Isolation of Echinochrome *A* from the Spines of the Sea Urchin, *Diadema setosum* (Leske)

THE naphthoquinone biochromes in the animal kingdom are only found in the group of sea urchins: the various colours (green, red, violet or black) of the spines and the tests of sea urchins arise from the calcium salts of these naphthoquinone pigments[1,2].

Echinochrome *A*[3,4] (7-ethyl-2, 3, 5, 6, 8,—penta-hydroxy—1, 4—naphthoquinone) which was recognized as the naphthoquinone pigment in the ovaries of the sea urchin *Arbacia lixula* (Linn.), has been found in the tests and the spines of the four species of sea urchins: *Strongylocentrotus purpuratus* (Stimpson)[5], *Paracentrotus lividus* (Lam.)[1,3], *Echinus esculentus* (Linn.)[6] and *Echinarachnius mirabilis* (Ag.)[7]. Recently, a naphthoquinone pigment isolated from the dark violet-black spines of the sea urchin, *Diadema setosum* (Leske) (Japanese name, 'gan gaze uni') was identified with echinochrome *A*.

Spines washed with water were dissolved in dilute hydrochloric acid and the pigment was extracted therefrom with ether and transferred into saturated sodium bicarbonate solution. The pigment was extracted again in ether, after acidification with dilute hydrochloric acid, and purified by column chromatography on calcium carbonate and recrystallization from dioxane-water. About 6 mgm. of the pure material were obtained from each 100 gm. of the spines. The pigment forms dark red-brown needles, m.p. 214°–215° and shows absorption maxima at 255, 340, 467, 490, 527 mμ in chloroform solution. Ferric chloride reaction gives a dirty black-violet colour and a violet precipitate appears when it reacts with methanolic lead acetate. The percentage of C and H was 53·94 and 3·90 respectively (calc. for $C_{12}H_{10}O_7$: C, 54·14; H, 3·79). The trimethyl-derivative was obtained by methylation with diazomethane in ethereal solution, as long red needles which were crystallized from dioxane-water. It melts at 130°, is not soluble in sodium bicarbonate solution, but dissolves in dilute sodium hydroxide with a blue colour. Absorption maxima were at 323, 476, 502, 537 mμ in chloroform solution. The percentage of C and H was 58·85 and 5·17 respectively (calc. for $C_{12}H_7O_4(O{\cdot}CH_3)_3$: C, 58·35; H, 5·29). Treatment with zinc dust, pyridine and acetic anhydride gave the leucoacetyl derivative as colourless fine rods, m.p. 240° (decomp.). The absorption maximum was at 295 mμ in methanol solution. The dehydro-derivative, formed by treatment with silver oxide, showed absorption maxima at 260, 319, 392 mμ in methanol solution.

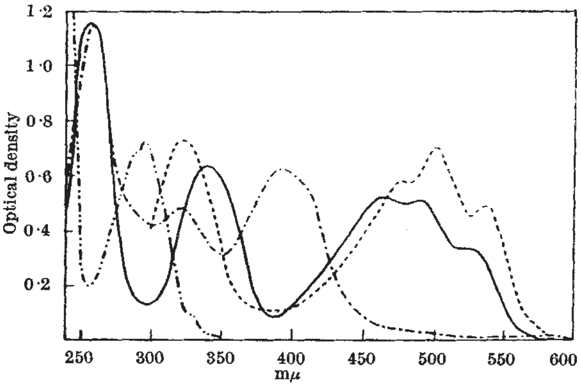

Fig. 1. Absorption spectra of echinochrome *A* of *Diadema setosum*. ———, Free pigment in chloroform solution; – – – –, trimethyl derivative in chloroform solution; —·—·—·—, dehydro-derivative in methanol solution; —··—··—··—, leucoacetyl derivative in methanol solution

Mixed melting point determinations have been carried out with echinochrome *A*, trimethylechinochrome *A* and leucoacetylechinochrome *A* (isolated from *E. mirabilis*)[7], and in each case no depression of the mixed m.p. was observed. The infra-red spectrum of this pigment and echinochrome *A*[7] was also fairly agreeable. Full details of this work will be published elsewhere.

I wish to express my gratitude to Prof. Y. Nakamura and Prof. T. Satto, of Hokkaido University for their encouragement and guidance. I am also grateful to Prof. R. Kamohara and Prof. T. Yatuzuka of Kochi University for offering me every possible assistance in collecting samples and to Dr. M. Inoue of Takeda Research Laboratory for the elemental and infra-red analysis.

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Occurrence of 4-Hydroxypipecolic Acid in *Acacia* Species

EXTRACTION of *Acacia excelsa* heartwood gave an imino-acid (0·2 per cent), m.p. 294° (decomp.), $[\alpha]_D^{20}$ —13·4° (1 per cent in water), characterized by an *N*-benzoyl derivative, m.p. 172°, and identified as *trans*-4-hydroxypipecolic acid. The acid was later isolated from the wood of other *Acacia* species, and was more conveniently obtained from the fresh leaves of *A. oswaldii* (0·25 per cent yield). The imino-acid fraction, isolated by means of the *N*-nitroso derivatives[1], sted alnsicomost entirely of proline, pipecolic acid, and the hydroxypipecolic acid, which crystallized readily from aqueous ethanol. The naturally occurring *trans*-isomer was epimerized by aqueous barium hydroxide (155°, 12 hr.) to a mixture of *cis*- and *trans*-4-hydroxypipecolic acid, and on paper chromatograms developed with butanol-acetic acid-water (4:1:5) the *cis*-acid (R_F 0·17) was indistinguishable from an authentic specimen, but was clearly separated from *cis*-3-hydroxypipecolic acid (R_F 0·24). A further distinction between the 3- and 4-hydroxypipecolic acids is that the former acid is decomposed when heated with alkali under conditions that cause epimerization of 4-hydroxypipecolic acid, and 3-hydroxypipecolic acid therefore resembles other β-hydroxy-α-amino-acids in its alkali-lability[2]. The naturally occurring *trans*-4-hydroxypipecolic acid ran in butanol-acetic acid-water with the same R_F(0·21) as 5-hydroxypipecolic acid from dates[1], but the two acids were separated on paper chromatograms developed with water-saturated phenol, and the 4-hydroxypipecolic acids were also distinguished by giving with ninhydrin a characteristic grey colour which showed deep red fluorescence under ultraviolet light.

Isolation of 4-hydroxypipecolic acid was first reported by Virtanen and Kari[3], and the same acid was isolated from *Armeria maritima* by Fowden[4] who tentatively revised its structure to 3-hydroxypipecolic acid. It now appears that 4-hydroxypipecolic acid is the true structure of the acid isolated by Virtanen and Kari and by Fowden as a sample provided by Dr. Fowden proved chromatographically indistinguishable from our *trans*-4-hydroxypipecolic acid, and its was similarly epimerized by hot baryta. Structural and stereochemical investigation of the *trans*-4-hydroxypipecolic acid from *Acacia* species is continuing, and details will be published elsewhere.

We thank Dr. L. Fowden for a sample of the acid from thrift and for comparing it with our imino-acid, and we are grateful to Dr. H. Plieninger and Dr. H. Vanderhaeghe respectively for samples of *cis*-3- and *cis*-4-hydroxypipecolic acids. This work was carried out during tenure of a General Motors Holden Fellowship (by P.I.M.).

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ANIMAL PHYSIOLOGY

Increase by Chlorothiazide of the Paralysing Activity of *d*-Tubocurarine Chloride

It is a well-established fact that chlorothiazide potentiates the hypotensive effects of ganglion-blocking agents[1-2]. However, the way this potentiation is brought about is not clear. It has been thought that chlorothiazide acts either by a direct hypotensive action[2], or by sodium depletion[3], or by reduction in plasma-volume[3], or as in the case of mecamilamine and possibly of pempidine, by a reduction in renal excretion[4] of the ganglion-blocking agents. From a pharmacological view-point there is a good deal of similarity between the neuromuscular junction and the gangliarsynapsis.

We have therefore investigated whether the paralysing activity in a rabbit, treated with *d*-tubocurarine chloride, could be modified by a previous intravenous injection of chlorothiazide.

In evaluating the paralysing activity of *d*-tubocurarine we have taken into account: (*a*) the appearance of muscular insufficiency that allows the animal, when set in a lateral position, quickly to resume its normal stand-up position (partial paralysis); (*b*) the appearance of a muscular insufficiency that deprives the animal of its ability to resume its stand-up position (total paralysis); (*c*) the animal's death owing to a respiratory insufficiency.

We have summarized our results in Table 1.

It is evident that chlorothiazide pretreatment potentiates the neuromuscular-blocking activity of *d*-tubocurarine. Hidrochlorothiazide, on the other hand, is ineffective in 10–100 mgm./kgm. dose intravenously in increasing *d*-tubocurarine paralysis.

Table 1. Paralysing activity of *d*-tubocurarine chloride in rabbits treated with chlorothiazide and hydrochlorothiazide

Pretreatment	mgm./kgm. i.v.	*d*-tubocurarine μgm./kgm. i.v.	Interval between the two treatments min.	Animals with partial paralysis/ treated animals	Animals with total paralysis/ treated animals	Dead animals/ treated animals
—	—	125	—	11/18	0/18	0/18
Chlorothiazide	100	125	10	7/7	7/7	2/7
,,	,,	125	15	2/2	2/2	0/2
,,	,,	125	30	3/3	0/3	0/3
Hydrochlorothiazide	100	125	1–14	7/9	1/9	0/9
,,	10	125	6–13	4/6	2/6	0/6

The mechanism of chlorothiazide action is not clear as yet.

Our results will be published elsewhere in detail.

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Are Mucosal Nerve Fibres Essential for the Peristaltic Reflex?

Recently Bülbring and co-workers[1,2] abolished the peristaltic reflex in an isolated piece of intestine by scraping off its mucous membrane and assigned an essential role in the initiation of the reflex to processes of sensory neurones which are distributed to the intestinal mucosa.

In the experiments reported here, an attempt was made to destroy the mucous membrane selectively by local administration of a protein-precipitating chemical. Silver nitrate and tannic acid were chosen as suitable chemical agents.

The method of eliciting the peristaltic reflex in an excised loop of guinea-pig ileum mounted in an organ bath[3], was modified so that the output of each peristaltic wave could be directly measured; this permitted to distinguish unequivocally between peristalsis which propelled fluid in a cephalocaudal direction, and pendular activity which did not. In order to avoid formation of silver chloride, the tubings and the intestinal lumen were thoroughly flushed with distilled water before and after the administration of silver nitrate.

Among various concentrations tried, a 30 per cent silver nitrate and a 20 per cent tannic acid solution proved suitable when left in contact with the mucosal surface for about 10 and 30 sec. respectively. After such treatment peristaltic activity continued in its normal pattern of co-ordinated contractions of the longitudinal and circular muscle layers. The amount of fluid expelled was generally slightly reduced and so was the size of the longitudinal contractions and at the same time the response of the longitudinal muscle to acetylcholine, indicating that some damage had occurred to all layers of the intestinal wall. As controls revealed, part of this could be accounted for by the mechanical strain exerted on the wall by forcing the solutions and wash fluid through the lumen in a specified time. However, in two experiments, peristaltic activity was even increased after treatment with 30 per cent silver nitrate solution.

Histological investigation of these preparations, carried out by Dr. M. R. Crompton of the Department of Histology, showed that most of the mucous membrane and parts of the muscularis mucosae were destroyed.