

BULLETIN

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Role of *V. cholerae* somatic antigens in the pathogenesis of cholera — A preliminary report

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Passive protection studies, in adult rabbit ileal loop model using various antisera against *V. cholerae* (Inaba) surface antigens, indicate that antismatic antibodies play a major role in protection. Antismatic antiserum which was prepared against boiled cells of *V. cholerae* 569B Inaba had no effect on *in vitro* proliferation or toxin production of 569B Inaba. However, the antiserum did inhibit adhesion of 569B Inaba to the intestinal epithelium in an *in vitro* system.

Introduction

V. cholerae is a non-invasive enteropathogen which proliferates in the small intestine and generates a powerful exotoxin, which induces fluid secretion. Consequently there are two possible immune mechanisms in the prophylaxis of the disease, one, antibacterial and the other, antitoxic. Conventional cholera vaccines are killed bacterial suspensions and obviously induce antibacterial immune responses. However, protection offered by these vaccines is limited and the duration of protection is also short (1). Cholera toxoid to induce antitoxic immunity was also tried in recent Bangla Desh trial with rather discouraging results (2). As a result, search for a more effective vaccine

has gained momentum in recent years. However, the mechanism of protection offered by the conventional vaccines has remained obscure to a great extent. Such knowledge, we feel may help to improve the present vaccines. Hence, attempts were made to understand the nature of surface antigens of *V. cholerae* which induce protective immune responses and the mechanism of protection offered.

Material and Methods

(A) Antisera :

(i) Antisera were raised in rabbits by intravenous immunisation with 0.5 ml, 1.0 ml, 1.5 ml, 2.0 ml and 2.5 ml of a suspension containing 10^9 organisms/ml. Two types of suspensions of *V. cholerae*

569B Inaba were employed, one containing live cells and the other boiled cells.

(ii) A portion of anti-live *V. cholerae* antiserum was absorbed with boiled cells of *V. cholerae* 569B Inaba to remove antiserum antibodies. Boiled cells of *V. cholerae* used for absorption were washed twice in phosphate buffered saline (PBS) and 1 ml. of packed cells were added to 5 ml. of the serum. This was incubated at 37°C for 1 hour and then centrifuged (3000 g for 30 minutes). The serum was absorbed thrice.

All the sera were de complemented before use. Bacterial agglutination titres were determined by using live cells of 569B Inaba by standard tube agglutination test (3). Anti-LPS antibody titrations were done employing sheep erythrocytes, sensitised with pure LPS of 569B Inaba in micro-haemagglutination trays. Anti-flagellar antibody titrations were done employing sheep erythrocytes sensitised with crude flagellar protein prepared as described by Yancey *et al* (10).

(B) Protection studies :

Adult rabbit ileal loop technique of De and Chatterjee (4) was used. Ileal loops of 10 cm length were prepared *in-vivo*. Loops were injected with 1 ml of appropriately diluted de complemented antiserum or normal rabbit serum 30 minutes prior to challenge with 5×10^5 cells of *V. cholerae* 569B Inaba. Loops were observed for fluid collection after 18 hours. Loops showing distinct fluid accumulation were considered as non-protected loops while those exhibiting no fluid accumulation were read as protected loops. Each dilution of the serum was injected in duplicate in each rabbit. Each antiserum was tested in three rabbits.

(C) *In vitro* proliferation and toxin production.

50 ml syncase medium in 250 ml conical flasks was inoculated with 10^6 live cells of *V. cholerae* 569B Inaba. Normal rabbit serum or anti-boiled *V. cholerae* antiserum was added to these flasks at 1:80 dilution and flasks were shaken at 200 RPM at 37°C for 8 hours. The samples from each flask were subjected for viable bacterial count and cholera toxin assay by cutaneous permeability method of Craig (5).

(D) *In vitro* adhesion

A portion of proximal ileum was taken out and ileal loops of 5 cm length were prepared *in vitro*. Two adjacent loops of the ileum were considered as pairs. One member of each pair received 0.5 ml of 1:200 dilution of de complemental normal rabbit serum and the other received 1:200 dilution of de complemented anti-boiled *V. cholerae* antiserum. After 10 minutes loops were injected with 5×10^5 live cells of 569B Inaba. Hank's gelatin solution was used for dilutions of culture and sera. After 30 min. at room temperature loops were rinsed in 10 ml of PBS (pH 7.2) and washed gently by swirling in three consecutive 250 ml conical flask containing 100 ml PBS in each flask. The loops were homogenised in a tissue homogeniser and suspended in 5 ml PBS and subjected to bacterial counts to get number of vibrios adherent to the epithelium. The results were analysed by Wilcoxon's paired rank sum test as described in (9).

Results :

Passive protection studies :

As shown in Table I anti-live *V. cholerae* antiserum could protect the challenge.

Table I
Comparison of antiserum and anti-flagellar antibody
titers with ileal loop protection titers.

Serum	Bact. aggl. titer	Anti-fla- gellar titer ^a	Anti- LPS titer ^b	Protec- tion titer ^c
Live <i>V. cholerae</i> antiserum	320	64,000	16,000	80
Boiled <i>V. cholerae</i> antiserum (anti- somatic antiserum)	320	3,200	16,000	80
Live <i>V. cholerae</i> antiserum after absorption with boiled cells of <i>V. cholerae</i>	< 2	32,000	< 2	0

a. passive haemagglutination titer against sheep red cells sensitised with crude flagellar protein

b. passive haemagglutination titer against sheep red cells sensitised with 569 B Inaba LPS.

c. *in vivo* ileal loop challenge with 10⁵ live cells of 569 B Inaba.

Table II
***In vitro* effect of antiserum on proliferation**
and toxin production of *V. cholerae* 569 B Inaba

Serum	Bacteria/ml	Total skin sensitising units
N R S	1.9 x 10 ⁸	22,625
V R S	1.8 x 10 ⁸	16,000
N R S	1.4 x 10 ⁸	11,310
V R S	1.4 x 10 ⁸	16,000

N R S : Normal rabbit serum

V R S : *V. cholerae* antiserum

Table III

in vitro adhesion of *V. cholerae* 569 B Inaba in the presence of rabbit antiserum

Set A No. of <i>V. cholerae</i> adherent in the presence of normal rabbit serum .	Set B No. of <i>V. cholerae</i> adherent in the presence of antiserum .
2,050	150
3,175	325
2,875	500
9,275	3,450
1,950	1,050
2,250	675
9,075	1,150
3,500	550

a : Rabbit anti-boiled *V. cholerae* 569 B Inaba antiserum.

b : Decomplemented sera at a dilution of 1/200.

Set A > Set B ($p < 0.005$)- Wilcoxon's paired rank sum test.

ged ileal loops upto 1 : 80 dilution. Anti-boiled *V. cholerae* antiserum which has predominantly antisomatic antibodies also protected the ileal loops to the same extent. Anti-live *V. cholerae* antiserum when absorbed with boiled cells of *V. cholerae* 569B Inaba was virtually free from antisomatic antibodies as seen from bacterial agglutination test and passive haemagglutination test using sheep erythrocytes sensitised with pure LPS of *V. cholerae* 569B Inaba but retained almost all anti-flagellar antibody activity as seen by passive haemagglutination test using sheep erythrocytes sensitised with crude flagellar protein (table I). Such absorbed serum failed to offer any protection. Thus, it seems that antisomatic antibodies play a major role in protection.

***In vitro* effect of anti-boiled *V. cholerae* antiserum on bacterial proliferation and toxin production :**

Bacterial proliferation and toxin production both in the presence of normal rabbit serum and anti-boiled *V. cholerae* antiserum remained almost the same as seen from the data in table II.

***In vitro* adhesion of 569B in the presence of anti-boiled *V. cholerae* antiserum :**

Number of *V. cholerae* 569 B Inaba adherent to the mucous membrane in the presence of normal rabbit serum and in the presence of anti-boiled *V. cholerae* antiserum are shown in table III. It can be clearly seen that the loops receiving antiserum showed much fewer adherent vibrios than the loops receiving normal rabbit serum ($P < 0.005$). Therefore it appears that anti-boiled *V. cholerae* antiserum inhibits adhesion of *V. cholerae* to the intestinal mucous membrane.

Discussion

Results on passive protection studies indicate that antisomatic antibodies play a major role in protection. Anti-boiled *V. cholerae* antiserum containing predominantly antibodies against somatic antigens (anti-LPS) could offer excellent protection. Anti-live *V. cholerae* antiserum, absorbed with boiled cells, containing predominantly antibodies against flagellar proteins failed to offer any passive protection. These data clearly indicate that antibodies against somatic antigens are responsible for passive protection observed. Role of antisomatic antibodies in protection against cholera has been suggested by various workers (7,8,9). Even the protection offered by conventional heat killed vaccine or purified somatic antigen vaccine (1) substantiates the role of somatic antigen in inducing protection.

However, in the present study 569 B Inaba which is a feebly motile strain, was used for challenge and it is likely to be less pathogenic, as suggested by Yancey *et al* (10). Hence, experiments on passive protection by the antisera were repeated using two other wild strains of *V. cholerae* Inaba showing active motility and the results were similar to those for 569 B Inaba (results not shown). The mechanism of protection offered by antisomatic antibodies is not known precisely. Complement mediated killing of *V. cholerae* is unlikely in this situation and we have shown that these antibodies have no effect on *in vitro* proliferation or toxin production of *V. cholerae*. However, *in vitro* adhesion of *V. cholerae* to the intestinal epithelium was inhibited by the anti-boiled *V. cholerae* antiserum. Freter (11) also report-

ed similar observations in an *in vitro* assay.

The antisomatic antiserum used in the experiments is "operationally" specific against somatic antigen. However, it is difficult to rule out antibody activity against other vibrio surface molecules, in the antisomatic antiserum. It is also possible that the adhesion is brought about by a bacterial surface molecule other than somatic antigen and that the antisomatic antibodies sterically hinder the interaction of this surface entity with

the mucous membrane. Studies designed to probe the various facets are in progress.

Thus, in conclusion our data suggest that antisomatic antibodies play a major role in inducing protection and these antibodies have no effect on bacterial proliferation and toxin production. However, they inhibit adhesion of *V. cholerae* to the intestinal epithelium. Experiments designed to understand the role of bacterial adhesion in the pathogenesis of cholera are in progress.

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Prevalence of Fertility Inhibition Negative type R Plasmids in Faecal Coliforms

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Out of 890 Gram negative isolates from the faeces of 848 individuals in Bombay, 36.7% isolates were multidrug resistant, and of these 17.3% harboured R plasmids transferable at 37°C, while 8.8% carried thermosensitive R plasmids transferable only at lower temperature, around 28°C. Over seventy five percent of the R plasmids were of fertility inhibition negative (fi-) type.

Introduction :

R plasmid bearing enterobacteria pose serious health problems for they can initiate endogenous infections and can, as well, transfer the plasmid borne multiple drug resistance to potential enteric pathogens like *Salmonella typhi* (1,2). The endemicity of typhoid in India (3) and the high incidence of R+ coliforms in the gut of Indians (4,5) point towards the possibility of conjugal transfer of R plasmids to *S. typhi* either in the gut or in voided faeces and sewage. Some R plasmids are thermosensitive and are not transferable at 37°C. Such R plasmids, transferable only around 28°C, are not likely to be involved in conjugal transfer in the gut but these may spread extensively by conjugation in sewage plants. Hence faecal coliforms from the local population were examined for R plasmids transferable at 37°C, as well as for thermosensitive R plasmids.

We have recently seen that fertility inhibition negative (fi-) type R plasmids are genetically stable in *S. typhi* (8). As stable association of R plasmid with *S. typhi* will ensure maintenance and spread of R plasmid, such a strain could possibly initiate drug resistant typhoid in the community. Hence, prevalence of fi- negative type R plasmids in the faecal coliforms was also studied.

Materials and Methods

Faecal samples from 848 individuals in Bombay were plated on MacConkey Agar and predominant colony-types were identified by standard biochemical tests (9). The isolates were then screened for antibiotic sensitivity by the disc diffusion technique of Bauer *et al* (10). Multi-drug resistant enterobacteria were examined by the method of Watanabe (11), for conjugal transferability of the drug resistance. *E. coli* K 12 Nal^r, kindly provid-

ed by Dr. B. Rowe, ERL, London, was employed as recipient and the multi-drug resistant enterobacteria were used as donors. Mating mixtures were plated on MacConkey Agar containing 60 mcg/ml nalidixic acid and donor drug-markers so that only transconjugants were selected. Plasmid nature of the drug-resistance markers was further confirmed by secondary crosses between *E. coli* K12 transconjugants and *S. typhi* and selection of *S. typhi* transconjugants on DEC Agar (12). To test whether multiple drug resistance (MDR) that was not transferable at 37°C in the above system could be transferred at lower temperature, mating experiments were conducted by incubating premating cultures and mating mixtures at 28°C.

Fertility inhibition typing of R plasmids was carried out by the method of Ander-

son and Threlfall (13). Briefly, R plasmids were transferred to *E. coli* K12HfrC Nal Transconjugant K12HfrC. Cultures were plated on Tryptose Yeast Extract Calcium Agar (14) over which spot inocula were made with F-pilus specific phage m_μ 2, and observed for lysis after incubation. K 12 HfrC without R plasmid was used as control.

Results

Out of 890 Gram-negative enterobacteria, 834 were *E. coli* while 49 were Enterobacter species and 4 were Proteus species. As seen in Table I, 36.7% isolates were multi-drug resistant and 17.3% could transfer the R plasmids at 37°C, as well as at 28°C, while in 8.8% isolates the MDR was transferable only at 28°C. All these R plasmids were introduced into K12 HfrC for further studies.

Table I

Incidence of multi-drug resistant and R⁺ enterobacteria.

No. of individuals	No. of isolates	No. of multi-drug resistant isolates	No. of isolates with MDR transferable at 37°C	No. of isolates with MDR transferable at 28°C
848	890	327 (36.7)	154 (17.3)	78 (8.8)

Figures in parentheses show percent values.

Studies with K 12 HfrC showed that out of 154 plasmids transferable at 37°C and at 28°C, 25.3 % rendered the strain insensitive to m_μ 2 phage, and hence were grouped as fertility inhibition posi-

tive (fi+) type. The remaining 74.7% did not alter the phage m_μ 2 sensitivity of K 12 HfrC and were grouped as fertility inhibition negative (fi-) type. Of the 78 thermosensitive R plasmids transferable only at 28°C, 20.5 % were of fi+ type and 79.5 % were of fi- type (Table II).

Table II :
Fertility inhibition character of R plasmids

Nature of R plasmid	No. of R plasmids examined	No. of fi + R plasmids	No. of fi — R plasmids
Transferable at 37°C	154	39 (25.3)	115 (74.7)
Thermosensitive ; transferable only at 28°C	78	16 (20.5)	62 (79.5)

Figures in parentheses show percent values.

Discussion

It is noteworthy that 8.8 % of the total isolates harboured MDR on plasmids transferable at 28°C but not at 37°C, indicating that the enzyme system involved in conjugal transmission was impaired at high temperature. Such thermosensitive R plasmids have been reported earlier by Smith (6), and Grant *et al* (7). The fi negative type R plasmids involved in chloramphenicol resistant typhoid outbreaks in Mexico, Thailand, South Vietnam and Kerala (India) also show accelerated transfer at 22°C and 28°C (15). Augmented transfer at lower temperature, around 28°C, may have special implications in the transmission of plasmids in extra-intestinal environment, particularly in voided faeces and sewage. Classification on the basis of fi character revealed that striking majority of the R plasmids were of fi negative type. This is indeed an alarming observation, for unlike fi+ type R plas-

mids which are spontaneously eliminated from *S. typhi* cells during cell division, the fi negative R plasmids are genetically stable in *S. typhi*, (8). Stable association in host-bacterium augments maintenance and transmission of the R plasmid. Considering this fact and the endemicity of typhoid in this country it seems that fi negative type R plasmids have greater potential of initiating typhoid outbreaks. Since the Vi-phage type of *S. typhi* does not influence the R plasmid receiving-ability or genetic stability of R plasmid (8), any strain, especially the type prevalent in the community (16) could initiate such an outbreak.

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Synthesis and Anthelmintic Activity of New Pyrido (3,2d) Thiazoles

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2-Substituted amino pyrido (3,2d) thiazoles are obtained either by reacting 2-chloro, 3-isothiocyanatopyridine with 4-amino,4'-substituted benzophenones, and N-heterocyclic aminophenyl anilines or by condensing 2-chloro, 3-aminopyridine with the corresponding isothiocyanates. These are tested against *N. dubius* and *H. nana* in mice. The results are discussed.

Introduction :

2-Substituted phenylaminopyrido (3,2d) thiazoles are reported to exhibit significant anthelmintic activity from our laboratory (1). Among the various benzimidazole derivatives, mebendazole and flubendazole have marked broad spectrum anthelmintic activity in which presence of benzoyl group is one of the essential factor for broad spectrum of activity (2). This fact encouraged us to synthesise 2-[4'-(substituted-benzoyl) phenylamino]-pyrido (3,2d)-thiazoles of the type V.

In addition to this, piperidine, morpholine and piperazine derivatives find their wide clinical applications in the therapy of functional diseases and exhibit anthel-

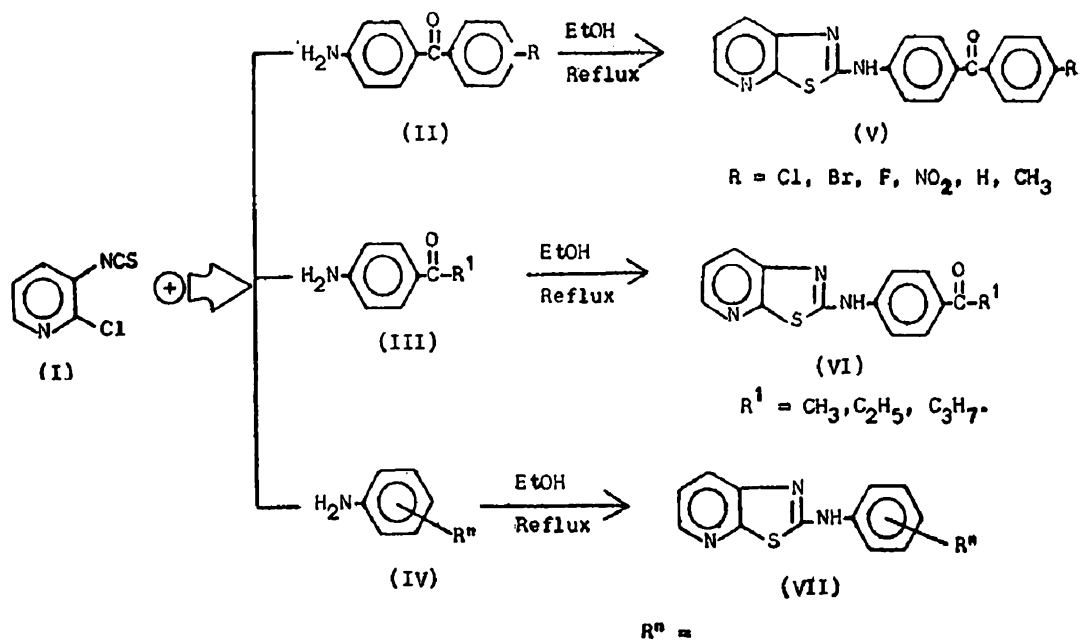
mintic, bactericidal and insecticidal activities (3). Therefore, efforts were also made to synthesise 2-[2'- and 4'-(N-heterocyclic amino) phenylamino]-pyrido (3,2d)-thiazoles of the type VII.

Chemistry :

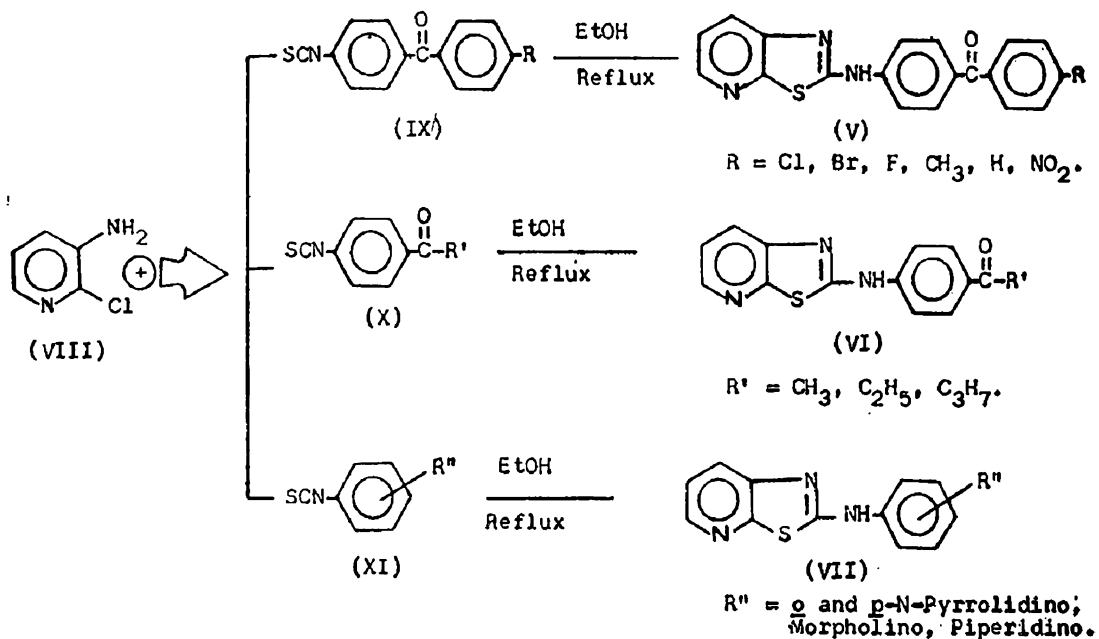
The starting material 2-chloro, 3-isothiocyanatopyridine was prepared according to the method of Arya *et al* (4). The desired 2-substituted-aryl amino pyrido (3,2d)-thiazoles could be prepared by reacting 2-chloro, 3-isothiocyanatopyridine (I) and 4'-substituted-4-amino-benzophenone (II), 4-aminoalkanophenones (III) and o- and p-N-heterocyclic amino anilines (IV) in reflux alcohol, as explained in Scheme — I or vice versa as in Scheme — II.

SYNTHESIS OF ANTHELMINTIC THIAZOLES

SCHEME - I



SCHEME - II



In few cases the hydrochloride salt obtained from the reaction mixture was neutralized in NH_3 solution to obtain free bases.

Experimental

2-[4'-(p-Nitrobenzoyl)phenylamino]-pyrido (3,2-d)-thiazole :

In a 100 ml round bottomed flask was taken 2-chloro-3-isothiocyanato pyridine (0.581 g; 0.0034 mole) in ethanol (25 ml). To it was added 4'-nitro, 4-aminobenzophenone (0.75 g; 0.0031 mole) in ethanol (20 ml). The reaction mixture was refluxed for six hours and the crystalline compound obtained was collected by filtration. It was crystallized from ethanol, m.p. 274°C .

Anal. calcd. for $\text{C}_{19}\text{H}_{12}\text{N}_4\text{O}_3\text{S} \cdot \text{HCl}$:

Found :

C, 55.25; H, 3.15; N, 13.57%

C, 55.41; H, 3.24; N, 14.00%

2-(4'-Butyryl phenylamino) pyrido (3,2-d) thiazole HCl :

In a 100 ml round bottomed flask was taken 2-chloro-3-aminopyridine (0.433 g; 0.0034 mole) in ethanol. To it was added 4-isothiocyanatophenyl butyl ketone (0.694 g; 0.0034 mole). The reaction

was refluxed for 18 hours and the crystalline compound obtained was collected by filtration. It was recrystallized from ethanol, m.p. 248°C .

Anal. calcd. for $\text{C}_{16}\text{H}_{15}\text{N}_3\text{OS}$:

Found :

C, 57.5; H, 4.78; N, 12.59%

C, 57.81; H, 4.83; N, 12.64%

Similarly, all other compounds prepared and tabulated in Tables I and II.

Biological Screening Results :

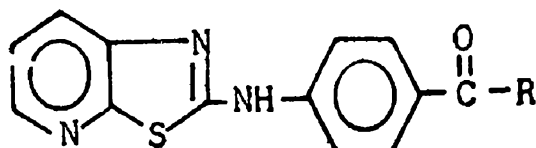
Most of the compounds were tested against *H. nana* and *N. dubius* in mice. Niclosamide, Jonit and Alcopar are the standard drugs. The activity shown by the compounds tested is given in Tables I and II.

Discussion :

Among all the compounds, 2-[4'-(p-nitrobenzoyl)-phenylamino]-pyrido (3,2-d) thiazole showed highest activity causing more than 90% mortality of the adult worms, at the dose of 25 mg/kg in mice against *N. dubius* (standard drug (Jonit)); however, 2-[4'-(N-p-nitrophenylamino) phenylamino]-Pyrido (3,2-d) thiazole was devoid of anthelmintic activity against *N. dubius*. If the nitro group is replaced by Cl, Br, CH_3 , H (type V), the activity is significantly reduced.

Table - I

2-Substituted-aminopyrido (3, 2-d) thiazoles.



Sr. No.	R	M.p. °C ^a (uncorr.)	Mol. formula ^b	Dose* (mg/kg)	% Mortality of adult 7 days of worms after drug admn.
1.	CH ₃	271	C ₁₄ H ₁₂ ClN ₃ OS	—	—
2.	C ₂ H ₅	270	C ₁₅ H ₁₄ ClN ₃ OS	—	—
3.	C ₃ H ₇	248	C ₁₆ H ₁₅ N ₃ OS	—	—
4.	Phenyl	263	C ₁₈ H ₁₄ ClN ₃ OS	—	—
5.	4-Tolyl	275	C ₂₀ H ₁₆ ClN ₃ OS	25	20
6.	4-Chlorophenyl	240	C ₁₉ H ₁₃ Cl ₂ N ₃ OS	25	20
7.	4-Bromophenyl	265	C ₁₉ H ₁₃ BrClN ₃ OS	25	30
8.	4-Fluorophenyl	220	C ₁₉ H ₁₃ FCIN ₃ O ₃ S	25	30
9.	4-Nitrophenyl	274	C ₁₉ H ₁₃ ClN ₄ O ₃ S	25	70
10.	Jonit			25	100

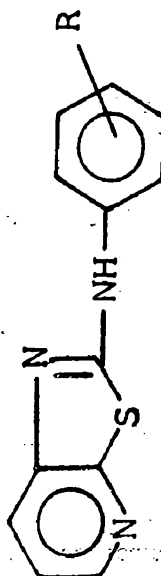
* Against *N. dubius* (standard drug 'Jonit').

a = Melting points are capillary melting points and are uncorrected.

b = Analysed correctly for C, H and N with $\pm 0.4\%$.

Table - II

2-Substituted aminopyrido (3, 2-d) thiazoles



No.	R	M.p. °C (uncorr.)	Mol. formula*	Dose (mg/kg)	% Mortality of adult worms after 7 days of drug administration. H. Nana N. dubius
1.	2'-(N-Pyrrolidino)	162	C ₁₆ H ₁₆ N ₄ S	200	Nil
2.	2'-(N-Piperidino)	143	C ₁₇ H ₁₈ N ₄ S	200	Nil
3.	2'-(N-Morpholino)	169.5	C ₁₈ H ₁₆ N ₄ S	200	Nil
4.	4'-(N-Pyrrolidino)	148	C ₁₆ H ₁₆ N ₄ S	200	Nil
5.	4'-(N-Piperidino)	186	C ₁₇ H ₁₈ N ₄ S.HCl.2H ₂ O	200	70.6
6.	4'-(N-Morpholino)	223	C ₁₈ H ₁₆ N ₄ OS	200	59.0
7.	2'-(N-Phenylamino)	235	C ₁₈ H ₁₅ ClN ₄ S	200	Nil
8.	2'-(N-p-Chlorophenyl)	265	C ₁₈ H ₁₄ Cl ₂ N ₄ S	—	—
9.	3'-Chloro-2'-(N-2, 4-dichlorophenylamino)	221 (d)	C ₁₈ H ₁₂ Cl ₄ N ₄ S	200	72
10.	4'-(N-p-Nitrophenyl-amino)	278	C ₁₈ H ₁₄ ClN ₄ O ₂ S	200	Nil
11.	Niclosamide**			200	100
12.	Alcopar**			200	100

* Analysed correctly for C, H, N within ± 0.4%.

** Standard drugs taken against H. nana = Niclosamide and against N. dubius = Alcopar.

From N-heterocyclic aminopyrido thiazole derivatives, (Type VII) almost all the compounds are inactive against *N. dubius* only compound No. 9 (Table II) had shown 72% mortality of the adult worms at the dose of 200 mg/kg. 2-[o- and p-N-piperidinophenyl amino]-pyrido(3, 2d)-thiazole had shown 65 and 70% mortality against *H. nana* in mice at dose 200 mg/kg, respectively (standard drug 'Niclosamide').

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Synthesis and Study of 2-Arylamino -4-(Substituted amino)-6- Methylpyrimidines as Possible Antimalarial Agents. I

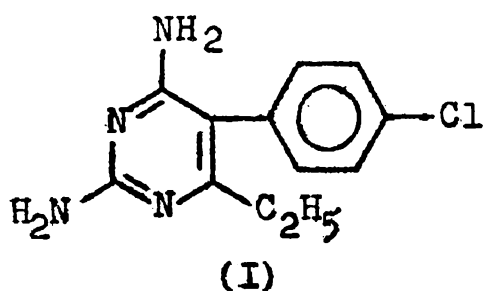
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In view of the broad spectrum of antimalarial activity displayed by pyrimidine derivatives, various new 2-arylamino-4-(substituted amino)-6-methyl pyrimidines have been synthesized. In all, twenty pyrimidines have been synthesized and fifteen representative members were screened for antimalarial activity against *P. yeolii* in mice. Most of them showed promising antimalarial activity.

Introduction

Pyrimethamine (Daraprim) (I), a pyrimidine derivative showed activity against strains of *Plasmodium falciparum* resistant to chloroquine in a recent study (1). Pyrimethamine arrests the development of malarial parasites at the stage of nuclear division (2). Pyrimethamine is effective suppressant of infection with *Plasmodium falciparum*, *P. vivax* and *P. malariae* in a dosage of 25 mg weekly which is much smaller than that of any available drug (3).



Recently, emphasis has been placed on evaluation of the closely related drug

trimethoprim, another pyrimidine derivative, which has shown varying degrees of activity against strains of *P. falciparum* resistant to chloroquine and quinine (4).

Curd and Rose (5) have synthesised some 2-(p-haloanilino)pyrimidine derivatives carrying (N', N'-dialkylamino)-alkylamino group at 4-position which possessed strong therapeutic effects against *P. gallinaceum* in chicks (6).

The antimalarial activity of the 4-amino quinolines viz., chloroquine and amodiaquine is mostly dependent on the presence of halogroup at the 7-position of quinoline nucleus and of the side chain.

Pyrimethamine, trimethoprim as well as the compounds reported by Curd et al. contain pyrimidine as a main nucleus. Similarly, drugs like chloroquine, amodiaquine, though having the

same basic nucleus of quinoline, have different amino side chain at 4-position of the quinoline moiety.

Taking into consideration the above facts, in the present work, it was decided to link pyrimidine nucleus of the above drugs with the side chain of chloroquine and amodiaquine to obtain new compounds (VI) and to study their anti-malarial activity.

Chemistry

The 2-aryl-amino-4-substituted-amino-6-methyl pyrimidines (VI) described in the present communication (Table I) were synthesized according to the route outlined in Scheme - I. These compounds (VI) were synthesized by the reaction of 2-aryl-amino-4-chloro-6-methyl pyrimidines (V) and appropriate dialkylamino-alkylamine using phenol as a catalyst (7). These chloropyrimidines were prepared by the fusion of substituted anilines (II) and 2-methylthio-4-hydroxy-6-methyl pyrimidine (III) at 120-130°C to get 2-aryl-amino-4-hydroxy-6-methyl pyrimidines (IV) and further chlorination of IV with phosphorus oxychloride gave V (5). In turn, the required 3,5-dihalo-4-alkoxyanilines (II) (8) and 2-methylthio-4-hydroxy-6-methyl pyrimidine (III) (9) were synthesised by the customary methods.

The final compounds VI were analysed either as free bases or as their picrate salts. The hygroscopic hydrochloride salts of these compounds (VI) were obtained in solid form and were utilized for determination of biological activity.

Experimental

2-[(3',5'-Dibromo-4'-ethoxy) anilino]-4-hydroxy-6-methyl pyrimidine :

3,5-Dibromophenetidine (8.85 g; 0.03 mole) and 2-methylthio-4-hydroxy-6-methyl pyrimidine (2.35 g; 0.015 mole) were ground together and the mixture heated at 130-35°C in an oil bath for about 24 hours. After cooling, the solid melt was then refluxed with alcohol (50 ml). The insoluble product was filtered off and dried. It was recrystallised from acetic acid from which it was separated in clumps of colourless needles of the desired compound.

Yield : 3.75 g (62.0%); m.p. 276-77°C.

Anal. calcd. for $C_{13}H_{13}Br_2N_3O_2$:

C, 38.71; H, 3.22; N, 10.42%

Found :

C, 38.80; H, 3.58; N, 10.73%

-2[(3',5'-Dibromo-4'-ethoxy) anilino]-4-chloro-6-methyl pyrimidine :

2-[(3',5'-Dibromo-4'-ethoxy) anilino]-4-hydroxy-6-methyl pyrimidine (3.6 g; 0.009 mole) was refluxed with phosphorus oxychloride (15 ml; 25.5 g; 0.17 mole) for 3 hours. After removing the excess of phosphorus oxychloride under reduced pressure at 50-60°C., the residue was treated with ice and rendered alkaline with ammonia solution. The resulting solid was filtered off, washed with water and recrystallized from the 2:1 mixture of hexane and benzene to obtain colourless needles of the titled compound.

Yield : 2.5 g (65.0%); m.p. 139-40°C.

Anal. calcd. for $C_{18}H_{12}ClBr_2N_3O$:

C, 37.01; H, 2.85; N, 9.96%

Found :

C, 36.62; H, 2.72; N, 10.35%

2-(3',5'-Dibromo-4'-ethoxyanilino)-4-[4-(diethylamino)-1-methylbutyl] amino]-6-methyl pyrimidine :

2-(3', 5'-Dibromo-4'-ethoxyanilino)-4-chloro-6-methyl pyrimidine (1.05 g; 0.0025 mole) and phenol (0.25 g; 0.0025 mole) were heated together at 125°C for two hours in oil-bath. To this mixture 4-(diethylamino)-1-methylbutyl amine (0.8 g; 0.005 mole) was added and it was again heated at a temperature of 135°C for 24 hrs. The reaction mixture was then poured into water containing 10 ml of hydrochloric acid and made alkaline with sodium hydroxide solution. A sticky mass was obtained. This sticky compound was extracted with benzene and purified using column chromatography over alumina. The free base obtained after this procedure was oily. This oil solidified on keeping it under vacuum for three days and was then analysed. The base of the title compound obtained was slightly hygroscopic.

Yield : 0.68 g (51.0%); m.p. 60-63°C.

Anal. calcd. for $C_{22}H_{33}Br_2N_3O.H_2O$:

C, 47.06; H, 6.25; N, 12.47%

Found :

C, 47.02; H, 6.01; N, 12.12%

2-(3',5'-Dibromo-4'-ethoxyanilino)-4-[[3-(diethylaminomethyl)-4-hydroxy] anilino]-6-methyl pyrimidine :

A mixture of 2-(3',5'-dibromo-4'-ethoxyanilino)-4-chloro-6-methyl pyrimidine (1.05 g; 0.0025 mole), p-aminodiethylamino-o-cresol (0.8 g; 0.004 mole). conc.

hydrochloric acid (0.5 ml) and alcohol (25 ml) was refluxed for 16 hours. The solution was evaporated under reduced pressure to a thick syrup which was then dissolved in water and made alkaline with ammonia solution. The precipitate obtained was filtered and recrystallized from hexane.

Yield: 0.8 g (55.0%); m.p. 96-98°C.

Anal. calcd. for $C_{24}H_{29}Br_2N_5O_2$:

C, 49.74; H, 5.01; N, 12.09%

Found :

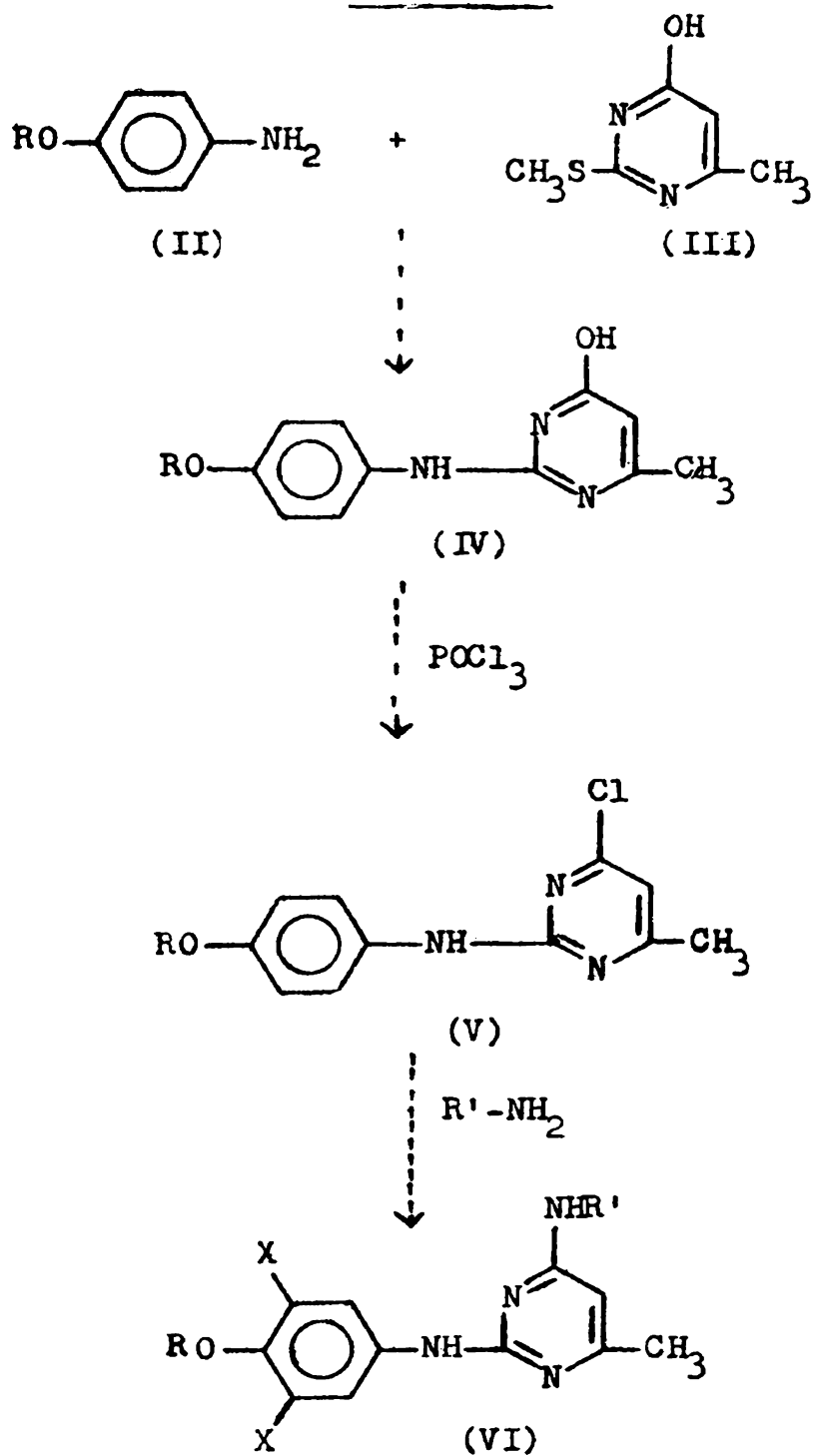
C, 49.37; H, 4.98; N, 12.26%

Biological Results and Discussion

Out of twenty pyrimidine derivatives, fifteen representatives were screened for their *in vivo* antimalarial activity against *P. yoelii* by Rane's blood Schizonticidal test (10).

Out of the fifteen compounds screened (Table II), eight compounds displayed promising antimalarial activity at dosages from 20 to 160 mg/kg. Compound No. 11 showed curative (4 cures) activity at 160 mg/kg. Also compound No. 13 and 14 gave activity at lower dose and in 2 and 3 cures were recorded respectively at higher dose. Compound No. 13, 15 and 17 to 20 were found toxic at 160 mg/kg. None of the compounds from 5 to 8 showed any significant activity.

Scheme - I

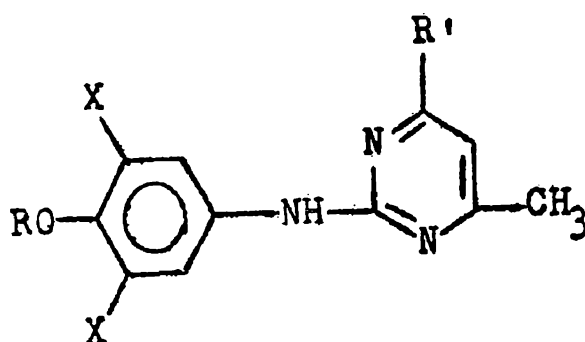


where,

$\text{R} = \text{CH}_3; \text{C}_2\text{H}_5; \text{X} = \text{Cl}, \text{Br}.$

$\text{R}' = (\text{CH}_2)_3\text{N}(\text{CH}_3)_2; \text{CH}(\text{CH}_3)-(\text{CH}_2)_3\text{N}(\text{C}_2\text{H}_5)_2;$
 $3,4-\text{CH}_2\text{N}(\text{C}_2\text{H}_5)_2(\text{OH})\text{C}_6\text{H}_3.$

Table I
2-Arylamino-4-substitued-6-methyl pyrimidines



No.	X	R	R'	M.p.°C ^a	Mol. formula ^b
1.	Cl	CH ₃	OH	284-85	C ₁₂ H ₁₁ Cl ₂ N ₃ O ₂
2.	Cl	C ₂ H ₅	OH	272-73	C ₁₃ H ₁₃ Cl ₂ N ₃ O ₂
3.	Br	C ₂ H ₅	OH	276-77	C ₁₃ H ₁₃ Br ₂ N ₃ O ₂
4.	Br	CH ₃	OH	259-60	C ₁₂ H ₁₁ Br ₂ N ₃ O ₂
5.	Cl	CH ₃	Cl	118-20	C ₁₂ H ₁₀ Cl ₃ N ₃ O
6.	Cl	C ₂ H ₅	Cl	117-18	C ₁₃ H ₁₂ Cl ₃ N ₃ O
7.	Br	C ₂ H ₅	Cl	139-40	C ₁₃ H ₁₂ ClBr ₂ N ₃ O
8.	Br	CH ₃	Cl	141-42	C ₁₂ H ₁₀ ClBr ₂ N ₃ O
9.	Cl	CH ₃	3,4-CH ₂ N(C ₂ H ₅) ₂ (OH)C ₆ H ₃ NH-	61-63	C ₂₃ H ₂₇ Cl ₂ N ₅ O ₂
10.	Cl	C ₂ H ₅	3,4-CH ₂ N(C ₂ H ₅) ₂ (OH)C ₆ H ₃ NH-	81-83	C ₂₄ H ₂₉ Cl ₂ N ₅ O ₂
11.	Br	C ₂ H ₅	3,4-CH ₂ N(C ₂ H ₅) ₂ (OH)C ₆ H ₃ NH-	96-98	C ₂₄ H ₂₉ Br ₂ N ₅ O ₂
12.	Br	CH ₃	3,4-CH ₂ N(C ₂ H ₅) ₂ (OH)C ₆ H ₃ NH-	79-81	C ₂₃ H ₂₇ Br ₂ N ₅ O ₂
13.	Cl	CH ₃	-NHCH(CH ₃)(CH ₂) ₃ N(C ₂ H ₅) ₂	87-90	C ₂₁ H ₃₁ Cl ₂ N ₅ O (Dipicrate)
14.	Cl	C ₂ H ₅	-NHCH(CH ₃)(CH ₂) ₃ N(C ₂ H ₅) ₂	72-75	C ₂₂ H ₃₃ Cl ₂ N ₅ O (Dipicrate)
15.	Br	C ₂ H ₅	-NHCH(CH ₃)(CH ₂) ₃ N(C ₂ H ₅) ₂	60-63	C ₂₂ H ₃₃ Br ₂ N ₅ O.H ₂ O
16.	Br	CH ₃	-NHCH(CH ₃)(CH ₂) ₃ N(C ₂ H ₅) ₂	73-75	C ₂₁ H ₃₁ Br ₂ N ₅ O (Dipicrate)
17.	Cl	CH ₃	-NH(CH ₂) ₃ N(CH ₃) ₂	82-84	C ₁₇ H ₂₃ Cl ₂ N ₅ O (Picrate)
18.	Cl	C ₂ H ₅	-NH(CH ₂) ₃ N(CH ₃) ₂	88-90	C ₁₈ H ₂₅ Cl ₂ N ₅ O (Picrate)
19.	Br	C ₂ H ₅	-NH(CH ₂) ₃ N(CH ₃) ₂	86-88	C ₁₈ H ₂₅ Br ₂ N ₅ O (Picrate)
20.	Br	CH ₃	-NH(CH ₂) ₃ N(CH ₃) ₂	82-84	C ₁₇ H ₂₃ Br ₂ N ₅ O (Picrate)

a) Melting points are capillary melting points and are uncorrected.

b) Analysed correctly for C,H and N within *0.4%.

TABLE II

Antimalarial activity against
P. yoelii in mice

Sr. No. *	Δ MST,	T or C ** (Dosage in mg/kg)			
	20	40	80	160	
5.	—	—	0.5	—	
6.	—	—	0.5	—	
7.	—	—	0.5	—	
8.	—	—	0.5	—	
9.	0.2	1.2	—	5.2 (A)	
10.	—	—	1.8	14.7 (A)	
11.	2.8	5.7 (A)	—	4 C	
12.	—	1.2	—	5.7 (A)	
13.	11.5 (A)	2 C, 1 T	—	5 T	
14.	—	18.2 (A)	—	3 C	
15.	—	0.5	0.5	2 T	
17.	—	7.2 (A)	14.9(A)	5 T	
18.	5.6 (A)	11.2 (A)	—	3 T	
19.	0.9	2.5	1.4	5 T	
20.	0.3	3 T	4 T	5 T	

* Numbers refer to those from Table I, hydrochlorides of these compounds were screened.

** Δ MST is the mean survival time (days) of treated mice (MSTT) minus the mean survival time (days) of control mice (MSTC). Death prior to MSTC is toxic (T), mice which live more than 40 days are regarded as cured (C). Drugs which double the survival time of mice compared to control are considered active (A). In each run pyrimethamine was included as standard drug (curative dose = 4.5 mg/kg).

(—) = Not tested.

Acknowledgement :

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Antituberculous Agents II

Synthesis of some new N-Substituted Pyrazinamides

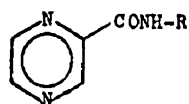
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A few N-substituted thiazolyl pyrazinamides and N-substituted phenyl pyrazinamides were synthesised and screened for antituberculous activity *in vitro*. One N-substituted thiazolyl pyrazinamide was found to be twice as active as pyrazinamide *in vitro*. One derivative of N-substituted phenyl pyrazinamide series was found to be eight times as active as pyrazinamide, while three derivatives from the same series were found to be fifty times as active as pyrazinamide, under identical conditions of *in vitro* testing.

Introduction :

Pyrazinamide (Ia) is a well-known antituberculous drug. It exhibits a moderate tuberculostatic activity *in vitro* at a concentration of 12.5 mg/ml. In spite of high potency, pyrazinamide suffers from the drawback of causing severe hepatic toxicity and has to be administered under strict medical supervision (1,2). Derivatization of pyrazinamide in the pyrazine nucleus generally leads to loss of activity.

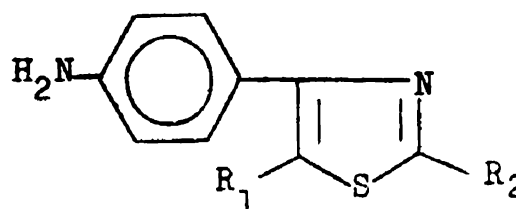


(I)

(a) R = H, (b) R = 2-Naphthyl, (c) R = -COCH₃

but N-2-naphthyl (Ib), and N-acetyl (Ic) pyrazinamides have been reported to possess antituberculous activity (3).

Earlier, derivatives of 4-(4'-amino-phenyl) thiazoles (II) have been reported from our laboratory to show good antituberculous activity *in vitro* (4). A few halo alkoxy anilines (III) have also been reported as intermediates in the preparation of biologically active compounds (5). With this view, we thought that molecular modification, involving incorporation of these thiazolyl amines (II) and haloalkoxy



(II)

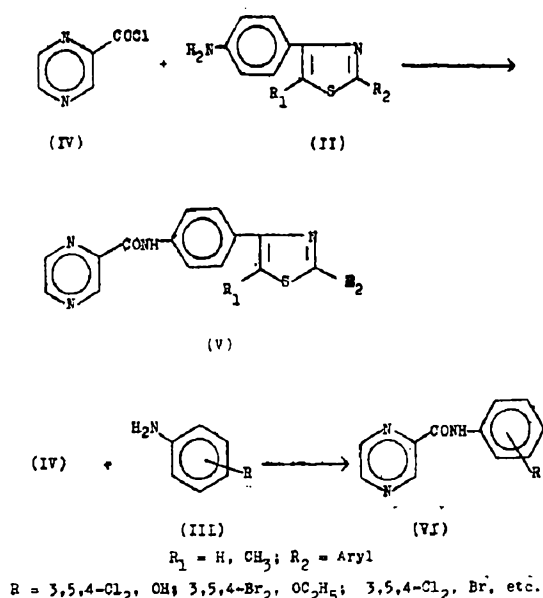
R = H, CH₃. R₂ = Aryl

anilines (III) in the pyrazinamide moiety may lead to more active and less toxic derivatives. We, therefore, prepared N-[p-(2,5-disubstituted-4-thiazolyl) phenyl] pyrazinamides (V) and N-(substituted phenyl) pyrazinamides (VI).

Chemistry :

The N-substituted pyrazinamide derivatives (V) and (VI) were obtained in good yields by condensation of appropriate 4-(4'-aminophenyl) thiazole (II) (4) or a halo alkoxy aniline (III) (5) and pyrazinoyl chloride (VI) (6,7) in boiling chloroform, in the presence of anhydrous potassium carbonate, as shown in the scheme.

Scheme :



N- p-[2-(3-methoxy-4-propoxy phenyl)-5-methyl-4-thiazolyl] phenyl pyrazinamide :

A mixture of 1.23 g (0.0035 mole) of 2-(3-methoxy-4-propoxy phenyl)-4-(4'-aminophenyl)-5-methyl thiazole, 470 mg

(0.0033 mole) of pyrazinoyl chloride and 5.5 g (0.04 mole) of anhydrous potassium carbonate were taken in 40 ml of dry chloroform in 100 ml round-bottomed flask fitted with reflux condenser and a guard tube. The mixture was refluxed for 3 hours. It was filtered and treated with charcoal and again filtered to get a clear solution. It was then concentrated (to about 15 ml) and cooled to yield fine needles of the pyrazinamide derivative

Yield : 0.98 g (65%); m.p. 185°C.

Analysis for C₂₅H₂₄N₄O₃S :

Calcd. : C, 65.21; H, 5.21; N, 12.17, S, 6.95%.

Found : C, 65.46, H, 5.11; N, 11.81; S, 7.11%.

N—(3,5-dichloro-4-bromophenyl) Pyrazinamide :

A mixture of 2.1 g (0.009 mole) of 3,5-dichloro-4-bromoaniline, 1.42 g (0.01 mole) of pyrazinoyl chloride and 8.3 g (0.08 mole) of anhydrous potassium carbonate were taken in 50 ml of dry chloroform in a 100 ml round-bottomed flask and the mixture refluxed for 3 hours. It was then filtered, treated with charcoal, refiltered and concentrated to 15 ml and cooled to afford rhombic crystals of N-(3,5-dichloro-4-bromophenyl) pyrazinamide.

Yield : 2 g (65%) ; m.p. 182-183°C.

Analysis for C₁₁H₆BrCl₂N₃O :

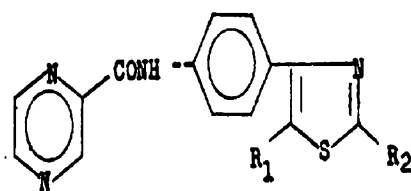
Calcd. : C, 38.05 ; H, 1.73 ; N, 11.94%

Found : C, 38.01 ; H, 1.64 ; N, 12.11%.

Biological Activity and Discussion :

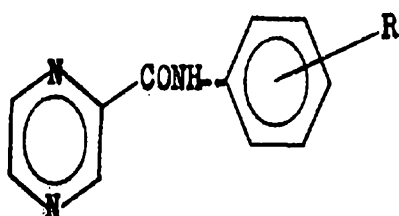
The *in vitro* tuberculostatic activity was determined on culture of *Mycobacterium tuberculosis* (H₃₇Rv) cultivated in

TABLE I
N-[P-(2,5-Disubstituted-4-Thiazolyl) Phenyl] Pyrazinamides



No.	R ₁	R ₂	Yield %	m. p. °C ^a (uncorr.)	Molecular formula ^b	Antitubercula ^c activity (mcg/ml) ^c
1.	CH ₃	3,4-MeO(PrO)C ₆ H ₃	65	185	C ₂₅ H ₂₃ N ₄ O ₃ S	—
2.	H	3,4-MeO(PrO)C ₆ H ₃	60	196-197	C ₂₄ H ₂₂ N ₄ O ₃ S	—
3.	CH ₃	3,4-EtO(MeO)C ₆ H ₃	64	179-180	C ₂₄ H ₂₂ N ₄ O ₃ S	100
4.	H	3,4-EtO(MeO)C ₆ H ₃	60	181-182	C ₂₃ H ₂₀ N ₄ O ₃ S	—
5.	CH ₃	3,4-MeO(PhCH ₂ O)C ₆ H ₃	63	190-191	C ₂₆ H ₂₄ N ₄ O ₃ S	..
6.	H	3,4-EtO(PrO)C ₆ H ₃	62	199-200	C ₂₅ H ₂₄ N ₄ O ₃ S	..
7.	H	2-(PhO) C ₃ H ₃ NC ₆ H ₃ N	62	249-250	C ₂₅ H ₁₇ N ₅ O ₂ S	—
8.	H	4-(oH)C ₆ H ₃	61	254-255	C ₂₁ H ₁₆ N ₄ O ₂ S	—

TABLE II
N-(Substituted Phenyl) Pyrazinamides



(VI)

No.	R	Yield	m.p. °C ^a (uncorr.)	Molecular Formula ^b	Antitubercular activity (mcg/ml) ^c
9.	3,5,4-Cl ₂ ,OCH ₃	68	193-194	C ₁₂ H ₈ Cl ₂ N ₃ O ₂	0.25
10.	3,5,4-Br ₂ ,OC ₂ H ₅	67	160	C ₁₃ H ₁₁ Br ₂ N ₃ O ₂	1.56
11.	3,5,4-Cl ₂ ,OH	68	260	C ₁₁ H ₇ Cl ₂ N ₃ O ₂	0.25
12.	3,5,4-Br ₂ ,OH	66	251-252	C ₁₁ H ₇ Br ₂ N ₃ O ₂	—
13.	3,5,4-Cl ₂ , Br	65	182-183	C ₁₁ H ₆ BrCl ₂ N ₃ O ₂	0.25
14.	3,4,5-Br ₃	70	216-217	C ₁₁ H ₆ Br ₃ N ₃ O	—
15.	3-CF ₃	69	108-109	C ₁₂ H ₈ F ₃ N ₃ O	25.00
16.	2,4-OCH ₃ ,NO ₂	18	214-215	C ₁₂ H ₁₀ N ₄ O ₄	—

(a) Melting points are capillary melting points and are uncorrected.

(b) Analysed correctly for C, H, N and/or S $\pm$ 0.4%

(c) Minimum Inhibitory Concentration *in vitro*. Antitubercular Action mcg/ml.
(For Isoniazid (INH) = 0.05 mcg/ml. ethionamide = 0.5 mcg/ml; para-Aminosalicylic acid = 0.4 mcg/ml; Pyrazinamide = 12.5 mcg/ml. (—) Compound found inactive up to 200 mcg/ml.

Note: The term activity in this paper refers to Minimum Inhibitory Concentration for the growth of *M. tuberculosis* (H₃₇Rv) *in vitro*

Youman's medium containing 10 per cent horse serum by a modified method of Doub and Youman (8) and previously reported from this laboratory (9). In each of the two series, eight compounds were screened. Out of the eight thiazolyl pyrazinamide derivatives, compound number 6 (Table I) was inhibitory to the growth of *M. tuberculosis* (H₃₇Rv) at a concentration of 6.25 mcg/ml. This activity is twice that of pyrazinamide, under identical conditions of *in vitro* testing (minimum inhibitory concentration for pyrazinamide = 12.5 mcg/ml). The *in vitro* testing results of the N-substituted pyrazinamide derivatives (Table II) were very encouraging. Compound number 10 was about eight times as active as pyrazinamide *in vitro*. The compounds 9, 11 and 13 were inhibitory to the growth of *M. tuberculosis* (H₃₇Rv)

at a concentration as low as 0.25 mcg/ml. These activities are nearly fifty times that of pyrazinamide *in vitro*. Pyrazinamide derivatives of this series with chloro substituents in the phenyl ring were found to be more active than corresponding bromo analogues.

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