Localization of Tritiated Tetracycline in Mitochondria of Rat Liver Cells.* (28430)

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Recently several authors have reported that tetracycline drugs of various configurations seem to localize preferentially, as detected by the characteristic fluorescence, in the implanted tumors of experimental animals(1) and certain human tumors(2). The basis of this apparent selectivity of tumor tissue has not been definitely elucidated, although Loo *et al.*(1) have reported the tetracycline in tumors apparently bound to a peptide. The affinity of bone for TTC† is a well-known phenomenon probably related to the chelation of calcium ions by TTC(3). Indication that lipoproteins may be involved in the partition of tetracycline derives from the observations that in the liver cells of TTC-treated rats typical fluorescence is seen emanating only from mitochondria(4) (exceedingly rich in lipoproteins), and that TTC, in the presence of calcium, is capable of complexing and quantitatively precipitating the B-lipoproteins of serum(5).

The availability of a tritiated TTC of high activity makes possible the accurate quantitation of the concentration of TTC in tissues such as the liver and subcellular fractions thereof, since it has been shown that TTC is not metabolized to a significant degree in rats(6). The partition of TTC among the subcellular fractions of the rat liver and the nature of its binding within the mitochondria were investigated.

Method. The livers of Sprague-Dawley rats, male and female, weighing 150-250 g, were used. Rats were injected intraperitoneally with 4-8 mg of tritiated TTC-HCl‡ (specific activity – 22.3 μc/mg) in 1 cc of normal saline buffered with ascorbic acid in ratio of 2 parts TTC to 3 parts ascorbic acid. Animals were fasted after injection and killed by decapitation about 12 hours after injection (duration yielding maximum activity in liver) and the livers perfused first with cold saline, then with cold 0.25 M sucrose. All tissues were maintained at 2-4°C throughout processing. Liver tissue was minced, and a 10% suspension in a medium containing 0.25 M sucrose and 7.5% polyvinylpyrrolidine, pH 7.6, was homogenized by means of motor-driven Teflon pestle in a tube with clearance of 0.04-0.06 mm twice for one minute with intermittent cooling. Ultracentrifugation of this homogenate was carried out according to the method of Novikoff *et al.*(7). Aliquots of each fraction were taken for radioactive counting. The mitochondrial fraction was resuspended in 5 ml of veronal sodium buffer (pH 8.6, $\mu = 0.1$) and 0.2 ml of Triton-X100 (a non-ionic detergent) was added(8). The solution was agitated intermittently for 15 minutes during which time the turbidity decreased markedly. Mitochondria were checked for integrity by vital staining with Janus Green B before and after disruption by the detergent. This mixture was centrifuged at 18,000 *g* for 15 minutes and the supernatant used immediately for electrophoresis. Sediment after this procedure was minimal and showed no electrophoretic mobility.

Paper electrophoresis was performed with simple glass plate apparatus with platinum electrodes on Whatman #3 MM filter paper with 450 v for 5 hours or 100 v for 16 hours in barbital buffer at pH 8.6, $\mu = 0.05$. Normal rat serum was used for a control. The papers were stained for proteins with bromphenol blue and for lipids with sudan black. Each paper was checked before staining by

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† Tetracycline.

‡ Kindly donated by Dr. Donald Buyske, Lederle Laboratories, Pearl River, N. Y., as 7-tritiotetracycline-HCl.

exposure to a light source§ producing u.v. light at 3660 A°, and the area of characteristic fluorescence outlined. Consecutive 0.5 cm segments of paper strips stained for protein were eluted in 1:1 methanol: 10% aqueous Na_2CO_3, and the resulting eluate was read at 595 mμ on a Coleman Jr. spectrophotometer for quantitation of proteins.

Starch block electrophoresis was performed at 5°C using 70 g purified potato starch for each block in barbital buffer at pH 8.6, μ = 0.1 at 400 v, for 24 hours. Starch blocks were then cut into 0.5 cm segments each of which was eluted in 5 cc of water and filtered. Each tube was observed under u.v. light and graded 0 to 4+ for fluorescence. The filtrate was then treated according to Lowry's technique(9) and absorbance read at 680 mμ to determine protein concentration.

For the lipoprotein precipitation studies, 0.45 M calcium chloride was added to an equal volume of the supernatant of the Triton-treated mitochondria and the resulting suspension was centrifuged at 1500 RPM for 5 minutes in a table centrifuge. The supernatant and precipitate were examined for fluorescence and used for paper electrophoresis as above.

Radioactive counting. Aliquots of the homogenate and the various fractions were dried under an infra red light in cellophane bags. These were then burned in an oxygen atmosphere in sealed flasks and the water vapor condensed by cooling the flasks in a dry ice-acetone bath(10). The tritium was taken up in the toluene-ethanol (80:20) counting medium of Kelly *et al.*(10).

Scintillation counting was performed by a Packard Tri-Carb Apparatus. Results were expressed (after counting with an internal standard and correcting for quenching) as disintegrations/minute(11). Papers containing fractions of electrophoretically separated proteins were combusted by the same technique, the results being expressed as absolute count minus background. The radioactivity of equal segments of paper strips, containing electrophoretically-separated proteins, was expressed as counts/min/cm of paper.

§ Mineralight, Ultraviolet Products, S. Pasadena, Calif.

TABLE I. Relative Concentrations of Tetracycline in Subcellular Fractions of Rat Liver 12 Hours After Injection.*

Fraction	% radioactivity† in fraction compared to homogenate	Standard deviation	Standard error of means
Nuclear	12.3‡	1.43	.54
Mitochondrial	6.6	.46	.17
Microsomal	6.7	.90	.37
Soluble	75.1	1.70	.64

* Each rat received 8 mg of tritiated TTC (178 μc) intraperitoneally.
† Disintegrations/min, corrected for quenching.
‡ Avg value for 4 rat livers, each run in duplicate.

Results. The concentration of TTC in each fraction of the rat liver cells is considerable (Table I). It would appear that each subcellular fraction of the rat liver is capable of sequestering a characteristic amount of TTC at a given interval after exposure. No attempt was made to quantitate the amount of TTC in each fraction with the volume of the fraction or the concentration of protein nitrogen in view of the disparity of constituents among the fractions. Since the particulate fractions were washed thrice with resuspension before counting, the constancy of TTC concentration in each fraction may well represent specific binding.

We invariably observed typical yellow fluorescence grossly in all subcellular fractions of the liver cell. This could not be attributed to the medium or autofluorescence of the liver tissue, since control observations were always negative. Repeated washing of the fractions with saline did not serve to deplete fluorescence significantly.

Although DuBuy and Showacre(4) demonstrated intracellular localization of tetracycline only within the mitochondria, their studies were performed 2 hours after injection in contrast to 12 hours in this study, when gross fluorescence was at a maximum.

Treatment of the mitochondrial fraction with the non-ionic detergent Triton X-100(8) resulted in complete disruption of mitochondria as judged by disappearance of visible particles with Janus Green B stain. Of the several methods of particle disruption employed by various authors, (digitonin, desoxycholate, sonication(12)) this one has seemed to produce the most uniform results.

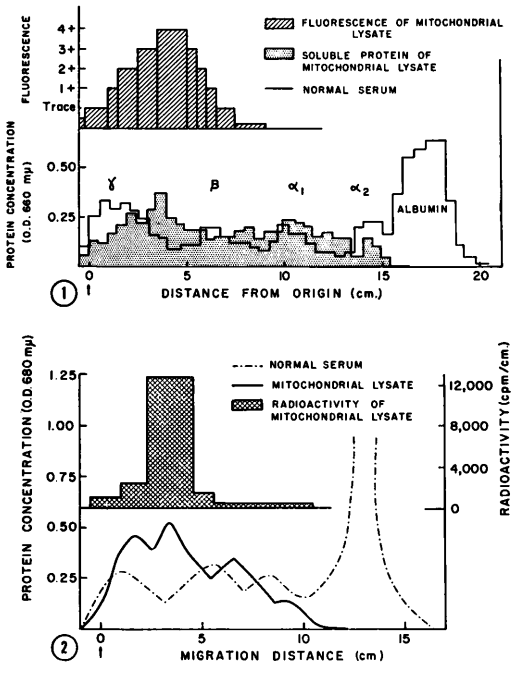

FIG. 1. Comparison of protein concentration and fluorescence in segments of starch block eluate of mitochondrial lysate. A—Pattern of distribution of soluble proteins of mitochondrial lysate on starch electrophoresis. B—Pattern of distribution of fluorescence in eluates of starch block used for protein determination.

FIG. 2. Quantitative measurement of proteins of mitochondrial lysate and normal serum correlated with measurement of radioactivity of segments of paper electrophoresis strips. Notice that maximal radioactivity coincides with the region falling between the Beta and Gamma peak of normal human serum. It coincides also with what are probably the beta-globulins of the mitochondrial lysate. This region corresponds to area of maximal fluorescence under ultraviolet light. Proteins measured by elution from paper strips. Radioactivity represented as a histogram.

Electrophoresis of the soluble lysate of liver mitochondria of rats treated with radioactive TTC resulted in demonstration of 4 bands corresponding to alpha, beta(2) and gamma protein mobilities as compared with simultaneously run serum proteins. The results were similar in both paper and starch block methods (Fig. 1).

When paper strips were examined under ultraviolet light, the fluorescent areas outlined and the strips then stained for lipid, the resulting stain invariably occupied the exact locale of the fluorescent area. This was in the slow-beta or beta region (Fig. 1). When concurrently run paper strips were cut into 1 cm segments, combusted and counted for radioactivity, the peak radioactivity was found in an area corresponding to the slow-beta and beta fraction (Fig. 2). When the lipoproteins were precipitated from the mitochondrial lysate by addition of calcium, all of the fluorescence appeared in the precipitate. Electrophoresis of the supernatant resulted in a strip free of fluorescence, stainable lipid and significant radioactivity.

Triton-treated nuclear, microsomal and soluble fractions never resulted in mobilization of TTC from the origin. Likewise, treatment of the nuclear fraction by freezing and thawing followed by extensive deoxyribonuclease digestion failed to solubilize the fluorescent material or convey on it electrophoretic mobility.

Discussion. Several methods have been evolved for fragmenting the complex lipoprotein envelope of the mitochondria, the most successful of which appears to be treatment with a non-ionic surface-active agent (Triton X-100). Congiu(13) was able to demonstrate 7 electrophoretically distinct protein components from mitochondria so treated, whereas lesser numbers have been isolated by other techniques (digitonin, deoxycholate and ultrasonically-treated particles)(12).

Utilizing Triton X-100 treatment followed by starch block electrophoresis, we were able to distinguish 5 distinct protein peaks. In addition, 2 lipoprotein fractions were distinguished by paper electrophoresis.

In the Triton-treated liver mitochondria of TTC-treated rats, the TTC, as determined by its characteristic fluorescence or its radioactive label, was found to be invariably associated with the beta lipoprotein fraction whether identified by electrophoresis on paper or in starch blocks (Fig. 2), or by solubility properties. This apparent association could be reproduced *in vitro* by addition of TTC to the lysate of mitochondria from the liver of an untreated rat. Subsequent electrophoresis of this mixture showed the TTC to migrate with the beta lipoproteins. In rat serum, TTC is also seen to migrate for the most part with the beta lipoproteins, although there was some detectable in the other protein fractions. This demonstration of a simi-

larity between serum and mitochondrial beta lipoproteins in their affinity for TTC parallels the affinity of the beta lipoproteins in these two locales to complex with a large variety of enzymes(14,15).

Summary. The localization of tetracycline labeled with tritium in the subcellular fractions of rat liver cells has been investigated. Ultracentrifugal analysis of cellular components revealed a significant and characteristic concentration of the drug in all fractions. Electrophoresis on paper and starch blocks of detergent-solubilized mitochondrial proteins revealed a distribution of the tetracycline, as determined by fluorescence and radioactivity, which coincided with the beta lipoprotein fraction of the mitochondrial lysate. Tetracycline in the presence of calcium ions is capable of precipitating the beta lipoprotein fraction of mitochondrial lysate as well as that of serum. The localization of TTC in fractions other than mitochondria is probably not related to complexing with lipoprotein.

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The Merocrine Component of Apocrine Secretion.* (28431)

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Apocrine sweat gland function has been divided into 2 separate and distinct activities, apocrine sweating and apocrine secretion(1). Apocrine sweating, the appearance of apocrine sweat on the skin surface, occurs when the apocrine myoepithelium is stimulated to contract, with resultant expression of preformed sweat from the apocrine tubule. Implicit in this concept is the belief that no active formation or secretion of sweat takes place at the time of apocrine sweating. Apocrine secretion, the formation of apocrine sweat by the secretory cells, is believed to be a slow, virtually continuous process, requiring hours to a few days to fill the tubular reservoir. It has been contrasted with the secretion of eccrine sweat which is not pre-formed but is rapidly produced at the time of eccrine sweating. Although apocrine secretion is presumed to be regulated or paced by hormonal substances, as yet not precisely defined, the cellular mechanisms involved in the formation of apocrine sweat have never been elucidated.

Recently we have been examining the excretion of locally introduced fluorescein sodium by the apocrine sweat glands. Within 3 minutes after its local intracutaneous injection, fluorescein may be detected in apocrine sweat. This prompt incorporation or excre-

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