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16. VERTEBRATE HORMONES AS DEFENCE SUBSTANCES IN DYTISCIDES

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Introduction

As we have shown (1), many arthropods defend themselves against their enemies with special glandular substances. The existing research has concerned itself with secretions which serve to repulse small invertebrates, vertebrates and microorganisms. In continuation of this work we have examined for the first time, in the case of *Dytiscus marginalis*, a group of protective substances which react specifically against vertebrates. This glandular protective system is found in the breast section of the beetle (Fig. 1). Figure 2 shows one of the two secretion reservoirs, each about 3.5 mm long and 1.4 mm wide, on which lie a thick layer of glandular cells.

Blunck (2), (3) describes the formation of the milk-white secretion in the glandular cells and its transmission to the reservoir. Merely by holding or by a light pressure to the head, the beetle secretes the glandular fluid through muscular pressure.

It was of particular advantage, for the successful identification of the toxic factor in the secretion, that this crystallised out without further assistance after several weeks from the secretion, absorbed in a glass capillary.

Identification of the defence substance as an steroid

In agreement with the already published analytical data of the toxic component of the defence secretion we also found, at the onset of our recontinued work, in the UV-spectrum of the secretion taken in optically pure ethanol, an absorption maximum at 240-241 mμ typical of a conjugated chromophore.

According to Woodward, mono-alkylated enones absorb on an average at 224 mμ. By the calculation of similar chromophores one adds to this value 11 mμ for an additional alkyl group or a cyclic residue in the β-position, and a further 5 mμ for the exo-cyclic position of the C = C-double bond. Thereby, e.g. a maximum of 240 mμ is obtained for testosterone which is almost identical with the maximum of the hormone as found by us. This agreement between testo-

sterone and the hormone from *Dytiscus* is especially good when one compares the values of the absorption bands of both infra-red spectra (Table 1).

TABLE 1 — COMPARISON OF THE IR-ABSORPTION BANDS OF TESTOSTERONE AND THE HORMONE

Type of vibration	Position of the bands with testosterone in cm^{-1}	Position of the bands with the hormone in cm^{-1}
ν O−H	3520	3476
ν C=O		1693
ν C=O	1668	1668
ν C=C	1612	1613
ν C−C=O		1272
ν C−C=O	1230	1233
ν C=O	1063	1074
ν C=O	1053	1061
γ C−H	867	870

Alone, a superficial consideration of Table 1 indicates that the *Dytiscus* secretion constituent could be a steroid. Also the position of the γ_{C-H} vibrational band at 870 cm^{-1} and the $\nu_{C=C}$ vibrational band at 1613 cm^{-1} as well as the $\nu_{C=O}$ vibrational band at 1668 cm^{-1} indicates a Δ^4-3-keto-steroid. Moreover, as with testosterone, an OH-group could be detected by an IR-spectrum taken in carbon disulphide (Fig. 5).

The intensity of these bands is very low and from this one could infer that the hydroxyl group is hydrogen bonded to a carbonyl oxygen atom. It is possible of course to rule out bonding with the carbonyl group in position 3. There is, however, a further discernable band at 1693 cm^{-1} in the IR-spectrum of the *Dytiscus* hormone which does not appear in the IR-spectrum of testosterone and must be assigned to a saturated aliphatic ketone (Fig. 6 & 7).

In the UV-absorption spectrum, however, this carbonyl chromophore was not detectable. It should, on the other hand be detectable with the help of circular dichroism (CD), when the hypothesis that it is a steroidal ketone is correct i.e. a ketone with several optically active centres. In fact, the CD-spectrum taken showed two extrema at 283 mμ with the $\Delta\varepsilon_{max} = +3.076$ and at 321 mμ with the $\Delta\varepsilon_{max} = -1.16$ (Fig. 8).

According to Velluz and Legrand (4), the Δ^4-3-keto-steroids possess a minimum at 334 mμ with the $\Delta\varepsilon_{max} = -1.35 \pm 0.12$ (ind dioxan) which conforms with our interpretation of the minimum at 321 mμ (in ethanol) found by us. This finding confirms again that the *Dytiscus* hormone may be a Δ^4-3-keto-steroid. For the second maximum at 283 mμ (in ethanol), two possibilities are indicated in the literature (4):

a) 20-keto-steroids: 295 mμ (dioxan)

b) 17-keto-steroids: 303 mμ (dioxan)

An assignation was made more difficult in that the two relevant $\Delta\varepsilon_{max}$ values at 3.459 $\pm$ 0.12 and 3.290 $\pm$ 0.10 are also not so very different. We must at this point, however, introduce a correction for the use of different solvents. Probably our compound should have its maximum at (283 + 13 =) 296 mμ in dioxan. Since the corrected value of 296 mμ lies nearer to 295 than to 303, we concluded that the hormone is a 20-keto-steroid.

On considering that the ketone group at C_{20} can be bridged and that its accompanying absorption band in the IR-spectrum occurs, not as usual, at 1710 cm^{-1} but at 1693 cm^{-1}, one may suppose that the OH-group is bonded to the C_{21} atom.

On the basis of the partial structure just proposed, we can thus assume that the *Dytiscus* hormone is Δ^4-pregnen-3,20-dion-21-01 (11-desoxycorticosterone, cortexon).

We confirmed this postulation by a comparison of the CD of cortexone (Fig. 9) and the secretion constituent.

Cortexone shows the two extrema at 282 and 322 mμ with the corresponding $\Delta\varepsilon_{max}$ values of 3.275 and -1.16. Small differences in the $\Delta\varepsilon_{max}$ values are known to occur — according to the literature (5), the influence of temperature frequently plays a big role.

In 1959, Heller published a paper on the IR-spectra of steroids (6). His information on hydroxyprogesterone can be referred to for the further identification of our natural product. He was concerned with the wave numbers of the bands to be found between 1000 and 1150 cm^{-1}. The curves obtained for the spectrum of the secretion are shown in Fig. 10.

A simple comparison of these spectroscopic data with those which were found by us for the secretion constituent reveals that the latter is most probably identical with cortexone (Table 2).

TABLE 2 — COMPARISON OF THE IR-SPECTRAL BANDS IN THE REGION OF THE ν-C—O -VIBRATION BETWEEN 1000 AND 1150 cm-¹

Bands found for Cortexone (cm-¹)	Bands found for the Hormone (cm-¹)
1005 (w)	1009
1039 (w)	1040
1060 (st)	1061
1072 (st)	1074
1093 (w)	1093
1117 (w)	1117

Since cortexone is commercially available, it was possible to obtain an IR-spectrum of a synthetic sample from Fluka Ltd. for further information.

The spectrum of this sample of cortexone (Fig. 11) agreed so completely with that of our compound, obtained by thin-layer chromatography, that an identity was thereby suggested.

The UV-spectrum of cortexone (Fig. 13) agreed likewise with that of the secretion constituent (Fig. 14) isolated by us.

We obtained a further agreement between cortexone and our natural product by the comparison of the physical data of the corresponding 2,4-dinitrophenyl-hydrazones (2,4-DNP). The 2,4-DNP of cortexone was prepared following the method of Reich and Samuels (7). In the literature, different melting points are recorded for this product — Wettstein *et al.* (8) give a decomposition temperature of 278 to 284°C and Reich and Samuels (7) record that the 2,4-DNP of cortexone melts at 251°C to 254°C. The 2,4-DNP of cortexone prepared by ourselves decomposed between 267° and 269°C. The UV-spectra had, however, the same appearance.

For the preparation of the 2,4-DNP of the beetle secretion, the secretion was added to a solution of 2,4-DNP in 2N hydrochloric acid, left to stand for several hours and the crystals formed were then filtered off. By the thin-layer chromatographic separation we obtained two zones which were scratched apart, eluted and crystallised from aqueous ethanol.

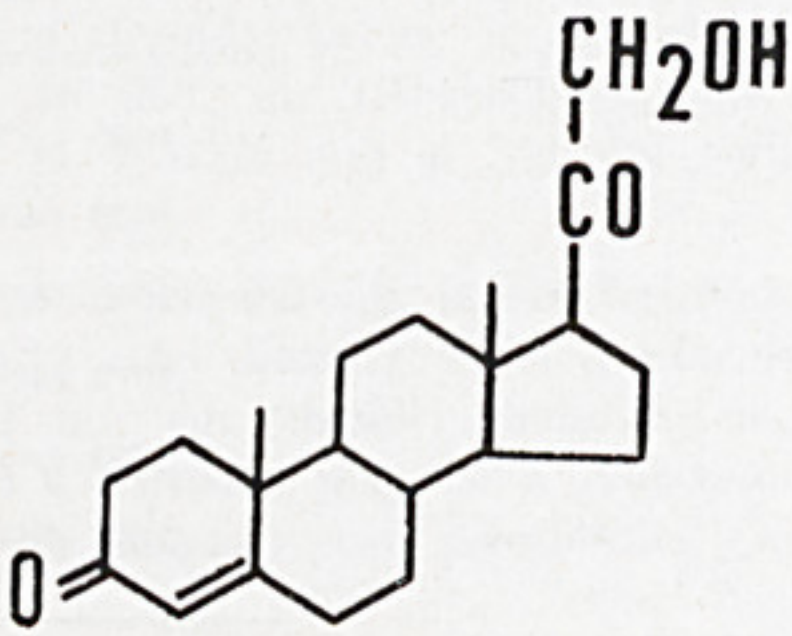
Reich and Samuels (7) record the UV — maxima and minima of the cortexone — 2,4-DNP as follows: maxima at 257 and 383 mμ and a minimum at 313 mμ. We found for the secretion — 2,4-DNP (decomp. 269°), maxima at 254 and 378 mμ and a minimum at 310 mμ. This is a satisfactory agreement.

Finally, there was still the agreement between the mass spectra of the synthetic cortexone and the material contained in the beetle secretion to be tested. In the mass spectrum of the beetle substance (Fig. 16), an additional mass peak at 316 was observed which could not be assigned. This may be caused by the contamination which also influenced the melting point. This was found at 135°C and not, as with cortexone, at 141°C.

The mass peak of 330 showed us the molecular weight of cortexone. Further, only the peak at 299 can be clearly assigned — the molecule having lost a CH_2OH group with mass 31. In then losses a carbonyl or an ethyl group of mass 28 resulting in a fragment of mass 271. An oxygen atom is removed as water leaving a residue of mass 253.

The mass spectrum of cortexone (Fig. 17) shows the same fragmentation pattern.

All these described results permit the conclusion that the non-volatile, ethanol-soluble compound from the water beetle's protective secretion is Δ^4-pregnen-3,20-dion-21-01.



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EXPERIMENTS ON THE PHYSIOLOGICAL EFFECT OF THE SECRETION AND ITS BIOLOGICAL IMPORTANCE

As previously mentioned in the introduction, the physiological effect of the beetle-secretion on various animal was investigated by Blunck (3). He found that it was a strong narcotic and poison effective in very small amounts, especially on cold-blooded vertebrates. On invertebrates, it produced only little or no effect. To begin with, we repeated some of Blunck's important experiments. In these, goldfish (*Carassius auratus*), **OLIGOCHAETA** (*Enchytreen*) and Dytiscides (*Colymbetes fuscus* and *Dytiscus marginalis*) were placed in an aqueous solution of the secretion. In agreement with Blunck's results, the goldfish were stupified whilst the worms and the beetles showed no reaction at all. The water beetle *Colymbetes* even ate meat, which had been treated with the poisonous substance, without harm.

Later, as it was found that the constituent of the beetle-secretion was cortexone, it was necessary to examine whether this substance had the same effect as the secretion. For this purpose we used cortexone from Fluka Ltd. The aqueous solution of cortexone is of 0.001% strenght and contains 10.39 mg of the substance per litre. From this, dilutions of 1:10 and 1:100 were prepared. Into these three solutions were placed the test fish (goldfish and young tench [*Finca vulgaris*]). The fish reacted to the concentrated solution exactly as to the natural secretion. After a short phase of excitement, the fish tired rapidly. After about eight minutes they could be turned onto their backs with a glass-rod and after twenty minutes they displayed severe equilibrium disturbances and lay immobile on their sides. Finally, they were completely motionless except for weak mouthing motions. When the stupified fish were placed in fresh water they recovered completely over a period of ten to twelve minutes and later could be used again in other experiments. Fish in the 1:10 dilution permitted themselves to be touched and turned over with a glass-rod without swimming away — Cortexone, in this instance, had no further action. In the case of the 1:100 dilution, the test fish showed no noticeable reaction.

Cortexone is a hormone of the cortex of mammalia. It possesses a mineralcorticoidal effect, i.e., it regulates the sodium-potassium content of the cells. On administration of an overdose, sodium is retained and potassium secreted, and so the ionic equilibrium is disturbed. This disturbance in the salt concentration corresponds to a disturbance in the sensitivity of the nerve and muscle cells by which, the physiological effect of the beetle-secretion and cortexone may be explained. This agreement of the biological effect of the secretion and cortexone again confirms the results of the chemical analysis.

In order to determine the biological importance of the secretion in nature, we carried out a series of further experiments. To this purpose we used synthetic cortexone as this is easier to administer and can be more easily obtained than the natural beetle-secretion.

There are two possibilities for the natural use of the poisonous secretion by the beetle. In the first instance, the predatory *Dytiscus* could stupify its prey with the cortexone containing secretion in order to overcome it more easily; one must then speak of an attacking-secretion. If, however, the beetle only delivers up the secretion when it is under attack in order to protect itself from an enemy, then it may be called a defence-secretion.

Initially we investigated the possibility of the *Dytiscus* secretion being used as an attacking weapon.

Experiment a: Water beetles were placed together with young tench and goldfish in small containers (100 ml) where the fish had no room to escape from the beetles. Soon after they were put together there was a fierce fight which ended in the death of the fish. In this experiment it was, however, impossible to observe the appearance of the white secretion. A following investigation of the water in order to detect cortexone by chemical means was also without success. In order to test whether the beetles had full reservoirs after all, they were so provoked after the experiment that they all gave up plentiful amounts of secretion.

Experiment b: In an experiment lasting several month, goldfish and water beetles were kept together in an aquarium (20 litre). Although the container was over-occupied by the water beetles and the beetles had not been fed for a long time, and already had begun to eat each other, the goldfish were not killed.

From these experiments it is concluded that the beetle-secretion is not used as an attacking weapon. It is therefore very probable that the beetle employs the secretion for protection from vertebrates. If a fish or an amphibian tries to eat a beetle, then the beetle immediately gives out a large quantity of secretion. This penetrates the gills of the fish and also, when the beetle has been swallowed, into the stomach-intestinal tract. The described experiments show that cortexone penetrates through the body surfaces and is effective.

In order to prove the effect, especially on the gills of the fish, we smeared 300 μg of cortexone on the gills of a large trout (*Salmo gairdneri*) of 750 g weight. After some time it was almost unable to move and could only with difficulty retain its balance. Only after more than six hours did it recover. It is interesting that this illness was immediately utilized by a small trout. Whilst before, this smaller fish had been continuously bitten by the larger one, now the order was reversed and the small fish attacked the larger continuously and pressed it into the very corner of the aquarium. Such examples could also be of importance in nature if a predatory fish ate a beetle.

Finally, we examined the effect of cortexone on stomach-intestinal tract of the fish. To this end, goldfish were fed with bread-crumbs in which 100 μg of cortexone was embedded. The same symptoms appeared as when cortexone penetrates through the body surfaces (skin and gills) of the organism. The effect takes place only a little later but on the other hand remains for a much longer time. Translated to the natural state, this means that a vertebrate that has eaten a water beetle temporarily suffers severe discomfort and probably will not capture further beetles in the future.

To sum up, it may be shown through the afore mentioned experiments that the prothoracic glandular secretion of the water beetle is a defence secretion that serves as protection against vertebrates. The physiological efficacy is caused by the contained cortexone.

Experimental Section

All IR-spectra quoted in the following work were taken on the Perkin-Elmer Spectralphotometer 221 and all UV-absorption spectra on the Beckman DB Spectrophotometer.

The concentration determination of cortexone in the beetle-secretion was carried out using a spectralphotometer PMQ II from Carl Zeiss Lts. Also, using this apparatus, the log ε value of cortexone was determined as 4.24.

A dichrograph from Roussel-Jouan Ltd., Paris was used for the circular dichroism measurements.

The mass spectra were taken with a mass spectrometer of the type CH 4 from the Atlas MAT Company, Bremen.

All melting points were determined on the Bock-monoscope.

The water beetle (*Dytiscus*) were compelled to give up the secretion by light pressure with the finger against the head. The secretion was taken up in a capillary and squirted into pure ethanol. After a few days the insoluble components collected on the bottom and could be centrifuged off using a microfuge. The insoluble components were dried in air and without preliminary treatment were examined spectroscopically in potassium bromide. The hormone was separated from the alcoholic solution and purified by the use of preparative thin-layer chromatography using plates coated with Kieselgel "G" and chloroform as the solvent. The plate was developed from a distance of 10 cm. Under a UV lamp of wave-length 366 mμ the compound is observable by fluorescence. Since the R_f-value is relatively low, the plate was redeveloped using chloroform. After this, the compound had moved 4 cm. The zone was scraped off and eluted with chloroform in a microcolumn. The compound so obtained was used without further preliminary treatment for spectral investigations.

Preparation of the 2,4-dinitrophenylhydrazones of the beetle-secretion and cortexone

The secretion of the *Dytiscus* was added to a solution of 2,4-DNP in 2N hydrochloric acid and allowed to stand for several days. The crystals formed were filtered off, dissolved in chloroform and the two 2,4-DNP derivatives preparatively separated on Kieselgel "G" thin-layer plates. As developing agent, chloroform was used and the plate was run for an hour. Although, after this time, the separation was noticeable we were unable to separate the zones by scraping them apart as they lay too closely together, and therefore the chromatography was carried out twice again each time for an hour. It was then easy to separate the two 2,4-DNP derivatives by scraping apart and eluting them separately on micro-columns. The products crystallised from aqueous ethanol with melting-points: 269°C.

The 2,4-DNP of cortexone was prepared following the method of Reich and Samuels (7). 10.7 mg of Cortexone and 15 mg of 2,4-DNP were dissolved in 1.8 ml of ethanol and 3 drops of concentrated hydrochloric acid were added. After 2.5 hours the crystals formed were filtered off, washed with ethanol and crystallised from chloroform/ethanol. Melting-point: 267-269°C.

Experiments to test the effects of the beetle secretion and cortexone

Experiments with the natural secretion were carried out on goldfish (*Carassius auratus*) and invertebrates. The secretion from seven beetles was dissolved in 100 ml of water and the goldfish was placed in the solution. After 30

minutes the excitation phase commenced. 75 minutes after the start of the experiment the reactions of the fish had been slowed down considerably, and after a further 90 minutes the fish was unable to react any more.

OLIGOCHAETA and *Dytiscus* exhibited no reaction. The effect of cortexone on fish was tested using young tench (*Finca vulgaris*).

Experiment a: A tench of 1.13 g weight was placed into a saturated solution of cortexone in water. As a blank-test, another fish was placed into tap-water. After 5 minutes the movements of the test-fish became unnatural. After a total of 9 minutes it swam on its back and had grown pale. 13 minutes after the start of the experiment it was unable to swim to the surface and lay on its side. After 18 minutes, it was unable to move and mouthed only seldom. After 24 minutes it lay, as if dead, on the bottom of the container. The control-fish was completely normal.

Experiment b: Young tench were tested in various concentrations of cortexone in water. A saturated solution of cortexone was made, and from this, dilutions of 1:10 and 1:100 were prepared.

250 ml of each of the three solutions of differing concentration were poured into separate containers and at the same time a young tench was placed into them. In container 1, with the concentrated cortexone solution, the fish swam immediately here and there in an agitated manner. After 5 minutes it reeled. It was possible after a period of 8 minutes to turn it onto its back with a glass-rod. After 11 minutes it lay down but now and then swam quite normally to the surface. 9 minutes later it had completely lost its sense of balance. The gills bled 28 minutes after the start of the experiment and after 60 minutes the fish lay unmoving on the bottom — it seldom made mouthing movements.

In container 2 with the dilution 1:10 the first reaction from the tench came after 11 minutes. It swam agitatedly, allowed itself to be touched with a glass-rod, and lay on its side. The influence of the cortexone did not proceed any further.

The fish in container 3 with the dilution 1:100 displayed no noticeable reaction throughout the duration of the experiment.

Experiment c: A young tench was placed in a saturated solution of cortexone and after some time was then placed into fresh water. 7 minutes after it was placed in the solution, the fish became disquitened. After a total of 16 minutes, it lay on its side and after a further 2 minutes it lay unmoving on the bottom. After 20 minutes it was mouthing only weakly and was then replaced in fresh water. Within 11 minutes the fish had recovered and then behaved again as normal. The experiment was repeated with the recovered-fish. In the cortexone solution it again displayed stupefaction symptoms and again recovered in fresh tap-water. This action may ostensibly be repeated as often as desired.

Experiment d: A young tench was kept for a longer time in a saturated cortexone solution.

After 18 minutes the fish tilted onto its side and after 26 minutes did not move any more. Soon it swam spontaneously (after 2 hours), but always lay down again. This lying-down occurred less and less frequently and the next day

the fish swam quite normally in its container. 26 hours after the commencement of the experiment a second fish was placed with the test-fish. This fish showed no reaction. After 10 minutes, both fish swam frequently to the surface probably due to an oxygen-shortage caused by the presence of two fish in the container.

Experiments to test the secretion as an attacking weapon

Experiment a: Eleven water beetles were placed singly into 100 ml of water together with goldfish and young tench. After a fierce battle the creatures were separated. The total amount of liquid (1100 ml) was reduced in a rotary evaporator and extracted with chloroform. The residue was treated with a solution of 2,4-DNP in 2N hydrochloric acid but a thin-layer chromatogram showed no evidence of the presence of cortexone-2,4-DNP.

Experiment b: Three goldfishes and up to 30 water beetles were kept in an aquarium, 39 by 25 by 22 cm with a capacity of ca. 20 litres. The fishes were fed continuously with dry forage and no beetle managed to kill a fish. If the fishes were not fed any more they soon became weak and unable to excape the beetles and were killed.

Experiments to test the secretion as a defence weapon

Experiment a: The effect of cortexone on the gills.

A trout (*Salmo gairdneri*) of 750 g weight was orally given 300 μg of cortexone in 10 ml water. The duration of the operation was one minute. After 9 minutes the fish began to pale and already after 13 minutes it succumbed in a battle with a smaller trout. After 85 minutes the trout stood inert on its head and it was possible to pull it out of the water by its tail.

Experiment b: The action of cortexone through the stomach-intestinal tract.

Different goldfishes were given cortexone embedded in bread by pushing it into their mouths with a glass-rod. A control fish was given only bread in the same amount.

In the case of the test-fishes they became weaker after 25 minutes. This weakening was so intense after 45 minutes that the fish was unable to turn back any more when one turned it over with a glass-rod. The fish was certainly not helpless but was constantly very slow to react. After 11 hours all the goldfishes behaved normally again.

The water beetles came from all parts of the Bundes-republik. They were kept in aquaria and were fed on meal-worms. As long as one changes the water frequently enough and give the beetles ample nourishment, also in form of meat and fish, they can be kept alive for a longer time. As previously stated, the beetles were "milked" by a light pressure on the head. They regenerated the secretion in about three weeks.

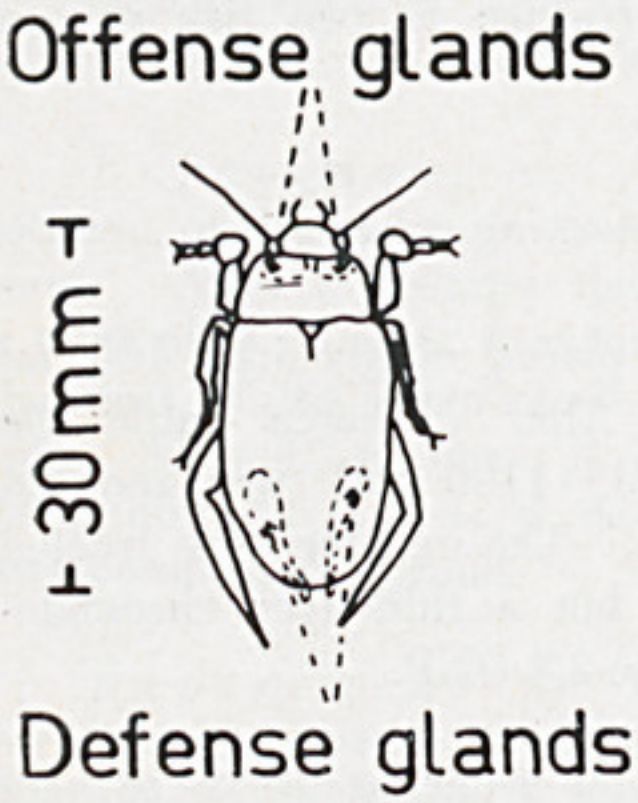

Fig. 1

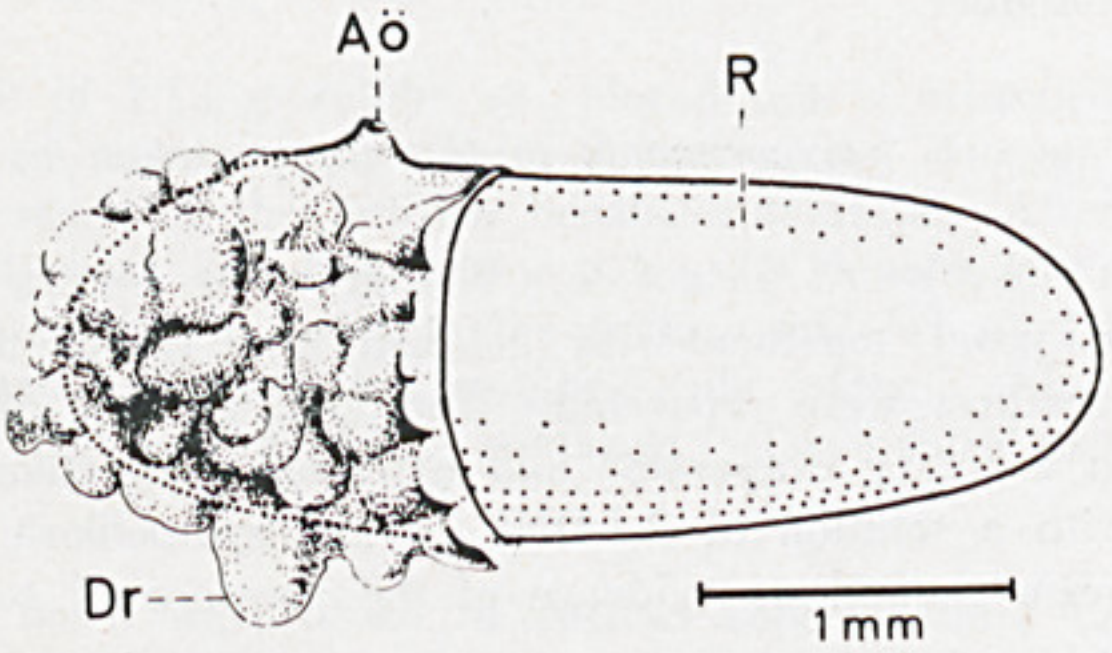

Fig. 2

Fig. 1 — Site of the protective glands of *Dytiscus*.

Fig. 2 — Secretion reservoirs with the overlying glandular cells

R = reservoir

Dr = glandular cells

Aö = secretary opening

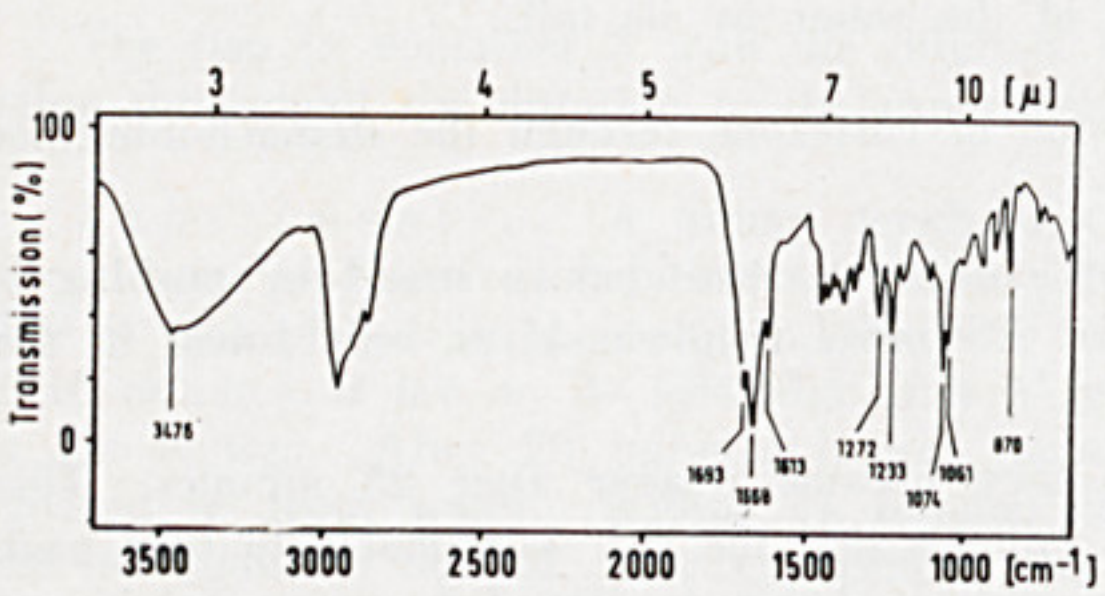

Fig. 3

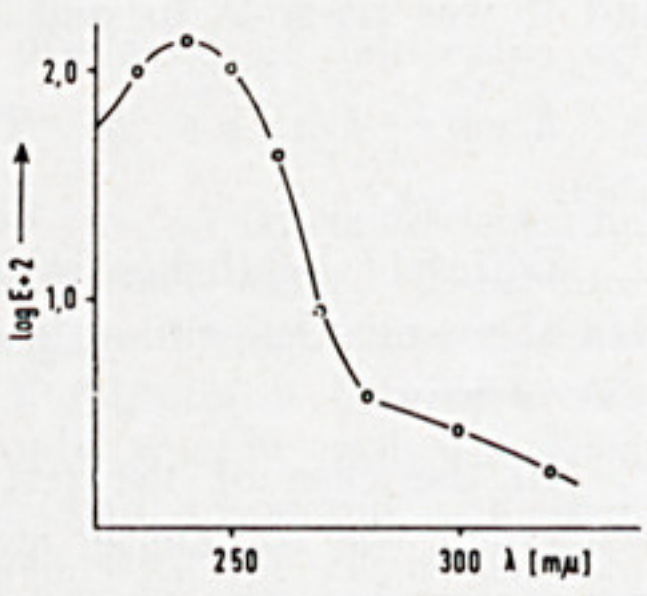

Fig. 4

Fig. 3 — IR-Spectrum of the crystalline crude secretion in KBr.

Fig. 4 — UV-Spectrum of the whole secretion in ethanol when taken immediately and after ¼ one years exposure to air.
λ_{max} 240-241 mμ

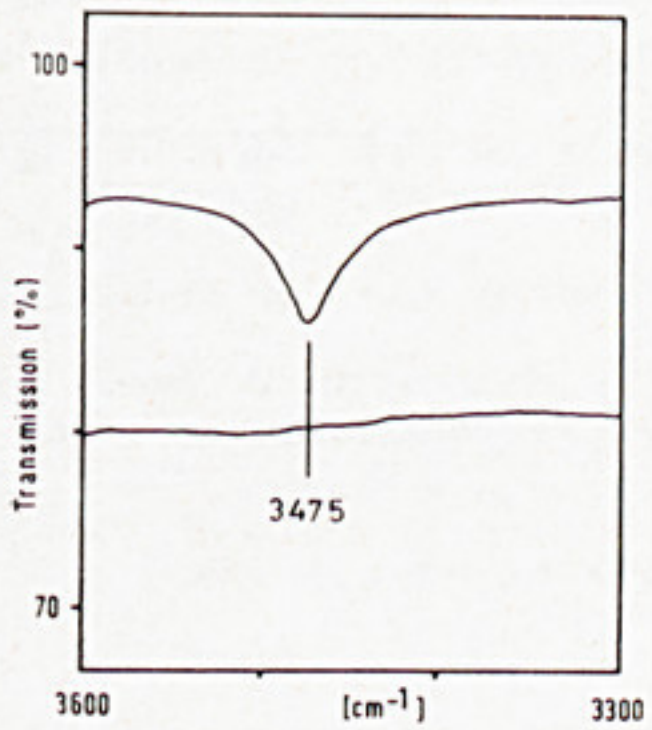

Fig. 5

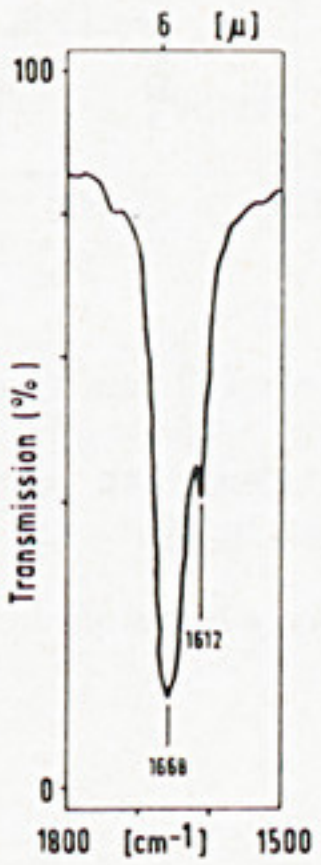

Fig. 6

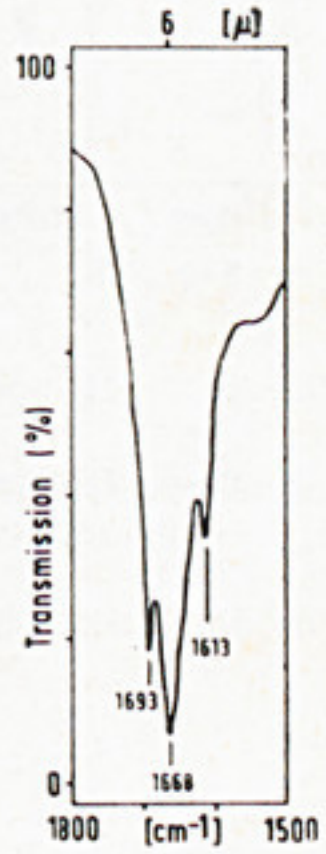

Fig. 7

Fig. 5 — IR-Absorption of the *Dytiscus* hormone in the region of the ν O—H vibration (upper curve in CS_2; lower curve pure CS_2).

Fig. 6 — IR-Absorption of testosterone in KBr.

Fig. 7 — IR-Absorption of the hormone in KBr.

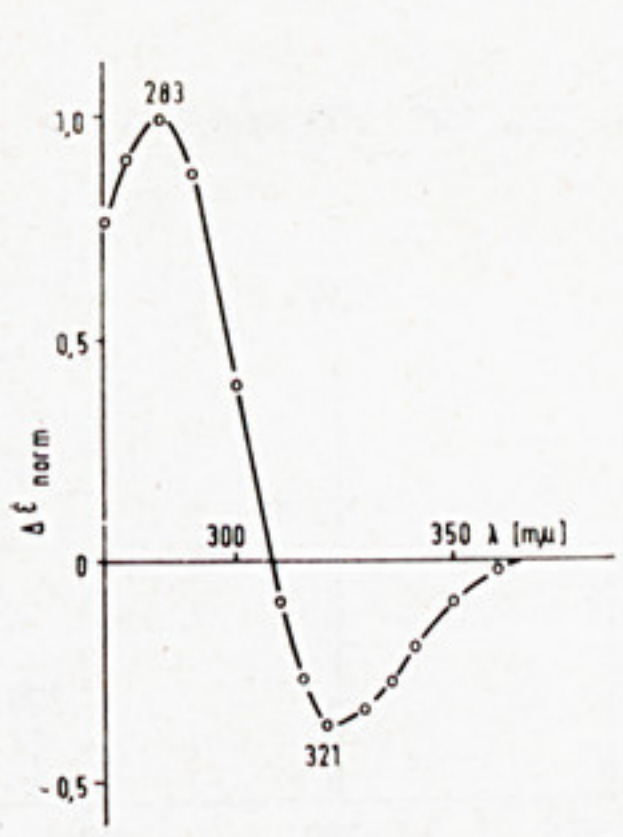

Fig. 8

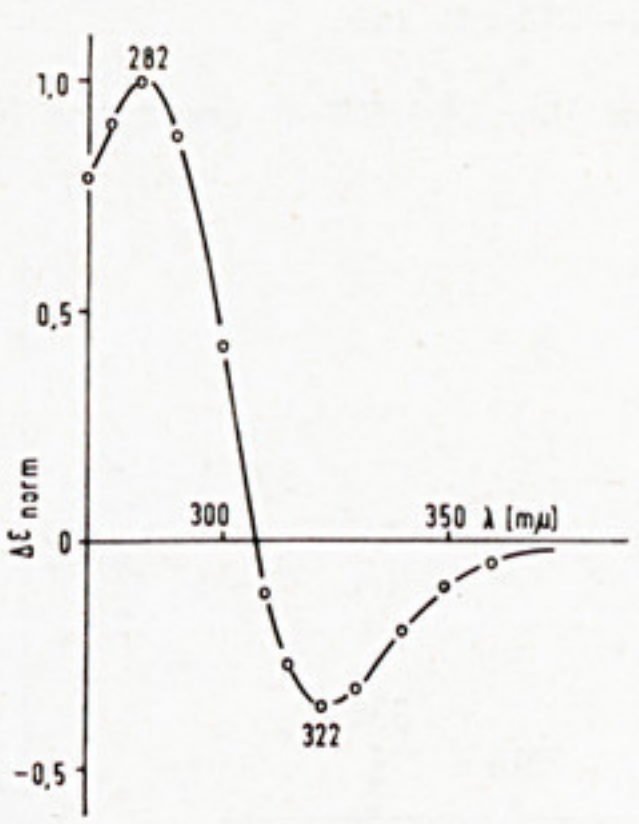

Fig. 9

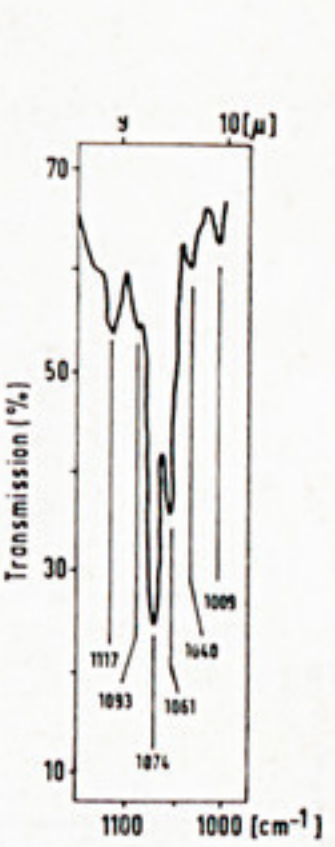

Fig. 10

Fig. 8 — The circular dichroism of the *Dytiscus* hormone.

Fig. 9 — The circular dichroism of cortexone.

Fig. 10 — IR-Absorption of the hormone in the region 1000 to 1150 cm-¹ taken in KBr.

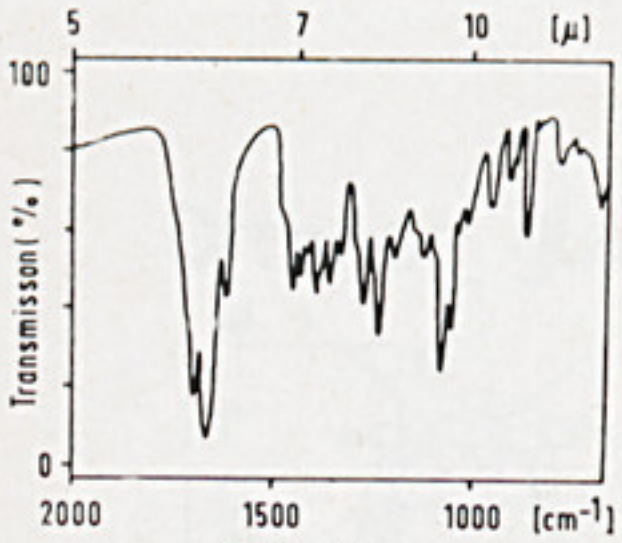

Fig. 11

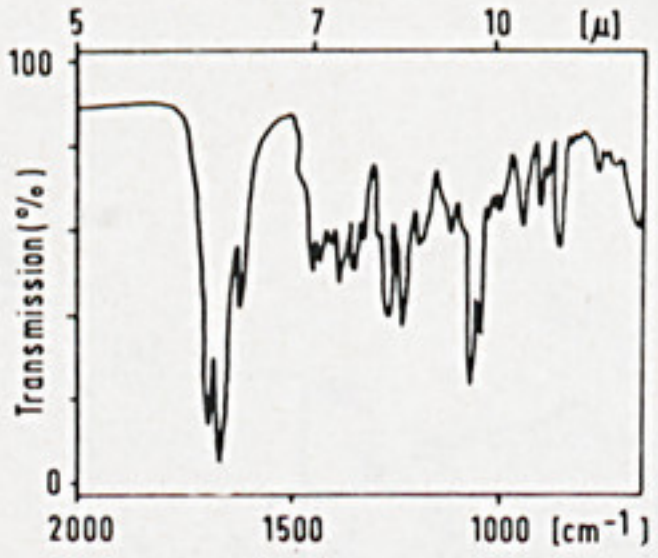

Fig. 12

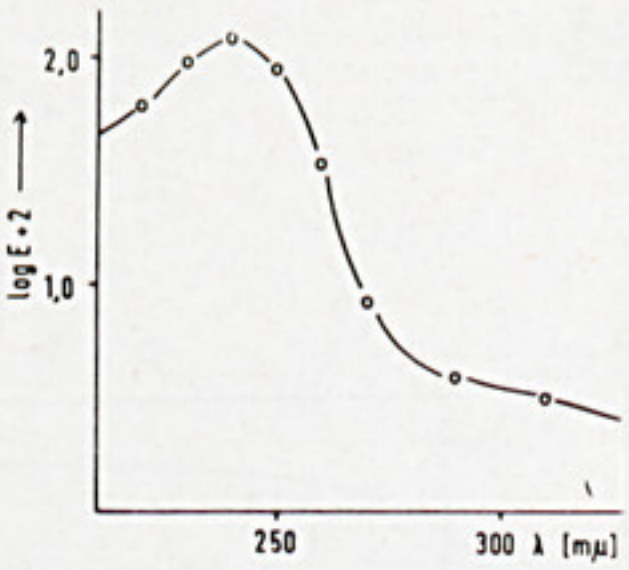

Fig. 13

Fig. 11 — IR-Spectrum of cortexone from Fluka Ltd., in KBr.

Fig. 12 — IR-Spectrum of the compound obtained by thin-layer chromatography using chloroform also taken in KBr.

Fig. 13 — UV-Spectrum of cortexone taken in ethanol λ_{max} 240 mμ, log ε = 4.24.

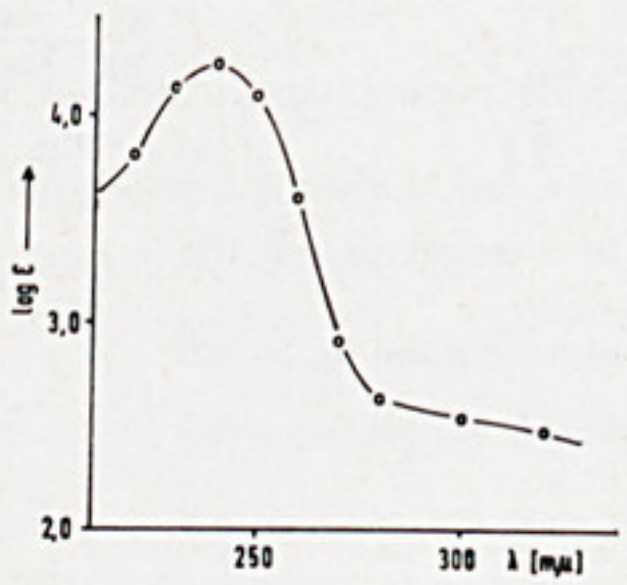

Fig. 14

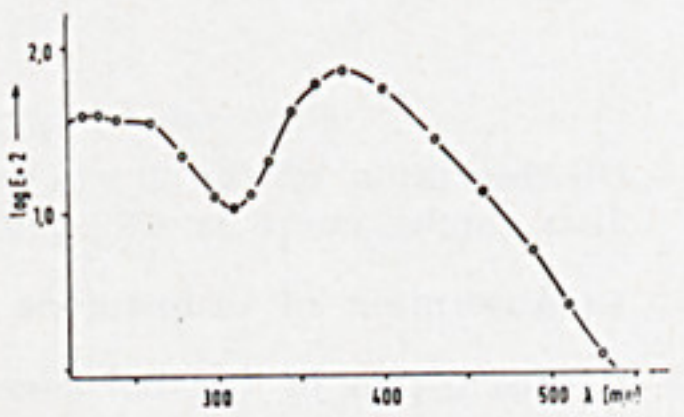

Fig. 15

Fig. 14 — UV-Spectrum of the compound obtained by thin-layer chromatography taken in ethanol λ_{max} 240-241 mμ.

Fig. 15 — UV-Spectrum of the 2.4-DNP of cortexone in ethanol λ_{max} 227, 256 and 376 mμ.

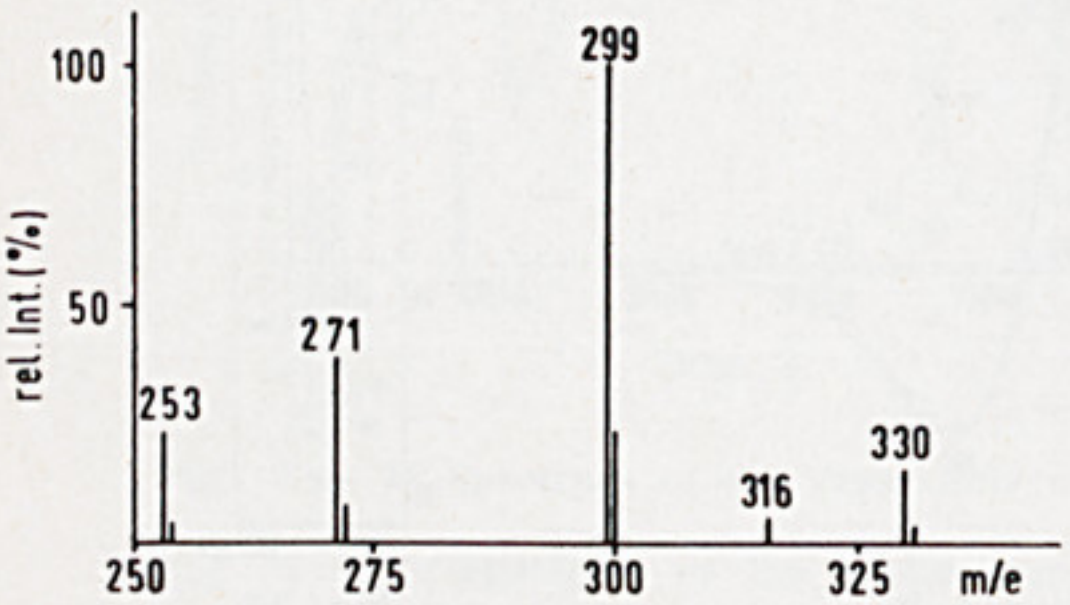

Fig. 16

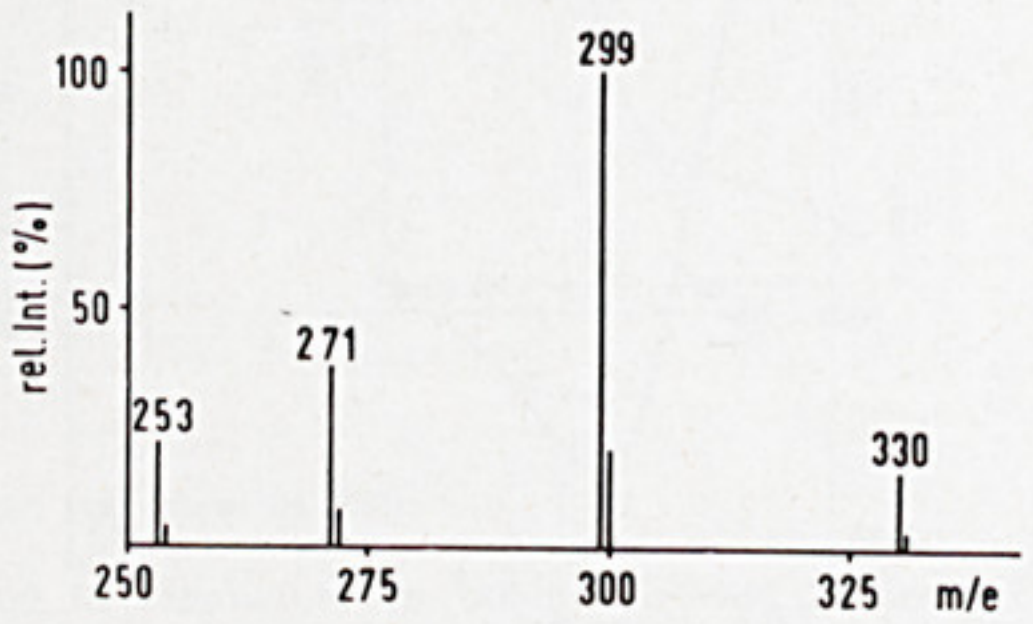

Fig. 17

Fig. 16 — Mass spectrum of the compound obtained by thin-layer chromatography.

Fig. 17 — Mass spectrum of cortexone.

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