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Transport and Storage of ^{14}C-Riboflavin in the Retina and Liver of Rats.* (32551)

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Riboflavin has been reported to exist in significant amounts as the free vitamin in parts of the eyes of various animals(1-3) in contrast to such other tissues as liver where the coenzyme forms; riboflavin-5′-phosphate (FMN) and especially flavin adenine dinucleotide (FAD), predominate(4). Riboflavin has been found to occur in the pigmented epithelium of fish eyes(1), as crystals in the tapetum of a particular species of lemur(2) and in choroid and iris of frogs(3). FMN has been found in frog retina(3) and FAD has been detected in ox retina(5). An unidentified flavin has been isolated from choroid of cats(6). Uptake of dietary riboflavin into corneas was demonstrated with riboflavin-deficient rats(7).

The present study was undertaken to follow the uptake and storage of injected riboflavin by retina as compared with liver of deficient and control rats. A further purpose was to compare the predominant form of flavin in these tissues.

Materials and methods. Riboflavin was

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purchased from Eastman Organic Chemicals; *D*-riboflavin-2-^{14}C was from Nuclear-Chicago Corp. Weanling, male, Sprague-Dawley rats, 45 to 55 g, from Blue Spruce Farms, Inc. (Altamont, N.Y.) were maintained on a riboflavin-deficient test diet‡ from Nutritional Biochemicals Corp. Half of the animals were fed the deficient diet, while the other half, as controls, were supplemented with 25 μg of riboflavin per 15 g of diet. Feed and water were given *ad libitum*.

After 2 weeks, both deficient and control animals were injected intraperitoneally with isotonic saline containing 25 μg (1.56 μC) of ^{14}C-riboflavin per 100 g of body weight. Three rats from each group were killed by decapitation at time intervals up to 7 days. Eyes were removed, cut in half along the limbus, and the posterior part everted. Both retinas from each rat were teased onto small glass rods and deposited in a scintillation vial. Average retinal weights were obtained by pooling the retinas from 5 to 6 animals of each group. Livers were removed, rinsed in water, and kept in ice until they were weighed, minced, and approximately 10 mg placed in a scintillation vial. After adding 0.5 ml of Hydroxide of Hyamine 10X (Packard Instrument Co.), the vials were capped and incubated at 40° for 18 hours to aid solubilization of the tissue. A toluene solution of scintillators was added and radioactivity determined as cpm in a Packard liquid scintillation spectrometer. A ^{14}C-benzoic acid standard was then added to each vial to obtain counting efficiencies for corrections to actual dpm.

To ascertain the distribution of ^{14}C in the flavins of retina, 10 rats weighing 100 to 150 g were injected with similar amounts of ^{14}C-riboflavin on each of 2 days and sacrificed on the third day. Subsequent steps were performed in semi-darkness. Retinas were quickly removed, placed in 3 ml of cold water, homogenized, and acidified to pH 3 with 1 N acetic acid. Liver was removed, homogenized in 2 volumes of cold water and similarly acidified. The mixtures were saturated with ammonium sulfate and centrifuged. To each supernatant solution was added 0.5 ml of 90% aqueous phenol. After shaking to extract the flavins, the layers were allowed to separate, and 50 μl of the phenol solution was spotted on Whatman No. 1 paper. The paper was subjected to ascending chromatography in *n*-butyl alcohol:acetic acid:water (4:1:5, v:v:v, upper phase) for 18 hours. The chromatograms were viewed under ultraviolet light to locate flavin-containing areas which were cut from the paper for liquid scintillation counting. R_f values were: riboflavin, .30; FMN, .12; FAD, .05.

Results. The deficient rats maintained their initial average body weight while the controls averaged a 30 g gain after two weeks.

Uptake of flavin radioactivity by retinas from both groups is illustrated in Fig. 1. No major difference between deficient and control animals was found when results were expressed per mg of retina. Maximal uptake occurred at 45 to 60 minutes. The level of radioactivity in retinas of both groups was relatively constant from 2 hours up to 7 days after injection.

Uptake of flavin radioactivity by livers of the animals is given in Fig. 2. The deficient group demonstrated a similar rate of uptake as did the controls, but obtained a slightly lower flavin content. By 24 hours after injection, the specific radioactivity in the livers of both groups had somewhat decreased. A gradual decline continued through the remaining experimental period. Approximately 25 percent of the injected radioactivity could be found in the livers of both groups after 24 hours.

The radioactive flavin in both liver and retina was found by paper chromatography to be greater than 90% FAD and FMN.

Discussion. Since a riboflavin deficiency did not increase the amount of an injected

‡ The diet contains the following in %: vitamin-free casein, 18; vegetable oil, 10; U.S.P. salt mixture no. 2, 4; sucrose and vitamins, 68. Vitamins are the following per 100 lbs of diet: vitamin A, 900, 000 units; vitamin D, 100,000 units; α-tocopherol, 5 g; ascorbic acid, 45 g; inositol, 5 g; choline chloride, 75 g; menadione, 2.25 g; *p*-aminobenzoic acid, 5 g; niacin, 4.5 g; pyridoxine hydrochloride, 1 g; thiamine hydrochloride, 1 g; calcium pantothenate, 3 g; biotin, 20 mg; folic acid, 90 mg; vitamin B_{12}, 1.35 mg.

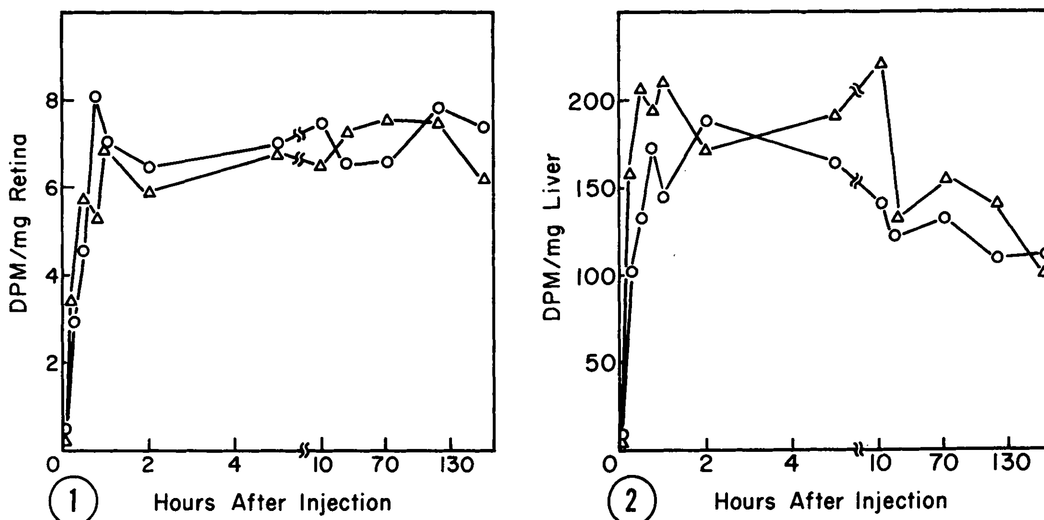

FIG. 1. Uptake of injected ^{14}C-riboflavin by retina of rats which received a diet deficient in riboflavin (○) and the same diet supplemented with 25 μg riboflavin per 15 g diet (△).

FIG. 2. Uptake of injected ^{14}C-riboflavin by liver of rats which received a diet deficient in riboflavin (○) and the same diet supplemented with 25 μg riboflavin per 15 g diet (△).

dose of the vitamin retained per unit weight of either retina or liver, this would suggest that such deficient tissues do not have unsaturated storage capacity. Rather, the slightly lower level of flavin in the deficient livers could reflect a small preference in depletion of flavoproteins with respect to total protein. Burch *et al*(8) reported that certain flavin-requiring enzymes decrease in liver and to a lesser extent in kidney and heart during riboflavin deficiency. These investigations further demonstrated an actual decrease in apoenzyme levels. Such a decrease in protein required to specifically bind FMN and FAD may account for less riboflavin retained by the deficient livers in the present study. The similarity between ^{14}C-flavin content of retinas from control and deficient animals could mean that there was no decrease in flavoprotein relative to total protein in retina. A further implication may be that retina is not affected as easily by riboflavin deficiency as is liver. Burch *et al*(8) reported that changes in enzyme and coenzyme levels in brain of riboflavin deficient animals were negligible. The gradual decline in radioactivity of liver as compared with retina may mean a faster turnover of flavin in the former. Retina may also be continually replenished with a supply of flavin released from other sites in the body.

The low level of free riboflavin in retina of rats is in contrast to that claimed for pigmented tissues in the eyes of nocturnal animals such as the lemur and some fish and amphibia. The presence of high free flavin levels in these latter animals has indicated involvement in photoreception. The results obtained in this study would indicate that this may not be the case with albino rats.

Summary. Deficient rats retain slightly less of an injected amount of ^{14}C-riboflavin per unit weight of liver than do controls, but levels in retina were similar for the two groups. A possible small preferential decrease in flavoproteins of liver, but not of retina, is suggested. No significant difference in rate of uptake by the tissues of the two groups was found. Riboflavin in the retinas of albino rats exists predominantly as FAD and FMN with free riboflavin levels comparable to those found in liver.

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Converted-Cell Focus Formation in Culture by Strain MC29 Avian Leukosis Virus.* (32552)

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Lack of a ready means for bioassay of avian tumor virus infectivity is a continuing impediment to quantitative studies of the agents. With procedures for titrating Rous sarcoma virus (RSV)(1) and a few leukosis agents(2,3) by host response *in vivo*, comprehensive experiments became feasible though tedious and expensive. Problems of RSV titration were immeasurably advanced by exploiting the phenomenon of focal morphologic conversion of chick embryo cells (CEC) infected by the agent in culture(4,5). The linear relation of focus number to virus concentration afforded the basis for simple and convenient infectivity assay(5). In marked contrast, CEC response to the leukosis viruses—those responsible for the leukemias and other hematopoietic diseases—was entirely different(6,7). Although these agents infected CEC, conversion was slow and uncertain(8), and none of them studied earlier produced distinctive foci. Assays by the Rous interference factor (RIF) test(6,9,10) or enumeration of infectious centers detectable with fluorescein-conjugated antibodies(6) yielded useful data but were inconvenient and time-consuming.

Recent studies, however, disclosed singular results with strain MC29 leukosis virus(11, 12). This agent which causes myelocytomatosis, renal growths and hepatomas(11-13) infects CEC cultures and, in high virus-cell multiplicity exposure, regularly effects rapid and massive cell conversion(14,15). On the other hand, with appropriate multiplicity and culture conditions, strain MC29 causes foci in CEC monolayers appearing at approximately the same rate and almost as distinct as those induced by RSV. This report illustrates the characteristics of strain MC29 leukosis virus focus formation and applicability of the phenomenon to bioassay of the agent.

Methods and materials. Strain MC29 was isolated(11) in 1960 in Sofia, Bulgaria. As a leukosis agent, the strain is characterized primarily(11,12) by the induction of myelocytomatosis with which there may be associated renal tumors and hepatomas(13). Virus for the present studies was from CEC cultures derived initially(15) by passage from the blood plasma of chickens diseased(12) with the agent. Assays were made on 4 culture-fluid pools—115, 202, 694 and 698, Table I—collected from conventional cultures(15) without agar.

Chick embryos were from eggs of chickens† free of Rous resistance inducing factor (RIF). Primary CEC cultures were prepared by trypsinizing 10- or 11-day old embryos with 0.25% trypsin in phosphate buffered saline (PBS), pH 7.4. Culture vessels, 100×20 mm plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.), were seeded with 8 to 10 $\times 10^6$ cells in 10 ml of growth medium like that used by Calnek(8). After 3 to 4 days,

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