C— BIOLOGICAL SCIENCES

Journal of Scientific & Industrial Research

THE COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH, NEW DELHI

J. sci. industr. Res., Vol. 21C, No. 9, Pp. 225-260

SEPTEMBER 1962

Journal of

Scientific & Industrial Research

Vol. 21C, No. 9, SEPTEMBER 1962

CONTENTS

J. sci. industr. Res., Vol. 21C, No. 9, Pp. 225-260. September 1962

Annual Subscription — All Sections (A, B, C and D): Rs 30 (Inland), £ 4 or $ 12.00 (Foreign). Individual Sections: Rs 10 (Inland), £ 1 or $ 3.00 (Foreign). Single Copies (Individual Sections): Re 1 (Inland), 2 sh. or 30 cents (Foreign)

Effect of Ultraviolet Radiation on the Incorporation of Radioactive Phosphorus in Growing & Non-growing Cells of *Vibrio cholerae*

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Manuscript received 23 *March* 1962

The incorporation of P^{32} in Vibrio cells is stimulated by glucose and inhibited by chloromycetin, dinitrophenol, azide and copper ions. In the trichloracetic acid-insoluble fraction, P^{32} is shared mostly by phospholipids and nucleic acids. Larger doses of ultraviolet radiation are required to inhibit the P^{32} incorporation in the non-growing cells. Incorporation of P^{32} in the growing cultures, however, is affected even at lower doses, and the effect is more pronounced in the DNA fraction. It has been suggested that P^{32} incorporation may be through macromolecular 'turnover' in the non-growing cells, which appears to be relatively insensitive to ultraviolet irradiation as compared to the net synthesis in the growing cultures.

STUDIES on the effect of ultraviolet irradiation on growth and nucleic acid synthesis (both DNA and RNA) in *Vibrio cholerae*[1] showed a marked inhibition of both processes when the cells were grown after irradiation. The effect on DNA synthesis, however, appeared to precede the inhibition of either RNA synthesis or growth. These observations, along with others reported[2-4], suggest that such initial arrest of DNA synthesis might be responsible for other manifestations of the action of ultraviolet irradiation.

Recent evidence has indicated that even in the non-growing bacteria synthesis of some new nucleic acid (and protein) molecules continues at the expense of the existing ones[5]. It, therefore, appeared of interest to study the action of ultraviolet irradiation on this phenomenon.

Materials and methods

Experiments with non-growing cells of Vibrio cholerae — *V. cholerae* (Ogawa 60) was grown on meat papain digest agar slants for 18-20 hr, the cells washed twice with normal saline by centrifugation, and finally suspended in the same medium to a standard turbidity (80 per cent absorption in a Lange's colorimeter model J, using a red, 660 mμ filter).

Reaction mixture — The reaction mixture consisted of 1 ml. each of *M*/15 phosphate buffer, *M*/20 glucose, and appropriately diluted radioactive orthophosphate (Na_2HPO_4) in buffered saline (obtained from Atomic Energy Establishment, Trombay) giving $8\text{-}10\times10^5$ counts/min. and 2 ml. of the cell suspension. The mixture was incubated at 37°C. for definite intervals and the reaction was stopped by the addition of cold trichloracetic acid (TCA; effective concentrations 5 per cent, wt/vol.).

Ultraviolet irradiation — The procedure of ultraviolet irradiation of the cells was the same as described earlier[1].

Fractionation of the cold TCA-insoluble precipitate of the cells — The precipitate obtained by the addition of TCA was centrifuged and washed twice with 5 ml. aliquots of *M*/60 phosphate buffer (*p*H 8·0). It was then suspended in 5 ml. of 75 per cent alcohol and 1 ml. aliquot was taken out for the determination of radioactivity. The remaining portion was fractionated according to Schneider's procedure as modified by Roberts *et al.*[6]. The separation of

RNA and DNA was done according to the method of Schmidt and Thanhauser[7].

Determination of radioactivity — Radioactivity was determined as reported earlier[8]. Fractions were counted directly after plating and adequate precautions were taken to avoid any error due to decay.

Experiments in the growing cells — Conditions of experiment were the same as described earlier[1]. Aliquots (10 ml.) of broth culture at start and 2 hr after incubation were mixed with an equal volume of 10 per cent cold TCA. Subsequent procedures for fractionation of the precipitate and determination of radioactivity were the same as above.

Results

Initial experiments showed that addition of glucose stimulated the incorporation of P^{32} in the cold TCA-insoluble fraction of Vibrio cells, though this increase was not markedly affected by its concentration (range: $M/200$-$M/25$). Further, the incorporation was found to be higher at alkaline *p*H (8·0-9·0). The incorporation, therefore, in all subsequent experiments was studied in presence of ($M/100$) glucose and at *p*H 8·0.

Incorporation of P^{32} in different fractions of V. cholerae — The incorporation of labelled phosphate (Fig. 1) in the cold TCA-insoluble material and its fractions was found to increase progressively up to 3 hr. The incorporated phosphorus seemed to be shared mostly by the phospholipids and nucleic acids. The protein fraction showed little uptake and was, therefore, not counted in subsequent experiments.

Effect of certain inhibitors on P^{32} incorporation — The data presented in Table 1 would show that chloromycetin (45 µg./ml.) inhibited the incorporation by 25 per cent, its effect being more or less uniform in different fractions. Sodium azide and 2,4-dinitrophenol at a concentration of $M/1000$ similarly brought about an inhibition of P^{32} uptake by about 50 per cent, whereas copper sulphate ($M/1000$) reduced the incorporation by about 70-75 per cent in different fractions. Further, the relative distribution of radioactivity between separated RNA and DNA fractions in the control indicated that more than 70 per cent of P^{32} incorporated in the ' nucleic acid ' was shared by RNA.

Effect of ultraviolet on P^{32} incorporation in non-growing cells — Irradiation of the cell suspension up to 10 min. was not found to have any significant effect on P^{32} incorporation in either cold TCA-insoluble material or its fractions (data not presented). At much higher doses (20 min. or more) of ultraviolet radiation, however, this incorporation was partly

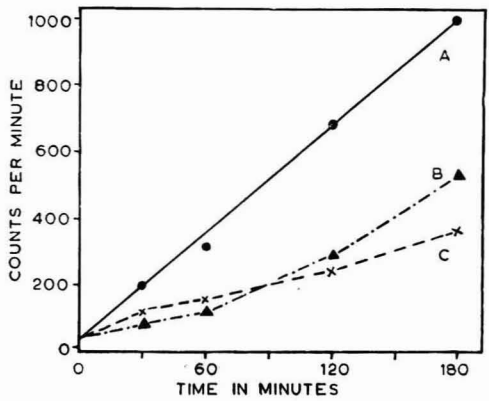

Fig. 1 — Kinetics of P^{32} incorporation in different fractions of non-growing cells of *V. cholerae* [(A) Cold TCA precipitate, (B) nucleic acids and (C) phospholipids]

TABLE 1 — EFFECT OF INHIBITORS ON INCORPORATION OF P^{32} IN NON-GROWING CELLS OF *V. CHOLERAE*

Inhibitor	Counts/min.			
	Cold TCA ppt.	Phospho-lipids	RNA	DNA
Control	2360	712	940	399
Chloromycetin	1680	400	560	242
Copper sulphate	660	176	247	176
Dinitrophenol	1220	376	482	210
Sodium azide	1135	352	527	220

TABLE 2 — EFFECT OF ULTRAVIOLET IRRADIATION ON INCORPORATION OF P^{32} IN NON-GROWING CELLS OF *V. CHOLERAE*

Irradiation time *min.*	Counts/min.			
	Cold TCA ppt.	Phospho-lipids	RNA	DNA
0 (control)	2360	712	940	399
20	1500	504	595	233
40	1365	448	538	220
60	1020	344	358	193

TABLE 3 — EFFECT OF ULTRAVIOLET IRRADIATION ON INCORPORATION OF P^{32} IN DIFFERENT CELLULAR FRACTIONS IN GROWING CELLS OF *V. CHOLERAE*

Irradiation time *min.*	Optical density after growth	Counts/min.			
		Cold TCA ppt.	Phospho-lipids	RNA	DNA
0	0·130	1140	552	255	296
2	0·100	920	462	230	176
4	0·088	755	438	160	124

*After irradiations the cultures were maintained in the growth medium for 2 hr; optical density before growth was 0·065.

inhibited, but the effect seemed to be more or less uniform in respect of phospholipid, RNA and DNA fractions (Table 2).

The effect of ultraviolet on the incorporation of P^{32} in growing Vibrio cholerae cells — The results presented in Table 3 would show that even in growing cultures the incorporated P^{32} was shared by the phospholipid and nucleic acid fractions and very little if at all by proteins. Between the two nucleic acids (DNA and RNA) its distribution was found to be approximately equal. The ultraviolet radiation was seen to progressively inhibit this incorporation with increase in dose, its effect being more pronounced on the incorporation in the DNA fraction.

Discussion

The present investigations show that non-growing cells of *V. cholerae* were able to incorporate inorganic P^{32} into protoplasmic constituents (cold TCA-insoluble products). That at least a major part of this incorporation is metabolic in nature is suggested, by its stimulation by glucose, its variability with *p*H and inhibition by chloromycetin, dinitrophenol, sodium azide and copper sulphate. In the absence of any external nitrogen source, incorporation of P^{32} in nucleic acids may perhaps be due to intracellular 'turnover' which has now been shown in a number of bacteria under non-growing conditions[5]. However, the possibility of exchange as postulated by Allfrey and Mirsky[9] cannot at present be entirely excluded.

In accordance with earlier results in *Esch. coli*[10], the incorporation of P^{32} into various fractions of non-growing *V. cholerae* cells was found to be less sensitive to ultraviolet as compared to its incorporation during growth. Assuming that the incorporation of phosphorus in non-growing cells occurs by way of nucleic acid turnover (postulated above), these observations would imply it to be relatively insensitive to the action of ultraviolet radiation as compared to the net synthesis in growing cultures.

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Asparaginase Activity of *S. typhosa*

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Manuscript received 23 *March* 1962

All the antigenic strains of *S. typhosa* used show considerable asparaginase activity. Soluble extracts of Vi-1 strain of *S. typhosa* hydrolyse only L-asparagine while urea, glutamine and D-asparagine are not hydrolysed. The optimum *p*H of the enzyme is 6·5-7·0 and Michaelis constant $5\times10^{-3}M$. The activity is linear with time and enzyme concentrations employed. The enzyme is strongly inhibited by Cu^{2+}, cyanide and arsenate while other substances like Hg^{2+}, Ag^{+}, Co^{2+}, Mg^{2+}, phosphate, azide, EDTA and *o*-phenanthroline produce no effect. Among the antibiotics used only chlorotetracycline, streptomycin and chloramphenicol inhibit the enzyme. A striking inhibition of the enzyme is observed by normal rabbit serum and the inhibitory factor is found to be heat stable and non-dialysable. The enzyme is stable when stored in deep freeze. Pre-incubation at *p*H 5·0 or at a temperature of 60-70°C. produces negligible inactivation and the enzyme is only completely inactivated when pre-incubated at 80°C. for 15 min.

THE presence of high concentration of asparagine in the proteins of *Salmonella typhosa* cells[1] is suggestive of an important metabolic role played by this amide. Saito[2] reported the presence of asparaginase in *S. enteritidis* which also showed deamination of aspartic acid. It has been demonstrated that *S. typhosa* can deaminate aspartic and glutamic acids[3]. In view of the relatively high content of asparagine in *S. typhosa* cells, investigation on the metabolic role of this amide appeared worth while. Experiments were also carried out to see whether glutamine and urea were also hydrolysed by the organism. Utjino *et al.*[4] observed slight hydrolysis of urea by strains of *S. typhosa* and recently Zimmerman and Graber[5] observed urease activity in young cultures of *S. choleraesuis* var. *Kunzendorf* which was rapidly lost on subculturing. However, the presence of this enzyme in *S. typhosa* is still a controversial issue.

A survey of the relevant literature did not reveal any information regarding the presence and activity of amidases in *S. typhosa*. The present work was, therefore, undertaken to determine whether *S. typhosa* possesses amidase activities. A preliminary account of this work has been published elsewhere[6].

Materials and methods

Enzyme preparation — The five antigenic strains of *S. typhosa* (H-901, O-901, Vi-1, Ty2 and Watson's V) obtained from the National Collection of Type Cultures, London, were maintained on Lablemco medium at *p*H 7·4 and 20 hr growths of these cultures were grown at 37°C. in Roux bottles containing buffalo heart infusion agar medium (*p*H 7·6) supplemented with 1 per cent Difco bacto peptone and 0·5 per cent sodium chloride.

For the preparation of cell-free extracts Vi-1 strain was used. Freshly harvested and washed cells were suspended in cold distilled water (100 mg. wet cells/ml.) and were disrupted for 20 min. intervals in a Mickle disintegrator (manufactured by H. Mickle, Gomshall, Surrey, England). The whole suspension thus obtained was centrifuged in the Spinco ultracentrifuge (model L) at 40,000 r.p.m. for 1 hr using rotor No. 40 and the supernatant was stored at −20°C. The protein nitrogen of this extract determined by the usual method[7] was found to be 0·42 mg. per ml. This extract served as the stock enzyme preparation and was diluted ten times before use.

Assay of enzyme activity — Enzyme activity was determined by measuring the amount of ammonia formed by a diluted enzyme preparation equivalent to 21·0 μg. protein nitrogen incubated for 30 min. in the presence of L-asparagine ($10^{-3}M$) or other substrates. All incubations were carried out in test tubes at 37°C. in 0·02*M* veronal buffer (*p*H 7·0) with a pre-incubation period of 15 min. unless stated otherwise, at the end of which the substrate was added. Ammonia formed was estimated as described previously[7] by direct nesslerization. When the effects of substances like inhibitors, antibiotics, etc. (the chemicals employed were of A.R., B.D.H. quality and the antibiotics were pure crystalline products supplied

by the manufacturers), were studied, the incubations were done in Warburg flasks, with a pre-incubation period of 30 min., at the end of which the substrate was added and the reaction was stopped after 30 min. Ammonia formed was determined as reported previously[8]. All results were corrected for suitable enzyme and substrate blanks.

Results

Asparaginase activity of different strains of S. typhosa — The comparative activities of cell suspensions and cell-free extracts of various strains of *S. typhosa* are recorded in Table 1. It was found that L-asparagine is markedly hydrolysed by all the strains when cell-free extract was employed whereas with cell suspension the rate of hydrolysis was comparatively less. It has been previously shown by paper chromatographic methods[6] that the deamidation of L-asparagine by *S. typhosa* involves direct hydrolysis of the amide giving rise to aspartic acid.

Deamidation activity of cell-free extracts — It appears from the results given in Table 2 that cell-free extract of *S. typhosa* can deamidate only L-asparagine, whereas urea and glutamine are not deamidated. Since deamidation of asparagine will give rise to aspartic acid, which may again be deaminated due to the presence of an active aspartic deaminase[3], experiments done with DL-aspartic acid showed that with the cell-free extract used such deamination is very slight. It was also found that deamidation of L-asparagine is not inhibited with equimolar or higher concentrations of D-asparagine.

*p*H *optimum* — The optimum *p*H of asparaginase of *S. typhosa* was found to be at 6·5-7·0 (Fig. 1).

Time-course of the reaction — The time-course of the reaction was found to be linear up to 90 min. with the enzyme concentration employed (Fig. 2).

Enzyme concentration — The activity was linear with respect to enzyme concentration up to 1·25 ml. of the diluted enzyme preparation corresponding to 52·5 μg. protein nitrogen (Fig. 3).

Substrate concentrations — On plotting the reciprocals of asparagine concentrations (*M*) and the velocity of reaction (μg. of ammonia released per hour), according to Lineweaver and Burk[9], the Michaelis constant of the enzyme was calculated from the curve presented in Fig. 4 and was found to be 0·005*M*.

Effect of different substances on the asparaginase activity — The results recorded in Table 3 show that Cu^{2+} markedly inhibits the enzyme while Hg^{2+}, Ag^{+}, Mn^{2+}, Co^{2+} and Mg^{2+} are without any effect. Cyanide and arsenate produce significant inhibition of the enzyme whereas phosphate, sulphate, azide, fluoride, iodoacetate, EDTA and glutathione are

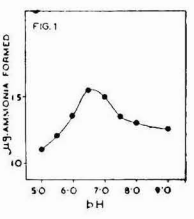
TABLE 1 — DEAMIDATION OF L-ASPARAGINE BY DIFFERENT STRAINS OF *S. TYPHOSA*

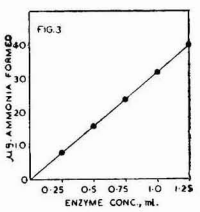
Strains of *S. typhosa*	Enzyme preparation*	Ammonia formed in 30 min. μg.
Vi-1	Cell suspension	9·8
	Cell extract	17·6
Ty-2	Cell suspension	9·9
	Cell extract	18·2
H-901	Cell suspension	9·5
	Cell extract	17·0
O-901	Cell suspension	9·6
	Cell extract	17·2
Watson's V	Cell suspension	7·8
	Cell extract	15·6

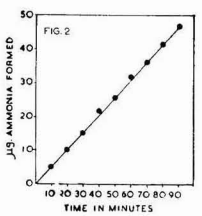
*Cell suspension adjusted to 40 per cent transmission at 650 mμ and 1 ml. of this was used. The cell extract was prepared by grinding the washed cells with acid-washed glass powder, extracting it with 0·9 per cent KCl and finally the supernatant was used after centrifugation at 10,000 r.p.m. One ml. of this extract, equivalent to 1 ml. of cell suspension described above, was used.

TABLE 2 — DEAMIDASE ACTIVITY OF CELL-FREE EXTRACTS OF *S. TYPHOSA* (Vi-1)

Substrates	Ammonia formed μg.
L-Asparagine ($10^{-3}M$)	15·0
D-Asparagine ($10^{-3}M$)	0·0
L-Asparagine ($10^{-3}M$) + D-asparagine ($10^{-3}M$)	15·0
L-Asparagine ($10^{-3}M$) + D-asparagine ($3\times10^{-3}M$)	15·0
L-Glutamine ($5\times10^{-3}M$)	0·0
Urea ($5\times10^{-3}M$)	0·0
DL-Aspartic acid ($2\times10^{-3}M$)	3·0







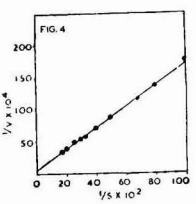


Fig. 1 — Optimum *p*H of asparaginase of *S. typhosa*

Fig. 2 — Incubation time and asparaginase activity of *S. typhosa*

Fig. 3 — Enzyme concentration and asparaginase activity of *S. typhosa*

Fig. 4 — Michaelis constant of asparaginase of *S. typhosa*

TABLE 3 — EFFECT OF VARIOUS SUBSTANCES ON THE ASPARAGINASE ACTIVITY OF CELL-FREE EXTRACTS OF *S. TYPHOSA* (Vi-1)

Additions*	Relative activity	Additions*	Relative activity
None	100·0	*o*-Phenanthroline	100·0
Mn^{2+}	100·0	α,α′-Dipyridyl	100·0
Co^{2+}	100·0	8-Hydroxyquinoline	96·0
Hg^{2+}	96·0	Glutathione	100·0
Ag^{+}	96·0	EDTA	100·0
Cu^{2+}	62·4	Iodoacetic acid	100·0
Mg^{2+}	100·0	Penicillin	96·0
Orthophosphate	98·4	Neomycin	100·0
Sulphate	96·0	Streptomycin	76·8
Arsenate	86·4	Chlorotetracycline	60·0
Cyanide	48·0	Tetracycline	96·0
Azide	96·0	Oxytetracycline	96·0
Fluoride	96·0	Chloramphenicol	84·0

*Final concentration of the substances added in the reaction mixture was 160 μg./ml. in the case of antibiotics, and $10^{-3}M$ in the case of other substances with the exception of orthophosphate, sulphate, arsenate, azide and fluoride where the concentration was $10^{-2}M$.

TABLE 4 — INHIBITION OF ASPARAGINASE ACTIVITY OF CELL-FREE EXTRACTS OF *S. TYPHOSA* (Vi-1) BY NORMAL RABBIT SERUM

Additions	Ammonia formed μg.	Inhibition %
None	15·0	—
Normal sera	5·4	64·0
48 hr dialysed normal sera	5·0	66·7
Boiled sera	6·0	60·0

without any effect. Similarly *o*-phenanthroline, α,α′-dipyridyl and 8-hydroxyquinoline have no effect. Among the antibiotics used, chlorotetracycline, streptomycin and chloramphenicol significantly inhibit asparaginase.

Inhibition by normal sera — It was observed that serum of normal rabbit contains a strong inhibitor of asparaginase of *S. typhosa* (Table 4). The inhibitor was found to be heat stable; heating the sera in a boiling water bath for 10 min. did not inactivate it. Prolonged dialysis of the sera for 48 hr in the cold failed to remove the inhibitor and consequently the inhibitor appeared to be non-dialysable.

Effect of heat, cold and acid treatment — The effect of temperatures of incubation on the asparaginase activity of *S. typhosa* is indicated by the results given in Table 5. It was found that the enzyme was quite active even at 60°C., although slight inactivation occurred between 60° and 70°C. The enzyme was completely inactivated at 80°C. within 15 min. of incubation. The optimum temperature of enzyme activity was found to be 55°C.

The enzyme was found to be stable and no loss of activity occurred when it was stored for more than a month in the deep freeze at −20°C. as shown in Table 6. The enzyme was also found to

TABLE 5 — EFFECT OF VARYING INCUBATION TEMPERATURE ON ASPARAGINASE ACTIVITY OF CELL-FREE EXTRACTS OF *S. TYPHOSA* (Vi-1)

Incubation temp. °C.	Ammonia formed μg.	Temp. range	Q_{10} values
15	3·2	—	—
25	7·6	15-25	2·375
35	14·0	25-35	1·842
45	21·2	35-45	1·514
55	24·6	45-55	1·160
65	20·6	55-65	0·837

TABLE 6 — EFFECT OF VARIOUS TREATMENTS ON THE STABILITY OF ASPARAGINASE ACTIVITY OF CELL-FREE EXTRACTS OF *S. TYPHOSA* (Vi-1)

Treatment*	Relative activity
None	100·0
Stored deep-frozen for 1 month	100·0
Heated at 60°C. for 15 min.	84·4
Heated at 70°C. for 15 min	73·3
Heated at 80°C. for 15 min.	1·4
Kept at *p*H 5 for 1 hr	84·3

*After the above treatments, the usual enzyme assay was carried out under the standard assay conditions.

be active when kept pre-incubated at *p*H 5·0 for 1 hr in the cold.

Discussion

The data presented in Tables 1-6 and Figs. 1-3 indicate that cell-free extracts of *S. typhosa* possess a very active asparaginase which can deamidate only L-asparagine but not D-asparagine. The extract also did not show any urease or glutaminase activity. Aspartic deaminase, though present, was very low in activity in the cell-free extracts employed. However, it was found that asparaginase is a common enzyme present in all the antigenic strains of *S. typhosa*.

The optimum *p*H of asparaginase of *S. typhosa* was found to be 6·5-7·0 whereas asparaginase from a variety of other sources[10-12] showed optimum *p*H at 8·0. It was also found that the hydrolysis of asparagine was linear to time and enzyme concentrations; this is similar to the finding of others with asparaginase from different sources[10,11,13,14]. There is a strong affinity of the enzyme for the substrate with a Michaelis constant of $5\times10^{-3}M$. Asparaginase of *S. typhosa* is strongly inhibited by Cu^{2+}, while Hg^{2+}, Ag^{+} and other metals failed to produce any effect. The strong inhibition of the enzyme by cyanide (as well as by arsenate) is similar to the observation made by Archibald[15] with kidney asparaginase. The enzyme was also inhibited by chlorotetracycline, streptomycin and chloramphenicol. The most striking inhibition of asparaginase activity of *S. typhosa* was observed when normal rabbit serum was used. It was found that the inhibitory factor was a

non-dialysable and heat-stable factor present in the normal serum. The exact nature of this factor remains unknown and further study to determine the nature of this factor would be of importance.

Another important property of asparaginase of *S. typhosa* is its highly stable nature. Grassmann and Mayr[14] found yeast asparaginase to be extremely unstable and Errera and Greenstein[12] observed that rat liver asparaginase (1) was also labile. On the other hand, asparaginase of *S. typhosa* is not inactivated on prolonged storage at −20°C. Even at 60-70°C. only slight inactivation of the enzyme occurred and the enzyme was only completely inactivated when pre-incubated at 80°C. for 15 min. From the above data, it appears that asparaginase is one of the most potent and stable enzymes of *S. typhosa*, although the significance of this enzyme in the metabolic cycle of this organism must await further study.

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Incipient Follicular Atresia & Acid Phosphatase Activity in Ovary & Serum of Rats*

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Manuscript received 30 *March* 1962

By the use of cadmium chloride which causes predictable regression of the follicles in prepuberal rats, it has been possible to substantiate that incipient atresia, not recognizable by routine histological procedures, could be detected by the appearance of histochemically demonstrable acid phosphatase in the granulosa. However, the activity of the enzyme in the granulosa does not attain a sufficiently high level during early atresia so as to register significantly on the overall activity in the ovary, as estimated biochemically. The evident atresia of the follicles detectable histologically is, however, accompanied by a marked rise in biochemically estimable enzyme activity in the ovary and in the necrosed granulosa. Parallel studies on serum show a decline in acid phosphatase activity only at the time when the rate of atresia is at its peak. The possibility of using serum acid phosphatase activity as a measure of the rate of regression of the follicles is discussed.

THE diagnosis of incipient follicular atresia by morphological criteria has not been achieved with any degree of certainty[1,2]. Routine histological procedures cannot detect early atresia. Deane[3] observed that lipid droplets appear in the granulosa of an atretic follicle. Recently, Lobel *et al.*[4] demonstrated that incipient atresia of the follicles and corpora lutea could be recognized by a rise in activity of hydrolytic enzymes (acid phosphatase and aminopeptidase) in the granulosa and lutein cells respectively. They based their studies on the premise that follicular (and luteal) regression

*Aided by a grant from the Population Council, the Rockefeller Foundation, New York.

is an example of physiologic autolysis engineered by enzymatic hydrolysis of endocellular substrates[5,6]; lysosomal hydrolases like acid phosphatase and cathepsins have been implicated in such intracellular digestion, including autolysis[5,7].

The mass atresia of the follicles caused by cadmium in prepuberal rats[8] has been utilized in the present study for an objective evaluation of the relationship between acid phosphatase activity and incipient follicular regression. The rate and course of follicular atresia with reference to time of administration of cadmium chloride are predictable and this is particularly suitable for the purpose of this investigation. Lobel *et al.*[4] demonstrated acid phosphatase (and aminopeptidase) activity in the follicles by histochemical procedures. In the present study, the overall activity of ovarian acid phosphatase has been estimated biochemically in addition to precise follicular localization by the histochemical method. Any meaningful parallel changes in serum acid phosphatase activity have also been sought.

Experimental procedure

Colony-bred albino rats of the Institute (5-6 weeks old), weighing 30-50 g., were injected with cadmium chloride by the subcutaneous route (0·04 mM/kg., a single injection). In animals which received cadmium chloride in combination with zinc acetate, the injections were given at different sites. The total dose of the latter salt[9] (6 mM/kg.) was split into three equal amounts of 2 mM/kg. each and administered in the following manner: (1) 5 hr before cadmium chloride, (2) simultaneously with cadmium chloride and (3) 19 hr after injection of cadmium chloride. In the animals which received zinc acetate alone (6 mM/kg.) the total dose was similarly divided into three of 2 mM/kg. each. The animals were sacrificed at 0, 3, 6, 24, 48 and 168 hr after administration of the salt. The animals injected with zinc acetate either alone or in conjunction with cadmium chloride, were sacrificed at one time interval (48 hr).

The ovaries were dissected out and weighed to the nearest 0·1 mg. in a Roller-Smith balance. For histological and histochemical studies the ovaries were fixed overnight in 10 per cent neutral formalin at 4°C. Serial paraffin sections were stained with Ehrlich's haematoxylin and eosinol. For demonstration of acid phosphatase activity, gelatin-embedded frozen sections (20 micra thick) were processed according to the method of Gomori[10]. Estimation of enzyme activity in the ovary and serum was carried out by the procedure laid down by Hawk *et al.*[11].

Results

Zero hour — The ovary was pale and inconspicuous. Histological examination revealed immature condition with primordial ovocytes and follicles in different stages of growth and atresia (Plate I, Fig. 1). The beginning of antrum formation was seen in some medium-sized follicles; the larger ones were more advanced in this respect. In occasional follicles of the latter type the antrum formation progressed considerably to give the ovum its characteristic disposition in relation to the formation of a definitive cumulus. In addition, there was an increased rate of accumulation of fluid in such follicles and thinning of the granulosa by several layers but without any signs of preovulatory changes[12]. The initial stages of thecal differentiation were seen only in the medium-sized follicles but not in the small ones. The follicular epithelium showed varying degrees of mitotic activity. The stroma contained fibroblast-like cells and epitheloid elements. Corpora lutea were absent.

Clearly, atretic follicles, recognized as such by histological criteria[4], like nuclear pycnosis and fragmentation of the granulosa cells bordering the antrum (vesicular follicles) or ovum (non-vesicular follicles), shrinkage and distortion of the entire follicle and nuclear changes in the ovum, showed high acid phosphatase activity. The enzyme was localized in the granulosa cells but not in the ovum. Even some of the apparently normal follicles showed varying degrees of phosphatase activity (Plate I, Fig. 1a). The primordial ovocytes and the interstitium were negative. The values for enzyme activity in the ovary and serum are given in Table 1.

Three hours — The macroscopic appearance of the ovary was the same as in 0 hr. Histologically,

TABLE 1 — ACID PHOSPHATASE ACTIVITY OF THE OVARY AND SERUM

(*Data based on pooled serum from two animals*)

Interval after $CdCl_2$ and/or $Zn(CH_3COO)_2$ injection *hr*	Acid phosphatase activity	
	Ovary *mg. phosphorus/ g./hr with S.E.*	Serum *mg. phosphorus/ 100 ml./hr with S.E.*
0	2·31 ± 0·34 (6)*	14·36 ± 1·61 (12)
3	2·50 ± 0·25 (7)	11·23 ± 0·65 (10)
6	2·82 ± 0·15 (13)	12·64 ± 1·18 (12)
24	4·70 ± 0·67 (6)	12·83 ± 1·53 (8)
48	4·74 ± 0·29 (7)	5·12 ± 0·48 (14)
	3·22 ± 0·46 (9)†	12·00 ± 1·57 (8)†
	2·13 ± 0·18 (6)‡	5·39 ± 0·54 (8)‡
168	0·72 ± 0·10 (7)	10·73 ± 0·76 (12)

*The figures given in parentheses refer to number of animals.
†Animals injected $CdCl_2 + Zn(CH_3COO)_2$.
‡Animals injected only $Zn(CH_3COO)_2$.

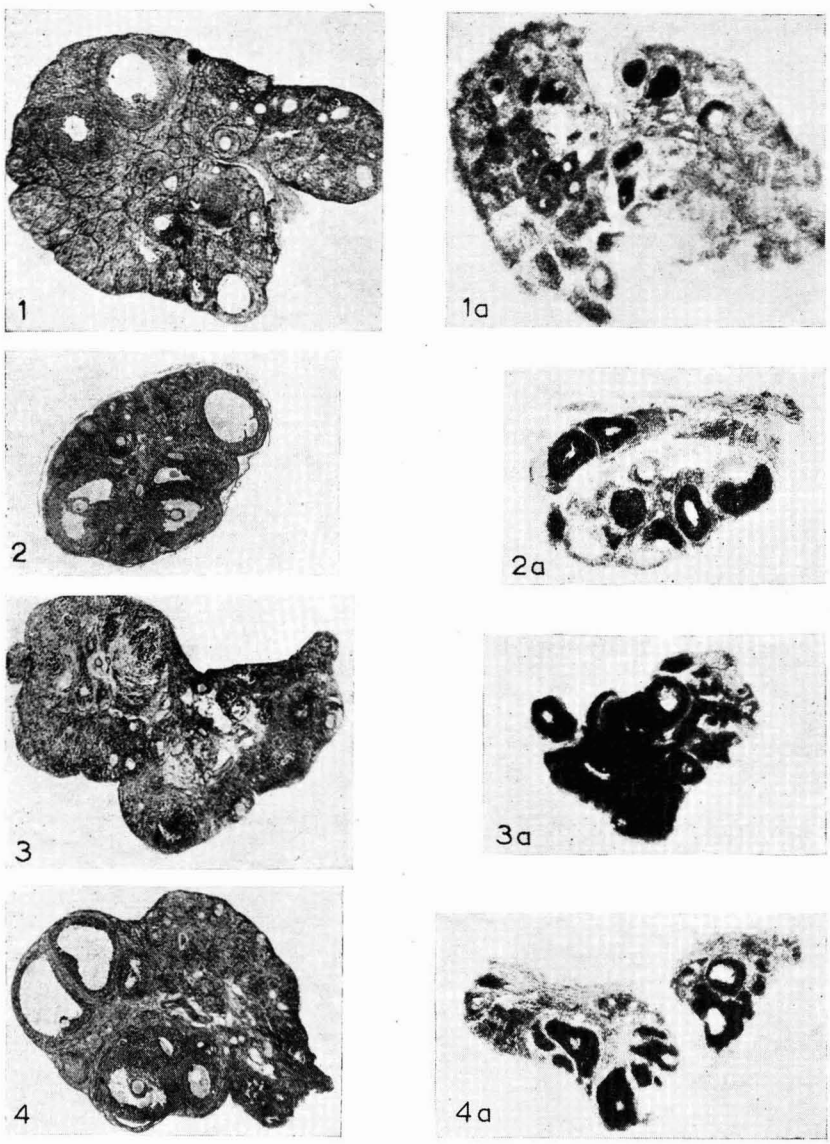

Plate I — Histochemical study of the ovaries of normal and cadmium chloride-treated rats × 72 [Fig. 1: The ovary of a normal rat. H and E. Note typical immature condition. Fig. 1a: The ovary of a normal rat. Gomori technique. Note varying degrees of acid phosphatase activity in some apparently normal small follicles. Fig. 2: The ovary of a rat 3 hr after the injection of $CdCl_2$. H and E. Note apparently normal appearance. Fig. 2a: The ovary of a rat 3 hr after the injection of $CdCl_2$. Gomori technique. More small and medium-sized follicles show high enzyme activity. Compare with Fig. 1a. Fig. 3: The ovary of a rat 48 hr after the injection of $CdCl_2$. H and E. Note degenerated condition of the ovary in general and follicles in particular. Fig. 3a: The ovary of a rat 48 hr after the injection of $CdCl_2$. Gomori technique. Note pronounced acid phosphatase activity in all the degenerated follicles. Fig. 4: The ovary of a rat 168 hr after the injection of $CdCl_2$. H and E. Note normal picture. Fig. 4a: The ovary of a rat 168 hr after the injection of $CdCl_2$. Gomori technique. Note reduction in overall enzyme activity as lesser number of follicles are reactive. Compare with Figs. 3a and 1a]

the ovary presented normal features with assortment of healthy and atretic follicles of different size. No increase in the rate of follicular atresia was seen. The stroma was essentially normal and no increase in vascularity was noticeable (Plate ,I Fig. 2).

The atretic follicles, recognizable by the histological criteria, were mostly of the large type and these showed marked phosphatase activity in the granulosum; the ovum was negative. Small and medium-sized follicles showing signs of regression were also similarly reactive. Some apparently normal follicles, too, showed varied distribution of high enzyme activity in the granulosa (Plate I, Fig. 2a). Many normal follicles, primordial ovocytes and the stroma did not, however, show any enzyme activity.

The acid phosphatase activity of the ovary and serum, as estimated biochemically, did not differ significantly from the respective 0 hr values (Table 1).

Six hours — The ovary was hyperaemic. Histological examination revealed atretic changes in virtually all of the large follicles and many of the medium-sized ones. Most of the small follicles were apparently normal; the primordial ovocytes were normal, too. Thecal blood vessels in most of the follicles were dilated and engorged. In some large follicles, the wall of the vessels collapsed and the theca appeared as an annular haemorrhagic area surrounding the atrophic granulosa cells. Similar vascular engorgement and haemorrhage were seen in many areas of the interstitium; atrophy of the stromal cells was also not uncommon in some portions.

Histochemically, the entire follicle population showed varying degrees of phosphatase activity. This ranged from intense activity in the clearly atretic large and medium-sized follicles to patchy localization in the apparently normal ones. The granulosa was the reactive site but the ovum was negative as before. The primordial ovocytes were non-reactive, too. Small patches of stroma with atrophied cells showed positive reactions for the enzyme. However, the overall activity of the enzyme, as determined biochemically, did not increase significantly (Table 1). Similarly, serum phosphatase did not show a significant change over the 3 hr value, although in comparison to 0 hr it was significantly less ($P < 0.02$; Table 1).

Twenty-four hours — The ovary was markedly hyperaemic. The regressive changes were widespread, affecting all types of follicles. Only an occasional apparently normal small follicle could be seen in the peripheral region. The granulosa, ovum and the theca lost their integrity in the majority of the large and medium-sized follicles. There was profuse haemorrhage in the stroma and focal degeneration of cellular components. However, plaques of healthy stromal tissue and primordial ovocytes withstood the toxic action of the salt.

Histochemical examination showed intense phosphatase activity in all the follicles. The granulosa seemed to be the specific reactive site. In some follicles the enzyme activity was so high that even their contour was obscured by black deposits. However, any localization of the enzyme in the ovum in such cases was probably due to diffusion artefact as indicated by Lobel *et al.*[4]. Patches of enzyme activity were also seen in the atrophied stroma.

The overall increase in ovarian phosphatase activity was reflected in the value which was significantly higher than that at any previous time interval ($P < 0.01$, Table 1). The serum enzyme activity, however, did not register any significant change from 0 and 6 hr level.

Forty-eight hours — Macroscopically, the ovary was merely a haemorrhagic mass. The degenerative changes were at their acme at this stage. The follicular atresia was total and the *entire* population of existing follicles was destroyed (Plate I, Fig. 3). Profuse haemorrhage was seen virtually throughout the organ and there was widespread disorganization of the stroma, so much so that the histological landmarks which characterize a normal ovary were almost obliterated. Deposits of extruded chromation together with yellow pigment were seen as detritus throughout the stroma. Most of the follicles were mere accumulations of degenerated granulosa cells in the stroma. In occasional small and medium-sized ones the degenerated ovum could be recognized; otherwise, any semblance to normal follicles was lost. The primordial ovocytes and plaques of stroma continued to withstand the toxic action of cadmium chloride. The ovary of zinc acetate-treated animals, either alone or in combination with cadmium chloride, presented normal macroscopic and histological features.

Histochemically, pronounced phosphatase activity was seen in all the follicles (Plate I, Fig. 3a). The other details about the distribution of the enzyme were virtually the same as in 24 hr. The ovary of animals treated with cadmium chloride plus zinc acetate continued to show marked enzyme activity in the large follicles and in some medium-sized ones; but others showed only a patchy reaction in the granulosa or no enzyme activity at all. The distribution of the enzyme in zinc acetate-treated animals was similar to that in the ovary at 0 hr.

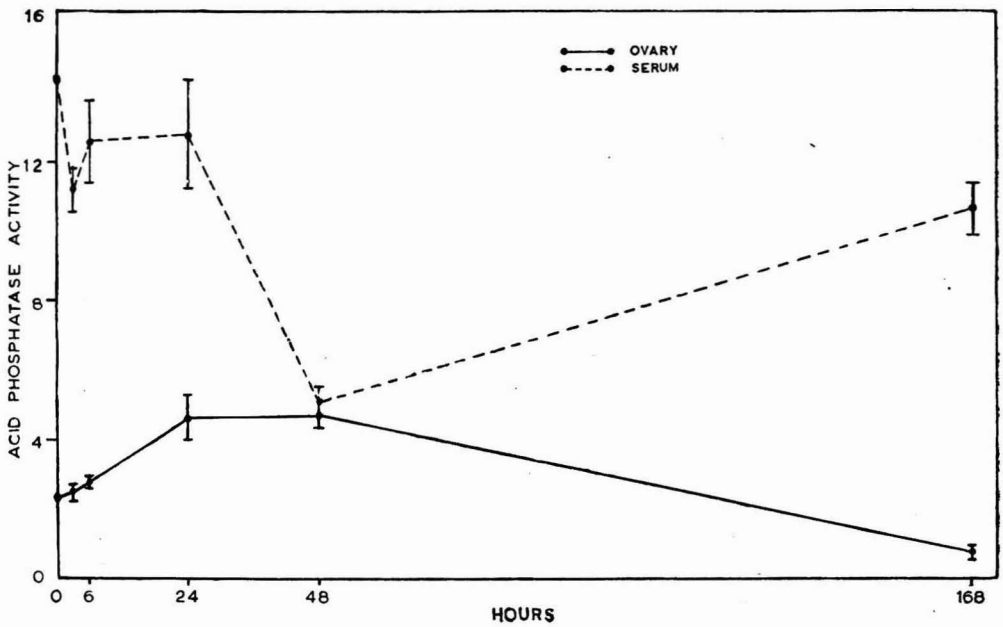

Fig. 1 — Ovarian and serum phosphatase activity in rats treated with cadmium chloride [Acid phosphatase activity expressed as mg. P/g. ovary/hr/100 ml. serum]

Biochemically, acid phosphatase activity in the ovary continued to be as high as in 24 hr (Table 1, Fig. 1). The enzyme activity in the ovary of animals treated with cadmium chloride plus zinc acetate was significantly higher than that at 0 hr ($P < 0.01$) but lower than that of 24 or 48 hr cadmium chloride-treated groups ($P < 0.01$). In the zinc acetate group the ovarian enzyme activity was of the same order as in 0 hr.

Serum acid phosphatase activity registered a marked decline at 48 hr (vs 0 hr-$P < 0.01$). In the animals treated with cadmium chloride plus zinc acetate the enzyme activity was not significantly different from that at 0 hr but was higher than that of the 48 hr group ($P < 0.01$). A low enzyme activity was also noticed in the serum of zinc acetate-treated animals (vs 0 hr-$P < 0.01$).

One hundred and sixty-eight hours — Macroscopically, the ovary was slightly hyperaemic; the haemorrhagic appearance noticed at 48 hr was absent. The histological features were indicative of recovery of the organ from toxic effects of cadmium chloride. Follicles in different stages of growth and atresia were seen and the stroma presented normal appearance. The overall histological picture was normal. There was, however, a little preponderance of vesicular follicles in the ovary at this stage (Plate I, Fig. 4).

The clearly atretic follicles showed high phosphatase activity (Plate I, Fig. 4a). Some apparently normal small and medium-sized follicles also gave positive reactions. The localization of the enzyme was mainly in the granulosa as before and the ovum continued to be negative. The overall activity of the enzyme in the organ was considerably reduced in comparison to 24 and 48 hr groups.

Biochemically, ovarian phosphatase activity registered a sharp decline; the value was significantly lower than that at any previous time interval (Fig. 1, Table 1). On the other hand, serum enzyme activity showed a significant rise over the 48 hr values ($P < 0.01$), but continued to be less than that of the 0 hr group although the difference was not statistically significant (vs 0 hr-$P < 0.01$).

In vitro studies — In preliminary *in vitro* studies, four ovaries from two rats were pooled together and homogenized in barbitone buffer (pH 5; 1 ml. buffer/5 mg. ovarian tissue). In one set of such preparations, 1 mg. of cadmium chloride (in 0·2 ml. sterile distilled water) was added/30 mg. of ovary or 6 ml. of the homogenate. The control set received 0·2 ml. of distilled water alone without cadmium chloride. The two sets (with or without cadmium chloride) were incubated side by side at 37°C. for 0, 30 and 60 min. The enzyme

TABLE 2 — EFFECT OF $CdCl_2$ ON OVARIAN ACID PHOSPHATASE ACTIVITY *IN VITRO*

Interval after incubation *min.*	Acid phosphatase activity *mg. phosphorus/g. ovary/hr*	
	Control	$CdCl_2$
0	2·26*	2·00
30	1·00	1·17
60	1·34	1·01

*Mean of two experiments.

activity was measured by the procedure of Hawk *et al.*[11]. The results are given in Table 2.

It will be seen that the acid phosphatase activity in the control and cadmium chloride preparations was more or less of the same order at a particular time interval. However, there was a slight reduction in enzyme activity at 30 and 60 min., but this was irrespective of the addition of the salt to the ovarian homogenate. These results, therefore, indicate that cadmium chloride had little effect on ovarian phosphatase activity *in vitro*.

Discussion

The results of the present study show that early atresia of the follicles not recognizable by conventional histological methods can be detected by an increase in acid phosphatase activity in the granulosa. The experimentally induced regression of the follicles by cadmium chloride is unique in that the rate and course of atresia can be predicted with accuracy[8]. Thus, at 3 hr after administration of the salt no evident increase in the rate of atresia is noticeable but at 6 hr, virtually all the large follicles and many of the medium-sized ones seem to be affected. At 24 hr all types of follicles (large, medium and small) except occasional apparently healthy small ones become clearly atretic. During the next 24 hr the entire population of existing follicles is destroyed. A new crop of follicles, presumably differentiated from the resistant ovocytes, is seen in the ovary at 168 hr. It is, therefore, evident that the large and medium-sized follicles succumb first to the toxic effect of cadmium chloride followed in quick succession by the small ones. The *rate* of atresia (as recognized by routine histological method) becomes higher than normal at 6 hr and then increases almost in a crescendo to become maximal at 48 hr.

The results of histochemical study indicate that incipient regression of the apparently healthy follicle population is foreshadowed as early as 3 hr after administration of cadmium chloride. At this stage no degenerative changes could, however, be detected in such follicles by conventional histological preparations, although an increase in acid phosphatase activity in varying degrees is clearly evident in the granulosa. Marked enzyme activity is, of course, seen in the granulosa of the clearly atretic follicles. The present findings are, therefore, in agreement with those of Lobel *et al.*[4] regarding the specific localization of the enzyme in the granulosa rather than its distribution in other components of the follicle. The eventual obliteration of all the available follicles is heralded at 6 hr when the entire population, including the apparently normal ones, shows enzyme activity of some degree. At 24 and 48 hr the histochemically demonstrable enzyme reaction is at its acme. This is followed by an overall reduction in enzyme activity at 168 hr; the picture becomes more or less similar as at 0 hr.

The total phosphatase activity in the ovary measured biochemically does not show any appreciable rise at 3 and 6 hr after administration of cadmium chloride, in spite of histochemical localization in varying degrees in the granulosa of a larger number of follicles. Possibly, the sum total increase in enzyme activity of all the histochemically reactive follicles is not of that high order as to register tellingly in biochemical estimations. Moreover, inherent defects in histochemistry of the phosphatases like diffusion artefacts which may exaggerate the true picture of enzyme activity[4], should also be taken into consideration. On the other hand, the peak period of follicular atresia (24 and 48 hr) is marked by a sharp rise in total enzyme activity. It is possible that the enzyme from the localized atrophic patches of the stroma also contributes to some extent to the total high activity seen during this period. However, this rise is specific and attributable to the destruction of primarily the granulosa (and to a less extent the stroma). This is borne out by the results of preliminary *in vitro* studies and the significant reduction of enzyme actlvity after combined administration of cadmium chloride and zinc acetate. The latter is known to prevent cadmium chloride-induced degenerative changes in ovary[9]. Moreover, no change in enzyme activity is seen after injection of zinc acetate alone; the histological and histochemical picture of the ovary continues to be similar to those at 0 hr. The disappearance of toxic manifestations in the ovary and a *pari passu* reduction in enzyme activity at 168 hr are also interesting pointers in this direction.

As indicated before, a focal point of the present study was to find out any parallel changes in serum phosphatase activity consequent upon an abnormal

increase in the rate of follicular atresia. The results obtained are suggestive but not quite conclusive. Thus, up to 24 hr (after administration of cadmium chloride) the enzyme activity in serum does not undergo any noteworthy change. It may be recalled that at 24 hr the rate of atresia is already very high; the ovarian enzyme activity shows a sharp rise, too. However, at 48 hr serum phosphatase activity registers a marked decline; this is reverse of the high enzyme activity in the ovary at this stage. Nevertheless, if this low activity in the serum is a true reflection of abnormally high rate of follicular atresia then it becomes a criterion of considerable diagnostic value. The increase in enzyme activity after combined administration of cadmium chloride and zinc acetate (cf. ovary) or spontaneously at 168 hr, tends to add to the specificity of serum picture in relation to follicular atresia. However, a drop in activity in zinc acetate (alone) treated animals at 48 hr, which is of the same magnitude as in the corresponding cadmium chloride group, is indeed a conundrum. It weighs against the possibility that a decrease in serum phosphatase activity is the true reflection of an increased rate of follicular atresia. Moreover, as mentioned earlier, zinc acetate alone does not cause any degenerative changes in the ovary. If it is assumed that zinc acetate *per se* has an intrinsic inhibitory effect on serum phosphatase activity, even then, the relative increase after combined administration with cadmium chloride remains anomalous. It can, however, be argued that the increase in enzyme activity at 48 hr is the expression of a mutual antagonism between the two metals, zinc acetate opposing any inhibitory effect of cadmium chloride on serum phosphatase primarily through a protective action on the ovary. On the other hand, the latter perhaps antagonizes the intrinsic depressive effect of zinc acetate on serum phosphatase. In point of fact, a mutual antagonism between the two metals with regard to their affinity for certain tissues in rodents is on record[13,14]. Nevertheless, these arguments do not help in establishing that a true correlation exists between high rate of follicular atresia and the characteristic depression in serum phosphatase activity. But at the same time, the potentiality of serum acid phosphatase change as a measure of the rate of follicular atresia cannot be dismissed altogether, either. It will be interesting to examine whether there is a comparable reduction in activity of this enzyme in conditions like pregnancy and lactation in which a spontaneous increase in the rate of follicular regression is known to occur[4].

Acknowledgement

The authors are grateful to the Population Council Inc., New York, for a generous grant and to Dr B. Mukerji for his interest in this study. Thanks are due to Shri R. P. Sinha for technical assistance and Shri S. Banerjee for the illustrations.

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Physico-chemical Studies on Indigenous Seed Proteins: Part VIII — Electrophoretic Characterization & Amino Acid Composition of Bengal Gram (*Cicer arietinum*) Meal Proteins & Its Globulin Fractions

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Manuscript received 23 *December* 1961

Optimum electrophoretic separation of Bengal gram meal proteins occurs between *p*H 8·0 and 9·0 and ionic strengths 0·1 and 0·2, when five components are obtained. Cooling and fractionation by isoelectric precipitation fractionation procedure yield a fraction which behaves as a single-protein boundary over the *p*H range 3-12. At *p*H 4·0 and 4·25, two and three components respectively are present. The isoelectric point of this fraction is around *p*H 4·5. Bengal gram meal and globulins lack serine and contain low amounts of cystine, methionine, tryptophan and tyrosine. The globulins contain good amounts of histidine and large amounts of essential amino acids like arginine, threonine, valine, leucines, phenylalanine and lysine which appear to account for the superior nutritive quality of this pulse.

BENGAL gram is the most important pulse crop in India and is extensively cultivated throughout the country and is regarded as a very nutritious dietary pulse. In continuation with our investigations on the physico-chemical aspects of seed proteins, Bengal gram has now been studied in a manner similar to that adopted for red gram[1-3] and black gram[4], in our earlier studies.

Materials and methods

Bengal gram meal was prepared by grinding the seeds and passing through 80 mesh B.S. sieve. The meal was defatted by soxhleting with light petroleum ether and the elementary composition of the meal was determined by standard methods.

The various fractionation procedures employed in the present study were more or less similar to those reported for red gram[2] and black gram[4] proteins. A brief description of the various methods are as follows.

Fractionation by heat coagulation — Sodium chloride (0·4*M*) extract of the meal was heated to different temperatures over a water bath (60-96°C.). The precipitates thus formed were removed by centrifugation.

Ammonium sulphate fractionation — Sodium chloride (0·4*M*) extract of the meal was fractionated by the use of different amounts of ammonium sulphate. The details of this method are described earlier[2,4]. The precipitates were electrophoretically analysed at *p*H 8·0 and ionic strength 0·1.

Cooling, isoelectric precipitation with hydrochloric acid, followed by ammonium sulphate fractionation — Sodium chloride (0·4*M*) extract of the meal was kept at 2-3°C. for 48 hr when a copious precipitate was obtained. The solution was centrifuged and the supernatant was adjusted to *p*H 5·6 with 0·1*N* HCl, when another precipitate was obtained. The solution was centrifuged and the supernatant was further adjusted to *p*H 4·5 which yielded one more precipitate. This precipitate was removed by centrifugation and the clear supernatant was further fractionated with varying amounts of ammonium sulphate saturation. All the precipitates obtained by the above procedures were dialysed free of salt against distilled water and dried over phosphorus pentoxide in cold, *in vacuo*. The details of this fractionation scheme are given in Chart I.

The experimental details and the buffers employed in the electrophoretic analysis have already been described[1,5].

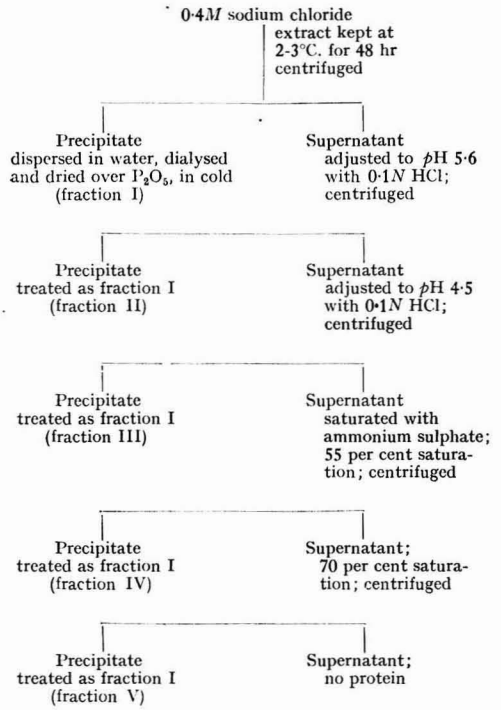


Chart I — Procedure for the fractionation of meal proteins

Bengal gram meal and the various globulin fractions obtained by cooling and isoelectric precipitation method were hydrolysed with acid and the hydrolysates were analysed by circular paper chromatography for their amino acid composition. The details of these procedures have been described earlier[3,6].

Results and discussion

The proximate analysis of the Bengal gram meal was as follows: nitrogen, 3·63; moisture, 10·93; fat, 4·90; fibre, 3·09 and ash, 2·05 per cent.

Maximum solubilization of meal proteins occurred when 0·4*M* sodium chloride solution was used for extraction of 80 mesh defatted meal, with the meal to solvent ratio of 1: 10, for 120 min.

In Table 1 are given the results of the electrophoretic studies with respect to mobility and percentage distribution of the meal protein components at various *p*H values. The results show that, as in the case of black gram meal proteins[4], optimum separation of the components occurs between *p*H 8·0 and 9·0 at ionic strength 0·1 when five components are observed. It appears that the naturally occurring protein system in Bengal gram is different from that of red gram[2] and black gram proteins[4], since only three components were observed in the former and four components in the latter.

TABLE 1 — ELECTROPHORETIC ANALYSIS OF BENGAL GRAM MEAL PROTEINS AT VARIOUS *p*H VALUES

(*Ionic strength*, 0·1; *temp.*, 25°C.)

*p*H	Mobility × 10^5 cm.^{2} V.$^{-1}$ sec.$^{-1}$									
	Components: I		II		III		IV		V	
	A	D	A	D	A	D	A	D	A	D
4·5	—	—	—	—	—	—	—	—	12·69 (100·00)	12·04 (100·00)
5·6	—	—	—	—	—	—	9·35 (24·40)	8·95 (20·30)	13·37 (74·60)	13·08 (76·70)
6·4	—	—	3·89 (25·30)	3·92 (23·50)	6·72 (18·40)	6·05 (19·90)	10·42 (25·50)	9·67 (24·40)	13·88 (30·80)	13·72 (33·20)
7·0	—	—	4·32 (29·00)	4·52 (31·20)	7·21 (16·00)	6·80 (17·20)	9·38 (20·30)	19·02 (15·60)	14·21 (34·70)	13·92 (36·80)
7·4	—	—	5·50 (24·30)	4·99 (20·40)	9·23 (14·90)	8·92 (21·80)	12·31 (28·30)	11·99 (22·10)	15·92 (32·50)	15·62 (35·70)
8·0	1·92 (10·00)	1·85 (8·60)	4·02 (13·20)	3·95 (16·30)	8·38 (20·30)	8·09 (23·60)	13·21 (20·10)	13·09 (20·00)	17·32 (36·40)	16·95 (31·50)
8·6	1·09 (9·40)	1·11 (11·30)	5·03 (11·40)	4·92 (16·50)	8·92 (25·30)	8·62 (25·30)	13·58 (22·70)	13·42 (18·70)	18·03 (31·20)	17·69 (28·20)
9·0	1·56 (9·90)	1·46 (8·80)	4·94 (15·70)	4·85 (17·20)	7·98 (21·20)	8·11 (23·30)	14·32 (18·40)	14·03 (19·80)	18·21 (34·80)	18·31 (31·90)
9·6	—	—	6·42 (25·40)	—	10·30 (22·63)	10·03 (40·40)	17·85 (20·50)	17·68 (23·40)	25·53 (31·50)	25·32 (36·20)
10·5	—	—	—	—	11·32 (30·90)	11·40 (30·40)	20·39 (31·60)	20·31 (36·90)	36·23 (37·50)	35·89 (32·70)

Figures in parentheses give percentage distribution of components.
A, ascending limb; D, descending limb.

The solubility of Bengal gram proteins below *p*H 4·5 was very low and only one component was observed. At *p*H 5·6, two components were apparent. Between *p*H range 6·4 and 7·4, four components were obtained. Also beyond *p*H 9·0, the number of components decreased and only three components were observed, indicating the possibility of complex formation outside *p*H range of 8-9. In Fig. 1 are shown some of the typical electrophoretic patterns of this meal.

The results on the effect of ionic strength on the electrophoretic separation of meal protein components at *p*H 8·0 have been given in Table 2. Unlike red gram and black gram proteins, at both 0·1 and 0·2 ionic strengths, Bengal gram meal proteins showed optimum separation.

By coagulation studies, four precipitates were obtained at 68-70°, 78-79°, 88-90° and 96°C. Only the fraction obtained at 68°C. was soluble in buffer of *p*H 8·0, ionic strength 0·1, and showed two components on electrophoretic analysis.

The ammonium sulphate fractionation gave five distinct fractions at 35, 45, 55, 70 and 90 per cent ammonium sulphate saturation. The results of electrophoretic analysis of these fractions at *p*H 8·0 and ionic strength 0·1 are given in Table 3. It can be noted from the mobility as well as percentage distribution of components that only the fraction obtained at 70 per cent ammonium sulphate saturation was fairly homogeneous.

Following the cooling and isoelectric precipitation procedure outlined in Chart I, five globulin

TABLE 2 — EFFECT OF VARYING IONIC STRENGTH ON THE ELECTROPHORETIC SEPARATION OF BENGAL GRAM MEAL PROTEINS

(*p*H, 8·0; *ionic strength*, 0·1; *temp.*, 25°C.)

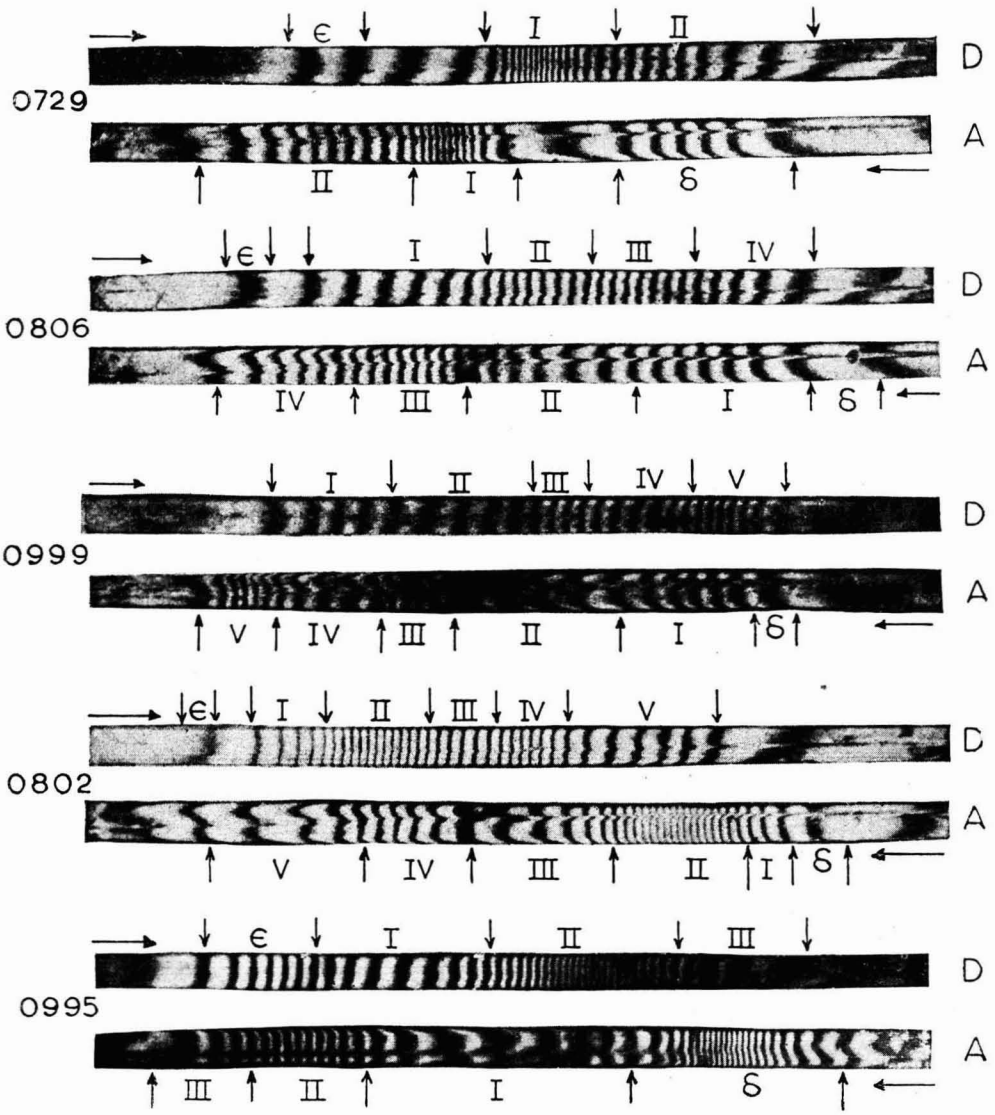
Ionic strength	Mobility × 10^5 cm.^{2} V.$^{-1}$ sec.$^{-1}$									
	Components: I		II		III		IV		V	
	A	D	A	D	A	D	A	D	A	D
0·01	—	—	—	—	6·21 (32·60)	—	10·52 (28·40)	10·37 (58·80)	16·31 (39·00)	16·09 (41·20)
0·05	—	—	5·21 (20·40)	—	8·41 (23·40)	8·31 (40·50)	11·62 (31·00)	11·92 (20·60)	14·22 (25·20)	14·05 (38·90)
0·10	1·92 (10·00)	1·85 (8·60)	4·02 (13·20)	3·95 (16·20)	8·38 (20·30)	8·09 (23·60)	13·29 (20·10)	13·01 (20·00)	17·32 (36·40)	17·39 (31·50)
0·20	1·76 (12·50)	1·62 (10·30)	4·61 (14·40)	4·62 (17·60)	8·05 (21·10)	8·15 (30·10)	12·86 (29·40)	12·32 (22·50)	17·52 (22·60)	17·31 (19·50)
0·50	—	—	—	—	6·23 (34·30)	6·20 (28·40)	14·92 (35·40)	14·59 (39·60)	19·46 (30·30)	19·24 (32·00)

Figures in parentheses give percentage distribution of components.
A, ascending limb; D, descending limb.

TABLE 3 — ELECTROPHORETIC ANALYSIS OF BENGAL GRAM PROTEIN FRACTIONS OBTAINED BY AMMONIUM SULPHATE FRACTIONATION

Ammonium sulphate saturation at which different fractions were obtained %	Mobility × 10^5 cm.^{2} V.$^{-1}$ sec.$^{-1}$									
	Components: I		II		III		IV		V	
	A	D	A	D	A	D	A	D	A	D
35	—	—	—	—	12·46 (30·40)	11·81 (25·60)	14·32 (28·60)	14·35 (23·40)	18·36 (41·00)	17·98 (51·00)
45	—	—	—	—	10·03 (21·50)	9·78 (24·20)	13·36 (44·30)	13·52 (40·80)	17·41 (34·20)	16·99 (35·00)
55	—	—	5·48 (25·80)	5·26 (26·30)	9·11 (20·20)	8·87 (25·30)	12·26 (17·50)	12·24 (19·60)	16·84 (36·50)	16·56 (35·80)
70	3·41 (20·40)	3·12 (15·30)	5·48 (79·60)	5·62 (84·70)	—	—	—	—	—	—
90	2·95 (35·40)	2·82 (45·30)	6·32 (30·60)	6·08 (54·70)	9·56 (43·40)	—	—	—	—	—

Figures in parentheses give percentage distribution of components.
A, ascending limb; D, descending limb.



Pattern No.	*p*H	Voltage gradient *V./cm.*	Duration *sec.*	Temp. °C.
0729	5·6	3·5	3900	25·5
0806	6·4	3·1	3300	29·5
0999	8·0	4·0	2400	29·3
0802	9·0	3·0	3600	30·0
0995	10·5	5·0	2220	25·5

Fig. 1 — Typical electrophoretic patterns of Bengal gram meal proteins at various *p*H values and ionic strength 0·1 [A, ascending pattern; D, descending pattern; I, II, III, IV and V are components]

TABLE 4 — ELECTROPHORETIC ANALYSIS OF BENGAL GRAM PROTEIN FRACTIONS OBTAINED BY COOLING, ISOELECTRIC PRECIPITATION AND AMMONIUM SULPHATE FRACTIONATION PROCEDURE

(*p*H, 8·0; *ionic strength*, 0·1; *temp.*, 25°C.)

Fraction	Mobility × 10^5 cm.^{2} V.$^{-1}$ sec.$^{-1}$									
	Components: I		II		III		IV		V	
	A	D	A	D	A	D	A	D	A	D
I	—	—	—	—	—	—	11·62 (40·50)	11·92 (35·90)	17·66 (59·50)	17·82 (64·10)
II	—	—	—	—	8·95 (20·40)	8·88 (25·50)	12·41 (60·80)	12·12 (62·30)	16·41 (18·80)	16·25 (12·20)
III	—	—	—	—	9·43 (100·00)	9·28 (100·00)	—	—	—	—
IV	—	—	5·62 (67·50)	5·45 (70·20)	8·94 (32·50)	8·90 (28·80)	—	—	—	—
V	2·40 (25·80)	2·36 (22·30)	6·41 (74·20)	6·25 (77·70)	—	—	—	—	—	—

Figures in parentheses give percentage distribution of components.
A, ascending limb; D, descending limb.

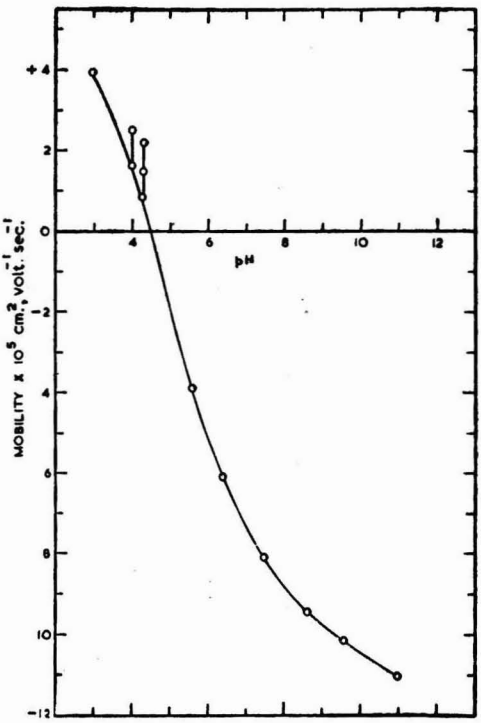


Fig. 2 — Mobility curve of fraction III at ionic strength 0·1

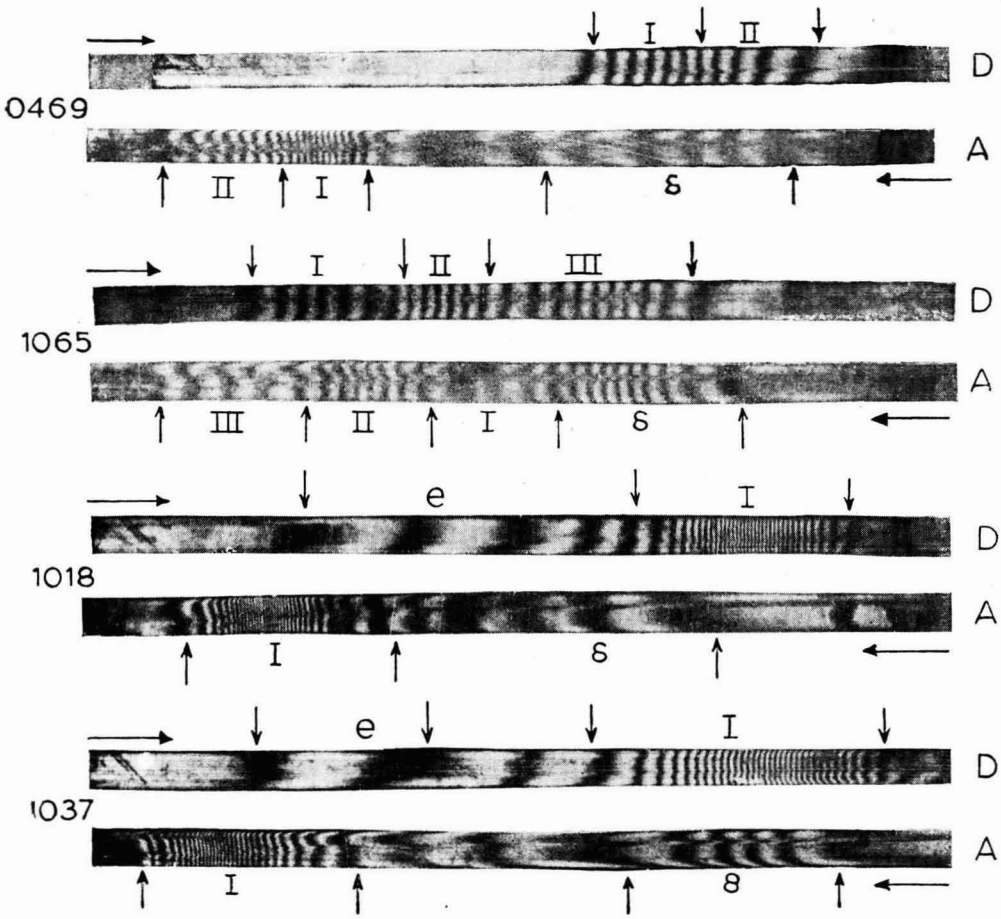
fractions were obtained. All the fractions were electrophoretically analysed at *p*H 8·0 and ionic strength 0·1 and the results have been presented in Table 4. Results show that fraction III obtained at *p*H 4·5 was electrophoretically homogeneous. This fraction was, therefore, analysed

TABLE 5 — ELECTROPHORETIC MOBILITIES OF FRACTION III AT DIFFERENT *p*H VALUES

*p*H	Mobility × 10^5 cm.^{2} V.$^{-1}$ sec.$^{-1}$	
	Components: Ascending	Descending
3·00	+3·97	+3·95
4·00	+1·55, +2·53	+1·42, +2·65
4·25	+0·70, +1·42, +2·32	+0·72, +1·46, +2·11
4·50	0·00	0·00
5·6	−4·10	−3·90
6·4	−6·21	−6·02
7·0	−7·23	−7·02
8·0	−8·68	−9·03
8·6	−9·42	−9·23
9·6	−10·36	−10·32
10·1	−10·59	−10·47
11·0	−11·13	−10·98
12·0	−11·45	−11·39

over a varied *p*H range of 3·0-12·0 and the results are given in Table 5. The *p*H-mobility curve is shown in Fig. 2 which indicates the isoelectric point of this fraction to be around *p*H 4·5. Though this fraction moved as a single boundary at most of the *p*H values, at *p*H 4·0 and 4·25, however, two and three boundaries respectively were apparent. Similar observations have been made by Nath and Giri[7] in the case of the homogeneous globulin fraction obtained from sesame seeds. Similar heterogeneity has been reported by Alberty[8] in the case of bovine serum albumin. This fraction, however, did not show heterogeneity with change in ionic strength at *p*H 7·0. Some of the electrophoretic patterns of this homogeneous fraction are shown in Fig. 3.

In Table 6 are given the amino acid composition of Bengal gram meal and its various globulin fractions.



Pattern No.	*p*H	Voltage gradient *V./cm.*	Duration *sec.*	Temp. °C.
0469	4·00	4·2	4020	25·5
1065	4·25	4·8	3900	25·2
1018	8·60	5·0	3360	26·5
1037	10·10	5·0	2760	27·0

Fig. 3 — Typical electrophoretic patterns of the homogeneous fraction (fraction III) obtained by cooling, isoelectric precipitation and ammonium sulphate fractionation method [A, ascending pattern; D, descending pattern; I, II and III are components]

The amino acid composition reported by Bagchi *et al.*[9] for this meal has been given for comparison as it has been obtained by paper chromatography. The amino acid values obtained in the present study were almost double those reported by Bagchi *et al.* (loc. cit.). As in the case of red gram[3] and black gram[4] proteins, Bengal gram meal and the globulin fractions are deficient in methionine and cystine. Cystine is present in fractions II and III and methionine is present in fractions IV and V while both these amino acids are absent in fraction I. Serine was absent in the meal and the fractions. Unlike red gram and black gram proteins, Bengal gram proteins contain considerable amounts of histidine. Among other essential amino acids, Bengal gram meal and globulins are deficient in tryptophan and are rich in lysine, phenylalanine, arginine, valine, threonine and leucines.

Comparing the results on the essential amino acid content of red gram, black gram and Bengal

TABLE 6 — AMINO ACID COMPOSITION OF BENGAL GRAM MEAL AND THE FRACTIONS OBTAINED BY THE COOLING, ISOELECTRIC PRECIPITATION AND AMMONIUM SULPHATE FRACTIONATION PROCEDURE

(*g. per* 100 *g. substance*)

Amino acid	Fraction I	Fraction II	Fraction III	Fraction IV	Fraction V	Meal	Meal*
Alanine	5·11	4·78	4·29	2·83	2·39	1·91	0·58
Arginine	8·42	10·78	7·89	9·21	7·76	1·01	1·12
Aspartic acid	10·97	12·22	11·53	10·00	13·61	2·36	1·60
Cystine	Traces	4·29	2·86	Traces	—	—	—
Glycine	5·33	5·56	5·11	4·89	4·78	1·33	0·61
Glutamic acid	14·00	14·09	12·63	13·09	16·99	3·00	1·84
Histidine	4·83	5·34	3·96	2·07	2·24	1·21	—
Isoleucine	7·64	6·39	5·32	6·56	6·92	2·88	1·62
Leucine	9·48	8·32	7·48	8·38	8·05	3·79	—
Lysine	13·15	10·65	9·77	7·01	11·53	2·88	1·97
Methionine	Traces	Traces	Traces	0·20	0·30	0·20	—
Phenylalanine	7·34	6·56	7·19	4·37	4·53	1·33	0·72
Proline	7·20	8·33	9·47	10·60	10·60	7·58	1·00
Serine	—	—	—	—	—	—	0·67
Threonine	17·75	12·36	11·66	10·97	10·69	2·50	0·67
Tryptophan	0·12	0·08	0·07	0·04	0·02	0·02	0·02
Tyrosine	2·50	Traces	1·78	—	1·16	0·80	0·34
Valine	10·74	10·74	9·42	9·23	8·65	2·78	0·68

*Values reported by Bagchi *et al.*[9].
Values are average of ten determinations.

gram meals and their globulins, it appears that while black gram proteins are nutritionally better than red gram proteins, Bengal gram proteins are even superior to the latter[10]. The differences in amino acid composition of these probably account for some variations in the electrophoretic characteristics of the naturally occurring protein systems of these pulses. It may be recalled that least number of protein components were present in the case of red gram[1] while Bengal gram gave maximum number of protein components.

Acknowledgement

The authors' thanks are due to the Council of Scientific & Industrial Research, New Delhi, for financial assistance.

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Screening of Antimalarials against *P. gallinaceum* in Chicks: Part VIII—Some Derivatives of Sulphaquinazolone, 4-Aminoquinoline & 1,2-Dihydro-*s*-triazines*

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Manuscript received 16 *March* 1962

Seventy-seven compounds have been screened against blood-induced *Plasmodium gallinaceum* infection in 7 days old chicks. Of the sulphaquinazolone derivatives only 2-ethyl-3-*p*-(2′-pyrimidyl)-sulphonamidophenyl-6-chloro-4(3)-quinazolone exhibits antimalarial activity at a dose 4 times the MED of quinine. The only 4-aminoquinoline derivative, 4-(7′-chloro-4′-quinolylamino)-α-1-pyrrolidyl-*o*-cresol dihydrochloride, tested is active at 1/24 MED of quinine. Among the 1,2-dihydro-*s*-triazine derivatives tested, only four compounds show antimalarial activity. Of these, 4,6-diamino-1-(*p*-methoxyphenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-1,2-dihydro-*s*-triazine hydrochloride exhibits antimalarial activity at 1/14 MED of quinine, while the other three compounds are feebly active (1/4 to 4 MED of quinine).

IN the previous communications[1-3], the results of screening of 260 potential synthetic antimalarials were reported. These compounds included substituted biguanides, sulphabiguanides, dihydro-*s*-triazines, guanidines, thioureas, sulphides, thiopegans, quinazolones and quinoline derivatives. Out of these compounds, the 1,2-dihydro-*s*-triazine derivatives (metabolites of analogues of chloroguanide) were found to be active and the activity varied according to the activity of the parent biguanide, whereas the compounds of other groups were either feebly active or inactive. In the present paper, the results of further studies on the screening of 77 compounds which include sulphaquinazolones, 4-aminoquinoline, 1,2-dihydro-*s*-triazine derivatives and miscellaneous compounds are reported.

Materials and methods

Minimum effective dose of quinine (MED) was taken as the standard for the comparison of the antimalarial activity of different compounds and, therefore, the doses tried were expressed in terms of the base of the compound and as multiples/fractions of the MED of quinine[4]. The compounds received from various sources were given the Malaria Institute Survey (MIS) numbers and were made into solutions or suspensions of suitable base content. Six to seven days old chicks were used for each dosage level and a control series was maintained in each experimental batch. A minimum of 75 per cent reduction in the parasites as compared to controls was taken as the criterion for declaring a particular dosage regime to be active[4,5]. The activity, if present, has been shown against the dosage expressed in terms of multiples/fractions of MED of quinine, e.g. a compound active at 1/4 MED of quinine was four times as active as quinine and hence had quinine equivalent of four. On the other hand, a compound active at a dose four times the MED of quinine had one-fourth quinine equivalent only. Most of the compounds were tested at 4Q, Q or lower dosages only.

Results and discussion

The results of screening of different compounds against *P. gallinaceum* in chicks are recorded in Table 1.

Sulphaquinazolone derivatives — Among the twenty-nine 2-alkyl-3-*p*-(substituted)-sulphonamidophenyl-6-alkyl or halo-4(3)-quinazolones[6] (I) (Table 1), only one compound, i.e. 2-ethyl-3-*p*-(2′-pyrimidyl)-sulphonamidophenyl-6-chloro-4(3)-quin-

*This work has been carried out under the research scheme 'Screening of Antimalarial Drugs' financed by the Council of Scientific & Industrial Research, New Delhi. Parts I to IV and VI have been published in *Indian J. Malariology*, **6** (1952), 145; **7** (1953), 117; **7** (1953), 311; **8** (1954), 1 and **10** (1956), 299 and Parts V and VII in *J. sci. industr. Res.*, **14C** (1955), 173 and **18C** (1959), 28 respectively.

TABLE 1 — SCREENING TESTS FOR ANTIMALARIALS AGAINST *P. GALLINACEUM* IN CHICKS

MIS No. of compound	Compound	Dosage in multiples/fractions of MED of quinine Q	Activity
	4(3)-Quinazolone derivatives*		
282	2-Methyl-3-*p*-(4′,6′-dimethyl-2′-pyrimidyl)-sulphonamidophenyl-	4	Inactive
		1	do
		1/4	do
283	2-Methyl-3-*p*-(2′-pyrimidyl)-sulphonamidophenyl-	4	do
		1	do
		1/4	do
306	2-Methyl-3-*p*-(2′-pyridyl)-sulphonamidophenyl-	4	do
		1	do
307	2-Methyl-3-*p*-(guanyl)-sulphonamidophenyl-	4	do
		1	do
308	2-Ethyl-3-*p*-(2′-thiazolyl)-sulphonamidophenyl-	4	do
		1	do
309	2-Ethyl-3-*p*-(4′,6′-dimethyl-2′-pyrimidyl)-sulphonamidophenyl-	4	do
		1	do
310	2-Ethyl-3-*p*-(2′-pyridyl)-sulphonamidophenyl-	4	do
		1	do
311	2-Ethyl-3-*p*-(2′-pyrimidyl)-sulphonamidophenyl-	4	do
		1	do
312	2-Methyl-3-*p*-(2′-pyridyl)-sulphonamidophenyl-6-chloro-	4	do
		1	do
313	2-Methyl-3-*p*-(2′-thiazolyl)-sulphonamidophenyl-6-chloro-	4	do
		1	do
314	2-Ethyl-3-*p*-(2′-thiazolyl)-sulphonamidophenyl-6-chloro-	4	do
		1	do
315	2-Ethyl-3-*p*-(2′-pyridyl)-sulphonamidophenyl-6-chloro-	4	do
		1	do
316	2-Ethyl-3-*p*-(2′-pyrimidyl)-sulphonamidophenyl-6-chloro-	4	Active
		1	Inactive
317	2-Ethyl-3-*p*-(4′,6′-dimethyl-2′-pyrimidyl)-sulphonamidophenyl-6-chloro-	4	do
		1	do
318	2-Methyl-3-*p*-(guanyl)-sulphonamidophenyl-6-bromo-	4	do
		1	do
319	2-Methyl-3-*p*-(2′-pyridyl)-sulphonamidophenyl-6-bromo-	4	do
		1	do
320	2-Methyl-3-*p*-(2′-thiazolyl)-sulphonamidophenyl-6-bromo-	4	do
		1	do
321	2-Methyl-3-*p*-(2′-pyrimidyl)-sulphonamidophenyl-6-bromo-	4	do
		1	do
322	2-Ethyl-3-*p*-(2′-pyridyl)-sulphonamidophenyl-6-bromo-	4	do
		1	do
323	2-Ethyl-3-*p*-(2′-thiazolyl)-sulphonamidophenyl-6-bromo-	4	do
		1	do
324	2-Ethyl-3-*p*-(2′-pyrimidyl)-sulphonamidophenyl-6-bromo-	4	do
		1	do
325	2-Ethyl-3-*p*-(guanyl)-sulphonamidophenyl-6-bromo-	4	do
		1	do
326	2,6-Dimethyl-3-*p*-(guanyl)-sulphonamidophenyl-	4	do
		1	do
327	2,6-Dimethyl-3-*p*-(2′-thiazolyl)-sulphonamidophenyl-	4	Inactive
		1	do
328	2,6-Dimethyl-3-*p*-(2′-pyridyl)-sulphonamidophenyl-	4	do
		1	do
329	2,6-Dimethyl-3-*p*-(2′-pyrimidyl)-sulphonamidophenyl-	4	do
		1	do
330	2-Ethyl-3-*p*-(guanyl)-sulphonamidophenyl-6-methyl-	4	do
		1	do
331	2-Ethyl-3-*p*-(2′-pyridyl)-sulphonamidophenyl-6-methyl-	4	do
		1	do
332	2-Ethyl-3-*p*-(2′-pyrimidyl)-sulphonamidophenyl-6-methyl-	4	do
		1	do
	4-Aminoquinolines†		
304	4-(7′-Chloro-4′-quinolylamino)-α-1-pyrrolidyl-*o*-cresol dihydrochloride	1	Active
		1/4	do
		1/16	do
		1/20	do
		1/24	do
		1/26	Inactive
	Hydrochlorides of 1,2-dihydro-*s*-triazine derivatives‡		
284	4,6-Diamino-1,2-(di-*p*-methoxyphenyl)-	4	Inactive
		1	do
		1/4	do
285	4,6-Diamino-1-(*p*-ethoxyphenyl)-2-(*p*-methoxyphenyl)-	4	do
		1	do
		1/4	do
286	4,6-Diamino-1-(*o*-chlorophenyl)-2-(*p*-methoxyphenyl)-	4	do
		1	do
		1/4	do
287	4,6-Diamino-1-(*m*-chlorophenyl)-2-(*p*-methoxyphenyl)-	4	do
		1	do
		1/4	do
288	4,6-Diamino-1-(2′,4′-dichlorophenyl)-2-(*p*-methoxyphenyl)-	4	do
		1	do
		1/4	do
289	4,6-Diamino-1-(*o*-ethylphenyl)-2-(*p*-methoxyphenyl)-	4	do
		1	do
		1/4	do
290	4,6-Diamino-1-(2′,6′-dimethylphenyl)-2-(*p*-methoxyphenyl)-	4	do
		1	do
		1/4	do
291	4,6-Diamino-1-(*p*-methylphenyl)-2-(*p*-methoxyphenyl)-	4	do
		1	do
		1/4	do
292	4,6-Diamino-1-(*p*-hydroxyphenyl)-2-(*p*-methoxyphenyl)-	4	do
		1	do
		1/4	do
293	4,6-Diamino-1-(*p*-methoxyphenyl)-2-(*p*-hydroxyphenyl)-	4	do
		1	do
		1/4	do
294	4,6-Diamino-1-(*p*-ethoxyphenyl)-2-(*p*-hydroxyphenyl)-	4	Active
		1	Inactive
		1/4	do
295	4,6-Diamino-1-(*o*-chlorophenyl)-2-(*p*-hydroxyphenyl)-	4	do
		1	do
		1/4	do
296	4,6-Diamino-1-(*p*-chlorophenyl)-2-(*p*-hydroxyphenyl)-	4	do
		1	do
		1/4	do

TABLE 1 — SCREENING TESTS FOR ANTIMALARIALS AGAINST *P. GALLINACEUM* IN CHICKS — *contd*

MIS No. of compound	Compound	Dosage in multiples/fractions of MED of quinine Q	Activity
297	4,6-Diamino-1-(2′,4′-dichlorophenyl)-2-(*p*-hydroxyphenyl)-	4	Inactive
		1	do
		1/4	do
298	4,6-Diamino-1-(*p*-methylphenyl)-2-(*p*-hydroxyphenyl)-	4	do
		1	do
299	4,6-Diamino-1,2-(di-*p*-hydroxyphenyl)-	4	do
		1	do
300	4,6-Diamino-1-(*o*-chlorophenyl)-2-(cyclohexylidene)-	4	do
		1	do
		1/4	do
301	4,6-Diamino-1-(2′,4′-dichlorophenyl)-2-cyclohexylidene)-	4	do
		1	do
		1/4	do
302	4,6-Diamino-1-(*o*-ethylphenyl)-2-(cyclohexylidene)-	4	do
		1	do
		1/4	do
303	4,6-Diamino-1-(*p*-hydroxyphenyl)-2-(cyclohexylidene)-	4	do
		1	do
		1/4	do
333	4,6-Diamino-1-(*p*-methoxyphenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	Active
		1	do
		1/4	do
		1/8	do
		1/12	do
		1/14	do
		1/16	Inactive
334	4,6-Diamino-1-(*p*-ethoxyphenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	Active
		1	do
		1/4	do
		1/6	Inactive
335	4,6-Diamino-1-(*p*-chlorophenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	do
		1	do
336	4,6-Diamino-1-(*p*-bromophenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	Toxic
		1	Inactive
337	4,6-Diamino-1-(*p*-iodophenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	do
		1	do
338	4,6-Diamino-1-(2′, 4′-dichlorophenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	do
		1	do
339	4,6-Diamino-1-(3′-chloro-4′-bromophenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	Toxic
		1	Inactive
340	4,6-Diamino-1-(3′-chloro-4′-iodophenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	do
		1	do
341	4,6-Diamino-1-(2′-methyl-4′-iodophenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	Toxic
		1	Inactive
342	4,6-Diamino-1-(3′,5′-dibromo-4′-hydroxyphenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	do
		1	do
343	4,6-Diamino-1-(*p*-hydroxyphenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	do
		1	do

TABLE 1 — SCREENING TESTS FOR ANTIMALARIALS AGAINST *P. GALLINACEUM* IN CHICKS — *contd*

MIS No. of compound	Compound	Dosage in multiples/fractions of MED of quinine Q	Activity
344	4,6-Diamino-1-(*p*-nitrophenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	Active
		1	Inactive
345	4,6-Diamino-1-(*p*-methylphenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	do
		1	do
346	4,6-Diamino-1-(2′,4′-dimethylphenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	do
		1	do
347	4,6-Diamino-1-(*o*-ethylphenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-	4	do
		1	do
348	4,6-Diamino-1-(*p*-methoxyphenyl)-2-methyl-2-dimethylvinyl-	4	do
		1	do
349	4,6-Diamino-1-(*p*-ethoxyphenyl)-2-methyl-2-dimethylvinyl-	4	do
		1	do
350	4,6-Diamino-1-(*p*-chlorophenyl)-2-methyl-2-dimethylvinyl-	4	do
		1	do
351	4,6-Diamino-1-(*p*-bromophenyl)-2-methyl-2-dimethylvinyl-	4	do
		1	do
352	4,6-Diamino-1-(*p*-iodophenyl)-2-methyl-2-dimethylvinyl-	4	do
		1	do
353	4,6-Diamino-1-(3′-chloro-4′-bromophenyl)-2-methyl-2-dimethylvinyl-	4	do
		1	do
354	4,6-Diamino-1-(3′,5′-dibromo-4′-hydroxyphenyl)-2-methyl-2-dimethylvinyl-	4	do
		1	do
355	4,6-Diamino-1-(*p*-nitrophenyl)-2-methyl-2-dimethylvinyl-	4	do
		1	do
356	4,6-Diamino-1-(*p*-methylphenyl)-2-methyl-2-dimethylvinyl-	4	do
		1	do
357	4,6-Diamino-1-(2′,4′-dimethylphenyl)-2-methyl-2-dimethylvinyl-	4	do
		1	do
	MISCELLANEOUS		
305	Nimbidin§	4	do
		1	do
		1/4	do
358	Antimalaria pills (indigenous drug — composition: Eruk and pepper)¶	20	do
		10	do
		1	do
		4	do

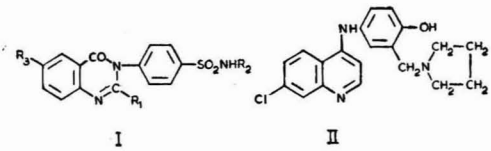
*All the compounds were supplied by the Chemistry Laboratory, Malaria Institute of India, Delhi 6.

†Supplied by Messrs Parke Davis & Co. Ltd, Bombay.

‡All the dihydro triazine derivatives were supplied by the Chemistry Department, Lucknow University, Lucknow.

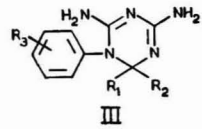
§Supplied by the Division of Organic Chemistry, National Chemical Laboratory, Poona.

¶Supplied by the Indian Council of Medical Research, New Delhi.



I II

R_1=CH_3, C_2H_5
R_2=guanyl, thiazolyl, pyridyl, pyrimidyl, etc.
R_3=H, CH_3, Cl, Br



III

R_1=H, CH_3
R_2=$CH_2-\overset{CH_3}{\overset{|}{C}}-(OH)-CH_3$, $CH=C(CH_3)_2$, cyclohexylidene, *p*-hydroxyphenyl, *p*-methoxyphenyl
R_3=Cl, Br, CH_3, OCH_3, OC_2H_5, etc.

azolone (MIS 316, I, R_1=C_2H_5, R_2=2′-pyrimidyl, R_3=Cl), exhibited only 1/4 the quinine equivalent. Though most of the sulpha drugs like sulphanilamide, sulphaguanidine, sulphathiazole, sulphapyridine, sulphadiazine, etc., have been found active against avian as well as simian malaria[7,10], the replacement of *p*-amino grouping with potential 4(3)-quinazolone nucleus of highly active febrifugine[11-13] results in complete loss of antimalarial activity. As a class, sulphaquinazolones[6] (I) have little potentiality as antimalarials. 3-Aryl-4(3)-quinazolone derivatives even previously had shown limited antimalarial activity[1,3,14].

4-Aminoquinolines — The only 4-aminoquinoline derivative, i.e. 4-(7′-chloro-4′-quinolylamino)-α-1-pyrrolidyl-*o*-cresol dihydrochloride (II, MIS 304), was found to be highly active; the MED of this drug was 1/24Q and was similar to that of chloroquin[15] (MED = 1/26Q).

1,2-Dihydro-s-triazine derivatives — Out of the forty-five, 4,6-diamino-1-substituted-phenyl-2-dialkyl, aryl or cyclohexylidene-1,2-dihydro-*s*-triazine derivatives[16] tested (Table 1), only four compounds showed antimalarial activity, while others were all inactive at a dose four times the MED of quinine. 4,6-Diamino-1-(*p*-methoxyphenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-1,2-dihydro-*s*-triazine hydrochloride [MIS 333, III, R_1=CH_3, R_2=$CH_2-\overset{CH_3}{\overset{|}{C}}(OH)-CH_3$, R_3=*p*-OCH_3] was found to be quite active and showed antimalarial activity at a dosage 1/14 times the MED of quinine. 4,6-Diamino-1-(*p*-ethoxyphenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-1,2-dihydro-*s*-triazine hydrochloride MIS 334, III, R_1=CH_3, R_2=$CH_2-\overset{CH_3}{\overset{|}{C}}(OH)-CH_3$, R_3=*p*-OC_2H_5] was found to be active at a dose 1/4 time the MED of quinine, while 4,6-diamino-1-(*p*-nitrophenyl)-2-methyl-2-[1′-(2′-metho-2′-hydroxypropyl)]-1,2-dihydro-*s*-triazine hydrochloride [MIS344, III, R_1=CH_3, R_2=$CH_2-\overset{CH_3}{\overset{|}{C}}(OH)-CH_3$, R_3=*p*-NO_2] was only 1/4 time as active as quinine. All the other 4,6-diamino-1-aryl-2,2-dialkyl-1,2-dihydro-*s*-triazine derivatives were inactive [III, R_1=CH_3, R_2=$CH_2-\overset{CH_3}{\overset{|}{C}}(OH)-CH_3$ or $CH=C(CH_3)_2$]. Of the 4,6-diamino-1-substituted-aryl-2-cyclohexylidene-1,2-dihydro-*s*-triazine derivatives [III, R_1 and R_2=cyclohexylidene] tested, none showed any antimalarial activity. Of the corresponding 2-aryl analogues [III, R_1=H, R_2=*p*-OH or OCH_3 phenyl], only 4,6-diamino-1-*p*-ethoxyphenyl-2-*p*-hydroxyphenyl-1,2-dihydro-*s*-triazine hydrochloride [MIS 294, III, R_1=H, R_2=*p*-hydroxyphenyl, R_3=*p*-OC_2H_5] was 1/4 time as active as quinine. These findings show that the replacement of 2,2-dimethyl radical in 1,2-dihydro-*s*-triazines with higher alkyl, cyclohexylidene or aryl radicals results in compounds with lower or no antimalarial activity.

Miscellaneous compounds — Nimbidine and antimalaria pills (an indigenous drug made of Eruk and pepper) did not show any antimalarial activity.

Acknowledgement

The authors wish to thank the Council of Scientific & Industrial Research, New Delhi, for a research grant and Sarvashri S. K. Sharma and S. Venkatesan for technical assistance.

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Identity of the 'Sweta Punarnava', *Boerhaavia punarnava spec. nov.* of the Ayurveda*

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Manuscript received 1 *February* 1962

In view of the confusion prevailing with regard to the existence and identity of 'Sweta Punarnava', a well-known drug plant mentioned in all indigenous systems of medicine, a systematic study of the problem has been undertaken and an attempt has been made to establish the existence of a white-flowered Boerhaavia as recorded in Sanskrit texts on Ayurvedic system of medicine. A white-flowered annual Boerhaavia, growing after the rains in the waste lands around Pondicherry and neighbouring areas, which differs in important vegetative and reproductive characters from all the six species of Boerhaavia mentioned in Indian flora and conforms to the 'Sweta Punarnava' described in ancient Sanskrit texts on Ayurvedic system of medicine, has been established as the 'Sweta Punarnava' of Ayurveda and identified to be a new species, viz. *Boerhaavia punarnava* Saha & Krishnamurthy *spec. nov.*

PUNARNAVA is a well-known drug plant mentioned in all indigenous systems of medicine for its diuretic properties and is recognized in *Indian Pharmacopoeia*. Three kinds of Punarnava bearing blue, red and white flowers, and having some therapeutic differences, are recorded in ancient medical texts. But it is the 'Sweta (white-flowered) Punarnava' that is preferred in the Ayurveda. The name Punarnava is equated by all to the genus Boerhaavia. In the crude drug trade, however, a white-flowered *Trianthema portulacastrum* L. belonging to a different family (*Aizoaceae*) is often sold in the market as 'Sweta Punarnava'. As such, there is confusion as to the existence and the botanical identity of 'Sweta Punarnava'. Indian flora mentions six species of Boerhaavia of which *B. verticillata* Poir. alone possesses white flowers, but it is neither used in indigenous medicine nor is it an "annual, springing forth after the rains" as the 'Sweta Punarnava' has been described in Sanskrit texts.

A white-flowered Boerhaavia differing in important vegetative and reproductive characters from all the six Indian species of Boerhaavia and conforming to the 'Sweta Punarnava' described in the Sanskrit texts on Ayurvedic system of medicine has been identified and established as a new species, viz. *Boerhaavia punarnava* Saha & Krishnamurthy *spec. nov.*

Punarnava is a well-known drug plant mentioned in ancient Ayurvedic texts. It is likewise mentioned in modern books on Indian medicine[1-6]. Various therapeutic properties are attributed to this plant by different authors. It is, however, universally recognized as a diuretic and is recorded in *Indian Pharmacopoeia*[7]. Of late, it has gained much popular interest because of its reported properties

*Paper presented at the Symposium on Production and Utilization of Medicinal and Aromatic Plants in India held from 27 to 29 November 1961, Regional Research Laboratory, Jammu.

as a cure for eye diseases, but extensive investigation and clinical trials have to be carried out before such a claim can be accepted.

Mention is commonly made of two kinds of Punarnava bearing red/pink and white flowers with some therapeutic differences between them. Literature on the Tibbi system of medicine mentions of a third kind of Boerhaavia with blue flowers. The red/pink-flowered ' Rakta Punarnava ' (*B. diffusa* L.) is the one that is widely distributed throughout the plains of the country and is described in the *Indian Pharmacopoeia*[7]. But it is the white-flowered, i.e. ' Sweta Punarnava ', that is referred to in ancient texts and preferred in Ayurvedic medicine. Another Sanskrit name for the ' Sweta Punarnava ' is ' shothagni ', which literally means a cure for dropsy.

The Sanskrit name ' Punarnava ' is equated with the botanical genus Boerhaavia. But the plant sold under the name of ' Sweta Punarnava ' in the crude drug trade does not even belong to the genus Boerhaavia. The herbalists attempt to pass the white-flowered *Trianthema portulacastrum* L. syn. *T. monogyna* L. belonging to a different family (*Aizoaceae*) as ' Sweta Punarnava'[8,9]. There is, thus, considerable confusion about the botanical identity of ' Sweta Punarnava '.

Indian flora[10] records the existence of six species under the genus Boerhaavia, of which four species are reported to occur in the peninsular region of India. The present investigation concerns about the occurrence of a white-flowered Boerhaavia which grows in the waste lands around Pondicherry and neighbouring areas and probably elsewhere in the Indian plains*. The plant is, however, much less abundant than the red-flowered Boerhaavia (*B. diffusa* L.), the latter being more common along mortar and brick dumps, road pavements and on old brick-walls.

This paper attempts to establish the existence of a white-flowered Boerhaavia of the kind recorded in the Ayurvedic texts. It also gives a critical botanical and pharmacognostic description of the plant pointing out the characters in which it differs from the common *B. diffusa L.* and the rare *B. verticillata* Poir.

Habit

The white-flowered Boerhaavia is an annual herb growing in open areas especially after the rains. The main shoot soon branches into 2-6, more or less equally prominent branches. The plant reaches, at times, a height of 100 cm. when in full bloom and spreads out 100-150 cm. at its extremities; ordinarily it is 60-75 cm. high when in blossom. The branches hardly bear any prominent tertiary branches. The main branches end in extensive inflorescences bearing minute white flowers in umbel-like 2- to 7-flowered loose groups (Plate I, Fig. 1).

The red-flowered Boerhaavia (*B. diffusa* L.) is a perennial, has a prostate, diffuse and trailing habit extending to a metre or more, and is divaricately branched with comparatively slender stems which have a tendency to ascend upwards at the tips (Plate I, Fig. 2). Both the lateral and terminal inflorescences are equally developed but they are not at all as extensive as in the white-flowered plant.

Root

The main root grows straight downwards but usually bears 2-3 strong lateral branches. The tertiary roots are slender and few. The root is whitish in colour, slender in diameter and grows to a depth of 15-18 cm., i.e. much less than that of the red-flowered plant (Plate I, Fig. 1).

The root of the red-flowered *B. diffusa* is narrowly conical, brown in colour, stouter, and bears hardly any lateral roots which, when present, are always slender. It grows straight and to a greater depth (30-40 cm.) in the soil (Plate I, Fig. 2).

Stem

It is distinctly pink on the upper side, particularly at the base of the main branches, but green elsewhere. It is woody and stout, almost glabrous, with nodes swollen but not knotted. Stem of the red-flowered *B. diffusa* is slender, more greenish, and the nodes are more swollen and knotted.

Leaf

Leaves are simple, opposite, and borne in unequal pairs. Larger leaves are 6 cm. long and 4 cm. broad, but ordinarily they are smaller. Petiole is 1-3 cm. in length, distinctly pinkish and grooved on the upper side and minutely puberrulous. Lamina is ovate to almost triangular with a mucronate apex; under surface whitish and minutely hairy. Venation is reticulate with 3-5 lateral veins on each side of the midrib. Margin is sinuate, particularly in young leaves, and is bordered with a pink outline which is distinctly evident when viewed from the under surface. The

*On 2 March 1962, while consulting in this connection the herbarium of the Botany Department of the Presidency College, Madras, two unnamed and unidentified herbarium sheets of this plant, said to have been collected years ago from a place 20 miles away from Madras city, were noticed.

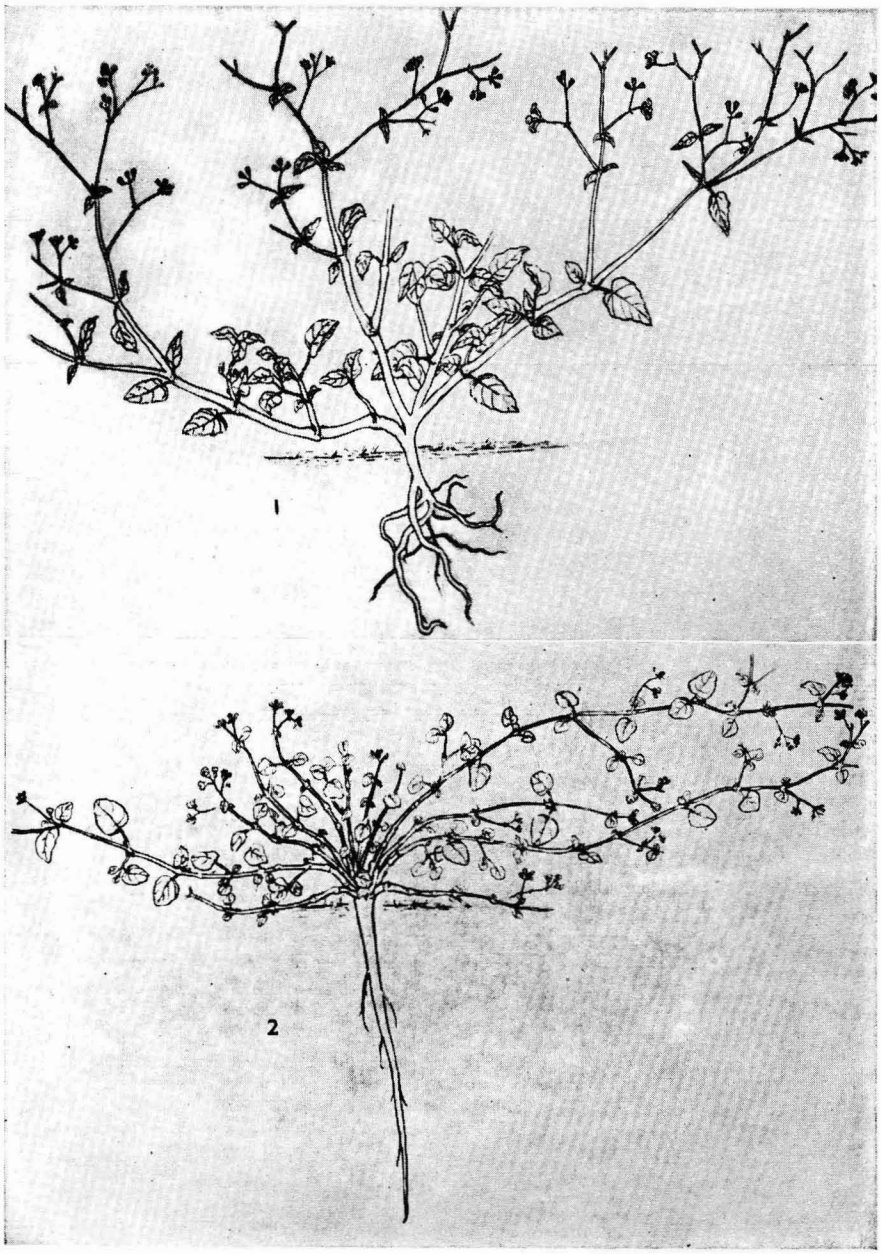

Plate I — Comparative habit sketches showing mode of branching and nature of inflorescence, stem and root of 'Sweta' and 'Rakta' Punarnavas (Boerhaavia) [Fig. 1: *B. punarnava sp. nov.* × $\frac{1}{5}$. Fig. 2: *B. diffusa* × $\frac{1}{5}$]

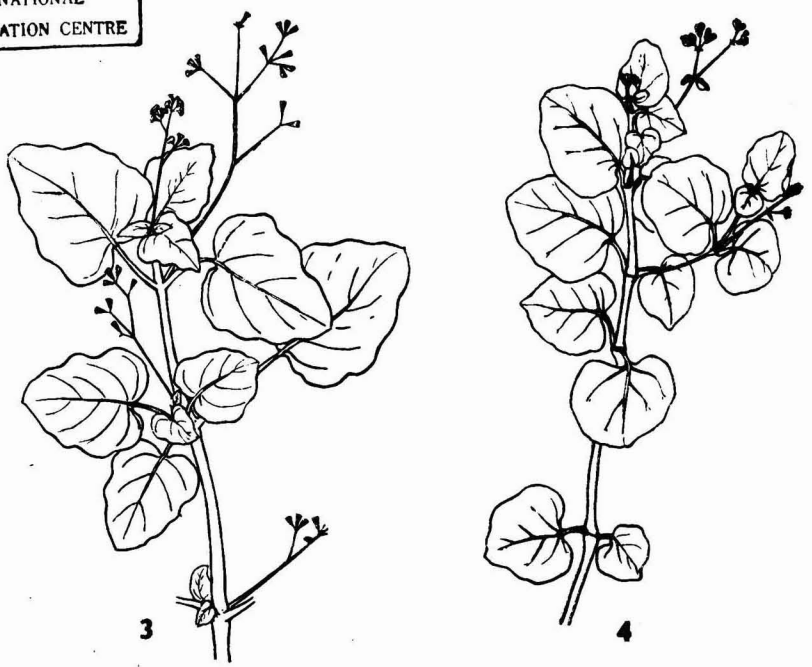

Plate II — Comparative morphological characters of leaf, inflorescence and fruits of 'Sweta' and 'Rakta' Punarnavas [Fig. 3: *B. punarnava sp. nov.* × ½. Fig. 4: *B. diffusa* × ½]

base of the lamina is, in most cases, unequal (Plate II, Fig. 3).

The red-flowered Boerhaavia bears ovate leaves (Plate II, Fig. 4); bigger ones are 4 cm. long and 3·5 cm. broad, but they are usually smaller. Base of the leaf is cordate and the apex rounded. But the shape of the leaf varies according to habitat, the leaves being reduced in size under dry situations. Petiole is smaller and greenish. Leaf margin is entire and not bordered with a pink outline as in the case of white-flowered Boerhaavia.

Inflorescence

It is terminal and extensively branched, and appears like an apparently dichotomous panicle (Plate I, Fig. 1). It bears small, stalked flowers (2-7 in number) in umbel-like loose clusters at the tips of slender pedicels (Plate II, Fig. 3). The axillary inflorescences, when present, are much smaller. The axis of the inflorescence is usually reddish brown, glabrous and bears swollen (but not knotted) nodes. The leaves on the basal portion of the inflorescence are narrow and distinctly lanceolate.

In contrast, the red-flowered *B. diffusa* bears nearly sessile flowers in 2-9 flowered small umbels (Plate II, Fig. 4), arranged in stalked corymbose, axillary or terminal panicles. Neither the axillary nor the terminal inflorescence (Plate I, Fig. 2) is as extensive and ramified as that of the white-flowered Boerhaavia. In contrast to the white-flowered plant, the extremities of its inflorescence axes are covered with viscid, glandular hairs. Furthermore, the leaves at the basal portion of the inflorescence, though small, are not different in shape from those of the vegetative region as is the case with the white-flowered Boerhaavia.

Flowers

They are small, white, about 3·5 mm. in length and 3 mm. in diam. at the top when fully open and are borne in 2-7 flowered umbel-like loose groups. Each flower has a stalk of about 0·5 mm. The flower (Plate III, Figs. 6a and 6b) is subtended by 2 minute, usually persistent, lanceolate bracteoles with fimbriate margins. The bracteole is as long as the anthocarpic portion of the perianth tube. Perianth is gamophyllous and is divided into a green, obconical, glabrous anthocarpic portion housing the ovary and a petaloid campanulate portion having 5 lobes. Each lobe is deeply cleft at the free end. There is a pink pigmented line on the

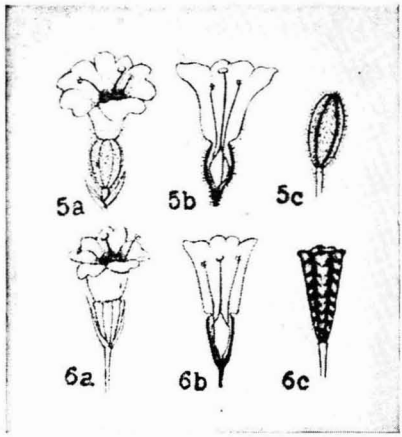

Plate III — Comparative floral morphology of 'Rakta' and 'Sweta' Punarnavas [Fig. 5: *B. diffusa*. Fig. 5a: Flower × 6. Fig. 5b: Flower, vertical section × 6. Fig. 5c: Fruit × 4. Fig. 6: *B. punarnava sp. nov.* Fig. 6a: Flower × 6. Fig. 6b: Flower, vertical section × 6. Fig. 6c: Fruit × 4]

dorsal surface of the petaloid portion in between the lobes. This sometimes imparts a faintly pinkish shade to the otherwise white flowers.

The petaloid campanulate portion (2·5 mm. in length) of the perianth is separated from the sepaloid anthocarpic portion (1 mm. long) by an abrupt straight constriction and is slightly gibbous at the base.

Stamens 2, rarely 3, usually exserted; filament white, filiform and a little longer than the style.

Pistil consists of one unilocular carpel with a single ovule. Ovary is completely included within the anthocarpic portion of the perianth. Style is single and filiform with a disc-like stigma.

Fruit is an achene inside the anthocarp, which bears 5 prominent ridges and furrows. The furrows show characteristic transverse wrinkles (Plate III, Fig. 6c). It is obconical with a truncated crown and is articulated to a stalk 2-3 mm. long; occasionally the stalk is almost absent. When the fruit is dry and shrunken, the furrows assume a characteristic pattern. The fruit is about 3·5 mm. long and at least 1·5 mm. broad at the crown.

In contrast, the red-flowered *B. diffusa* bears distinctly pink/red flowers on very short pedicels in compact umbels. The campanulate corolla (Plate III, Figs. 5a and 5b) is more spreading, being about 4 mm. in diameter when fully open. Corolla and stamens are light red or distinctly pink. Style is pinkish. The anthocarpic portion of the perianth is smaller and ovoid, and has ridges and furrows. It is covered with viscid glandular hairs which extend upward to the base of the petaloid campanulate portion and persist in the mature fruit (Plate III, Figs. 5a, 6b and 5c). The anthocarpic wall of the fruit does not bear any such characteristic transverse wrinkles as in the case of the white-flowered Boerhaavia. The fruit is smaller, about 2·7 mm. long and 1·5 mm. broad and is not truncated at the crown (Plate III, Fig. 5c). The fruit is almost sessile or bears a much shorter stalk of about 1 mm.

Anatomy

In anatomical features there is not much of difference between the white-flowered and the red-flowered Boerhaavia. The root of the former is whitish in colour, shows much less secondary growth and there is no formation of distinct bark. In contrast, the red-flowered *B. diffusa* has stouter root which shows more secondary growth and forms distinct brown bark.

A Latin rendering of the description of the plant is given below as required under Article 35 of the International Code of Botanical Nomenclature.

Boerhaavia punarnava Saha *et* Krishnamurthy *spec. nov.*— Herba annua, caule lignoso, ad 100 cm. longo; stirps princeps saepe in duos tresve caules aeque dividitur qui desinunt in paniculas extensas, terminales, iterum iterumque furcatas, 30-60 cm. longas. Radix principalis alba est, nec conica, nonnumquam ornata duplici vel triplici radice laterali robusta. Folia simplicia, opposita, petiolata, inaequalia in unoquoque jugo, ovata vel fere triangularia, 6×4 cm. sed saepius minora, apice mucronato, nervis reticulatis, marginibus sinuatis et linea angusta rosea ornatis, quae in pagina inferiore multo clarior evadit. Flores umbellato-fasciculati, fasciculis 2-7 florum, minuti, albi, gamophylli, parte anthocarpica glabra obconica viridi 1 mm. longa, parte petaloidea campanulata 5 loba, 2·5 mm. longa. Stamina 2, raro 3, parum exserta; filamenta alba. Ovarium uniloculare, uniovulatum, penitus inclusum in anthocarpo. Stylus unus, stigmate discoideo. Fructus anthocarpicus, obconicus, jugis et sulcis quinis distinctus, cum stipite 2-3 mm. longo articulatus, extus glaber, 3·5 mm. longus, 1·5 mm. latus ad coronam; fructus siccus typicum aspectum sulcorum profert.

Typus lectus a Saha ad Pondicherry in India meridionali, die 26 junii anni 1961, et positus in Herbario Indico Nationali sub numero collectionis Saha 1/1961. Paratypi positi in Horto Indico Nationali ad Lucknow, in Forest Research Institute ad Dehra Dun, in Herbario Kewensi in Anglia, et in Herbario Biologica Occidentalis Virginiae Academiae ad Morgantown, W. Va., U.S.A.

Discussion

The white-flowered Boerhaavia differs in habit and in important vegetative and reproductive characters from all the six species of Boerhaavia recorded so far in this country[10-12]. Out of the six species of Indian Boerhaavia, only one, namely *Boerhaavia verticillata* Poir., bears white flowers. But no medicinal properties are attributed to this plant according to traditional belief nor is it known to be used for medicinal purposes in the Ayurveda. Moreover, *B. verticillata* Poir. differs from the white-flowered *Boerhaavia punarnava* Saha & Krishnamurthy *spec. nov.* in its habit of being a perennial "decumbent or climbing among bushes" and in bearing "flowers in long-peduculate racemes arranged in few-flowered distant whorls along a slender rachis; . . . . . pedicels very variable in length, $\frac{1}{4}$-$\frac{3}{4}$ in. (= 6-19 mm.) long; . . . . perianth $\frac{1}{3}$ in. (= 8 mm.) long; . . . . ovarial part of tube $\frac{1}{12}$ in. (= 2 mm.) long; . . . . fruit $\frac{1}{6}$ in. (= 4 mm.) long, clavate, furnished with large semi-globose glands round the crown"[11] (Plate IV, Figs. 7-10). Furthermore, the white flowered *B. verticillata* Poir. is restricted to Western India in the dry Carnatic and Deccan districts extending northward along West Pakistan, Afghanistan, Baluchistan and then westward to Syria and tropical Africa. No white-

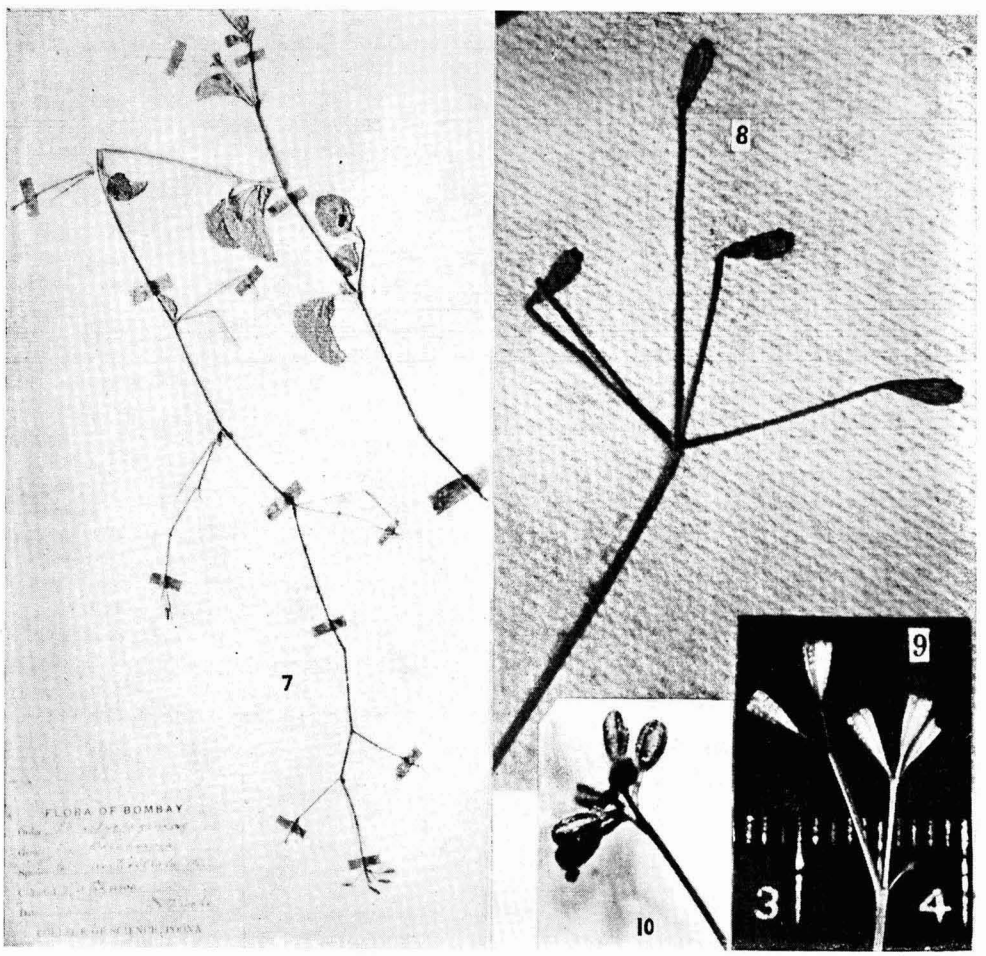

Plate IV — Fruits of *B. verticillata*, *B. punarnava sp. nov.* and *B. diffusa* [Fig. 7: Photograph of a herbarium specimen of *B. verticillata* × $\frac{1}{3}$. Fig. 8: A cluster of fruits of *B. verticillata* (note the long pedicels) × 3. Fig. 9: Two clusters of short pedicelled fruits (note the characteristic shape and transverse wrinkles) of *B. punarnava sp. nov.* (background scale in cm.) × 3. Fig. 10: A cluster (note its compact nature) of fruits of *B. diffusa* × 3]

flowered annual Boerhaavia commonly used for medicinal purposes has so far been recorded, so far as the authors are aware, nor described in modern literature until now.

It may be stressed here once again that a plant called 'Sweta Punarnava' bearing white flowers is universally recognized in the Ayurvedic medicine for its diuretic properties. The description given in ancient Sanskrit texts is unfortunately not interpretable in precise botanical terms. But the terms 'Sweta Punarnava' in the ancient description of the plant refers to the white colour of the flower, while the term 'varshabhu' used in Sanskrit description refers to its habit of springing up after the first showers of the rain and thereby indicating its annual habit. In these two specific characters mentioned in the description of the plant in Sanskrit texts as well as its continued use, wherever available, in Ayurvedic medicine, the white-flowered annual *Boerhaavia punarnava* Saha & Krishnamurthy *spec. nov.* described here conforms to the 'Sweta Punarnava' and differs from all the species of Boerhaavia recorded so far in the Indian floras.

Acknowledgement

The genesis of this investigation dates back to January 1959 when the senior author had the benefit of a stimulating discussion on the prevailing confusion on this topic with Dr J. C. Sen Gupta, the then Director of the Botanical Survey of India, during the latter's official visit to this area.

Sincere thanks are due to Reverend Father Dr H. Santapau, Director of the Botanical Survey of India, for his permission to the senior author to study the collection of Indian Boerhaavia in the Central National Herbarium at Shibpur (Calcutta) and for Latin rendering of the specific description of the plant. Grateful acknowledgement is made of the help rendered by Dr S. K. Mukherjee, Keeper of the said Herbarium, and particularly for his kindly furnishing two photographs of herbarium materials of *B. verticillata*.

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Short Communications

Distribution of Amino Acid Decarboxylases in the Cucurbitaceae Family

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Manuscript received 8 *July* 1962

The presence of active glutamic and aspartic acid decarboxylases has been detected in ten species of the Cucurbitaceae family by manometric and chromatographic methods.

THE results of a survey for the presence of amino acid decarboxylases[1] in ten common members of the Cucurbitaceae family are summarized in this communication.

The vegetables were obtained from the market in as fresh a condition as possible and after washing in running tap water were grated in a glass grater or cut into 1 cm. cubes by a stainless steel knife. Aliquots were soaked in an equal part of 0·01*M* phosphate buffer (*p*H 5·7) and homogenized in the Waring blender for 5 min. The homogenate was filtered through a double layer of cheese cloth. All these operations were performed at 5-8°C. Enzyme activity was assayed manometrically at 38°C. with air as gas phase in the flasks. Protein content of the extracts was estimated by the Folin method[2].

The results presented in Table 1 indicate that decarboxylase activity on glutamic and aspartic acids is present in all the materials examined. The common sweet pumpkin (*Cucurbita maxima*) showed the highest specific activity and compared favourably with summer squash reported to be the best source of glutamic acid decarboxylase[3]. Next in order of activity was the field cucumber. The activity of vegetable marrow (*Lagenaria vulgaris*), sweet melon (*Cucumis melo*) and *Parval* (*Trichosanthes dioica*) was of the same order. Appreciable activity was also present in *Tinda* (*Citrullus vulgaris fistulosus*), *Torai* (*Luffa acutangula*) and *Karela* (*Momordica charantia*).

The differences noticed between the specific activity of decarboxylase calculated on the basis of carbon dioxide release or γ-aminobutyric acid formation (followed by paper chromatography[4]) are apparently due to further metabolism of the amine by the crude extracts. In chromatograms run with aspartic acid as the substrate, a spot corresponding to β-alanine was observed and the intensity of this spot increased with time of incubation concomitantly with the decrease in the intensity of the aspartic acid spot.

The enzyme activity of homogenates prepared as above was found to be extremely unstable even when preserved in the frozen state (−18°C.). Activity in the cut pieces of the vegetables could, however, be preserved at −18°C. for over two weeks. Addition of pyridoxal phosphate or sodium metabisulphite did not result in the recovery of the lost activity in the homogenates. A freshly prepared homogenate was found to lose nearly 50 per cent of its enzyme activity within 30 min. of exposure to 37°C. in the absence of the substrate. This instability has rendered purification of the enzyme rather difficult. Attempts to purify the glutamic acid decarboxylase of *Torai* (*Luffa acutangula*) and *Lauki* (*Lagenaria vulgaris*) by ammonium sulphate fractionation were not successful. Activity with respect to glutamic acid was found to be readily adsorbed on calcium phosphate gel from which it could be eluted by a higher molarity phosphate

TABLE 1 — DISTRIBUTION OF AMINO ACID DECARBOXYLASES IN CUCURBITACEAE

Source material	Protein content of homogenate *mg./ml.*	Specific activity*		Release† of GABA
		L-Glutamate	DL-Aspartate	
Cucumis sativus (Hindi: *Kheera*)	1·8	2·3	—	—
Cucurbita maxima (Hindi: *Kadoo*)	2·7	3·0	1·0	1·0
Citrullus vulgaris (Hindi: *Turbooz*)	2·1	1·0	1·3	1·4
Cucumis melo (Hindi: *Karbuza*)	5·5	0·9	0·6	0·5
Cucumis utilissimus (Hindi: *Kakri*)	2·8	2·8	0·7	—
Lagenaria vulgaris (Hindi: *Lauki*)	4·0	0·8	0·8	0·9
Citrullus vulgaris fistulosus (Hindi: *Tinda*)	3·7	0·5	—	—
Luffa acutangula (Hindi: *Torai*)	3·7	0·5	—	—
Momordica charantia (Hindi: *Karela*)	5·8	0·4	0·6	—
Trichosanthes dioica (Hindi: *Parval*)	5·2	1·0	0·4	—

*Specific activity is μmole CO_2 released per mg. protein in 30 min. at 37°C. Manometric flasks contained in the main chamber 1·0 ml. homogenate, 1·5 ml. 0·1*M* phosphate buffer (*p*H 5·7) and 0·4 ml. 0·1*M* substrate and 0·1 ml. pyridoxal phosphate (0·1 mg./ml.) inside arm. The substrate and pyridoxal phosphate were tipped in after temperature equilibration for 5 min. Carbon dioxide liberated was corrected for 1 mg. endogenous production of CO_2 in the absence of substrate. Heated enzyme controls were always run for checking any non-enzymatic decarboxylation.

†Release of GABA (γ-aminobutyric acid) is expressed as μmole GABA per *mg.* enzyme protein in 2 hr reaction at 37°C.

*On deputation from the Chemistry Department, Aligarh University, Aligarh.

buffer resulting in about three-fold purification. The proteolytic activity of the fresh homogenates was negligible and could be ruled out as a factor responsible for the instability of the glutamic acid decarboxylase. Further work on the properties of decarboxylases in purified plant extracts is in progress.

The author is grateful to the Council of Scientific & Industrial Research for the award of a fellowship, to Dr B. Mukerji, Director, Central Drug Research Institute, Lucknow, for the provision of laboratory facilities and to Dr C. R. Krishna Murti for helpful guidance.

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The Presence of a Chromatophorotropic Principle in the Visceral Ganglia of the Oyster, *Crassostrea virginica*

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Manuscript received 18 *June* 1962

A principle influencing the pigment concentration in the red and the white chromatophores of the crayfish, *Orconectes clypeatus*, is found in the extracts of the visceral ganglia of the oyster, *Crassostrea virginica*.

THE colour changes in crustaceans are accomplished by means of the chromatophores which are found directly underneath or within the hypodermis. The chromatophores are amoeboid cells which contain one, two or more pigments. The movement of pigment within the chromatophores is under the control of hormones produced in the eyestalk and the central nervous system of the crustaceans. Chromatophorotropic substances have also been found in the crude extracts of the corpora cardiaca of the cockroach, *Periplanata*[1], in the nervous system of the horseshoe crab, *Limulus*[2], and in the central nervous system of the earthworm, *Lumbricus*, and the mollusc, *Venus*[3].

The cerebral and visceral ganglia of the oyster, *Crassostrea virginica*, possess neurosecretory cells (Nagabhushanam, R., unpublished data). To learn whether the neurosecretory material of the oyster could affect the chromatophores of the crustaceans, the following experiment was conducted with the chromatophores of the crayfish, *Orconectes clypeatus*, as the test object. Red and white chromatophores in the portion of the carapace dorsal to the heart were staged according to the system of Hogben and Slome[4] with the aid of stereoscopic dissecting microscope and lamp. According to their scheme, stage 1 represents maximal pigment concentration, stage 5 maximal dispersion, and stages 2, 3 and 4 the intermediate conditions.

Extracts of visceral ganglia were prepared as follows. The ganglia to be assayed were dissected out and placed in van Harreveld's solution. When the desired number of ganglia was available, they were transferred to a glass mortar, triturated, and resuspended in a sufficient volume of van Harreveld's solution such that the final concentration was one ganglion per 0·02 ml. of extract.

Orconectes that received injections of extracts had had one of their eyestalks removed at least 24 hr prior to the experiment. Fingerman[5] found that the responses to chromatophorotropins of one-eyed specimens were greater than the responses of intact specimens, probably because the presence of both eyestalks makes the crayfish more capable of antagonizing injected hormone.

Five one-eyed *Orconectes* were placed into each of two white enamelled pans containing aerated tap water. These crayfish had been conditioned on a white background for at least 60 min. The average chromatophore stage of the five crayfish in each pan was determined prior to injection of extract or saline to assure that the red and the white chromatophores were in the proper state of background adaptation. The animals in one pan received an injection of 0·02 ml. of the extract, while in the remaining pan the animals were injected with 0·02 ml. van Harreveld's solution as a control. The average red and white chromatophore stages for the specimens in each pan were then determined 15, 30, 45 and 60 min. after injecting into the crayfish. The experiment was performed twice.

The results are presented in Fig. 1 where each point represents the average of ten specimens. As is evident from inspection of the figure, the neurosecretory material in the visceral ganglion produced concentration of the pigment in the red and the white chromatophores of *Orconectes*.

The presence of chromatophorotropins in the visceral ganglia of the oyster might seem at first glance rather extraordinary because of the absence of functional chromatophores in bivalves. But in many other organisms without physiological colour change these materials were also extracted[1,2,3,6,7]. These studies suggest that the chromatophorotropic actions of these organs are only one of a number of

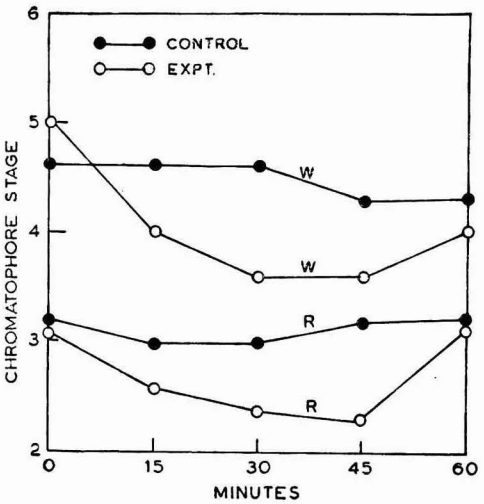

Fig. 1 — Responses of the red (R) and the white (W) chromatophores of one-eyed *Orconectes* on a white background to the extracts of the visceral ganglia of *Crassostrea virginica*

functions, many of which are far more basic in the life processes of the animals than that of chromatic adaptation.

The author wishes to express his thanks to Dr M. Fingerman for providing the facilities to carry out this investigation.

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Removal of Phosphorus from Sewage Flowing in Natural Channels

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Manuscript received 18 *June* 1962

During the flow of sewage in natural channels, purification proceeded and the phosphorus from the liquid was rapidly removed; the purified effluents contained very little or traces of phosphorus. An important factor influencing the rapid removal of phosphorus and other changes in these channels carrying sewage for many years appeared to be microbial activity, notably the activity of Vorticellids.

It was observed that during filtration of sewage through sand and soil, phosphorus was removed from the liquid by chemical reaction and by microbial activity[1]. Such a removal of phosphorus, particularly by microbial activity, which is of sanitary significance as well as of agricultural importance, has recently been observed in natural channels carrying an increasing volume of sewage during the last four decades. By the constant flow of sewage and with the passage of time, most of the reactive iron and aluminium in the soil have apparently reacted with the sewage phosphorus, and this chemical reaction is now not so evident as the microbial activity in the removal of phosphorus.

About 16 million gallons of sewage from a population of about 1·4 millions in Bangalore are now daily taken to three main outfalls and allowed to flow down in natural channels on the outskirts of the city. The sewage purifies itself naturally as it covers a distance of about 1·3-5 miles, depending upon the gradient of the channel, 1-in-50, 1-in-100 or 1-in-800, the extent of agitation or turbulence and other related conditions. The purified effluents are comparable in quality to the effluent from the activated sludge process and have been found to be used by the villagers in the neighbourhood for washing purposes[2-6]. This natural purification of flowing sewage is of unusual interest as there seems to be no record of such purification except a rather empirical statement in a paper[7] published as early as 1860, " that if sewage ran away for a distance of about 10 miles, it was no longer sewage, but almost plain water ". The recent work in this laboratory on this aspect included a study of the phosphorus changes, a brief account of which is given in this communication.

The water-soluble and total phosphorus contents of samples of sewage from the Bangalore City sewerage system, which contained more silica, iron and aluminium, were less than those of the sewage samples from the sewerage system at this Institute, which contained less silica, iron and aluminium, although the sewage from this source generally contained less organic matter than the sewage from the city. At periodical intervals samples of the city sewage flowing in the three channels at various points were taken and examined for their quality, including water-soluble and total phosphorus, by the usual methods[8-12]. The results obtained for the three channels were similar, and one set of results relating to the channel having 1-in-50 gradient and taking daily about 4 million gallons are given in Table 1. In view of the observation on the occurrence of relatively large numbers of colonial Vorti-

TABLE 1 — REMOVAL OF PHOSPHORUS FROM THE FLOWING SEWAGE AND OTHER CHANGES

	Outfall	Distance from outfall (*miles*)*				
		0·17	0·38	0·67	0·92	1·29
Turbidity						
Minimum	165	115	85	70	25	18
Maximum	230	160	137	125	90	32
Dissolved oxygen, p.p.m.						
Minimum	0·0	0·9	1·6	1·8	4·2	6·4
Maximum	1·2	3·0	3·4	3·9	6·8	8·4
3-min. permanganate value, p.p.m.						
Minimum	21·0	19·2	13·6	13·4	6·0	2·0
Maximum	52·5	35·0	25·2	19·4	13·0	5·0
4-hr permanganate value, p.p.m.						
Minimum	41·4	38·2	27·4	23·2	15·2	6·0
Maximum	112·0	72·0	44·2	31·4	19·0	14·0
Water-soluble phosphorus (P), p.p.m.						
Minimum	6·1	5·4	5·1	4·8	1·1	Trace
Maximum	7·6	6·3	5·6	5·2	3·0	0·8
Total phosphorus (P), p.p.m.						
Minimum	9·3	8·9	8·7	8·0	1·9	Trace
Maximum	16·4	15·6	12·3	11·0	3·2	1·1
Phosphorus content of soil (mg./100 g.)						
In sewage channel	137·4	81·2	82·5	100·0	92·4	57·6
30 ft away from channel	45·0	54·0	52·0	40·0	42·0	40·0
Number of Vorticella per ml. of aerated soil suspension	1038	330	333	963	367	143

The average value for B.O.D. of the final effluent was 15 p.p.m.
*Between 0·67 and 0·92 mile, large numbers of colonial Vorticellids (species of Carchesium and Epistylis) were found to occur. The cells of Carchesium sp. were picked out, washed and added to raw sewage and the mixture artificially aerated. The protozoan cells removed phosphorus from the sewage in a manner similar to that observed with activated sludge.

cellids in the region of rapid clarification of sewage in the channels, samples of the soil from one of the channel beds (1-in-50 gradient) were taken, air dried and 1 g. each of the samples was added to heat-sterilized sewage (autoclaved at 15 lb./sq. in. for 30 min.) and aerated for 48 hr. The numbers of Vorticella sp. that developed in these aerated soil suspensions are also given in Table 1. The total phosphorus contents of these soil samples and of those from the corresponding points (not in contact with sewage) about 30 ft away from the side of the sewage channel are also included in the table. The amounts of iron and aluminium in the Bangalore red soil have already been given[1].

The results given in Table 1 indicate that: (1) during flow of sewage up to the stage of clarification in the natural channel there was a somewhat steady removal of phosphorus from the sewage and its deposition in the soil below; (2) in the region of rapid clarification of the sewage, comparatively more phosphorus was removed and deposited in the soil than at other regions; (3) the rate of removal of phosphorus seemed to have a close bearing on other facets of purification of the sewage and the number of Vorticella sp.; (4) the purified effluent contained very little or a trace of phosphorus; and (5) the influence of the Vorticellid examined (Carchesium sp.) on the removal of phosphorus from sewage and other changes was similar to that of activated sludge.

Thus, in the rapid removal of phosphorus from continuously flowing sewage, the influence of microbial activity was striking. While the chemical reaction of the sewage phosphorus with the iron and aluminium in the natural channels was limited in the course of time, the microbial activity was greatly enhanced as probably in activated sludge which was reported to remove rapidly the phosphorus from sewage[8].

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An Inhibitor of Hexokinase in the Spores of *Aspergillus niger*

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Manuscript received 19 *July* 1962

The addition of extracts of resting and germinated spores of *A. niger* completely suppressed the hexokinase activity of the mold mycelium. It is concluded that an inhibitor of hexokinase is present in the spores of *A. niger*. This inhibitor appears to be heat stable and soluble to a considerable extent.

BHATNAGAR and Krishnan[1] failed to demonstrate hexokinase activity in the resting spores of *Aspergillus niger*, NRRL 599. Even more significant was the observation that the activity was not demonstrable when phosphoglucomutase, phosphohexoisomerase and aldolase activities were just detectable in the spores germinated in a synthetic medium. We have now found a powerful inhibitor of hexokinase activity in the spores, both resting and germinated.

The collection of spores was as described by Bhatnagar and Krishnan[2]. The desiccator-dried spores were cultured for 12 and 18 hr at 27-30°C. with continuous agitation on a Gump shaker in synthetic medium adjusted to *p*H 4·0. Microscopic examination revealed formation of germ tubes at the end of 18 hr. Both resting and germinated spores were ground with acid-washed sand and 0·4*M* tris buffer of *p*H 7·8 containing 0·06*M* sodium fluoride to give a 10 per cent suspension. Similar homogenates were prepared from the mycelium of *A. niger* grown on composite medium[3]. The homogenates were clarified by centrifugation in the cold at 600 *g* for 15 min. and the supernatant used for hexokinase estimation. The cell debris from spores was taken up in buffer and adjusted to the same volume as the supernatant. The assay system consisted of 0·5-1·0 ml. aliquot of enzyme preparation, 0·1 ml. of glucose solution containing 2·5 μmoles, 0·1 ml. of $MgCl_2$ containing 5 μmoles, 0·2 ml. of ATP containing 5 μmoles, 0·6-1·1 ml. of water or appropriate supplement, the total volume being 2·0 ml. The enzyme preparation consisted of either the ground resting spores, germinated spores, or the mycelium or a mixture made up of equal volumes of the mycelial preparation and the resting or the germinated spores. The activity of enzyme was expressed in terms of μmoles of glucose disappearing under the assay conditions.

TABLE 1 — INHIBITION OF HEXOKINASE ACTIVITY OF MYCELIUM OF *A. NIGER*

Mycelial extract *ml.*	Spore homogenate		Glucose disappearing *μmoles*
	Supernatant *ml.*	Residue *ml.*	
	RESTING SPORES		
0·5	—	—	0·174
—	1·0	—	nil
0·5	0·5	—	nil
0·5	—	—	0·182
—	—	1·0	nil
0·5	—	0·5	0·084
	GERMINATED SPORES		
0·5	—	—	0·182
—	1·0	—	nil
0·5	0·5	—	nil
	HEAT-TREATED SPORES		
0·5	—	—	0·192
—	1·0	—	nil
0·5	0·5	—	nil
0·5	—	—	0·182
—	—	1·0	nil
0·5	—	0·5	0·046

The results are presented in Table 1.

Whereas hexokinase activity could be demonstrated in the mycelial extracts, the supernatants from broken cell preparations of resting and germinated spores were negative. On mixing the two, the enzyme activity of the mycelium was completely suppressed. In other experiments, the ground resting spores were tested both in the supernatant and the cell debris fractions. The inhibition of the hexokinase activity of mycelial extract was 100 per cent with supernatant, while with the residue it was 54 per cent. In yet other experiments, resting spores were suspended in water and subjected to heat treatment at 70°C. for 15 min. On grinding these spores and separating the supernatant and cell debris, it was found that the inhibition of mycelial hexokinase activity was 100 per cent by the supernatant and 75 per cent by the residue. The incorporation of EDTA, final concentration 0·005*M*, in hexokinase assay system did not affect the results.

The above findings indicate the occurrence of a hexokinase inhibitor in the spores of *A. niger*. The nature of the inhibitor is under study.

References

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Regd. No. P-842