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Mode of Action of Psoralen in Pigment Production: Part II—Inactivation of —SH Groups by Irradiated Psoralen*

RASHID ALI & S. C. AGARWALA
Central Drug Research Institute, Lucknow

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Solar irradiation of psoralen in aqueous ethanol solution has been shown to produce degradation products which inactivate the —SH groups as indicated by the inhibition of succinic dehydrogenase of rat kidney and the reversal of thiourea inhibition of potato tyrosinase. The possible significance of this inactivation has been discussed vis-à-vis the role of psoralen in pigment production.

ALTHOUGH furocoumarins are known to potentiate the action of solar[1-2] or ultraviolet[3] irradiation in bringing about increased pigmentation, little information is available regarding their mode of action. Lerner *et al.*[4] reported that methoxsalen as such had no effect on tyrosinase, although Chakraborty *et al.*[5] observed *in vivo* enhancement of tyrosinase and melanin formation in the skin and liver tissues of male toads after oral administration of psoralen. In view of the well-known observations[6-8] regarding the inhibition of tyrosinase and hence melanin formation, brought about by —SH groups, it was considered of interest to investigate the inactivation, if any, of such groups by normal and irradiated psoralen. The results of such study are reported in the present communication. The inhibition of succinic dehydrogenase, a —SH enzyme, and the reversal of thiourea inhibition of potato tyrosinase were taken as an index of the inactivation of —SH groups.

Experimental procedure

Irradiation of psoralen — The irradiation of psoralen and the production of other compounds have been reported elsewhere[9]. For the present studies a suspension of pure psoralen† (1 mg./ml.) in alcohol-water (1 : 1, vol./vol.) was irradiated in 10 ml. stoppered cylinders, kept horizontally, under bright sun for the requisite period. The yellowish turbid solution thus obtained was concentrated three-fold under an infrared lamp. Methods for the chromatographic separation of products and determination of their ultraviolet absorption were the same as previously described[9].

Succinic oxidase — Rat kidney[10] was taken as the source of enzyme. A 10 per cent (wt/vol.) homogenate was made in Potter Elvehjem homogenizer (previously chilled) in cold *M*/5 phosphate buffer (*p*H 7·0). Manometric method of Slater[11] was used for the assay of enzyme activity. Two ml. of the homogenate were added to 2 ml. of concentrated irradiated solution (containing 6 mg.) of psoralen and 1 ml. of this homogenate was added to each Warburg vessel and the succinic oxidase activity was determined as reported earlier[12]. Controls without psoralen were included in each experiment.

Tyrosinase — Pieces of fresh frozen (—13°C.) potato were ground in chilled pestle and mortar and the juice extracted by squeezing through a fine cloth, which served as the source of enzyme. In actual experiments 1 ml. of thiourea (10 μg.) solution was incubated with 1 ml. of irradiated concentrated psoralen (6·0 mg.) solution for 2 hr. To this were added 2 ml. of potato juice. One ml. of this mixture was used in each Warburg flask and

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†Psoralen was extracted from *Psoralea corylifolia* Linn. in the Medicinal Chemistry Division of this Institute.

tyrosinase assay was done manometrically as described elsewhere[13]. Controls (i.e. without thiourea and psoralen or with thiourea alone) were run with each experiment for evaluation of the reversal brought about by irradiated psoralen.

Results

The R_f values of products obtained by solar irradiation of psoralen, under different conditions, are recorded in Table 1. Even irradiation for 30 min. produced products having different R_f values. The major product formed under the present conditions appears to be one having bluish fluorescence (R_f 0·26) and another having greenish bright fluorescence (R_f 0·34). The intensity of these spots (as seen by enhanced fluorescence) increased with the time of irradiation up to about 2 hr, after which no further increase was seen. The other products were minor in nature showing weak fluorescence. After longer exposure some more new fluorescent products were also observed which have been included in Table 1. The absorption spectrum of eluates of spots having R_f values of 0·26, 0·34 and 0·57 indicated a generalized absorption getting stronger towards the shorter wavelength region, essentially different from that of the dimer or furocoumaric acid as reported by Fowlks[14], but similar to that reported earlier[9]. In the absorption spectra of all these products, no distinct maxima were obtained between 230 and 420 mμ, though the eluate of psoralen (R_f 0·90) showed[15] the usual three maxima at 245, 290 and 330 mμ.

Inhibition of succinic oxidase by irradiated psoralen — Since succinic dehydrogenase, a component of succinic oxidase complex, is known to be an —SH containing[16] enzyme, indirect proof for the inactivation of —SH groups by irradiated products of psoralen was sought by studying their effect on kidney succinic oxidase. The results presented in Fig. 1 show that the inhibition of succinic oxidase brought about by irradiated products of psoralen increased with the period of irradiation. The average inhibition after 1 hr exposure was 14 per cent which increased to 55 per cent after psoralen was exposed for 2 hr to solar radiations and this value rose up to 61, 68 and 70 per cent at the end of 3, 4 and 6 hr exposures respectively. It may be mentioned that unirradiated psoralen or that irradiated only for 30 min. did not bring about any inhibition, but on the contrary caused slight activation of the enzyme.

Reversal of thiourea inhibition of potato tyrosinase by irradiated psoralen — Further evidence for the inactivation of —SH groups by products of irradiated psoralen was attempted by incubating thiourea (10 μg.) with psoralen irradiated for different periods and seeing its effect on potato tyrosinase. The

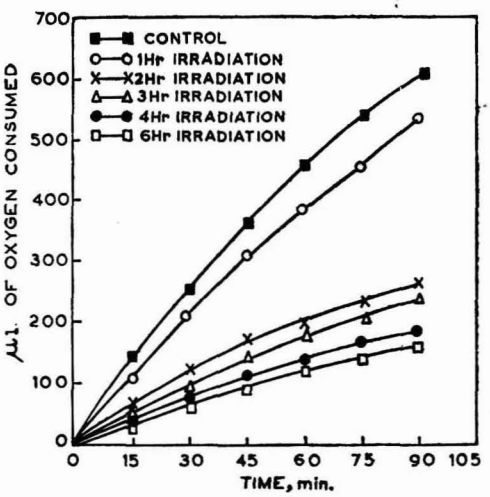

Fig. 1 — Inhibition of succinic oxidase (rat kidney) by irradiated psoralen

TABLE 1 — R_f VALUES OF PRODUCTS OBTAINED AFTER SOLAR IRRADIATION OF PSORALEN* FOR DIFFERENT PERIODS

Irradiation period hr	Mean R_f of fluorescent products						
	1	2	3	4	5	6	7
0·5	0·015 (+)†	—	—	0·28 (+)	0·35 (+)	0·57 (+)	0·88 (+++)
1·0	0·012 (++)	—	—	0·26 (++)	0·34 (+++)	0·57 (++)	0·90 (+++)
2·0	0·014 (++)	0·060 (+)	—	0·27 (+++)	0·35 (+++)	0·57 (++)	0·86 (++)
4·0	0·010 (++)	0·062 (+)	0·14 (+)	0·26 (+++)	0·33 (+++)	0·55 (++)	0·88 (++)
6·0	0·013 (++)	0·066 (+)	0·12 (+)	0·26 (+++)	0·35 (+++)	0·53 (++)	0·90 (++)

*Psoralen in water-alcohol (1 : 1, vol./vol.) was irradiated.
†The intensity of fluorescent bands given in parentheses is indicated as follows: +, faint; ++, medium; +++, strong.

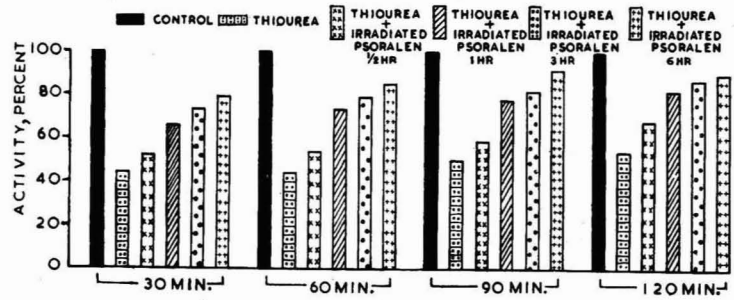

Fig. 2 — Reversal of thiourea inhibition of tyrosinase by psoralen irradiated for different periods

results presented in Fig. 2 indicate that thiourea (in the concentration used) brought about an inhibition of about 58 per cent in the tyrosinase activity (as seen by reduced oxygen uptake). However, when thiourea incubated with psoralen was added, the inhibition was significantly reduced and it became only about 20 per cent when psoralen irradiated for 6 hr was used. The results presented in Fig. 2 show the extent of reversal to be greater in the later stages of tyrosinase estimation than in the beginning, almost complete reversal (92 per cent) being achieved in 120 min. This may be because the estimation of tyrosinase activity in the presence of thiourea and irradiated products of psoralen, in itself, involves further incubation of these materials together.

Discussion

In spite of the fact that —SH groups have been considered important *in vivo* determinants of tyrosinase activity, little information is available regarding their exact mechanism vis-à-vis the role of ultraviolet light and furocoumarins in pigment production. Rothman[17] has pointed out that the effect of ultraviolet irradiation was chiefly the inactivation of inhibitors, like —SH compounds, in the skin leading to increased tyrosinase activity and better pigmentation. Following ultraviolet irradiation of the skin, the level of the inhibitory —SH groups (which form strong covalent bonds with copper[18]) was also found[19] to be reduced.

The results of the present study would show that solar irradiation of psoralen results in the formation of certain products, other than psoralen, which have the capacity to inhibit succinic oxidase, an —SH containing enzyme. That this is caused by the action of some of these products on —SH groups of the enzyme is indicated by the abolition of thiourea inhibition of tyrosinase by irradiated psoralen. Since tyrosinase is known to be involved in melanin formation it seems plausible that the pigmentation by psoralen may depend on the interaction of psoralen or more correctly its irradiation product or products with —SH groups inhibitory to tyrosinase. This would explain the role of ultraviolet or solar radiations as potentiators of psoralen effect. The inability of psoralen *per se* to bring about pigment production and the observations of Lerner *et al.*[4] that methoxsalen as such has no effect on tyrosinase are in conformity with the present observations. In this connection it might also be interesting to mention that arsenic, an —SH group inactivator, causes the well-known hyper-pigmentation[20].

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Utilization of Fish Byproducts as Cattle Feed: Influence of the Method of Manufacture on the Digestibility & Nutritive Value of *Manthal* Fish Meal

S. S. NEGI & N. D. KEHAR*
Division of Animal Nutrition,
Indian Veterinary Research Institute, Izatnagar

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The influence of the method of manufacture on the nutritive value of *manthal* fish (*Cyanoglossus semifasciatus* Day) meal has been studied. The beach-dried meal has been found to be superior to the cooked and pressed meal in overall nutritive value as evaluated on cattle.

THE possibility of the utilization of fish meals in the dietary of cattle has been indicated by the authors[1]. Since various contradictory opinions are held about the influence of the method of manufacture on the nutritive value of fish meal, it was decided to investigate the effect of the two main methods of the preparation of fish meals followed in this country on their nutritive value as evaluated on cattle. A brief description of these methods of preparation of fish meals, viz. 'beach drying' and 'cooking and pressing', has been given earlier[2]. *Manthal* fish (*Cyanoglossus semifasciatus* Day) meals processed by these two methods were received through the courtesy of the Fisheries Technological Station, Calicut.

Experimental procedure

The technique followed in the nutritive evaluation of fish meals has been described previously[1]. The *ad lib.* wheat *bhoosa* basal ration of the experimental animals was supplemented with a calculated quantity of beach-dried (BD) meal in group I and cooked and pressed (C and P) meal in group II, on equivalent digestible crude protein basis, assuming the protein digestibility in the meals to be 60 per cent as obtained in the case of mackerel meal[1]. Salt (0·5 oz. per head per day) was supplied to the animals mixed with their fish meal quota. The metabolic trials of ten days duration were carried out towards the end of the feeding period of 28 days. The blood of the experimental animals was examined, at the start and end of the feeding period, for haemoglobin, cell volume and red blood cells[3], and also for serum calcium[4], blood phosphorus[5] and serum protein fractions — total globulins and albumin[6].

Results and discussion

Due to the practical difficulty in procuring fish meals and the experimental animals, the feeding trials with BD and C and P meals could not be taken up simultaneously. However, both the trials were carried out during the same season. The average composition of feeds fed to the two groups and sampled as weighed out from day to day for feeding during the metabolic periods is given in Table 1. It is evident from Table 1 that both the meals were well balanced with regard to protein, lime and phosphate. It was observed by Negi[2] that the crude protein content of the beach-dried meals was lower due to high content of externally picked up silica in these meals. In the present case, the contamination with silica has been avoided and hence the crude protein content in the BD meal has not been lowered and is even higher than in the C and P sample. The crude fibre content of 1·85 per cent in C and P meal is rather high for a protein feed like fish meal or tankage, and seems to have been included during the course of processing. However, Morrison[7] has recorded a figure of 1·2 per cent fibre in air-dry samples of fish liver oil meal and sardine fish meal and the compilation of Schneider[8] includes a high figure of 4·8 per cent crude fibre in a sample of moisture-free tankage.

The average composition of wheat *bhoosa* fed to the two groups of animals shows that the quality varied particularly with regard to the crude protein content from one part of the mow to the other

*Present address: Department of Animal Science, U.P. Agricultural University, Pantnagar.

TABLE 1 — COMPOSITION OF FEEDS ON DRY BASIS

(*Values expressed in percentages*)

Group		Crude protein	Ether extract	Crude fibre	Nitrogen-free extract	Total carbo-hydrates	Total ash	CaO	P_2O_5
I	BD *Manthal* meal	71·95	3·52	0·14	3·96	4·10	20·43	5·94	6·17
	Wheat *bhoosa*	2·13	0·64	45·53	43·03	88·56	8·67	0·34	0·15
II	C and P *Manthal* meal	71·40	1·56	1·85	4·67	6·52	20·52	6·77	5·35
	Wheat *bhoosa*	2·76	0·64	46·59	42·50	89·09	7·51	0·38	0·17

(the wheat *bhoosa* in the two cases was from the same bulk lot of supply stored in the barn). Variations in the composition of feed stuffs in bulk storage have also been noted by Armsby and Fries[9]. However, the wheat *bhoosa* fed to both the groups is of a poor quality. Sen[10], in compiling the data on the chemical composition and digestibility coefficients of wheat straw, has noted that in the range of 1·8 to 2·7 per cent crude protein on dry basis the digestibility of crude protein is always negative. It has been conventional, however, to assume the digestibility of crude protein in wheat *bhoosa* to be zero on the basis of the average data given by Sen[11].

The animals got used to the fish meals within the first few days but they started relishing the BD meal much earlier, indicating its better palatability. The data for the daily dry feed consumed voluntarily by the animals in the two groups during the metabolic period are given in Table 2. The dry matter intake of the animals per 100 lb. live-weight was 887 g. in group I and 652 g. in group II, giving a 35 per cent higher consumption of the total ration in favour of the group fed the BD meal. This difference tested statistically according to Snedecor[12] is highly significant. As the "protein very definitely increases the palatability of food"[13], the lower consumption of wheat *bhoosa* by animals in group II in spite of the comparatively higher crude protein content in the stuff fed to this group, gives added significance to this observation.

It appears at the very outset that the use of C and P *Manthal* meal in place of the BD meal in wheat *bhoosa* basal ration lowers the feeding value of the entire ration, since "it is almost universally true that the nutritionally poor diets are consumed in smaller quantities"[14]. The difference in the overall dry matter digestibility in the two groups tested in the usual manner[12] is statistically not significant.

The digestibility coefficients of the nutrients in the whole ration under the conditions of feeding in this test are given in Table 3.

TABLE 2 — AVERAGE DRY FEED CONSUMPTION DURING THE METABOLIC PERIOD

	Group I	Group II
Live-weight of animals, lb.	296±20	315±5
Feed consumed, g./head/day		
Wheat *bhoosa*	2456±227	1856±94
Manthal meal	175±10	198±0
Total	2631±236	2054±92
Feed consumed/100 lb. live-weight*	886±31	652±19

*Difference between groups highly significant.

TABLE 3 — DIGESTIBILITY OF NUTRIENTS IN THE WHOLE RATION

(*Values expressed in percentages*)

Constituents	Group I	Group II
Dry matter	48·3±1·1	49·0±1·6
Crude protein*	47·5±1·7	61·8±0·5
Ether extract	49·5±0·5	51·5±3·3
Crude fibre	60·8±1·7	64·3±1·7
Nitrogen-free extract	46·5±2·3	40·3±2·5
Total carbohydrates	53·8±0·5	53·0±1·9

*Difference between groups highly significant.

The most striking difference was noted in the digestibility of crude protein. For the BD meal ration, the average digestibility of protein was as low as 48 per cent while it averaged 62 per cent in the C and P meal ration. This difference is highly significant. The observed differences in the digestibility of ether extract, crude fibre and nitrogen-free extract in the two groups are statistically not significant.

As the carbohydrates supplied by fish meals constitute only a negligible proportion of those in the entire ration, the digestibility of this constituent in the two meals was not considered. The digestibility coefficients of crude protein and ether extract in the meals, arrived at by the conventional method of elimination of the nutrients digested from wheat *bhoosa* on the basis of the average digestibility data given by Sen[11], are given in Table 4.

There is a marked difference in the average digestibility of both the nutrients in the two meals — the coefficients for both being lower in the BD meal. The difference though highly significant in respect of crude protein is not significant in respect of ether extract. However, since the amount of ether extract in quality fish meals should be negligible to avoid fishy taint in the animal product, the digestibility of this nutrient is of little importance.

The level, the biological value and the true digestibility of proteins for the two groups evaluated according to Mukherjee and Kehar[15] are presented in Table 5.

Due to a higher consumption of the basal ration by animals in the BD meal group, the protein level in the two groups was not the same and as such a comparison of the proteins on the basis of their biological values is not possible. Though the apparent digestibility of proteins in the BD meal ration was 23 per cent below that in the C and P meal ration, the true digestibility was only 5 per cent lower.

TABLE 4 — DIGESTIBILITY OF NUTRIENTS IN *MANTHAL* FISH MEAL

(*Values given in percentages*)

Constituents	Group I	Group II
Crude protein*	67·0±1·6	84·3±1·4
Ether extract	83·0±1·2	117·0±21·2

*Difference between groups highly significant.

TABLE 5 — LEVEL, BIOLOGICAL VALUE AND TRUE DIGESTIBILITY OF PROTEINS IN WHOLE RATION

Protein	Group I	Group II
Level in the ration	6·8±0·2	9·4±0·3
Biological value	69·5±3·5	43·2±0·8
True digestibility	84·0±1·2	89·0±0·5
Apparent digestibility	47·5±1·7	61·8±0·5

The values for nitrogen, lime and phosphate balance for the two groups are given in Table 6. The intakes of nitrogen by animals in the two groups from the two meals were almost the same on the live-weight basis. The disposition of animals in the two cases in regard to the utilization of this constituent was, however, different. As evident from the average daily retentions, the animals on the BD meal ration made a better utilization of this nutrient. The difference in the average retention in the two cases is highly significant. The lower faecal output of this nutrient by animals on the C and P meal ration resulted in an increase in the apparent digestibility of protein from that meal but the large excretion of absorbed nitrogen via the kidney suggests that cooking may have an injurious effect on the protein complex of fish meals. Because energy requirements are supplied preferentially to protein requirements[16], it may be argued that on the C and P meal ration, the overall consumption of which was of a lower order, the large excretion of urinary nitrogen may be due to the utilization of at least a part of the absorbed protein to supply energy for maintenance. There is no doubt that the daily ingestion of total digestible nutrients by an animal of an average live-weight of 300 lb. (136 kg.) works out to only 2·12 lb. on the C and P meal ration as compared to 2·83 lb. on the BD meal ration. But it is only below a certain critical level that the calorie intake determines the efficiency of protein synthesis and above that level the additional calories merely serve to lay down additional fat[17]. For the breed of animals used in these experiments, it has been shown that the average daily maintenance requirement for an average animal of 133 kg. live-weight is 2·2 ± 0·29 lb. of total digestible nutrients[18]. Obviously the maintenance calorie requirement of the animals on the C and P

TABLE 6 — NITROGEN, LIME AND PHOSPHATE BALANCE

(*Values expressed in g./head/day*)

Nutrient	Fish meal in ration		Total intake	Output in			Balance
				Faeces	Urine	Total	
N*	BD	Av.	28·49	14·95	10·08	25·03	+3·46
		SE ±	1·92	1·25	1·21	1·81	0·55
	C and P	Av.	30·77	11·86	18·46	30·32	+0·44
		SE ±	1·31	0·25	0·31	0·39	0·06
CaO	BD	Av.	18·76	16·29	0·77	17·05	+1·70
		SE ±	1·35	1·25	0·09	1·18	0·49
	C and P	Av.	20·45	16·32	0·71	17·03	+3·42
		SE ±	1·13	0·38	0·09	0·45	0·70
P_2O_5†	BD	Av.	15·22	11·19	0·09	11·28	+3·94
		SE ±	1·02	0·57	0·00	0·57	0·61
	C and P	Av.	13·74	11·97	0·07	12·04	+1·70
		SE ±	0·16	0·40	0·00	0·40	0·52

Difference between groups: *highly significant; †significant.

meal ration was adequately met with. The experimental animals were all adults but all of them recorded positive nitrogen balances indicating further that sufficient energy and protein were consumed to meet the maintenance requirements and that the body proteins were not called upon for their energy value[19].

The average lime and phosphate balance in both the groups of animals is well maintained. The difference in the average lime retention in the two groups is not significant but in case of phosphate the difference is significant indicating in some degree the association of the metabolism of proteins and phosphates. The uniformly positive character of the retention of other major minerals, viz. magnesia, soda, potash and chlorine, by animals in both the groups (the data are not presented here) indicated that in amounts needed to supply the digestible crude protein requirements, fish meals would make available to the animals an abundance of all the major minerals.

The percentage of digestible nutrients in BD and C and P meals are given in Table 7. Though the meals had nearly the same protein contents the amount of digestible crude protein in the C and P meal was much higher than in the BD meal. As the content of digestible ether extract was only small, the relation holding between the digestible crude protein content of the two meals also holds good for the starch equivalent and total digestible nutrient contents.

TABLE 7—DIGESTIBLE NUTRIENTS IN FISH MEALS

(*Values expressed in lb.*/100 *lb. dry matter*)

	Beach-dried meal	Cooked and pressed meal
Digestible crude protein	48·93	59·98
Digestible ether extract	2·93	1·82
Starch equivalent	52·50	60·50
Total digestible nutrients	55·40	64·10

In spite of the superiority of the C and P meal in digestible nutrients, the general condition of the animals was better on the BD meal ration. During the feeding period of 28 days, the animals in the BD and C and P meal groups recorded average body gains of 15 per cent and 2 per cent respectively. The animals in group I also put on a comparatively fine bloom, developed a lustrous smooth coat and were found to be more vigorous when left out in the field.

The average data for the morphological and chemical composition of the blood of the animals in the two groups at the pre- and post-feeding stages presented in Table 8 also indicate in a general way the superior conformation on the blood picture on feeding of the BD meal diet. Fish meals are mainly protein feeds and their evaluation on the basis of the conformation on quality in blood proteins is a better criterion than the mere digestibility determination. The effect of the BD meal in raising the globulin to albumin ratio also points to a better body integrity obtaining on this diet rather than on the C and P meal diet. It appears that the protein fission products in the C and P meal might have been broken down to different stages of degradation and rendered unfit for efficient anabolism. The protein breakdown, however, was not indicated by the true protein estimation in the two meals.

As had been contended by the various workers, heat treatment seems to be responsible for the difference in the nutritive value of the two meals. Clifford[20] observed that the cooked meat was digested much more rapidly than raw meat and in conformity with his observations, it appears from the present work that cooking may improve the digestibility of proteins in fish meals. It has been observed before that the BD meal was more palatable. Evidently the palatability does not go hand in hand with digestibility. Barrick *et al.*[21] in experiments in rats with different fish meals also concluded that a more palatable meal is not necessarily

TABLE 8—AVERAGE DATA FOR THE PRE- AND POST-FEEDING BLOOD COMPOSITION OF ANIMALS

	Group I			Group II		
	Stage of feeding		% change	Stage of feeding		% change
	Pre	Post		Pre	Post	
Haemoglobin, g./100 ml. blood	7·20	7·20	—	9·60	8·10	−15·6
RBC, millions/cu. mm.	6·10	7·30	+19·7	5·50	6·10	+10·9
Cell vol., %	37·20	43·90	+48·3	40·20	35·80	−10·9
Calcium, mg./100 ml. serum	9·90	10·90	+11·1	9·70	10·90	+12·4
Phosphorus, mg./100 ml. blood	4·70	5·90	+25·5	4·40	4·70	+6·8
Total serum proteins, g./100 ml.	7·03	7·45	+5·6	7·56	7·77	+2·8
Globulin/albumin ratio	1·00	1·34	+34·0	1·27	1·12	−11·8

more nutritious. The injurious effect of heat on the protein complex of substances, including fish meals, has been surveyed by Fixen[22]. Jones *et al.*[23] emphasized the deleterious effect of heat on proteins but held that under proper exposure dry heat might improve the biological value of certain feeds including fish meals. Prolonged drastic treatments such as steaming, drying and grinding break down the protein fission products to different stages of degradation[24] and a high drying temperature increase the humin and volatile basic nitrogen content along with a diminution in arginine and cysteine nitrogen[25]. Nitrogen balance experiments with rats demonstrated the superior quality of vacuum-dried over flame- or steam-dried manhaden fish meals[26] indicating that the heat treatment was at least partially responsible for the nutritive differences found[27]. Honcamp *et al.*[28] observed no difference in the digestibility of air-dried and steam-dried cod fish meals. Record *et al.*[29], in growth experiments with chicks, observed no difference in protein value between meals prepared by wet and dry rendering processes. According to Oshima and Itaya[30], the digestibility of fish meals was highest for steam-dried and least for roasted meals. Rhiam *et al.*[31] showed in experiments with chicks that in general a wet process dog fish meal was of a higher nutritive value than the dry process dog fish meal and Evans *et al.*[32] observed that the difference was due to the content of protein that was higher in the wet processed meal. The latter workers also observed a greater proportion of decomposition products and hot water-soluble proteins of low nutritive value in the dry processed meal. On the other hand, cooking and pressing may amount to hydrolytic extraction, a process which results in the leaching out of fish solubles that supply vitamin like supplementary nutrients[33].

From the foregoing discussion it seems that the proteins from the C and P meal ration are absorbed better from the alimentary tract of animals but those absorbed from the BD meal ration are retained better. Thus, the observed higher biological value of proteins on the beach-dried meal ration may be more real than apparent. The large excretion of nitrogen via the kidney on the C and P meal ration indicates that 'cooking' may have an injurious effect on the protein complex of fish meals resulting in their easy post-absorptive deaminization.

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Influence of Feeding Curds on the Intestinal Synthesis of Thiamine in Caecectomized Rats*

B. R. BALIGA & R. RAJAGOPALAN
Central Food Technological Research Institute, Mysore

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Supplementation of curds to rats on a casein-starch diet leads to increased intestinal synthesis of thiamine. Such an increase does not take place when curds supplement is offered to caecectomized rats. A part of the observed effect may be due to protection and/or efficient absorption of thiamine intake due to the curds supplement.

In an earlier communication, Baliga *et al.*[1] reported that feeding a supplement of curds, along with a basal diet containing 18 per cent casein and all vitamins at optimum levels with the exception of thiamine which was provided at half-optimum level, improved the thiamine nutrition status of rats by way of enhanced intestinal synthesis of the vitamin. To find out whether the observed effect was due to enhanced intestinal synthesis or a protective action of curds supplement against destruction or inactivation of thiamine, and whether the unaltered anatomy of the gastro-intestinal tract is necessary for the development of this phenomenon, experiments with caecectomized rats were undertaken, and the results are reported here.

Materials and methods

Adult rats weighing 150-160 g. were used. Caecectomy and ileocolostomy were performed as described by Farris and Griffith[2].

Three days after the operation, the experiment was started. Two comparable groups, each of normal and caecectomized rats, were fed an 18 per cent casein diet and vitamins at optimum level with the exception of thiamine which was restricted to 10 μg./rat/day. The experimental diet, vitamin supplement and experimental technique were the same as those in the previous paper of this series[1]. These studies were conducted only for 4 weeks as it is well known that a part of the large gut gets enlarged and takes over the functions of the caecum in the caecectomized rats[3].

Results and discussion

Growth data — There is no difference in the growth response between the control and curds-fed group either in normal or caecectomized rats (Table 1). While the normal rats gained 30 g. in weight, the caecectomized rats gained only 10 g. in the same period. The food intake of the caecectomized rats is lower than that of the normal rats. But the intakes are not too far apart to account for the difference in weights gained. This is clearly apparent on comparing the feed efficiency of the normal and caecectomized rats. It is pertinent to point out here that the thiamine intake of all the rats was equal. A similar growth failure was reported by Taylor *et al.*[4] in caecectomized rats on a casein-sucrose diet.

Urinary thiamine excretion — The excretion in the normal curds-fed group was observed to increase steadily, while the excretion in the control was steady (Table 2). The influence of curds on the excretion is observed from the second week onwards. The urinary excretion in the caecectomized control is less than the excretion in the normal control. The caecectomized curds-fed rats also excrete more of thiamine than the caecectomized control. The increase was not of the same magnitude as obtained in the normal rats. The level of excretion in the caecectomized curds-fed rats reached the level of excretion prevailing in the normal control. This is probably due to the difference in the factors responsible for the increase in excretion in the two cases. Schweigert *et al.*[5,6] reported a reduction in the excretion of riboflavin in caecectomized rats, equivalent to the reduction brought about by sulphasuxidine in normal rats.

Faecal thiamine excretion — The curds-fed normal rats excreted more of thiamine than the normal control (Table 3). The increase due to curds feeding reached the steady level by about the second week. The increase in excretion was not merely a reflection of the faecal bulk. This becomes evident on comparing the excretion of thiamine calculated as μg./g. of dry faeces. The caecectomized rats

*A part of this work was done at the Indian Institute of Science, Bangalore.

TABLE 1 — GROWTH RESPONSE OF NORMAL AND CAECECTOMIZED RATS TO CURDS FEEDING

Group	Initial wt g.	Final wt g.	Increase in wt g.	Food intake g.	Feed efficiency
I Normal control	151	180	29	275	0·1067±0·001
II Normal curds	152	182	30	284	0·1033±0·001
III Caecectomized control	152	162	10	198	0·05052±0·001
IV Caecectomized curds	152	163	11	208	0·05297±0·001

TABLE 2 — INFLUENCE OF FEEDING CURDS TO NORMAL AND CAECECTOMIZED RATS ON THE URINARY EXCRETION OF THIAMINE

(*Values expressed in μg./day*)

Group	I week	II week	III week	IV week
I Normal control	1·60±0·02	1·47±0·02	1·62±0·01	1·69±0·03
II Normal curds	1·56±0·03	2·09±0·06	2·14±0·03	2·25±0·02
III Caecectomized control	0·93±0·03	1·21±0·06	1·23±0·02	1·21±0·04
IV Caecectomized curds	0·94±0·02	1·46±0·03	1·57±0·03	1·67±0·09

TABLE 3 — INFLUENCE OF FEEDING CURDS TO NORMAL AND CAECECTOMIZED RATS ON FAECAL EXCRETION OF THIAMINE

Group	I week		II week		III week		IV week	
	μg./day	μg./g.	μg./day	μg./g.	μg./day	μg./g.	μg./day	μg./g.
I Normal control	3·46±0·06	4·09±0·03	3·41±0·03	3·94±0·04	3·39±0·06	4·07±0·04	3·45±0·04	4·00±0·04
II Normal curds	3·69±0·03	4·65±0·03	4·77±0·04	6·24±0·04	5·11±0·04	6·56±0·06	5·14±0·04	6·40±0·03
III Caecectomized control	1·79±0·05	2·40±0·04	1·56±0·04	2·49±0·03	2·15±0·03	2·93±0·03	2·32±0·03	3·54±0·02
IV Caecectomized curds	1·85±0·03	2·22±0·04	2·22±0·04	2·51±0·04	2·74±0·04	3·02±0·04	3·01±0·03	3·42±0·05

excreted less of thiamine than the normal rats, the control as well as the curds-fed group. Comparing the control and the curds-fed group in the caecectomized rats, the curds-fed group shows an apparent increase of thiamine excretion. But there is no difference if the same is calculated as excretion per g. dry faeces, which shows that it is only due to an increase in faecal bulk. The excretion in the caecectomized rats, control as well as curds-fed, increased week by week at about the same rate. This is probably a manifestation of the establishment of the gut flora reduced suddenly on caecectomy. Another mechanism conducive to this increase is the out-pouching of a part of the colon as a compensatory organ for the caecum.

Considering the urinary and faecal excretion data together, it is observed that the higher urinary excretion in the case of the curds-fed normal rats is due to the absorption of a part of the thiamine from the increased intestinal synthesis. In the case of the curds-fed caecectomized rats, there is a small increase in urinary excretion even though no increase in faecal concentration is apparent. The explanation of this observation probably is that curds has some protective action towards thiamine intake, thereby rendering all or a substantial part of the intake available for absorption. Another mechanism whereby a similar response could be explained is that the curds facilitates the absorption of thiamine or the bacterial activity takes place higher up in the intestines in the case of caecectomized rats with the result that the thiamine synthesized is absorbed and not traceable through faecal excretion. However, in normal rats, the increased intestinal synthesis is by far more important than protection and/or better absorption.

Liver storage of thiamine — The data presented in Table 4, in general, support the conclusion drawn from the urinary and faecal excretion. The storage in the livers of normal curds-fed rats is higher than that in the normal control. In the case of the caecectomized rats also, curds supports an increased liver storage, but the magnitude of the increase is less. This is due to the difference in the causative factor responsible for the increase in the normal and caecectomized rats just as was the case in the urinary excretion.

Bacterial counts in faeces — The data presented in Table 4 also agree with the previous observations. While curds feeding supported a luxuriant flora,

TABLE 4 — INFLUENCE OF FEEDING CURDS TO NORMAL AND CAECECTOMIZED RATS ON LIVER STORES OF THIAMINE AND BACTERIAL COUNTS IN FAECES

Group	Liver stores		Bacterial counts $10^8/g.$†	
	Total $\mu g.$	$\mu g./g.$*	Total	Coliform
I Normal control	10·88±0·29	1·46±0·03	1·9	0·9
II Normal curds	16·55±0·37	1·62±0·04	3·1	1·4
III Caecectomized control	9·68±0·37	1·42±0·04	1·3	0·7
IV Caecectomized curds	10·18±0·15	1·56±0·03	1·8	0·7

*Fresh weight. †Dry weight.

particularly coliforms, in the case of normal rats, no such effect was observed in the case of the caecectomized rats.

The failure of curds to evoke an increased intestinal synthesis of thiamine in caecectomized rats parallels the observation of Wostmann *et al.*[7] on the absence of thiamine-sparing effect due to penicillin supplementation in germ-free rats. The observation of Kon *et al.*[8] that they were unable to produce refection in a group of more than 50 rats after caecectomy, also supports the present observation.

One of the first questions that is raised in respect of increased intestinal synthesis of thiamine is the lowered digestibility of curds brought by the supplement in question, thereby making available a richer medium for development of bacterial flora. In the present experiment, reduced digestibility due to curds supplementation is unlikely. The gain in weight during the experimental period and the food intake are nearly equal in the control and curds-fed group in the normal as well as caecectomized rats.

The observed phenomenon may be due to the coprophagus tendency of rats as shown by Barnes *et al.*[9]. These workers reported that, even though the site of maximum intestinal synthesis in the rat does not precede the site of absorption, as in the case of ruminants, functionally, it could be taken as preceding absorption in view of coprophagy. The same group of workers[10] have also reported that penicillin spares thiamine when coprophagy is allowed. Similar results were obtained by Morgan and Yudkin[11] who studied the thiamine-sparing action of sorbitol in rats. Further work is necessary to verify whether coprophagy exists under the experimental conditions employed here.

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The Efficacy of Blends of Groundnut Protein Isolate, Casein & Skim Milk Powder Fortified with Essential Amino Acids in Meeting the Protein Requirements of Protein Depleted Rats

P. K. TASKER, KANTHA JOSEPH, M. NARAYANA RAO, M. SWAMINATHAN, N. NATARAJA, A. SREENIVASAN & V. SUBRAHMANYAN

Central Food Technological Research Institute, Mysore

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The efficacy of groundnut protein isolate, protein blend III (a mixture of groundnut protein isolate, 66 parts and skim milk powder, 33 parts) and of protein blend IV (a mixture of groundnut protein isolate, 50 parts and casein, 50 parts, fortified with L-lysine hydrochloride, 2 parts and DL-methionine, 1 part) in meeting the protein requirements of protein depleted albino rats has been studied and compared with that of skim milk powder. When fed on a nitrogen-free diet for a period of 21 days, there is loss of *c.* 50 per cent of total body nitrogen and 55 per cent of total liver nitrogen. Xanthine oxidase activity is completely lost. Rehabilitation of the protein depleted animals by feeding for 21 days on diets containing 20 per cent protein from the different protein foods restores xanthine oxidase activity, serum proteins, haemoglobin and red blood cell counts to normal levels. The mean gains in body weight per gram of protein intake on diets containing the different protein foods are as follows: groundnut protein isolate, 2·28 g.; protein blend III, 2·31 g.; protein blend IV, 2·31 g.; and skim milk powder, 2·29 g. The mean protein retentions (expressed as percentage of intake) are 37·4, 39·0, 42·2 and 42·4 respectively on the different diets. The results show that groundnut protein isolate by itself is slightly less effective while a 2 : 1 blend of groundnut protein isolate and skim milk powder is almost as effective as skim milk powder in meeting the protein requirements of protein depleted rats.

In view of the inadequate production of milk and other protein-rich foods of animal origin in several developing countries, investigations have been carried out by several workers on the use of protein-rich foods of vegetable origin, e.g. oilseed meals and legumes, in the treatment of kwashiorkor and as supplements to the diets of children[1-4]. Recent investigations have shown that blends of isolated proteins from groundnut and soyabean along with skim milk or casein are highly effective in the treatment of kwashiorkor[5,6]. Groundnut protein by itself, though effective in curing the clinical signs and symptoms of protein malnutrition, is, however, inferior to milk proteins or blends of groundnut protein and skim milk powder in its capacity to regenerate serum proteins[6]. This may be due to the fact that groundnut proteins are partially deficient in certain essential amino acids, viz. methionine, lysine and threonine, as compared with milk proteins. Bhagavan *et al.*[5] and Dean[7] reported that blends of groundnut protein and skim milk powder or casein are almost as effective as skim milk powder in regenerating serum proteins in cases of kwashiorkor.

The object of the present investigation is to assess the relative efficacy of groundnut protein isolate and blends of groundnut protein isolate with casein or skim milk powder in meeting the protein requirements of protein depleted rats.

Materials and methods

Diets — The protein foods tested in this investigation were: (i) groundnut protein isolate; (ii) protein blend III (a mixture of 66 parts of groundnut protein isolate and 33 parts of skim milk powder); (iii) protein blend IV (a mixture of groundnut protein isolate,

50 parts and casein, 50 parts, fortified with L-lysine hydrochloride, 2 parts and DL-methionine, 1 part); and (iv) skim milk powder.

Groundnut protein isolate was prepared from edible quality groundnut meal according to Anantharaman *et al.*[8]. The protein isolate was powdered to pass through 60 mesh sieve and used. Skim milk powder (spray dried) and casein were commercial products of good quality. The nitrogen contents of the groundnut protein isolate and protein blends III and IV, determined by micro-Kjeldahl method, are given in Table 1. The amino acid composition of groundnut protein isolate and of protein blends III and IV, calculated using the values given in literature[9,10], as compared with that of milk proteins and FAO reference protein pattern is also given in Table 1.

Animal experiments — Male albino rats weighing about 100-110 g. from the laboratory stock colony were grouped according to a randomized block design, ignoring litters, into six groups of six rats each. The animals were housed in individual cages having raised wire screen bottoms. The initial values for haemoglobin, red blood cell count, total serum proteins, electrophoretic serum protein pattern, xanthine oxidase activity of the liver and the moisture, fat and protein contents of the carcass and liver were determined in one group of rats. The haemoglobin, red blood cell count and serum protein contents were determined according to Joseph *et al.*[11]. The electrophoretic pattern of serum proteins was studied by the method of Durram[12], using a veronal buffer of ionic strength 0·05. The animals were then sacrificed and the moisture, fat and protein contents of the livers and carcass determined according to Joseph *et al.*[11]. Xanthine oxidase activity of the livers was determined according to Dhungat and Sreenivasan[13].

The remaining five groups of rats were depleted of body protein by feeding a protein-free purified diet (Table 2) for a period of 3 weeks. The animals lost about 45-50 g. body weight at the end of the period. One group of depleted rats was sacrificed at this stage and blood, liver and carcass were analysed for the different constituents according to the methods mentioned above. The remaining four groups were rehabilitated by feeding on diets containing 20 per cent protein from different sources (Table 2) for a period of 3 weeks. Records of the body weight and food intake of individual rats were maintained. After feeding for 3 weeks, the rats were sacrificed and blood, liver and carcass were analysed for the different constituents according to methods referred to above. From the results obtained, the retentions of nitrogen in the four groups of animals during the rehabilitation period were calculated.

Results

Effect of protein depletion on body weight and composition of blood, liver and carcass — The results in Table 3 show that the rats fed on a protein-free purified diet for a period of 3 weeks lost on the average about 47 per cent body weight and 51 per cent of liver weight. The mean loss of total nitrogen of liver was about 55 per cent and that of the body was about 50 per cent. There were also significant reductions in the levels of serum protein, haemoglobin and red blood cell counts of the blood (Table 4). The xanthine oxidase activity in the livers was completely lost (Table 3).

TABLE 1 — AMINO ACID COMPOSITION* OF PROTEIN BLENDS, GROUNDNUT PROTEIN ISOLATE AND SKIM MILK POWDER AS COMPARED TO FAO REFERENCE PROTEIN PATTERN

(*Values in g. per* 16 *g. N*)

	Protein blend III	Protein blend IV	Groundnut protein isolate	Skim milk powder	FAO reference protein pattern
Lysine	3·8	7·7	3·1	7·4	4·2
Tryptophan	1·0	1·2	0·9	1·4	1·4
Methionine	1·3	2·9	1·0	2·8	2·2
Cystine	1·5	1·0	1·6	1·1	—
Methionine + cystine	2·8	3·9	2·6	3·9	4·2
Threonine	3·1	3·5	2·9	4·6	2·8
Phenylalanine	4·9	5·2	4·7	5·5	2·8
Leucine	7·6	7·9	6·7	12·1	4·8
Isoleucine	4·3	5·2	3·9	6·7	4·2
Valine	5·6	6·1	4·6	7·1	4·2
Protein (N × 6·25), %	68·2	83·3	85·4	33·9	—

*Calculated from data taken from Orr, M. L. & Watt, B. K., *Amino Acid Content of Foods* (U.S. Dept. of Agriculture), 1959, and Altschul, A. M., *Processed Plant Protein Foodstuffs* (Academic Press Inc., New York), 1958.

Effect of rehabilitation with diets containing different proteins on body weight and the composition of blood, liver and carcass — The results in Tables 3 and 6 show that the protein depleted rats when fed on diets containing 20 per cent protein rapidly gained in body weight. The mean gains in body weight per gram of protein intake on diets containing the different protein foods were as follows: groundnut protein isolate, 2·28 g.; protein blend III, 2·31 g.; protein blend IV, 2·31 g.; and skim milk powder, 2·29 g. The mean gains in body weight per gram of protein retained were 6·12, 5·96, 5·61 and 5·41 g. respectively. The mean protein retentions per 100 g. increase in body weight were 16·4, 16·9, 18·4 and 18·5 g. respectively on the different diets indicating thereby that the increase in body weight was accompanied by a corresponding increase in body protein. The mean protein retentions (expressed as per cent of intake on the different diets) were 37·4, 39·0, 42·2 and 42·4 respectively. There were no significant differences in the mean moisture, fat and protein contents of the livers and carcasses of rats rehabilitated on diets containing different protein foods.

TABLE 2 — PERCENTAGE COMPOSITION OF EXPERIMENTAL DIETS*

Constituents	Diet I (N-free diet)	Diet II (containing protein blend III	Diet III (containing protein blend IV)	Diet IV (containing groundnut protein isolate)	Diet V (containing skim milk powder)
Protein blend III	—	29·3	—	—	—
Protein blend IV	—	—	24·0	—	—
Groundnut protein isolate	—	—	—	23·4	—
Skim milk powder	—	—	—	—	59·0
Corn starch	87·0	57·7	63·0	63·6	28·0
Groundnut oil	10·0	10·0	10·0	10·0	10·0
Salt mixture†	2·0	2·0	2·0	2·0	2·0
Vitaminized starch‡	1·0	1·0	1·0	1·0	1·0

*Diet I did not contain any protein while diets II to V contained *c.* 20 per cent protein.
†Hubbel, Mendel and Wakeman salt mixture.
‡Vitaminized starch of Chapman *et al.*[14].

TABLE 3 — COMPOSITION OF LIVER AND CARCASS OF RATS DEPLETED* ON N-FREE DIET AND AFTER REHABILITATION† ON DIETS CONTAINING DIFFERENT PROTEIN FOODS

(*Mean values for 6 males per group; level of protein in the diet, 20 per cent*)

Group	Diet	Initial body wt *g.*	Final body wt *g.*	Wt of fresh liver *g.*	Composition of liver			Liver xanthine oxidase activity	Composition of carcass			
					Moisture %	Fat %	Protein %	*µl. O_2/g./hr*	Moisture %	Fat %	Protein %	Protein (on fat-free basis) %
A	Initial control (before depletion)	104·5	—	4·5	70·90	4·00	18·90	352·5	68·00	7·80	19·30	—
B	N-free diet (after depletion)	55·6	—	2·2	73·10	2·10	17·30	nil	68·40	4·70	18·30	19·20
C	Protein blend III (after rehabilitation)	55·8	161·3	6·2	70·30	3·50	18·30	361·3	55·40	20·40	17·40	21·80
D	Protein blend IV (after rehabilitation)	55·8	164·3	6·1	69·60	3·60	18·80	361·3	55·40	19·60	18·30	22·80
E	Groundnut protein isolate (after rehabilitation)	55·7	152·7	5·8	70·10	3·30	18·20	400·8	56·80	19·70	17·30	21·50
F	Skim milk powder (after rehabilitation)	55·1	136·9	5·8	70·30	3·50	19·20	388·0	56·40	18·10	18·40	22·50
	Standard error of the mean (15 d.f.)	—	—	—	±0·23	±0·16	±0·32	±19·5	±0·70	±0·67	±0·46	±0·56
	Critical difference at 5% level for differences among groups C, D, E and F	—	—	—	0·70	0·49	0·96	58·7	2·11	2·02	1·39	1·70

*Period of depletion, 21 days.
†Period of rehabilitation, 21 days.

TABLE 4 — COMPOSITION OF BLOOD OF RATS DEPLETED* ON N-FREE DIET AND AFTER REHABILITATION† ON DIETS CONTAINING DIFFERENT PROTEIN FOODS

(*Mean values for* 6 *males per group; level of protein in the diet,* 20 *per cent*)

Group	Diet	Red blood cell count 10^6/*cu. mm.*	Haemoglobin *g.*/100 *ml.*	Total serum protein %	Electrophoretic serum protein pattern				
					Albumin %	Alpha-1 globulin %	Alpha-2 globulin %	Beta globulin %	Gamma globulin %
A	Initial control (before depletion)	7·82	15·85	6·26	34·20	18·60	11·20	24·20	11·70
B	N-free diet (depleted)	5·59	13·85	5·12	22·50	19·10	18·60	18·70	21·00
C	Protein blend III (after rehabilitation)	7·84	15·85	6·21	34·50	21·50	10·20	22·50	11·20
D	Protein blend IV (after rehabilitation)	7·96	16·05	6·32	36·80	18·20	11·10	24·20	10·00
E	Groundnut protein isolate (after rehabilitation)	6·81	14·95	5·92	35·40	20·80	10·30	24·60	12·10
F	Skim milk powder (after rehabilitation)	7·88	14·95	6·31	32·10	20·60	11·30	23·00	9·60
	Standard error of the mean (15 d.f.)	±0·16	±0·25	±0·09	±0·78	±0·86	±1·09	±0·71	±1·91
	Critical difference at 5% level for differences among groups C, D, E and F	0·48	0·75	0·26	2·34	2·60	3·28	2·13	5·80

*Period of depletion, 21 days.
†Period of rehabilitation, 21 days.

TABLE 5 — WEIGHT AND NITROGEN CONTENT OF BODY AND LIVER OF RATS DEPLETED ON N-FREE DIET AND AFTER REHABILITATION ON DIETS CONTAINING DIFFERENT PROTEIN FOODS

(*Mean values for* 6 *males in each group*)

Group	Diet		Body wt *g.*	Total N in body (including liver) *mg.*	Wt of fresh liver *g.*	Total N in liver *mg.*
	Original	Rehabilitation				
A	Stock diet (initial control)	—	104·5	3350	4·5	142
B	N-free diet	—	55·6	1680	2·2	63
C	do	Protein blend III	161·3	4645	6·2	189
D	do	Protein blend IV	164·3	4775	6·1	191
E	do	Groundnut protein isolate	152·7	4665	5·8	175
F	do	Skim milk powder	136·9	4625	5·8	186

TABLE 6 — INTAKE AND RETENTION OF PROTEIN IN PROTEIN DEPLETED RATS AFTER REHABILITATION* ON DIETS CONTAINING DIFFERENT PROTEIN FOODS

(*Mean values for* 6 *males in each group; level of protein in the diet,* 20 *per cent*)

Group	Diet	Protein intake *g.*	Gain in body wt *g.*	Gain in body wt per g. of protein intake (3 weeks)	Initial body protein† *g.*	Total body protein *g.*	Protein retained *g.*	Gain in wt per g. protein retained *g.*	Protein retained as % of intake	Protein retained per 100 g. gain in body wt *g.*
C	Protein blend III	45·66	105·5	2·31	10·22	27·96	17·74	5·96	38·98	16·88
D	Protein blend IV	46·77	108·5	2·31	10·22	29·22	19·75	5·61	42·16	18·40
E	Groundnut protein isolate	42·67	97·0	2·28	10·20	26·12	15·92	6·12	37·38	16·40
F	Skim milk powder	35·57	81·8	2·29	10·10	25·24	15·14	5·41	42·35	18·53
	Standard error of the mean (15 d.f.)	—	±4·2	±0·11	—	—	—	±0·20	±2·14	±0·70
	Critical difference at 5% level	—	12·6	0·32	—	—	—	0·61	6·46	2·12

*Period of rehabilitation, 21 days.
†Calculated from the protein content of the carcass of rats depleted on N-free diet for 21 days (cf. Table 3).

The mean xanthine oxidase activity of the livers and the mean haemoglobin, red blood cells and serum protein levels in blood of the protein depleted rats were restored to normal levels by rehabilitation for 3 weeks on diets containing 20 per cent protein, there being no significant difference in the response of rats to diets containing the different protein foods.

Discussion

The results indicate that groundnut protein isolate, which is partially deficient in lysine, methionine and threonine, can meet the protein requirements of protein depleted rats when fed at 20 per cent level. Nevertheless, groundnut protein isolate, by itself, is somewhat inferior to blends of groundnut protein isolate with skim milk powder or casein. The results are in conformity with the earlier observations of Gopalan[6], in his investigations on the treatment of protein malnutrition in children using blends of groundnut protein isolate and skim milk powder.

Acknowledgement

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Studies on Some Umbelliferous Fruits: Part I—Pharmacognosy of the Fruits of *Trachyspermum roxburghianum* Benth. & Hook.

S. M. J. S. QADRY & C. K. ATAL

Institute of History of Medicine & Medical Research, Delhi &
Pharmacy Department, Panjab University, Chandigarh

The market sample of 'Ajmod' fruit, a drug used in Ayurvedic and Unani systems of medicine, has been identified to be that of *Carum roxburghianum* Benth. & Hook., although in literature the name 'Ajmod' is given to several different umbelliferous fruits. The macroscopical and microscopical characters of the drug have been described.

THE vernacular name 'Ajmod' refers to several different umbelliferous fruits[1-5] as (i) *Trachyspermum roxburghianum* Benth. & Hook. f.; (ii) *Trachyspermum ammi* Linn. and (iii) *Apium graveolens* Linn. Chemical and pharmacological studies on *Trachyspermum roxburghianum* have been done[3-4] but data on its pharmacognostic characters are not available. The present paper deals with the same.

'Ajmod' is a reputed drug of the Ayurvedic and Unani systems of medicine[6]. It is much used as a carminative, cardiotonic and emmenagogue. The active principle is an essential oil which has been chemically characterized[3-4]. Gujral[3] studied the chemical and pharmacological properties of a crystalline ketonic substance isolated from the fruits and found it to be strongly antispasmodic in nature.

Materials and methods

The material was obtained from drug dealers in Delhi and Amritsar and its authenticity was established in the Minor Forest Products Branch, Forest Research Institute, Dehra Dun.

Rotary microtome and free hand sections were taken, stained and mounted, and the cell contents and the cell-wall structure studied according to Trease[7] and Johanson[8]. Five per cent potassium hydroxide was used for isolating the entire vittae.

Macroscopy

'Ajmod' fruits occur mainly as entire cremocarps with the pedicel attached or detached and a bifid stylopod, the free ends of which curve along the dorsal sides. A small proportion of the drug occurs as separate mericarps. They are (Figs. 1 and 2) glabrous, ovoid-conical, about 1·5-3 mm. long and 1·2-2·8 mm. wide, and are yellow to yellowish green in colour. The separated mericarps are broadly ovoid, more or less curved, dorsal surface convex with five equally distinct longitudinal primary ribs and at the summit curved stylopodium; commissural surface flat, showing darker and lighter coloured longitudinal bands, the former representing the position of vittae and vascular bundles (Fig. 3). Odour aromatic, taste at first slightly bitter becoming strongly aromatic and producing a slight numbness to the tongue.

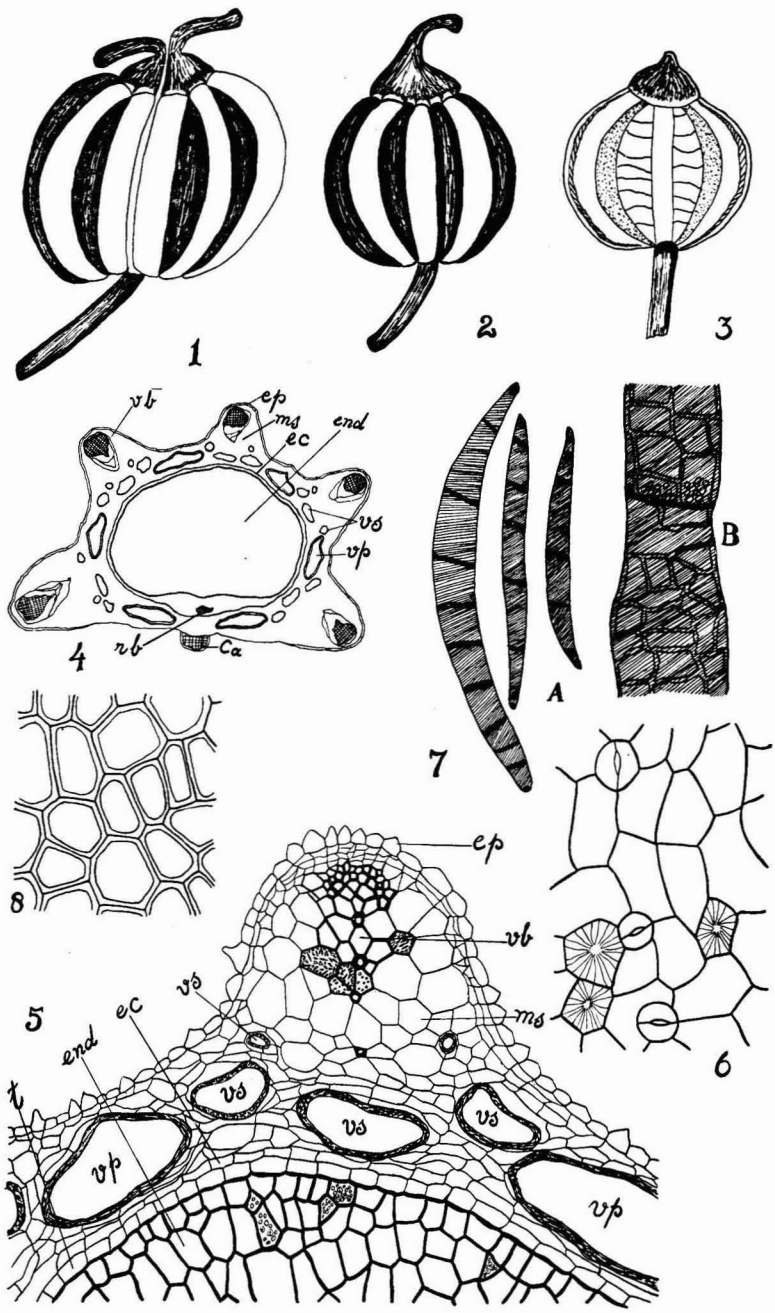
Microscopy

Mericarps have 4 large vittae on the dorsal surface and 2 on the commissural surface and 5 primary ridges on the dorsal surface. Three to five secondary oil canals are present under each primary ridge, and they are present also between the ridges. Carpophore is present on the commissural surface.

The cells of the epicarp have thin striated cuticle and the outer walls are drawn into papillae. Dimensions: T = 17-**35**-53 μ, R = 9-**21**-30 μ and L = 26-**40**-55 μ. Ranunculaceous stomata up to 35 μ in diameter are present among these cells.

The mesocarp consists of polygonal parenchyma plus thickened and lignified cells having oval to round pits. These thickened cells measure 30-**62**-95 μ in diameter in t.s. and are found mostly around the vascular strands in the region of ridges.

Collateral vascular bundles lie beneath the epicarp. The tracheids are 25-**202**-388 μ in length with spiral scalariform or reticulate thickenings. Xylem fibres measure 66-**150**-264 μ in length and the xylem parenchyma is lignified, elongated and with elliptical pits. They measure 52-**118**-176 × 13-**30**-44 μ.



Figs. 1-8 — Macroscopic and microscopic characters of the fruit of *T. roxburghianum* [Fig. 1: Lateral view of cremocarp × 15. Fig. 2: Dorsal view of cremocarp × 15. Fig. 3: Commissural surface of a mericarp × 15. Fig. 4: A diagrammatic representation of the mericarp in a t.s. × 33. Fig. 5: T.s. of a portion of mericarp in the region of the rib × 115. Fig. 6: Epicarp cells in surface view showing the papillose outgrowth areas and striations × 265. Fig. 7: A, primary and secondary vittae isolated by maceration × 33; B, a portion of vitta showing segmentation and epithelium × 265. Fig. 8: Cells of testa in surface view × 265. *ca*, carpophore; *ec*, endocarp; *end*, endosperm; *ep*, epicarp; *ms*, mesocarp; *rb*, raphe bundle; *t*, testa; *vb*, vascular bundle; *vp*, primary vittae; *vs*, secondary vittae]

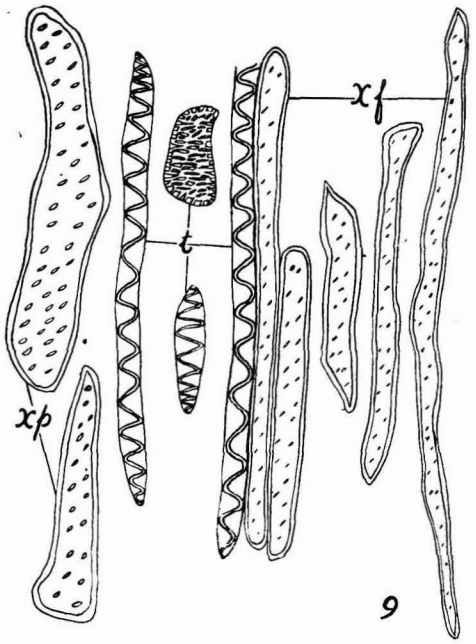


Fig. 9 — Macerated elements of the mesocarp bundle × 318 [*t*, tracheids; *xf*, xylem fibres; *xp*, xylem parenchyma]

The larger secondary vittae towards endosperm measure up to 123 μ in width and the smaller towards the periphery have the smallest diameter of 18 μ. The measurement of single vittae is L = 1820-**1960**-2000 μ, T = 240-**277**-308 μ and R = 52-**80**-102 μ. Each vitta is divided into small chambers by transverse partitions and is lined by an epithelium of square to polygonal cells.

The endocarp cells measure T = 17-**70**-155 μ and R = 6-**22**-23 μ. The testa consists of tangentially elongated cells attached to pericarp and measure T = 26-**44**-80 μ, R = 12-**17**-22 μ and L = 15-**40**-58 μ.

The raphe is made up of parenchyma cells surrounding a vascular strand, just outside the centre of the commissural region of the seed coat.

The endosperm is of the usual umbelliferous type and its cells measure 22-**48**-75 μ in t.s. The embryo lies at the centre of the endosperm.

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Long-acting Pharmaceutical Preparations: Part I—Ephedrine & Chlorpheniramine Resin Complexes

E. D. BHARUCHA & Y. K. HAMIED
Cipla Research Laboratories, Bombay-8

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A study has been made of the release of ephedrine and chlorpheniramine from their respective resin complexes in artificial gastric and stimulated intestinal juice. The results show that, under the experimental conditions, nearly 75 per cent of ephedrine and 73 per cent of chlorpheniramine are released in a total time of 6 hr.

BOTH ephedrine and chlorpheniramine as the hydrochloride and maleate respectively are widely used as therapeutic agents. The medicinal use of ephedrine is as a sympathomimetic agent for allergic disorders, stimulation of the central nervous system, prophylaxis and treatment of hypotensive and hypertensive sinus. It is usually administered in single doses of 30 mg., the maximum daily requirement being in the region of 100-150 mg. Chlorpheniramine is a potent antihistamine with minimum toxicity and is extensively used for local and generalized allergic reactions. It is given in doses of 4 mg., the daily requirement being in the region of 12 mg. Ephedrine and chlorpheniramine, being extremely soluble in stomach and intestinal fluids, exert their action soon after being administered. There is hence a need to control the action of ephedrine and chlorpheniramine so that the drugs are slowly and uniformly liberated by the stomach and intestinal juice at an effective therapeutic dosage during the time when the drug is in the stomach and intestine.

Oral long-acting pharmaceutical preparations have recently become increasingly popular as they provide a steady, prolonged action which cannot be achieved by the drug in its usual form, especially if the drug acts for a very short time. Further, it reduces the frequency of doses. A number of methods are known by which long-acting oral drugs have been made[1]. One of these consists of forming a complex of the drug with a suitable ion-exchange resin. Some important factors have to be considered before such a complex can be formulated, viz. rate of release in the stomach and intestine, toxicity, superiority over normal administration and effectiveness.

The ion-exchange resin used for this investigation was a sulphonic acid cation-exchange resin of high exchange capacity, stability and good chemical resistance over a wide *p*H range. It is supplied in fine particle size (100-325 mesh) with a cross-linkage of 8-9. The resin is first activated by stirring with excess of 5 per cent hydrochloric acid, filtered, washed free of acid and dried. A number of experiments were conducted taking varying amounts of ephedrine hydrochloride and chlorpheniramine maleate with resin and observing the amount of ephedrine and chlorpheniramine bonded to the resin. A slurry of the resin was added to a stirred solution of the drug in water at room temperature, and stirring continued for 6 hr. The complex is filtered, washed and finally dried at 60°C. As the resin does not contain any nitrogen, the amount of bonded drug was determined by nitrogen analysis using a modified Kjeldahl technique.

When the ratio of ephedrine to resin was 8:92, all the ephedrine was bonded to the resin and the release of ephedrine in the stomach and gastric fluids was suitable for the use of the complex as a long-acting ephedrine drug. Similarly, it was observed that the most suitable ratio of chlorpheniramine to resin was 27:73. The suitability of the complexes as long-acting drugs was studied by observing the release of ephedrine and chlorpheniramine from the respective complexes in artificial gastric juice consisting essentially of 0·03*N* sodium chloride and 0·07*N* hydrochloric acid without pepsin, followed by its release in stimulated intestinal juice consisting essentially of 0·039*N* sodium hydroxide and 0·05*N* monobasic potassium phosphate without pancreatin[2]. The method adopted was one of slow percolation of the gastric and intestinal juice through the resin complex and estimating the

TABLE 1 — RELEASE OF EPHEDRINE AND CHLORPHENIRAMINE FROM RESIN COMPLEXES

Drug complex	Gastric juice			Intestinal juice			Total % released
	First hour	Second hour	Third hour	First hour	Second hour	Third hour	
Ephedrine-resin complex (8% ephedrine)	23·5	19·75	13·85	4·26	4·56	9·27	75·19
Chlorpheniramine-resin complex (27% chlorpheniramine)	23·5	7·85	6·14	4·90	14·34	16·13	72·86

amount of ephedrine and chlorpheniramine released spectrophotometrically in terms of the hydrochloride and maleate respectively. The results obtained are given in Table 1.

It is observed that during the first hour nearly 25 per cent of ephedrine and chlorpheniramine are released from their respective complexes. In the case of ephedrine-resin complex, the release in 3 hr in gastric juice is approximately 57 per cent, the subsequent release in 3 hr in intestinal juice being approximately 18 per cent, giving a total release in 6 hr of approximately 75 per cent. Unlike ephedrine, 37·5 per cent of chlorpheniramine is released in 3 hr in gastric juice and approximately 35 per cent in 3 hr in intestinal juice, giving a total release of nearly 73 per cent. The rate of release of both ephedrine and chlorpheniramine from the resin complexes containing 8 and 27 per cent of ephedrine and chlorpheniramine respectively are suitable for the use of their resin complexes as therapeutic agents.

Experimental procedure

Cation-exchange resin — Resin (100 g.) was suspended in 5 per cent hydrochloric acid (500 ml.) and the mixture vigorously stirred for 5 hr at room temperature. The supernatant solution was decanted, the resin filtered, thoroughly washed with water and dried at 60°C. to give activated resin (98 g.).

Ephedrine-resin complex — A slurry of activated resin (9·2 g.) in water was slowly added to a solution of ephedrine hydrochloride (980 mg.) in water (250 ml.). The mixture was vigorously stirred for 6 hr at room temperature, the supernatant solution decanted, the product filtered, thoroughly washed with water and dried at 60°C. to give ephedrine-resin complex; yield 9·6 g. [Found: N, 0·65. Ephedrine-resin complex (8 per cent ephedrine) requires N, 0·67%].

Chlorpheniramine-resin complex — A slurry of activated resin (7 g.) was added to a solution of chlorpheniramine maleate (3·85 g.) in water (250 ml.). Following the method described above, chlorpheniramine-resin complex (9·5 g.) was obtained [Found: N, 2·67. Chlorpheniramine-resin complex (27 per cent chlorpheniramine) requires N, 2·7%].

Release of ephedrine from ephedrine-resin complex — Ephedrine-resin complex (1·25 g.) equivalent to ephedrine (100 mg.) was taken on a sintered glass filter and percolated with gastric juice at the rate of 50 ml. per hour for 3 hr. The solution at the end of each hour was removed and the ultraviolet absorption taken at the maxima 262·5 mμ. The percentage of ephedrine eluted was calculated in terms of standard ephedrine hydrochloride in gastric juice (Table 1). Intestinal juice was then percolated through the complex at the rate of 50 ml. per hour for 3 hr. The solution was removed at the end of each hour and the ultraviolet absorption taken at 262·5 mμ. The percentage of ephedrine eluted was calculated in terms of standard ephedrine hydrochloride in intestinal juice (Table 1).

Release of chlorpheniramine from chlorpheniramine-resin complex — Chlorpheniramine-resin complex (371 mg.) equivalent to chlorpheniramine (100 mg.) was released by both gastric and intestinal juices by the methed described above. The ultraviolet absorption readings were taken at the maximum 264 mμ and the results of the release of chlorpheniramine are recorded in Table 1.

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The Pharmacology of Certain Terpene Alcohols & Oxides

B. J. NORTHOVER & J. VERGHESE

Christian Medical College, Vellore

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Terpene alcohols and oxides produce a vasodilation both in the anaesthetized intact dog and in the decerebrated and despinalized dog. This effect is due to a direct action upon the blood vessels, or structures in their immediate vicinity, and is probably not mediated via the central nervous system. The direct vasodilator action of these compounds has been demonstrated in the perfused hind limb of the anaesthetized dog as well as in the isolated-perfused ear preparation. Terpene alcohols have been shown to dilate the small blood vessels of the exposed mesorchium of the anaesthetized mouse stimulated electronically.

MUCH work has been done on the pharmacology of the lower members of the aliphatic series of alcohols[1] and to a less extent on the higher members of the series. Very little work, however, appears to have been carried out on the pharmacology of the terpene series of alcohols and oxides unless one counts the studies carried out with the complex mixtures of these compounds which occur in many natural terpenoid essential oils. Studies of the latter type yield very little information on the pharmacology of the individual constituents of these oils. It was, therefore, of interest to study the properties of terpene alcohols and oxides, in particular their action on blood vessels, and correlate their chemical structure and biological action.

Experimental procedure

All the compounds studied were tested for their ability to produce a lowering of the arterial blood pressure in the dog maintained under chloralose anaesthesia with positive pressure artificial respiration through a tracheal cannula. The blood pressure was recorded from the central end of the ligated common-carotid artery, using a mercury manometer and a float-operated stylus marking a revolving smoked paper. The drugs were administered intravenously via a cannula into the femoral vein, in the form of an oil-in-water emulsion with acacia as the emulgent and washed in with a stream of normal saline from a burette.

In some animals the brain and spinal cord (CNS) were destroyed by performing a laminectomy on the axis vertebra, and inserting a long probe both up into the cranial cavity and down the length of the spinal cord. The haemorrhage was then controlled by packing the remnant of the axis vertebra with a piece of wood sealed round with plasticine.

The hind legs of anaesthetized dogs were also perfused in some experiments with oxygenated Locke solution warmed to 37°C. containing 5 per cent of low molecular weight dextran and 0·1 U./ml. heparin. The perfusate was fed under gravity into the distal portion of the ligated femoral artery and collected and measured from a cannula inserted in the ligated femoral vein. The leg was isolated from all other vascular connections with the body of the animal by the ligation of the iliac artery close to the bifurcation of the aorta, and the tight application of a length of rubber around the thigh, taking care to exclude the sciatic nerve from the tornique.

In some experiments the isolated ear of the rabbit was perfused under gravity through the central artery with the same perfusion fluid as described above, and the rate of venous drainage from the ear determined by placing the ear over a collecting funnel and cylinder, so that all drops emerging from the central and marginal ear veins were measured.

Mice were anaesthetized with pentobarbitone and the exposed mesorchium draped over a Perspex transilluminating rod. The mesorchium was kept warm and moist by flooding it with a slow stream of Tyrode solution containing 1 per cent gelatin at 38°C. Platinum microelectrodes were applied to a suitable arteriole and the diameter of the vessel observed under the microscope with an eyepiece micrometer. The frequency of square wave pulses, each of 0·01 V. and 1 msec. duration, was found,

which caused the diameter of the vessel to contract to half its original calibre.

Acute toxicity of the drugs was determined in mice. Groups, each of 10 animals, were set up; the animals received intramuscular injections of the substances under test, and the number of animals which died in the course of the next 48 hr period was noted. In those groups where the animals died, the death usually occurred within the first 12 hr from the time of giving the drug, but the recording of death was postponed to 48 hr to avoid missing the few animals which died later than the rest. Graded doses of each substance were administered and the number of animals which died was recorded as in the case of linalool (Table 1). A graph was plotted of the dose administered against number of animals which died and from this graph the dose which would kill 50 per cent of the animals in a group was calculated. These calculated LD_{50} doses are recorded in Table 2.

TABLE 1 — NUMBER OF MICE WHICH DIED IN GROUPS OF 10 FOLLOWING INTRAMUSCULAR INJECTION OF GRADED DOSES OF LINALOOL

Dose *g./kg.*	No. dead at the end of 48 hr
4	1
6	2
8	6
10	9
12	10

TABLE 2 — HYPOTENSIVE ACTION OF ALCOHOLS AND OXIDES WITH THEIR LD_{50} DOSES

Substance	Dose (*mg./kg.*) required to produce a 25% fall in blood pressure	LD_{50} *g./kg.*
Linalool	9·2	8
Citronellol	14·2	4
Nerol	16·7	3
Cyclohexene oxide	18·4	1
Cyclohexanol	18·4	1
Geraniol	20·4	4
m-Methylcyclohexanol	23·0	1
o-Methylcyclohexanol	23·0	1
Nopol	23·0	0·5
Rhodinol	23·0	4
Limonene monoxide	26·3	0·1
α-Pinene oxide	26·3	0·1
α-Terpineol	26·3	2
1,8-Cineole	26·3	1
Limonene dioxide	30·7	0·6

Results

Hypotensive action in the anaesthetized dog — All the alcohols and oxides tested gave a fall in arterial blood pressure when administered, the fall being rapid, and the recovery somewhat slower, reaching pre-injection levels within 3 min. if the dose had not been excessive. With very large doses some permanent damage was inflicted and the blood pressure never fully regained the pre-injection value. The possibility of the fall in blood pressure being a non-specific action of the injection of an oil-in-water emulsion was excluded, since the emulsions of nopol acetate, which is an ester, and also of cyclohexane, which is a hydrocarbon, were without any effect in the dose range used.

In the case of each substance tested, graded doses were administered intravenously to the dog and the dose which would produce a maximum fall in systolic arterial pressure of 25 per cent of the pre-injection pressure was determined. It was considered that the only valid comparison in hypotensive action would involve the expression of actual fall in pressure as a percentage of the pre-injection value. In other words, a maximum fall of 20 mm. of Hg on a pre-injection blood pressure of 100 mm. of Hg would be described as a 20 per cent fall in blood pressure as also would a fall of 30 mm. of Hg on a pre-injection blood pressure of 150 mm. of Hg. The doses of the various substances which were necessary to produce a maximum fall in systolic arterial pressure of 25 per cent of the pre-injection values are recorded in Table 2.

Hypotensive action in the decerebrated and despinalized dog — All the alcohols and oxides tested in this investigation were still active in the decerebrated and despinalized animal, but the magnitude and duration of the responses were considerably less. At first sight this would indicate that the alcohols were partly active in lowering blood pressure by means of some central action, and partly by a peripheral action. It is well known, however, that hypotensive drugs show a much greater effect in the animal with a high blood pressure than in the animal with a pre-existing low blood pressure such as in the decerebrated and despinalized dog. The destruction of the central nervous system, therefore, cannot be said to have definitely removed a site of action of these compounds in the body in so far as the hypotensive response is concerned.

Perfused hind limb of dog — When alcohols and oxides were added to the perfusion fluid on the arterial side the effect was to reduce the resistance to flow in the vascular bed of the limb and the rate of outflow of perfusate was increased.

Fifty mg. of each of the substances being tested were given and the outflow measured by noting the volume which flowed out each minute. The minute during which the greatest volume of fluid appeared

was taken as the time of maximum action, and the volume collected in that minute, expressed as percentage of the volume collected in the 1 min. period, immediately prior to giving the drug, is recorded in Table 3. There seemed to be very little point in recording the duration of action of these compounds since the duration of their action seemed to be mainly dependent upon the rapidity of the circulation. This would be expected since the more rapidly the perfusion fluid flows the more rapidly is the substance washed out of the perfusion circuit and the more rapidly would the action of the compound cease. The rapidity of the circulation varied considerably from one preparation to another and so there can be no valid comparison of this aspect of the action of these compounds.

When alcohols were injected into the systemic venous circulation of the animal, there was no comparable vasodilation in the perfused leg, despite the fact that the nervous connection of the limb with the CNS had been preserved. To test the competence of the nervous pathway between the perfused limb and the CNS, the animal was given doses of nicotine on the one hand and of hexamethonium bromide on the other. In the former case there was vasoconstriction and in the latter vasodilation. The nature of the preparation excludes the possibility that these drugs could have been exercising a local effect in the perfused limb, since the substances could not gain entrance to it, and hence confirming its physiological integrity with CNS. The inactivity of the alcohols when injected systemically in this preparation is strong evidence that the alcohols do not produce vasodilation by a centrally mediated pathway, but only by a direct action of the substances in, on or around the blood vessels themselves.

Isolated-perfused ear of the rabbit — The direct vasodilator action of the various alcohols and oxides on the blood vessels (Table 3) has been confirmed on this *in vitro* preparation.

Response of electronically stimulated blood vessels — It can be seen from Table 4 that a number of terpene alcohols lower the threshold to stimulation.

TABLE 3 — VASODILATOR ACTION OF TERPENE ALCOHOLS AND OXIDES

Substance	Maximum increase (%) in venous outflow over pre-injection values with a dose of 0·05 g.	
	Perfused leg of dog	Isolated perfused ear of rabbit
Linalool	120	90
Citronellol	120	85
Geraniol	95	85
Nerol	95	80
Cyclohexene oxide	90	80
Nopol	85	70
Cyclohexanol	80	70
m-Methylcyclohexanol	80	70
o-Methylcyclohexanol	80	70
Rhodinol	70	70
Limonene monoxide	70	60
α-Terpineol	65	55
α-Pinene oxide	65	55
1,8-Cineole	65	55
Limonene dioxide	50	45

TABLE 4 — RESPONSE OF ELECTRONICALLY STIMULATED BLOOD VESSELS TO TERPENE ALCOHOLS

Substance	Threshold for stimulation *pulses/sec.*
Control	35
Linalool	2
Citronellol	5
Geraniol	9
Nerol	13

Discussion

It is not easy to draw conclusions regarding structure-activity relationships in this series of compounds since it is not possible to say with certainty whether a difference in potency is a reflection of a difference in physical properties, such as viscosity, ability to be emulsified in the blood, volatility, aqueous solubility, or rate of mixing in the blood stream, or whether it is a true reflection of the chemical properties of the molecule. Within the limitations of this reservation, however, there do seem to be a few general observations which should be recorded.

Linalool, the only tertiary alicyclic alcohol tested, was considerably more potent as a hypotensive agent than the primary alcohols in the series. Nerol and geraniol are *trans* and *cis* isomers respectively and the fact that they show somewhat different potencies suggests that *cis-trans* isomerization can modify activity in this series of compounds. The open chain alcohols seem to be more potent than the monocyclic ones. It seems from the case of cyclohexanol and its homologues that methyl substitution reduces activity. It is, therefore, suggested that the most satisfactory explanation of the activity of the various oxides studied is that they are broken down in the blood to the corresponding alcohol, and it is the latter compound which produces the characteristic actions. This is suggested by the fact that cyclohexene oxide and cyclohexanol have similar potencies, as also have 1,8-cineole and α-terpineol, and in both of these pairs of compounds it is reasonable to expect that

they undergo hydration in the blood to give the corresponding alcohols.

Acknowledgement

We wish to thank the following for kindly supplying various terpenoids: linalool (Hoffmann-La Roche Ltd); nopol (Glidden Co.); nopol acetate (Schimmel & Co. Inc.); and d-limonene monoxide (Prof. E. Earl Royals). Thanks are due to the Council of Scientific & Industrial Research, New Delhi, for the grant on the scheme 'A study of the pharmacological action of certain terpenoids' under which the work reported in this paper was carried out.

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Studies on the Pharmacology of *Terminalia chebula* Retz.

M. C. INAMDAR & M. R. RAJARAMA RAO

Department of Chemical Technology, University of Bombay, Bombay 19

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The dried fruits of *T. chebula* contain a non-nitrogenous neutral principle possessing antispasmodic activity resembling that of papaverine. The active principle, chebulin, is isolated in a pure state by treating an alcoholic extract of the fruits with caustic alkali and then extracting the mixture with ether.

THE fruits of *Terminalia chebula* Retz. (Combretaceae) are used in Ayurvedic and Unani systems of medicine for eye and skin diseases, dysentery, leucorrhoea, epilepsy[1], hepatic congestion, dyspepsia, biliousness and diarrhoea, and recommended as cardio-tonic[2]. Purgative activity[3,4] and antibacterial and antifungal activity[5] of the drug have already been reported. Besides tannins and gallic acid, the presence of chebulinic acid has also been reported by Schmidt and Nieswandth[6]. The investigations reported here were incidental to the finding of antispasmodic activity in an alcoholic extract of the fruits[7].

Isolation and characterization of the active principle

The fruits of *T. chebula* were obtained from an authorized dealer in vegetable drugs. They were freed from seeds and powdered to pass through a 64 mesh. The powdered drug (1 kg.) was defatted with petroleum ether (60-80°) and extracted by percolation with 80 per cent ethanol. The alcoholic extract was concentrated *in vacuo*, suspended in 1·5 litres of 95 per cent alcohol and mixed with a 60 per cent (wt/vol.) solution of potassium hydroxide[8] so that the *p*H was nearly 10. The mixture was kept for 48 hr at room temperature and then diluted to four times its volume with water and extracted repeatedly with ether. The ether extract was washed with water, dried over anhydrous sodium sulphate, and concentrated. The crude 'chebulin' thus obtained was treated with animal charcoal and crystallized from 95 per cent alcohol (0·1 per cent yield).

Chebulin is a white crystalline material (m.p. 249-50°) and optically inactive. It is insoluble in water, dilute acids and dilute alkalies. It is soluble in ether, ethanol, methanol, acetone and pyridine. Tests for nitrogen are negative. Chebulin decolourizes bromine water and a dilute solution of potassium permanganate. Acetyl derivative of chebulin melts at 160°C.

On analysis chebulin was found to contain C, 75·3; H, 10·4; and O (by subtraction), 14·3 per cent; molecular weight (by Rast method), 459; suggested molecular formula $C_{28}H_{48}O_4$.

Infrared spectra of chebulin showed a band at 2·95 mμ in the region of hydroxyl group. In the

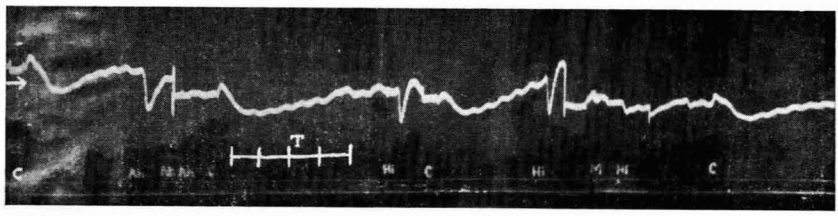

Fig. 1 — Blood pressure and respiration of cat after previous administration of atropine and mepyramine [Arrow indicates normal blood pressure, 110 mm. Hg; T, time interval, one division represents 80 sec.; Ah, acetyl choline 2 μg./kg.; Hi, histamine 2 μg./kg.; At, atropine 2 mg./kg.; M, mepyramine 2 mg./kg.; and C, chebulin 2 mg./kg.]

double bond region there were two bands, viz. at 6·2 and 8·6 mμ. The band at 5·8 mμ suggested the presence of ester (—CO—) linkage. This was supported by the fact that there was a band at 9·4 mμ which possibly was due to the presence of an acetate group. The infrared spectra of acetyl derivative of chebulin showed absence of band at 2·95 mμ which was due to hydroxyl group, thereby showing acetylation of the group.

Pharmacological studies

The effect of chebulin on the blood pressure, respiration and intestinal movements of cat was studied by conventional methods. The cats were anaesthetized with phenobarbitone sodium, administered intraperitoneally at a dosage level of 100 mg. drug per kg. body weight. Blood pressure was recorded on a kymograph through a mercury manometer connected to a cannula in the carotid artery. Respiration was recorded through a tambour connected to a tracheal cannula. Intestinal movements were recorded by a tambour connected to a small balloon inserted in the jejunum through an abdominal dissection. Spinal cats were prepared according to a method described by Burn[9].

Studies on blood vessels were made on the hind limbs of rats and isolated ears of rabbits. The abdominal aorta of the rat was cannulated and the body cut off above the level of the cannula. The hind limbs were perfused with warm (37°C.) oxygenated Ringer's solution. The outflow of the perfusate was counted as drops per minute in both these studies.

The heart of rabbit was removed from the body and perfused with warm (37°C.) Ringer's solution through a cannula in the aorta. The perfusate circulated through the coronary arteries and its outflow was measured as ml. per minute.

Small bits of the duodenum of rabbits and the ileum of guinea-pigs were mounted in organ baths of 40 ml. capacity and bathed in warm oxygenated Tyrode solution. Their tone and contractions induced by drugs such as acetylene choline, barium chloride and histamine were recorded on a smoked drum.

Acute toxicity of chebulin was studied on young mice weighing 20-25 g. The drug was injected intraperitoneally to groups of 10 mice in varying doses and the mortality rate measured.

Chebulin was used as a suspension in saline for animal experiments.

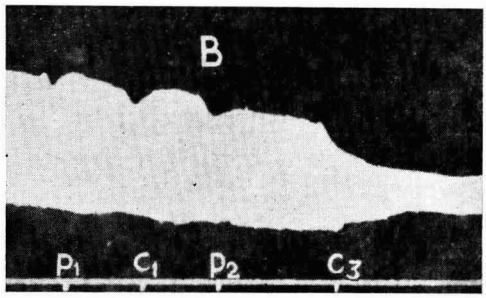

Fig. 2 — Isolated perfused heart of rabbit [C_1 and C_3, chebulin 0·2 and 0·6 mg.; and P_1 and P_2, papaverine 0·2 and 0·6 mg.]

TABLE 1 — EFFECT OF CHEBULIN AND PAPAVERINE ON CORONARY OUTFLOW OF ISOLATED HEART OF RABBIT

Drug	Dose *mg.*	Rate of coronary outflow *ml./min.*		Increase %
		Before administering the drug	After administering the drug	
Chebulin	0·2	6	9	50
	0·6	6	12	100
Papaverine	0·2	6	10	66
	0·6	6	12	100

Results

Effect on heart and blood vessels — Chebulin depressed the blood pressure of the cat; with 2 mg./kg. body weight the fall was 20-30 per cent. This fall was sharp and rather prolonged (Fig. 1).

TABLE 2 — EFFECT OF CHEBULIN AND PAPAVERINE ON PERFUSED HIND LIMB OF RAT

Drug	Dose *mg.*	Outflow *No. of drops/min.*		Increase %
		Before administering the drug	After administering the drug	
Chebulin	0·1	16	22	38
	0·25	16	25	56
	0·3	17	30	76
Papaverine	0·1	14	22	57
	0·25	15	24	60
	0·3	15	30	100

TABLE 3 — EFFECT OF CHEBULIN AND PAPAVERINE ON PERFUSED EAR OF RABBIT

Drug	Dose *mg.*	Outflow *No. of drops/min.*		Increase %
		Before administering the drug	After administering the drug	
Chebulin	0·1	27	32	19
	0·25	28	36	29
	0·3	27	38	41
Papaverine	0·1	26	32	23
	0·25	26	35	35
	0·3	24	35	46

There was no change in this fall of blood pressure even after the cat was atropinized (2 mg. atropine/kg. body weight) and mepyraminized (2 mg. mepyramine/kg. body weight). This fall of blood pressure was seen even in spinal cats. The amplitude of contraction of the isolated heart of rabbit was greatly diminished after the administration of chebulin (Fig. 2). In this experiment, however, introduction of chebulin into the perfusion fluid just before it entered the heart increased the rate of flow of the perfusate through the heart (Table 1). This effect was compared with identical doses of papaverine. In the case of the perfusion of the hind limbs of rats and the ears of rabbits, introduction of chebulin into the perfusion fluid just before it entered the perfused organs produced an immediate increase in the rate of the outflow (Tables 2 and 3). This effect was compared with identical doses of papaverine.

Effect on respiration — In the case of anaesthetized cat, injection of chebulin produced rapid, shallow breathing for a short period of time.

Effect on intestine — A moderate inhibition of peristalytic movements was observed in the intestines of anaesthetized cat on injection of 3 mg. chebulin/kg. body weight (Fig. 3). This effect was

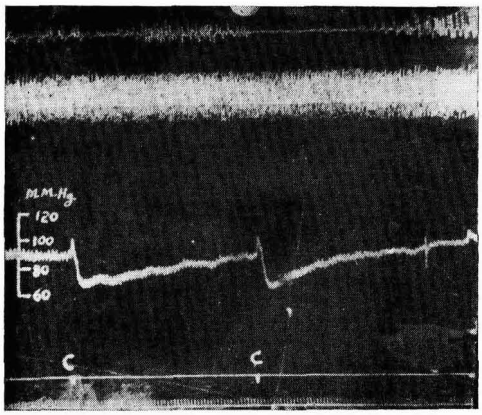

Fig. 3 — Blood pressure (bottom), respiration (middle) and intestinal movements (top) of cat [C, chebulin 3 mg./kg.]

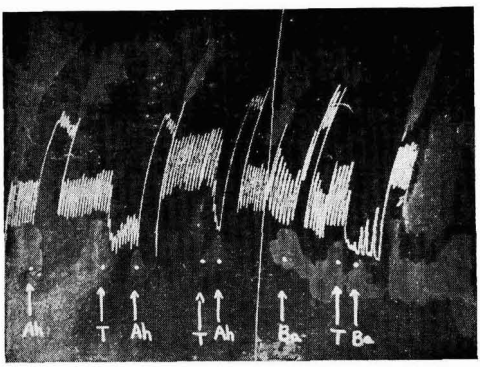

Fig. 4 — Isolated duodenum of rabbit [Ah, acetyl choline 2 μ; Ba, barium chloride 2 mg.; and T, chebulin 1 mg.]

studied in greater detail on isolated strips of rabbit duodenum and guinea-pig ileum.

In the case of rabbit duodenum there was a diminution of amplitude of spontaneous contractions as well as of tone on the addition of 1 mg. chebulin to the organ bath (capacity 40 ml.). A powerful spasm induced by barium chloride was completely inhibited by chebulin (Fig. 4).

In the case of guinea-pig ileum spasms were induced by acetyl choline, histamine and barium chloride. These were opposed by varying doses of chebulin. The action of chebulin was compared with those of atropine, mepyramine and papaverine. This comparison was made on a quantitative basis. It was observed that while chebulin was very much weaker than atropine in opposing the spasms induced by acetyl choline and weaker than mepyramine in opposing the spasms induced by histamine, it was nearly equal to papaverine in its

TABLE 4 — INHIBITION OF ACETYL CHOLINE, HISTAMINE AND BARIUM CHLORIDE INDUCED SPASMS OF GUINEA-PIG ILEUM BY CHEBULIN, PAPAVERINE AND ATROPINE

Drug	Conc. of drug	Contractions		Inhibition %
		Before administering the drug *mm.*	After administering the drug *mm.*	
		ACETYL CHOLINE		
Chebulin	$5{\cdot}0\times10^{-6}$	44	32	27·20
	$10{\cdot}0\times10^{-6}$	45	17	62·20
	$15{\cdot}0\times10^{-6}$	51	12	76·40
Papaverine	$2{\cdot}5\times10^{-6}$	53	20	62·20
	$5{\cdot}0\times10^{-6}$	55	16	70·09
	$7{\cdot}5\times10^{-6}$	57	3	94·70
Atropine	$5{\cdot}0\times10^{-8}$	58	34	32·87
	$1{\cdot}0\times10^{-7}$	61	35	42·60
	$2{\cdot}5\times10^{-7}$	45	18	60·00
		HISTAMINE		
Chebulin	$7{\cdot}5\times10^{-6}$	49	35	28·57
	$12{\cdot}5\times10^{-6}$	43	14	67·44
	$20{\cdot}0\times10^{-6}$	40	9	77·50
Papaverine	$2{\cdot}5\times10^{-6}$	65	39	40·00
	$5{\cdot}0\times10^{-6}$	58	15	43·00
	$6{\cdot}25\times10^{-6}$	63	7	56·00
Mepyramine	$5{\cdot}0\times10^{-8}$	51	36	29·40
	$1{\cdot}0\times10^{-7}$	43	27	37·20
	$1{\cdot}5\times10^{-7}$	48	12	75·00
		BARIUM CHLORIDE		
Chebulin	$2{\cdot}5\times10^{-5}$	59	46	22·00
	$6{\cdot}25\times10^{-5}$	60	29	51·60
	$1{\cdot}0\times10^{-4}$	77	33	57·10
	$1{\cdot}25\times10^{-4}$	66	13	80·30
Papaverine	$2{\cdot}5\times10^{-5}$	50	36	28·00
	$6{\cdot}25\times10^{-5}$	53	15	51·70
	$7{\cdot}5\times10^{-5}$	58	2	96·70

TABLE 5 — ACUTE TOXICITY OF CHEBULIN ON MICE

(*The drug was administered intraperitoneally; No. of mice used,* 10)

Dose *mg./kg.*	No. of animals dying	Mortality %	Dose *mg./kg.*	No. of animals dying	Mortality %
400	1	10	600	6	60
450	3	30	650	8	80
500	4	40	700	8	80
550	5	50			

opposition to spasms induced by barium chloride (Table 4).

Toxicity — LD_{50} of chebulin was found to be 550 mg. of drug/kg. body weight from acute toxicity experiments on mice (Table 5).

Discussion

The results of the experiments with chebulin on the circulatory system indicate to a depressant action on the heart and blood vessels. The amplitude of contraction of the isolated rabbit heart is greatly diminished after the administration of chebulin. There is a vasodilatation in the coronary artery and the peripheral blood vessels of rabbits and rats. And in the case of studies on blood pressure a moderate fall is observed in the anaesthetized cat as well as in the spinal cat indicating the fall to be due to peripheral action. Further, since the fall in blood pressure is not blocked by either atropine or mepyramine, it is presumed that the fall is not due to a cholinergic or a histaminic action. Therefore, the depressant action on the vascular system is considered to be due to a direct action on the musculature.

Disturbances of respiration are probably due partly to a fall of blood pressure and partly to a direct action on the bronchial muscles. This view is supported by the antispasmodic nature of chebulin, as seen in other organs also.

The antispasmodic nature of chebulin as seen in the various experiments on the intestines of cat, rabbit and guinea-pig indicates that it is of a non-specific nature. Opposition of the spasms induced by acetyl choline are in no way comparable to that of atropine. Similarly, antihistaminic action is very poor in comparison to mepyramine. The drug appears to be nearly equal to papaverine in its capacity to oppose barium chloride. It is, therefore, inferred that chebulin exerts its antispasmodic activity directly on the smooth muscles.

Acknowledgement

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Physiological Activity of the Leaves of *Dodonaea viscosa* Linn.

V. M. SUKKAWALA & V. B. DESAI*
L.M. College of Pharmacy, Ahmedabad

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Aqueous and alcoholic extracts of *D. viscosa* exhibit cardio-inhibitory and coronary constricting properties, and spasmolytic activity on smooth muscles and intestine. The alcoholic extract is more effective than the aqueous extract in counteracting the spasm induced by barium chloride, histamine acid phosphate and acetylcholine. It has also a sedative action on the uterus of virgin guinea-pig and also exhibits a hypotensive effect which is unaffected by atropine sulphate. Both the extracts possess slight anthelmintic activity while the alcoholic extract alone exhibits antibacterial activity.

THE leaves of *Dodonaea viscosa* Linn. (Sapindaceae) are reported to be used as alterative, laxative and in rheumatism[1]. They are also used as febrifuge and stimulant, and considered as sudorific as well. Powdered leaves are applied over wounds, burns and scalds, and as a poultice[2]. Leaf juice is used as a wash on swellings[2]. Since the physiological activity of the leaves of *D. viscosa* have not so far been reported, the present investigation was undertaken.

Materials and methods

Preparation of extracts — Leaves of *D. viscosa* Linn. were collected at the time of flowering, dried in shade and powdered (40 BS mesh).

Aqueous extract of the leaves was prepared by imbibing the powdered drug with chloroform water and setting aside for 4 hr. It was then placed in a percolator and macerated with sufficient water for 24 hr and then percolated till complete exhaustion. The percolate was concentrated under reduced pressure and the concentration of the extract was finally adjusted so as to represent 2 g. of the drug per 1 ml. of the extract.

Alcoholic extract of the powdered drug was prepared by exhausting the drug in a soxhlet with 90 per cent (vol./vol.) alcohol. Saponin traces were removed in the usual manner. The strength of alcohol in the extract was adjusted to 70 per cent and the concentration of the extract was finally adjusted so as to represent 2 g. of the drug per 1 ml. of the extract.

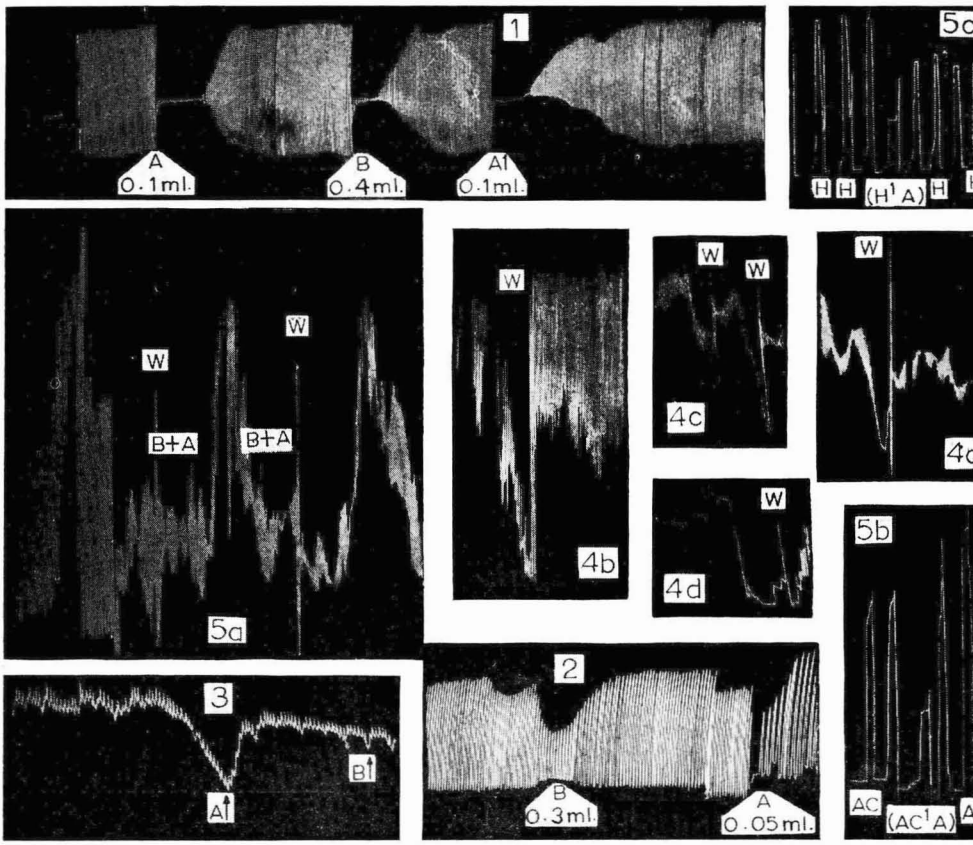
*Present address: Department of Pharmacology, Indian Institute of Science, Bangalore.

Results and discussion

Effect of the extracts on isolated frog's and rabbit's heart — A Syme's cannula was inserted into frog's heart through the aorta. The sinus venosus, carotid arch and the aorta were ligatured. Records were made on a smoked kymograph. The cannula contained frog-Ringer solution maintained at the room temperature. Similarly, rabbit's heart was isolated and attached to Lagendroff's heart perfusion assembly and perfused with oxygenated Ringer's solution at 37°C. The response of the heart to graded concentrations of the extracts was recorded. The results recorded in Fig. 1 show that alcoholic and aqueous extracts caused immediate temporary cardiac inhibition of frog's heart, which was unaffected by pretreatment with atropine sulphate. In the case of rabbit, the effect of 0·1 and 0·3 ml. of the alcoholic and the aqueous extracts showed temporary inhibition of the heart (Fig. 2). Coronary outflow was depressed by 41 per cent on injecting 0·05 ml. of the alcoholic extract and 43 per cent on injecting 0·3 ml. of the aqueous extract.

Effect on carotid blood pressure — Healthy cats of either sex were anaesthetized with pentobarbitone (30 mg./kg.) intraperitoneally and carotid blood pressure, after administration of the extracts, was recorded. The results presented in Fig. 3 show that on injecting 0·2 ml. of the alcoholic extract into the femoral vein of cat, a slight fall in carotid pressure is registered; the aqueous extract up to a dose of 0·6 ml. had no effect.

Effect on rabbit's duodenum, and guinea-pig's and rat's ileum — The tissues (2·5 cm. long) were



Figs. 1-5 — Physiological activity of aqueous and alcoholic extracts of the leaves of *D. viscosa* [Fig. 1: Effect on frog's heart. Fig. 2: Effect on rabbit's heart. Fig. 3: Effect on blood pressure of cat. Fig. 4: Action on (a) rat's duodenum, (b) rabbit's duodenum, (c) guinea-pig's ileum and (d) action on the uterus of guinea-pig. Fig. 5: Action on (a) barium chloride induced spasm; (b) acetylcholine induced spasm and (c) histamine induced spasm on guinea-pig's ileum. A, effect of alcoholic extract. B, effect of aqueous extract. W, washing]

suspended in a bath (50 ml. capacity) containing oxygenated Tyrode's solution at 37°C. The extracts were kept in contact with the gut for 30 sec. before the addition of spasmogenic drugs (barium chloride, acetylcholine and histamine acid phosphate) and then allowed to contract to the maximum. The bath was then drained and the muscle washed. After allowing the tissue to stand for 4-10 min., its contractions were recorded.

Both the alcoholic and the aqueous extracts caused a marked relaxation of rat's and rabbit's duodenum and guinea-pig's ileum (Figs. 4a, 4b and 4c) in a dose of 0·2 ml. and 0·5 ml. respectively.

When the rabbit's duodenum was treated with 0·2 ml. of the alcoholic extract, it completely counteracted the effect of 1 mg. of barium chloride (Fig. 5a). Similarly, treating rat's ileum with 0·2 ml. of the extract inhibited the spasm induced by treating it with acetylcholine (1: 5 million) by 50 per cent (Fig. 5b) and the spasm induced by histamine acid phosphate (1 : 50 million) by 55 per cent.

Effect on uterine horn of virgin guinea-pig — The uterine horn previously stimulated with pitocin (1·0 I.U.) was suspended in Dale's solution at 35°C. and the contractions recorded. The results presented in Fig. 4d show that the aqueous extract up to a dose of 0·4 ml. had no effect on the contraction of the uterus and on the spasm induced by acetylcholine. The alcoholic extract, on the other hand, showed a relaxation effect.

Depressant or stimulating effect of the extracts — Both the alcoholic and the aqueous extracts did not show any depressant or stimulating effect on albino rats when injected subcutaneously (with doses up

to 0·5 ml. and 0·8 ml. respectively of the two extracts).

Anthelmintic activity — The anthelmintic activity of the extracts was determined according to Shah and Bhattacharya[3]. The dried alcoholic extract of the leaves dissolved in propylene glycol solution (1·5 ml. in 48·5 ml. in distilled water) was used in the tests. The aqueous extract was used as such. Six earthworms (*Pheretima postuma*) of almost equal size were kept in 50 ml. of the drug solution and the time taken for killing them was noted. It was observed that 1 per cent aqueous and alcoholic extracts took 480 and 360 min. respectively for killing all the worms as compared to 280 min. taken by 1 : 1000 solution of santonin.

Antibacterial activity — The concentrations of the extracts were adjusted so as to represent 250 mg. of the drug in 1 ml. of the test solution and then tested for antibacterial activity by the cup-plate method on 18-24 hr old cultures of gram-negative and gram-positive bacteria listed below. The aqueous extract was found to be inactive against all the organisms whereas the alcoholic extract inhibited the growth of the organisms. The antibacterial activity of the alcoholic extract against different organisms was as follows (values given in parentheses represent mm. of clear zone) : *Micrococcus pyogenes* var. *albus* (22); *Micrococcus pyogenes* var. *citreus* (18); *Bacillus megatherium* (18); *Bacillus mycoides* (18); *Bacillus subtilis* (24); *Corynebacterium diphtheriae* (26); *Sarcina lutea* (19); *Escherichia coli* (20); *Salmonella typhi* (24); *Salmonella paratyphi* 'A' (20); and *Salmonella paratyphi* 'B' (25). The alcoholic extract exhibited no antibacterial activity against *Salmonella paratyphi* 'C', *Micrococcus pyogenes* var. *aureus*, *Pseudomonas pyocyneae*, *Pseudomonas vulgaris* and *Pseudomonas aeruginose*.

Bhatnagar *et al.*[5] have reported that the leaves of *D. viscosa* possess no physiological activity. The present work shows that both the alcoholic and the aqueous extracts of the leaves are physiologically active. Both the extracts exhibit cardio-inhibitory activity and coronary constriction. The action of the extracts on the heart muscle is a direct one as pretreatment of the heart does not change their inhibitory action. Both the extracts show spasmolytic activity. The alcoholic extract exhibits antihistaminic, antibarium and antiacetylcholine effects. The hypotensive effect of the extract may be attributed to cardio-inhibitory action. Spasmolytic activity of the extracts may be due to their direct action as well as neurotropic effect.

Acknowledgement

The authors wish to express their thanks to Dr R. P. Patel, Principal, L.M. College of Pharmacy, Ahmedabad, for his interest in the work.

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Short Communications

Interconversion of N-Acetyl Glucosamine 6-Phosphate & N-Acetyl Glucosamine 1-Phosphate in Rat Brain

T. N. PATTABIRAMAN & B. K. BACHHAWAT

Neurochemistry Laboratory, Department of Neurology & Neurosurgery, Christian Medical College Hospital, Vellore

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The enzymic interconversion of N-acetyl glucosamine 6-phosphate and N-acetyl glucosamine 1-phosphate has been demonstrated in the rat brain. By the coupled action of UDP-acetyl glucosamine pyrophosphorylase and mutase, N-acetyl glucosamine 6-phosphate has been obtained from UDP-acetyl glucosamine. The product has been identified by paper chromatography and by its enhancement of the activity of glucosamine 6-phosphate deaminase as N-acetyl glucosamine 6-phosphate.

THE presence of the biologically important aminosugar containing macromolecules, gangliosides and mucopolysaccharides in nervous tissue has been established by the work of Bogoch[1], Brante[2] and Guha *et al.*[3]. Work in this laboratory has established the enzymic synthesis of glucosamine 6-phosphate ($GlcNH_2$ 6-P), N-acetyl glucosamine 6-phosphate (GlcNHAc 6-P) and UDP-acetyl glucosamine (UDP-GlcNHAc)[4-6]. The presence of these enzymes suggested that the synthesis of uridine derivative of acetyl aminosugar is occurring in brain. However, the presence of the mutase which interconverts GlcNHAc 6-P and N-acetyl glucosamine 1-phosphate (GlcNHAc 1-P) has not been reported in brain. This interconversion has been shown in kidney and *Neurospora crassa*[7,8]. It has also been observed that the mutase from *N. crassa* may be specific for the phosphorylated acetyl glucosamine. The existence of a similar enzymic interconversion in rat brain is presented in this communication.

The adult rat brain obtained (albino rats of CFTRI strain) immediately after killing the animals was homogenized. with 0·03*M* tris (hydroxymethyl) amino methane (tris) buffer (*p*H 7·4; twice the volume per weight) in a mortar with the aid of equal weight of analytical grade sand. The homogenate was centrifuged at 20,000×*g* for 20 min. and the supernatant, designated as crude extract, was used for further experiments.

For the identification of GlcNHAc 6-P formed from UDP-GlcNHAc, the following experiments were carried out.

UDP-GlcNHAc (0·3 μmole), cysteine (1 μmole), sodium pyrophosphate (1·2 μmoles), magnesium sulphate (0·1 μmole), purified UDP-GlcNHAc pyrophosphorylase (70 μg.) from sheep brain[6] and the crude extract (1·1 mg.) in a total volume of 0·3 ml. were incubated for 1 hr at 37°C. The reaction was stopped by the addition of 0·1 ml. of 30 per cent trichloroacetic acid (TCA). The precipitated protein was centrifuged off. The supernatant was assayed for acetyl aminosugar as described elsewhere[9]. 0·06 μmole of GlcNHAc 6-P was formed.

In another experiment, after the same incubation period, the reaction was stopped by the addition of 0·3*N* barium hydroxide and 5 per cent zinc sulphate to precipitate the sugar phosphate and the free acetyl aminosugar was estimated. Virtually no free acetyl aminosuga was found in the reaction mixture. Since GlcNHAc 1-P is not chromogenic, the product formed in the reaction appeared to be only GlcNHAc 6-P.

To identify the product, 3 μmoles of UDP-GlcNHAc, and correspondingly higher concentrations of the reactants and the crude extract, in a total volume of 3 ml., were incubated as described above. The reaction was stopped by the addition of TCA and after removal of the protein by centrifugation, the nucleotides were removed by absorption on activated charcoal. Excess TCA in the supernatant was removed by repeated extraction with ether. The solution was then passed through Dowex-50 in H^+ form. N-Acetyl hexosamine phosphate was eluted with water and lyophilized. N-Acetyl hexosamine phosphate formed was 0·56 μmole.

An aliquot was taken and incubated with 15 μg. of prostatic phosphomonoesterase which cleaves GlcNHAc 1-P to GlcNHAc which is chromogenic[10]. Acetyl aminosugar was estimated before and after phosphomonoesterase treatment. There was an enhancement of 10 per cent in colour which accounts for GlcNHAc 1-P present in the reaction mixture.

The product was identified as GlcNHAc 6-P by paper chromatography. Twenty-five μg. of the product isolated were plotted on Whatman 3MM paper and irrigated in the basic water-miscible solvent system of Hanes and Isherwood[11] (propanol-saturated ammonia-water) (6:3:1) and ascending chromatography was performed for 16 hr at room

TABLE 1 — IDENTIFICATION OF ISOLATED PRODUCT AS GlcNHAc 6-P BY ITS ENHANCEMENT OF $GlcNH_2$ 6-P DEAMINASE ACTIVITY

[*0·2 μmole of $GlcNH_2$ 6-P, 10 μmoles of tris buffer (pH 7·4), 82 μg. of partially purified $GlcNH_2$ 6-P deaminase and the additions as indicated in a volume of 0·2 ml. were incubated for 1 hr at 37°C. and aminosugar degraded was determined as described elsewhere*[4]]

Additions	$GlcNH_2$ 6-P disappeared μmole
0·02 μmole of GlcNHAc 6-P	0·0625
0·02 μmole of the isolated product*	0·0610
0·02 μmole of GlcNHAc 6-P + 0·1 μmole of manganese sulphate	0·1120
0·02 μmole of the isolated product* + 0·1 μmole of manganese sulphate	0·1105
0·02 μmole of GlcNHAc 6-P + 0·1 μmole of mercuric chloride	0·1140
0·02 μmole of the isolated product* + 0·1 μmole of mercuric chloride	0·1020

*Calculated as GlcNHAc 6-P based on the assay for acetyl aminosugars described elsewhere[9].

temperature. The paper was then dried and sprayed with 0·16*M* sodium tetraborate solution to ensure complete wetting. The paper was kept in an air oven maintained at 100°C. for 10 min. after which Ehrlich reagent (10 g. of *p*-dimethylaminobenzaldehyde in 90 ml. of acetic acid and 10 ml. of 10*N* hydrochloric acid) was sprayed. A purple spot was formed immediately. The test sample was found to have the same R_f value as an authentic sample of GlcNHAc 6-P. The R_f value for GlcNHAc 6-P was found to be 0·33.

The isolated product was further identified as GlcNHAc 6-P by its enhancement of the activity of $GlcNH_2$ 6-P deaminase to the same extent as an authentic sample of GlcNHAc 6-P when used in equimolar amounts[4]. The results are presented in Table 1.

These observations indicate the following sequence of reactions:

$$\text{UDP-Acetyl glucosamine} + \text{Pyrophosphate} \xleftarrow{\text{Pyrophosphorylase}} \text{N-Acetyl glucosamine 1-phosphate} + \text{UTP}$$

$$\text{N-Acetyl glucosamine 1-phosphate} \xleftarrow{\text{Mutase}} \text{N-Acetyl glucosamine 6-phosphate}$$

GlcNHAc 1-P could be identified when GlcNHAc 6-P was incubated with the crude extract. GlcNHAc 6-P (0·6 μmole), cysteine (2 μmoles), magnesium sulphate (0·2 μmole) and the crude extract (6·6 mg.) in a total volume of 0·8 ml. were incubated for 1 hr at 37°C. The reaction was stopped by the addition of 0·2 ml. of 30 per cent TCA. The precipitated protein was centrifuged off. An aliquot was kept in a boiling water bath for 10 min. to hydrolyse GlcNHAc 1-P

TABLE 2 — FORMATION OF GlcNHAc 1-P FROM GlcNHAc 6-P

	Acetyl aminosugar μmole	Ratio of GlcNHAc 6-P to GlcNHAc 1-P in the reaction mixture
Before acid hydrolysis	0·216	
After acid hydrolysis	0·243	8·0: 1·0
Before phosphomonoesterase treatment	0·207	
After phosphomonoesterase treatment	0·231	8·6: 1·0

formed. After neutralizing with 20 per cent potassium bicarbonate aliquots, before and after acid hydrolysis, were assayed for acetyl aminosugar content. The increase in colour after acid hydrolysis gives the amount of GlcNHAc 1-P. The ratio of GlcNHAc 6-P to GlcNHAc 1-P in the reaction mixture was found to be 8: 1.

GlcNHAc 1-P formation from GlcNHAc 6-P was further confirmed with the help of prostatic phosphomonoesterase. After the incubation as mentioned above the reaction was stopped by keeping the tube in a boiling water bath for 3 min. The precipitated protein was centrifuged off. An aliquot of the supernatant was further incubated with 15 μg. of prostatic phosphomonoesterase. The amount of acetyl aminosugar was estimated in this aliquot as well as another aliquot which was not subjected to phosphomonoesterase action. The increase in colour after phosphomonoesterase treatment can be taken as a measure of GlcNHAc 1-P formed. The ratio of GlcNHAc 6-P to GlcNHAc 1-P was found to be 8·6: 1·0. The results are recorded in Table 2.

An attempt was made to isolate UDP-GlcNHAc by coupling the mutase action with UDP-GlcNHAc pyrophosphorylase. The results indicated that due to the presence of UDP-GlcNHAc pyrophosphatase and nucleotide phosphatase (Sekhara Varma, T. N. & Bachhawat, B. K., unpublished data), the primary product obtained was uridine and GlcNHAc 1-P. It was not possible to isolate UDP-GlcNHAc in the crude incubation mixture using GlcNHAc 6-P as the starting material.

Considering the earlier reports from this laboratory on the enzymes related to aminosugar metabolism, the identification of this enzymic interconversion is very vital since it bridges the gap between hexose 6-phosphate and UDP-GlcNHAc. It may be noted that all the enzymes which are known to involve in the biosynthesis of aminosugars are present in brain. On the basis of these facts an

outline of the enzymic sequence in the synthesis of aminosugars in brain may be given as follows:

$$\text{Fructose 6-phosphate} + \text{Ammonia} \xleftarrow{\text{Deaminase}} \text{Glucosamine 6-phosphate}$$

$$\text{Glucosamine 6-phosphate} + \text{Acetyl CoA} \xrightarrow{\text{Acetylase}} \text{N-Acetyl glucosamine 6-phosphate} + \text{CoA}$$

$$\text{N-Acetyl glucosamine 6-phosphate} \xleftrightarrow{\text{Mutase}} \text{N-Acetyl glucosamine 1-phosphate}$$

$$\text{N-Acetyl glucosamine 1-phosphate} + \text{UTP} \xleftrightarrow{\text{Pyrophosphorylase}} \text{UDP-Acetyl glucosamine} + \text{Pyrophosphate}$$

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Botanical Identity of *Ratanjot*

P. V. BOLE

Botany Department, St Xavier's College, Bombay

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The red dye-yielding root sold in Indian bazaars under the name *Ratanjot* is derived from the plant *Arnebia nobilis* Rech. f. and not *Onosma echioides* Linn. as reported by several workers. The plant is not a native of India. It grows wild in Afghanistan from where the roots are imported to India.

ALTHOUGH *Ratanjot* is one of the colouring matters permitted by the Government of India for use in foodstuffs[1], its botanical source is not defined. In an earlier communication from this laboratory[2], we had stated that as many as 15 different plant species are reported in the literature under this vernacular name. These various *Ratanjots* belong to at least four different families of flowering plants, namely Apocynaceae, Boraginaceae, Geraniaceae and Rosaceae, the one which was submitted to us for botanical identification being a red dye-yielding root material derived from a Boraginaceous plant. The Indian bazaar samples are sterile, thus precluding any definite conclusions being drawn regarding their botanical identity. A comparison of our sample with herbarium specimens of all the 15 species mentioned in the literature, supplemented by references to several leading international authorities, had suggested that it probably originated from *Arnebia nobilis* Rech. f. which is a native of Afghanistan.

According to sale tender notices for 1959 and 1960 from the Conservator of Forests, Timber Utilization Circle, Srinagar, very little. (< 1 seer) *Ratanjot* is produced in the country. An extensive market survey conducted by the author during 1960-61 revealed that *Ratanjot* is chiefly imported from Kabul, Afghanistan. This was confirmed by the customs officials posted at Amritsar as well as by a reference to Public Notice No. 82 ITC(PN)/59 dated 5 August 1959 issued by the Government of India in the Ministry of Commerce & Industry[3] which relates to imports of fruits, asafoetida, cumin seeds and medicinal herbs from Afghanistan. Included in the list of medicinal herbs is '*Ratanjot*' (Hindi) for which the equivalent vernacular name in Afghanistan is given as '*Yarlang*'.

In order to obtain fertile specimens of the plant(s) forming the source of *Ratanjot* of Indian bazaars, which is essential for establishing the botanical identity and also for verifying whether Afghanistan is in fact the source country, the author undertook a tour of Afghanistan and some parts of North and North-west India during the summer of 1961. In this note are given the observations made on the tour and the results of a study conducted in the author's laboratory.

When a sample of red dye-yielding *Ratanjot* root from the Bombay bazaar was shown to a number of Afghan tradesmen, it was promptly identified as *Yarlang* or *Yella-rang*. That was also the reaction of several laymen whom the author consulted. The museum attached to the Ministry of Agriculture, Royal Afghan Government, Kabul, has a specimen of *Yarlang* roots which matched the bazaar sample of *Ratanjot* from Bombay on visual examination. Enquiries in the Kabul market as well as in the Ministry of Agriculture, Royal Afghan Government, revealed that there are 4 or 5 areas from where *Yarlang* is collected and sent to the Kabul market. The author visited these areas and personally collected many fertile specimens of the *Yarlang* plant.

Their roots compared very well with the Boraginaceous *Ratanjot* roots sold in the Indian bazaars. Incidentally, while there do not appear to be any survey records in Afghanistan regarding the potential availability of *Yarlang*, the author was told at Amritsar that about 6 tons of roots were imported into India in 1959.

Like *Ratanjot* in India, *Yarlang* in Afghanistan is a vernacular name under which at least three species are known. However, the *Yarlang* of commerce in Kabul is derived from one source only.

The whole plant specimens collected by the author in Afghanistan were fertile and could be identified easily. These plants are quite definitely *Arnebia nobilis* Rech. f., a conclusion which was subsequently confirmed by all the international authorities consulted by the author. It may be mentioned here that while Dr K. H. Rechinger and Dr H. O. Volk consider *Arnebia nobilis* Rech. f. as a distinct species forming the source of *Yarlang* of Afghanistan and the Boraginaceous *Ratanjot* of Indian bazaars, Prof. S. Kitamura considers *Arnebia nobilis* Rech. f.[4] and *Macrotomia speciosa* (Aitch. & Hemsl.) Kitamura[5] to be synonymous (private communication). We also find that the *Yarlang* plant does not differ greatly from the characters described for *Macrotomia speciosa*[6]. This might involve some adjustments in its nomenclature according to the International Code of Botanical Nomenclature and we are at present engaged in settling this matter jointly with several international authorities. From all available information, *Arnebia nobilis* Rech. f. occurs only in Afghanistan and perhaps in some contiguous areas in West Pakistan, but not in India.

The chief areas which the author visited in North and North-west India were Kashmir and Chamba. The Forest Department of the Jammu & Kashmir State offered small quantities of *Ratanjot* for sale. The plants growing in these areas and known locally as *Ratanjot* belong to *Geranium rectum* Trautv. and *Potentilla nepalensis* Hook. and their roots, although reddish in tinge, bear no resemblance whatsoever to the Boraginaceous root material sold in Indian bazaars. Furthermore, their annual production is too small to be of interest to trade or industry. No other species of plants reported in the literature as *Ratanjot*, namely those of *Arnebia* (or *Macrotomia*) or *Onosma*, appear to be available commercially in these areas and in fact they are difficult to obtain even for experimental purposes.

Several authors[7-10] who have studied the toxicity of *Ratanjot* and its application as a visible colour for vanaspati, have reported that the material used in their experiments was *Onosma echioides* Linn. More recently, Bisht *et al.*[11] have reported a pharmacognostical study of the roots of what they consider to be *Onosma echioides* Linn. The material used in their studies " was obtained from the local market where it is sold under the Hindi name *Ratanjot* ", and no cogent reasons have been offered to support the assumption that the *Ratanjot* of Indian markets is in fact *Onosma echioides* Linn. Elsewhere in their paper, these authors state that *Onosma echioides* Linn. " is found in Kashmir and Kumaon at an altitude of 5000-9000 ft ". This has not been supported by any evidence and appears to be based on information contained in some old publications which is not critical as far as this plant is concerned. The *Ratanjot* roots from the Lucknow market, which were assumed to be *Onosma echioides* Linn. by the team at Central Drug Research Institute, Lucknow, were obtained by the author and have been found to be identical with the roots of *Arnebia nobilis* Rech. f.

According to well-known international authorities, notably Johnston[12], *Onosma echioides* Linn. is a polymorphic species. In fact, very different species from various countries have been designated by this name and the true delimitation of the species can be fixed only by a critical revision of the entire group. In a restricted sense, *Onosma echioides* Linn. does not occur in India, the local plant being *Onosma hispidum* Wall. Even this does not grow widely and it is certainly not the source of *Ratanjot* of Indian bazaars today. We have been able to obtain fertile specimens of the Indian plant (*Onosma hispidum* Wall.) as well as herbarium specimens of the plants from Spain, France and Italy known to local botanists as *Onosma echioides* Linn. All these specimens are different from one another and none bears roots matching the *Ratanjot* of Indian bazaars.

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Isolation of *Penicillium janthinellum* Biourge from Parachute Nylon Fabric

H. M. DAYAL, K. L. MAHESHWARI, P. N. AGARWAL & S. S. NIGAM

Defence Research Laboratory (Stores), Kanpur

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The fungus *Penicillium janthinellum* Biourge, isolated from damaged parachute nylon fabric, has been found to be capable of degrading synthetic fabrics such as nylon, terylene and orlon bright.

FABRICS made from synthetic fibres are claimed to be resistant to mildew[1]. Although nylon fabric/cordages showed loss in tensile strength under soil burial[2] or on exposure to sunlight[3] under tropical conditions, no specific organism has so far been reported to have been isolated from the synthetic fabrics. Recently, a piece of parachute nylon with pinkish stains was received in this laboratory from a depot for examination and ascertaining the cause of the stains on it. Microscopic examination of the stained regions revealed the presence of some fungal hyphae. The fungus was isolated on potato-dextrose-agar[4] (PDA) and Waksman[4] media, and identified as *Penicillium janthinellum* Biourge*.

The experimental nylon fabric was of three shades, white, green and greenish brown, used in the manufacture of parachutes. Yarns of terylene and orlon bright were used in the experiments. Three media, viz. potato-dextrose-agar, potato-dextrose-broth and Waksman's medium, were used in these tests.

Pieces (2×2 in.) of the three fabrics, sterilized with methyl alcohol vapours[5], were placed on the 7 days old fungal mat grown on PDA medium in sterilized 4 in. Petri dishes. The dishes were incubated at 30°±2°C. After 18 days of incubation, all the fabrics showed colouration in the regions having fungal growth. While green and greenish brown nylon developed pinkish brown stains, white nylon developed pink colour. The colour was similar to those observed on the original samples.

In order to study the degrading effect of the fungus on the nylon fabric, pieces (11·0×2·5 in.) of the fabrics, after sterilization with methyl alcohol vapours, were placed on potato-dextrose-broth and on potato-dextrose-agar medium and inoculated with the organism. After 6 weeks of incubation at 30°± 2°C., the pieces were taken out, washed with tap water, dried in shade and then subjected to breaking strength determinations. The fabric kept on the PDA medium showed 14 per cent loss in tensile strength as compared to only 1·6 per cent for pieces kept in the broth. Similar tests were carried out with other synthetic yarns and 15 and 18 per cent loss in tensile strength was observed in the case of terylene and orlon bright respectively when these yarns were inoculated with this fungus and incubated at 30°±2°C. for 6 weeks.

Detailed studies on the microbial degradation of synthetic fabrics and its prevention are in progress.

The authors are indebted to Dr J. N. Nanda, Director of this laboratory, for encouragement in the work and to Shri S. K. Ranganathan for initiation of the work. Thanks are also due to the Commonwealth Mycological Institute, Kew, U.K., for identifying the fungus.

*Identification was done by the Commonwealth Mycological Institute, Kew, U.K.

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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.

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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).

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