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Distribution and Seasonal Variation of *allo*Hydroxy-L-proline in *Santalum album* L.

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In the course of extensive investigations on the occurrence of free amino acids in plants (Radhakrishnan, Vaidyanathan & Giri, 1955), the presence of high amounts of free hydroxyproline was noticed on paper chromatograms of extracts of the leaves of sandal, *Santalum album* L., by Giri, Gopalkrishnan, Radhakrishnan & Vaidyanathan (1952). This observation led to the isolation of the imino acid from leaves of sandal tree by Radhakrishnan & Giri (1954), who identified it as *allo*-hydroxy-L-proline.

The distribution of *allo*hydroxy-L-proline in the sandal tree under a variety of conditions and preliminary results concerning its formation *in vivo* are now given.

EXPERIMENTAL

Sampling. For studying the variation of *allo*hydroxy-L-proline content in leaves from different trees, branches were collected in the morning and the eight pairs of youngest leaves were detached from each branch, shredded, dried in an oven at 50° for 48 hr. and powdered (100 mesh/in.).

For distribution studies the branches were obtained as before and the pair of leaves at each node was detached and mixed with the corresponding leaves from other branches. Thus the leaves from the apical nodes of all branches were pooled and the sample was designated '1st node'. Increasing sample number indicates pro-

gressively older leaves. The flowers and buds were dried and powdered similarly. The fruits were dried and powdered after splitting off the pericarp from the seed. The seed was defatted with light petroleum (b.p. 40–60°) before analysis.

The influence of storage upon the *allo*hydroxy-L-proline content of leaves was determined by collecting a large number of leaves from a healthy sandal tree. Separate portions were then kept in a moist chamber under normal laboratory conditions of temperature (24–26°) and illumination. After 1, 3, 4 and 6 days, the *allo*hydroxy-L-proline content of the leaves was determined, before and after drying.

Effect of amino acids on formation of *allo*hydroxy-L-proline by leaves of excised branches of sandal. Branches containing an equal number of nodes were cut from healthy sandal trees, and the excised ends were placed immediately in distilled water. Each experimental sample contained five branches selected from the main stock. The basal ends of the branches were cut to expose fresh surfaces. The branches were placed in a sterile solution of the appropriate amino acid (200 p.p.m.). A control sample was placed in water. After 54 hr. the leaves were detached and their *allo*hydroxy-L-proline contents were determined after drying. All experiments were conducted in triplicate.

Determination of *allo*hydroxy-L-proline. The method followed was essentially that of Neuman & Logan (1950) as modified by Martin & Axelrod (1953). Each powdered sample (50 mg.) was extracted with aqueous 75% (v/v) ethanol (5 ml.) for 15 min. with occasional stirring. After centrifuging, the residue was again extracted with 75% (v/v) ethanol (5 ml.) and finally washed with 75% (v/v) ethanol (1 ml.). The combined ethanol extracts were evaporated to dryness over CaCl₂ *in vacuo*. The residue was extracted with water and after filtration the volume was

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adjusted to 100 ml. This aqueous extract was free from chlorophyll and ethanol. Measured portions (0.5 ml.) of each extract were placed in tubes for the estimation of *allohydroxy-L-proline*. Then 0.5 ml. each of distilled water, 0.01M-CuSO₄, 2.5N-NaOH and 6% H₂O₂ were added in that order. The mixtures were shaken intermittently and, after exactly 5 min., 0.1 ml. of 0.05M-FeSO₄ was added to each, when they were shaken again until no more O₂ was evolved, usually 10 min. 4N-H₂SO₄ (2 ml.) and 4% (w/v) *p*-dimethylaminobenzaldehyde solution in propan-1-ol (1 ml.) were then added, and the tubes were heated at 70° for 16 min. After cooling, the colour intensities were determined in a Klett-Summerson photoelectric colorimeter with a 54 filter. Simultaneous additions of CuSO₄ and NaOH and of H₂SO₄ and *p*-dimethylaminobenzaldehyde as suggested by Martin & Axelrod (1953) did not increase the colour intensities. In each experiment three known concentrations of hydroxy-L-proline were also included. *alloHydroxy-L-proline* and hydroxy-L-proline gave identical standard curves in this procedure. *alloHydroxy-L-proline* values have been expressed throughout on the basis of dry weight.

Two precautions are essential for ensuring maximum sensitivity in hydroxyproline estimation. The final extract should be free of ethanol, which decreases the colour yield. Ethanol has been used as a solvent in the preparation of the *p*-dimethylaminobenzaldehyde solution (Waldschmidt-Leitz & Akabori, 1934), but the colour obtained with this reagent was less than that obtained in the above procedure where propan-1-ol was the solvent. It is also necessary to destroy H₂O₂ completely before the addition of 4N-H₂SO₄. Pyrrole-2-carboxylic acid is formed from hydroxyproline under the assay conditions used (Radhakrishnan & Meister, 1957), but the intermediate, 4-hydroxy- Δ^1 -pyrrole-2-carboxylic acid, formed in this reaction is decarboxylated by H₂O₂ at acidic pH values to give γ -amino- β -hydroxybutyric acid. Pyrrole-2-carboxylic acid itself is rather unstable in acid solutions.

Total N was determined by the micro-Kjeldahl procedure.

RESULTS

Variation in the content of allohydroxy-L-proline in leaves. The 'spike' disease of sandal, presumably caused by viral infection (Sreenivasaya & Gopalaswamy Naidu, 1930), is characterized by spike-like and unusually small leaves and shortening of the internodes with an enhanced vegetative growth resulting in a total inhibition of the reproductive phase (Varadaraja Iyengar, 1938). The content of *allohydroxy-L-proline* in leaves from a number of spiked and healthy trees located in different parts of Mysore State was determined. The analysis of all the samples could not be carried out simultaneously, and, in view of the observation made later that the content of *allohydroxy-L-proline* in leaves shows large variations depending on the season, a strict comparison cannot be made. However, when the results are expressed as a percentage of *allohydroxy-L-proline* N to total N, more *allohydroxy-L-proline* is present generally in the spiked samples (Table 1).

Table 1. *Variation in the content of allohydroxy-L-proline in sandal leaves*

Contents are expressed as g. of N present in *allohydroxy-L-proline*/100 g. of total N. The difference between the mean values was found to be highly significant ($t = 3.37$; $n = 22$; $P < 0.01$).

	No. of samples	<i>alloHydroxy-L-proline</i>	
		Range	Mean
Healthy	9	4.99-15.15	9.37 $\pm$ 3.94*
Spiked	13	12.20-52.16	21.4 $\pm$ 10.9

* Standard deviation of mean.

Distribution of allohydroxy-L-proline in the sandal tree. The distribution of *allohydroxy-L-proline* in various parts of a healthy tree during the preflowering and reproductive periods of growth is given in Tables 2 and 3. In the early stages of fruit formation the seed contained only traces of *allohydroxy-L-proline*, but the content increased during ripening of the fruit as is evident from results given here.

Although the amount of *allohydroxy-L-proline* in the wood and bark of the root is rather high on a nitrogen basis, it does not constitute a significant fraction of the dry weight of the whole plant.

Seasonal variation in the distribution of allohydroxy-L-proline in the sandal tree. In view of the above results, which indicate a possible seasonal variation in the distribution of *allohydroxy-L-proline* in sandal trees, one apparently healthy tree and two spiked trees were chosen for detailed observation and the hydroxyproline contents in the different parts studied between the months of March and December. The sandal tree has normally two flowering periods in a year, the first around March-April and the second in July-August, although variations are not uncommon. The trees were located in Marigalvali near Bangalore and were about 100 yards apart, the healthy tree being in the midst of a spiked locality.

Table 4 reveals the pattern of seasonal changes in the content of hydroxyproline. In March the highest concentration of *allohydroxy-L-proline* in the healthy tree was present in the flowers and in the pericarp of the green fruit, with a very low content in the leaves. The content of *allohydroxy-L-proline* in leaves was lowest in May. In June, the second preflowering period, the concentration of *allohydroxy-L-proline* in leaves had suddenly increased. In July, when buds and flowers appeared, they contained a high concentration of *allohydroxy-L-proline*; the concentration in leaves decreased. The formation of green fruit in August was accompanied by elevated *allohydroxy-L-proline* content in flowers and in the pericarp of the fruit; the amount in leaves was still small. In the

Table 2. *Nodal distribution of allohydroxy-L-proline in the leaves from a healthy sandal tree in the vegetative phase*

Node no.	<i>allo</i> Hydroxy-L-proline (g./100 g. of dry plant tissue)	Total N (g./100 g. of dry plant tissue)	$\frac{\text{alloHydroxy-L-proline N}}{\text{Total N}} \times 100$
1	2.88	3.4	9.05
2+3	3.12	3.7	9.01
4+5	2.56	3.6	7.60
6+7	2.16	3.3	7.00
8+9	1.72	3.0	6.14

Table 3. *Distribution of allohydroxy-L-proline in the sandal tree in the reproductive phase*

Part of the tree	<i>allo</i> Hydroxy-L-proline (g./100 g. of dry plant tissue)	Total N (g./100 g. of dry plant tissue)	$\frac{\text{alloHydroxy-L-proline N}}{\text{Total N}} \times 100$
Pericarp of the green fruit	10.64	2.38	47.90
Flowers	8.00	2.77	30.90
Root bark	3.80	1.40	29.01
Root wood	0.48	0.20	25.70
Young stem	3.20	1.96	17.42
Leaves:			
Node no. 1	1.20	2.94	4.37
2+3	1.15	2.87	4.31
4+5	0.84	2.10	4.28
6+7	0.76	2.07	3.92
8+9	0.54	1.96	2.95
Seed from the green fruit	Traces	—	—

Table 4. *Seasonal variation in the distribution of allohydroxy-L-proline in healthy and spiked sandal trees*

	<i>allo</i> Hydroxy-L-proline (g./100 g. of dry material)						
	March	May	June	July	August	October	December
Healthy							
Leaves*	0.61	0.27	2.4	0.85	0.57	0.61	0.65
Flower buds	—	—	—	2.88	—	—	—
Flowers	3.28	—	—	2.72	2.48	—	—
Pericarp of green fruit	6.16	—	—	—	4.00	3.51	3.12
Spiked†							
Leaves*	3.3	2.14	2.84	0.97	1.52	2.10	2.54

* Average value for three pairs of youngest leaves.

† One of the two spiked trees under observation succumbed in the second month of this study.

healthy tree the *allohydroxy-L-proline* concentration of leaves remained fairly constant thereafter until December.

In December fruits were collected from the healthy tree in different stages of ripeness. The *allohydroxy-L-proline* concentrations in the pericarp of the fruits decreased as ripening proceeded, whereas those of the seed increased. A typical set of values for *allohydroxy-L-proline* content (g./100 g. of dry plant material) of pericarp were: green fruit, 3.12; ripe ones, 3.04; dried ones (plucked

from the tree), 2.4 and dried fruits that had fallen from the tree, 1.92. In another experiment, the *allohydroxy-L-proline* content of the pericarp decreased from 2.64 in the green fruit to 1.12 in the ripe ones; correspondingly that of the endosperm rose from 1.52 to 2.8. These results are very interesting in view of the fact that the endosperm constitutes the reserve food source for the developing embryo.

In the spiked tree, the leaves had a uniformly higher *allohydroxy-L-proline* content than those

from the healthy tree (except in June and July when the content was nearly the same), and the seasonal variation was somewhat analogous to that mentioned above. Although the content in the leaves of the healthy tree remained fairly constant during the months August to December, that in the spiked leaves showed a slight rise in August, probably due to new leaf growth.

Effect of time of storage on the content of allohydroxy-L-proline in detached leaves. When freshly collected sandal leaves from a healthy tree were dried at 50°, an increase in their allohydroxy-L-proline content occurred. The allohydroxy-L-proline contents of freshly detached leaves kept in a moist chamber for 24 hr. at room temperature also increased. The effect of keeping detached leaves and of drying them after various intervals of time on their contents of allohydroxy-L-proline is given in Table 5. The content of allohydroxy-L-proline in fresh leaves after keeping the leaves for 24 hr. showed a 54 % increase, and almost the same increase (59 %) was observed in fresh leaves dried at 50°. When leaves were kept for prolonged periods (144 hr.) there was a slight decrease in the content of allohydroxy-L-proline and also in total N. Pearsall & Billimoria (1937, 1938) have noted losses of N in excised daffodil leaves kept under different experimental conditions. The synthesis of allohydroxy-L-proline in the above case simulates

that of proline in rye grass under conditions of wilting as observed by Kemble & Macpherson (1954).

Formation of allohydroxyl-L-proline in sandal. The experiments conducted with excised sandal branches were designed to study the possible precursors utilized for allohydroxy-L-proline synthesis. The content of allohydroxy-L-proline in the leaves was determined after infiltration with the amino acid solutions for 54 hr. Ornithine and glutamic acid produced toxic symptoms after 24 hr. manifested as a 'burning' of the leaf along the principal veins and browning of the green twigs.

The results obtained on branches from two healthy trees, one of them bearing fruits and the other in the preflowering period, are given in Table 6. alloHydroxy-L-proline increased in leaves from the tree in the preflowering period when ornithine or glutamic acid was supplied. The effect of various ornithine concentrations was determined in branches taken from the tree during the preflowering phase. The results presented in Table 7 indicate that the optimum concentration was about 125 p.p.m. Although there was a slight increase brought about by proline in branches from fruit-bearing trees, the results were negative for leaves taken at the preflowering stage. Only slight changes were observed with solutions of other amino acids: γ -aminobutyric acid (–6 %), glutamine (+13 %) and arginine (+2 %).

Table 5. *Effect of time of storage at 24–26° on the content of allohydroxy-L-proline in detached leaves of sandal*

After storage, determinations were made on either (a) fresh leaves or (b) leaves dried at 50°.

Time (hr.)	Moisture (%)	Total N (%)	(a) Fresh leaves		(b) Dried leaves	
			alloHydroxy- L-proline (g./100 g. of dry plant tissue)	alloHydroxy- L-proline N Total N × 100	alloHydroxy- L-proline (g./100 g. of dry plant tissue)	alloHydroxy- L-proline N Total N × 100
0	64	2.94	2.0	7.3	3.2	11.6
24	65	2.77	2.9	11.2	2.9	11.2
72	62	2.74	2.9	11.2	2.9	11.2
96	61	2.69	2.8	11.1	2.9	11.2
144	63	2.66	2.5	10.0	2.6	10.0

Table 6. *Influence of amino acids on the synthesis of allohydroxy-L-proline by leaves of excised branches of sandal*

alloHydroxy-L-proline contents were expressed as g./100 g. of dry leaf material. Amino acids were supplied at 200 p.p.m. Period of infiltration, 54 hr.

	Branches from the tree in the vegetative phase		Branches from the tree in the reproductive phase	
	alloHydroxy- L-proline	% Difference over control	alloHydroxy- L-proline	% Difference over control
Water	2.08	—	2.16	—
L-Proline	2.64	+27	2.08	–4
L-Glutamic acid	1.92	–8	2.84	+31
DL-Ornithine-HBr	2.40	+12	3.52	+63

Table 7. *Effect of concentration of ornithine on synthesis of allohydroxy-L-proline by leaves of excised branches of sandal*

Period of infiltration, 54 hr.; the tree was in the vegetative phase.

DL-Ornithine-HBr (p.p.m.)	alloHydroxy-L-proline (g./100 g. of dry leaf material)	Increase over control (%)
0	2.24	—
8	2.53	13
16	3.11	39
32	3.18	42
64	3.50	58
125	3.61	61
250	3.56	59
500	2.46	11

DISCUSSION

*allo*Hydroxy-L-proline is distributed in all parts of the sandal tree, although the concentrations present in various organs differ markedly. In the preflowering period the content is highest in leaves from the apical node when expressed on either a dry weight or total nitrogen basis. Flowers and pericarp of the green fruit contain exceedingly high amounts of free *allo*hydroxy-L-proline and so constitute rare examples of excessive accumulation of a free amino acid in biological materials. *allo*Hydroxy-L-proline tends to accumulate in the apical leaves (meristematic regions) of a branch in the vegetative phase and in flowers and pericarp of the green fruit in the reproductive phase. It is not known whether *allo*hydroxy-L-proline is simply translocated from the leaves to the flowers and thence to the pericarp or whether it is partly synthesized *in situ* in these parts.

The *allo*hydroxy-L-proline content of leaves from a spiked tree is higher than that of leaves from a healthy tree. This may be partly attributed to the fact that in spiked trees reproductive mechanisms are known to be inhibited and the normal mode of transport of *allo*hydroxy-L-proline from the leaves to the flowers and fruits does not occur in diseased trees.

Drying of detached leaves at relatively low temperatures (50°) or storing them at room temperature in a humid chamber causes an increase in the content of *allo*hydroxy-L-proline. Since acid hydrolysis of fresh or dried leaves did not show any marked increase (less than 8%) in total *allo*hydroxy-L-proline, it is unlikely that the increase is due to protein breakdown in the excised leaves. Kemble & Macpherson (1954) have observed formation of proline in excised rye grass; under conditions of 'moist wilting', proline was not synthesized, indicating that a certain degree of desiccation was essential. It is conceivable that the increase in

*allo*hydroxy-L-proline is due to synthesis from precursors, as yet unrecognized, originally present in the leaves.

Infiltration experiments with dilute solutions of amino acids indicated that glutamic acid and ornithine were probable precursors of *allo*hydroxy-L-proline in the sandal tree. Proline was effective only for the tree in the reproductive phase. At the present time it is not certain whether the increase in the content of *allo*hydroxy-L-proline is due to net synthesis from, or to some kind of stimulation by, these amino acids.

There is much information on the interrelationships between the metabolism of ornithine, arginine, proline, hydroxyproline, glutamic acid and aspartic acid (Stetten, 1949, 1955; Stetten & Schoenheimer, 1944). Hydroxy-L-proline is converted into alanine (Wolf, Heck & Leak, 1956) and also into glutamic acid and aspartic acid (Stetten, 1949); γ -hydroxyglutamic γ -semialdehyde is a possible intermediate in the latter conversion (Gianetto & Bouthillier, 1954; Lang & Mayer, 1953; Adams, Friedman & Goldstone, 1958). By analogy with proline synthesis, it is possible that the conversion of ornithine into *allo*hydroxy-L-proline in sandal proceeds through this intermediate. An alternative pathway is also indicated by the work of Radhakrishnan & Meister (1957), who have shown that 4-hydroxy- Δ^1 -pyrroline-2-carboxylic acid, the keto analogue of hydroxyproline, is formed from γ -hydroxyornithine. Another analogous reaction is the formation of 5-hydroxypipercolic acid from δ -hydroxy-DL-lysine (Lindstedt & Lindstedt, 1959).

The mode of distribution of *allo*hydroxy-L-proline in the tree points to an intimate connexion with growth, development and perhaps the post-fertilization stages of the plant. Kemble & Macpherson (1954) suggested that the role of proline synthesis during wilting of rye grass is probably similar to that usually provided by amide formation, and Virtanen & Miettinen (1952) regard the occurrence of large amounts of free citrulline in alder (*Alnus*) as a mode of nitrogen storage.

Santalum album is native to India and *allo*hydroxy-L-proline has been detected in all the samples of sandal, healthy and spiked, examined by us. McKee & Urbach (1955) confirmed the presence of *allo*hydroxy-L-proline in the Australian varieties of *Santalum obtusifolium* and *Santalum murrayanum*. Other Australian varieties, *Santalum yassi* (Forest Research Lab., Bangalore) and *Santalum austrocaledonicum* (obtained from Dr H. S. McKee), were also found to contain free *allo*hydroxy-L-proline, and the free amino acid compositions of the Australian varieties were similar to that of *S. album*. Since sandal is a root parasite, a

number of host plants usually growing close to sandal, e.g. *Pongamia glabra*, *Acacia farnesiana*, *Casuarina equisetifolia* and *Bambusa arundinaceae* were examined chromatographically for the presence of allohydroxy-L-proline. No allohydroxy-L-proline, free or combined, was detected, indicating clearly that the amino acid was not obtained from the haustoria but is a normal constituent of sandal. Hydroxy-L-proline was not detected in the free amino acid fraction of sandal leaves by the procedure of Friedberg (1954) for the chromatographic separation of the two isomers of hydroxyproline as their copper salts.

Previously allohydroxy-L-proline was recognized in bound form as part of a hexapeptide isolated from *Amanita phalloides* and *Agaricus phalloides* (Wieland & Witkop, 1940). Besides the well-known occurrence of hydroxy-L-proline in collagens and gelatins evidence for its limited distribution in plant proteins is now available (Steward, Thompson & Pollard, 1958; Steward, Bidwell & Yemm, 1958), although the presence of allohydroxy-L-proline in sandal in the free state is rather conspicuous. However, significant amounts of hydroxy-L-proline are found predominantly in proteins of rapidly proliferating tissues, e.g. leaf tumours and growing tissue cultures of carrot and potato, even when such tissues normally do not contain this amino acid. Surprisingly, both hydroxy-L-proline and allohydroxy-L-proline strongly inhibit the growth of carrot explants (Steward, Pollard, Patchett & Witkop, 1958). Hydroxy-L-proline has been reported to be toxic to the growth of tobacco plants (Steinberg, Bowling & McMurtrey, 1949; Steinberg, 1951), of micro-organisms (Robbins & Ma, 1945) and even of rats (Womack & Rose, 1947). The presence of large amounts of allohydroxy-L-proline as a normal constituent of sandal raises the interesting possibility of a difference in the metabolic behaviour between the two naturally occurring isomers of hydroxyproline.

SUMMARY

1. alloHydroxy-L-proline is widely distributed in the genus *Santalum*, existing largely in the free state.

2. The distribution of allohydroxy-L-proline in the sandal tree has been studied in the vegetative and reproductive phases of growth. In the former stage the imino acid content is highest in the apical leaves of any one branch, whereas in the latter stage the pericarp of the green fruit contains a remarkably high concentration of the free imino acid. Buds and flowers also contain large amounts of the imino acid. Fruit ripening is accompanied by a steady decline in the allohydroxy-L-proline content of the pericarp and a concomitant increase in that of the seed.

3. The seasonal distribution of allohydroxy-L-proline in a healthy and in a diseased ('spiked') tree has been studied. The content in the leaves of the spiked tree was uniformly higher than that of leaves from the healthy one. The amount of allohydroxy-L-proline present in healthy leaves reached a peak value just before the onset of flowering.

4. Drying of sandal leaves at relatively low temperatures (50°) or storing them at room temperature causes an increase in the content of allohydroxy-L-proline. The increase is apparently not due to protein breakdown.

5. Preliminary investigations suggest that ornithine and glutamic acid and, under certain conditions, proline are the likely precursors of allohydroxy-L-proline in the sandal tree.

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Hydrolysis of Polysaccharide Sulphate Esters by a Sulphatase Preparation from *Charonia lampas*

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The large marine gastropod, *Charonia lampas* (*Tritonia sauliae*), is known to be a source of glucosulphatase (Soda & Hattori, 1933), arylsulphatase (Soda & Egami, 1933), chondrosulphatase (Soda & Egami, 1938), myrosulphatase (Ishimoto & Yamashina, 1949), cellulose polysulphatase (Takahashi & Egami, 1960) and steroid sulphatase (N. Takahashi & F. Egami, unpublished observation).

Takahashi (1960*a, b, c*) has shown that cellulose polysulphate, containing glucose residues sulphated at positions 6 and 2 and/or 3, can be degraded to glucose by the combined action of cellulose polysulphatase, a polysaccharase and glucosulphatase, all of which are present in crude extracts of the liver of *Charonia*. It was suggested that cellulose polysulphate was first partially desulphated by cellulose polysulphatase, ester sulphate bonds at positions 2 and 3 being preferentially attacked. Subsequently the polysaccharide chain of the partially desulphated product was degraded by the polysaccharase to yield glucose and glucose monosulphates (principally the 6-*O*-sulphate) and, finally, the glucose monosulphate residues were desulphated by glucosulphatase, an enzyme which preferentially attacks glucose 6-*O*-sulphate.

The present work describes the activity of a cellulose polysulphatase preparation, from which the associated polysaccharase has been removed,

towards a number of polysaccharide sulphate esters, and provides additional support for the suggestions of Takahashi (1960*a, b, c*).

MATERIALS AND METHODS

Enzyme preparation. The cellulose polysulphatase of *Charonia* liver was concentrated and separated from associated polysaccharases, by a method based on the procedures described by Takahashi (1960*a, b*). All operations were carried out at 0°. The fresh liver of *Charonia* (20 g.) was macerated in 100 ml. of 0.1M-sodium acetate-acetic acid buffer, pH 6.0. The suspension was centrifuged, and sufficient acriflavine was added to the clear supernatant to give a final concentration of 1% (w/v). Precipitated material was separated by centrifuging and chilled ethanol was added (with stirring) to the clear supernatant until the concentration of ethanol was 70% (v/v). The resulting precipitate was separated by centrifuging and dissolved in 50 ml. of water. The solution was dialysed overnight and insoluble material was removed by centrifuging. Sufficient M-sodium acetate-acetic acid buffer, pH 5.2, was added to the clear supernatant to give a final buffer concentration of 0.01M and 8.0 ml. of the solution (containing 37 mg. of protein, as determined by the method of Lowry, Rosebrough, Farr & Randall, 1951) was passed through a column (0.7 cm.³ × 30 cm.) of carboxymethylcellulose (approx. 3 g. of adsorbent, buffered with 0.01M-sodium acetate-acetic acid buffer, pH 5.2). The column was then washed with 100 ml. of the 0.01M-acetate buffer before applying, from a 250 ml. mixing chamber, a buffer gradient increasing from 0.01M, pH 5.2, to 0.5M, pH 6.0. The flow-rate was 2 ml./hr. and the eluate was collected in 5 ml. fractions. The enzyme was eluted within the range 0.1–0.2M-buffer (cf. Takahashi, 1960*b*) and the appropriate fractions were pooled and dialysed against water before freeze-drying. The final preparation contained darylsulphat-

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