Properties of Density Gradient-Separated Liver Mitochondria from Triiodothyronine-Treated Rats[1] (37191)

THOMAS S. RUH,[2] MARY F. RUH,[3] AND HOWARD M. KLITGAARD

Department of Physiology, Medical College of Wisconsin, Milwaukee, Wisconsin 53233

Greif, Alfano and Eich (1) reported the appearance on sucrose density gradients of two distinct populations of rat liver mitochondria from triiodothyronine (T_3)-treated animals. The mechanism by which T_3 altered some mitochondria to become more dense and separate from other mitochondria on the density gradient has remained unanswered. There are at least two basic factors which could determine mitochondrial density equilibrium in a linear density gradient—permeability of mitochondria to the density gradient media and the density of the mitochondrial components themselves. The T_3-induced changes in either one or both of these factors could account for changes in density by the mitochondria.

This invesigation is concerned with the study of the two populations of rat liver mitochondria which Greif, Alfano and Eich (1) reported were obtainable when rats were treated with high doses of T_3.

Materials and Methods. Male Sprague–Dawley rats weighing between 350 and 375 g were divided into two groups. Experimental rats were given daily sc injections of 1 mg 3,3′,5-triiodo-L-thyronine/kg body wt in 0.01 *N* NaOH–0.9% NaCl for 7 days. Normal rats were injected with the vehicle over the same time period. Animals were fed Rockland complete rat diet *ad libitum*.

After decapitation the livers were removed and chilled at 4° in 0.3 *M* sucrose–0.002 *M* Tris, pH 7.4. Mitchondria were isolated from 4 g of liver which were homogenized in 2 g portions in 3 ml of the sucrose–Tris medium by a Potter-Elvehjem ground glass homogenizer. Forty milliliters of a 10% homogenate were prepared with the sucrose–Tris medium and centrifuged at 970*g* for 10 min in a Sorvall refrigerated centrifuge at 4°. The top 30 ml of supernatant were carefully removed and centrifuged at 5200*g* for 10 min to isolate the mitchondria.

Highly purified mitochondria were prepared and tested for microsomes using the methods of O'Brien and Kalf (2). Microsomal contamination was found to be less than 0.8 μg microsomal protein/mg mitochondrial protein. The final mitochondrial pellet was suspended in the sucrose–Tris medium to a final volume of 1 ml.

Linear sucrose density gradients of 28 ml were prepared at 4° in a concentration range between 37 and 52% (w/w) using a Buchler gradient mixing chamber and polystaltic pump. Gradient linearity was verified by using indocyanine green.

One-half milliliter of the mitochondrial suspension was layered on the top of the sucrose density gradients and centrifuged at 50,000*g* for 180 min at 4° in the SW 25.1 rotor of the Spinco Model L ultracentrifuge.

Successive 1 ml aliquots were removed at 4° from the bottom of the gradient tubes. The 3 ml which corresponded to each mitochondial band were combined and used for further analyses. Protein concentrations were determined in duplicate by the method of Lowry *et al.* (3). Succinic dehydrogenase, a mitochondrial marker, was assayed in duplicate using 2-*p*-iodophenyl-3-(*p*-nitro-

[1] This research was supported by U.S. Public Health Service Grants (5 F01 GM 36827 and 5 T01 HE05366), a Grant-in-Aid from the Wisconsin Heart Association, and a National Science Foundation Traineeship (GZ202 and GZ987).

[2] Present address: Department of Obstetrics & Gynecology, Washington University, St. Louis, MO 63110.

[3] Present address: Department of Physiology, St. Louis University School of Medicine, St. Louis, MO 63104.

phenyl)-5-phenyltetrazolium chloride (4).

In vivo incorporation of amino acids into mitochondrial protein was studied by injecting 30 μCi/kg body wt of uniformly labeled ^{14}C-L-leucine (New England Nuclear, sp act 2 mCi/mg) in 0.25 ml of saline into the dorsal penis vein of the rat at 5, 15, 30, 60, 120 min before decapitation. The mitochondria were isolated and subjected to sucrose density gradient partition as previously described. The amount of radioactivity was determined using 2 ml aliquots from each sucrose density gradient mitochondrial band. The protein was precipitated and washed on Millipore filters according to the method of Buchanan and Tapley (5). The filters were dried and added to glass counting vials containing 15 ml of a modification of Kinard's (6) xylene-dioxane-ethanol scintillation counting medium in which 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene was substituted for α-naphthylphenyloxazole and 40 g thixotropic gel powder (Packard Instrument Co.) were added/liter. After partial disintegration of the filters at room temperature, mixing on a vibrator completely dissolved the filters and dispersed the protein into homogeneous suspensions. Radioactivity was determined in a Packard Tri-Carb liquid scintillation counter, Model 3380, at an efficiency of 85% and a SD of less than 1.5%.

In vivo incorporation of ^{32}P-orthophosphate (Amersham-Searle Corp.) into the phospholipids of mitochondria was studied by injecting 150 μCi/kg body wt of ^{32}P (sp act 77.4 Ci/mg) in 0.16–0.25 ml of saline into the dorsal penis vein. Rats were decapitated at 15, 30, or 60 min after injection, and the liver mitochondria were prepared as before.

Aliquots of 2.5 ml of the mitochondrial band were slowly diluted to 10 ml with 0.002 *M* Tris buffer (pH 7.4) and centrifuged at 12,000*g* for 30 min to obtain a mitochondrial pellet. Lipids were extracted according to Folch, Lees and Stanley (7). Phosphate was determined by the method of Chen, Toribara and Warner (8) in triplicate on an ashed aliquot of lipid extract (9). Radioactivity was determined on the remaining evaporated lipid extract in 17 ml of a toluene-phosphor scintillation counting media (10). The radioactivity was determined in a Packard Tri-Carb liquid scintillation counter, Model 3380, at an efficiency of 90% and a SD of less than 1.5%.

For fatty acid determinations phospholipids were precipitated with acetone from the lipid extract (11), hydrolyzed, and the fatty acids were extracted with petroleum ether (12). The petroleum ether extracts were evaporated to dryness under nitrogen and the fatty acids were redissolved in *n*-hexane. The fatty acids were then determined using a Barber-Colman Model 15 gas chromatograph.

Results. Optimum separation of the mitochondria into two distinct bands was obtained after 7 days of T_3 treatment. The upper and lower mitochondrial bands from the T_3-treated animals were positioned in the density gradient at a sucrose molarity of 1.50 (1.119 density) and 1.73 (1.229 density) respectively, whereas, mitochondria from normal animals formed one band at a sucrose molarity of 1.55 (1.205 density).

Succinic dehydrogenase analysis of successive gradient fractions clearly showed that the enzyme marker for mitochondrial presence coincided with the protein bands.

Determinations of ^{14}C-L-leucine uptake into the mitochondrial protein *in vivo* revealed a classic incorporation pattern with time (Fig. 1). There were no detectable differences in leucine incorporation among the

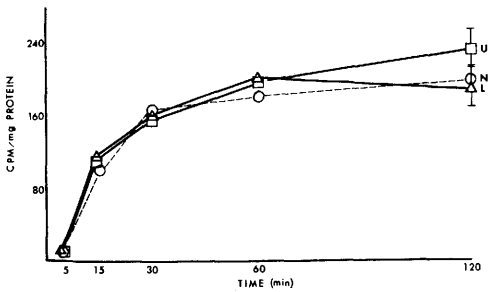

FIG. 1. The *in vivo* ^{14}C-L-leucine incorporation into rat liver mitochondrial protein. Each point is the mean of 3–5 animals, except for 5 min values where each point represents a single value. At 120 min, the standard error of the mean is given. L = lower mitochondrial band from T_3-treated rats; U = upper mitochondrial band from T_3-treated rats; N = mitochondrial band from normal rats.

bands either during the maximum rate of incorporation (15 min) or during the plateau phase of incorporation.

The ^{32}P-orthophosphate incorporation into phospholipids *in vivo* was nearly linear with time in all mitochondrial bands studied (Fig. 2). The experimental lower mitochondrial band consistently had the highest rate of incorporation. A significant difference was found at 60 min between the upper and lower mitochondrial bands from T_3-treated rats ($p < 0.005$). Incorporation of ^{32}P into mitochondrial phospholipids from normal rats did not differ significantly from incorporation into the upper mitochondrial band phospholipids from T_3-treated rats.

A significant difference in phospholipid-phosphorus:protein ratios (Table I) was found between the upper and lower mitochondrial bands of the T_3-treated group ($p < 0.001$). The phospholipid phosphorus:protein ratio of mitochondrial bands from normal rats is given for comparison.

In the T_3-treated rats, no significant difference was found in the fatty acids present in the mitochondria of either the upper or lower bands (Table II).

Discussion. The density differences of the mitochondrial bands from the T_3-treated rats might be explained by alterations in the phospholipid:protein ratios since changes in these ratios correlate with mitochondrial densities. The more dense mitochondrial bands had lower phospholipid:protein ratios than the less dense mitochondrial bands. Both Luck (13) and Goldhor (14) have found a similar relationship between lipid:protein ratios and mitochondrial density. It would seem that the changes in the phospholipid:protein ratios were due more to variations in the amount of phospholipid present in the mitochondria than to any changes in protein, since in the present study no differences were found in the rate of ^{14}C-L-leucine incorporation into mitochondrial protein. Likewise, Luck (15) found no change in the activities of five mitochondrial proteins in *Neurospora crassa* when he induced a doubling of the lipid:protein ratio by a high choline diet. Other investigators (16–19) have shown that the turnover of mitochondrial proteins is nearly constant, but the turnover of mitochondrial lipids can be very heterogeneous. Gross (20), however, has recently suggested that mitochondrial protein turnover may be altered with thyroid state.

In our studies there were significantly higher rates of ^{32}P-orthophosphate incorporation into the phospholipids of the more dense mitochondria than that of the less dense mitochondria. One might speculate that an initial T_3 induced loss of phospholipids from one population of mitochondria caused these mitochondria to become more dense. In these dense mitochondria a more rapid mitochondrial phospholipid synthesis was stimulated, but this increase in synthesis was not sufficient to offset the phospholipid loss.

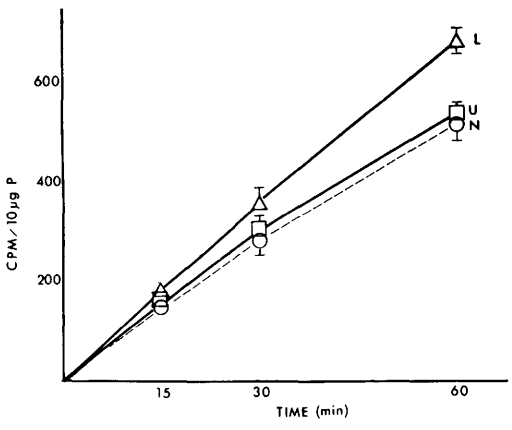

FIG. 2. The *in vivo* ^{32}P-orthophosphate incorporation into rat liver mitochondrial phospholipids. Each point for normal rats is the mean of 3–4 animals. Each point for T_3-treated rats is the mean of 4–6 animals. At 60 min, the standard error of the mean is given. L = lower mitochondrial band from T_3-treated rats; U = upper mitochondrial band from T_3-treated rats; N = mitochondrial band from normal rats.

TABLE I. Mitochondrial Phospholipid:Protein Ratio.[a]

Mitochondrial band	No. of animals	Mean ± SEM
Experimental upper	10	6.38 ± 0.22
Experimental lower	10	4.82 ± 0.18
Normal	6	5.51 ± 0.22

[a] Values expressed as micrograms of phospholipid-phosphorus:milligrams of protein ± standard error of the mean.

TABLE II. Fatty Acid Determination on Mitochondrial Phospholipids.[a]

Fatty acids	Mitochondrial band from normal rats	Mitochondrial upper band from T_3-treated rats	Mitochondrial lower band from T_3-treated rats
Palmitic	32.01 ± 1.78	25.70 ± 1.20	22.98 ± 1.39
Palmitoleic	7.23 ± 1.14	7.58 ± 1.52	6.22 ± 1.03
Stearic	15.96 ± 2.75	18.66 ± 2.39	15.85 ± 1.82
Oleic	10.46 ± 1.33	12.58 ± 1.07	12.51 ± 1.03
Linoleic	11.42 ± 3.23	14.44 ± 3.85	14.95 ± 3.40
Arachidonic	4.22 ± 0.30	4.81 ± 1.30	6.57 ± 1.21

[a] Values are expressed as percentage of the total fatty acids recovered. Mean ± SEM. Number of animals = 5.

Since the mitochondrial phospholipids did not reveal an increase in either the saturated or unsaturated fatty acids, it is not possible to relate the density separation of the mitochondria with membrane stability. However, preliminary electron micrographs of the mitochondria from the T_3-treated rats have revealed that the more dense mitochondria have a highly condensed matrix, whereas, the less dense mitochondria have a normal matrix. The above observation agrees with the report of Greif, Alfano and Eich (1) using T_3-treated rats and that of Pollak and Munn (21) who also found two populations of mitochondria using starved rats. These findings seem to indicate a change in the inner membranes of the more dense mitochondria.

Numerous investigators (22–25) have inferred that there is a heterogeneous population of mitochondria across the rat liver lobule. The suggestion that there are at least two mitochondrial populations in the normal rat liver is supported by our own findings since some rat liver mitochondria are quite sensitive to T_3 treatment *in vivo*. This is shown by the decreased phospholipid:protein ratio, increased ^{32}P incorporation, and increased density in some of these mitochondria. Based on these findings, this induced density separation of rat liver mitochondria by T_3 treatment should prove useful in the further study of the biochemical differences between these mitochondrial populations.

Summary. Two populations of liver mitochondria from T_3-treated rats, which were separated on sucrose density gradients, and normal rat liver mitochondria were studied. The more dense mitochondrial band from T_3-treated rats consistently had the highest *in vivo* rate of ^{32}P-orthophosphate incorporation into phospholipids and was significantly different from ^{32}P incorporation into the phospholipids of the less dense mitochondrial band at 60 min. No differences in the *in vivo* rate of ^{14}C-L-leucine incorporation into mitochondrial protein or in the major mitochondrial fatty acids were found among the bands studied. Significant differences in phospholipid:protein ratios were found between the two mitochondrial bands of the T_3-treated group. The density differences of the mitochondrial bands might be explained by alterations in the phospholipid:protein ratios since changes in these ratios correlate with mitochondrial densities.

The authors thank Dr. Donald Greiff for the use of his ultracentrifuge, and Drs. Roberto Galindo and Kenneth Chun for the fatty acid analyses.

1. Greif, R. L., Alfano, J. A., and Eich, E., Endocrinology **78**, 733 (1966).

2. O'Brien, T. S., and Kalf, G. F., J. Biol. Chem. **242**, 2172 (1967).

3. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

4. Shelton, E., and Rice, M. E., J. Nat. Cancer Inst. **18**, 117 (1957).

5. Buchanan, J., and Tapley, D. F., Endocrinology **79**, 81 (1966).

6. Kinard, F. E., Rev. Sci. Instrum. **28**, 293 (1957).

7. Folch, J., Lees, M., and Stanley, S., J. Biol. Chem. **226**, 497 (1957).

8. Chen, P. S., Toribara, T. Y., and Warner, H., Anal. Chem. **28**, 1756 (1956).

9. Ames, B., and Dubin, D., J. Biol. Chem. **235**, 769 (1960).

10. Meade, R. C., and Stiglitz, R. A., Int. J. Appl. Radiat. Isotop. **13**, 11 (1962).

11. Hanahan, D. J., Dittmer, J., and Warashina, E., J. Biol. Chem. **228**, 685 (1957).

12. Swelley, C. C., and Moscatelli, E. C., J. Lipid Res. **1**, 40 (1959).

13. Luck, D. J., J. Cell Biol. **24**, 461 (1965).

14. Goldhor, S., J. Cell Biol. **37**, 823 (1968).

15. Luck, D. J., J. Cell Biol. **24**, 445 (1965).

16. Bailey, E., Taylor, C. B., and Bartley, W., Biochem. J. **104**, 1026 (1967).

17. Beattie, D. S., Biochem. Biophys. Res. Commun. **35**, 67 (1969).

18. Beattie, D. S., Basford, R. E., and Koritz, S. B., J. Biol. Chem. **242**, 4584 (1967).

19. Fletcher, M. J., and Sanadi, D. R., Biochim. Biophys. Acta **51**, 356 (1961).

20. Gross, N. J., J. Cell Biol. **48**, 29 (1971).

21. Pollak, J. K., and Munn, E. A., Biochem. J. **117**, 913 (1970).

22. Shank, R., Morrison, G., Cheng, C., Karl, I., and Schwartz, R., J. Histochem. Cytochem. **7**, 237 (1959).

23. Bahr, G. F., and Zeitler, E., J. Cell Biol. **15**, 489 (1962).

24. Swick, R. W., Stange, J. L., Nance, S. L., and Thomson, J. F., Biochem. **6**, 737 (1967).

25. Wilson, M. A., and Cascarano, J., Biochem. J. **129**, 209 (1972).

Received Dec. 13, 1972. P.S.E.B.M., 1973, Vol. 142.