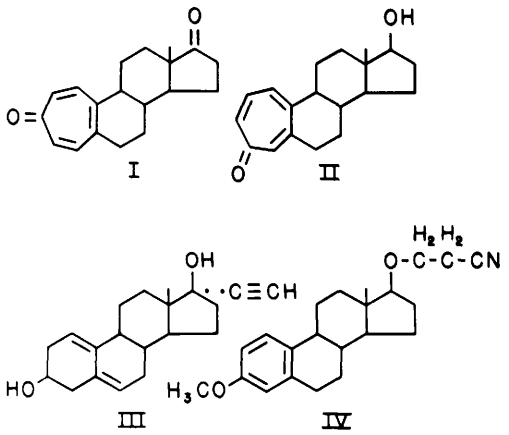


FIG. 1. Compounds with activities separation ratio (ASR) of 3 or greater: I—(A-Homoandrosta-1,4,5(10)-triene-3,17-dione) ASR = >120. II—(17β-Hydroxy-A-homoandrosta-1(10),2,4a-trien-4-one) ASR = 10. III—(17α-Ethynylestra-1(10),5-diene-3β,17β-diol) ASR = 5. IV—(3-Methoxy-1,3,5(10)-triene-17β-cyanoethoxy) ASR = 3.

responding total doses of 0.2 and 0.9 μg of estradiol-17β were required to produce minimum uterine weight stimulation and the minimum dose to produce a maximum response, respectively. Four steroids showed significantly greater pituitary inhibition than uterotrophic activity; 17α-ethynylestra-1(10),5-diene-3β,17β-diol, A-homoandrosta-1,4,5(10)-triene-3,17-dione, 3-methoxyestra-1,3,5(10)-triene-17β-cyanethoxy and 17β-hydroxy-A-homoandrosta-1(10),2,4a-trien-4-one.

1. Kincl, F. A., Ringold, H. J., Dorfman, R. I., *Acta Endocrinol.*, 1961, v36, 83.

2. Kincl, F. A., Dorfman, R. I., *Methods in Hormone Research,* Ralph I. Dorfman, Ed., Academic Press, Inc., New York, vIV, in press.

3. Meyer, R. K., Hertz, R., *Am. J. Physiol.,* 1937, v120, 232.

4. Biddulph, C., Meyer, R. K., Lumbreck, L. G., *Endocrinology,* 1940, v26, 280.

5. Byrnes, W. W., Meyers, R. K., *ibid.,* 1951, v48, 133.

6. Dorfman, R. I., Dorfman, A. S., *ibid.,* 1963, v72, 115.

7. Shipley, E. G., *Methods in Hormone Research,* Ralph I. Dorfman, Ed., Academic Press, Inc., New York, 1962, vII, 179.

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## Lipid Composition of *Trypanosoma ranarum.* (29635)

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Very little is known of the lipid biochemistry of trypanosomes(1), although it has been shown that some trypanosomes contain considerable amounts of lipid material(2). A basic knowledge of the lipid composition of trypanosomes is important for studies of lipid metabolism and immunological investigations. While most trypanosomes require the presence of serum or blood in their medium *Trypanosoma ranarum* can be grown for several generations in absence of serum. This makes possible the study of its lipid synthesizing capacities without the interference of serum lipids. The lipid composition of *T. ranarum* is described in this paper.

*Materials and methods.* A strain of *Trypanosoma ranarum* F-13 was used in this study (by courtesy of Dr. F. G. Wallace, Dept. of Zoology, Univ. of Minnesota). Stock cultures of this organism were maintained in a medium composed of lactalbumin hydrolysate (Nutritional Biochemical Co., Cleveland, Ohio) 2%, yeast extract (Difco Lab., Detroit, Mich.) 0.2%, bovine albumin powder 0.05%, hemin 1 mg%, and triethanolamine 0.25%. The ingredients were dissolved in Hanks' balanced salt solution without sodium bicarbonate, and the pH was adjusted to 7.8. 5% rabbit serum was added to the medium, which was dispensed in 10 ml portions into 4 oz rectangular bottles. This medium without serum, (designed "serumless" medium) was used in our experiments.

An aliquot of cells grown in the presence of serum was centrifuged, washed with physiological saline and inoculated into "serum-

less" medium. Towards the end of the logarithmic growth phase, an aliquot (about 2% of the final volume) was centrifuged, washed with saline, and inoculated into a 250 ml Erlenmeyer flask containing 100 ml of "serumless" medium. The cells were incubated for 3 days (end of the logarithmic growth phase) at 28°C, with shaking at about 250 oscillations per minute. The cells were then centrifuged and washed with saline. For isolation of the sterol, cells were grown with shaking in 2 litre Erlenmeyer flasks containing 1 litre of medium.

For lipid determinations cells were extracted with chloroform methanol(3) and a measured aliquot of this extract was evaporated under nitrogen and fractioned by thin layer chromatography (TLC). The layers contained 0.8 $\mu$g of 2′, 7′-dichlorofluorescein per square cm of silica Gel G(4). Various lipid fractions were located under ultraviolet light, removed from the plate and extracted with chloroform methanol. Appropriate lipid free areas of the plate were used as blanks. The eluted fractions were analyzed for the amounts of glycerides, esterified sterols(3), and long chain free fatty acids, using palmitic acid as standard(5). The amount of protein was determined with Nessler reagent. As free sterols overlap diglycerides in this chromatographic system, the sterol was determined separately after precipitation with digitonin (3). The average results of 3 separate determinations are presented.

For isolation and identification of the sterol, free sterol was precipitated with digitonin, the digitonide was dissolved in hot pyridine and the liberated sterol was acetylated with acetic anhydride(6). The crude sterol acetate thus obtained was recrystallized from methanol and further purified by thin layer chromatography using benzene as solvent, and recrystallized from ethyl acetate. The sterol acetate isolated was characterized by the following criteria: Liberman Burchard color test (LB)(3), $R_f$ values on TLC(4), ultraviolet (UV) absorption spectrum(7), infrared (IR) absorption spectrum, and melting point determination.

*Results and discussion.* *T. ranarum* contains "fast acting" sterols mostly in a free form (Table I). Spraying of the plate with $SbCl_3$ showed the presence of a "fast acting" sterol only. The amount of triglycerides was slightly greater than that of diglycerides. Only traces of monoglycerides, and considerable amounts of free fatty acids were present. An unidentified sterol-like spot was seen under ultraviolet light between the free fatty acids and triglycerides ($R_f$ 0.76). All tests for sterols were, however, negative, and the small amount of the material made identification difficult.

TABLE I. Lipid Composition of *T. ranarum*.

| Sterol esters | Sterols* | Diglycerides | Triglycerides | Free fatty acids |
|---|---|---|---|---|
| | | ←——$\mu$g/mg protein——→ | | |
| Traces | 35.6 | 1.5 | 2.1 | 14.5 |

* Sterol was determined with LB reagent(3) after 1½ min, and calculated as ergosterol.

The nature of the "fast acting" sterol was elucidated further by its isolation and identification. As most of the sterol was present in a free form, it was precipitated from the extract with digitonin without prior hydrolysis.

Preliminary experiments have shown that the various compounds present in lipid extract of *T. ranarum* did not interfere with precipitation of the sterol by digitonin. In the LB test, *T. ranarum* sterol acetate showed characteristic colors, and its rate of development was similar to that of ergosterol (Table II), as was its $R_f$ value (0.96) on TLC. UV and IR absorption spectra (Fig. 1), and melting point temperature were similar to those of authentic ergosterol, and in accord with data present in the literature(7,8,9). Though the medium contained only traces of lipids, small amounts of "fast acting" sterols were present in the medium, probably as ergosterol, which contaminates yeast extract. It was calculated that even if this contaminating sterol was absorbed by the cells, it would constitute less than 0.4% of the ergosterol isolated from the cells.

Various similarities are known to exist between fungi and protozoa. They include resistance of both groups to most bacterial antibiotics, and a parallel susceptibility to chema-

TABLE II. Comparison of Chemical and Physical Properties of Ergosterol and Unknown Sterol.

| | | Ergosterol acetate | | | | *T. ranarum* sterol acetate | | | |
|---|---|---|---|---|---|---|---|---|---|
| Rate of color development | min | 1½ | 10 | 20 | 35 | 1½ | 10 | 20 | 35 |
| with LB reagent | % | 100 | 96 | 83 | 72 | 100 | 96 | 85 | 73 |
| $R_f$ value on TLC | | | .96 | | | | .96 | | |
| UV absorption spectra | mμ | 262 | 271.5 | 282 | 294 | 262 | 271.5 | 282 | 294 |
| Melting point temperature | | | 172-173°C | | | | 172-173°C | | |
| Mixed melting point | | | | | not depressed | | | | |

therapeutic agents(10). To these general similarities one may add that, like fungi(11), phytoflagellates contain ergosterol as their major sterol, as has been shown with *Euglena gracillis* and *Ochromonas donica*(8,12). The phytoflagellates are, however, a heterogeneous group occupying a systematic position at intersections of plant and animal lines of descent(13). The finding that *T. ranarum* contains ergosterol as its major sterol may possibly indicate a closer phylogenetic relation between fungi, phytoflagellates and parasitic flagellates. Ergosterol metabolism has been studied so far mainly in yeast and fungi(14). Studies of its biochemistry in phytoflagellates and trypanosomes may possibly shed new light on the relationship between flagellates and fungi.

In view of the presence of ergosterol in *T. ranarum* it is of interest to compare the sterol biochemistry of two important groups of parasitic flagellates, namely Trypanosomidae and Trichomonadiae. Nutritional studies with Trypanosomatids have shown that they do not require external sources of sterol for their growth(15). This observation could be further supported by the presence of ergosterol, which is synthesized by the cells of *T. ranarum*. Furthermore, it has been shown that sterols were synthesized from $C^{14}$-labeled sodium acetate by other Trypanosomidae, such as *Leptomonas culucidarum, Herpetomonas musciradum, Leishmania tropica* and *Trypanosoma cruzi*. These strains contain considerable quantities of "fast acting" sterols, possibly ergosterol(16). "Fast acting" sterols in small amounts have been shown to be present in *T. cruzi* by Von Brand *et al*(17), though the major sterol of this trypanosoma has been identified as cholesterol. This finding, however, does not contradict the possibility that the major sterol synthesized by trypanosomes might be ergosterol. As for the cholesterol present in *T. cruzi*, it could have been absorbed from the medium, which contained considerable amounts of it.

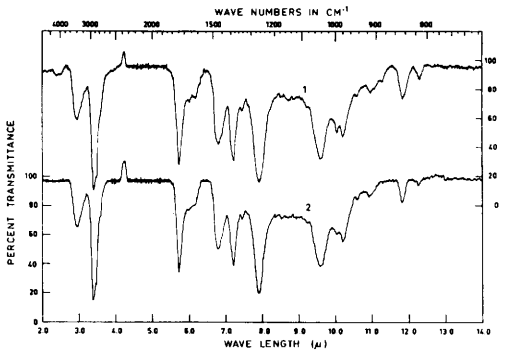


FIG. 1. Infrared spectra of: 1, ergosterol acetate; 2, sterol acetate prepared from sterol isolated from *T. ranarum*. The sample and powdered KBr were ground to a homogeneous mixture and fused.

Nutritional studies with Trichomonadinae have so far indicated that they require external sources of sterol(18). From biochemical studies performed with *Trichomonas foetus*(3), it became apparent that this organism does not synthesize sterol from $C^{14}$-labelled sodium acetate, although it contains considerable amounts of sterols, similar to cholesterol derived from the medium. It seems therefore that there is a pronounced difference in sterol metabolism between Trypanosomatidae and Trichomonadinae.

Most of the sterol present in *T. ranarum* was in a free form, as in animal tissue(11). Free fatty acids are generally found in minimal amounts in living tissue, and the glycerides present are triglycerides(19). In these respects, *T. ranarum* differs from animal tissues, as it contains free fatty acids and the amount of triglycerides is only slightly higher than that of diglycerides.

*Summary.* The lipid composition of *Trypanosoma ranarum* was determined. The cells contained traces of monoglycerides and about equal amounts of di- and triglycerides. Free fatty acids and "fast acting" sterols, mostly in free form, were present. The sterol was isolated and identified as ergosterol. The implications of these findings are discussed.

1. Seaman, G. R., Reifel, R. M., *Ann. Rev. Microb.*, 1963, v17, 1963.

2. Von Brand, T., *Chemical physiology of endoparasitic animals,* Academic Press, Inc., 1952.

3. Halevy, S., PROC. SOC. EXP. BIOL. AND MED., 1963, v113, 47.

4. Brown, J. L., Johnston, J. M., *J. Lip. Res.*, 1962, v3, 480.

5. Duncombe, W. G., *Biochem. J.*, 1963, v88, 7.

6. Avigan, J., Goodman, D. S., Steinberg, D., *J. Lip. Res.*, 1963, v4, 100.

7. Shaw, W. H., Jefferies, J. P., *Analyst,* 1953, v78, 509.

8. Stern, A. I., Schiff, J. A., Klein, H. P., *J. Protozool.*, 1960, v7, 52.

9. Dobriner, K., Katzenellenbogen, E. R., Jones, R. N., *Infrared absorption spectra of steroids.* Interscience Publishers, Inc., 1953.

10. Hutner, S. H., *Biochemistry and Physiology of Protozoa,* Hutner, S. H., Lwoff, A., Ed., Academic Press, Inc., 1955, Vol. 2.

11. Cook, R. P., *Cholesterol,* Cook, R. P., Ed., Academic Press, Inc., 1958.

12. Aaronson, S., Baker, H., *J. Protozool.*, 1961, v8, 274.

13. Hutner, S. H., Provasoli, L., *The Biochemistry and Physiology of Protozoa,* Lwoff, A., Ed., Academic Press, Inc., 1951.

14. Gould, R. G., *Cholesterol,* Cook, E. P., Ed., Academic Press, Inc., 1958.

15. Guttman, H. N., *Exp. Parasitol.*, 1963, v13, 129.

16. Halevy, S., *Bull. Res. Council Israel,* 1962, v10E, 65.

17. Von Brand, T., McMahan, P., Tobie, E., Thompson, N. J., Mosetting, E., *Exp. Parasitol.*, 1959, v8, 171.

18. Hutner, S. H., Holz, G. G., *Ann. Rev. Microb.*, 1962, v16, 189.

19. Shorland, F. B., *Comparative Biochemistry,* Florkin, M., Mason, H. S., Ed., Academic Press, Inc., 1962, v3.

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## Effect of $D_2O$ on Uptake in Yeast.* (29636)

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It has been shown that deuterium oxide ($D_2O$) inhibition of the rates of growth and glucose catabolism in yeast were similar(1). The rates of these functions are dependent, to some extent, on rate of uptake of substrate into the cell. Thus, the possibility existed that $D_2O$ effects on growth and glucose catabolism were due to uptake inhibition. In this report, the effects of $D_2O$ on uptake of glucose, potassium and phosphate are described.

*Materials and methods.* Unless otherwise stated, the haploid yeast strain and growth medium were the same as those described before(1). For uptake experiments, cells were grown to mid-log phase, washed free of growth medium, suspended in distilled water and starved for 3 hours with vigorous aeration. After the starvation period, cells were washed and resuspended in 0.02 M tris buffer (pH 7.2) to a final concentration of approximately 25 mg per ml wet weight (5-7 mg dry weight). After temperature equilibration at 25°C, an equal volume of a solution containing per ml: 2 $\mu$moles potassium as KCl, 2 $\mu$moles phosphate as $K_2HPO_4$, 6 nc $P^{32}O_4^{\equiv}$, and 10 mg glucose was added. The cell suspensions were incubated at 25°C with constant shaking and at time intervals indicated in Fig. 1 and 2, 5 ml samples taken. Cells and medium were separated by centri-

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