

BULLETIN

Of

Haffkine Institute



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

EDITORIAL BOARD

Chairman

Dr. B. B. GAITONDE, M.D., M.Sc. (Med.), F.A.Sc.
Director, Haffkine Institute for Training, Research and Testing, Bombay

Editor

Dr. M. V. N. SHIRODKAR, M.B., B.S. (Bom.); A.B., Sc.D. (Johns Hopkins); P.B.K. (U.S.A.)
Head, Department of Virology, Haffkine Institute for Training, Research and Testing, Bombay

Assistant Editor

Dr. H. J. MANSHARAMANI, M.B., B.S. (Bom.); Ph.D. (Bom.)
Senior Scientific Officer, CI-I, Department of Virology, Haffkine Institute for Training, Research and Testing, Bombay

Members

Dr. C. G. PANDIT, M.B., B.S., Ph.D., D.P.H., D.T.M.
Ex-Director, Indian Council of Medical Research

Dr. H. I. JHALA, M.D., F.C.P.S., D.T.M.
Superintendent, Sir Harkisondas N. Hospital, Prarthana Samaj, Bombay

Dr. K. G. NAIR, M.D., F.C.P.S. (Chicago)
Director-Professor of Medicine, Seth G. S. Medical College, Bombay

Dr. D. D. BANKER, M.D., Ph.D.
Chief Bacteriologist, Glaxo Laboratories, Bombay

Dr. F. FERNANDES, M.Sc., Ph.D.
Head of Microbiology Department, Ciba Research Centre, Bombay

Dr. S. S. RAO, M.Sc., Ph.D.
Assistant Director (Retd.), Haffkine Institute, Bombay

Mr. PERCY E. D'SOUZA
Managing Editor, 'Medical Times', Sandoz (India) Ltd., Bombay

Dr. C. V. DELIWALA, M.Sc., Ph.D.
Deputy Director (Retd.), Haffkine Institute, Bombay

Dr. M. V. SANT, M.D., F.C.P.S., D.E. (Prague)
Deputy Director (Retd.), Haffkine Institute, Bombay

Dr. A. N. D. NANAVATI, M.D.
Assistant Director (Retd.), Haffkine Institute, Bombay

Dr. M. B. BHIDE, M.D.
Assistant Director, I/c. Pharmacology Department, Haffkine Institute for Training, Research and Testing, Bombay

Dr. F. L. SALDANHA, M.Sc., Ph.D., F.I.C. (Cal.)
Deputy Manager (Quality Control), Haffkine Bio-Pharmaceutical Corporation Ltd., Bombay

CONTENTS

	PAGE
<i>Effects of Environmental Conditions on the Culture of Giardia lamblia</i> S. NATARAJAN IYER AND B. B. GAITONDE	77
<i>Potential Anticancer Agents, IV : Schiff bases derived from 4-[N, N-BIS-[3-Methylsulphonyl) Oxypropyl] Amino] Benzaldehydes</i> D. T. CHAUDHARI AND S. S. SABNIS	85
<i>Synthesis of Thiazolyl Derivatives of Possible Therapeutic Value (Anti-inflammatory Agents-I)</i> B. R. COLAH, S. S. SABNIS, NIRMALA D. VAIDYA AND M. B. BHIDE	93
<i>Effect of Disodium Cromoglycate on the Formation of Bradykinin</i> R. B. GHOOI AND M. B. BHIDE	99
<i>Studies on the Interaction of Piperazine and Sennosides on Isolated Rat Colon</i> B. B. GAITONDE, M. B. BHIDE AND R. S. JOSHI	105
<i>Substituted Salicylanilides and Their Antimicrobial Activity</i> R. P. KINI, MADHUBEN R. PATEL AND M. H. SHAH	109
Short/Preliminary Communications	
<i>Synthesis and Study of 2-Heterocyclic Aminopyrido-(3, 2-d) Thiazoles as Possible Anthelmintic Agents</i> B. G. KHADSE, M. H. SHAH, M. B. BHIDE AND VIJAYALAXMI M. BHAT	113
<i>Studies on Sensitive Colorimetric Method for Determination of Ethinyl Estradiol</i> LALITA S. SHIRSAT AND M. H. SHAH	115

EFFECTS OF ENVIRONMENTAL CONDITIONS ON THE CULTURE OF GIARDIA LAMBLIA

S. NATARAJAN IYER* AND B. B. GAITONDE

Haffkine Institute, Parel, Bombay 400 012

Environmental conditions for optimum growth of *G. lamblia* in the modified Pavlova medium were studied. Optimum growth occurred at pH range of 7 to 7.4 at 37° C. Generation time was 5.4 at twenty-four hours and 7.8 at forty-eight hours. It was found that a close relation existed between the growth of giardia and oxidation-redox potential developed in the culture medium; best growth occurring at a potential between -350 and -450 mV.

Introduction

A simple method for the cultivation of *G. lamblia* *in vitro* using modified Pavlova medium was described earlier (1). Since the optimum growth of any microorganism is mainly influenced by the environmental conditions in which it is cultivated, the following important aspects were studied: for their role in the cultivation of giardia: (i) The effect of hydrogen ion concentration, (ii) Temperature of incubation and generation time, (iii) Use of rice starch in the culture of giardia and (iv) Oxidation-reduction potential.

Materials and Methods

I. *The Effect of Hydrogen Ion Concentration*—It is observed that *G. lamblia* lives in an environment of pH 6.3 to 7.02 which the duodenum and upper jejunum provide. Growth is one criterion that can be used as a measure of the effects of pH (2). In the present study, giardia was cultivated in association with bacteria and a study of the effects of pH on the growth of giardia provided a possible pH range in which giardia could be conveniently cultivated.

Giardia was cultivated in modified Pavlova medium at different values of pH. Media with different pH values were prepared as follows: Buffer salt solutions were made by mixing up M/15 solution of disodium hydrogen phosphate and M/15 solution of potassium dihydrogen phosphate in normal saline. Before inoculation, the appropriate amount of horse serum and rice starch were added under aseptic conditions. A seventy-two hours' old culture of giardia was inoculated with sterile pipette into the media tubes. The initial count of giardia in the inoculum as well as counts at the end of seventy-two hours' incubation were made using haemocytometer counting chamber. Any change in pH value of the medium was also recorded. To record the pH of the growing culture the supernatant solution was poured out and passed through Seitz filter and the readings were taken on the pH meter.

II. *Temperature of Incubation and Generation Time*—In most studies the optimum temperature for growth is based on the rate of growth and on the size of the crop of microorganism produced. It was found that the temperature of incubation had its effect not only on the growth but also on

(Received for publication February 19, 1975)

*Present Address : Hoechst Research Centre,
L. B. Shastri Marg, Mulund, Bombay 400 080.

the individual microbial metabolic activities. These microbial metabolic activities include rate of synthesis of enzyme or pigment production. Temperature of incubation affects the rate of growth, rate of death, the length of growth period, generation time and the total growth. The rate of multiplication of the organism is increased by the rise in temperature and this is measured by calculating the generation time of the organism.

The generation time of the culture can be calculated by measuring the initial and final growth after a fixed period of incubation. The formula for calculating the generation time of the culture is as follows (3) :

$$n = 3.3 \times \log b/B \text{ and } g = t/n$$

Where: b = final growth,

B = initial inoculum,

n = the number of generations,

t = time in hours, and

g = generation time.

In the present study above formula was used to calculate the generation time of *G. lamblia* at different periods of incubation. Also, the growth of giardia at various temperatures of incubation were studied.

Media tubes were inoculated with known number of giardia and the culture was incubated at different temperatures. The temperatures selected for this study were 4 °C, 27–28 °C, 30°, 35°, 37°, 40° and 45° C. At the end of seventy-two hours' incubation giardia population per tube was estimated by making counts on a haemocytometer counting chamber and the per cent growth in each tube was calculated. Highest growth was considered as 100 per cent and the growth in other tubes was compared with this tube. For the determination of generation time and the effect of temperature of incubation on generation time the counts of giardia were taken every twenty-four and forty-eight hours' of incubation. When it was found that giardia could not

multiply at 4° C, the culture tubes were transferred to the incubator at 37° C after twenty-four hours and it was noted that giardia could grow well at 37° C after the initial static stage at 4° C.

III: *Use of Rice Starch in the Culture of G. lamblia*—The incorporation of rice starch in the cultivation of protozoan parasite is known for a long time. One well known example is that of *E. histolytica* in the cultivation of which rice starch is routinely used. *H. meleagridis*, another parasite causing blackhead in turkey and chicken, is cultivated in a medium containing 1 to 2 mgm rice starch (4). Since *G. lamblia* also belongs to the group of intestinal parasites it was decided to study the effect of rice starch in the medium for the growth promoting activity.

Modified Pavlova medium was used for the experiment and rice starch was added to each tube serially in quantities varying from 0 to 100 mgm. A known volume of inoculum containing definite number of giardia was inoculated into the tubes. At the end of seventy-two hours' incubation counts of giardia were taken and the results were recorded.

IV. *Oxidation-reduction Potential in the Culture of G. lamblia*—Oxidation-reduction potential is one of the important physico-chemical aspects to be considered in the cultivation of microorganisms. This is usually determined in volts or in millivolts and this value gives the information about the formation of redox potential in the culture of microorganisms. In the study of redox potentials in relation to the cultivation of intestinal protozoa, Jahn (5) stated that the redox potential might be a factor of very great importance in culturing protozoa free from bacteria. It was demonstrated by Jahn (6) that in a culture of *Chilomunax paramecium* changes in redox potential were similar to those found in aerobic culture.

Since in the present studies *Giardia lamblia* was cultivated in association with bacteria *in vitro*, the dependency of giardia can be explained in terms of oxidation-reduction potential developed in the medium by the bacteria. The possibility that the bacteria produce the necessary metabolic products for the culture of giardia was noticed when giardia failed to grow in the absence of bacteria. Of possible influential factors the following seems to be most important : (a) the bacterial flora may provide suitable conditions such as anaerobiosis, for the metabolic activity of giardia, (b) the bacteria may provide essential growth factors and enzyme system in utilising the food materials in the medium.

In the present paper the results obtained with the measurements of redox potential in the culture of giardia are presented. Also, the effect of redox potential on the growth and optimum value at which the peak growth of giardia occurred have been studied.

Electrode—The measuring electrode consisted of bright platinum metal. This was used in combination with calomel electrode which was used as standard reference electrode. The potential of the calomel electrode is determined by the concentration of chloride ions. In the present study saturated solution of potassium chloride was used.

Culture Vessel for the Measurement of Redox Potential—To prepare culture vessel for the estimation of potential a rubber stopper was equipped with four holes one each for fitting platinum and calomel electrodes, one for passing oxygen and one hole which was plugged with cotton for the outlet of gas. The stopper was fitted to the mouth of a bottle in which the culture medium was taken for the cultivation of giardia. Electrodes were passed through the respective holes and two tight fitting glass tubes were passed through the

remaining holes. These tubes were plugged with cotton when not in use. The culture vessel was clamped to a stand and kept in a water bath at 37 °C, the temperature of which was regulated by a thermostat. Thus continuous measurement or redox potential could be taken at any desired time during the period of incubation. The potential of the medium was recorded after the medium was warmed upto 37 °C. The medium was inoculated with the culture containing known number of giardia. Potential developed in the medium was measured with the use of pH meter.

Measuring Instrument—The measurements of redox potential were taken on a pH meter with one scale graduated in millivolts. The platinum and calomel electrode of the culture vessel were connected to the respective terminals of the pH meter, and the knob was turned to the scale indicating mV. Ordinarily the potential of the culture is negative (reducing) to that of calomel half-cell. The reading of the potential, thus, observed will be that referred to that of calomel half-cell and this observed potential E_{obs} . can be converted to E_h , the potential referred to that of standard normal hydrogen electrode, by adding E_{obs} . and E_{cal} ., the potential of calomel half-cell, algebraically, thus:

$$E_h = E_{obs} + E_{cal}.$$

The potential developed in the culture vessel at different periods of incubation was measured as E_{obs} . and E_h was calculated from the above formula. Also, the growth of giardia was estimated by counts at different intervals of time. When oxygenation was desired the gas was introduced into the culture vessel through the tube provided. With this type of arrangement continuous measurement of redox potential of the culture could be made at any desired stage of incubation.

Development of Redox Potential in the Medium—100 ml of modified Pavlova

medium was taken in the culture vessel and the electrodes were inserted. The culture vessel was transferred to the water bath at 37°C and the redox potential measurements were taken as described above. Inoculum consisted of seventy-two hours' old culture of giardia and the readings were taken every one hour intervals. The readings indicated the development of potential in the culture medium and a time-potential curve was plotted using these readings.

Time-potential curve for giardia-bacteria culture was compared with that of parallel bacterial cultures. The associated bacterial flora was grown in the medium without giardia and the redox potential readings were taken. From these readings time-potential curve was plotted for the bacteria alone and the comparison was made.

Results and Discussion

I. The Effect of Hydrogen Ion Concentration—In Table I is given the initial and final pH of the medium and the growth at different pH values. From the above table it is clear that maximum growth was obtained with the initial pH value of 7.2. At higher pH values of 7.2 to 9.0 giardia could be seen multiplying but a pH value beyond 10 could not support the growth. Though giardia could survive in media of wide pH range it was found that a pH of 7.2 was favourable for maximum growth. The present study indicated that a lower pH range of 5 to 6 could help propagation of giardia with only reduced growth. Also, there is not much change in the pH value after incubation at the optimum pH range.

In many cases, pH is a function of interaction between the associated bacterial flora and the substrate especially in mixed cultures. From this study it is obvious that the medium with initial pH of 7.2 can well support good growth of giardia.

II. Temperature of Incubation and Generation Time—The relation between the incubation temperature and the generation time

at the end of twenty-four and forty-eight hours' incubation time is recorded in Table II. The mean generation time for *G. lamblia* in modified Pavlova medium at the end of twenty-four and forty-eight hours' incubation was found to be 5.4 and 7.8 hours respectively. The similar figures for generation time in modified Karapetyan's medium were found to be 10.05 and 8.73 hours respectively. The higher values of generation time in modified Karapetyan's medium indicated a lessened growth as compared to the growth in modified Pavlova medium. In the previous studies it was found that modified Pavlova medium supported the growth of giardia better than modified Karapetyan's medium. Best growth of giardia occurred at 37°C and at higher temperature of 40°C the growth was so less that a satisfactory count not be made. At 27–28°C the growth was minimum. At 4°C there was no multiplication of cells, but when the culture tubes were transferred to 37°C after twenty-four hours the culture could be revived and grew well indicating thereby that temperature of 4°C was unfavourable for the growth leaving the organism in a static stage.

TABLE I
Growth of *G. lamblia* at different pH value

Initial pH of the medium	Final pH of the culture	Growth of giardia 10 ⁶ /ml	S. E.
5.0	5.0	40.0	2.74
6.0	6.0	51.5	1.39
6.5	6.6	96.0	1.04
7.0	7.25	104.5	1.93
7.2	7.4	136.0	2.74
7.4	7.4	121.0	3.03
7.6	7.6	64.0	1.52
7.8	7.7	59.0	1.08
8.0	7.65	52.0	2.14
8.5	7.7	50.0	2.06
9.0	7.9	46.0	1.58
9.5	8.25	26.0	1.32
10.0	8.1		

TABLE II

Optimum temperature of incubation for the culture of G. lamblia and the relationship with the generation time

Temperature °C	Giardia population $\times 10^4/\text{ml}$ (S.E.)	Per cent growth	Generation time (hours)
27-28	4.08 (0.35)	4.3	18.5
30	31.6 (1.25)	33.36	5.6
35	87.5 (2.44)	92.45	4.2
37-38	94.75 (1.16)	100.0	4.1
40-41	*		
45	—		

*Few giardia seen.

III. *Use of Rice Starch in the Culture of G. lamblia*—The inoculum for this study was pooled from the supernatant solution of different culture tubes and giardia population was determined before inoculating into the medium. Care was taken to see that the inoculum did not contain any starch particles from the previous tubes. At the end of seventy-two hours' incubation the result indicated that the tube containing starch of 60 mgm and above showed good growth. When the medium did not contain starch the growth was upto the extent of 7.1 per cent as compared to that of the tube containing 60 mgm starch. Giardia could be seen growing even in the absence of starch indicating thereby that it is not an essential component of the medium for the growth of giardia but it enhances the growth considerably when used in the concentration of 60 mgm and above. Results are recorded in Table III.

In the present studies it was noticed that the degree and the duration of giardia growth depend upon the amount of rice starch incorporated in the medium. Better yield of giardia was not obtained with higher amount of rice starch than the optimum.

TABLE III

Rice starch in the cultivation of G. lamblia

Amount of rice starch (mgm)	Giardia population $10^4/\text{ml}$ (S.E.)	Per cent growth
0	3.5 (0.25)	7.1
20	8.3 (0.57)	16.84
40	19.5 (1.52)	39.56
50	37.3 (3.21)	75.67
60	49.3 (2.78)	100.0
80	48.5 (3.71)	98.37
100	48.8 (2.54)	99.0

IV. *Oxidation-reduction Potential in the Culture of G. lamblia*—The significance of redox potential measured during the cultivation of microorganisms can be discussed in various ways. They can be caused by the metabolic products developed in the solution and the action of different enzymes. Variations in oxygen pressure resulting in oxygen uptake by the microorganisms may be responsible for the change in redox potential value.

In the present work the culture of giardia was maintained at more or less fixed level of potential by passing oxygen at a different rate with fluctuation of potential within 30 mV. By leaving the culture undisturbed the lowest potential was maintained. Cultures of high potential were prepared as follows: seventy-two hours' old culture of giardia was pooled, centrifuged and washed with normal saline. After repeated washing of the culture three to four times inoculum was taken in normal saline. Measurements of redox potential were taken with these cultures at reduced conditions.

Counts of giardia were taken at different periods of incubation along with the recording of potential value. Accurate determination of the number of giardia could not be made using haemocytometer counting chamber as in the previous studies because the process of shaking and stirring the culture interfered with the potential determination due to oxygenation. Hence

the number of giardia was determined per field under low power magnification by observing a drop of the culture from the sediment and they are indicated as follows:

- + Very poor growth of giardia, 1 to 10 organisms per field.
- ++ Moderately good number of giardia present, 10 to 30 per field.
- +++ Heavy growth, showing numerous giardia in the smear.

Eh, the redox potential with reference to hydrogen electrode was calculated by adding 238 mV, which is the potential of standard calomel electrode at 37°C to the observed potential Eobs.

Oxygen was passed for nearly ten hours and the potential was kept constant at various levels. At the potential levels between -100 and -150 mV, and between -190 and -250 mV growth was very less denoted by +. But between the potential levels of -350 and -450 mV there was heavy growth of giardia denoted by +++. The potential range of -350 to -450 mV was obtained by leaving the culture undisturbed since oxygenation could not bring down potential to such low levels (Table IV).

TABLE IV

Growth of G. lamblia at different potential levels

Potential range (mV)	Growth
-100 to -150	+
-190 to -250	+
-250 to -350	++
-350 to -450	+++

+ Very poor growth, 1 to 10 giardia/low power field.

++ Moderately good growth, 10 to 30 per field.

+++ Heavy growth, showing numerous giardia in the smear.

In Table V is given the changes of redox potential developed in the medium as the incubation proceeded. As the incubation progressed the change in redox potential was slow during the first three hours of incubation. In three to four hours time the potential changed from positive to negative values. Within a period of seventy-two hours the potential dropped to a minimum (mean) value of -442 mV (S. E. 6.548). Then the potential remained at this value more or less constant. The results are presented graphically (see Figures 1 and 2).

TABLE V

Time-potential relationship in the culture of G. lamblia

Time (Hours)	Potential of giardia-bacteria culture (mV)	Potential of bacteria culture alone (mV)
0	38	36
1	33	33
2	28	23
3	-32	23
4	-232	-232
5	-232	-232
6	-232	-242
24	-392	-392
48	-422	-432
72	-442	-432
96	-422	-432
120	-402	-412
144	-312	-342

Similar values for potential were taken for the accompanying bacterial culture alone. From these readings time-potential curve for the bacteria culture was plotted. It was found that the time-potential curve for bacteria culture was similar to that of giardia-bacteria culture. Apparently the redox potential of the medium was not either affected by giardia or the extent to which was affected was not detectable in the presence of bacteria. The minimum redox potential value obtained with the bacteria culture was -432 mV (S.E. 5.0) in

a period of seventy-two hours. This potential value was close to the figure obtained with giardia-bacteria culture. Also, not much change was noticed in the redox potential value reached when inocula containing varying number of giardia were used. From these observations it can be concluded that the redox potential developed in the culture medium could be due to the presence of bacterial flora.

potential value. A high rate of multiplication was seen at a final pH range of 6.5 to 6.8. The redox conditions developed in the culture medium and the fall in pH could be due to the presence of bacterial flora.

The present study indicated that giardia grow in anaerobic conditions requiring a reduced state for its metabolic activity and possibly the reducing conditions were provided by the bacterial flora.

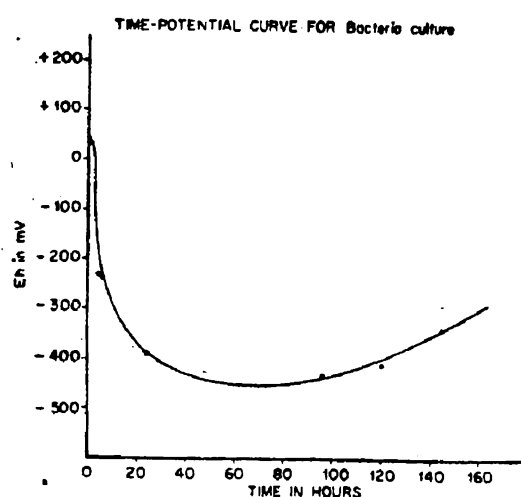


Figure 1

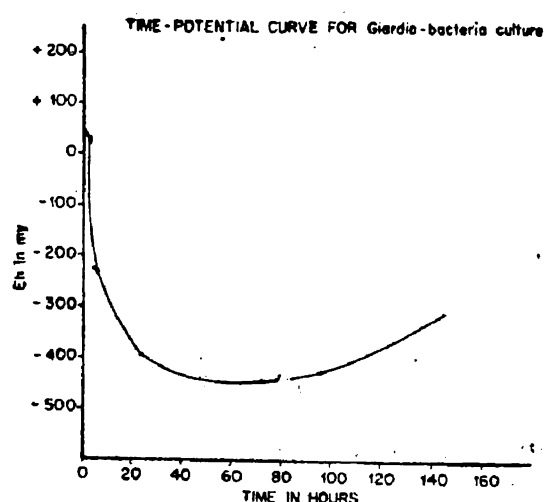


Figure 2

Counts of giardia were made at various intervals of time to see if any correlation existed between the potential levels observed and the multiplication of giardia. Results showed that good growth was obtained during 72-96 hours period when the value of redox potential value was around -400 mV.

In the present work the medium employed had an initial pH of 7.2 to 7.4. After twenty-four hours of incubation the pH value of the culture dropped to 6.5. After this period the pH readings remained more or less constant at the minimum redox

TABLE VI

Effect of varying inoculum of G. lamblia on the redox potential developed in the medium

Initial inoculum $\times 10^4$ /ml	Potential developed at the end of seventy-two hours mV (S. E.)
73	-422 (6.83)
64	-372 (12.39)
40.5	-402 (9.66)
31.5	-422 (5.77)
20.5	-402 (12.39)
16	-412 (8.56)

TABLE VII

Growth of G. lamblia in relation to the potential developed in the medium

Time (hours)	Potential mV (S.E.)	Final growth $\times 10^4/\text{ml}$ (S.E.)
48	-412 (3.65)	17.5 (0.79)
72	-382 (3.65)	51.25 (0.26)
96	-382 (6.32)	52 (2.18)
120	-302 (10.3)	22 (2.14)

Summary and Conclusions

The effect of hydrogen ion concentration on the effect of the growth of giardia was studied *in vitro* using modified Pavlova medium for the cultivation of giardia. It was found that an initial pH range of 7 to 7.4 favoured the growth of giardia. A pH value beyond 10 could not support the growth, while in lower acidic pH values giardia could survive only with poor growth.

Another growth parameter studied was the incubation temperature. The optimum temperature of incubation of giardia was found to be 37°C and at higher temperatures of 40 to 41°C very few giardia could be seen and a temperature of 45°C was lethal to giardia.

The generation time of culture of giardia in modified Pavlova medium was 5.4 and 7.8 hours at the end of twenty-four and

forty-eight hours of incubation respectively. It was found that generation time varied with the incubation temperature.

Use of rice starch was studied in the cultivation of giardia to find out whether rice starch could enhance the growth. When starch was added to the medium at a concentration of 60 mgm and above best growth of giardia occurred. Though giardia was found to grow even in the tube without starch, the growth was minimum.

It was found that a close relation existed between the growth of giardia and oxidation-reduction potential developed in the culture medium. Redox potential measurements were made in the culture of giardia using platinum electrode and pH meter. The best growth of giardia occurred at potential between -350 and -450 mV. At the higher positive values of redox potentials, no multiplication of the cells could occur. The potential curves were plotted for giardia-bacteria culture as well as for bacteria culture alone. The curve for giardia-bacteria culture was parallel to that of bacterial culture. Along with the measurement of redox potentials a fall in pH value of the medium was also observed. Further, with the varying number of giardia in the inoculum, the redox potential observed remained more or less constant.

REFERENCES

- 1 Iyer, S. N. and Gaitonde, B. B. (1975)—*Giardia lamblia*: A simple method of cultivation, *Bull. Haff. Instt.*, 3, 2, 114-18.
- 2 Gale, E. F. and Epps, H. M. R. (1942)—The influence of the presence of glucose during growth on the enzymic activities of *E. coli*, Comparison of the effect with that produced by fermentation acids, *Biochem. J.*, 36, 600.
- 3 Geiman, G. M. and Becker, C. E. (1953)—*In vitro* growth and metabolism of *E. histolytica*, *Ann. N. Y. Acad. Sci.*, 56, 1048.
- 4 De Volt, H. M. (1943)—A new medium for the cultivation of *H. meleagridis*, *J. Parasitol.*, 29, 353.
- 5 Jahn, T. L. (1934)—Respiratory metabolism. In 'Protozoa in Biological Research by Calkins, G. N. and Summers, F. M., pp. 352 (Quoted by Jacobs, 1950).
- 6 Jahn, T. L. (1935)—Studies on the O—R potential of protozoan cultures. I. The reduction potential of cultures *Chilomonas paramecium*. *Arch. Protistenk.*, 86, 225 (Quoted by Jacobs, 1950).
- 7 Jacobs, L. (1950)—Oxidation-reduction potentials in the cultivation of *E. histolytica*, *Amer. J. Trop. Med.*, 30, 803.

POTENTIAL ANTICANCER AGENTS, IV: SCHIFF BASES DERIVED FROM 4-[N, N-BIS-[3-(METHYLSULPHONYL) OXYPROPYL] AMINO] BENZALDEHYDES

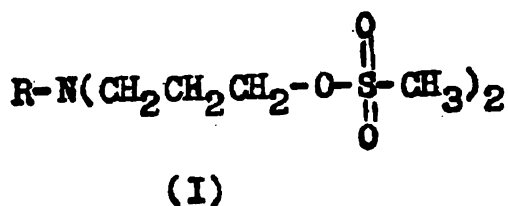
D. T. CHAUDHARI AND S. S. SABNIS

Department of Chemotherapy, Haffkine Institute, Bombay 400 012

Schiff bases from various (a) aromatic amines and (b) 4-(*p*-aminophenyl) thiazoles and 4-[N, N-bis-[3-(methylsulphonyl) oxypropyl] amino] benzaldehydes have been synthesized. Nineteen representative compounds were screened by NCI, Bethesda, U.S.A. for antitumor activity against L1210 lymphoid leukemia and in KB cell culture. None of these compounds showed activity against L1210 lymphoid leukemia, although these were non-toxic even on large dosage. Two compounds were reported active in KB cell culture.

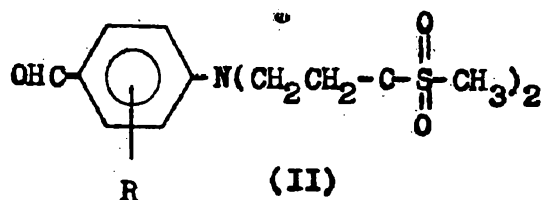
Introduction

Sakurai *et al.* have reported some new derivatives (I) in methanesulphonic acid esters series possessing high degree of activity against Yashida Sarcoma (1-4).



R - H; CH₃PhCH₂ ; Ph, etc.

Earlier, we have also reported the Schiff bases from 4-[N, N-bis-[2-(methylsulphonyl)-oxyethyl] amino] benzaldehydes (II) active against L1210 lymphoid leukemia and P 388 lymphocytic leukemia (5, 6).



In the present work, 4-[N, N-bis-[3-(methylsulphonyl)-oxypropyl] amino]benzaldehydes (V) were used to prepare the Schiff bases (VIII and IX), with those amines which afforded the active Schiff bases with II in antileukemic screening (5, 6). These are methanesulphonic acid esters of substituted N, N-bis-(3-hydroxypropyl) amines in place of reported N, N-bis-(2-hydroxyethyl) amines (5-7).

Chemistry

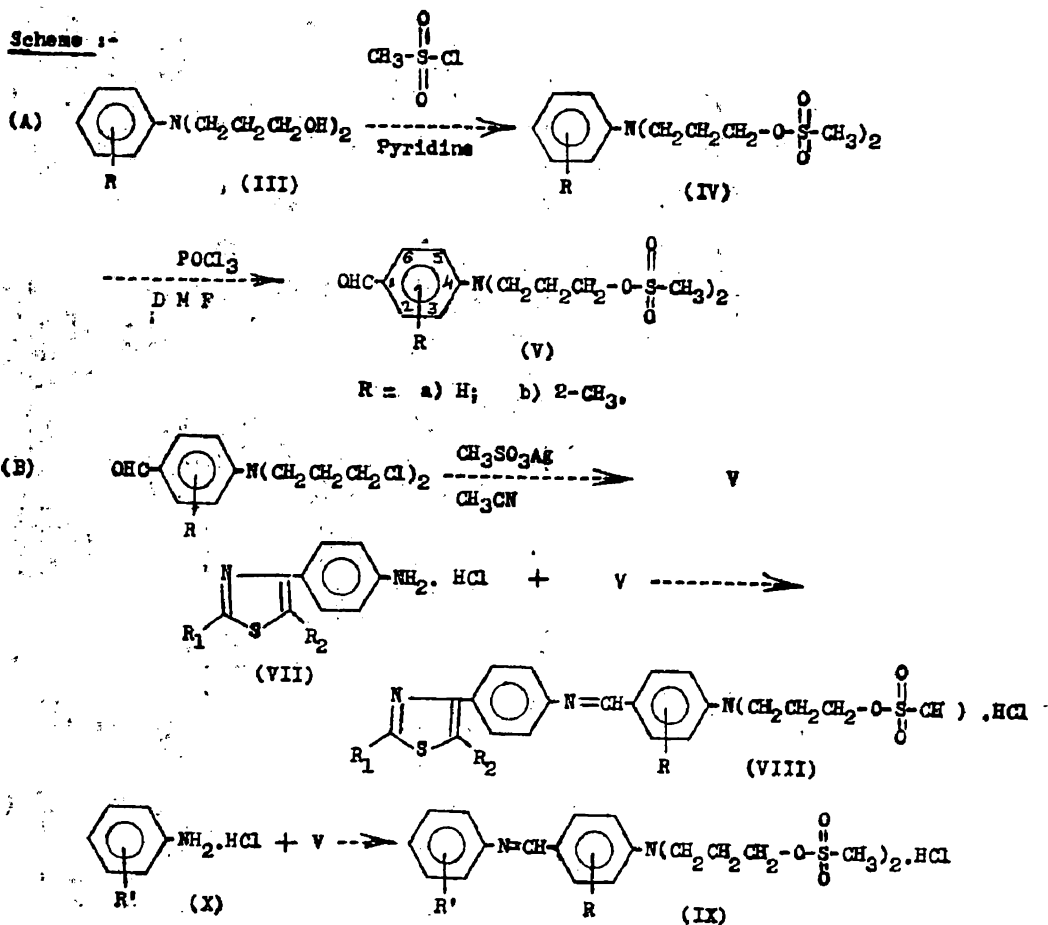
4-[N, N-bis-[3-(methylsulphonyl)-oxypropyl] amino] benzaldehydes (V) were prepared through the formylation of N, N-bis-[3-(methylsulphonyl) oxypropyl] anilines (IV) with POCl₃ in DMF (5-7). The required N, N-bis-[3-(methylsulphonyl) oxypropyl] anilines were prepared by the action of methanesulphonyl chloride on

corresponding N, N-bis-(3-hydroxypropyl) anilines (III) (8) in pyridine below 10° C and were used for further reaction without further purification. Alternatively these benzaldehyde dimethanesulphonates were prepared in dark, starting from 4-[N, N-bis-(3-chloropropyl) amino] benzaldehydes (8) by the action of silver salt of methanesulphonic acid in acetonitrile (7). The benzaldehyde dimethanesulphonates were characterised as 2, 4-dinitrophenylhydrazones.

The general method adopted for the preparation of Schiff bases VIII and IX (Tables I, II and III) is the heating of mixture of 4-(*p*-aminophenyl) thiazole hydrochloride (VII) (9) or aromatic amine hydrochloride with aldehydes (V) in alcohol as shown in scheme.

Experimental

N, N-bis-[3-(methylsulphonyl)-oxypropyl] aniline (III, *R*=H)—To an ice-cold solution of N, N-bis-(3-hydroxypropyl)-aniline (10.5 g; 0.05 mole) in dry pyridine (100 ml) was added methanesulphonyl chloride (12.6 g, 0.11 mole) gradually with stirring at -5° to 10° C. Ice-cold chloroform (200 ml) was then added followed by 10 per cent ice-cold sulphuric acid (400 ml). The organic layer was separated, washed successively with water, 10 per cent sodium bicarbonate solution and then with water. After drying on anhydrous sodium sulphate, the solution was distilled and thick residue was freed from traces of chloroform by applying vacuum giving 13.1 g (72 per cent) of viscous title compound (4).



p-[*N*, *N*-bis-[3-(methylsulphonyl) oxypropyl] amino]-benzaldehyde, (V, a)—A solution of 11.0 g (0.03 mole) of *N*, *N*-bis-(3-methylsulphonyl)-oxypropyl aniline in 20 ml of *N*, *N*-dimethylformamide (DMF) was added dropwise during one hour with stirring to a solution of 6 ml (0.066 mole) of phosphorus oxychloride in 20 ml of *N*, *N*-dimethylformamide cooled to -5°C . The cooling bath was then removed and stirring was continued for 100 minutes. Finally the mixture was poured on 1 kg of crushed ice giving greenish solution which was made basic by addition of solid sodium bicarbonate. It was kept overnight in ice-box. Next day a green-reddish viscous oil separated. The aqueous layer was removed by decantation and the oil taken up in chloroform (75 ml). The chloroform solution was washed with ice-cold water and dried over anhydrous sodium sulphate. The oil obtained on removal of solvent was successively washed with hot benzene (30 ml $\times$ 3) and the last traces of solvent were removed in rotary evaporator under vacuum leaving reddish-green viscous compound. The yield was 7.2 g (62 per cent).

Alternatively, the title aldehyde was prepared in dark from *p*-[*N*, *N*-bis-(3-chloropropyl) amino] benzaldehyde (VI) by the action of silver methanesulphonate using acetonitrile as solvent (7, 8.)

2, 4-Dinitrophenylhydrazine was prepared in ethanol solution and recrystallised from methanol-acetone (2 : 1) mixture into dark purple crystals, m.p. $129-131^{\circ}\text{C}$.

Anal. calcd. for $\text{C}_{21}\text{H}_{27}\text{N}_5\text{O}_{10}\text{S}_2$: C, 43.87; H, 4.71; N, 12.22 per cent.

Found : C, 43.64; H, 4.39; N, 12.10 per cent.

4-[*N*, *N*-bis-[3-(methylsulphonyl) oxypropyl] amino]-2-methylbenzaldehyde, (V, b)—The title aldehyde was prepared as

syrup from *N*, *N*-bis (3-hydroxypropyl)-*m*-toluidine as described above for V (R=H) in 44 per cent yield. 2, 4-Dinitrophenylhydrazine was prepared in usual way, m.p. $150-152^{\circ}\text{C}$.

Anal. calcd. for $\text{C}_{22}\text{H}_{29}\text{N}_5\text{O}_{10}\text{S}_2$: C, 44.98; H, 4.94; N, 11.93 per cent.

Found : C, 44.89; H, 4.68; N, 11.90 per cent.

2-(2, 4-dibromophenoxyethyl)-4-[*p*-[4-[*N*, *N*-bis-[3-(methylsulphonyl)-oxypropyl] amino]-2-methylbenzylidene]-amino]phenyl] thiazole hydrochloride—A solution of 2-(2, 4-dibromophenoxyethyl)-4-(*p*-amino-phenyl) thiazole hydrochloride (1.2 g, 0.0025 mole) in 8 ml of hot ethanol was added to warm solution of 4-[*N*, *N*-bis-[3-(methylsulphonyl)-oxypropyl] amino]-2-methylbenzaldehyde (1.1 g, 0.0025 mole) in ethanol (10 ml) and boiled for fifteen minutes. The resulting clear red solution on standing overnight at room temperature in absence of sunlight deposited shining reddish crystalline solid of desired Schiff base which was collected by filtration 1.7 g (79 per cent). It was recrystallised from ethanol, m.p. $118-121^{\circ}\text{C}$.

Anal. calcd. for $\text{C}_{32}\text{H}_{35}\text{Br}_2\text{N}_3\text{O}_7\text{S}_3\cdot\text{HCl}$: C, 44.36; H, 4.16; N, 4.85 per cent.

Found : C, 43.97; H, 4.44; N, 4.89 per cent.

Results and Discussion

Out of fifty-seven Schiff bases nineteen representatives were screened at National Cancer Institute (NCI), Bethesda, U.S.A., against L1210 lymphoid leukemia cells implanted intraperitoneally in mice on single dose and chronic schedules and in KB cell culture. None of these compounds passed the criteria of activity as specified in Cancer Chemotherapy Report (10), however majority of these compounds were found to be non-toxic even at high dose of 400 mg/kg/day (Table IV). Compounds 7

and 28 showed activity in KB Cell culture and had $ED_{50}=4.9 \times 10(0)$ and $5.4 \times 10(0) \mu\text{g/ml}$, respectively (ED_{50} designates the effective dosage which inhibits the growth to 50 per cent of control) (10).

A search through the literature shows that a compound though inactive to one particular tumor system, yet possesses high order of activity against a different neoplasm. For example *p*-[bis(2-hydroxyethyl)-amino] benzaldehyde, 6-purinyldimethanesulphonate is exceptionally active against Yoshida sarcoma but inactive against many other neoplasms (11). The Schiff bases VIII and IX have no activity against L1210, however, may be active against other tumor systems. Their screening against Yoshida sarcoma would

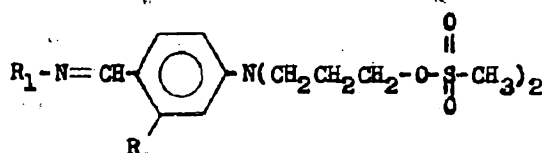
be of special interest, since related compounds (I) are reported to be highly active against Yoshida sarcoma (1-4).

Acknowledgements

The authors are greatly indebted to Dr. C. V. Deliwala, Ex-Deputy Director, Haffkine Institute for his interest and guidance and to Dr. Harry B. Wood (Jr.) of National Cancer Institute, Bethesda, U.S.A., for his co-operation and for making available the screening data. Thanks are due to Dr. M. H. Shah, Asstt. Director, Department of Chemotherapy for his keen interest in present work and to Shri M. T. Jaokar for elemental microanalysis. They are also grateful to Dr. B. B. Gaitonde, Director, Haffkine Institute, Bombay for providing facilities to carry out the present work.

TABLE I

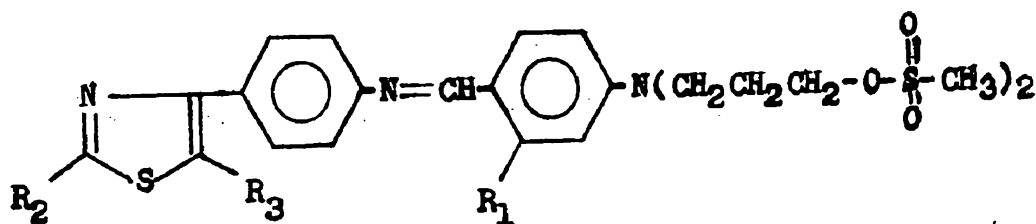
Schiff bases from arylamines and benzaldehyde dimethanesulphonates



Serial No.	R ₁	R	M.p. ° C* (Uncorr)	Molecular formula†
1	3, 5-Dichloro-4-hydroxyaniline	H	109-12	C ₂₁ H ₂₆ Cl ₂ N ₂ O ₇ S ₂ · HCl
2	3, 5-Dichloro-4-hydroxyaniline	CH ₃	196-99	C ₂₂ H ₂₈ Cl ₂ N ₂ O ₇ S ₂ · HCl
3	3, 5-Dibromo-4-hydroxyaniline	H	90-94	C ₂₁ H ₂₆ Br ₂ N ₂ O ₇ S ₂ · HCl
4	3, 5-Dibromo-4-hydroxyaniline	CH ₃	202-05	C ₂₂ H ₂₈ Br ₂ N ₂ O ₇ S ₂ · HCl
5	β-Naphthylamine	CH ₃	200-05	C ₂₈ H ₃₂ N ₂ O ₆ S ₂ · HCl
6	<i>m</i> -Chloroaniline	H	94-97	C ₂₁ H ₂₇ ClN ₂ O ₆ S ₂ · HCl
7	<i>m</i> -Chloroaniline	CH ₃	140-45	C ₂₂ H ₂₉ ClN ₂ O ₆ S ₂ · HCl
8	<i>m</i> -Bromoaniline	H	88-91	C ₂₁ H ₂₇ BrN ₂ O ₆ S ₂ · HCl
9	<i>m</i> -Bromoaniline	CH ₃	160-63	C ₂₂ H ₂₉ BrN ₂ O ₆ S ₂ · HCl

TABLE II

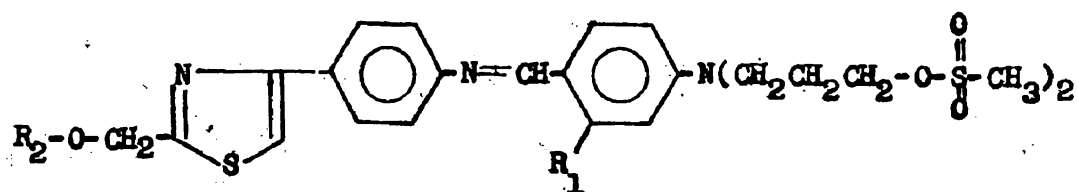
Schiff bases from 2, 5-disubstituted-4-(p-aminophenyl) thiazoles and benzaldehyde dimethanesulphonates



Serial No.	R ₁	R ₂	R ₃	M.p.° C* (uncorr.)	Molecular formula†
10	H	CH ₃	H	97-100	C ₂₅ H ₃₁ N ₃ O ₆ S ₃ ·HCl
11	H	C ₆ H ₅	H	95-100	C ₃₀ H ₃₃ N ₃ O ₆ S ₃ ·HCl
12	H	4-CH ₃ C ₆ H ₄	H	102-105	C ₃₁ H ₃₅ N ₃ O ₆ S ₃ ·HCl
13	H	2-ClC ₆ H ₄	H	124-28	C ₃₀ H ₃₂ ClN ₃ O ₆ S ₃ ·HCl
14	H	3-ClC ₆ H ₄	H	128-32	C ₃₀ H ₃₂ ClN ₃ O ₆ S ₃ ·HCl
15	H	4-ClC ₆ H ₄	H	108-11	C ₃₀ H ₃₂ ClN ₃ O ₆ S ₃ ·HCl
16	H	2-HOC ₆ H ₄	H	132-36	C ₃₀ H ₃₃ N ₃ O ₇ S ₃ ·HCl
17	H	4-HOC ₆ H ₄	H	128-30	C ₃₀ H ₃₃ N ₃ O ₇ S ₃ ·HCl
18	H	4-MeOC ₆ H ₄	H	135-38	C ₃₁ H ₃₅ N ₃ O ₇ S ₃ ·HCl
19	H	4-(n)C ₃ H ₇ OC ₆ H ₄	H	111-115	C ₃₂ H ₃₉ N ₃ O ₇ S ₃ ·HCl
20	H	4, 3-HO(EtO)C ₆ H ₃	H	107-110	C ₃₂ H ₃₇ N ₃ O ₈ S ₃ ·HCl
21	H	4, 3-(MeO) ₂ C ₆ H ₃	H	97-100	C ₃₂ H ₃₇ N ₃ O ₈ S ₃ ·HCl
22	H	4, 3-PhCH ₂ O (MeO)C ₆ H ₃	H	111-115	C ₃₆ H ₄₁ N ₃ O ₈ S ₃ ·HCl
23	H	4, 3-PhCH ₂ O (EtO)C ₆ H ₃	H	85-88	C ₃₉ H ₄₃ N ₃ O ₈ S ₃ ·HCl
24	CH ₃	4, 3-PhCH ₂ O (MeO)C ₆ H ₃	H	90-93	C ₃₉ H ₄₃ N ₃ O ₈ S ₃ ·HCl
25	CH ₃	4, 3-PhCH ₂ O (EtO)C ₆ H ₃	H	78-81	C ₄₀ H ₄₅ N ₃ O ₈ S ₃ ·HCl
26	CH ₃	4-ClC ₆ H ₄	H	186-90	C ₃₁ H ₃₄ ClN ₃ O ₆ S ₃ ·HCl
27	CH ₃	4-CH ₃ C ₆ H ₄	H	212-15	C ₃₂ H ₃₇ N ₃ O ₆ S ₃ ·HCl
28	H	C ₆ H ₅ CH ₂	H	70-73	C ₃₁ H ₃₅ N ₃ O ₆ S ₃ ·HCl
29	H	4-ClC ₆ H ₄ CH ₂	H	95-97	C ₃₁ H ₃₄ ClN ₃ O ₆ S ₃ ·HCl
30	H	4-MeOC ₆ H ₄ CH ₂	H	93-95	C ₃₂ H ₃₇ N ₃ O ₇ S ₃ ·HCl
31	H	C ₆ H ₅	CH ₃	117-21	C ₃₁ H ₃₅ N ₃ O ₆ S ₃ ·HCl
32	H	4-CH ₃ C ₆ H ₄	CH ₃	105-10	C ₃₂ H ₃₇ N ₃ O ₆ S ₃ ·HCl
33	H	3, 4-(MeO) ₂ C ₆ H ₃	CH ₃	98-102	C ₃₃ H ₃₉ N ₃ O ₆ S ₃ ·HCl
34	H	C ₆ H ₅ CH ₂	CH ₃	195-98	C ₃₂ H ₃₇ N ₃ O ₆ S ₃ ·HCl
35	H	4-ClC ₆ H ₄ CH ₂	CH ₃	190-95	C ₃₂ H ₃₈ ClN ₃ O ₆ S ₃ ·HCl
36	CH ₃	C ₆ H ₅ CH ₂	CH ₃	230-34	C ₃₃ H ₃₉ N ₃ O ₆ S ₃ ·HCl
37	H	4-CH ₃ OC ₆ H ₄ CH ₂	CH ₃	110-15	C ₃₃ H ₃₉ N ₃ O ₇ S ₃ ·HCl
38	H	CH ₃	CH ₃	159-61	C ₃₂ H ₃₅ N ₃ O ₁₃ S ₃ ·(picrate)
39	CH ₃	CH ₃	CH ₃	186-90	C ₂₇ H ₃₅ N ₃ O ₆ S ₃ ·2HCl

TABLE III

Schiff bases from 2-aryloxymethyl-4-(p-aminophenyl) thiazoles and benzaldehyde dimethanesulphonates



Serial No.	R ₁	R ₂	M.p. °C* (uncorr)	Molecular formula†
40	H	C ₆ H ₅	103-105	C ₃₁ H ₃₅ N ₃ O ₇ S ₃ ·HCl
41	H	2-CH ₃ C ₆ H ₄	107-110	C ₃₂ H ₃₇ N ₃ O ₇ S ₃ ·HCl
42	H	3-CH ₃ C ₆ H ₄	104-106	C ₃₂ H ₃₇ N ₃ O ₇ S ₃ ·HCl
43	CH ₃	C ₆ H ₅	172-175	C ₃₂ N ₃ O ₇ S ₃ ·HCl
44	H	4-ClC ₆ H ₄	105-108	C ₃₁ H ₃₄ ClN ₃ O ₇ S ₃ ·HCl
45	H	4-BrC ₆ H ₄	107-110	C ₃₁ H ₃₄ BrN ₃ O ₇ S ₃ ·HCl
46	H	2, 4-Cl ₂ C ₆ H ₃	100-103	C ₃₁ H ₃₅ Cl ₂ N ₃ O ₇ S ₃ ·HCl
47	CH ₃	2, 4-Cl ₂ C ₆ H ₃	125-128	C ₃₂ H ₃₆ Cl ₂ N ₃ O ₇ S ₃ ·HCl
48	H	2, 4-Br ₂ C ₆ H ₃	120-124	C ₃₁ H ₃₃ Br ₂ N ₃ O ₇ S ₃ ·HCl
49	CH ₃	2, 4-Br ₂ C ₆ H ₃	90-92	C ₃₂ H ₃₅ Br ₂ N ₃ O ₇ S ₃ ·HCl
50	H	2, 4-Br(CH ₃)C ₆ H ₃	90-92	C ₃₂ H ₃₆ BrN ₃ O ₇ S ₃ ·HCl
51	CH ₃	2, 4-Br(CH ₃)C ₆ H ₃	130-132	C ₃₂ H ₃₆ BrN ₃ O ₇ S ₃ ·HCl
52	H	2, 4, 6-Br ₂ (CH ₃)C ₆ H ₂	85-88	C ₃₂ H ₃₅ Br ₂ N ₃ O ₇ S ₃ ·HCl
53	CH ₃	2, 6, 4-Br ₂ (CH ₃)C ₆ H ₂	105-107	C ₃₃ H ₃₇ Br ₂ N ₃ O ₇ S ₃ ·HCl
54	H	2, 5-iso-C ₃ H ₇ (CH ₃)C ₆ H ₃	134-138	C ₃₅ H ₄₃ N ₃ O ₇ S ₃ ·HCl
55	H	β-Naphthyl	106-109	C ₃₆ H ₃₇ N ₃ O ₇ S ₃ ·HCl
56	CH ₃	β-Naphthyl	130-135	C ₃₆ H ₃₉ N ₃ O ₇ S ₃ ·HCl
57	CH ₃	4-CH ₃ CONHC ₆ H ₄	150-153	C ₃₄ H ₄₀ N ₄ O ₈ S ₃ ·HCl

* Melting points are capillary melting points and are uncorrected.

† Analysed correctly for C, H and N.

TABLE IV
*Screening Data**
 (Test System L1210 Lymphoid Leukemia)

No.†	Dose (mg/kg)	Survivors	Animal Wt. differenced‡ (T-C)	Survival days (T/C)	T/C§ per cent
2	400	5/6	-0.9	9.2/9.9	92
4	400	5/6	-1.1	9.4/9.9	94
5	400	6/6	-0.8	9.3/9.9	93
7	400	6/6	-0.1	9.2/9.9	92
9	400	6/6	-1.1	8.5/9.3	91
19	400	6/6	-0.4	8.8/9.8	89
22	400	6/6	-1.2	7.8/8.7	89
23	400	6/6	-2.5	8.3/9.0	92
25	400	6/6	-0.5	8.8/9.8	89
28	100	6/6	-4.3	10.5/9.8	107
36	200	6/6	-0.4	9.2/9.3	98
39	200	6/6	-2.2	8.7/9.3	93
43	400	6/6	-0.5	8.8/9.8	89
44	400	6/6	-0.4	8.3/8.7	95
45	400	6/6	-0.4	7.7/8.7	88
46	400	6/6	-2.5	8.3/9.3	89
47	100	6/6	-0.4	8.5/9.3	91
48	400	6/6	-0.7	8.5/9.8	86
49	200	6/6	-2.4	8.5/9.3	91

* Only part of the data is presented.

* Assays were performed according to specifications established by National Cancer Institute (Formerly CCNSC) as reported (Ref. 10).

† Numbers refer to those from Table I, II and III.

‡ Average weight change of test group (T) *minus* average weight of control (C) animals (mice).

§ The index of evaluation for L1210 is T/C [(mean survival time of treated mice)/(mean survival time of control mice)] × 100 and T/C ≥ 125 is considered positive test i.e., compounds demonstrating T/C ≥ 125 per cent are considered active on the ground that it is a reproducible effect.

REFERENCES

- 1 Sakurai, Y. and El-Merzabani, M. M. (1964)—Carcinostatic methanesulphonic acid esters of some amino glycols, *Chem. Pharm. Bull. (Tokyo)*, **12**, (8), 954-8.
2. El-Marzabani, M. M. and Sakurai, Y. (1965)—A new alkylating antitumor agent effective on experimental tumors resistant to nitrogen mustard, *Gann.*, **56**, 589-98.
- 3 El-Merzabani, M. M. (1965)—Synthesis of 3, 3'-Dimesyloxy-N-methyl (14C) dipropylamine hydrochloride, *Chem. Pharm. Bull. (Tokyo)*, **13** (11), 1362-63.
- 4 Sakurai, Y. and El-Merzabani, M. M. (1965)—Carcinostatic activity of several new derivatives of sulphonic acid esters of some amino glycols, *Chem. Pharma. Bull. (Tokyo)*, **13**, 1363-65.
5. Chaudhari, D. T., Sabnis, S. S. and Deliwala, C. V. (1975)—Potential anticancer agents-I, Schiff bases from benzaldehyde dimethanesulphonates, *Bull. Haff. Instt.*, **3**(1), 20-26.
- 6 Chaudhari, D. T., Sabnis, S. S. and Deliwala, C. V. (1975)—Potential anticancer agents-II, Schiff bases from benzaldehyde dimethanesulphonates, *Bull. Haff. Instt.*, **3**(2), 81-90.
- 7 Cohen, A. and Tipson, R. S. (1963)—Some derivatives of 2, 2'-(phenylimino) diethanol, *J. Med. Chem.*, **6**, 822.
- 8 Chaudhari, D. T., Sabnis, S. S. and Deliwala, C. V. (1975)—Potential anticancer agents-III, Schiff bases from 4-[N, N-bis (3-chloropropyl) amino] benzaldehydes, *Bull. Haff. Instt.*, **4**(1), 20-27.
- 9 Chaudhari, D. T., Sabnis, S. S. and Deliwala, C. V. (1975)—Synthesis and Study of 4-(p-aminophenyl) thiazoles for *in vitro* antitubercular activity and as useful chemotherapeutic intermediates, *Bull. Haff. Instt.*, **4** (1), 8-15.
10. Geran, R. I., Greenberg, N. H., MacDonald, M. M., Schumacher, A. M. and Abbot, B. J. (1972)—*Cancer Chemotherapy Report (Part 3)*, No. 2, Vol 3.
- 11 Elderfield, R. C., Prasad, R. N., and Lico, T. K. (1962)—Synthesis of potential anticancer agents, XIV, *J. Org. Chem.*, **27**, 573.

■ ■

SYNTHESIS OF THIAZOLYL DERIVATIVES OF POSSIBLE THERAPEUTIC VALUE (ANTI-INFLAMMATORY AGENTS-I)

B. R. COLAH AND S. S. SABNIS

Department of Chemotherapy

AND

NIRMALA D. VAIDYA AND M. B. BHIDE

Department of Pharmacology, Haffkine Institute, Parel, Bombay 400 012

A series of Schiff bases incorporating 2-substituted thiazoles and 1, 2, 5-substituted indole moieties has been synthesized and screened for antiinflammatory activity. One of the compounds (46) showed over 30 per cent inhibition of edema in the rat paw edema test.

Introduction

Over the past decade, there has been an increased interest in non-steroidal anti-inflammatory drugs which exhibit less side-effects and possess good therapeutic effectiveness.

Thiazole and indole moieties have become increasingly important in both pharmaceutical and biochemical fields. Thiazoles showing significant antiinflammatory activity include 2-(4-chlorophenyl)-thiazol-4-yl-acetic acid (fenclozic acid, I) (1, 2) and 2-phenyl-4-(4-chlorophenyl)-thiazol-5-yl-acetic acid (II) (3). Among the indole derivatives, indomethacin (III) (4), is a well known non-steroidal antiinflammatory drug which is in wide clinical use today.

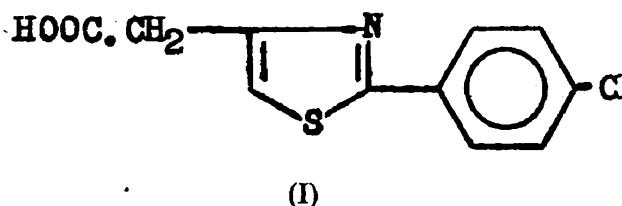
Schiff bases are known to have a high degree of biological activity including antiinflammatory activity (5, 6).

In the present work, Schiff bases (IV) possessing both the thiazole and indole moieties have been synthesized and screened for thier antiinflammatory activity.

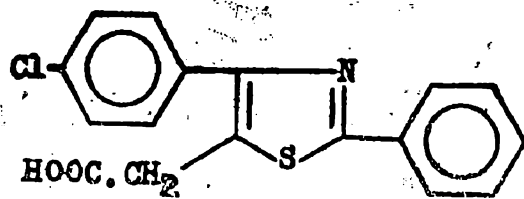
Chemistry

The Schiff bases were prepared as their hydrochlorides by heating a mixture of the 4-(4-aminophenyl)thiazole hydrochloride and indole-3-aldehyde in alcohol.

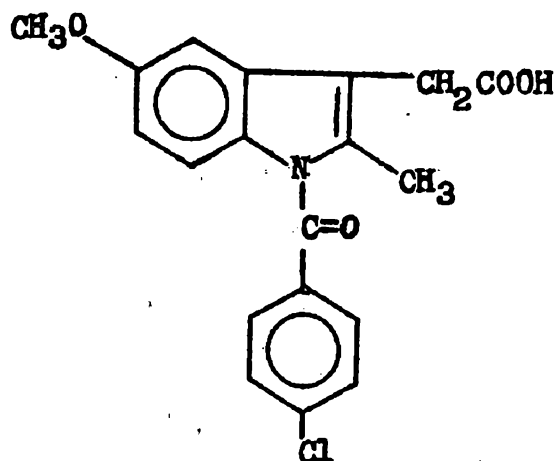
Indole and substituted indole-3-aldehydes were prepared from the appropriate indoles and dimethylformamide and phosphorus oxychloride (7).



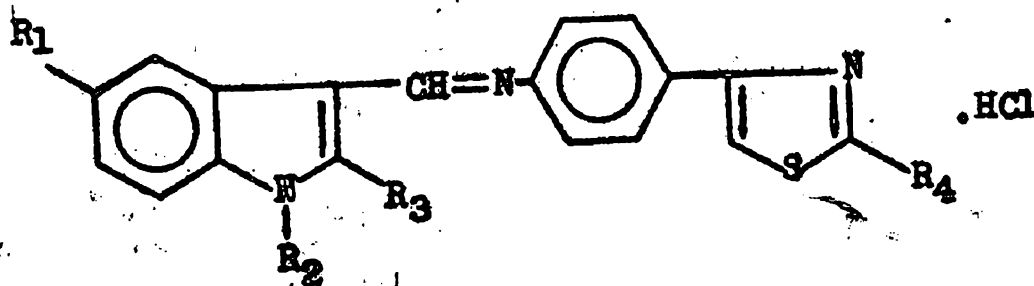
(Received for publication December 12, 1975)



(II)



(III)



(IV)

R_1, R_2, R_3, R_4 = Alkyl or aryl.

The required 4-(4-aminophenyl)thiazoles were prepared as their hydrochlorides by condensing equimolecular quantities of 2-chloro-4'-aminoacetophenone and various thioamides in boiling ethanol. The thiazole hydrochlorides were obtained in good yields and in a sufficiently pure state for further reactions (8).

Experimental

2-Phenyl-4-[4-(3-indolydine) amino]phenylthiazole hydrochloride—To a solution of 2-phenyl-4-(4-aminophenyl)thiazole hydrochloride (1.45 g, 0.005 mole) dissolved in a minimum quantity of dry ethanol, was added a hot ethanolic solution of indole-3-aldehyde (0.7 g, 0.005 mole). The resulting solution was boiled for two-three minutes. On cooling it deposited a crystalline solid

which was filtered and washed with dry alcohol and ether. Yield 1.65 g, (80 per cent), m.p. 256–259° C.

The other Schiff bases were similarly prepared and are listed in Table I.

Results

The compounds were screened for their antiinflammatory activity by an adaption of the carrageenin induced rat paw edema test of Winter *et al.* (9). Compound numbers 8, 26, 30, 31, 35, 46 and 56 showed over 25 per cent inhibition of edema. Compound 46 was the most active of the series showing an activity of 30.7 per cent.

These results show that the 5-CH₃O group in indole and the 4-ClC₆H₄CO group in the 1-position of the indole ring

(as in III) appeared to enhance the anti-inflammatory activity while the 5-CH₃ group in indole did not result in any enhanced activity.

Acknowledgements

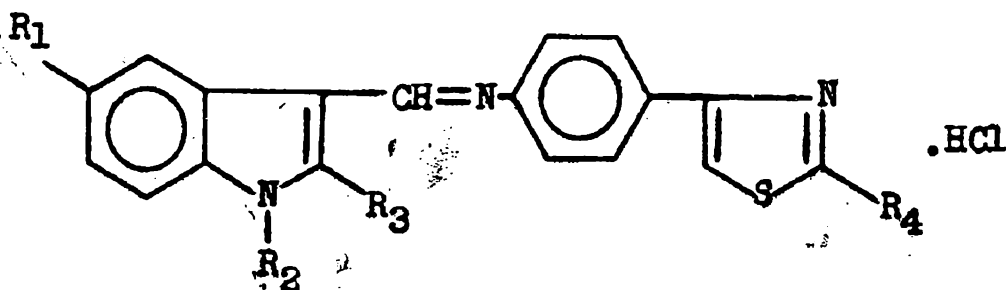
The authors are deeply indebted to Dr. M. H. Shah, Assistant Director, Department of Chemotherapy, for his helpful suggestions and encouragement and to Mr. M. T. Jaokar and his associates for

the elemental microanalyses. They are pleased to acknowledge the gift of some intermediates from F. Hoffmann-La Roche and Co. Ltd. Company, Basle, Switzerland and Dr. U. Kucklander of the Pharmazeutisches Institut der Frelen Universitat Berlin, Berlin, Germany.

They are grateful to Dr. B. B. Gaitonde, Director, Haffkine Institute, Bombay for providing facilities to carry out the present work.

TABLE I

Schiff bases from 2-substituted-4-(4-aminophenyl)thiazoles and 1, 2, 5-substituted-indole-3-aldehydes



Serial No.	R ₁	R ₂	R ₃	R ₄	M.p. °C* (Uncorr.)	Molecular formula†	per cent inhibition of edema‡
1	H	H	H	C ₆ H ₅	256-259	C ₂₄ H ₁₇ N ₃ S · HCl	9
2	H	H	H	4-ClC ₆ H ₄	277-280	C ₂₄ H ₁₆ ClN ₃ S · HCl	5
3	H	H	H	C ₆ H ₅ CH ₂	239-241	C ₂₅ H ₁₉ N ₃ S · HCl	
4	H	H	H	3, 4-(CH ₃ O) ₂ C ₆ H ₃	176-180	C ₂₆ H ₂₁ N ₃ O ₂ S · HCl	7.7
5	H	H	H	3, 4-(C ₂ H ₅ O) ₂ C ₆ H ₃	154-157	C ₂₆ H ₂₅ N ₃ O ₂ S · HCl	11.1
6	H	H	H	3, 4, 5-(CH ₃ O) ₃ C ₆ H ₂	255-258	C ₂₇ H ₂₃ N ₃ O ₃ S · HCl	5
7	H	H	H	4-CH ₃ CONHC ₆ H ₄	235-240	C ₂₆ H ₂₀ N ₄ OS · HCl	2.1
8	H	CH ₃	H	C ₆ H ₅	237-240	C ₂₅ H ₁₈ N ₃ S · HCl	26
9	H	CH ₃	H	4-ClC ₆ H ₄	233-235	C ₂₅ H ₁₆ ClN ₃ S · HCl	11.4
10	H	CH ₃	H	C ₆ H ₅ CH ₂	130-134	C ₂₆ H ₂₁ N ₃ S · HCl	3.3

TABLE 1—*contd.*

Serial No.	R ₁	R ₂	R ₃	R ₄	M.p. °C* (Uncorr.)	Molecular Formula†	per cent inhibition of edema‡
11	H	CH ₃	H	3, 4-(CH ₃ O) ₂ C ₆ H ₃	256-259	C ₂₇ H ₂₃ N ₃ O ₂ S·HCl	10
12	H	CH ₃	H	3, 4-(C ₂ H ₅ O) ₂ C ₆ H ₃	230-235	C ₂₉ H ₂₇ N ₃ O ₂ S·HCl	
13	H	CH ₃	H	3, 4, 5-(CH ₃ O) ₃ C ₆ H ₂	230-233	C ₂₈ H ₂₅ N ₃ O ₃ S·HCl	10.6
14	H	CH ₃	H	4-CH ₃ CONHC ₆ H ₄	215-217	C ₂₇ H ₂₂ N ₄ OS·HCl	7.6
15	H	CH ₃ CO	H	C ₆ H ₅	204-207	C ₂₆ H ₁₉ N ₃ OS·HCl	
16	H	CH ₃ CO	H	4-ClC ₆ H ₄	206-208	C ₂₆ H ₁₈ ClN ₃ OS·HCl	16.7
17	H	CH ₃ CO	H	C ₆ H ₅ CH ₂	197-200	C ₂₇ H ₂₁ N ₃ OS·HCl	16.7
18	H	CH ₃ CO	H	3, 4-(CH ₃ O) ₂ C ₆ H ₃	203-205	C ₂₈ H ₂₃ N ₃ O ₂ S·HCl	12
19	H	CH ₃ CO	H	3, 4-(C ₂ H ₅ O) ₂ C ₆ H ₃	205-208	C ₃₀ H ₂₇ N ₃ O ₂ S·HCl	12.5
20	H	CH ₃ CO	H	3, 4, 5-(CH ₃ O) ₃ C ₆ H ₂	189-191	C ₂₉ H ₂₅ N ₃ O ₄ S·HCl	10
21	H	CH ₃ CO	H	4-CH ₃ CONHC ₆ H ₄	206-209	C ₂₆ H ₂₂ N ₄ O ₂ S·HCl	6.2
22	H	C ₆ H ₅ CH ₂	H	C ₆ H ₅	240-244	C ₃₁ H ₂₃ N ₃ S·HCl	8
23	H	C ₆ H ₅ CH ₂	H	4-ClC ₆ H ₄	239-242	C ₃₁ H ₂₂ ClN ₃ S·HCl	15
24	H	C ₆ H ₅ CH ₂	H	C ₆ H ₅ CH ₂	174-177	C ₃₂ H ₂₅ N ₃ S·HCl	1.7
25	H	C ₆ H ₅ CH ₂	H	3, 4-(CH ₃ O) ₂ C ₆ H ₃	230-234	C ₃₃ H ₂₇ N ₃ O ₂ S·HCl	8
26	H	C ₆ H ₅ CH ₂	H	3, 4-(C ₂ H ₅ O) ₂ C ₆ H ₃	214-216	C ₃₅ H ₃₁ N ₃ O ₂ S·HCl	26.1
27	H	C ₆ H ₅ CH ₂	H	3, 4, 5-(CH ₃ O) ₃ C ₆ H ₂	223-226	C ₃₄ H ₂₉ N ₃ O ₃ S·HCl	
28	H	C ₆ H ₅ CH ₂	H	4-CH ₃ CONHC ₆ H ₄	215-219	C ₃₃ H ₂₆ N ₄ OS·HCl	12.6
29	H	4-ClC ₆ H ₄ CO	H	C ₆ H ₅	148-152	C ₃₁ H ₂₀ ClN ₂ OS·HCl	18.6
30	H	4-ClC ₆ H ₄ CO	H	4-ClC ₆ H ₄	250-255	C ₃₁ H ₁₉ Cl ₂ N ₃ OS·HCl	25
31	H	4-ClC ₆ H ₄ CO	H	C ₆ H ₅ CH ₂	180-183	C ₃₂ H ₂₂ ClN ₃ OS·HCl	29.4
32	H	4-ClC ₆ H ₄ CO	H	3, 4-(CH ₃ O) ₂ C ₆ H ₃	144-147	C ₃₃ H ₂₄ ClN ₃ O ₂ S·HCl	20.4
33	H	4-ClC ₆ H ₄ CO	H	3, 4-(C ₂ H ₅ O) ₂ C ₆ H ₃	153-156	C ₃₅ H ₂₈ ClN ₃ O ₂ S·HCl	1.5
34	H	4-ClC ₆ H ₄ CO	H	3, 4, 5-(CH ₃ O) ₃ C ₆ H ₂	140-144	C ₃₄ H ₂₆ ClN ₃ O ₄ S·HCl	7.8

TABLE 1—*contd.*

Serial No.	R ₁	R ₂	R ₃	R ₄	M.p. °C* (Uncorr.)	Molecular Formula†	per cent inhibition of edema‡
35	H	4-ClC ₆ H ₄ CO	H	4-CH ₃ CONHC ₆ H ₄	144–147	C ₃₃ H ₂₃ ClN ₄ O ₂ S·HCl	25
36	CH ₃	H	H	C ₆ H ₅	242–245	C ₂₅ H ₁₆ N ₃ S·HCl	3.3
37	CH ₃	H	H	4-ClC ₆ H ₄	262–265	C ₂₆ H ₁₈ ClN ₃ S·HCl	
38	CH ₃	H	H	C ₆ H ₅ CH ₂	226–230	C ₂₆ H ₂₁ N ₃ S·HCl	
39	CH ₃	H	H	3, 4-(CH ₃ O) ₂ C ₆ H ₃	250–254	C ₂₅ H ₂₀ N ₃ O ₂ S·HCl	
40	CH ₃	H	H	3, 4-(C ₂ H ₅ O) ₂ C ₆ H ₃	173–176	C ₂₉ H ₂₇ N ₃ O ₂ S·HCl	4.6
41	CH ₃	H	H	3, 4, 5-(CH ₃ O) ₃ C ₆ H ₂	258–261	C ₂₈ H ₂₅ N ₃ O ₃ S·HCl	1.5
42	CH ₃	H	H	4-CH ₃ CONHC ₆ H ₄	218–220	C ₂₇ H ₂₂ N ₄ OS·HCl	22.3
43	CH ₃ O	H	H	C ₆ H ₅	245–249	C ₂₅ H ₁₉ N ₃ OS·HCl	14.7
44	CH ₃ O	H	H	4-ClC ₆ H ₄	256–258	C ₂₅ H ₁₈ ClN ₃ OS·HCl	23.7
45	CH ₃ O	H	H	C ₆ H ₅ CH ₂	231–233	C ₂₆ H ₂₁ N ₃ OS·HCl	9.7
46	CH ₃ O	H	H	3, 4-(CH ₃ O) ₂ C ₆ H ₃	258–260	C ₂₇ H ₂₀ N ₃ O ₃ S·HCl	30.7
47	CH ₃ O	H	H	3, 4-(C ₂ H ₅ O) ₂ C ₆ H ₃	247–250	C ₂₉ H ₂₇ N ₃ O ₃ S·HCl	12.3
48	CH ₃ O	H	H	3, 4, 5-(CH ₃ O) ₃ C ₆ H ₂	254–256	C ₂₈ H ₂₅ N ₃ O ₄ S·HCl	10.7
49	CH ₃ O	H	H	4-CH ₃ CONHC ₆ H ₄	200–203	C ₂₇ H ₂₂ N ₄ O ₂ S·HCl	12.6
50	CH ₃ O	H	CH ₃	C ₆ H ₅	202–205	C ₂₆ H ₂₁ N ₃ OS·HCl	7.7
51	CH ₃ O	H	CH ₃	4-ClC ₆ H ₄	182–185	C ₂₈ H ₂₀ ClN ₃ OS·HCl	
52	CH ₃ O	H	CH ₃	C ₆ H ₅ CH ₂	150–152	C ₂₇ H ₂₃ N ₃ OS·HCl	
53	CH ₃ O	H	CH ₃	3, 4-(CH ₃ O) ₂ C ₆ H ₃	176–179	C ₂₈ H ₂₅ N ₃ O ₃ S·HCl	14.9
54	CH ₃ O	H	CH ₃	3, 4-(C ₂ H ₅ O) ₂ C ₆ H ₃	166–170	C ₃₀ H ₂₉ N ₃ OS·HCl	16.8
55	CH ₃ O	H	CH ₃	3, 4, 5-(CH ₃ O) ₃ C ₆ H ₂	178–181	C ₂₉ H ₂₇ N ₃ O ₄ S·HCl	11
56	CH ₃ O	H	CH ₃	4-CH ₃ CONHC ₆ H ₄	230–233	C ₂₈ H ₂₄ N ₄ O ₂ S·HCl	26.7

*All melting points are capillary melting points and are uncorrected.

†Analysed correctly for C, H and N.

‡Reference drug—Phenylbutazone (50.6 per cent inhibition of edema); Dose—100 mg/kg; Number of rats used—6 rats per group; Test samples—Each 100 mg/kg.

REFERENCES

- 1 Hepworth, W., Newbould, B. B., Platt, D. S. and Stacey, G. J. (1969)—2-(4-chlorophenyl)thiazol-4-yl-acetic acid (Myalex): a new compound with antiinflammatory, analgesic and antipyretic activity, *Nature*, **221**, 582.
- 2 Newbould, B. B. (1969)—The pharmacology of fenclozic acid, 2-(4-chlorophenyl)thiazol-4-yl-acetic acid: I.C.I. 54, 450; (Myalex); a new compound with antiinflammatory, analgesic and antipyretic activity, *Brit. J. Pharmacol.*, **35**, 487.
- 3 Brown, K. *et al.* (1974)—Nonsteroidal antiinflammatory agents—I. 2, 4-diphenylthiazole-5-acetic acid and related compounds, *J. Med. Chem.*, **17**, 1177.
- 4 Shen, T. Y. *et al.* (1963)—Nonsteroid antiinflammatory agents, *J. Am. Chem. Soc.*, **85**, 488.
- 5 Pala, G. *et al.* (1971)—Synthesis and pharmacological properties of naphthalene Schiff bases, *Chem. Abstr.*, **75**, 33578w.
- 6 Negrevergne, G. (1974)—Pharmaceutical Schiff bases and phthalides, *Chem. Abstr.*, **80**, 3232q.
- 7 James, P. N. and Snyder, H. R. (1963)—Indole-3-aldehyde. 'Organic Syntheses', Coll. Vol. IV, Rabjohn, N. (Ed.), John Wiley and Sons, Inc., New York, p. 539.
- 8 Chaudhari, D. T., Sabnis, S. S., Patel, M. R. and Deliwala, C. V. (1976)—Synthesis and study of 4-(*p*-aminophenyl)thiazoles for *in vitro* antitubercular activity and as useful chemotherapeutic agents, *Bull. Haff. Instt.*, **4**, 8.
- 9 Winter, C. A., Risley, E. A. and Nuss, G. W. (1962)—Carrageenin induced edema in hind paw of the rat as an assay for antiinflammatory drugs, *Proc. Soc. Exp. Biol. Med.*, **111**, 544.

■ ■

EFFECT OF DISODIUM CROMOGLYCATATE ON THE FORMATION OF BRADYKININ

R. B. GHOOI* AND M. B. BHIDE

Pharmacological Research Unit, C. C. R. I. M. & H., Circuit No. 2,
Department of Pharmacology, Haffkine Institute, Bombay 400 012

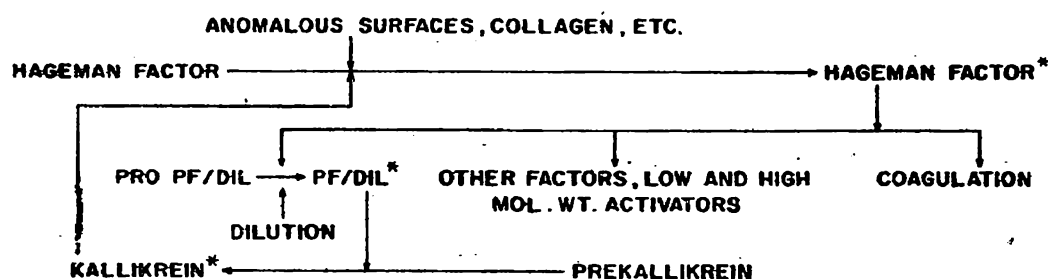
Disodium cromoglycate (DSCG) is a specific inhibitor of antigen antibody reactions of the reaginic type, its effects on the formation of bradykinin from human plasma have been studied. The formation of kinin was induced by activation of endogenous factors, or reaction with exogenous kallikrein. It was observed that DSCG inhibits the activation of PF/Dil by activated Hageman factor; however the activation of Hageman factor or its constitution were unaffected. These results were reproducible when cell free samples of plasma were used.

It is suggested that DSCG inhibits the formation of kinin from human plasma *in vitro*, by interfering with the activation of PF/Dil by activated Hageman factor and that, this is not mediated by the cyclic nucleotide system.

Introduction

Bradykinin is one of the mediators of anaphylaxis (1, 2) which is formed by the action of kallikrein on plasma kininogen (3). Though both, kininogen and kallikrein are present in the blood, kinin formation is

normally negligible (4), this is due to the presence of kallikrein in an inactive form—prekallikrein. The activation of the kallikrein precursor can be brought about in a number of ways, Hageman factor and the Plasma factor for dilution play important



ASTERIK * DENOTES THE ACTIVE FORM OF THE FACTOR.

Figure 1

(Received for publication December 3, 1975)

* Present Address : Abteilung Biochemische Pharmakologie Max-Planck-Institut für Experimentelle Medizin, Hermann-Rein-Strasse 3, Göttingen, Federal Republic of Germany.

roles in the activation of prekallikrein (5,6, 7). Douglas (1975) has suggested a scheme which is operative in the formation of bradykinnin (27), (see Figure 1).

Disodium Cromoglycate is a specific inhibitor of antigen antibody mechanisms (8). It has been demonstrated to inhibit the appearance of the mediators of anaphylaxis during experimental anaphylaxis (9). It has been suggested to afford protection against anaphylaxis, by preventing the release of mediators, which follows an antigen antibody reaction of the reaginic type. Bradykinin is one of the substances, whose appearance in the blood is regulated by its formation and destruction. An attempt has therefore been made to study the formation of bradykinin from human plasma, using various methods, and the influence of disodium cromoglycate (DSCG) on these mechanisms.

Materials and Methods

All the glassware used for this study were siliconized. All chemicals used were of analytical grade.

Agents used in this study—Heparin (Biological Evans), atropine sulphate (B.D.H.) methysergide bimalate (Sandoz), disodium cromoglycate (Fisons Ltd.), synthetic bradykinin-BRS640 (Sandoz), kaolin (E. Merck), Kallikrein-Padutin (Bayer). Whole human blood was collected from the departmental staff, in siliconized test tubes containing heparin. The plasma was separated by centrifugation at 4,500 rpm for twenty-five minutes. It was stored at 4°C, and used within twenty-four hours of collection. Four samples of plasma were filtered through a milipore filter of pore size 25 microns, to render them cell free.

The plasma samples were subjected to the following procedures :

1. *Acid Deactivation*—0.2 ml of plasma was heated with 1.8 ml of 0.02 per cent acetic acid, at 95°C for thirty minutes. This procedure causes the destruction of plasma kallikrein rendering it suitable for the study of exogenously added kallikrein (10, 11).

2. *Kinin Formation*—For studies on the formation of kinins, plasma was incubated with appropriate agents. The incubations were carried out on a Dubnoff metabolic shaker, maintained at 37°C. The reactions were stopped by the addition of 1 ml of boiling physiological saline.

3. *Kinin Assays*—Assays of bradykinin were performed on the isolated rat uterus, bathed in DeJalon's solution, at 30°C. The perfusion fluid contained atropine sulphate (100 µg/L) and methysergide bimalate (100 µg/L).

4. *Effect of DSCG on the Formation of Kinins*—Kinin formation was induced by various methods, and for each of these methods, the effects of DSCG were studied using a wide range of doses (0.125 to 100 µg/ml). The following procedures were used to induce kinin formation in human plasma :

(a) *Activation of Hageman Factor*—Hageman factor can be activated by anomalous surfaces (4), kaolin in suspension, provides a large surface area for contact (12). Plasma was incubated with kaolin suspension to achieve activation of this factor.

The incubation mixture contained: 0.1 ml plasma + 0.1 ml saline (or DSCG) + 0.1 ml kaolin suspension (50 mg/ml).

(b) *Activation of PF/Dil*—The pre-PF/Dil can be activated by either activated Hageman factor or by a 10- or 20-fold dilution with saline (6, 7). Plasma was incubated with saline in order to study this mechanism.

The incubation mixture contained: 0.1 ml plasma + 0.9 ml saline (control samples), in test samples it contained 0.1 ml DSCG + 0.8 ml saline.

(c) *Formation of Kinins by Kallikrein*—Acid deactivated plasma was used for this study, incubation was carried out with exogenous kallikrein. Plasma was neutralized before use.

The incubation mixture contained: 1.0 ml of deactivated plasma + 0.1 unit of kallikrein.

The results of the pilot experiments suggested that peak formation of kinin is at 2 to 4 minutes of incubation, all incubations were stopped at the end of four minutes.

Kaolin cephalin time has been used to study the activity of Hageman factor (13, 14, 15). This parameter was used to study the effect of DSCG on the activation of Hageman factor. The incubation was carried out using citrated plasma, and

the following procedure was adopted:

0.1 ml citrated plasma + 0.1 ml saline (or DSCG) + 0.1 ml cephalin incubate mixture for two minutes + 0.1 ml kaolin suspension (50 mg/ml) + 0.1 ml of 0.025 M CaCl_2 . Note time required for the formation of clot.

Results

The peak formation of kinin was found to be between 2 and 4 minutes of incubation time. Average amount at the peak was found to be 0.55 ± 0.36 ng/ml.

Effect of DSCG on the Formation of Kinin—DSCG was studied in the doses, ranging from 0.125 to 100 $\mu\text{g/ml}$. DSCG in doses ranging from 0.125 to 2.0 $\mu\text{g/ml}$ caused a graded inhibition of the bradykinin formation. Figure 3 gives a graphic representation of this effect. Figure 2 shows the recording of the assay.

EFFECT OF DSCG ON KININ FORMATION BY KAOLIN

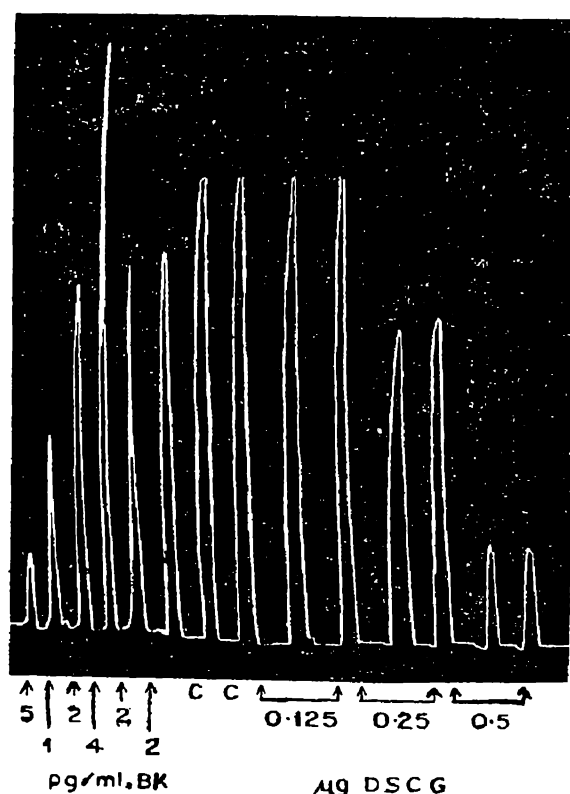


Figure 2.

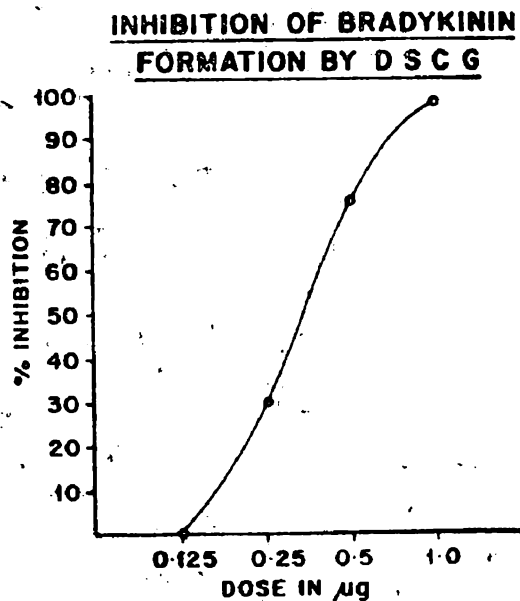


Figure 3

It was observed that DSCG was without effect, on the kinin formation induced by dilution, or pure kallikrein enzyme.

Figure 4 shows comparative effects of DSCG (2 µg/ml) on kinin formation induced by all the methods. It can be observed that DSCG in that dose does not inhibit formation of kinin induced by dilution, or kallikrein, total inhibition of formation induced by kaolin, and negligible inhibition when plasma was incubated with saline and kaolin.

DSCG in doses upto 100 µg/ml was without effect on kaolin cephalin clotting time. All the afore mentioned results were reproducible using cell free samples of plasma.

Discussion

Disodium cromoglycate was found to inhibit the formation of kinin from human plasma. However, it did so when the formation was induced by kaolin only, it had no inhibitory effects when other mechanisms were employed. The fact that DSCG does not prolong the kaolin cephalin time suggests that DSCG though not

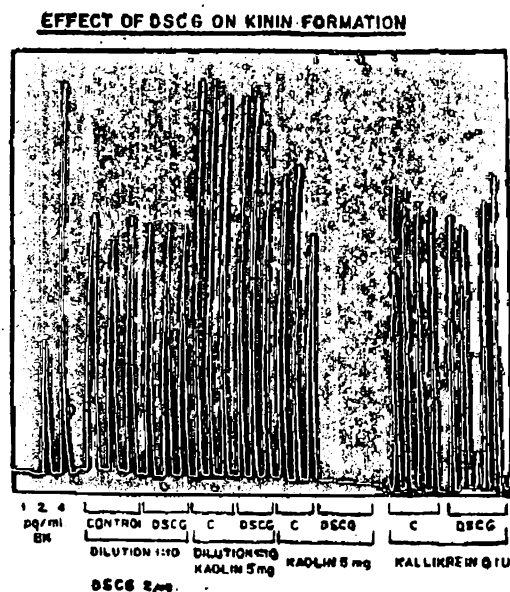


Figure 4

affecting the activation of the Hageman factor, inhibits its activity on the PF/Dil. The constitution of PF/Dil appears to be unaffected, for it can be activated by dilution even in the presence of DSCG. Therefore DSCG probably acts by interfering with the activation of PF/Dil by activated Hageman factor.

The suggested mechanism for the action of DSCG, is that it inhibits the enzyme phosphodiesterase, thus causing an accumulation of 3', 5'-adenosine monophosphate, cyclic. This cyclic nucleotide is believed to inhibit and interfere with the release of mediators of anaphylaxis (8,16). This hypothesis adequately explains the effect of the drug on preformed mediators like histamine and serotonin, whose appearance in the body fluids can be prevented by interfering with their release from storage sites. It however fails to explain the effects of the drug on the mediators which are formed prior to their release, and whose appearance in the body fluids is controlled by the rate of their synthesis. Bradykinin (2), Prostaglandins (17, 18, 19),

and the slow reacting substance of anaphylaxis (20), are known to be formed before their release.

The experiments using cell free samples of plasma yielded identical results. The cyclic nucleotide system essentially operate intracellularly, adenylyl cyclase being a part of the cell membrane (21). We therefore suggest that the effect of DSCG on kinin formation may not be mediated by the inhibition of phosphodiesterase enzyme.

In conclusion we suggest that DSCG inhibits the formation of kinin from human

plasma, *in vitro*, by interfering with the activation of PF/Dil by activated Hageman factor, and that this inhibition may not be mediated by the cyclic nucleotide system.

Acknowledgements

The authors thank the Director, Haffkine Institute, for the facilities provided for the work. Thanks to the Director, CCRIM & H, for the financial assistance to one of us (R. B. G.) and to our colleagues who donated their blood for the study.

REFERENCES

- 1 Beraldo, W. T. (1950)—Formation of bradykinin in anaphylactic and peptone shock, *Am. J. Physiol.*, **163**, 283.
- 2 Brocklehurst, W. E. and Lahiri, S. C. (1962)—The production of bradykinin in anaphylaxis, *J. Physiol.*, **160**, 15.
- 3 Elliot, D. F. (1963)—Bradykinin and its mode of release, *Ann. N. Y. Acad. Sci.*, **104**, 35.
- 4 Ratnoff, O. D. and Miles, A. M. (1964)—The induction of permeability increasing activity in human plasma by activated Hageman factor, *Brit. J. Exp. Pathol.*, **45**, 328.
- 5 Schachter, M. (1956)—A delayed slow contracting effect of serum and plasma due to the release of the substance resembling kallidin and bradykinin, *Brit. J. Pharmacol.*, **11**, 111.
- 6 Schachter, M. (1963)—Kinins of different origin, *Ann. N. Y. Acad. Sci.*, **104**, 108.
- 7 Morgolis, J. (1963)—The interrelationship of coagulation of plasma and release of peptides, *Ann. N. Y. Acad. Sci.*, **104**, 133.
- 8 Cox, J. S. G. (1967)—Disodium cromoglycate (FPL 670) (Intal). A specific inhibitor of reagenic antibody antigen mechanisms, *Nature*, **216**, 1328.
- 9 Vane, J. R. (1971)—Mediators of the anaphylactic reactions, Identification of Asthma, *CIBA Symp.*, No. 38, Churchill Livingstone, London.
- 10 Prado, J. L., Prado, S. E., Brandi C. M. W. and Kathburian, A. V. (1963)—Some properties of highly purified horse urinary kallikrein, *Ann. N. Y. Acad. Sci.*, **104**, 187.
- 11 Diniz, C. R. and Carvalho, I. F. (1963)—A micro method for determination of bradykininogen under several conditions, *ibid.*, **104**, 77.
- 12 Margolis, J. (1958)—Activation of plasma by contact with glass, Evidence for a common reaction which releases plasma kinins and initiates coagulation, *J. Physiol.*, **144**, 1.
- 13 Ratnoff, O. D. and Devie, E. W. (1962a)—The purification of activated Hageman factor, *Biochemistry*, **1**, 67.
- 14 Ratnoff, O. D. and Davie, E. W. (1962b)—The activation of Christmas factor by activated plasma thromboplastin antecedent, *Biochemistry*, **1**, 967.
- 15 Nossel, H. L. (1969)—The early stages of blood coagulation, *Recent Advances in Blood Coagulation*, 39–67 J. & A. C. Churchill Ltd., London-2.
- 16 Mongar, J. L. (1974)—Secretion of histamine from Mast cells, Aspects of Allergy and Immunology, *VIII Annual Conference, I. C. A. I., Bhopal*.
- 17 Kunze, H. and Vogt, W. (1971)—A significance of phospholipase A for prostaglandin formation, *Ann. N. Y. Acad. Sci.*, **180**, 123.

- 18 Kunze, H., Bohn, E. and Vogt, W. (1974)—Effects of local anaesthesia on prostaglandin biosynthesis *in vitro*, *Biochem. Biophys. Acta.*, **360**, 260.
19. Kunze, H., Nahas, N. and Wurl, M. (1974)—Phospholipases in human seminal plasma, *Biochem. Biophys. Acta.*, **348**, 35.
- 20 Lewis, R. A., Wasserman, S. I., Goetz, E. and Frank Austen, K. (1975)—Formation of slow reacting substance of anaphylaxis in human lung tissue and cells before release, *J. Exp. Med.*, **140**, 1133.
- 21 Sutherland, E. and Robison, G. A. (1966)—Metabolic effects of catecholamines, *Pharmac. Rev.*, **18**, 145.
- 22 Rochae Silva M., Beraldo, W. T. and Rosenfield, G. (1949)—Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and trypsin, *Am. J. Physiol.*, **156**, 261.
- 23 Rochae Silva M. (1963)—Physiological significance of bradykinin, *Ann. N. Y. Acad. Sci.*, **104**, 190.
- 24 Feldberg, W. and Kellaway, C. H. (1938)—Liberation of histamine and formation of lysolecithin like substances by cobra venom, *J. Physiol.*, **94**, 187.
- 25 Habermann, E. (1963)—Snake venoms, Kallikreins and Kininogens, *Ann. N. Y. Acad. Sci.*, **104**, 130.
- 26 Khairallah, P. A. and Page, I. H. (1963)—Effects of bradykinin angiotensin on smooth muscle, *Ann. N. Y. Acad. Sci.*, **104**, 212.
- 27 Douglass, W. W. (1975)—Polypeptides and prostaglandins, *The Pharmacological basis of therapeutics*, Goodman, L. S. and Gilman, A., ed. V. McMillan Publishing Co. Inc., New York.

■ ■

STUDIES ON THE INTERACTION OF PIPERAZINE AND SENNOSIDES ON ISOLATED RAT COLON*

B. B. GAITONDE, M. B. BHIDE AND R. S. JOSHI†

Department of Pharmacology, Haffkine Institute, Parel, Bombay 400 012

During our study on the bioassay of senna in a piperazine-sennosides preparation, piperazine was found to inhibit the action of sennosides. Hence this *in vitro* study of interaction of piperazine and sennosides on isolated rat colon was undertaken.

Sennosides produced a dose dependent contraction of the rat colon in a dose range of 50 to 800 $\mu\text{g/ml}$. This action was not blocked by atropine or mecamlamine. Histamine, 5-HT and bradykinin failed to produce the contraction of the rat colon.

Piperazine blocked the contractions induced by sennosides but failed to block the actions of acetylcholine or barium chloride. The action of sennosides appears to be on specific receptors. Mechanism of action of sennosides and the nature of block produced by piperazine are discussed.

Introduction

A number of piperazine-sennosides preparations are available for the treatment of round worm infestation. Sennosides are added to such preparations in order to produce purgation and thus expel paralysed worms. During our study on the bioassay of sennosides in one such preparation, using Lou's method (1), it was observed that piperazine inhibited the action of sennosides *in vivo*. As the colon is the site of action of senna (2), rat colon was chosen as the preparation for this study.

Materials and Methods

Rats of either sex weighing between 100-150 g were used. Food was withdrawn twenty-four hours before the experiment, allowing water *ad lib*. The animals were

sacrificed by cervical dislocation. Colon was dissected and lumen washed using DeJalon's solution. A piece, 2-3 cm in length, was suspended in an organ bath containing DeJalon solution maintained at $29 \pm 1^\circ \text{C}$ and bubbled with oxygen. Drugs were added into the bath and responses recorded on a smoked drum using frontal writing lever with a magnification of $\times 15$. Action of sennosides on guinea-pig ileum rat stomach fundus and rat uterus was also studied.

Drugs used—Calcium sennosides A & B; piperazine citrate; acetylcholine; histamine; 5-hydroxytryptamine, bradykinin; barium chloride; atropine and mecamlamine.

The concentrations of the drugs are expressed in terms of their salts. Number of experiments (N) are stated under results.

Results

Sennosides A & B produced a dose related contraction of isolated rat colon in doses

(Received for publication December 18, 1975)

*The paper was presented at the VII Annual Conference of Indian Pharmacological Society held at Ahmedabad from 27 to 29 December 1974.

†Unichem Research Fellow.

ranging from 50 to 800 $\mu\text{g/ml}$ (fig. 1, $N=6$). The onset of action was within 10-15 seconds and the maximum effect was seen within sixty seconds. Atropine did not block the action of sennosides in a dose upto 10 $\mu\text{g/ml}$, although it abolished the responses to acetylcholine (fig. 2a). Mecamylamine (10 $\mu\text{g/ml}$) also had no effect on the responses to sennosides ($N=3$). Histamine, 5-HT and bradykinin did not produce contraction of the isolated rat colon in a concentration as high as 500 $\mu\text{g/}$

ml. Sennosides failed to produce any action on isolated guinea-pig ileum, rat stomach fundus and rat uterus.

Piperazine, upto a dose of 800 $\mu\text{g/ml}$, did not produce any effect on the isolated rat colon. But, when the tissue was exposed to the action of piperazine for three minutes prior to the addition of sennosides, the action of sennosides was blocked (fig. 2b, $N=6$). The degree of block was related to the concentration of piperazine. Increased

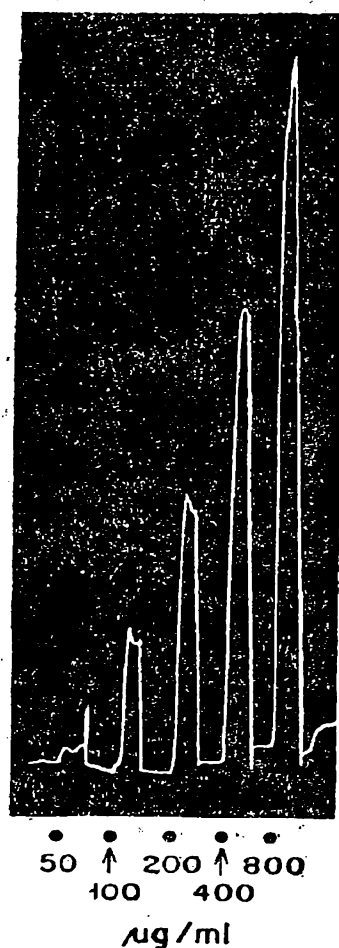


Figure 1—Dose response curves of sennosides (●) on isolated rat colon.

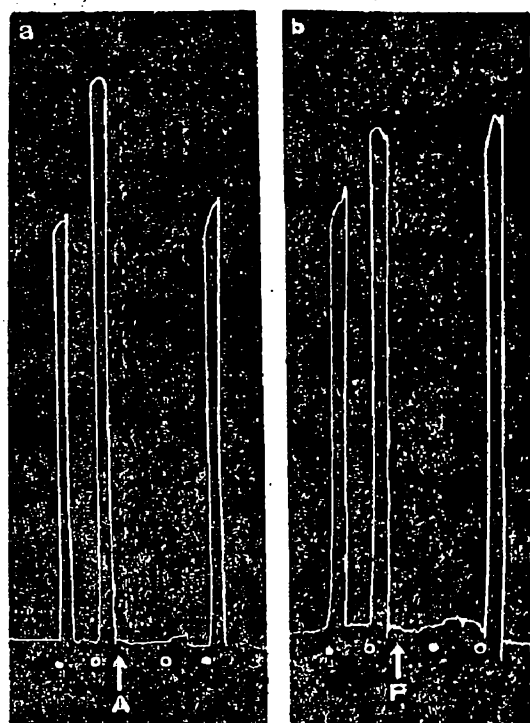


Figure 2—Effect of atropine (A—1.0 $\mu\text{g/ml}$) and piperazine (p—200 $\mu\text{g/ml}$) on the responses induced by acetylcholine (O—20 ng/ml) and sennosides (●—200 $\mu\text{g/ml}$) on the isolated rat colon.

concentration of sennosides overcame the block. Piperazine failed to block the actions of acetylcholine or barium chloride in concentrations as high as 800 $\mu\text{g/ml}$ ($N=3$).

Discussion

We have shown that sennosides produce contractions of the smooth muscles of the colon. This appears to be a specific action on colon, since the drug does not produce contraction of other smooth muscle preparations such as guinea-pig ileum, rat stomach fundus or rat uterus in doses as high as 500 $\mu\text{g/ml}$.

Action of anthracene purgatives has been attributed to the presence of anthraquinones which are absorbed and resecreted in the large colon where the bacterial flora are said to hydrolyse the glycoside into an active aglycone. Our results, *in vitro*, demonstrate clearly that formation of aglycone may not be necessary.

The smooth muscle activity of the colon is under the control of the parasympathetic nerves. The stimulation of the muscle by the drug can be either through the action on the parasympathetic ganglia or on the cholinergic receptor. Both these mechanisms do not seem to be involved in the action of sennosides, since neither mecamlamine nor atropine blocks the contractions induced by the drug. Further, the action does not seem to be mediated through the release of histamine, 5-HT or bradykinin. The colon is not sensitive to the action of these substances in the concentration upto 500 $\mu\text{g/ml}$. It is therefore concluded that sennosides act directly on the smooth muscle of the colon.

Piperazine does not have any action on the smooth muscle. It has been shown to block the action of acetylcholine on the

nicotinic site (3). Further, piperazine blocks acetylcholine receptors on the ascaris muscle (4). Piperazine failed to block the contraction of the colon induced by acetylcholine and also barium chloride. However, it specifically blocked the smooth muscle stimulation induced by sennosides. This block is dose dependent and reversible. The piperazine block can be overcome by increasing the concentration of sennosides in the bathing fluid and maximum effect of sennosides can be obtained in the presence of piperazine. All this evidence goes in favour of a competitive type of antagonism as described by Arunalakshana and Schild (5). However, further work will be needed to elucidate the mechanism of this block.

Sennosides seem to act on specific receptors on colon and its action is blocked by piperazine.

The results indicate that giving piperazine with senna in clinical practice is not rational since the action of senna is likely to be blocked by piperazine. The failure of senna to produce purgation in animals given piperazine with senna combination is explained by this antagonism.

Summary

Sennosides produce dose dependent contraction of colon.

The drug acts on specific receptors since its action is not blocked by atropine or mecamlamine. Colon is not sensitive to histamine, 5-HT or bradykinin.

Piperazine does not block the action of acetylcholine or barium chloride.

Piperazine specifically blocks the action of sennosides. The block is reversible and dose dependent.

REFERENCES

- 1 Lou, T. C. (1949)—The biological assay of vegetable purgatives. *J. Pharma. Pharmacol.*, **1**, 673-82.
 - 2 Dipalma, J. R. (1965)—Drill's pharmacology in medicine, 3rd ed., 747, McGraw Hill Book Company, New York.
 - 3 Norton, S. and De Beer, E. J. (1957)—Investigations on the action of piperazine on the ascaris lumbricoides, *Am. J. Trop. Med. Hyg.*, **6**, 898-905.
 - 4 Gaitonde, B. B. and Mahajani, S. S. (1974)—Pharmacology of nerve muscle preparation of human *Ascaris lumbricoides*, *Bull. Haff. Instt.*, **2**, 54-60.
 - 5 Arunalakshana, O. and Schild, H. O. (1959)—Some quantitative uses of drug antagonism, *Brit. J. Pharmacol.*, **14**, 48.
- ■

SUBSTITUTED SALICYLANILIDES AND THEIR ANTIMICROBIAL ACTIVITY

R. P. KINI, MADHUBEN R. PATEL AND M. H. SHAH

Department of Chemotherapy, Haffkine Institute Parel, Bombay 400 012

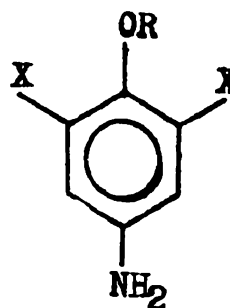
A series of substituted salicylanilides were prepared and tested for antimicrobial activity. Majority of the compounds showed *in vitro* antituberculosis activity in the range of 1-5 $\mu\text{g/ml}$. None of them showed any activity against other pathogenic bacteria and fungi.

Introduction

Derivatives of salicylic acid have long been known to possess fungistatic activity; and some of these have been employed as both industrial and medicinal chemicals. It has been shown in the literature that, conversion of salicylic acids into anilides markedly increased antibacterial activity (1, 2). Halonitrosalicylanilides have been patented for their antibacterial activity (3, 4).

Mayer's hypothesis (5), postulates that the mycobacteria and fungi being phylogenetically closely related, substances acting as antifungal agents are likely to show antituberculosis activity also. In the present work, the lead was taken from 5-bromo-4'-chlorosalicylanilide (Multifungin), which is being used clinically for the fungus infection of the skin (6).

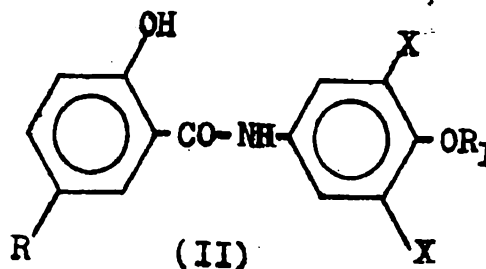
Besides, some of the substituted anilines with general formula (I), tested in our laboratory have shown *in vitro* antituberculosis activity upto 20 $\mu\text{g/ml}$ (unpublished data).



(I)

Where $\text{X} = \text{Cl}$ or Br ; $\text{R} = \text{H}$, CH_3 , or C_2H_5 .

The synthesis of a series of compounds of the general formula (II) was, therefore, undertaken in an effort to develop improved drug activity and define further the structural parameters leading to activity within the class.



(II)

Where $\text{R} = \text{Br}$ or NO_2 ; $\text{X} = \text{Br}$ or Cl ;
 $\text{R}_1 = \text{H}$, CH_3 or C_2H_5 .

Chemistry

The proposed anilides have been obtained by reacting 5-substituted salicylic acid chloride with appropriate 3,5-dihalo-4-alkoxyanilines in a nonpolar solvent. These compounds could also be obtained directly from the acids, by reacting them with the appropriate aniline in presence of thionyl chloride although with lower yields. The requisite amines were prepared from *p*-nitrophenol as per the standard procedures.

Experimental

2,6-Dichloro-4-nitrophenol was prepared in 86 per cent yield by chlorination of 4-nitrophenol (7). The corresponding 2,6-dibromo derivative was obtained in quantitative yield by using bromine in acetic acid (8). 2,6-Dihalo-4-nitro-anisole was prepared by the action of dimethyl sulphate on the requisite 2,6-dihalo-4-nitrophenol (9). 2,6-Dibromo-4-nitrophenetole was prepared by the action of diethyl sulphate on the corresponding phenol; however, 2,6-Dichloro-4-nitrophenetole could only be obtained (65 per cent yield), by using ethyl iodide in presence of anhydrous potassium carbonate. Nitro compound was reduced to amino compound using granulated tin and concentrated hydrochloric acid (12).

3',5'-Dichloro-4'-methoxy-5-bromosalicylanilide : Method A—A mixture of 5-bromosalicylic acid (2.17 g, 0.01 mole), thionyl chloride (8 ml) and benzene (30 ml) was refluxed for four hours. The solvent along with excess of thionylchloride was removed by distillation under reduced pressure. To the residue (acid chloride), a solution of 2,6-dichloro-4-aminoanisole (1.92 g; 0.01 mole) in benzene (30 ml) was added and refluxed for four hours. The solution was concentrated and filtered. The residue was dried, pulverised and then suspended in

100 ml of 10 per cent hydrochloric acid. The product was filtered, washed well with water and crystallised from aqueous ethanol (m.p. 266–268°C).

3',5'-Dibromo-4'-methoxy-5-bromosalicylanilide : Method B—A mixture of 5-bromosalicylic acid (2.17 g; 0.01 mole), 2,6-dibromo-4-aminoanisole (2.81 g, 0.01 mole) and benzene (20 ml) was treated dropwise with thionyl chloride (6 ml), and then refluxed for eight hours. Solvent was then removed and the residue was treated with dilute hydrochloric acid and subsequently with sodium bicarbonate. The solid was washed with water and recrystallised from aqueous ethanol, m.p. 260°C. The salicylanilides thus prepared are listed in Table I.

Biological Results and Discussion

The various anilides were screened against three strains of pathogenic fungi viz., *C. albicans*, *T. rubrum* and *T. mentagrophytes*, in the liquid medium of Schraufstatter *et al.* (10). Simultaneously, these compounds have also been tested *in vitro* against *M. tuberculosis* (H₃₇Rv) in Youmans' medium (11), using serial dilution method. The results are shown in the last column in Table I. The compounds were also tested against a few pathogenic gram-positive and gram-negative bacteria.

None of the compounds from this series showed any antifungal action against three strains of pathogenic fungi. Majority of the compounds showed *in vitro* activity against *M. tuberculosis* (H₃₇Rv) in the range of 1–5 µg/ml. In the series, 5-nitrosalicylanilides were comparatively more active than 5-bromosalicylanilides. Introduction of alkoxy group in 4'-position increased the activity. It was observed that when 4'-methoxy group (No. 4) was replaced by 4'-ethoxy group (No. 5), the

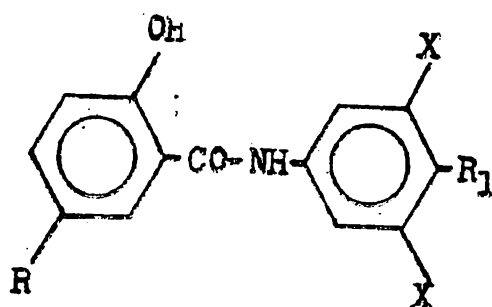
activity markedly increased. The most active compound encountered in the series was 5-nitro-3',5'-dibromo-4'-ethoxy salicylanilide (No. 8) showing activity at 1 µg/ml. Also, the compounds in this series showed no activity against gram-positive (*S. aureus*) and gram-negative (*S. typhi*, *E. coli* and *V. comma*) bacteria.

Acknowledgements

The authors wish to thank Dr. C. V. Deliwala, Ex-Deputy Director (Research), for his keen interest and Mr. M. T. Jaokar and Associates for microanalyses. Thanks are also to the Director, Haffkine Institute, Bombay for facilities to carry out this work.

TABLE I

Substituted salicylanilides



Serial No.	R	X	R ₁	M.p.° C*	Method followed	Molecular formula	Analyses†	Lowest <i>in vitro</i> anti-T.B. conc. µg/ml
1	Br	Br	OCH ₃	260	B	C ₁₄ H ₁₀ Br ₃ NO ₃	C, H, N	100
2	Br	Br	OC ₂ H ₅	248	B	C ₁₅ H ₁₂ Br ₃ NO ₃	C, H, N	5
3	Br	Cl	OH	220-221	A	C ₁₃ H ₈ BrCl ₂ NO ₃	‡C, H, N	5
4	Br	Cl	OCH ₃	266-268	A	C ₁₄ H ₁₀ BrCl ₂ NO ₃	C, H, N	100
5	Br	Cl	OC ₂ H ₅	236-239	B	C ₁₅ H ₁₂ BrCl ₂ NO ₃	C, H, N	5
6	NO ₂	Br	OH	233-236	A	C ₁₃ H ₈ Br ₃ N ₂ O ₅	C, H, N	40
7	NO ₂	Br	OCH ₃	277-280	A	C ₁₄ H ₁₀ Br ₂ N ₂ O ₅	C, H, N	2
8	NO ₂	Br	OC ₂ H ₅	265-266	A	C ₁₅ H ₁₂ Br ₂ N ₂ O ₅	C, H, N	1
9	NO ₂	Cl	OCH ₃	270-272	B	C ₁₄ H ₁₀ Cl ₂ N ₂ O ₅	C, H, N	2
10	NO ₂	Cl	OC ₂ H ₅	250-252	A	C ₁₅ H ₁₂ Cl ₂ N ₂ O ₅	§C, H, N	2

*Melting points are capillary melting points and are uncorrected.

†The analytical results of the elements indicated by symbols were within ±0.4 per cent of the theoretical values.

‡Found, C, 42.10 per cent; requires C, 41.27 per cent.

§Found C, 49.42 per cent; requires C, 48.52 per cent.

REFERENCES

- 1 Taborsky, R. G., Darkar, G. D. and Kaye, S. (1959)—Substituted salicylanilides with antimicrobial activity, *J. Am. Pharm. Assoc.*, **48**, 503.
- 2 Lemaire, H.; Schramm, C. H. and Cahn, A. (1961)—Synthesis and germicidal activity of halogenated salicylanilides and related compounds, *J. Pharm. Sci.*, **50**, 831.
- 3 Hok, A. K., Hamilton, A. Y., Pilcher, S. K. and Newman, R. (1956)—Antifungal activity of a new group of salicylamide derivatives for dermatophytes, *Antibiotics and Chemotherapy*, **6**, 456.
- 4 Rotimistrow, M. M., Kulik, G. V. and Nevkipila, O. S. (1965)—Synthetic antimicrobial preparations, *Chem. Abstr.*, **63**, 6043g.
- 5 Mayer, R. L. (1942)—Investigations on the chemotherapy of mycoses and of tuberculosis, *Chem. Abstr.*, **36**, 5199.
- 6 Knoll, A. G. (1958)—Halogenated salicylic acid arylamide, *Chem. Abstr.*, **52**, 18320g.
- 7 Bogdanov, S. V. and Frenkel, S. P. (1945)—2-chloro and 2, 6-dichloro-4-nitrophenols, *Chem. Abstr.*, **39**, 948.
- 8 Hartman, W. W., and Dickey, J. B. (1944)—2, 6-dibromo-4-nitrophenol, 'Organic Syntheses', Coll. Vol. II, Blatt, A. H. (Ed.), John Wiley and Sons, Inc., New York. p. 173.
- 9 Clement de Traz (1947)—Surdeux trichloro-anisidines, *Helv. Chimica Acta.*, **30**, 232.
- 10 Schraufstatter, E., Richter, R. and Ditscheid, W. (1955)—Method III. Fungistatic method according to Schraufstatter, Richter and Ditscheid (1949), *Acta. Pathol. Microbiol. Scand. Suppl.*, **104**, 49.
- 11 Doub, L. and Youmans, G. P. (1950)—Studies in tuberculosis chemotherapy, *Amer. Rev. Tuber.*, **61**, 407.
- 12 Clarke, H. T. and Hartman, W. W. (1944)—Phloroglucinol, 'Organic Syntheses', Coll. Vol. I, Blatt, (Ed.), John Wiley and Sons, Inc., New York, p. 455.

■ ■

SYNTHESIS AND STUDY OF 2-HETEROCYCLIC AMINOPYRIDO-(3, 2-d) THIAZOLES AS POSSIBLE ANTHELMINTIC AGENTS

B. G. KHADSE, M. H. SHAH, M. B. BHIDE AND VIJAYALAXMI M. BHAT

Haffkine Institute, Parel, Bombay 400 012

2-heterocyclic aminopyrido-(3, 2-d) thiazoles are synthesized and tested against *N. dubius* in mice. 2-adamantyl aminopyrido-(3, 2-d) thiazole is the most active.

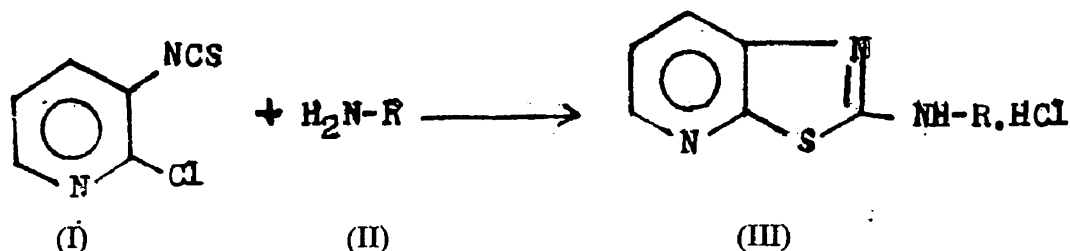
The presence of various heterocyclic units like thiazole, thiazoline, adamantane, quinoline, pyrimidyl, etc. in many anti-parasitic (1, 2), particularly anthelmintic (3,4), compounds tempted us to incorporate these biologically important moieties into pyridothiazole moiety to get the compounds of type III.

These compounds were prepared by reacting the corresponding heterocyclic amines with 2-chloro-3-isothiocyanato-pyridine as previously described (5).

refluxed on a water bath for three hours. A solid product separated out which was filtered, washed with cold ethanol and recrystallised from ethanol. The various compounds thus prepared are listed in Table I along with their analytical and activity data.

Biological Discussion

All the compounds in this series were insoluble in water except the 2-aminothiazine derivative. Therefore, this was the only compound that could be tested against



Where R = 2-thiazolyl, 2-thiazoline, 2-thiazine, 1-adamantyl, 3-quinoline, 8-quinoline, 2-furfuryl, 2-(4, 6-dimethyl) pyrimidyl.

Preparation of 2-(substituted-amino) pyrido(3,2-d)-thiazole—2-Chloro-3-isothiocyanato-pyridine (0.01 mole) in ethanol (25 ml), was treated with the appropriate amine and the reaction mixture was

A. lumbricoides using the known method (6). It showed activity at a minimum inhibitory concentration of 1 µg/ml (33 per cent). However, the compounds were tested against *N. dubius in vivo* and all of them exhibited anthelmintic activity. The most active compound in this series was the 2-amino-adamantane derivative which showed mortality of the adult worm upto the extent of 92 per cent at 0.2 mg dose level.

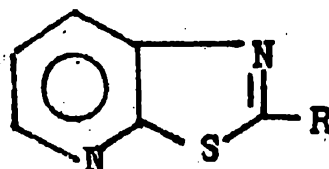
(Received for publication September 1, 1975)

Acknowledgements

The authors are thankful to Mr. M. T. Jaokar, and his associates for microanalyses. Thanks are also due to the Director,

Haffkine Institute, Parel, Bombay 400 012, for his interest and providing facilities to carry out the present work.

TABLE I
2-heterocyclic amino-pyrido (3, 2-d) thiazole



Serial No.	R	M.p. °C* (uncorr.)	Yield Per cent	Molecular formula†	Dose (mg)	Per cent Mortality‡
1	2-Aminothiazoline ..	255-57	45	C ₉ H ₈ N ₄ S · HCl	0.1 0.5	67 71
2	2-Aminothiazolyl ..	175-77	40	C ₉ H ₈ N ₄ S ₂ · HCl	0.1	
3	2-Amino (8-quinoline)	183-84	50	C ₁₅ H ₁₀ N ₄ S · (F.B.)	0.1 0.5	55 62
4	2-Amino (3-quinoline)	297-98	50	C ₁₅ H ₁₀ N ₄ S · (F.B.)	0.1 0.5	54 78
5	2-Amino (4,6-dimethyl)-pyrimidyl	285-86	15	C ₁₂ H ₁₁ N ₅ S · (F.B.)		
6	2-Amino (furfuryl)	198-200	60	C ₁₁ H ₉ N ₃ OS · HCl	0.1 0.5	55 65
7	2-Amino (1-adamantyl)	265-66	60	C ₂₆ H ₁₉ N ₃ S · HCl	0.1 0.2	79 92
8	2-Amino-5, 6-dihydro-1, 3, 4-(H) thiazine.	124-25	55	C ₁₀ H ₁₀ N ₄ S ₂ · HCl	0.1 0.5	61 79

* Melting points are capillary melting points and are uncorrected.

† Analysed correctly for C, H and N up to ± 0.4 per cent.

‡ Per cent mortality of the adult work at the seventh day, after administration of the drug.

Standard drug = L-Tetramisole.

(Mortality rate at the dose of 0.1 mg = 100 per cent).

REFERENCES

- 1 Peter, J. Islip, Michael, D. Johnson, M. R. and Martin, C. Neville (1972)—Substituted thiazolyl ureas, *J. Med. Chem.*, **15**, 101.
- 2 Leslic, M., Werbel, Margavet, Deganan, Gail, F., Harger, David, B. Capps (1972)—1-alkyl-3-(3-alkyl-5-nitro-4-thiazolin-2-yl-idene) ureas and related compounds as schistosomicides, *J. Med. Chem.*, **15**, 955.
- 3 Costakis, E. and Canonne (1971)—Antimicrobials, *J. Med. Chem.*, **14**, 1222.
- 4 Austin, W. C., Courtney, W., Danilewics, J., Morgan, D. H., Conover, L. H. and Lowes, H. L. (Jr.), Lynch, J. E., MacFarland, J. W., Cornwell, R. L. and Theodorides, V. J. (1966)—Anthelmintic Agents, *Nature, London*, **212**, 1273.
- 5 Khadse, B. G., Shah, M. H. and Deliwala, C. V. (1975)—Anthelmintic Agents, *Bull. Haff. Instt.*, **1**, 27.
- 6 Gaitonde, B. B. and Mahajani, S. S. (1974)—Pharmacology of nerve muscle, Preparation of human, *A. lumbricoides*, *Bull. Haff. Instt.*, **2**, 2.

STUDIES ON SENSITIVE COLORIMETRIC METHOD FOR DETERMINATION OF ETHINYL ESTRADIOL

LALITA S. SHIRSAT AND M. H. SHAH

*Department of Pharmaceuticals, Haffkine Bio-Pharmaceutical Corporation Ltd.,
Parel, Bombay 400 012*

The diazotized *m*-nitroaniline is coupled with ethinyl estradiol for its rapid colorimetric estimation. The yellow colour obtained in alkaline condition is quantitatively measured at 450 nm. The application of this procedure to pharmaceutical products is satisfactory.

Estimation of ethinyl estradiol has posed a problem, in view of the very small dosage usually recommended. Particularly, in programme of family planning, estimation of every tablet containing ethinyl estradiol individually has become important. There have been a number of methods employed, most of them having certain disadvantages. The very sensitive fluorometric method offered by James (1) is based on Liebermann Burchard reaction. This method can estimate 0.5 µg/ml but often its fluorescence is quenched by other ingredients (2). However, reasonably sensitive and reliable colorimetric methods have recently been reported (2) (3). These methods are claimed to show minimal interference by other common adjuvants. The method using 4-amino-6-chloro-*m*-benzenedisulphonamide offers a stable colour which could be measured at 500 nm; however, the minimum quantity estimated is 50 µg of estrogen per milliliter.

In 1975, diazotized 5-chloro-2,4-dinitroaniline has been reported to couple with ethinyl estradiol (or any phenolic compound) to give extremely stable colour. We were attracted by this method for adopting for our routine estimations;

however, in our hands the 5-chloro-2,4-dinitroaniline failed to get diazotized, under the conditions reported and we, therefore, searched for an alternate reagent which could successfully be used to the required sensitivity and reliability. We observed that *m*-nitroaniline when diazotized and coupled with ethinyl estradiol produced a stable yellow colour which could be measured at 450 nm. This reagent could be utilized to estimate phenolic steroids i.e. ethinyl estradiol in the dosage recommended for daily use. To minimize the instrumental error we could successfully add known standard quantity of phenol in each of our estimations with no loss of accuracy of the results. By this, the readings were brought to the centre of the scale of the spectrophotometer.

Materials and Methods

Coleman Spectrophotometer model 14 was used to determine the absorbance values reported.

Reagents and Solutions—*m*-Nitroaniline (m.p. 112–113°C) 0.04 per cent solution in ethanol, 1N hydrochloric acid, 3 per cent aqueous sodium nitrite, 2M sodium acetate, 0.1 N and 1.0 N sodium hydroxide, approximately 0.001 per cent phenol and distilled absolute ethanol.

Standard Ethinyl Estradiol Solution—Accurately weighed about 5 mg pure ethinyl estradiol is dissolved in 5 ml ethanol in a 50 ml volumetric flask and volume made up with 0.1 N NaOH.

Preparation of the Sample—Extract an accurately weighed sample of the powder or powdered tablets, preferably equivalent to about 0.5 mg of ethinyl estradiol with three 5 ml portions of ethanol. Centrifuge each portion, filter the ethanol extracts into a small beaker, and evaporate to dryness. Transfer the contents of the beaker quantitatively with 0.1 N sodium hydroxide to a 10 ml volumetric flask and dilute to volume with additional 0.1 N sodium hydroxide. If solution is not clear, centrifuge it and take supernatant as the assay solution.

Single Tablet Analysis—Extract finely powdered tablet (about 50 μ g of ethinyl estradiol) thrice with 3 ml, 2 ml and 2 ml portions of ethanol centrifuging each portion separately in the same centrifuge tube and collect the extract in a small beaker. Evaporate the extract to dryness. Add 1.5 ml of 0.1 N NaOH and again centrifuge this solution. Take 1 ml from the supernatant and add 1 ml of standard solution of phenol to make the assay solution.

Colour Development—Into separate flasks, pipette successively 1 ml of each of *m*-nitroaniline, hydrochloric acid and sodium nitrite solutions and mix well. Allow to stand for two minutes and add 1 ml of ethanol, and either 2 ml of standard solution, 2 ml of the assay solution or 2 ml of 0.1 N sodium hydroxide solution to separate flasks. Add 2 ml of sodium acetate solution to each flask and allow to stand for twenty minutes. Add 3 ml of 1 N sodium hydroxide. Allow to stand for thirty minutes and determine the absorbance of the standard and the sample solution at 450 nm against the blank.

TABLE

Commercial two component sample of ethinyl estradiol

Name	Amount labelled in μ g.	Amount found in μ g	Content in per cent
Orlest 28 ..	50	50	100
	50	50	100
	50	50	100
	50	50	100
Norlestrin ..	50	51	102
	50	51	102
Ovulon	50	52.5	105
	50	52.5	105

Results and Discussion

It is observed that 0.04 per cent ethanolic solution of the reagent and 3 per cent sodium nitrite gave the most satisfactory results. Excess sodium nitrite did not interfere and so it was not necessary to destroy the excess nitrous acid. Use of 0.1 N NaOH to dissolve ethinyl estradiol and diluted with 1 N NaOH after coupling was found to offer most intense and reproducible colour.

For extraction of ethinyl estradiol from tablets the *iso*-octane-benzene mixed solvent as suggested by National Formulary was not necessary since ethanol or methanol worked well. Thus extraction of ethinyl estradiol from tablets with ethanol is preferred. No difficulties were encountered in estimation of Orlest 28, as well as Norlestrin (both Parke Davis) or Ovulan 50 (Searle). In colour development, addition of 1 ml of ethanol was found necessary to avoid turbidity which appeared in its absence.

The colour intensity increases for the first twenty minutes at pH 5 and then remains constant for at least sixty minutes before taking readings at 450 nm at pH 12.

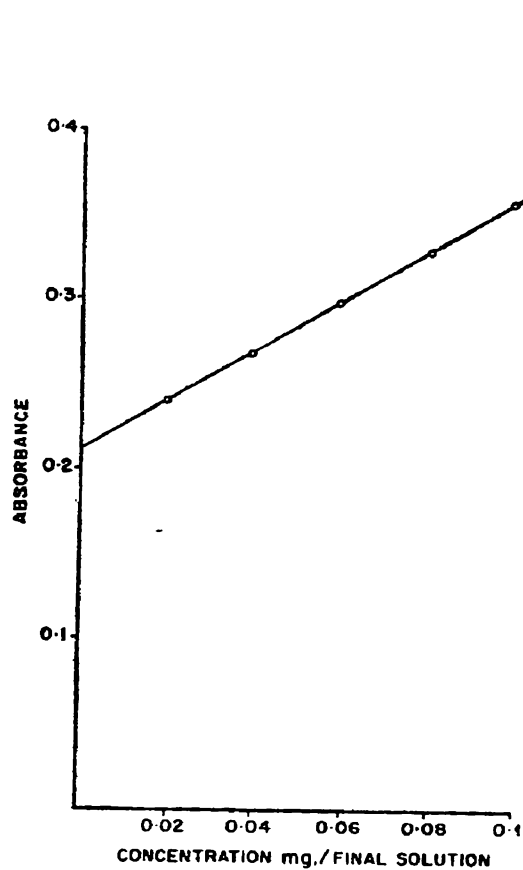


Figure 1—Absorbance vs. concentration for diazo product of ethinyl estradiol with addition of 9.85 μ g phenol in each concentration.

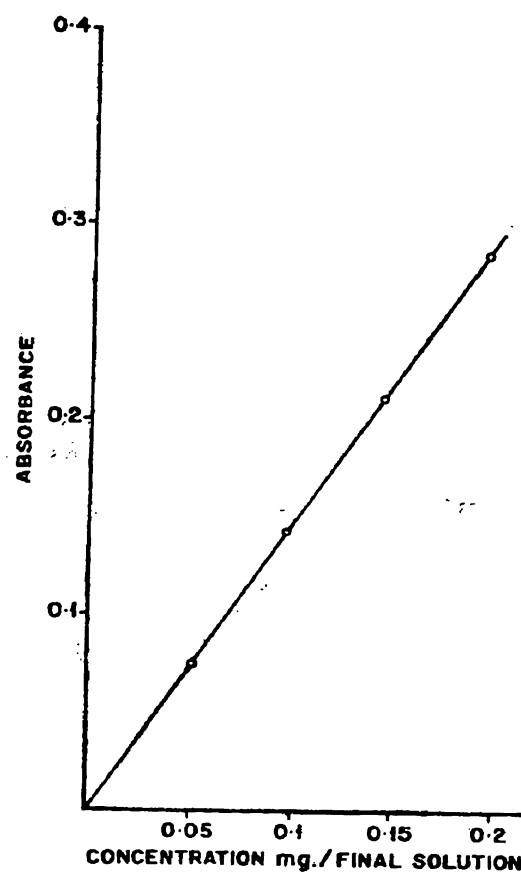


Figure 2—Absorbance vs. concentration for diazo product of ethinyl estradiol.

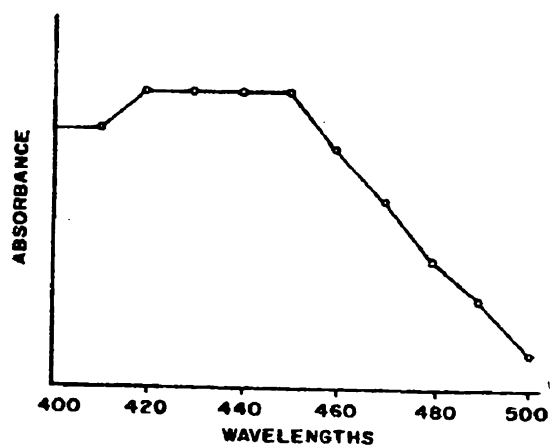


Figure 3—Absorption spectrum of ethinyl estradiol with diazotized *m*-nitroaniline.

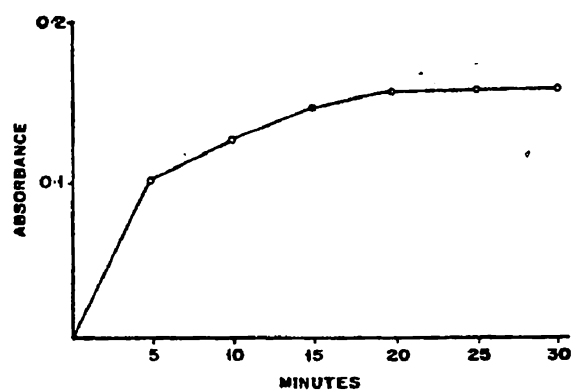


Figure 4—Effect of coupling time on the absorbance of the final solution.

The relationship between absorbance at 450 nm and concentration is linear upto 20 µg/ml concentration of ethinyl estradiol. The sensitivity of this method permits a reliable assay on a single tablet with 50 µg of ethinyl estradiol.

The recoveries (table) in all cases was 100–102 per cent with a good spectrophotometer preferably with a voltage stabilizer

Acknowledgments

Our thanks are to Dr. Yusuf K. Hamied, Director, CIPLA Laboratories Limited, Bombay, for supplying pure ethinyl estradiol. The authors are also thankful to the Managing Director, Haffkine Bio-Pharmaceutical Corporation Limited, Parel, Bombay 400 012 for his keen interest.

REFERENCES

- 1 James Theron (1972)—Fluorometric determination of ethinyl estradiol in tablets, *J. Pharm. Sci.*, **61**, 1306.
- 2 Eldawy, M. A., Tawfik, A. S. and Elshabouri, S. R. (1975)—Rapid sensitive colorimetric method for determination of ethinyl estradiol, *J. Pharm. Sci.*, **64**, 1221.
- 3 Urbanyi Tibor and Rehm, C. R. (1966)—Rapid sensitive colorimetric method for the determination of estrogens, *J. Pharm. Sci.*, **55**, 501.

■ ■

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 by Dr. M. V. N. Shirodkar, Head, Department of Virology, Haffkine Institute for Training, Research and Testing. Published by the Director, Haffkine Institute for Training, Research and Testing, Bombay 400 012 and printed at the Government Central Press, Bombay 400 004

