a Research Officer is a Parasitologist working in the Laboratory Animal Centre of the Medical Research Council of England. In her first talk she described the set-up, functions and the functioning of the centre. In her second talk she discussed the common parasites of laboratory

animals, animal breeding and care.

Shri S. P. Gawade of the Director's Research Cell spoke on the Sea-Snake Antivenine before the Critics' Club. He discussed the distribution of the various species of sea-snakes, the toxicity of their venoms, and methods for the preparation of antivenine. Using the procedure employed for the production of land snake antivenine the workers have obtained a monovalent sea-snake antivenine. Though prepared against *Enhydrina schistosa* venom the antivenine showed considerable activity against *Enhydrina schistosa* venom, some other sea-snake venoms. In the lively discussion which followed the presentation, many suggestions were offered to render the antivenine fail-safe for clinical use.

R. B. Ghooi
Scientific Secretary

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. K. D. Sharma, Director, Haffkine Institute for Training Research and Testing, Bombay-400 012 and printed at the Nive Arts
Bombay 400 031

