C —BIOLOGICAL SCIENCES

Journal of Scientific & Industrial Research

THE COUNCIL OF SCIENTIFIC & INDUSTRIAL RESEARCH, NEW DELHI

J. sci. industr. Res., Vol. 21C, No. 11, Pp. 293-320

NOVEMBER 1962

Journal of

Scientific & Industrial Research

Vol. 21C, No. 11, NOVEMBER 1962

CONTENTS

J. sci. industr. Res., Vol. 21C, No. 11, Pp. 293-320. November 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)

Purification & Properties of 3′-Nucleotidase of Green Gram (*Phaseolus radiatus*)

(Miss) RANJEETH K. AHLUWALIA & R. K. MALLER

Department of Biochemistry, Indian Institute of Science, Bangalore 12

Manuscript received 27 *July* 1962

Properties of 3′-nucleotidase purified about 27-fold from extracts of green gram (*Phaseolus radiatus*) seedlings are described. The enzyme has maximal activity at about *p*H 7·8 and 70°, its thermal stability being strikingly high. It is specific for 3′-ribonucleotides; adenosine 3′-monophosphate is readily attacked. The enzyme is not metal dependent and is of non-sulphydryl nature. The preparation has ribonuclease activity unlike that of the bovine pancreatic type. In several of its properties it resembles the rye grass 3′-nucleotidase of L. Shuster and N. O. Kaplan [*J. biol. Chem.*, Vol. 201 (1953), 535].

3′-NUCLEOTIDASES are specific phosphatases which cleave the phosphomonoester bond at carbon 3′ of the sugar moiety of the nucleotide molecule to form nucleosides and inorganic phosphate. The first 3′-nucleotidase to be purified and studied in detail was isolated by Shuster and Kaplan[1] from germinating rye grass seeds. These authors had also demonstrated its occurrence in the crude extracts of several kinds of leaves, a number of cereals and a few microorganisms. Since then, certain acid phosphatases of *Escherichia coli* and white lupine seedlings have been reported, by Rogers and Reithel[2] and Newmark and Wenger[3] respectively, to have pronounced 3′-nucleotidase activity. Earlier, Krishna Murti and Shrivastava[4] had shown its presence in *Vibrios* among other nucleotidase activities. Recently, Stockx and Parijs[5] briefly reported the finding of a specific 3′-nucleotidase with an alkaline *p*H optimum in the seeds of *Phaseolus aureus* Roxb. But, so far, there has not been any comprehensive study of the enzyme from sources other than rye grass.

During a study of the ribonuclease in germinating green gram (*Phaseolus radiatus*), it was observed that a 50-80 per cent saturated ammonium sulphate fraction of the crude aqueous extracts of the hypocotyl and root portions of 48 hr old seedlings, when incubated with purified yeast RNA at an alkaline *p*H, liberated appreciable amounts of inorganic phosphate which could not be ascribed to non-specific alkaline phosphatase activity in the fraction. Subsequently, it was noted that the phosphate release was pronounced with 3′-ribonucleotides and negligible with their 2′ and 5′-isomers. The purification of this nucleotidase and some of its properties are reported here.

Experimental procedure

Materials — Of the chemicals used, the 3′-monophosphates of adenosine (3′-AMP), cytidine (3′-CMP) and uridine (3′-UMP) and the 2′-monophosphates of cytidine (2′-CMP) and uridine (2′-UMP) were laboratory preparations (*see* below). 2′-,3′- and 5′-monophosphates of guanosine (2′-, 3′- and 5′-GMP) were gifts from Dr D. R. Sanadi (Gerontology Branch, NHI, Baltimore, U.S.A.) and adenosine 2′-monophosphate (2′-AMP) from Dr C. Heidelberger (University of Wisconsin, U.S.A.). 2′,3′-Cyclic phosphates of adenosine and cytidine were products of Schwarz Bioresearch Inc., yeast adenylic (2′,3′-AMP), guanylic (2′,3′-GMP), cytidylic (2′,3′-CMP) and uridylic (2′,3′-UMP) acids, cytidine, uridine, *p*-nitrophenyl phosphate, glucose-1-phosphate and glucose- and fructose-6-phosphates were obtained from Nutritional Biochemicals Corporation.

Adenosine 5′-monophosphate (5′-AMP) was a product of Pabst Laboratories and fructose-1-phosphate, ribose-1-phosphate and tris of Sigma Chemicals Co. Yeast RNA and *p*-chloromercuribenzoate were preparations of Light & Co., adenosine, guanosine, cysteine-HCl, GSH, iodoacetate, EDTA and sodium β-glycerophosphate that of the British Drug Houses. 8-Hydroxyquinoline was obtained from E. Merck. Sodium pyrophosphate was a Baker's analysed product. Calcium bis-*p*-nitrophenyl phosphate was a gift from Dr S. S. Rao (Haffkine Institute, Bombay). The rest of the chemicals were all of analytical grade. Green gram seeds were purchased from the local market.

Cytidine and uridine 2′- and 3′-monophosphates — 2′,3′-CMP (30 mg.) was first converted into a 1:1 mixture of the isomers according to Brown *et al.*[6]. The individual isomers were then isolated from a Dowex-1-formate (8 X, 200-400 mesh) column, in the manner described by Cohn[7] and modified by Brown *et al.*[6]. The solid samples obtained on evaporation of the appropriate fractions at 45-50°, under reduced pressure, were checked for purity and material yield by making use of the values for ratios of extinction at 250, 280, 290 and 260 mμ and ε 260, at *p*H 2·0, provided by Volkin and Cohn[8]. The yields of the separated 2′- and 3′-CMP were respectively 9·3 and 11·4 mg.

One of the lots of the separated isomers was chemically deaminated to the corresponding 2′- and 3′-UMP and isolated by the method of Shuster and Kaplan[1]. The ultraviolet absorption spectra of these products accorded with those of 2′- and 3′-UMP respectively and their yields were more than 75 per cent. 3′-UMP on chromatographing from a Dowex-1-formate column (Brown *et al.*[6]) was recovered almost completely and the elution diagram exhibited a single peak thereby confirming the presence of only one isomeric species in the recovered material.

Adenosine 3′-monophosphate — 3′-AMP was isolated from 2′,3′-AMP using ion-exchange column chromatography in essentially the same manner as described for the separation of 3′-CMP from 2′,3′-CMP. However, no pretreatment for conversion into a 1:1 mixture of the isomers was given in the present instance. Instead, 1*M* urea was added before chromatography because it improved the separation. In one run, 28 mg. of 2′,3′-AMP gave 14·7 mg. of pure 3′-AMP.

Yeast ribonucleic acid — This was purified according to Frisch-Niggemeyer and Reddi[9] and the phosphorus content estimated by the method of Fiske and Subbarow[10] after digestion with sulphuricnitric acid mixture.

Buffers — Citrate, barbital and tris buffers were prepared according to Gomori[11] and checked in each case by a Beckman *p*H-meter.

Enzyme assay — I. Reaction mixtures were prepared by adding the following components in the order stated: 2 μmoles of substrate in 0·2 ml. of water adjusted to *p*H 7·4 with $NaHCO_3$, 30 μmoles of 0·05*M* tris buffer, *p*H 7·4 (0·6 ml.), enzyme solution (0·1 ml.) containing 5 μg. of protein, and water to make up a total volume of 1 ml. Reaction mixtures without substrate and without enzyme served as controls. After 30 min. at 37°, the reaction was stopped by the addition of 2*N* perchloric acid, centrifuged if necessary, and inorganic phosphate (Pi) was determined by the methods of Fiske and Subbarow[10] or Bartlett[12], using a Klett-Summerson photoelectric colorimeter. Appropriate corrections, based on control values, were made for non-enzymic hydrolysis.

II. Wherever Pi estimations could not be performed, the enzymic activity was determined by measuring the release of adenosine from its 3′-monophosphate. In such cases the reaction was stopped by boiling the reaction mixture for 2-3 min., the same treatment being given to the controls. The reaction mixtures were then brought to *p*H 8-10 by the addition of aqueous ammonia solution and an aliquot shaken with Dowex-1-chloride (8 X, 200-400 mesh, 0·5 ml. wet packed resin). After keeping for 10 min. with intermittent shaking, the supernatant was collected by centrifuging and the resin washed three times with 1 ml. portions of water, followed by three times with 2 ml. portions of 0·01*M* ammonium chloride, and the washes added to the main supernatant. The total solution was then made to 10 ml., filtered and the adenosine concentration therein determined by ultraviolet spectrophotometry according to Volkin and Cohn[8]. Corrections were applied for non-enzymic action as in assay I. In test experiments, using adenosine and 2′,3′-AMP, either singly or in mixture, the latter was retained on the resin whereas the former could be recovered almost quantitatively. Further, the activity of the enzyme in a typical reaction mixture was repeatedly found to be almost the same whether assayed on the basis of phosphate or adenosine release. Use of this procedure obviated the necessity for employing enzymic method for estimation of adenosine as was adopted by Shuster and Kaplan[1].

One unit of enzyme activity is defined as that which, under the standard conditions, liberated 0·1 μmole of Pi. Specific activity is the number of units per mg. of the protein. Protein was determined by the method of Lowry and coworkers[13] using

crystalline bovine serum albumin (Armour) as the standard.

Purification of enzyme

All the steps were carried out at 4° unless otherwise stated. Glass distilled water was used throughout.

Crude extract — Hand-picked, well-formed seeds with healthy green colour were kept in approximately 0·01*M* potassium permanganate for 10 min. and then washed repeatedly with sterile water till the washes were colourless. The seeds were then spread in a single layer on a filter paper circle placed over a pad of moist cotton wool in a sterile petri dish and allowed to germinate under laboratory conditions of light, humidity and temperature for 48 hr. The hypocotyledonous parts (hypocotyl and root) from 10 g. of seeds were ground in a mortar into a thin slurry with water (10 ml.) and squeezed through muslin cloth. The filtrate was centrifuged for 10 min. at 1600 *g* and the supernatant thus obtained designated as crude extract.

Heat treatment — The crude extract (*p*H 5-6) was kept for 20 min. in a water bath maintained at 60-65° and then quickly chilled and the precipitate removed by centrifuging for 10 min. at 1600 *g*.

First ammonium sulphate fraction — The slightly green-tinged but almost clear supernatant from step 2 was made 80 per cent saturated with solid ammonium sulphate and after standing for 1 hr the precipitate was collected by centrifuging for 15 min. or more at 1600 *g* till a clear supernatant resulted. The precipitate was dissolved in 10 ml. of water and the solution dialysed against several changes of water (total of 2 litres). The dialysis residue was then centrifuged to remove precipitated material, if any, and the clear supernatant used for the subsequent step.

Calcium phosphate gel treatment — The gel prepared according to Keilin and Hartree[14], dry weight 12 mg./ml., was added to the above supernatant, the gel-protein ratio being 10: 1. After keeping for 10 min., with constant shaking, the suspension was centrifuged and the gel pellet eluted with *M* $NaHCO_3$ (5 ml.) for the enzyme.

Second ammonium sulphate fraction — The eluate from the gel was treated with solid ammonium sulphate to 80 per cent saturation as in step 3. The isolated precipitate was dissolved in 2 ml. of water and dialysed against repeated changes of water (total 1 litre). The water-clear supernatant obtained on centrifuging the non-diffusible material was used for all enzyme studies after appropriate dilutions. The preparation when kept frozen retained its full activity for periods up to 3 months and above.

Results

Purification of 3′-nucleotidase — Table 1 summarizes the results of the purification procedure which has been repeated several times and also with larger lots of starting material (40 g. of seeds). It has consistently provided not less than 27-fold purification of the enzyme in an overall yield of about 50 per cent. At no step was there need for correction for the presence of non-specific alkaline phosphatase activity since it was either negligibly small or undetectable against *p*-nitrophenyl phosphate throughout. Occasionally the final fraction had shown lowered nucleotidase activity which on storage, however, returned to the normal level and at times even exceeded it, probably due to destruction of any inhibitor-protein present. Use of higher speeds of centrifuging, up to 12,000 *g*, for obtaining the crude extracts did not influence the specific activity of the final preparation.

Table 2 shows the relative rates of hydrolysis of the various 2′,3′-nucleotides by fractions at different steps of enzyme purification.

Properties of purified fraction

Optimum pH — The green gram 3′-nucleotidase showed maximum activity at about *p*H 7·8 (Fig. 1). The concomitant non-specific phosphatase (optimal *p*H 5·6-6·2) was inactive in the range of maximal activity of the nucleotidase.

Period of incubation — With the purified enzyme and under standard assay conditions, maximal dephosphorylation of 3′-AMP took place within 40 min. (Fig. 2). The reaction followed zero order

TABLE 1 — SUMMARY OF PURIFICATION OF 3′-NUCLEOTIDASE FROM GREEN GRAM SEEDLINGS

Step No.	Fraction	Vol. *ml.*	Protein *mg.*	Total activity *units*	Specific activity *units/mg.*	Yield %
1	Crude extract	40	109	11141	102	—
2	Heated extract	39	33	9002	274	81
3	I $(NH_4)_2SO_4$ ppt.	10	6	7091	1223	64
4	II $(NH_4)_2SO_4$ ppt. from calcium phosphate gel eluate	3·5	2	5510	2755	50

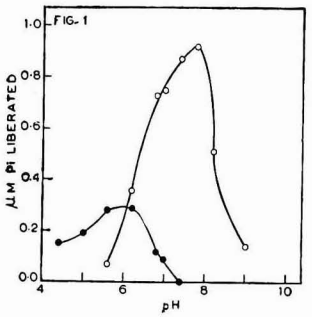

Fig. 1 — Effect of *p*H on nucleotidase activity [Standard assay conditions were used. 30 μM of each buffer (0·05*M*) at the required *p*H was present in each reaction mixture. The buffers were citrate-Na (*p*H 3·0-6·2), veronal-HCl (*p*H 6·8-7·0) and tris-HCl (*p*H 7·4-9·0). The system contained 2′,3′-AMP (○) or *p*-nitrophenyl phosphate (●) as substrate]

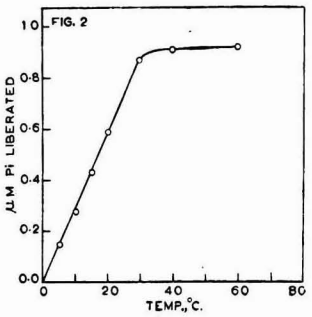

Fig. 2 — Time course of hydrolysis of 3′-AMP by green gram 3′-nucleotidase [Standard conditions except for time were used. Reaction tubes were removed at the intervals specified and treated with 2*N* perchloric acid. Pi released was determined as described in the text]

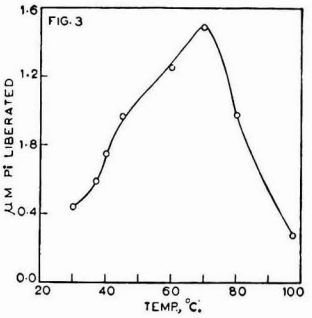

Fig. 3 — Effect of temperature on the hydrolysis of 3′-AMP [Conditions of standard assay except for temperature and incubation time, which was 10 min., were followed]

TABLE 2 — RELATIVE RATES OF 3′-NUCLEOTIDASE ACTIVITY AGAINST SUBSTRATES DURING ENZYME PURIFICATION

[*The regular assay method was employed, the concentration of substrate being 2 mM in all cases for all the fractions. Enzyme conc. was 20 μg. per reaction mixture for the first fraction and 5 μg. for the remaining. The relative activities were calculated using 2′,3′-AMP as reference substrate* (=100)]

Enzyme fraction	Substrates		
	2′,3′-AMP	2′,3′-GMP	2′,3′-UMP
Step 1	100	48	11
Step 2	100	36	11
Step 3	100	50	14
Step 4	100	57	21

kinetics up to 30 min. The other substrates gave a similar behaviour. With 3′-UMP and 3′-CMP, and especially with the former, the release of Pi, however, was appreciably enhanced by longer periods of incubation (2 hr) but it was nevertheless considerably less than from their purine counterparts.

Influence of temperature — Fig. 3 shows that the activity was highest at 70° above which it decreased rapidly.

Stability — About one-third of the activity was lost in 10 min. at 80° and *p*H 7·4, but on boiling the enzyme preparation at the same *p*H there was complete inactivation within 2-3 min.

Substrate specificity — The dephosphorylating activity of the preparation against ribonucleotides and their isomers and several non-nucleotidic phosphomonoesters is shown in Table 3. The enzyme was inactive against the non-nucleotidic compounds tested and among the nucleotides themselves it acted only on their 3′-isomers, under the specified conditions. The rate of their cleavage was in the order, 3′-AMP > 3′-GMP > 3′-UMP. Although 3′-CMP proved resistant to the enzyme at the standard assay level, at the elevated concentration of 15 μg. per reaction mixture, there was activity although relatively small. Use of more enzyme did not alter its specificity. The presence of Mg^{2+} (1 mM) in reaction mixtures containing *p*-nitrophenyl phosphate or Fe^{3+} (2·9 mM) in those with sodium β-glycerophosphate as substrates did not cause the liberation of any Pi under the standard assay procedures.

Phosphodiesterase activity — There was a high activity to adenosine 2′,3′-cyclic phosphate but none to cytosine 2′,3′-cyclic phosphate or bis-*p*-nitrophenyl phosphate (Table 4). It showed only slight activity towards yeast RNA. With ten times more enzyme, incubated for 18 hr, about 12 per cent of yeast RNA phosphorus was released as Pi. In separate experiments, on incubation for 18 hr, there was appreciable liberation of Pi from the cytidine cyclic phosphate at the standard assay level of enzyme.

Substrate concentration and enzyme activity — The Michaelis-Menten constant, K_m, for the three nucleotides was evaluated from the Lineweaver and Burk[16] plots and the values are as follows: 3′-AMP, 12 mM; 3′-GMP, 3·3 mM; and 3′-UMP, 2 mM (Fig. 4, a and b).

Effect of combining different 3′-nucleotides — No additive effects were observed (Table 5) in the simultaneous presence of two substrates in different combinations.

Inhibitors and activators — The products of hydrolysis of 3′-AMP did not appreciably affect the

TABLE 3 — RELATIVE ACTIVITY OF GREEN GRAM NUCLEOTIDASE TOWARDS VARIOUS PHOSPHOMONOESTERS

[Each compound as its sodium salt (2 μM) was tested, under the conditions of standard assay and also at an enzyme conc. of 15 μg. per reaction mixture. Analytical methods were as described in the text]

Compound	Activity with enzyme/reaction mixture*	
	5 μg.	15 μg.
3′-AMP	1·00	—
2′,3′-AMP	1·00	1·00
2′-AMP	0·00	0·00
5′-AMP	0·00	0·00
3′-GMP	0·70	—
2′,3′-GMP	0·43	0·76
2′-GMP	0·00	0·00
5′-GMP	0·00	0·00
3′-UMP	0·11	—
2′,3′-UMP	0·30	0·64
2′-UMP	0·00	0·00
5′-UMP	0·00	0·00
3′-CMP	0·00	—
2′,3′-CMP	0·00	0·18
2′-CMP	0·00	0·00
5′-CMP	0·00	0·00
p-Nitrophenylphosphate	0·00	0·11
β-Glycerophosphate	0·00	0·07
Glucose-1-phosphate	0·00	—
Glucose-6-phosphate	0·00	—
Fructose-1-phosphate	0·00	—
Fructose-6-phosphate	0·00	—
Ribose-5-phosphate	0·00	—
Pyrophosphate	0·00	0·04
Hexametaphosphate	0·00	0·03

*Activity relative with respect to 3′-AMP which was used as the reference substrate.

TABLE 4 — PHOSPHODIESTERASE ACTIVITY OF THE GREEN GRAM 3′-NUCLEOTIDASE PREPARATION

(Each compound as its sodium salt and in amounts specified was tested at two enzyme conc. and under the remaining conditions of standard assay. When yeast RNA was used as substrate the reaction was stopped by 0·75 per cent uranyl acetate in 25 per cent perchloric acid[15]. Rest of the analytical method followed as for standard assay in every case)

Compound	Total organic phosphate	Pi released (%) with enzyme per reaction mixture	
		5 μg.	15 μg.
YRNA (1·25 mg.)	100 μg.	1·3	3·0
bis-*p*-Nitrophenyl phosphate	0·5 μM	0·0	0·0
Adenosine 2′,3′-cyclic phosphate	2·0 μM	19·3	19·6
Cytidine 2′,3′-cyclic phosphate	2·0 μM	0·0	0·0

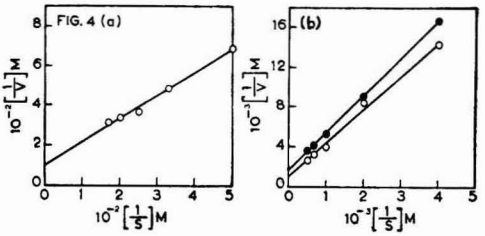

Fig. 4 (a and b) — Lineweaver and Burk plot for green gram 3′-nucleotidase [S, conc. of substrate expressed as μM/ml. and V, velocity of reaction expressed as total Pi (μM) liberated during 30 min. Values for 1/S and 1/V computed from separate experiments. Substrates were 3′-AMP, ○ (Fig. 4a), 3′-GMP, ○ (Fig. 4b) and 3′-UMP, ● (Fig. 4b)]

TABLE 5 — HYDROLYSIS OF COMBINATIONS OF TWO DIFFERENT 3′-NUCLEOTIDES BY THE GREEN GRAM NUCLEOTIDASE

(The conc. of each substrate was 2 mM whether used singly or in combination with another. The conditions of regular assay were followed as also the analytical methods)

Substrate	Pi liberated μM
2′,3′-AMP	1·10
2′,3′-GMP	0·63
2′,3′-UMP	0·21
2′,3′-AMP+2′,3′-GMP	0·82
2′,3′-AMP+2′,3′-UMP	0·96
2′,3′-GMP+2′,3′-UMP	0·52

TABLE 6 — EFFECT OF ADDED PHOSPHATE, NUCLEOSIDES AND ISOMERIC NUCLEOTIDES ON GREEN GRAM NUCLEOTIDASE ACTIVITY AGAINST 3′-AMP

[The reaction mixture in each case comprised 1 μM of 3′-AMP and the added compound at the specified final conc. The rest of the components and conditions of assay as for standard procedure except that when phosphate was the added compound the activity was determined based on adenosine release (see enzyme assay II)]

Compound	Control activity (%) at final conc.	
	5 mM	10 mM
Monosodium phosphate	92	90
Adenosine	98	90
Guanosine	63	—
Uridine	95	94
Cytidine	93	91
2′-AMP	88	79
5′-AMP	70	23

enzymic activity at 10 mM. Among the other nucleosides tested only guanosine seemed to inhibit the enzyme (Table 6). 5′-AMP markedly inhibited the enzyme and 2′-AMP had considerably less effect and their inhibitions were reversed by the use of excess substrate (10 μmoles).

The effect of a number of metal ions and two metal complexing agents, EDTA and 8-hydroxyquinoline, on the hydrolytic activity of green gram 3′-nucleotidase is shown in Table 7. The metal concerned was added to the reaction mixture as its sulphate or chloride. Of the various cations tested, Mn^{2+} appeared to be the most inhibitory at the level specified, Fe^{3+} and Mg^{2+} were only slightly inhibitory, other metals tested having little or no effect. Pre-incubation of the metal ion either with the enzyme or substrate did not alter significantly

TABLE 7 — EFFECT OF METALS AND METAL CHELATING AGENTS ON GREEN GRAM 3′-NUCLEOTIDASE

[*The test compound at the conc. specified was in every case incubated with the enzyme and buffer at 37°C. for 10 min. prior to the addition of substrate (2′,3′-AMP, 2 μM). Conditions of standard assay were followed throughout. In the case of metal chelating compounds 120 μM of buffer per reaction mixture was present. Except for EDTA all the agents were tested at final conc. of 1 mM*]

Substance	Control activity %	Substance	Control activity %
Mn^{2+}	56	K^{+}	109
Fe^{3+}	66	Na^{+}	111
Mg^{2+}	74	Co^{2+}	113
Zn^{2+}	81	8-Hydroxy-quinoline	71
Ba^{2+}	96		
Fe^{2+}	100	EDTA	85
Ca^{2+}	107	EDTA (5 mM)	53

TABLE 8 — EFFECT OF OTHER INHIBITORS AND ACTIVATORS ON GREEN GRAM NUCLEOTIDASE

[*Each compound neutralized where necessary and at the required conc. was preincubated for 10 min. at 37°C. with the enzyme and buffer (120 μM) and then the substrate added (2′,3′-AMP, 2 μM). Conditions and analytical methods were as for standard assay except that when arsenate was employed, the enzymic activity was determined on the basis of adenosine release*]

Compound	Final conc. *mM*	Control activity %
p-Chloromercuribenzoate	1	90
Iodoacetate	1	100
Cysteine	1	52
GSH	1	90
Sodium sulphite	1	105
Potassium permanganate	1	66
Potassium cyanide	5	32
Sodium fluoride	10	100
Sodium arsenate	10	68
Sodium molybdate	10	91
Sodium tungstate	5	55

the degree of its inhibition. But the removal of the inhibitory cation, by dialysis of treated enzyme against water, led to the complete restoration of enzymic activity. 8-Hydroxyquinoline and EDTA both inhibited at 1 mM but not at 0·1 mM. On removal of the chelating agent by dialysis against water, original activity was restored.

Of the other inhibitors tested (at 1 mM) under similar conditions, *p*-chloromercuribenzoate, iodoacetate, GSH and sodium sulphite showed virtually no inhibition of enzymic activity while cysteine at the same level decreased it by about half (Table 8). Potassium permanganate was also significantly inhibitory.

Discussion

The preparation is evidently different from the alkaline phosphatase which Appaji Rao *et al.*[17] had purified from the extracts of green gram seeds and shown to have an absolute specificity for sodium β-glycerophosphate in the presence of ferric ions, but it had several properties in common with the rye grass 3′-nucleotidase of Shuster and Kaplan[1].

Both the 3′-nucleotidases showed the same order of activity against the substrates: 3′-AMP > 3′-GMP > 3′-UMP > 3′-CMP. But our enzyme showed larger differences in the rates of dephosphorylation of these nucleotides. The K_m was 12 mM for 3′-AMP for green gram nucleotidase whereas Shuster and Kaplan[1] reported 0·3 mM for their 3′-nucleotidase. The K_m values for 3′-UMP are more similar.

The absence of any additive effects in the experiments on the simultaneous hydrolysis of the two substrates occurring in various combinations in the reaction mixture (Table 5) and also the fair degree of constancy exhibited in the relative rates of hydrolysis of three susceptible substrates during the course of enzyme purification (Table 2), both favour the belief that the properties of the green gram nucleotidase are those of a single enzyme.

The behaviour of rye grass nucleotidase and our preparation was similar towards cysteine, cyanide and fluoride (Table 8) but totally different with orthophosphate which even at 10 mM had little inhibitory effect on our preparation (Table 6). The isomeric nucleotides, however, competitively inhibited the enzyme just as in the case of rye grass preparation.

The inhibitory effects of metals like Mn^{2+}, Fe^{3+}, Mg^{2+} and to some extent Zn^{2+} as well as those of metal chelators tested (Table 7) on the green gram 3′-nucleotidase are seemingly contradictory. However, the restoration of original activity in both cases, on dialysis of the inhibitory agent, is strongly suggestive of the possibility that in either case the complex with the enzyme, if at all formed, was loose and easily disrupted. Therefore, it seems likely that the enzyme is not metal dependent and the apparent inhibition by some metal ions is probably due to their binding with the substrate. Smith and Alberty[18] had shown that metal ions like Mn^{2+} and Mg^{2+} do form stable complexes with nucleotides such as adenosine phosphates.

The lack of inhibition by *p*-chloromercuribenzoate and iodoacetate and also the absence of stimulation by thiols and sodium sulphite argue for non-sulphydryl nature of the green gram nucleotidase (Table 8). The profound inhibition elicited by cysteine is probably due to activation of concomitant inhibitor protein.

Our preparation appears to contain a ribonuclease (Table 4) as did Shuster and Kaplan's[1] preparation of 3′-nucleotidase from rye grass. The

rye grass nuclease has been purified by Shuster[19] and studied by Shuster *et. al.*[20] and shown to be unlike the bovine pancreatic ribonuclease in its action. Preliminary data indicate that the activity of green gram ribonuclease resembles that of rye grass preparation.

Acknowledgement

We wish to record our gratefulness to those who helped us with generous gifts of chemicals. We are deeply thankful to Prof. P. S. Sarma for his kind interest in this work and to our colleagues for their valuable advice and criticism. One of us (R.K.A.) was the recipient of a research scholarship from the University Grants Commission.

References

1. SHUSTER, L. & KAPLAN, N. O., *J. biol. Chem.*, **201** (1953), 535.
2. ROGERS, D. & REITHEL, F. J., *Arch. Biochem. Biophys.*, **89** (1960), 97.
3. NEWMARK, M. Z. & WENGER, B. S., *Arch. Biochem. Biophys.*, **89** (1960), 110.
4. KRISHNA MURTI, C. R. & SHRIVASTAVA, D. L., *J. sci. industr. Res.*, **14C** (1955), 12.
5. STOCKX, J. & PARIJS, R. V., *Chem. Zbl.*, **133** (1962), 7559.
6. BROWN, D. M., DEKKER, C. A. & TODD, A. R., *J. chem. Soc.*, (1952), 2719.
7. COHN, W. E., *J. Amer. chem. Soc.*, **72** (1950), 2811.
8. VOLKIN, E. & COHN, W. E., cited in *Methods of Biochemical Analysis*, Vol. 1, edited by D. Glick (Interscience Publishers Inc., New York), 1959, 304.
9. FRISCH-NIGGEMEYER, W. & REDDI, K. K., *Biochim. biophys. Acta*, **26** (1957), 40.
10. FISKE, C. H. & SUBBAROW, Y., *J. biol. Chem.*, **66** (1925), 375.
11. GOMORI, G., in *Methods in Enzymology*, Vol. 1, edited by S. P. Colowick & N. O. Kaplan (Academic Press Inc., New York), 1955, 140, 144.
12. BARTLETT, G. R., *J. biol. Chem.*, **234** (1959), 466.
13. LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J., *J. biol. Chem.*, **193** (1951), 265.
14. KEILIN, D. & HARTREE, E. F., *Proc. roy. Soc.*, **B124** (1938), 397.
15. ANFINSEN, C. B., REDFIELD, R. R., CHOATE, W. L., PAGE, J. & CARROLL, W. R., *J. biol. Chem.*, **207** (1954), 201.
16. LINEWEAVER, H. & BURK, D., *J. Amer. chem. Soc.*, **56** (1934), 658.
17. APPAJI RAO, N., CAMA, H. R., KUMAR, S. A. & VAIDYANATHAN, C. S., *J. biol. Chem.*, **235** (1950), 3353.
18. SMITH, R. M. & ALBERTY, R. A., *J. Amer. chem. Soc.*, **78** (1956), 2376.
19. SHUSTER, L., *J. biol. Chem.*, **229** (1957), 289.
20. SHUSTER, L., KHORANA, H. G. & HEPPEL, L. A., *Biochim. biophys. Acta*, **33** (1959), 452.

A Study of Terramycin Action on the Uptake of Vitamin B_{12} by Intestinal Microorganisms

T. BALAKRISHNA RAO, D. V. TAMHANE & A. SREENIVASAN*
Department of Chemical Technology, University of Bombay, Bombay 19

Manuscript received 17 April 1962

Uptake of vitamin B_{12} by *Esch. coli* cells during a period of 3 hr is inhibited *in vitro* by terramycin. Ageing reduces vitamin B_{12} uptake, which is restored in the presence of an oxidizable substrate. Vitamin B_{12} uptake is not altered on subsequent exposure to terramycin for 3 hr. Terramycin-treated cells have a reduced capacity for vitamin B_{12} uptake than aged cells. Vitamin B_{12} uptake is reduced in the presence of cyanide or DNP and under anaerobic conditions. Lysozyme-treated *Esch. coli* cells possess the capacity for vitamin B_{12} uptake under hypertonic conditions; this is also reduced in the presence of terramycin. Coliforms from terramycin-fed rats have a much reduced capacity for vitamin B_{12} uptake as compared to those from animals not fed the antibiotic. Unlike vitamin B_{12}, there is a release of PGA from *Esch. coli* cells in suspension and this release is augmented in the presence of terramycin.

IT has been reported by Stokstad[1] that, with rats, antibiotics cause an increase in the bacterial synthesis of vitamin B_{12} in the intestine. An increase in the blood and liver levels of vitamin B_{12} has been observed by us earlier[2]. The report by Lichtman *et al.*[3] that aureomycin or combination of antibiotics may produce haematological responses in pernicious anaemia as well as the observations by Davis and Mingioli[4] that *Esch. coli* cells take up vitamin B_{12} from solutions would imply that

*Present address: Central Food Technological Research Institute, Mysore.

antibiotics may increase the availability of dietary or intestinally synthesized vitamin B_{12} by preventing its uptake by *Esch. coli* and related organisms. The present work is an extension of a preliminary report[5] and relates to the effect of terramycin on vitamin B_{12} uptake by *Esch. coli* cells and by intestinal coliforms. Observations on the behaviour of *Esch. coli* cells to PGA solutions in the presence of terramycin are also included.

Experimental procedure

A strain of *Esch. coli* (Macleod) was maintained by fortnightly transfers on agar slopes of a salts-dextrose medium of Green and Sevag[6] containing per litre: KH_2PO_4, 2·0 g.; NaCl, 4·0 g.; $(NH_4)_2HPO_4$, 2·0 g.; $FeSO_4(NH_4)_2SO_4.6H_2O$, 0·1 g.; $CaCl_2$, 0·1 g.; $MgSO_4.7H_2O$, 0·1 g.; and dextrose, 15·0 g. The organism was grown at 30°C. in shake flask cultures using 200 ml. aliquots of the medium in 500 ml. conical flasks. Cells were harvested after 18 hr by centrifugation, thoroughly washed with ice-cold distilled water and made into a suspension to give 20 mg. dry weight cells per ml.

For the isolation of intestinal coliforms, male albino rats (45 g. initial weight), reared on a low methionine low choline Bengal gram diet[2] with or without additions of terramycin (25 mg./kg.) were used. After a six-week period on these diets, during which growth stimulation by terramycin was observable, the faeces were collected and suspended in sterile water. Coliforms were isolated in McConkey's medium from which they were carried into salts-dextrose medium. Cell suspensions were obtained as for *Esch. coli*.

For studies on vitamin B_{12} uptake the system consisted of: 1 ml. of vitamin B_{12} solution containing 1000 mμg. of the vitamin; 3 ml. of *M*/20 phosphate buffer (*p*H 7·4); 1 ml. of cell suspension and other additions where indicated, in a total volume of 6 ml. At the end of 3 hr incubation at 37°C., the cells were removed by centrifugation, washed once and resuspended in ice-cold distilled water. Vitamin B_{12} was liberated after papain digestion in acetate buffer (*p*H 4·5) for 18 hr, and assayed using *L. leichmanni* as test organism[7].

Where lysozyme-treated *Esch. coli* cells were used, the procedure followed was similar to that of Prestidge and Pardee[8]. Cultured and harvested cells were washed with 0·03*M* sodium phosphate solution (*p*H 7·0), containing 0·2*M* sucrose, and resuspended in the same medium. Lysozyme (Nutritional Biochemical Corporation) was added at 0·1 mg./ml. of suspension and the system kept cold for 30 min. when the cells settled down; these were isolated by centrifugation at 5000 *g* for 10 min., washed with suspending medium and resuspended in the same medium. Vitamin B_{12} uptake by lysozyme-treated cells was studied in buffered hypertonic sucrose. A control for *Esch. coli* cells in buffered sucrose was kept alongside.

In experiments on the behaviour of *Esch. coli* cells in the presence of PGA solutions the procedure was the same as for vitamin B_{12} uptake studies except that pteroylglutamic acid (PGA) solutions (280-440 mμg.) were substituted for vitamin B_{12}. After incubation at 37°C. for 3 hr, cells were removed by centrifugation. The supernatants and the washings were combined and steamed for 10 min. The samples were then diluted to convenient volume and assayed for PGA using *S. faecalis* R as the test organism and improved assay procedure of Mitbander and Sreenivasan[9]. The results reported are the averages of at least three independent observations.

Results

Freshly harvested cells of *Esch. coli* showed in several experiments an uptake of 520-600 mμg. of vitamin B_{12} per 20 mg. dry cells in 3 hr. This uptake was markedly decreased in the presence of 100 μg. of terramycin[5]. The vitamin B_{12} content of *Esch. coli* cells depends on the nature and the vitamin B_{12} content of the medium in which the cells are harvested. Cells harvested in the vitamin B_{12}-free salts-synthetic medium used in these experiments analysed to 1·25 to 1·38 mμg. per 100 mg. dry cells. The values for the vitamin B_{12} content of the cells in different incubation systems reported in this paper thus represent an uptake of the vitamin from solutions.

When cells were aged by suspension in phosphate buffer at 37°C. for 3 hr, vitamin B_{12} uptake by the cells was decreased by 43 per cent, the presence of 50 and 100 μg. of terramycin caused a further reduction to 60 and 72 per cent respectively in vitamin B_{12} uptake. However, when the cells were incubated in the presence of vitamin B_{12} for 3 hr and then incubated with terramycin (100 μg.) for a further 3 hr period, there was no decrease in the uptake of the vitamin. This would mean that vitamin B_{12} taken up by the cells is not released or lost from the cells if they are exposed to terramycin solution.

Vitamin B_{12} uptake by respiring *Esch. coli* cells is not affected if an oxidizable substrate like succinate or citrate is included in the system, but is considerably increased if they are added to aged cells (Table 1).

That the uptake of vitamin B_{12} by resting cells of *Esch. coli* is energy dependent is inferable from

the inhibitory effects of cyanide and of 2,4-dinitrophenol (Table 2). The uptake is significantly reduced in an atmosphere of nitrogen; sodium fluoride ($4 \times 10^{-4}M$) and iodoacetic acid ($8{\cdot}8 \times 10^{-5}M$) are without effect.

Esch. coli cells possess the ability to take up vitamin B_{12} under hypertonic conditions. Lysozyme-treated cells, however, show reduced uptake under these conditions. Terramycin suppresses the vitamin B_{12} uptake by the lysozyme-treated cells by about 40 per cent (Table 3).

Coliforms isolated from the faeces of terramycin-fed rats have considerably reduced capacity for vitamin B_{12} uptake as compared to those isolated from rats not receiving the antibiotic in the diet (Table 4).

TABLE 1 — EFFECT OF OXIDIZABLE SUBSTRATE ON VITAMIN B_{12} UPTAKE BY CELLS OF *ESCH. COLI*

System	Vitamin B_{12} uptake by 20 mg. dry wt cells ($m\mu g.$ *in* 3 *hr*)	
	Freshly harvested cells	Aged cells*
Cells alone	600	310
Cells + sodium succinate (40 μM)	620	540
Cells + sodium citrate (40 μM)	585	525

*Ageing was in phosphate buffer for 3 hr at 37°C.

TABLE 2 — EFFECT OF INHIBITORS ON VITAMIN B_{12} UPTAKE BY CELLS OF *ESCH. COLI*

System	Vitamin B_{12} uptake by 20 mg. dry wt cells ($m\mu g.$ *in* 3 *hr*)
Cells alone	610
Cells + DNP ($2 \times 10^{-4}M$)	400
Cells + KCN ($2{\cdot}4 \times 10^{-3}M$)	415
Cells + iodoacetic acid ($8{\cdot}8 \times 10^{-5}M$)	610
Cells + NaF ($4{\cdot}0 \times 10^{-3}M$)	625
Cells under nitrogen atmosphere	187
Cells under nitrogen atmosphere + iodoacetic acid ($8{\cdot}8 \times 10^{-5}M$)	180

TABLE 3 — VITAMIN B_{12} UPTAKE BY LYSOZYME-TREATED CELLS OF *ESCH. COLI*

System	Vitamin B_{12} uptake by 20 mg. dry wt cells ($m\mu g.$ *in* 3 *hr*)
Cells alone	
In phosphate buffer (*p*H 7·4)	620
In buffered sucrose [0·03*M* sodium phosphate solution (*p*H 7·0) containing 0·2*M* sucrose]	680
Cells treated with lysozyme (0·1 mg./ml.)	
In buffered sucrose	625
In buffered sucrose + terramycin (100 μg.)	360

TABLE 4 — UPTAKE OF VITAMIN B_{12} BY FAECAL COLIFORMS

Vitamin B_{12} in incubation system $m\mu g.$	Vitamin B_{12} uptake by 20 mg. dry wt cells ($m\mu g.$ *in* 3 *hr*)	
	Coliforms from control rats	Coliforms from terramycin-fed rats
1000	580	150
2000	905	210
3000	1040	240
4000	1045	310

TABLE 5 — EFFECT OF TERRAMYCIN ON RELEASE OF PGA FROM *ESCH. COLI* CELLS

PGA in incubation system $m\mu g.$	PGA released from 20 mg. dry wt cells ($m\mu g.$ *in* 3 *hr*)	
	Cells alone	Cells + terramycin (100 μg.)
280	90	175
360	90	180
440	80	160

Unlike vitamin B_{12}, there is a release of PGA from the cytoplasmic material of *Esch. coli* cells into the surrounding medium. The release is enhanced in presence of terramycin (Table 5).

Discussion

The decreased uptake of vitamin B_{12} by *Esch. coli* cells in the presence of terramycin as well as by the coliform organisms isolated from antibiotic-fed animals would point to improved availability of the vitamin to the host organism. This impairment in the property of the organism could arise from the known slowing down of its metabolic activities. A reduction in oxygen uptake by intestinal bacteria from chicks receiving aureomycin in the diet has been noted[10].

The vitamin B_{12} taken up by *Esch. coli* cells is apparently firmly bound and is not released unlike other metabolites like folic acid. Oginsky[11] also reports that uptake of Co^{60}-labelled vitamin B_{12} by *Esch. coli* appears to be a specific binding because of its resistance to simple elution techniques. The reduced uptake of vitamin B_{12} by aged cells could be due to their decreased respiratory capacity as a result of depletion of endogenous substrate. Ageing in the presence of terramycin further reduces the vitamin B_{12} uptake by *Esch. coli*. While the addition of small amounts of oxidizable substrates like succinate and citrate have no effect on the vitamin B_{12} uptake by resting cells, there appears to be considerable stimulation when these are added to the

aged cells. The stimulation by succinate could be due to the availability of an oxidizable substrate sufficient to step up the enzymatic activity in the cells as there was no significant increase in the dry weight of the cells after incubation period. The results obtained with citrate, a substrate which is not used by intact cells, point to a breaking up of the permeability barrier in aged cells probably arising out of partial lysis of cell wall. The results, however, bring out a probable linking of vitamin B_{12} uptake with the oxidative metabolism of the organism.

The inhibitory effect of DNP would also bear out the energy-dependent nature of vitamin B_{12} uptake and this can be explained on the basis that, like terramycin[12,13], DNP is a powerful inhibitor of oxidative phosphorylation[14].

The marked reduction in vitamin B_{12} uptake in the presence of KCN and in a nitrogen atmosphere also points to the linking of this process with oxidative metabolism in the organism. The work of Lichstein and Waller[15] reveals that biotin accumulation by *L.arabinosus* also appears to be an energylinked process. The non-interference by iodoacetate, both under aerobic and anaerobic conditions, would suggest that both aerobic and anaerobic glycolysis are not involved in the process of vitamin B_{12} uptake by *Esch. coli*.

Esch. coli cells take up more vitamin B_{12} under hypertonic conditions than when incubated in phosphate buffer indicating more favourable conditions for the transport of the vitamin to cellular mileu. The reduced uptake shown by lysozyme-treated cells under the same conditions could be due to the action of lysozyme on the cellular surface as evidenced by clumping and settling of cells during lysozyme treatment. Terramycin further reduces vitamin B_{12} uptake by these cells.

The decreased vitamin B_{12} uptake by faecal coliforms from terramycin-fed rats as compared with control animals would indicate an alteration in the metabolism of the organism as a result of antibiotic feeding. Recent report by Netrawali *et al.*[16] indicates that coliforms isolated from terramycin-fed rats have altered morphological characteristics, reduced metabolic activity and developed a marked tolerance to terramycin. Other work also suggests that development of resistance to aureomycin could result in a reduction of vitamin B_{12} uptake by *Esch. coli*[17].

The release of PGA from the cytoplasmic material to the surrounding medium on incubation in phosphate buffer may possibly be due to slow lysis of the cells as a result of ageing. Such release of PGA from *Esch. coli* cells has been reported by Alimchandani and Sreenivasan[18] and by Lichstein and White[19]. Terramycin would seem to enhance this release presumably by acceleration of catabolic processes in the cell. Although the increase in release is not very significant, it affords some presumptive proof for a slight increase in PGA levels in the liver of rats fed terramycin in the diet[2].

Acknowledgement

Our thanks are due to the Council of Scientific & Industrial Research, New Delhi, for a research grant and to Chas Pfizer & Co. Inc. for a gift of crystalline terramycin hydrochloride.

References

1. Stokstad, E. L. R., *Proceedings of Third International Congress of Biochemistry, Brussels*, edited by Claude Lièberg (Academic Press Inc., New York), 1956 445-52.
2. Rao, T. Balakrishna, Tamhane, D. V. & Sreenivasan, A., *Antibiotics — A Symposium* (Council of Scientific & Industrial Research, New Delhi), 1958, 215.
3. Lichtman, M., Ginsberg, V. & Watson, J., *Proc. Soc. exp. Biol., N.Y.*, **74** (1950), 834.
4. Davis, B. D. & Mingioli, E. S., *J. Bact.*, **60** (1950), 17.
5. Rao, T. Balakrishna, Tamhane, D. V. & Sreenivasan, A., *Antibiotics — A Symposium* (Council of Scientific & Industrial Research, New Delhi), 1958, 221.
6. Green, M. N. & Sevag, M. G., *Arch. Biochem. Biophys.*, **9** (1946), 129.
7. Skeggs, K. R., Nepple, K. H., Valenlik, R. A. *et al.*, *J. biol. Chem.*, **184** (1950), 211.
8. Prestidge, L. S. & Pardee, A. B., *J. Bact.*, **74** (1957), 48.
9. Mitbander, V. B. & Sreenivasan, A., *Arch. Microbiol.*, **21** (1955), 60.
10. Sieburth, J., Gutierrez, J., Mcginnis, J. *et al.*, *Proc. Soc. exp. Biol., N.Y.*, **76** (1951), 15.
11. Oginsky, E. L., *Arch. Biochem. Biophys.*, **36** (1952), 71.
12. Miura, Y., Nakamura, Y., Matsudaira, H. & Komeiji, T., *Antibiot. & Chemother.*, **2** (1952), 152.
13. Brody, T. M., Hurwitiz, R. & Bain, J. A., *Antibiot. & Chemother.*, **4** (1954), 864.
14. Loomis, W. F. & Lipmann, F., *J. biol. Chem.*, **173** (1948), 867.
15. Lichstein, H. C. & Walter, J. R., *J. Bact.*, **81** (1961), 65.
16. Netrawali, M. S., Rege, D. V. & Sreenivasan, A., *J. sci. industr. Res.*, **21C** (1962), 31.
17. Peterson, G. E. & Johansson, K. R., cited in *Antibiotics Ann.* (Medical Encyclopedia Inc., New York, N.Y.), 1956-57.
18. Alimchandani, H. R. & Sreenivasan, A., *J. Bact.*, **74** (1957), 171.
19. Lichstein, H. C. & White, J. E., *J. Bact.*, **59** (1950), 751.

Amino Acid Composition of Indian Silk Fibroins & Sericins: Part II — Sericins*

R. S. DHAVALIKAR
National Chemical Laboratory, Poona

Manuscript received 1 *June* 1962

The amino acid composition of sericins derived from four different Indian silks, viz. mulberry, tussah, muga and eri, has been determined by ion-exchange chromatography. The amino acid pattern of the sericins has been found to be distinctly different from that of the corresponding fibroin filaments, the sericins containing all the amino acids of the fibroins, but with markedly lower contents of glycine and alanine and higher contents of lysine and the dicarboxylic acids. Differences in the amino acid compositions are also apparent between the different sericins.

SERICIN is the major protein of the gummy material associated with silk fibroin and is formed within the silk-producing glands. Sericin is non-fibrous and is dissolved and removed from the fibroin by hot water during the process of degumming[1]. The sericin of mulberry silk (*Bombyx mori*) has been studied in some detail[1-3], while little is known of the properties and composition of those of other silks such as tussah, muga and eri. The sericin isolated from mulberry silk has been shown to be heterogeneous containing probably three distinct proteins[3], although there seems to be some doubt as to whether the heterogeneity exists within the silk gland itself or is produced during the extraction procedure[3,4]. The quantitative determinations of particular amino acids in mulberry sericin have been reported by a number of workers[5-9], and qualitative paper chromatograms of the protein hydrolysate have been described by Shaw and Smith[10], Zahn[11] and Bryant[3]. Fukuda[12] has recently reported the analysis of mulberry sericin for its amino acid content by microbiological assay procedures and accounted for approximately 80 per cent of the total nitrogen. The present paper describes the analysis of the sericins of mulberry, tussah, muga and eri silks for their amino acid content.

Experimental procedure

The sericin samples were prepared by degumming raw silk as described by Dunn *et al.*[13]. The nitrogen content in successive extracts of the raw silk samples is presented in Table 1. Other analytical methods were the same as those described in Part I of this series[14], except that the ninhydrin procedure for the estimation of proline was found to be insensitive for the low level of the amino acid in the sericins. The determination of proline by Chinard's method[15] was also found to be inaccurate on account of the high levels of lysine in the sample. Proline was, therefore, estimated in the protein hydrolysates by paper chromatography, using *n*-butanol-acetic acid-water system and isatin for development as described by Pasieka and Morgan[16]. The amino acid was estimated by comparisons with standard runs made with graded concentration of proline. Cystine was estimated by paper chromatography using butanol-acetic acid-water system and ninhydrin as the developing reagent.

Results and discussion

It will be seen from the results given in Table 1 that the different types of raw silks vary considerably in their sericin content; mulberry silk has the highest sericin content (*c.* 21 per cent) followed by tussah and muga silks (*c.* 7-8 per cent), and eri silk has the lowest sericin content (3 per cent). Similar differences between the sericin contents of mulberry and tussah silks have been reported in literature[17]. It will also be evident from the results given in Table 1 that the extractability of the gum from the mulberry and the non-mulberry groups varies markedly, the sericin of mulberry being leached out almost completely with a single extraction, while those of the non-mulberry group require more rigorous and drastic treatment for extraction of gum[18]. In the present investigation it was considered more advantageous to avoid severe conditions during extraction, such as boiling with sodium carbonate or sodium carbonate soap mixture[18], in order to minimize the risk of fibroin degradation by hydrolysis of the more labile peptide bonds[19] during the isolation of sericin.

The amino acid composition of the sericins from the four silks presented in Table 2 shows that the sericins

*Communication No. 512 from the National Chemical Laboratory, Poona.

TABLE 1 — SERICIN EXTRACTED FROM RAW SILK DURING DEGUMMING

(*Amount of raw silk used for extraction, 5 g.*)

Silk	N in raw silk %	N extracted, %				Total N extracted %
		1st ext.	2nd ext.	3rd ext.	4th ext.	
Mulberry	18·34	17·84	1·22	1·00	0·67	20·73
Tussah	18·08	2·79	1·51	1·39	1·03	6·72
Muga	18·75	3·09	2·97	1·26	0·70	8·02
Eri	17·55	0·87	0·80	0·76	0·56	3·00

TABLE 2 — AMINO ACID COMPOSITION OF SERICINS

(*Values expressed as per cent amino acid N/total nitrogen on moisture-free basis*)

Amino acid	Mulberry sericin	Tussah sericin	Muga sericin	Eri sericin
Glycine	9·85	11·61	13·27	18·62
Alanine	3·21	4·67	1·57	2·09
Valine	2·20	4·18	2·46	0·82
Leucine	1·12	2·00	0·60	1·24
Isoleucine	0·67	1·07	0·55	0·60
Serine	19·31	9·86	15·48	7·15
Threonine	5·90	8·84	9·48	2·60
Aspartic acid	10·30	8·11	9·78	6·77
Glutamic acid	2·34	5·50	4·56	3·88
Phenylalanine	0·27	1·56	—	0·49
Tyrosine	1·51	5·40	4·75	4·34
Lysine	26·83	26·38	20·51	26·27
Histidine	5·97	5·05	2·81	3·86
Arginine	1·21	5·47	3·38	14·19
Proline	0·02	0·60	0·30	0·20
Tryptophan	0·24	0·43	0·19	0·16
Cystine	0·01	0·03	0·08	0·00
	90·96	100·76	89·77	93·28

contain the same amino acids as the fibroins but with significant differences in their proportions. The sericins are characterized by a markedly higher content of lysine but lower contents of glycine and alanine as compared to fibroins. Lysine constitutes 20-27 per cent of the total nitrogen in the sericins as compared to 0·05-0·6 per cent in the fibroins. The sericins contain only 13-20 per cent of glycine and alanine nitrogen while these two amino acids contribute 60-75 per cent of the nitrogen in fibroins. Some differences are also apparent in the dicarboxylic and hydroxy amino acids contents, the values being 10-15 per cent and 10-25 per cent of the total nitrogen respectively in the sericins, while these two groups of amino acids are present to the extent of only 2·5-6·0 per cent and 5·5-11·0 per cent respectively in the fibroins.

It is interesting to note that while in silk fibroins the dicarboxylic acids, threonine and lysine constitute only minor proportions of the fibre, they form the major constituents of the sericins. On the contrary, glycine and alanine which together make up the bulk of the fibroins are present in much lower proportions in the sericins. The sericins are similar to the fibroins, however, in having very low contents of imino and sulphur-containing amino acids.

Mulberry sericin is deficient in tyrosine as compared to the non-mulberry sericins, the values being 1·51 per cent for the mulberry sericin and 4·34-5·40 per cent for the non-mulberry sericins. In the case of the fibroins, however, mulberry and the non-mulberry silks have 4·60-5·40 per cent tyrosine contents[14]. Mulberry sericin also contains less of arginine, glycine and glutamic acid than the corresponding non-mulberry sericins. Eri sericin is characterized by a markedly higher amount of arginine than the other sericins. The existence of more than one sericin component in various types of silks has been reported. This factor has to be borne in mind while considering the differences in the amino acid make-up of the gums from raw silk samples.

Acknowledgement

The author wishes to express his gratitude to Drs V. Jagannathan, C. Siva Raman and B. V. Ramachandran for valuable advice.

References

1. Kodama, K., *Biochem. J.*, **20** (1926), 1208.
2. Rouch, W., *Melliand Textilber*, **28** (1947), 307.
3. Bryant, F., *Text. Res. J.*, **22** (1952), 523.
4. Rutherford, H. A. & Harris, M., *Text. Res.*, **10** (1940), 17.
5. Abderhalden, E. & Zumstein, O., *Z. physiol. Chem.*, **207** (1932), 141.
6. Sasaki, S. & Miyauchi, M., *J. Fac. Agr. Kyushu Univ.*, **9** (1940), 167.
7. Lang, O., *Biochem. Z.*, **319** (1949), 283.
8. Nicolet, B. H. & Saidel, L. J., *J. biol. Chem.*, **139** (1941), 477.
9. Ito, T., *J. agri. Chem. Soc., Japan*, **25** (1951), 411.
10. Shaw, J. T. B. & Smith, S. G., *Nature, Lond.*, **168** (1951), 745.
11. Zahn, H., *Melliand Textilber*, **33** (1952), 1076.
12. Fukuda, F., *J. Biochem., Japan*, **42** (1955), No. 3, 341.
13. Dunn, M. S., Camien, M. N., Rockland, L. S. *et al.*, *J. biol. Chem.*, **155** (1944), 591.
14. Dhavalikar, R. S., *J. sci. industr. Res.*, **21C** (1962), 261.
15. Chinard, F. P., *J. biol. Chem.*, **199** (1952), 91.
16. Pasieka, A. F. & Morgan, J. F., *Proc. Soc. exp. Biol., N.Y.*, **93** (1956), 54.
17. Matthews, J. H., *Textile Fibres*, edited by H. R. Mauersberger (John Wiley & Sons Inc., New York), 1954, 792.
18. Lucas, F., Shaw, J. T. B. & Smith, S. G., *J. Text. Inst.*, **46** (1955), T440.
19. Lucas, F., Shaw, J. T. B. & Smith, S. G., *Advanc. Protein Chem.*, **13** (1958), 127.

Antitubercular Activity of Capparis Fruits

(Miss) AMINA E. BUNDEALLY, M. H. SHAH,
R. A. BELLARE & C. V. DELIWALA
Department of Chemotherapy, Haffkine Institute, Bombay

Manuscript received 4 *June* 1962

Preparations of Konkan and Khandala varieties of Capparis (*Capparis moonii* Wight) fruits, viz. aqueous extracts of fresh fruits, dried whole fruits, dried fruit chips, dried fruits powdered and stored for one year, Rudanil tablets (a proprietary product stated to contain 300 mg. of Konkan variety Capparis fruits per tablet) and the juice of fresh fruits have been examined for *in vitro* antitubercular activity. Activity of a low order is present in the fresh fruit juice, aqueous extracts of the fruits and dried whole fruits. Although heat stable, the activity is reduced or lost on storage of the fruits in the form of chips or powder. Similar results have been obtained in serum antitubercular activity studies in guinea-pigs fed with these preparations. The feeding of dried whole Capparis fruits to guinea-pigs experimentally infected with tuberculosis, however, fails to show any curative action.

IN 1958 Krishna Murty[1] described favourable results with *Capparis moonii* fruits (under the name *Rudanti*) in the clinical treatment of tuberculosis. In a subsequent paper, Sheth and Krishna Murty[2] recorded two different varieties, one grown around Khandala (Maharashtra State) and the other in Mysore State (Konkan variety). These two varieties were reported to differ in the shape and size of their fruits, leaves, etc., as well as in their antitubercular activity, but both were called by these authors as *Capparis moonii*. In 1959 Shah and Sukkawala[3] published the results of pharmacognostic study of *Rudanti* fruits but did not distinguish between the two varieties (Khandala and Konkan). These facts were discussed by two of us (Miss A. E. Bundeally and R. A. Bellare) in a previous communication[4] wherein it was reported that the serum of guinea-pigs fed with Konkan variety of dried Capparis fruits showed antitubercular action while the serum of guinea-pigs fed with the Khandala variety did not show such activity. Recently, Shah and Sukkawala[5] have identified the Khandala variety as *Capparis moonii* Wight and the Konkan variety as *Capparis horrida* Linn.*.

The present work describes the *in vitro* tuberculostatic activity of the different Capparis fruit preparations, tuberculostatic activity of the sera of guinea-pigs fed with these different preparations and the effect of feeding the Konkan variety of dried Capparis fruits on the experimental tuberculosis of guinea-pigs.

Materials and methods

In vitro antitubercular testing was carried out in Youmans' medium containing 10 per cent horse serum, using human virulent strain (H37Rv) of *Mycobacterium tuberculosis*, according to the method of Doub and Youmans[6]. Different dilutions of test solutions were added aseptically (after Seitz filtering or autoclaving) to sterile media contained in borosilicate test tubes (150 × 20 mm.) to give a final volume of 4·9 ml. The tubes were then seeded with 0·1 ml. of an inoculum, prepared by suspending in Youmans' basic medium a loopful of 7 days growth of *M. tuberculosis* on Loewenstein Jensen slant and adjusting the opacity of the suspension to Browns opacity tube No. 4 (approximately 10^6 bacilli per ml.) and incubated at 37°C. for 21 days.

Preliminary trials with different solvent extracts of Capparis fruits showed that only the aqueous extracts show tuberculostatic activity, and, therefore, all the subsequent work was done with aqueous extracts, which were prepared as follows:

1. Fresh fruit juice of Capparis fruits (Khandala variety) was collected during the preparation of chips from the fruit. The juice thus obtained was stored in a refrigerator. It contained 6·5 per cent solids.

2. Fresh Capparis fruits (Khandala variety; stored in a refrigerator for about one week; 600 g.)

*Just prior to sending this paper for publication, we were able to get the help of Shri R. S. Rao, Regional Botanist, Botanical Survey of India, Western Circle, Poona, to identify the Konkan variety of the fruits used by us. These have been identified as *Capparis moonii* Wight, i.e. same as Khandala variety and not *Capparis horrida* Linn., as stated by Shah and Sukkawala[5].

were comminuted and boiled with 1 litre distilled water for 45 min., cooled to room temperature, kept overnight at 0°C. and filtered through muslin cloth. The clear brown solution contained 3·58 per cent of solid matter.

3. Dried chips of Capparis fruits (Khandala variety; 600 g.) on treatment with 1 litre of water as above became swollen and gave very little filtrate. Therefore, additional 500 ml. water were added for extraction. The filtrate contained 5 per cent solids.

4. Dried whole Capparis fruits (Konkan variety; 600 g.), finely powdered, on boiling with 1·5 litres water gave a filtrate containing 14·9 per cent solids.

5. Dried Capparis fruits (Konkan variety), which was stored for one year after powdering (600 g.), on extraction with 1·5 litres by boiling, etc. (as in 2), gave an extract containing 4·0 per cent solids.

6. Rudanil tablets (stated to contain 300 mg. of *Capparis moonii* fruit of Konkan variety/tablet), 33-1/3, equivalent to 10 g. of fruits, on extraction with 50 ml. water gave a filtrate containing 8·4 per cent solids. (The use of water in the same proportion as used for dried fruits gave a paste, probably due to the presence of binding material used for tabletting.)

All the above aqueous extracts were divided into two parts; one part was autoclaved at 15 lb./sq. in. pressure for 15 min. and the other was seitz-filtered, and were separately added to the tubes containing media at the following dilutions: 2:5, 1:5, 1:10, 1:25, 1:50 and 1:100.

Serum antitubercular activity was tested as described previously[4]. Solutions 1 to 5 mentioned above were thoroughly mixed with the animal stock diet (consisting of wheat bran and germinated grams) to give a final concentration of 10 per cent (vol./wt). The powdered fruits, Rudanil tablets as well as the dried residue obtained after extraction of fruits with water were mixed with the stock diet to give a final concentration of 5 per cent (wt/wt). The diets so prepared, containing the various Capparis products, were separately fed to groups of guinea-pigs *ad libitum*. After various intervals (in days) the sera collected from the animals were pooled, added to Youmans' basic medium, inoculated with *M. tuberculosis* and incubated for 21 days at 37°C.

Experimental tuberculosis of guinea-pigs — Guinea-pigs, Haffkine Institute breed, weighing 350-400 g., were tuberculin tested with 100 units of old tuberculin. All animals giving negative test were taken for the experiment and caged in groups of five. Fifteen animals from these were infected intraperitoneally with 5 ml. of a suspension of *M. tuberculosis* (H37Rv) in physiological saline, containing about 10^6 organisms per ml. Five animals were kept as normal, non-infected controls. After 23 days, fifteen infected animals were again tuberculin tested and all were found to give highly positive tuberculin reaction. These fifteen animals were divided into three groups of five each.

Group I was treated with Konkan variety of Capparis fruits, administered by incorporating the powdered fruits with the stock diet at a concentration of 5 per cent by weight. Group II was kept as a standard drug control by feeding the animals of this group with a diet containing isonicotinic acid hydrazide at a concentration of 0·1 per cent (wt/wt). Group III constituted the untreated, infected control.

The drug-diet was fed *ad libitum* for 60 days. During this period the animals were weighed twice, 30 and 60 days after starting the drug treatment. After 60 days of treatment all the animals were sacrificed. The autopsy findings with respect to gross appearance of the lungs, liver, spleen and omentum were recorded according to the method described by Soltys[7]. These organs were also weighed according to the method of Gupta *et al.*[8] and studied histopathologically.

Results

In vitro study — From the results given in Table 1 it can be seen that the fresh fruit juice of Khandala variety and the aqueous extract of the dried whole fruits of Konkan variety inhibit the growth of *M. tuberculosis* up to a dilution of 1:25. The extracts from fresh Capparis fruits (Khandala) and Rudanil tablets inhibited the growth up to a dilution of 1:5 only indicating much poorer action, while the extracts of dried chips of fruits from Khandala or the powder of Konkan variety of dried fruits that had been stored in powdered condition for one year showed no inhibiting action against tubercle bacilli even at the highest concentration tested.

Serum tuberculostatic activity — The results given in Table 2 show that the growth of *M. tuberculosis* (H37Rv strain) was completely inhibited only by the sera of animals fed with diet containing fresh fruit juice (Khandala) and freshly powdered dried fruits of Konkan variety at the drug concentration used.

Effect of feeding dried whole Capparis fruits (Konkan) on the experimental tuberculosis of guinea-pigs — In Table 3 are presented data on the gross appearance, average weights of the organs and omental index of the three groups of infected animals in comparison to normal, uninfected animals. It is seen that the weights and gross appearance of all the organs, except the omentum, of the isonicotinic

TABLE 1 — *IN VITRO* ANTITUBERCULAR ACTIVITY OF DIFFERENT CAPPARIS FRUIT PREPARATIONS

Nature of preparation	Highest dilution inhibiting growth of H37Rv strain		Inhibiting conc. in terms of solid content of the preparation
	Seitz filtered	Autoclaved	
Fresh juice of Capparis fruits (Khandala variety)	1 : 25	1 : 25	2·6 mg./ml.
Aqueous extract of fresh Capparis fruits (Khandala variety)	1 : 5	1 : 5	7·2 mg./ml.
Aqueous extract of dried chipped fruits (Khandala variety)	No inhibition even at 2 : 5	No inhibition even at 2 : 5	No inhibition up to 20 mg./ml.
Aqueous extract of whole dried Capparis fruits (Konkan variety)	1 : 25	1 : 25	5·96 mg./ml.
Aqueous extract of dried whole Capparis fruits (Konkan) powdered and stored for one year	No inhibition even at 2 : 5	No inhibition even at 2 : 5	No inhibition up to 16 mg./ml.
Aqueous extract of Rudanil tablets	1 : 5	1 : 5	16·87 mg./ml.
Isonicotinic acid hydrazide	1 : 25,000,000	1 : 25,000,000	0·04 µ/ml.

TABLE 2 — SERUM ANTITUBERCULAR ACTIVITY OF GUINEA-PIGS FED ON DIET CONTAINING DIFFERENT CAPPARIS FRUIT PREPARATIONS

Nature of preparation	Conc. of preparation in diet	*In vitro* growth of H37Rv in Youmans' medium containing 10% (vol./vol.) of serum from guinea-pigs receiving the drug-diet for				
		Days: 3	7	14	21	28
Dried whole Capparis fruits (Konkan)	5% w/w	++++	++	nil	nil	nil
Dried chips of Capparis fruits (Khandala)	5% w/w	++++	++++	++++	++++	++
Rudanil tablets	5% w/w	++++	++++	++++	++++	++++
Fruit juice of fresh Capparis fruits (Khandala)	10% v/w	++++	++++	++++	++++	nil
Aqueous extract of dried whole Capparis fruits (Konkan)	10% v/w	++++	++++	++++	++	++
Aqueous extract of fresh Capparis fruits (Khandala)	10% v/w	++++	++++	++++	++++	++
Aqueous extract of Rudanil tablets	10% v/w	++++	++++	++++	++++	++++
Residue of dried chips of Capparis fruits (Khandala) after aqueous extraction	5% w/w	++++	++++	++++	++++	++++
Residue of dried whole Capparis fruits (Konkan) after aqueous extraction	5% w/w	++++	++++	++++	+++	+++

Nil, no growth; +, slight growth; ++, moderate growth; ++++, maximum growth.

TABLE 3 — EXTENT OF INVOLVEMENT OF ORGANS IN THE EXPERIMENTAL TUBERCULOSIS OF GUINEA-PIGS BY THE INCORPORATION OF CAPPARIS FRUITS (5%) AND INH (0·1%) IN THE DIET

Organ	Non-infected controls		Infected, untreated controls		Infected, treated with INH (0·1%)		Infected, treated with Capparis fruits (5%)	
	Gross appearance	Weight *g.*	Gross appearance	Weight *g.*	Gross appearance	Weight *g.*	Gross appearance	Weight *g.*
Lungs	Normal	4·12	++++	6·75	Normal	5·61	++	4·88
Liver	do	15·67	++++	35·81	do	21·81	++++	38·72
Spleen	do	0·8	++++	5·06	do	0·8	++++	6·42
Omentum	do	1·8	++++	8·45	+	2·92	+	3·42
Omental index	3·54		18·78		6·21		7·43	

+, organ involvement less than 10 per cent; ++, organ involvement more than 10 per cent but less than 25 per cent; +++, organ involvement more than 25 per cent but less than 50 per cent; ++++, organ involvement over 50 per cent.
Omental index = the ratio of the weight of the excised omentum in grams to the weight of the animal in kilograms.

acid hydrazide-treated animals were similar to normal, non-infected controls, indicating that it prevented the spread of the disease. The omentum, which was thick, white and fibrous, indicated that the disease was in the process of being checked. In the group of animals treated with dried Capparis fruits (Konkan), the gross appearance of all the organs, except the omentum, indicated the same state of spread of infection as in the infected, untreated control animals. The spleen was highly enlarged and discoloured. The liver was also enlarged and studded with tubercles. The lungs were

hardened, greyish in colour and full of tubercles. Only the omentum of these animals showed the least involvement (similar in appearance to that of the animals treated with INH) whereas the omentum of the infected, untreated animals was highly enlarged and contained bunches of yellow tubercular nodules.

Histopathological examination revealed that all the organs of animals treated with Capparis fruits showed signs of progressive tuberculosis similar to those observed in the infected untreated controls. Nothing abnormal was noted in the organs of INH-treated animals except that the omentum showed slight evidence of tuberculosis.

Discussion

In our previous communication[4] it was shown that the serum of guinea-pigs fed on a diet containing *Capparis moonii* (Konkan variety) possessed antitubercular action while this could not be demonstrated with the sample of Capparis fruits of Khandala variety received by us. There was, however, a difference in the mode of drying of the fruits of the two varieties. In Konkan variety the whole fruit was dried but in the Khandala variety the fruits were cut into small chips and then dried. Our subsequent work with the active Konkan variety having shown that the activity was lost on storage of the powdered fruits for a long time, it was necessary to know whether the fruits of Khandala variety did not possess any activity at all or whether the activity originally present was lost due to drying in chopped condition. Previous workers[9,10] investigating the different solvent extracts reported no *in vitro* tuberculostatic activity in Capparis fruits. We have shown that aqueous extracts of the fruits show *in vitro* antitubercular action. This action is found not only in the dried whole fruits of Konkan variety but also in the fresh fruits (juice and water extract) of Khandala variety. The residue remaining after the aqueous extraction was found to be devoid of antitubercular activity as tested by feeding to guinea-pigs and studying their serum for inhibition of *M. tuberculosis*. Heat-sterilized and seitz-filtered samples of extracts inhibited the growth at the same dilutions, indicating that the active material is heat stable, but the activity was reduced and lost on long storage of powdered fruits. The content of water-extractable components in the powdered and stored fruits was also found to have decreased in comparison to the fruits stored whole, possibly due to polymerization of the active material. Rudanil tablets (stated to be prepared from Konkan variety of *Capparis moonii* fruits and recommended by the manufacturers for the treatment of tuberculosis) showed poor *in vitro* antitubercular action and the serum of guinea-pigs fed with these tablets or their aqueous extract did not inhibit the growth of *M. tuberculosis* whereas the whole dried fruits of Konkan showed activity by *in vitro* and the serum inhibition tests. However, the dried whole Capparis fruits (Konkan) when fed to guinea-pigs infected with *M. tuberculosis* failed to show any curative action, which is in agreement with the findings of Prof. G. N. Pershin[11] using white mice. In view of the failure of Capparis fruits to show any action on the experimental tuberculosis of laboratory animals, even when fed at a high dosage level, and the low order of *in vitro* activity in comparison to better known antitubercular drugs like INH, streptomycin, etc., the use of Capparis fruits for the treatment of tuberculosis does not appear justifiable.

Acknowledgement

We are thankful to Shri A. V. Modi of Unichem Laboratories for the generous supply of dried whole Capparis fruits (Konkan variety), to Messrs R. S. Vats & Sons of Khandala for the supply of fresh fruits and juice of Capparis (Khandala) and to Dr G. Krishna Murty and Dr S. C. Sheth for making available Rudanil tablets. One of the authors (Miss A.E.B.) is grateful to Dr S. S. Rao for guidance and valuable suggestions. The authors also wish to express thanks to Shri R. S. Rao for the identification of Capparis fruits (Konkan), Dr M. V. Sant for histopathological examinations and to Dr H. I. Jhala, Director, Haffkine Institute, for encouragement and interest in the work.

References

1. Murty, G. K., *Curr. med. Pract., India*, **2** (1958), 117.
2. Sheth, S. C. & Murty, G. K., *Curr. med. Pract., India*, **3** (1959), 179.
3. Shah, C. S. & Sukkawala, V. M., *Indian J. Pharm.*, **21** (1959), 305.
4. Bundeally, A. E. & Bellare, R. A., *J. sci. industr. Res.*, **19C** (1960), 128.
5. Shah, C. S. & Sukkawala, V. M., *J. sci. industr. Res.*, **20C** (1961), 100.
6. Doub, L. & Youmans, G. P., *Amer. Rev. Tuberc.*, **61** (1950), 407.
7. Soltys, M. A., *Tubercle Bacillus and Laboratory Methods in Tuberculosis* (E. & S. Livingstone Ltd, Edinburgh and London), 1952, 172.
8. Gupta, S. K. & Sen, N., *Indian J. med. Res.*, **47** (1959), 382.
9. Bhatnagar, S. S., *Symposium on Chemotherapy* (Council of Scientific & Industrial Research, New Delhi), 1958, 12.
10. Gupta, S. K. & Mukerji, B., *Symposium on Chemotherapy* (Council of Scientific & Industrial Research, New Delhi), 1958, 90.
11. Pershin, G. N., cf. Kanthamani, S., Narayanan, C. R. & Venkataraman, K., *J. sci. industr. Res.*, **19B** (1960), 409.

Effect of Quinazolones, Substituted Hydroxyquinolines, Substituted Diamines, Purine & Nucleoside Antagonists on Vaccinia Virus in Chick Embryo

UMA AGARWAL, B. M. GUPTA, S. K. KHAN, I. CLIFFORD & K. CHANDRA
Central Drug Research Institute, Lucknow

Manuscript received 25 *June* 1962

Forty-five compounds comprising quinazolones, substituted hydroxyquinolines, substituted diamines, purine and nucleoside antagonists have been tested against vaccinia virus (Bangalore strain) in chick embryo. 8-Hydroxy-4-quinazolone has been found to give the best results followed by 3-deazapurine riboside and 5-chloro-7-iodo-8-hydroxyquinoline in suppressing pock formation and in reducing the mortality of chick embryos.

IN a previous communication 8-hydroxy-4-quinazolone, a potential amoebicide synthesized at this Institute, was reported to be effective in suppressing vaccinial pock formation in chorioallantoic membrane (CAM) of chick embryo[1]. In view of this finding it was decided to test more compounds of this series and of other series prepared in this Institute for antiviral activity. This paper presents results of tests done with 45 compounds.

Materials and methods

Drugs — The water-soluble compounds were dissolved in glass distilled water (*p*H 7·2). Insoluble drugs were triturated in pestle and mortar to get a fine suspension in water. Toxicity of all the 45 drugs (Table 1) was then assessed by mortality of embryo caused by the aqueous suspension or solution of 2 mg. of the substance in 72 hr. The drugs which proved non-toxic by this standard were then tested for antiviral activity. Maximum tolerated dose was defined as maximum quantity which when administered into CAM did not cause mortality of chick embryo in 72 hr.

Virus infection — Egg-adapted vaccinia virus (Bangalore strain) obtained from the Vaccine Institute, Bangalore, was passaged serially several times on CAM of the chick embryo. The stock virus[2] having $2{\cdot}5\times10^{14}$ PFU/ml. was diluted ten-fold in 0·01*M* phosphate buffer saline (*p*H 7·2) and 0·1 ml. of the diluted virus was inoculated on the dropped CAM of 10-12 days old chick embryo. After 72 hr of incubation at 37°C., the number of pocks were counted. The mean pock count on six membranes was determined.

Antiviral assay — Eggs were inoculated on the dropped CAM of fertile 10-12 days old chick embryo first with the appropriate virus dilution that caused discrete pocks, followed by 0·2 ml. of drug suspension. The antiviral effect of different concentrations of the drug was judged by their ability to suppress pock formation caused by the multiplication of the inoculated virus. The virus activity in presence of a drug is expressed as logarithm of the factor by which initial pock count in chorioallantois is reduced, and is described by the equation, log N—log (N—X), where N is the initial count and X the final (Table 2). A compound was considered partially effective if it saved at least 30 per cent of the infected eggs, or permitted log virus activity not exceeding 0·221.

Results

Out of the 45 compounds tested, 21 were non-toxic (Table 1). Of the 21 non-toxic compounds, only 3-di-*n*-heptylaminoethyl-8-hydroxy-4-quinazolone, 3-β-dimethylaminoethyl-4-quinazolone allyl bromide, 5-chloro-7-iodo-8-hydroxyquinoline, 3-deazapurine riboside, pyridine-4-hexylamide; α-methyl-β-(3,4-methylenedioxyphenyl) and 2-(2′-hydroxyphenyl)-2-thiazoline-4-carboxylic acid, proved partially effective in suppressing the pock formation (Table 2) and in reducing the death of the embryos. However, complete absence of pocks was not observed with any of these compounds. 5-Chloro-7-iodo-8-hydroxyquinoline was the most effective among all these compounds. This compound was able to reduce mortality caused by lethal dose of virus by about 70 per cent (Table 3); it is

TABLE 1 — TOXICITY OF DIFFERENT COMPOUNDS TESTED

No.	Code No.	Compound	Solubility in water	Toxicity 2 *mg./embryo*
		QUINAZOLONES		
1	I148	3-Di-*n*-heptylaminoethyl-8-hydroxy-4-quinazolone	WI	Non-toxic
2	BK1	3-β-Dimethylaminoethyl-4-quinazolone	WI	Toxic
3	BK2	3-β-Di-*n*-butylaminoethyl-quinazolo-4-thione	WI	Non-toxic
4	BK3	3-γ-Di-*n*-butylaminopropyl-4-quinazolone	WI	Toxic
5	BK4	3-β-Di-isobutylaminoethyl-4-quinazolonephenyl ethyl bromide	WI	Non-toxic
6	BK5	3-β-Dimethylaminoethyl-4-quinazolone benzbromide	WI	Toxic
7	BK6	3-β-Dimethylaminoethyl-4-quinazolone allyl bromide	WS	Non-toxic
8	BK7	3-γ-Diethylaminopropyl-4-quinazolone benzbromide	WS	Toxic
9	BK8	3-β-Di-isobutylaminoethyl-4-quinazolone methiodide	WS	do
10	BK9	3-β-Dimethylaminoethyl-4-quinazolonephenylethyl bromide	WS	do
11	BK10	3-β-Dimethylaminoethyl-4-quinazolone-*p*-nitrobenzyl bromide	WS	do
12	BK11	3-β-Di-*n*-propylaminoethyl-4-quinazolone	WI	do
13	BK12	3-γ-Di-*n*-amylaminopropyl-4-quinazolone	WI	do
14	BK13	3-β-Dimethylaminoethyl-4-quinazolone-*n*-butyl iodide	WS	Non-toxic
15	BK14	3-β-Diethylaminoethyl-quinazolo-4-thione	WI	do
16	BK15	3-γ-Diethylaminopropyl-quinazolo-4-thione	WI	Toxic
		SUBSTITUTED HYDROXYQUINOLINES		
17	QV1	5-Chloro-7-iodo-8-hydroxyquinoline	WI	Non-toxic
18	QV2	5-Hydroxyquinoline	WI	Toxic
19	QV3	4-Hydroxyquinoline	WI	do
20	QV4	8-Hydroxyquinoline	WI	Non-toxic
		SUBSTITUTED DIAMINES		
21	P1	N-Ethyl-3,4-dimethoxybenzylamino-5-tetrahydrofurfuryl aminopentane	WI	Toxic
22	P2	1-Piperidino-5-3,4-diethoxybenzylaminopentane	WI	do
23	P3	1-Piperidino-5-ethylaminopentane	WI	do
24	P5	*pp'*-Di-*p*-methoxybenzylidiene amino-*mm'*-dimethyldiphenyl methane	WI	Non-toxic
25	P6	*pp'*-Dimethylamino-*mm'*-dimethyldiphenyl methane	WI	do
26	P7	*pp'*-Di-veratrylamino-*mm'*-dimethyldiphenyl methane	WI	do
27	P8	*pp'*-Di-vanillylamino-*mm'*-dimethyldiphenyl methane	WI	do
28	P9	*pp'*-Propylbutylamino-*mm'*-dimethyldiphenyl methane	WI	Toxic
29	P10	N-1-(3,4-Dimethoxyphenylethyl)-4-(6,7-dimethoxy-1,2,3,4-tetrahydro-isoquinolyl)-piperidine-2HCl	WS	Non-toxic
30	P14	1-(N-Methyl)-4-(phenylethyl propionamido) piperidine	WI	Toxic
31	P15	1-(N-Phenylethyl)-4-(phenylethyl carboxamido) piperidine	WI	do
32	P16	1-(N-3,4-Dimethoxyphenylethyl)-4-(3,4-dimethoxyphenylethyl carboxamido) piperidine	WI	do
		INH DERIVATIVES		
33	P4	INH-cholest-4-en-3-one	WI	Non-toxic
34	P12	N2-Pipiridino-isonicotinic acid hydrazide	WS	Toxic
35	P13	N2-Tetrahydro pyrrolo-isonicotinic acid hydrazide	WS	do
		PURINE ANTAGONISTS		
36	SC6	Pyrido(b)triazole	WI	Non-toxic
37	SC7	2-(α-Hydroxybenzyl)-imidazo pyridine	WI	do
		NUCLEOSIDE ANALOGUES		
38	PC2	1-Chloro-1-deazapurine riboside	WI	Non-toxic
39	PC4	1-Bromo-1-deazapurine riboside	WI	Toxic
40	SC5	3-Deazapurine riboside	WS	Non-toxic
		MISCELLANEOUS		
41	I175	3-Piperidinomethyl-4-hydroxyquinaldine	WI	Toxic
42	CN2	2-Piperidyl-3-phenyl propanol	WS	do
43	P11	Pyridine-4-hexylamide	WI	Non-toxic
44	P17	α-Methyl-β-(3,4-methylenedioxyphenyl)	WI	do
45	M14	2-(2'-Hydroxyphenyl)-2-thiazoline-4-carboxylic acid	WS	do

WI, water insoluble; WS, water soluble.

TABLE 2 — ANTIVACCINAL ACTIVITY OF SOME SELECTED CHEMICALS

(Activity expressed by logarithm of the factor by which initial pock count in CAM is reduced)

No.	Code No.	Compound	M.T.D.* *mg./egg*	Dose *mg./egg*	No. of eggs	Virus activity *log N − log (N − X)*
1	I148	3-Di-*n*-heptylaminoethyl-8-hydroxy-4-quinazolone	>10	2·5	4	0·669
				5·0	4	0·448
				7·5	6	0·266
				10·0	6	0·105
2	BK6	3-β-Dimethylaminoethyl-4-quinazolone allyl bromide	7·5	2·5	4	0·698
				5·0	4	0·443
				7·5	8	0·221
3	QV1	5-Chloro-7-iodo-8-hydroxyquinoline	>7·5	2·5	4	0·397
				5·0	6	0·167
				7·5	6	0·055
4	SC5	3-Deazapurine riboside	>10	2·5	4	0·619
				5·0	6	0·397
				7·5	6	0·221
				10·0	—	—
5	P11	Pyridine-4-hexylamide	10	2·5	4	0·552
				5·0	6	0·318
				7·5	6	0·193
				10·0	6	0·142
6	P17	α Methyl-β-(3,4-methylenedioxyphenyl)	>10	2·5	4	0·544
				5·0	4	0·368
				7·5	6	0·222
				10·0	8	0·129
7	M14	2-(2′-Hydroxyphenyl)-2-thiazoline-4-carboxylic acid	>7·5	2·5	4	0·397
				5·0	6	0·221
				7·5	6	0·119
8	I8	8-Hydroxy-4-quinazolone†	>10	2·5	6	0·398
				5·0	6	0·114
				7·5	6	0·023
				10·0	6	0·005

*Maximum tolerated dose. †Standard.

TABLE 3 — EFFECT OF SOME SELECTED CHEMICALS ON POCK TITRE AND MORTALITY OF CHICK EMBRYO INOCULATED WITH LETHAL DOSE OF VACCINIA VIRUS

(Virus conc. employed, 10^{-8} obtained by diluting the stock virus)

No.	Code No.	Compound	Without drug		Drug conc. *mg./embryo*	With drug	
			Mortality*	Mean pock count		Mortality	Mean pock count
1	I148	3-Di-*n*-heptylaminoethyl-8-hydroxy-4-quinazolone	6/6	Cp	10·0	3/6	20
2	BK6	3-β-Dimethylaminoethyl-4-quinazolone allyl bromide	6/6	Cp	7·5	4/6	25
3	QV1	5-Chloro-7-iodo-8-hydroxyquinoline	6/6	Cp	7·5	2/6	18
4	SC5	3-Deazapurine riboside	6/6	Cp	10·0	3/6	22
5	P11	Pyridine-4-hexylamide	6/6	Cp	10·0	4/6	30
6	P17	α-Methyl-β-(3,4-methylenedioxyphenyl)	6/6	Cp	10·0	3/6	25
7	M14	2-(2′-Hydroxyphenyl)-2-thiazoline-4-carboxylic acid	6/6	Cp	7·5	3/6	23

*Mortality/number inoculated. Cp: confluent pocks.

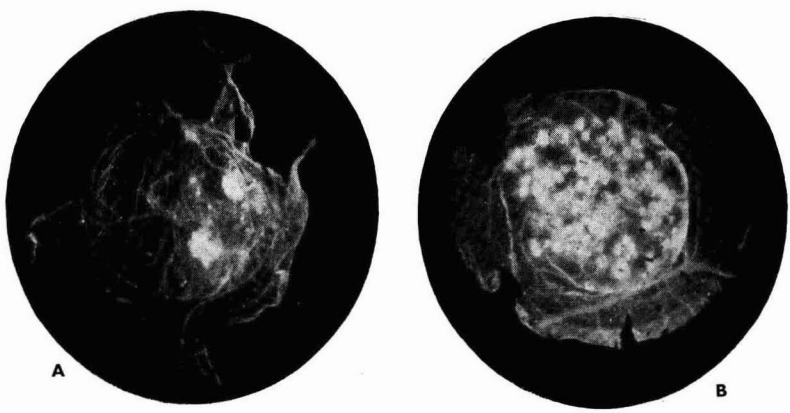

Fig. 1 — Effect of 8-hydroxy-4-quinazolone on vaccinia virus [(A) Effect of 7·5 mg. of the drug on pock formation in chick CAM; (B) control (no drug)]

one-tenth less active than 8-hydroxy-4-quinazolone (Table 3, Fig. 1).

Discussion

A large series of analogues of purine and pyrimidine bases and related substances, various substituted amino acids, halogenated purines, benzimidazoles have been tested in tissue culture, mouse and chick embryo for their ability to interfere with multiplication of vaccinia virus. Many of these substances, quite unrelated to each other, have been demonstrated to have high activity[1,3-5]. Quite recently certain derivatives of thiosemicarbazone have given extremely encouraging results in mice infected intracerebrally with neurovaccinia virus[6]. It was reported from this Institute that 8-hydroxy-4-quinazolone, originally synthesized as a potential amoebicide, causes significant decrease in pock count[1]. In this paper seven new compounds have been shown to possess the ability to suppress viral pock formation and in reducing mortality of chick embryos.

Acknowledgement

The authors wish to thank Dr B. Mukerji and Dr B. N. Singh for their interest in the work. Thanks are also due to Dr V. N. Krishnamurthy of the Vaccine Institute, Bangalore, for the supply of egg-adapted Bangalore strain of vaccinia virus.

References

1. GUPTA, B. M., KHAN, S. K. & AGARWAL, U., *J. sci. industr. Res.*, **21C** (1962), 189.
2. AGARWAL, U. & GUPTA, B. M., *J. sci. industr. Res.*, **20C** (1961), 43.
3. HURST, E. W., *Brit. med. Bull.*, **9** (1953), 180.
4. TAMM, I. & OVERMAN, J. R., *Virology*, **3** (1957), 185.
5. MATTHEWS, R. E. F. & SMITH, J. D., *Advanc. Virus Res.*, **3** (1955), 51.
6. BAUER, D. J. & SADLER, P. W., *Lancet*, i (1960), 1110.

Further Studies on the Antiviral Effect of 3-β-D-Ribofuranosyl Imidazo(b)pyridine* on Ranikhet Disease Virus

O. P. BABBAR & B. L. CHOWDHURY
Central Drug Research Institute, Lucknow

Manuscript received 3 *July* 1962

It has been shown that 3-β-D-ribofuranosyl imidazo(b)pyridine had no antiviral activity against Ranikhet disease virus *in vitro*, but it inhibits virus multiplication in CAM cultures completely, provided it is given 1-2 hr before or after infection. In chick embryos this compound (3 mg./embryo) stops virus multiplication completely and gives 100 per cent protection to the embryos when the virus and the drug are given together. When the compound is administered 1 or 2 hr after infection it gives 100 per cent protection to the embryos and there is marked inhibition of viral multiplication. The compound has no antiviral effect when given 5 hr after infection.

IT was shown[1] that 3-β-D-ribofuranosyl imidazo-(b)pyridine (1-deaza-7-β-D-ribofuranosyl purine) inhibited Ranikhet disease virus multiplication in stationary chorioallantoic membrane (CAM) cultures. This communication reports the results of further studies of the antiviral properties of this compound and related ribofuranosyl imidazo pyridine against Ranikhet disease virus.

Materials and methods

The strain of Ranikhet disease virus[1] used and the methods of testing the antiviral properties of compounds in chick embryos and in stationary

*Previously named as 1-deaza-7-β-D-ribofuranosyl purine[2].

cultures of minced chorioallantoic membranes are given in the earlier publications[1-3].

Results

Twenty batches of CAM cultures[1] (six cultures/batch) were divided into two groups of ten each. One group was infected with 0·00032 HA unit of the virus/culture and the other with 0·032 HA unit/culture. Nine batches of CAM cultures in each group were given, along with the virus, different concentrations of the compound (Table 1). The remaining two batches served as the virus control. The cultures were incubated for 48 hr. The multiplication of the virus was measured by haemagglutination (HA) titre of the culture fluid collected after 48 hr. 0·14 mg. of the compound completely inhibited virus multiplication when 0·00032 HA unit/culture of the virus was inoculated (Table 1). When the virus inoculum was increased to 0·032 HA unit/culture, 0·625 mg. of the compound was needed to inhibit virus multiplication completely (Table 1). The culture fluids, which gave no titre (Table 1), were non-infective when passaged serially in CAM cultures.

In order to investigate the *in vitro* effect of the compound on the virus, equal volumes of the virus suspension (0·064 or 0·00064 HA unit/ml.) and the drug dilution (1·25 mg. and 2·5 mg./ml.) were mixed and incubated for 3 hr at 35°C. One ml. of 10^{-2} dilution of the mixture was inoculated into each of the six CAM cultures. After 1 hr, the membranes were washed, fresh nutrients added and incubated for 48 hr. The HA titres of the culture fluids showed that the *in vitro* treatment had no effect on the multiplication of the virus (Table 2).

TABLE 1 — EFFECT OF 3-β-D-RIBOFURANOSYL IMIDAZO(b)PYRIDINE ON THE MULTIPLICATION OF RANIKHET DISEASE VIRUS IN CAM CULTURE

Conc. of compound *mg./ml.*	Virus multiplication in CAM cultures infected with	
	0·032 HA unit/culture	0·00032 HA unit/culture
0·0035	1·53*	1·25*
0·0070	1·53	0·30
0·0140	1·53	0·00†
0·0280	1·53	0·00†
0·0560	1·61	0·00
0·312	0·30	0·00
0·625	0·00†	0·00
1·250	0·00†	0·00
2·500	0·00	0·00
0 (virus control)	1·53	1·35

*HA titre (mean log value).
†10^{-2} dilution of culture fluid was passaged serially in CAM cultures and was found to be non-infective.

TABLE 2 — ANTIVIRAL EFFECT OF 3-β-D-RIBOFURANOSYL IMIDAZO(b)PYRIDINE AGAINST RANIKHET DISEASE VIRUS *IN VITRO* AND IN THE CAM CULTURES

Conc. of compound *mg./ml.*	Interval between virus infection and compound administration *hr*	Virus multiplication in CAM culture treated with compound: After virus infection	Virus multiplication in CAM culture treated with compound: Before virus infection	Multiplication of *in vitro* treated virus in CAM culture
CONC. OF VIRUS: 0·032 HA UNIT/CULTURE				
0·625	1	0*	0	1·26*
	2	0	0†	
	3	1·26		
1·25	1	0		1·26
	2	0		
	3	1·26		
2·5	1	0	0	
	2	0	0†	
	3	1·26		
CONC. OF VIRUS: 0·00032 HA UNIT/CULTURE				
0·625	1	0	0	1·25
	2	0	0	
	3	1·25		
1·25	1	0		1·35
	2	0		
	3	1·35		
2·5	1	0	0	
	2	0	0	
	3	1·35		
Virus control		1·35	1·35	1·35

*HA titre (mean log value).
†CAM cultures treated with the compound were washed and incubated for 4 hr with fresh nutrient medium before infecting with the virus.

Six CAM cultures were incubated for 1 or 2 hr in the presence of 0·625 mg. or 0·25 mg. or 2·5 mg./ml. of the compound. The membranes were washed thoroughly and infected with virus (0·032 or 0·00032 HA unit/culture), either immediately or after incubation for 4 hr. The culture fluids were collected after 48 hr. The HA titres showed that there was no demonstrable multiplication of the virus in the CAM cultures. This suggests that the compound is taken up by the cells, and its presence interferes with the multiplication of the virus.

Six CAM cultures infected with virus (0·032 or 0·00032 HA unit/culture) were incubated for 1, 2 and 3 hr (Table 2). The CAM pieces were washed thoroughly to get rid of the unadsorbed virus particles and incubated for 48 hr after adding fresh nutrient fluid containing the compound. The compound inhibited the multiplication of the virus when given 1 or 2 hr after infection (Table 2). It

had no effect on virus multiplication when given after 3 hr of infection.

Ten to eleven days old chick embryos (WLH) were infected with virus (0·000032 HA unit/embryo) by the method described earlier[3] and then the compound was injected (1·25, 2·5 and 3·0 mg./embryo) into the allantoic sac at different intervals. The antiviral effect of the compound was judged by the percentage mortality[3] of the embryos and HA titre of the allantoic fluid (Table 3). The embryos which received the virus and the compound simultaneously showed marked reduction in viral multiplications and significant decrease in the percentage mortality; 2·5 mg./embryo gave 100 per cent protection of the embryos and 3·0 mg./embryo not only protected the embryos but also stopped viral multiplication. When the compound (3·0 mg./embryo) was given 1 or 2 hr after infection, there was 100 per cent protection of the embryos and marked inhibition of the virus multiplication. The compound had no effect when given 5 hr after infection (Table 3).

TABLE 3 — ANTIVIRAL EFFECT OF 3-β-D-RIBOFURANOSYL IMIDAZO(b)PYRIDINE AGAINST RANIKHET DISEASE VIRUS* IN CHICK EMBRYOS

Conc. of compound *mg./embryo*	Interval between virus infection and compound administration *hr*	No. of embryos	Virus multiplication		Mortality %
			No. of embryos showing haemagglutination	HA titre (mean log value)	
1·25	nil	7	7	2·59† (2·34-2·84)	14·3
2·50	nil	9	9	2·34 (2·02-2·41)	0
3·00	nil	10	0	0‡	0
Virus control		9	9	3·14 (2·91-3·36)	100
3·00	1	6	5	1·24 (1·04-1·47)	0
3·00	2	6	5	1·36 (1·30-1·65)	0
3·00	3	6	6	2·55 (2·34-2·84)	33·3
3·00	5	6	6	2·8 (2·64-2·87)	100
Virus control		9	9	2·89 (1·65-2·91)	90

*0·0000032 HA unit/embryo.
†Geometric mean of the HA titres with 95 per cent fiducial limits.
‡When 10^{-2} dilution of each allantoic fluid was passaged further in embryo (4 embryos/fluid) they were non-infective.

Six new imidazopyridines [2-azaimidazo(b)pyridine, 1(-3)-β-D-ribofuranosyl imidazo(c)pyridine, 2-(α-hydroxybenzyl) imidazo(b)pyridine, 6-bromo-3-β-D-ribofuranosyl imidazo(b)pyridine, 6-chloro-3-β-D-ribofuranosyl imidazo(b)pyridine, and 7-nitro-1-β-D-ribofuranosyl imidazo(c)pyridine] were tested for their antiviral activity. The CAM cultures were given the virus (0·0000032 HA unit/culture) and the compounds to be tested simultaneously. None of these compounds inhibited virus multiplication although their concentration was 40 times more than that of 3-β-D-ribofuranosyl imidazo(b)pyridine.

It has been reported[4] that introduction of halogen atom increases markedly the antiviral activity of ribofuranosyl benzimidazole. The halogen derivatives (6-chloro, 6-bromo) of 3-β-D-ribofuranosyl imidazo(b)pyridine have no antiviral activity against Ranikhet disease virus.

Discussion

It has been shown that 3-β-D-ribofuranosyl imidazo(b)pyridine does not inactivate the virus particle *in vitro*, but its presence in the cell interferes with the multiplication of the virus. The compound has been found to inhibit virus multiplication in the CAM cultures and in chick embryos when administered 1-2 hr after infection, but has no effect when given 5 hr after infection. These findings suggest that the presence of the compound in the cell inhibits viral multiplication by blocking the RNA synthesis before it has been orientated and switched on to the synthesis of the viral materials.

Acknowledgement

The authors wish to thank Dr B. N. Singh and Dr M. L. Dhar for their keen interest in the work and Drs Nitya Anand and S. K. Chatterjee for the supply of the compounds.

References

1. Babbar, O. P., *J. sci. industr. Res.*, **20C** (1961), 216.
2. Babbar, O. P., *J. sci. industr. Res.*, **20C** (1961), 232.
3. Babbar, O. P. & Dhar, M. M., *J. sci. industr. Res.*, **15C** (1956), 249.
4. Tamm, I., Folker, K., Shunk, C. H. & Horsfall, F. L. (Jr), *J. exp. Med.*, **99** (1954), 227.

Fungicidal & Insecticidal Activity of Some Organic Fluorine Compounds Containing Aryloxy, Benzamido, Acetamido & Thiazole Ring Systems

K. C. JOSHI, S. GIRI & S. C. BAHEL
Department of Chemistry, University of Gorakhpur, Gorakhpur

Manuscript received 19 *March* 1962

Forty-seven organic fluorine compounds comprising fluoroaryloxy acids (6), and their mercury (6) and amide (15) derivatives, N-substituted fluorobenzamides (10) and 2-arylamino substituted thiazoles (10) have been screened for their fungicidal activity against *Alternaria solani*. While a number of compounds exhibit antifungal activity at higher concentrations, all the compounds, with the exception of five mercury derivatives, exhibit no activity at concentrations of 50 and 500 p.p.m. Amide derivatives of fluoroaryloxy acids (7) and N-substituted fluorobenzamides (5) show poor insecticidal and larvicidal activity against adult mosquitoes and mosquito larvae as compared to DDT.

IN earlier communications[1,2], the synthesis of some new organic fluorine compounds, likely to possess pesticidal activity, was described. In this paper, the results of screening of some of these compounds which include fluoroaryloxy acids (and their mercury and amide derivatives), N-substituted fluorobenzamides and 2-arylamino substituted thiazoles for fungicidal and insecticidal activity are reported. In all, 54 compounds have been screened, 47 for fungicidal activity and 12 for insecticidal activity.

Experimental procedure

Fungicidal activity — The compounds listed in Table 1 were screened for their fungicidal activity against the test organism, *Alternaria solani*, by the agar plate method[3]. Preliminary tests were carried out by placing the test compound (0·1 g.) directly over 1 sq. cm. of agar surface inoculated with the test organism and incubating the plate for 5 days at 25°C. Those compounds which exhibited significant activity were tested at dilutions of 50 and 500 p.p.m. The compounds were dissolved or suspended in N,N-diethyl formamide and made up to volume with distilled water as required. For comparison, iron thiocarbonate was used as the standard.

Insecticidal and larvicidal activity — The following compounds were screened for insecticidal and larvicidal activity employing adult mosquitoes (*C. fatigans*) and larvae as the test insects: (1) N-ethyl-*p*-fluoroaryloxy acetamide, (2) N-phenyl-*p*-fluoroaryloxy acetamide, (3) N-(*o*-chlorophenyl)-*p*-fluorophenoxy acetamide, (4) N-(*o*-phenetyl)-*p*-fluorophenoxy acetamide, (5) N-phenyl *o*-fluorophenoxy acetamide, (6) N-(*o*-bromophenyl)-*o*-fluorophenoxy acetamide, (7) N-(*m*-tolyl)-*o*-fluorophenoxy acetamide, (8) N-(*m*-bromophenyl)-*p*-fluorobenzamide, (9) N-(*o*-chlorophenyl)-*o*-fluorobenzamide, (10) N-ethyl-*p*-fluorobenzamide, (11) N-(*o*-bromophenyl)-*p*-fluorobenzamide, and (12) N-(*o*-chlorophenyl)-*p*-fluorobenzamide. Topical application method was employed for testing the compounds against adult mosquitoes which were reared in the laboratory and were 2-3 days old. Alcoholic solutions of the compounds were applied to the mosquitoes (10 mg./mosquito) by means of a micrometer syringe on the dorsum of the anaesthetized insect. The treated insects were kept under observation for 24 hr and the percentage mortality was recorded. For comparison, DDT (1 mg./mosquito) was used as the standard.

The compounds were tested for their larvicidal activity by exposing the larvae to suspensions of the compounds in water. The suspensions were prepared by mixing 1·0 ml. of a solution of the compound in alcohol (0·1 g.) with 250 ml. of water so as to give 40 p.p.m. of insecticide concentration. The percentage mortality was recorded after exposing the larvae for 24 hr and compared with the percentage mortality produced by DDT (1·0 p.p.m.).

TABLE 1 — ANTIFUNGAL ACTIVITY OF VARIOUS ORGANIC FLUORINE COMPOUNDS*

Sl No.	Name of compound	Width of inhibition zone *mm.*
	FLUOROARYLOXY ACID	
1	*o*-Fluorophenoxyacetic acid	16
2	*o*-Fluorophenoxypropionic acid	20
3	*o*-Fluorophenoxybutyric acid	20
4	*p*-Fluorophenoxyacetic acid	12
5	*p*-Fluorophenoxypropionic acid	15
6	*p*-Fluorophenoxybutyric acid	22
	MERCURY DERIVATIVES OF FLUOROARYLOXY ACIDS	
7	$F.C_6H_3$-(O.CH_2.COO)-Hg (*o*)	16
8	$F.C_6H_3$-(O.CH_2.COO)-Hg (*p*)	5
9	$F.C_6H_3$-(O.CH.CH_3COO)-Hg (*o*)	19
10	$F.C_6H_3$-(O.CH.CH_3COO)-Hg (*p*)	6
11	$F.C_6H_3$-(O.CH.C_2H_5COO)-Hg (*o*)	12
12	$F.C_6H_3$-(O.CH.C_2H_5COO)-Hg (*p*)	14
	AMIDE DERIVATIVES OF FLUOROARYLOXY ACIDS (F = fluoroaryloxy acetamide; F′ = α-fluoroaryloxy propionamide)	
13	*p*-F	12
14	N-Ethyl-*p*-F	—
15	N-(*n*-Butyl)-*p*-F	6
16	N-Benzyl-*p*-F	0
17	N-Phenyl-*p*-F	0
18	N-(*p*-Chlorophenyl)-*p*-F	0
19	N-Cyclohexyl-*p*-F	10
20	*o*-F	3
21	N-Ethyl-*o*-F	12
22	N-(*m*-Bromophenyl)-*o*-F	0
23	*p*-F′	12
24	N-Ethyl-*p*-F′	10
25	N-Phenyl-*p*-F′	0
26	N-(*p*-Fluorophenoxyacetyl)-*p*-amino-salicylic acid	12
27	N-(*p*-Fluorophenoxypropionyl)-*p*-amino-benzoic acid	10
	N-SUBSTITUTED FLUOROBENZAMIDES (F = fluorobenzamide)	
28	N-Ethyl-*p*-F	—
29	N-(*n*-Butyl)-*p*-F	3
30	N-Phenyl-*p*-F	0
31	N-Benzyl-*p*-F	0
32	N-(*p*-Bromophenyl)-*p*-F	2
33	N-(*o*-Bromophenyl)-*p*-F	0
34	N-(*o*-Chlorophenyl)-*p*-F	0
35	N-(*o*-Tolyl)-*p*-F	0
36	N-Benzyl-*o*-F	5
37	N-(*p*-Chlorophenyl)-*o*-F	0
	2-ARYLAMINO SUBSTITUTED THIAZOLES	
38	2-(*m*-Chlorophenylamino)-4-(2′-hydroxy-5′-fluorophenyl)	3
39	2-(*p*-Tolylamino)-4-(2′-hydroxy-5′-fluorophenyl)-5-methyl	3
40	2-Phenylamino-4-(2′-hydroxy-5′-fluorophenyl)-5-methyl	0
41	2-Phenylamino-4-(2′-hydroxy-5′-fluorophenyl)	2
42	2-(2′,5′-Dichlorophenylamino)-4-(2′-hydroxy-5′-fluorophenyl)	6
43	2-(2′,5′-Dichlorophenylamino)-4-(2′-hydroxy-5′-fluorophenyl)-5-methyl	1
44	2-(*m*-Chlorophenylamino)-4-(2′-hydroxy-5′-fluorophenyl)-5-methyl	2
45	2-(*p*-Anisylamino)-4-(2′-hydroxy-5′-fluorophenyl)	0
46	2-(2′,4′,6′-Tribromophenylamino)-4-(2′-hydroxy-5′-fluorophenyl)	11
47	2-(2′-Pyridylamino)-4-(2′-hydroxy-5′-fluorophenyl)	13

*The results of screening the compounds at concentrations of 50 and 500 p.p.m. showed that only mercury derivatives (Sl Nos. 7-11) exhibit activity at these concentrations and the rest were inactive. The width (mm.) of inhibition zone at 50 and 500 p.p.m. concentrations respectively for the compounds are Sl No. 7: 2 and 13; Sl No. 8: 8 and 17; Sl No. 9: 7 and 21; Sl No. 10: 11 and 21 and Sl No. 11: 12 and 13.

Results and discussion

Fungicidal activity of the compounds — The results of preliminary screening recorded in Table 1 show that of the compounds tested five amide derivatives of fluoroaryloxy acids (Sl Nos. 16-18, 22 and 25), six N-substituted fluorobenzamides (Sl Nos. 30, 31, 33-35 and 37) and two 2-arylamino substituted thiazoles do not possess any fungicidal activity even at high concentrations. All the compounds, with the exception of five mercury derivatives of fluoroaryloxy acids (Sl Nos. 7-11), showed no activity even when employed in concentrations of 500 p.p.m.

Of the fluoroaryloxy acids tested (Sl Nos. 1-6), *p*-fluorophenoxy butyric acid exhibits maximum fungicidal activity, indicating thereby that the activity of this series of compounds is dependent on the chain length of the aliphatic moiety. Of the six mercury derivatives tested, five (Sl Nos. 7-11) show high activity even at low concentrations (50 p.p.m.). Eight of the fifteen amide derivatives of fluoroaryloxy acids exhibit sufficient activity only at high concentrations (Sl Nos. 13, 14, 19, 21, 24, 26 and 27) of these only two compounds (Sl Nos. 26 and 27) exhibit activity at 500 p.p.m. Of the N-substituted fluorobenzamides, which generally show poor activity, the *p*-derivative, in which one of the amide hydrogen is replaced by ethyl group (Sl No. 27), shows maximum activity. All the thiazole derivatives are fairly active and maximum activity is exhibited by the compounds in which the amino hydrogen at 2-position is replaced by pyridyl or tribromophenyl radicals.

Insecticidal and larvicidal activity — None of the compounds gave 100 per cent mortality in the case of adult mosquitoes and larvae even at high

concentrations. The percentage mortality in the case of adult mosquitoes varied from 5 to 88 per cent at the concentrations (10 mg./mosquito) of the compounds employed whereas DDT gave 100 per cent mortality at a concentration of 1 mg./mosquito. The percentage mortality in the case of larvae varied from 0 to 87 per cent at concentrations of 40 p.p.m. of the compounds tested whereas D.D.T. at a concentration of 1 p.p.m. gave 100 per cent mortality.

Acknowledgement

The authors wish to express their thanks to Dr R. A. Kubista of the Wisconsin Alumni Research Foundation, U.S.A., for valuable technical assistance in screening the fungicidal activity of compounds, the Director, Malaria Institute of India, for assistance in assessing their insecticidal and larvicidal activity, the Council of Scientific & Industrial Research, New Delhi, for partly financing the project and to Dr R. C. Mehrotra, Head of the Department of Chemistry, University of Gorakhpur, for providing facilities to carry out this work.

References

1. JOSHI, K. C. & BAHEL, S. C., *J. Indian chem. Soc.*, **37** (1960), 365, 685.
2. JOSHI, K. C. & GIRI, S., *J. Indian chem. Soc.*, **37** (1960), 423; **39** (1962), in press.
3. *U.S. Department of Agriculture, Cir. No.* 198, 1931.

Botanical Identity of 'Gaozaban'

S. M. J. S. QADRY & ABDUL HAMID
Institute of History of Medicine & Medical Research,
Delhi 6

Manuscript received 23 *June* 1962

An attempt has been made to establish the botanical identity of the Unani drug 'Gaozaban' imported into India. 'Gaozaban' is supposed to be indigenous to India and is mentioned in literature under six species of plants. A critical examination of the market samples and study of the morphological characters of the various plants mentioned in the literature have led to the conclusion that the drug does not belong to any of the plants mentioned in the literature. Reference to the international authorities and examination of herbarium specimens have led to the conclusion that the drug 'Gaozaban' may belong to *Anchusa strigosa* Labill., a species not mentioned in the literature and not indigenous to India. The market sample, available under the name of 'Kashmiri Gaozaban', has been identified as *Macrotomia benthami* Boiss.

THE drug 'Gaozaban' (Persian: Cow's tongue) is extensively used in the Unani system of medicine. The drug is said[1-8] to be stimulant, tonic, diuretic, laxative, demulcent, useful in cough, asthma, in cases of stones in the bladder and kidney and in complaints due to black bile. It is useful[6-8] in cases of jaundice, melancholia, madness, anxiety and pain of the chest and lungs. It is used[1,2,4,6-8] in syphilis, rheumatism and leprosy.

Its identity has not yet been established. It has been supposed to be an Indian indigenous drug. However, our enquiries revealed that it is imported from Persia (and probably from Afghanistan) in large quantities into India. Our attention was drawn to it as a result of a number of enquiries, from some of the leading medical and research institutions in India, concerning the supply of the genuine drug. We were also given to understand that the plant yielding 'Gaozaban' grows at higher altitudes in Kashmir, especially near Khillanmarg. In order to confirm this a survey trip to Kashmir valley was undertaken in 1961. But no plant resembling the imported market drug could be traced. The non-availability of the real plant in India, the disputed identity and authenticity of the drug and the supply of different drugs under the same name from the market were the causes

why no pharmacognostic, chemical or pharmacological investigations could be undertaken on such an important drug. The books and old manuscripts of Unani medicine[6-8] have helped us in the study of the problem.

In literature six species of plants are associated with the name of 'Gaozaban'. The botanical names of different species which are given the name 'Gaozaban' together with references are listed in Table 1. These six species can be categorized under two groups: (i) plants belonging to the family *Boraginaceae*, i.e. plants 2-6; (ii) plant not belonging to the family *Boraginaceae*, i.e. plant 1, belonging to the family *Labiatae*. The latter is an entirely different plant called 'Catmint', the leaves of which do not have any resemblance with the imported market 'Gaozaban' and so the plant does not merit further consideration.

A critical study of the morphological character of the leaf of the plants 2-6 was made[1,9]. (Herbarium specimens from the Division of Botany, Forest Research Institute, Dehra Dun, were also compared.) Since the 'Gaozaban' plant could not be obtained, samples of the imported 'Gaozaban' (Plate I, Fig. 1) from Iran were soaked in water for a short

TABLE 1 — SPECIES REFERRED TO AS 'GAOZABAN'

Sl No.	Botanical name	Ref.
1	*Anisomeles malabarica* R.Br.	2
2	*Macrotomia benthami* Boiss.	1, 2, 5
3	*Caccinia glauca* Savi.	1, 2, 4, 5
4	*Onosma bracteatum* Wall.	1, 2, 3, 5
5	*Trichodesma indicum* R.Br. (=*Borago indicum* Br. =*B. spinulosa* Roxb.[9])	1, 2
6	*Trichodesma zeylanicum* R.Br.	2

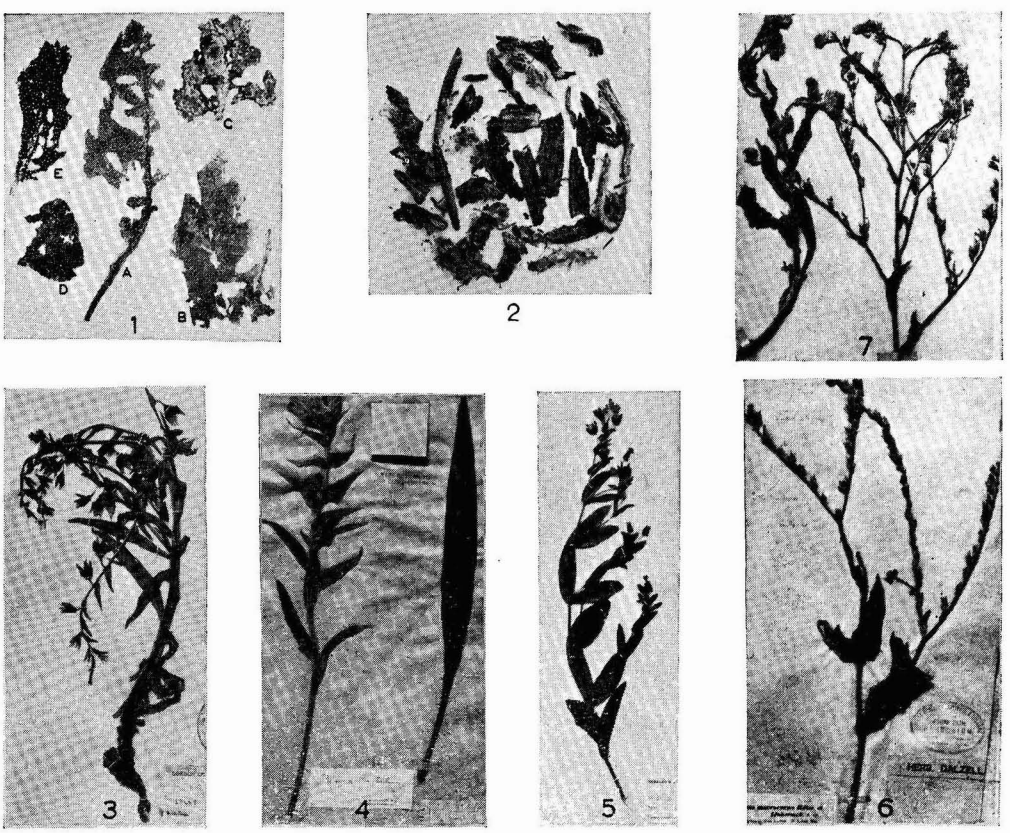

Plate I — Macroscopic characters of leaf fragments of genuine market 'Gaozaban' and leaves of *Macrotomia benthami* Boiss., and herbarium specimens of *Caccinia glauca* Savi., *Onosma bracteatum* Wall., *Trichodesma indicum* R.Br., *Anchusa macrocarpa* Boiss. and *Anchusa italica* Retz. [Fig. 1: Leaf fragments of genuine market 'Gaozaban' sent to Kew for re-examination and identification (B, C and D show the upper surface; A and D show the lower surface of leaf). Fig. 2: Leaves of *M. benthami* sold in Kashmir under the name of 'Gaozaban' or 'Kashmiri Gaozaban'. Fig. 3: *C. glauca*. Fig. 4: *O. bracteatum*. Fig. 5: *T. indicum*. Fig. 6: *A. macrocarpa*. Fig. 7: *A. italica*]

TABLE 2 — DIFFERENCES IN THE MORPHOLOGICAL CHARACTERS OF THE LEAVES OF PLANTS REFERRED TO AS 'GAOZABAN' AND GENUINE MARKET SAMPLE OF 'GAOZABAN'

Sl No.	Name of plant	Size and shape of leaf	Nature of trichomes or hair	Fig. No.
1	*Macrotomia benthami* Boiss.	Up to 28 by 1·3 cm. linear to narrow-lanceolate, sessile	Covered all over with dense soft hair	2
2	*Caccinia glauca* Savi.	Up to 18 by 5 cm. linear to lanceolate, sessile	Short white conical spines are present on the margin, on the upper side and on the midrib on the lower side, where the spines are arranged in rows	3
3	*Onosma bracteatum* Wall.	Up to 15 by 2·5 cm. lanceolate to ovate-lanceolate, petioled	Upper surface hispid with tubercular based hair	4
4	*Trichodesma indicum* R.Br.	Up to 10 by 5 cm. ovate or oblong or lanceolate oblong, sessile	Clothed above with stiff hair springing from white tubercles, less harshly hairy and more or less densely villous beneath	5
5	*Trichodesma zeylanicum* R.Br.	Up to 10 by 2·5 cm. lanceolate oblong, shortly petioled	Bristly with hairs springing from tubercles on the upper surface	
6	Commercial drug	Up to 25 by 15 cm. oval lanceolate or ovate	Thick hooked spines, springing from fine white to light brown thick tubercle bases, are present on both the sides of the leaf. Some spines may fall off leaving the tubercular bases intact	1

In case of plants 1 and 3-5 data collected from a study of herbarium specimens at the Forest Research Institute, Dehra Dun; data for plant 2 from the herbarium of the Regional Research Laboratory, Jammu.

time, pressed and dried for morphological study, and for sending the leaves to different herbaria for identification.

The difference in the leaves of the plants 2-6 and the commercial drug are given in Table 2.

From the data given in Table 2 it is clear that the leaf of the imported market drug differs from the leaves of the plants 1-5 (Table 2) in shape, size and nature and occurrence of the spines.

Samples of market 'Gaozaban' were submitted for identification to the following authorities: (1) Director, Royal Botanic Gardens, Kew; (2) Chairman, Department of Botany, University of Peshawar, Peshawar; and (3) Keeper, Central National Herbarium, Botanical Survey of India, Calcutta.

The Director, Royal Botanic Gardens, Kew, examined the leaves morphologically and anatomically and provisionally identified the plant as *Anchusa strigosa* Labill., a species which occurs in Iran but is not confined to that country. According to him this species shows much closer resemblance to commercial 'Gaozaban' than with any of the species mentioned in the literature. The Chairman, Department of Botany, University of Peshawar and Keeper, Central National Herbarium, Calcutta, identified 'Gaozaban' as the leaves of *Caccinia crassifolia* O. Kuntze (= *C. glauca* Savi.) and *Caccinia strigosa* Boiss. respectively. Thus, these different findings made the confusion worse confounded, especially because 'Gaozaban' was nowhere mentioned as *Anchusa strigosa* Labill. or *Caccinia strigosa* Boiss. As a result, more critical examination of the market samples was undertaken with a view to finding whether the market drug was uniform or a mixture of the leaves of above three species. It was observed that there were differences in the nature and distribution of spines, colour, thickness and size of leaves within one market sample. Five individual leaf fragments, representing each variant, were selected from this specimen and, after proper treatment and mounting (Fig. 1), resubmitted to the Director, Royal Botanic Gardens, Kew. The following report was received.

"The material has been compared anatomically with herbarium specimens of *Caccinia strigosa* Boiss. and *Anchusa strigosa* Labill. Evidence would point to all the material being *Anchusa strigosa*. Important points of agreement with *A. strigosa* are: (a) the arrangement of the palisade layer in the lamina; (b) the nature of the petiolar bundles; and (c) the distribution of the collenchyma. The anatomical structure of the leaves does not agree with *Caccinia strigosa* Boiss. as represented in the Kew herbarium."

Examination of herbarium specimens at the Forest Research Institute, Dehra Dun, revealed that 'Gaozaban' did not belong to any of the plants mentioned in literature, i.e. plants 1-6, Table 1. Figs. 2-5 show clearly the wide difference between them and the genuine market 'Gaozaban' (Fig. 1). Though the herbarium specimen of *Anchusa strigosa* Labill. was not available in the F.R.I., Dehra Dun, yet two other species of the genus *Anchusa*, namely *A. macrocarpa* Boiss. (Fig. 6) and *A. italica* Retz. (Fig. 7), were found having leaves resembling 'Gaozaban' and very nearly support the finding of

the Director, Royal Botanic Gardens, Kew, and suggest that the market drug may consist of the leaves of *Anchusa strigosa* Labill.

Besides the genuine ' Gaozaban ', another drug of Indian market offered as ' Gaozaban ' or ' Kashmiri Gaozaban ' was also investigated by us. This drug (Fig. 2) was found to be derived from *Macrotomia benthami* Boiss. based on our comparison of commercial sample and herbarium specimens at the Regional Research Laboratory, Jammu. The preliminary chemical examination also showed that the leaves of *Macrotomia benthami* Boiss. commonly sold in Kashmir under the name of ' Gaozaban ' contained less amount of mucilage, the chief water-soluble content, as compared to the imported drug.

Further, in Indian literature ' Gaozaban ' is wrongly given the name ' Shankhahuli '[1,3,7], which is quite a different drug.

On the basis of the facts presented in the foregoing, it appears that the true source of ' Gaozaban ' may be *Anchusa strigosa* Labill. Further attempts are being made to obtain an authentic specimen of the plant from Iran for pharmacognostic comparison with the aim of finally confirming the true source of ' Gaozaban '.

Acknowledgement

The authors gratefully acknowledge the generous help kindly rendered by the authorities of the various institutions mentioned in the text. Thanks are also due to Dr C. K. Atal, Head of the Section of Pharmacognosy, Department of Pharmacy, Panjab University, Chandigarh, for his help during the survey in Kashmir and keen interest throughout the course of this investigation.

References

1. KIRTIKAR, K. R. & BASU, B. D., *Indian Medicinal Plants*, Vol. III (Lalit Mohan Basu, Allahabad), 1933, 1692-9.
2. NADKARNI, K. M., *Indian Materia Medica*, Vol. I (Popular Book Depot, Bombay), 1954, 114, 225, 759, 871, 1233.
3. CHOPRA, R. N., CHOPRA, I. C., HANDA, K. L. & KAPUR, L. D., *Chopra's Indigenous Drugs of India* (U. N. Dhur & Sons, Calcutta), 1958, 517.
4. *The Wealth of India, Raw Materials*, Vol IIC (Council of Scientific & Industrial Research, New Delhi), 1950, 1.
5. CHOPRA, R. N., NAYAR, S. L. & CHOPRA, I. C., *Glossary of Indian Medicinal Plants* (Council of Scientific & Industrial Research, New Delhi), 1956, 43, 159, 180.
6. GHANI, M. N., *Khazanatul Adviya*, Vol. III (Munshi Navil Kishore, Lucknow), 1921, 460.
7. KARIM, N., *Makhzanul Adviya*, Vol. II (Munshi Navil Kishore, Lucknow), 306.
8. KHAN, M. A., *Muheet-e-Azam*, Vol. IV, Part II (Maktaba-Nizami, Kanpur), 1894, 13.
9. HOOKER, J. D., *Flora of British India*, Vol. IV (L. Reeve & Co., Ashford, Kent), 1885, 177-8, 153-4.

INSTRUCTIONS TO CONTRIBUTORS

Manuscripts should be typewritten in double space and on one side of the paper; the *original and one carbon copy are to be submitted.*

Names of chemical compounds and not their formulae should be used in the text. Greek letters should be written plainly and explained by marginal notes. Superscripts and subscripts should be legibly and carefully placed. Footnotes should be avoided as far as possible.

Abstract — The abstract should indicate the scope of the work and the principal findings of the paper. It should not exceed 3 per cent of the length of the paper, and, except in exceptional cases, it should not exceed 200 words. The abstract prepared should be in such a form that abstracting periodicals can use it without modification.

Tables — Tables should be typed on separate sheets of paper without any text matter on the page. They should be numbered consecutively in Arabic numerals and should bear brief titles. Column headings should be brief. Units of measurement should be abbreviated, typed in small letters (underlined) and placed below the headings. Nil results should be indicated and distinguished clearly from absence of data. Graphs as well as tables, both representing the same set of data, must be strictly avoided. Inclusion of structural formulae inside the tables should be avoided as far as possible.

Illustrations — All illustrations must be numbered consecutively in Arabic numerals. Captions and legends to the figures should be typed on a separate sheet of paper and attached at the end of the manuscript. Line drawings should be made with Indian ink on white drawing paper (preferably Bristol board), cellophane sheet or tracing cloth. The lettering should be done in pencil. In addition to the originals, a set of blue prints or photostat copies should be sent. For satisfactory reproduction, the graphs and line drawings should be drawn to approximately twice the printed size. The size of letters, numbers, dots, lines, etc., should be sufficiently large to permit reduction to the page or the column size as required in the journal without loss of detail. In the case of photographs, prints must be on glossy paper and contrasty.

References — References to literature, numbered consecutively, must be placed at the end of the article. In the text they should be indicated by numbers placed above the line (superior).

In citing references to research papers, names and initials of authors should be followed, in order, by the title of the periodical in the abbreviated form (underlined), the volume number (two lines underneath), the year within circular brackets and the page reference [e.g. RAMACHANDRAN, B. V. & SIVARAMAN, C., *J. sci. industr. Res.*, **19C** (1960), 244]. For names of periodicals, the standard abbreviations listed in the *World List of Scientific Periodicals* edited by William Allan Smith and Francis Lawrence Kent (Butterworths Scientific Publications, London) should be used.

Reference to a book must include, in the following order, names and initials of authors, the title of the book (underlined), name of publisher and place of publication within circular brackets and year [e.g. VENKATARAMAN, K., *The Chemistry of Synthetic Dyes*, Vol. II (Academic Press Inc., New York), 1952, 966].

Proceedings of conferences and symposia should be treated in the same manner as books. Reference to a paper presented at a conference, the proceedings of which are not published, should include, in the following order, names and initials of authors, title of the paper (underlined), name of the conference, place where the conference was held and date (e.g. THATHACHARI, Y. T., *Structure of Collagen*, paper presented to the Symposium on Solid State Physics, Indian Institute of Science, Bangalore, 1-3 Feb. 1960).

Reference to a thesis should include the name of author, title of the thesis (underlined), university or institution to which it was submitted and year of submission (e.g. CHANDRASEKHARAN, K. S., *Studies on Crystal Structure and Absolute Configuration of Crystals*, Ph.D. Thesis, Madras University, 1956).

Reference to a patent should include names of patentees, country of origin (underlined) and patent number, the organization to which the patent has been assigned within circular brackets, date of acceptance of patent and reference to an abstracting periodical [e.g. TREPAGNIER, J. H., *U.S. Pat.* 2,463,219 (to E.I. du Pont de Nemours & Co.), 1 March 1949; *Chem. Abstr.*, **43** (1949), 7258].

Even if a reference contains more than two authors, the names of all the authors should be given. The abbreviations *et al.*, *idem*, *ibid.* should be avoided.

Unpublished papers and personal communications should not be listed under references but should be indicated in the text. Thus: (Pande, A. B., unpublished data); (Pande, A. B., personal communication).

Printed and published by Shri B. N. Sastri, Council of Scientific & Industrial Research, New Delhi,
at the Catholic Press, Ranchi, India

Regd. No. P-842