The fluid, collected from the nephridial bladders under aseptic conditions, was used for plating and attempts were made to grow the bacteria on Bacto nutrient agar plates, ordinary nutrient agar plates, simple blue plates containing bromothymol blue, blood agar plates (blood of both sheep and cattle used), Loeffler's serum medium, Dorset's egg medium, Aronson's medium, alkaline peptone water, papain digest broth, cooked meat medium, Christenson's medium, aerobically, anaerobically and also in an atmosphere rich in carbon dioxide at 20°C to 37°C. Alteration in pH, temperature and even the media made with Seitz-filtered leech extract with an addition of some of the common growth factors did not promote the growth of these bacteria.

For studying the chemical characteristics of the bacteria, the fluid was collected and divided into two parts. One part was incubated at 37°C for 12 h. In the other, the bacteria were killed by ultraviolet irradiation, autoclaving or addition of toluine and then the containers were incubated at 37°C for 12 h. Both the parts were analysed after the incubation for ammonia nitrogen and urea nitrogen by Conway's microdiffusion technique[4]. Total amount of ammonia nitrogen and urea nitrogen was almost constant in the two cases. Quantity of ammonia nitrogen, however, was significantly higher and of urea nitrogen lower in the untreated fluid as compared with the respective values of the other in which the bacteria were killed before incubation. This appears to indicate that the bacteria are capable of breaking urea into ammonia. The bacteria seem to differ from the genus *Corynebacterium*, two species (*C. vesiculare* and *C. hirudinis*) of which have been reported by BÜSING et al.[5,6] from the nephridial bladders of *Hirudo medicinalis*.

Deproteinized vesicular fluid, analysed chromatographically, contained approximately 50 to 60 µg of amino acid nitrogen per ml. The supernatant fluid of the centrifugated vesicular fluid gave a positive reaction for proteins (albumin and globulin). The amino acids and proteins, which presumably leak out during the process of excretion, provide a rich substrate and promote the floccose growth of bacteria in the nephridial bladders. The growth is richest at the point from where the fresh excretory fluid, containing a sizeable quota of amino acids and proteins, trickles into the nephridial bladder. A thick felt-like covering on the inner wall of the bladder, and the macroscopic clumps are formed to avoid the escape of the bacteria through the nephridiopores.

*Zusammenfassung.* Die Nephridia von *Hirudinaria granulosa* weisen an ihrer inneren Oberfläche keine Cilia, wie es von BHATIA angenommen wurde, sondern Bakterien auf, die makroskopisch sichtbare Klumpen bilden. Ihr Wachstum wird durch grosse Quantitäten von Aminosäuren und Eiweisskörpern des Urins gefördert. Die Bakterien zeigten einen Unterschied gegenüber der Spezies der Corynebakterien. Sie konnten auf verschiedenen Nährböden nicht gezüchtet werden und spalteten Urea in Ammoniak.

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[4] E. J. CONWAY, *Microdiffusion Analysis and Volumetric Error* (Crosby Lockwood & Son, London 1957), p. 465.
[5] K. H. BÜSING, W. DÖLL, and K. FREYTAG, Arch. Mikrobiol. *19*, 52 (1953).
[6] K. H. MANN, *Leeches (Hirudinea)—Their Structure, Physiology, Ecology, and Embryology* (Pergamon Press, Oxford 1962), p. 201.

## The Fatty Acids of the Deposit Lipids in the Hibernant

It is known that hibernating animals form lipid deposits during the summer months, to supply the requirements of basal metabolism, which are notably reduced in lethargy[1,2].

The biochemical and physiological modifications that occur during hibernation were studied by various authors, but today many points still need further clarification.

With regard to the fatty acids, LYMAN[3] has observed an increase in the iodine number during hibernation. An evaluation of the spectrum of fatty acids and of their possible variations is the object of the present research.

The investigation was conducted on the lipid deposits of *Rinolophus ferrum equinum* at three different periods durring hibernation: immediately before (October), after some time (January) and at the end (June).

The methyl esters of the fatty acids have been analysed by means of a gas chromatograph with ionization chamber using a celite column with PGA as stationary phase. The temperature was 181°C and the carrier gas was argo with a rate of flow of 50 ml/min.

The results obtained from the assays of 3–4 pools of two individuals for each period are in agreement: mean values are reported in the Table and show that the saturated fatty acids do not vary appreciably, the monounsaturated fatty acids are in greater amount before and at the end of

Spectrum of the fatty acids in the subcutaneous adipose tissue of the bat (*Rinolophus ferrum equinum*) in different periods of hibernation

| Fatty acids[a] | Before hibernation | At the beginning of hibernation | At the end of hibernation |
|---|---|---|---|
| Myristic | 0.25 | 1.38 | 0.28 |
| Palmitic | 19.30 | 17.46 | 21.94 |
| Palmitoleic | 8.37 | 5.95 | 9.44 |
| Stearic | 1.09 | 3.42 | 1.03 |
| Oleic | 59.37 | 48.75 | 53.24 |
| Linoleic | 5.64 | 8.82 | 5.63 |
| Linolenic | 5.85 | 11.33 | 8.41 |
| Saturated | 20.64 | 22.26 | 23.25 |
| Monounsaturated | 67.74 | 54.70 | 62.68 |
| Polyunsaturated | 11.49 | 20.15 | 14.04 |
| Ratio: 16:0/16:1 | 2.30 | 2.93 | 2.32 |
| 18:0/18:1 | 0.18 | 0.70 | 0.19 |

[a] Fatty acids are expressed in % of chromatographed fatty acids.

[1] F. G. BENEDICT and R. C. LEE, *Hibernation and Marmot Physiology* (Carnegie Inst. Wash. Publ., 1938), n. 497.
[2] C. KAYSER, Thesis, Fac. Sci. Strasbourg (1949).
[3] C. P. J. LYMAN, Exper. Zool. *109*, 55 (1958).

hibernation than at the beginning, while the greatest variations concern the polyunsaturated fatty acids that are lower before the hibernation than at the beginning, and again fall in the last period. About polyunsaturated acids the most evident increase is that of the linolenic acids; in these analyses arachidonic acid was not found.

As the hibernation coincides with the period of low temperature, our results agree with those of LYMAN.

*Riassunto.* Gli autori hanno studiato le variazioni degli acidi grassi dei lipidi del tessuto sottocutaneo del *Rinolophus ferrum equinum* in differenti periodi dell'ibernazione ed hanno riscontrato modificazioni quantitative nei vari acidi grassi e particolarmente nei polinsaturi.

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## Isolation and Characterization of a Polyhydroxy Compound in Adult *Tribolium confusum* Duval[1]

DEVI et al.[2] while studying the variation in the contents of DNA, RNA and nucleotide in *Tribolium confusum* Duval[3], during the various stages of growth and development suggested the presence of a hydroxylated aromatic compound (or hydroxylated aromatic amino acid) in the adult insects. The present paper is primarily concerned with the isolation and characterization of the hydroxylated aromatic compound (or compounds). Insects of 28–30 days old were taken from a pure stock continually reared for the last ten years on a diet composed of whole wheat flour, previously passed through sieve No. 80 and, 5% dried brewer's yeast. The cultures are kept in a room maintained at 28±1°C and at the constant humidity of 70±5%. The insects were homogenized for 5 min at 0°C in cold glass distilled water (10 ml/100 mg of insects) in an Elvehjem-Potter homogenizer, and then filtered through a sintered glass funnel in a cold room. The filtrate was freeze-dried. A spongy mass of light brown colour which changed to deep brown on exposure to air, was obtained. The yield was 5 mg for every 100 mg of the insect used.

For the identification of the compound a 5% solution of this material in water was used; (a) with $FeCl_3$ solution it gave a deep purple colour, (b) on warming with Millon's reagent it gave a red colour and (c) on heating with a secondary amine in the presence of sulphuric acid and on subsequent alkylation it gave a deep blue indophenol. These chemical tests indicated the compound under question was most probably an aromatic amino acid with free hydroxyl groups or a polyhydroxyphenol.

Since the material was not obtained at this stage to the requisite degree of purity, identification of the compound was carried out by two dimensional ascending paper chromatography on a sheet of Whatman No. 1 filter paper (16″ × 20″) in the solvent systems butanol: acetic acid: water (4:1:5 by volume) and phenol:water (4:1 by volume). Between application of the material on chromatogram, the spot was dried in a stream of $N_2$. The chromatogram was run until the solvent front reached the end of the paper (it usually takes 16–18 h). Solvents were removed by keeping the paper in an air oven maintained at 40–50°C. The dried chromatogram sprayed with 0.1% ninhydrin solution made in 95% ethanol, revealed the presence of nine amino acids. According to their Rf values they appeared to be cysteine, arginine, glycine, tryptophane, alanine, leucine, proline, phenylalanine and valine. Since only nine amino acids in water extractable material could be detected by ninhydrin test, we assume that there are only nine amino acids present as such in adult insect.

Since the use of ninhydrin is not promising to detect the hydroxylated aromatic compounds except those containing $-NH_2$ groups in the α position, one-dimensional paper chromatography was carried out by the ascending method in the solvent system butanol, acetic acid, water (4:1:5 by volume). The reference substances used were dopa (dihydroxy phenyl alanine), catechol and tyrosine, and the amount used was 10μl (0.1%) of the known substances and 50 μl for the test substance. The dried chromatogram was dipped into a solution containing 0.44*M* $K_3Fe(CN)_6$ in 0.1*M* phosphate buffer pH 7.8. Three coloured spots immediately appeared on a yellow background. In the Table the results are given. The colour of the ensuing spots and the Rf values gave easy identification as catechol. Tyrosine did not give any spot, owing to the absence of two hydroxyl groups. A similar chromatogram was also run thereafter and the area corresponding to catechol (based on the Rf value obtained from the previous chromatogram) was cut out, dipped into chloroform and allowed to stand for 4 h. The liquid was decanted off,

Rf values of catechol, DOPA and the compound extracted from adult insects with water, when subjected to one-dimensional ascending chromatography in a solvent mixture containing butanol:acetic acid:water (4:1:5); the characteristic colours as revealed on the dried chromatogram dipped into $K_3Fe(CN)_6$ solution prepared in phosphate buffer pH 7.8, identified the compound tested.

Rf values and colour reaction of catechol, DOPA, tyrosine, and the extract obtained from adult insects

| Compound tested | Rf System: butanol/acetic acid/water 4:1:5 | Colour, characteristic of the compound after ferricyanide reaction in phosphate buffer pH 7.8 |
|---|---|---|
| Catechol | 0.840 | brownish black which changed to permanent blue |
| DOPA | 0.250 | orange brown changed to dark blue colour afterwards |
| Tyrosine | no spot | no colour |
| Extract of adult insect | 0.820 | light brownish black changed to light blue |

[1] This work has been supported from a grant (A-1388) received from the National Research Council of Canada.

[2] ANIMA DEVI, A. LEMONDE, and N. K. SARKAR, Exp. Cell Res. *29*, 443 (1963).

[3] The life cycle of *Tribolium confusum* Duval is subdivided into five well defined phases such as (a) embryonic stage (—6–0 days), (b) larval stage (0–13 days), (c) prepupal stage (14–17 days), (d) pupal stage (18–22 days), and (e) adult stage.