(6) G. W. Snedecor, "Statistical Methods," The Iowa State University Press, Ames, Iowa, 1961.

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Whole Body Measurements of ^{131}I-Tetracycline as an Index of Skeletal Growth

R. G. WOLFANGEL*, S. M. SHAW, and J. E. CHRISTIAN

Abstract □ A derivative of tetracycline was tagged with ^{131}I and administered to rats. Whole body retention of the tetracycline was determined by sequential measurements of whole body radioactivity. Statistically significant differences of whole body burdens were found for two age groups of rats (100 g. *versus* 200 g.); the younger animals retaining a greater portion of the administered tetracycline. Subsequent distribution analysis indicated that whole body radioactivity measurements did not precisely assess skeletal burdens of ^{131}I-labeled tetracycline because variable amounts of tetracycline persisted in soft tissue for prolonged intervals after injection, but did provide estimates of skeletal burdens which could be used to recognize differences in skeletal growth rate between groups of young and mature rats. The whole body counting technique may be applicable for the study of metabolic skeletal disorders.

Keyphrases □ ^{131}I-Tetracycline—skeletal growth index, whole body measurements □ Growth, skeletal—index, radioiodinated tetracycline □ Paper chromatography—analysis, identity □ UV spectrophotometry—analysis, identity □ Scintillation counting, whole body—analysis

Since the initial observations of tetracycline-induced fluorescence of bones by Rall *et al.*, investigators have been examining tetracycline fixation in mineralized tissue (1). The following has been reported concerning the deposition of tetracycline in bones and teeth; deposition occurs after introduction by any route, but is greatest following parenteral administration (2); tetracyclines are actively deposited at all sites of newly mineralizing bone and are relatively permanently fixed in the bone until resorption occurs (3, 4). The quantity of tetracycline deposited in bone is proportional to animal age and the dose administered (5, 6), and the presence of tetracycline in bone or teeth can be readily detected by the appearance of a bright yellow fluorescence under UV irradiation (7, 8).

Tetracycline bone labeling, followed by microscopic measurements of the width, area, or volume of yellow fluorescent zones found in bone sections, is used as an index of skeletal metabolic activity (9–11) such as appositional growth rate, radial rate of osteon closure, and osteon maturation rate. Direct determinations of the total quantity of tetracycline bound to the skeleton might also provide an index of skeletal metabolic activity; if so, the necessity of skeletal biopsy, sectioning, and tedious fluorescence microscopy currently employed for tetracycline skeletal observations would be alleviated. Thus, ^{131}I-labeled tetracycline was prepared and used to conduct animal studies. Whole body measurements of tetracycline retention, following administration of labeled tetracycline, were investigated for possible value to assess skeletal metabolic activity in young growing rats as compared to older mature rats. The accuracy of whole body counting for the determination of the total quantity of tetracycline bound to the skeleton was established by the direct measurement of labeled tetracycline bound to the entire skeleton of the two age groups of rats, as well as the residual amount of tetracycline remaining in the soft tissue of the animals.

METHODS

Synthesis and Purity—Hlavka *et al.* (12) reported the preparation of 7-iodo-6-demethyl-6-deoxytetracycline (766 tet) by dissolving 6-demethyl-6-deoxytetracycline (66 tet) and *N*-iodosuccinimide (NIS) in concentrated sulfuric acid at 0°. By substitution of ^{131}I for stable iodine, 7-radioiodo-6-demethyl-6-deoxytetracycline (*766 tet) was prepared in this laboratory according to Hlavka's directions. The ^{131}I-label was introduced by the preparation of *N*-^{131}iodosuccinimide (N*IS) by modification of the method of Benson *et al.* (13). Aqueous solutions of Na^{131}I[1] (25–75 mc.) were transferred to a test tube containing 2 ml. of carbon tetrachloride, and 1 ml. of NaI carrier (5 mg./ml.) was added. The test tube was fitted with a rubber stopper through which a dropping pipet, filled with concentrated nitric acid, had been inserted. Nitric acid was then added to the water–carbon tetrachloride mixture. The closed tube was left for 18–24 hr., during which time free iodine was formed and dissolved in the organic liquid. The aqueous overlayer was removed with a micropipet allowing the $^{131}I_2$ in the carbon tetrachloride to remain in the test tube. Stable elemental iodine (1 g.) was placed in an amber 5-dr. vial, the cap lined with Teflon, and 5 ml. of sodium-dried, distilled dioxane added. The solution of carbon tetrachloride, containing $^{131}I_2$, was transferred to the vial. The test tube was rinsed with 1 ml. additional carbon tetrachloride, and the 1 ml.

[1] Purchased from Nuclear Science and Engineering Corp., Pittsburgh, Pa.

rinse added to the reaction vial. Then 1.2 g. of *N*-silver succinimide was added in one portion, the lined cap replaced, and the closed vial agitated continuously for 90 min. The closed vial was immersed in a water bath (50°) for 5 min., removed, and the contents filtered through a sintered-glass filter. The reaction vial and silver iodide precipitate were washed with 2-, 2-, and 1-ml. portions of hot dioxane. The dioxane wash was filtered and added to the filtrate. Carbon tetrachloride (25 ml.) was added to the filtrate, the flask stoppered, covered with aluminum foil, and placed in a freezer for 12–18 hr. The contents of the flask were filtered to obtain $N^{131}IS$ crystals which were washed with 30-, 20-, and 10-ml. portions of carbon tetrachloride. The $N^{131}IS$ crystals were stored in a vacuum desiccator, in the dark, for at least 24 hr. prior to utilization for the preparation of *766. The *766 was prepared with the 66 tet and the $N^{131}IS$ according to the procedures of Hlavka (12).

Analyses of the *766 tet for chemical purity were performed as described by Hlavka *et al.* (12). These analyses included the preparation of spectral absorbance curves and paper chromatographic procedures. Solutions of known concentration (2–15 mcg./ml.) were prepared by dissolving the labeled tetracycline in 0.1 *N* HCl. A Bausch and Lomb Spectronic 505 was used to record a continuous spectral absorbance curve, between the wavelengths of 220 and 370 mμ, for each solution of *766 tet. The absorbance peaks were located and log epsilon values calculated. For the chromatographic procedures, Whatman No. 1 chromatography paper was immersed in a 0.2 *M* phosphate buffer (pH 2) solution and allowed to dry. Labeled tetracycline samples were applied to the buffer-treated chromatography paper as 1–2% solutions (aqueous and/or methanolic). Volumes of 2–25 μl. of the tetracycline solutions were applied to the paper. The tetracycline-spotted chromatography paper was placed in a standard chromatography jar and developed by the descending movement of *n*-butanol saturated with 0.2 *M* phosphate buffer (pH 2). Developing the paper for 19–20 hr. afforded a solvent movement of approximately 45 cm. The atmosphere of the chromatography jar was saturated with *n*-butanol and water vapor at least 24 hr. before using the jar to develop a chromatogram. Location of the tetracycline on the developed chromatograms was performed by UV light irradiation.

Hlavka *et al.* (12) reported maximum absorbance at 230 and 345 mμ with log epsilon values of 4.48 and 4.12, respectively, for 766 tet dissolved in 0.1 *N* HCl. UV spectral absorbance curves of the *766 tet prepared in this laboratory showed peaks at 239 and 345 mμ. The log epsilon values calculated for the absorbance peaks of *766 tet were 4.35 and 4.14, respectively. The UV absorbance and log epsilon values were not in exact agreement with the literature. This disagreement is attributed to chemical contamination of the *766 tet with minute quantities of 66 tet. The small amount of chemical contaminant appeared on the developed chromatograms and was identified as 66 tet using R_f values. The degree of chemical contamination was considered insignificant.

To determine the radiochemical purity of the *766, paper chromatographic procedures utilized for the determination of chemical purity were employed. Autoradiography was used to detect the labeled tetracycline. The developed chromatograms were overlayed with No Screen Kodak Medical X-Ray film.[2] After sufficient exposure time (10^8 d./cm.^{2}), the film was developed in accordance with the recommendations supplied by the Kodak X-ray developer.[2] The area of the chromatogram bearing the *766 tet (as observed from the autoradiogram) was sectioned. The remaining portion of the chromatogram was considered to contain radiochemical impurities. The sections were counted individually in a 7.6-cm. (3-in.), sodium iodide, thallium-activated well crystal scintillation counting system. Ninety-nine percent of the radioactivity appeared at the R_f of 766 tet.

Whole Body Retention—Two groups of female Sprague-Dawley[3] strain albino rats were utilized in this phase of the investigation. The first group of six animals was comprised of young, rapidly growing animals (100 ± 5 g.) and the second group of six rats contained adult rats (200 ± 10 g.). Animals were housed in individual metabolism cages for 24 hr. prior to *766 tet administration and during the remainder of the study. Food and water were allowed *ad libitum*. A dose of 20 mg. of *766 tet (0.25 μc./mg.) per kilogram of body weight was given to each rat intraperitoneally. Distilled water served as the solvent for the labeled tetracycline.

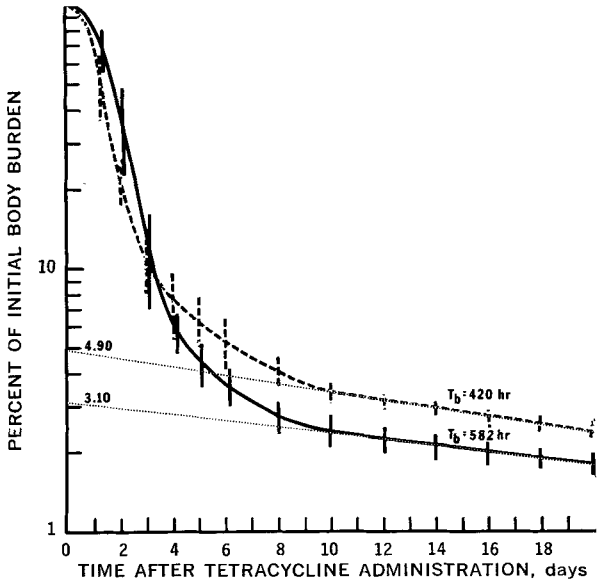

Figure 1—*Whole body retention of labeled tetracycline in rats. Key: - - -, 100 g.; and ——, 200 g.*

Whole body radioactivity counts of each rat were taken using an Armac liquid scintillation counting system.[4] Just prior to placing the rats in the counter, they were imprisoned in a cylindrical cage which inhibited virtually all motion and allowed for reproducible positioning of the rats within the detector. The initial measurement of whole body radioactivity was made 45–60 min. after the dose of radioiodinated tetracycline was given and was considered as the initial or 100% body burden. Whole body counts were repeated at various time intervals for 480 hr. after drug administration. Subsequent counts were corrected for variation in counter efficiency, background, and radioactive decay to the time of the initial determination. Corrected counts were expressed as a percent of the initial body burden.

During the course of the whole body counting study, urine and feces were collected separately from each rat at 24-hr. intervals. Each intact 24-hr. specimen was counted in the Armac liquid scintillation counting system. The fraction of the administered *766 tet excreted per time interval was calculated in a manner similar to the whole body counts and the data expressed as a percent of the initial body burden.

Distribution—The distribution and retention of *766 tet were determined in young and adult rats at various intervals after intraperitoneal administration of the labeled tetracycline. Sixteen 40-day-old female Sprague-Dawley rats (100 ± 10 g.) were administered a dose of 20 mg. of *766 tet (0.40 μc./mg.) per kilogram of body weight. Similarly, eighteen 90-day-old female rats (205 ± 10 g.) were each injected with the labeled tetracycline solution. The rats were housed, watered, and fed as in the previous study. Three[5] animals from each age group were sacrificed 24 hr. after tetracycline injection, and at 72-hr. intervals thereafter, with the final group sacrificed 384 hr. after tetracycline administration. The rats were ether anesthetized and a 1–3-ml. blood sample withdrawn by intracardiac puncture. The rats were then sacrificed by an overdose of ether. Each rat was weighed to the nearest 0.5 g. The following samples were excised for analysis: heart, liver, lung, kidney, spleen, thyroid, humerus, femur, and a 1–3-g. sample of muscle. Each sample, except blood, was rinsed in distilled water and weighed. In most cases, the entire organ was removed and weighed. Each tissue sample was analyzed for tetracycline content by counting in the 7.6-cm. (3-in.), well crystal scintillation counting system. The counting data were expressed as the micrograms of tetracycline in the tissue specimen. Subsequent calculations, using the weight of the tissue specimen and the milligrams of tetracycline initially administered to each rat, were performed to express the data as the percent of

[2] Eastman Kodak Co., Rochester, N. Y.
[3] Sprague-Dawley, Inc., Madison, Wis.
[4] Packard Model 440 Armac Scintillation detector in conjunction with a Packard model 410A Auto-Gamma spectrometer, Packard Instrument Co., La Grange, Ill.
[5] Only two rats were sacrificed at 24 and 96 hr. for the younger group.

Table I—Percent of the Dose of Iodinated Tetracycline (20 mg./kg.) Retained in Various Tissues[a] of Young Female Rats (100 g.) following Drug Administration

Organ	Time after Administration, hr.[b]					
	24	96	168	240	312	384
Heart	0.173 ± 0.010	0.007 ± 0.003	—	—	—	—
Liver	3.223 ± 0.616	1.556 ± 1.052	0.304 ± 0.404	0.437 ± 0.406	0.218 ± 0.170	0.129 ± 0.183
Lung	0.207 ± 0.010	0.012 ± 0.003	0.006 ± 0.002	—	—	—
Kidney	1.593 ± 0.172	0.036 ± 0.003	0.019 ± 0.006	0.009 ± 0.002	0.007 ± 0.002	0.006 ± 0.002
Spleen	0.133 ± 0.000	0.170 ± 0.052	0.038 ± 0.019	—	—	—
Thyroid	0.128 ± 0.054	0.204 ± 0.018	0.126 ± 0.040	0.088 ± 0.016	0.055 ± 0.017	0.048 ± 0.026
Skeletal[c]	3.748 ± 0.145	3.030 ± 0.266	2.794 ± 0.228	2.759 ± 0.601	2.333 ± 0.130	1.856 ± 0.032

[a] Data expressed as the percent of dose per entire organ or tissue. [b] Intervals of 24 and 96 hr. represent the means of two rats; all others are the means of three rats. [c] Skeletal is average percent per gram of right humerus and femur multiplied by the number of grams of skeletal tissue estimated from body weight.

Table II—Percent of the Dose of Iodinated Tetracycline (20 mg./kg.) Retained in Various Tissues[a] of Mature Female Rats (200 g.) following Drug Administration

Organ	Time after Administration, hr.[b]					
	24	96	168	240	312	384
Heart	0.212 ± 0.019	0.007 ± 0.004	—	—	—	—
Liver	2.974 ± 0.850	1.092 ± 0.692	0.322 ± 0.350	0.235 ± 0.172	0.198 ± 0.155	0.254 ± 0.262
Lung	0.364 ± 0.135	0.013 ± 0.003	0.005 ± 0.112	—	—	—
Kidney	1.504 ± 0.162	0.051 ± 0.019	0.017 ± 0.003	0.010 ± 0.002	0.007 ± 0.000	0.006 ± 0.001
Spleen	0.178 ± 0.089	0.009 ± 0.004	0.004 ± 0.002	—	—	—
Thyroid	0.138 ± 0.052	0.167 ± 0.033	0.115 ± 0.006	0.104 ± 0.019	0.066 ± 0.020	0.084 ± 0.038
Skeletal[c]	1.821 ± 0.205	1.754 ± 0.194	1.614 ± 0.232	1.447 ± 0.198	1.578 ± 0.163	1.216 ± 0.178

[a] Data expressed as the percent of dose per entire organ or tissue. [b] Data represent the means of three rats. [c] Skeletal is average percent per gram of right humerus and femur multiplied by the number of grams of skeletal tissue estimated from body weight.

the administered dose of tetracycline retained per gram of tissue or organ, as well as per the entire tissue or organ.

Skeletal Retention—Following the distribution study, six 40-day-old female Sprague-Dawley rats (100 ± 5 g.) and six 90-day-old rats (200 ± 10 g.) received *766 tet in the same manner as described for the distribution study. Three of the rats from each age group were sacrificed at 312 hr. (13 days) and 384 hr. (16 days) after injection of the labeled tetracycline. The entire skeleton was excised from each rat and the total mass of nonskeletal tissue assembled. The skeleton and composited soft tissue from each rat were separately assayed for tetracycline by counting in the Armac liquid scintillation counting system. The quantity of the administered dose of labeled tetracycline retained by the skeletal mass and by the nonskeletal tissue was expressed as a percent of the initial body burden.

Excretion—Examination of excreta obtained from rats dosed with *766 tet was undertaken in order to determine the metabolic fate of the tetracycline. Three 40-day-old female Sprague-Dawley rats (100 ± 10 g.) were injected intraperitoneally with a dose of 20 mg. of *766 tet (2.4 μc./mg.) per kilogram of body weight. Three 90-day-old female rats (200 ± 10 g.) were also given a dose of 20 mg. of *766 tet (2.4 μc./mg.) per kilogram of body weight. A higher specific activity tetracycline was necessary for this phase of the investigation in order to ensure the detection of metabolic products, if any, in the excreta. Following labeled-tetracycline administration, the rats were housed in individual metabolism cages and the excreta collected 48 hr. after injection of the tetracycline. Radioactive products appearing in the 48 hr. excreta were qualitatively analyzed using chromatographic techniques. Thick-layer chromatograms were prepared with an aqueous slurry of MN-Cellulose powder-300.[6] The cellulose-coated plates were air dried and impregnated with a mixture of 0.2 *M* phosphate buffer (pH 2.0) and glycerol (19:1). The plates were allowed to air dry before spotting. Untreated urine was applied to the thick-layer chromatograms in amounts ranging from 2.5 to 30 μl. per application. Following thorough homogenation of feces, portions of the fecal homogenates were applied to the thick-layer chromatograms. Each plate was also spotted with a sample of the *766 tet used to dose the rats. After solvent development,[7] the chromatograms were autoradiographed for 4 weeks.

RESULTS AND DISCUSSION

Whole body retention of tetracycline for the two different age groups of rats is presented in Fig. 1. Whole body retention of tetracycline was similar during the first 4 days of the experiment, but then the body burden of tetracycline in the younger (100 g.) rats became 1% greater than the mature (200 g.) animals. A Student's *t* difference of means test showed the body burden of tetracycline to be statistically greater ($p = 0.005$) in the younger rats from Day 8 until termination of the study at Day 20. The curves shown in Fig. 1 were fitted by a standard exponential analysis in the sense of least squares. The intercepts and slopes are reported for the long-lived exponentials. The young rats intercept is 1.8% greater and has a half-time 162 hr. faster than the mature rats. The elevated whole body tetracycline retention shown by the young rats was considered to be the result of increased tetracycline fixation to the faster growing skeletal tissue of the younger rats, but this remained to be demonstrated from the distribution and skeletal studies.

The results of the distribution study, expressed in terms of the percent of the administered dose of tetracycline retained by the entire organ or tissue, are presented in Tables I and II and in Fig. 2. In Fig. 2 the curves were fitted by a standard exponential analysis in the sense of least squares. The percent of the administered dose of tetracycline present in the entire skeleton was calculated according to the method of Myers and Jaffe (6). They averaged the micrograms of tetracycline retained per gram of humerus bone and femur bone, then multiplied the value by the total skeletal weight. The values of total skeletal weight were obtained from the body weight of the rats by using data from a quantitative study of skeletal growth in the albino rat performed by Donaldson (14). As may be observed from Tables I and II, the amount of tetracycline decreased to undetectable levels by Day 7 or 10 for the spleen, lung, and heart. The skeleton, liver, thyroid, and kidney persistently contained tetra-

[6] Brinkmann Instruments, Inc., Westbury, N. Y.

[7] The organic phase of an *n*-butanol–0.2 *M* phosphate buffer (pH 2.0) mixture.

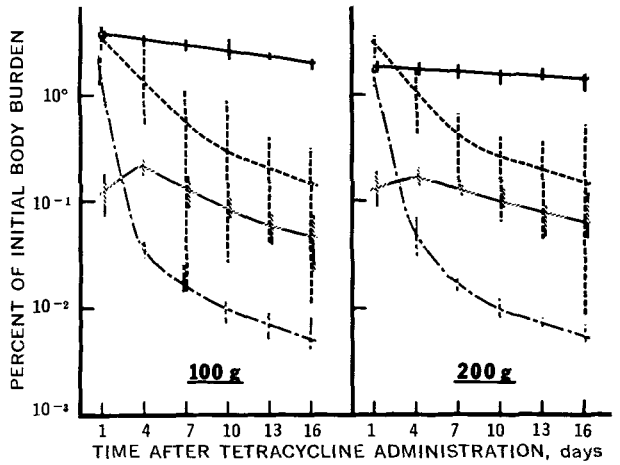

Figure 2—*Retention of labeled tetracycline in rat organs. Key: ——, skeleton; - - -, liver; —|||—, thyroid; and - — - —, kidney.*

cyline until the termination of the study. For both age groups, bone contained greater amounts of the tetracycline than any of the other tissues studied. After Day 10, the quantity of tetracycline in the skeleton of either age group exceeded the level of all other organs by a factor of 10 or greater.

The intercepts and biological half-times of the whole body retention curves (Fig. 1) and organ distribution curves (Fig. 2) have been summarized and compared in Table III. Both the intercepts, as percent of the dose, and half-times, in hours, are listed for the long-lived exponentials. Differences have been computed by subtracting the value of the mature rats from that of the young rats. Whole body retention curves show an intercept difference of 1.80%. Intercepts of the liver, kidney, and thyroid are very similar for young and adult rats; however, the skeleton shows an intercept difference of 1.92% which is very comparable to the intercept difference seen in whole body retention. Examination of the biological half-time data shows that the long-term whole body retention half-time of the younger rats was 162 hr. shorter than that observed for the older animals. Age differences in the biological half-times of the liver and kidney were not great. Thyroids show a difference in biological half-times between the age groups, but the skeleton, by far, displayed the greatest difference (the younger group 319 hr. faster). Although the intercepts and half-times obtained for whole body retention would not seem to be precise measurements of the tetracycline bone compartment, there is a high correlation between whole body retention intercepts and the skeletal intercepts obtained from the distribution study. This correlation becomes less evident when biological half-times are examined in the same manner.

In addition to the distribution analyses previously described, the levels of tetracycline in entire skeletons were investigated in that time domain where the whole body burdens were statistically greater in the young rats as compared to the mature rats. At Day 13 and Day 16 postinjection, complete skeletons of three females per age group were separated from their respective soft tissue. The skeletons and soft tissues were counted separately for tetracycline content. In Table IV, skeletal and soft tissue retention as a

Table III—Summary of Age-Related Differences of Long-Term ^{131}I-Tetracycline Retention Intercepts and Half-Times[a]

	Intercepts, % of Initial Body Burden			Biological Half-Times, hr.		
	100 g.	200 g.	Δ[b]	100 g.	200 g.	Δ
Whole body retention	4.90	3.10	1.80	420	582	−162
Liver	1.01	0.80	0.21	125	180	− 55
Kidney	0.03	0.03	—	135	150	− 15
Thyroid	0.20	0.16	0.04	180	327	−147
Skeleton	3.82	1.90	1.92	405	724	−319

[a] Obtained from the long-lived exponentials of the whole body retention and organ distribution curves. [b] Value from mature rat substracted from that of the young rat.

Table IV—Percent of the Initial Body Burden of Tetracycline Retained by the Skeleton and Nonskeletal Tissue

Time, hr.	Skeletal Retention,[a] % Young, 100 g.	Mature, 200 g.	$t_{obs.}$[b]
312	1.600 ± 0.021	1.098 ± 0.088	9.61
384	1.520 ± 0.058	0.998 ± 0.171	5.012
	Retention by Nonskeletal Tissue,[a] %		
312	1.780 ± 1.144	1.110 ± 0.626	1.182
384	0.901 ± 0.351	0.634 ± 0.153	1.207

[a] Mean and standard deviation of three rats per age group. [b] Statistic calculated from the experimental data according to a Student's t difference of means test. Any $t_{obs.}$ exceeding 4.60 provides statistical proof (4 degrees of freedom, 99.5 percentile point) of greater retention in the younger animals.

percent of the initial body burden is shown for the young and mature rats both at 312 hr. (Day 13) and 384 hr. (Day 16). The younger rats retained more tetracycline in both hard and soft tissue compartments at each time interval. A difference of means test indicated that the skeleton of young rats contained significantly greater amounts of tetracycline than the skeleton of older rats. Skeletal values were also noted to be highly consistent as evidenced by the low standard deviations. Regarding soft tissue retention of tetracycline, considerable variations were present among the members of each group resulting in standard deviations much larger than those obtained for skeletal retention. A difference of means test did not indicate that the observed differences in soft tissue retention between age groups were statistically significant.

The results of this phase of the study indicate that the statistically greater whole body retention of tetracycline previously shown by the young group of rats resulted from localization of increased amounts of tetracycline in the skeletons of the young. This supports the hypothesis that increased skeletal mineralization in young animals can be determined using whole body measurements of radioiodinated tetracycline, but points out that the validity of the test remains dependent upon obtaining either a complete or uniform clearance of tetracycline from the soft tissue compartment prior to making whole body measurements.

Figure 3 graphically represents the percent of the administered tetracycline accumulated in the urine and the feces of rats which were used in the whole body retention study. No significant difference in excretion of the tetracycline was noted between age groups. The data show clearly the importance of fecal excretion as the route of tetracycline elimination.

As described in the methods section, examination of excreta obtained from both age groups was undertaken in order to deter-

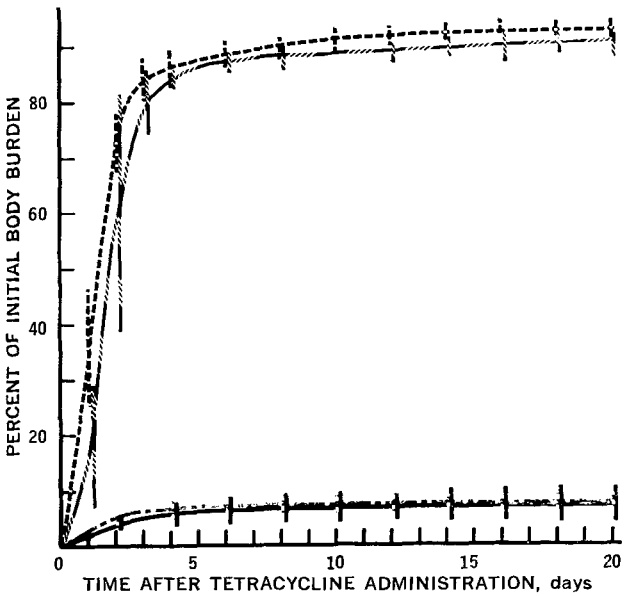

Figure 3—*Accumulated excretion of labeled tetracycline in the rat. Key: - - -, feces, 100 g.; —|||—, feces, 200 g.; - — - —, urine, 100 g.; and ——, urine, 200 g.*

Table V—Comparison of *In Vivo* Properties of ^{131}I-Labeled Tetracycline to Commerical Tetracyclines

Criteria of Comparison, %	Chlortetracycline	Demethyl-chlortetracycline	Tetracycline	^{131}I-Labeled Tetracycline
Whole body retention	3–23% at 72 hr.[a] (18)	10% at 168 hr.[a] (16)	9.5% at 72 hr.[a] 6.7% at 168 hr.[a] (15)	10% at 72 hr. 3–4% at 168 hr.
Dose eliminated in feces	50% at 168 hr. (18)	15% at 168 hr. (16)	23–54% at 168 hr. (15)	90% at 168 hr.
Dose in skeleton	—	—	3–5% in 60-g. male rats at 4 days; 1–2% in 340-g. males (6)	3% in 100-g. female rats at 4 days; 1.8% in 200-g. females
Dose per gram of bone after intraperitoneal injection of 20 mg./kg.	—	—	0.16% per gram of bone at 24 hr. in adult males (19)	0.15% per gram of bone at 24 hr. in adult females
Dose recovered unmetabolized in excreta	Not metabolized to any significant extent (18)	93% (16)	95% (15)	Not metabolized to any significant extent[b]

[a] Values listed are based upon quantitative recovery of excreta products. Numbers in parentheses are references. [b] Analysis performed on excreta collected within 48 hr. after administration of the drug.

mine the metabolic fate of the labeled tetracycline. For both age groups the autoradiograms prepared from chromatograms of urine disclosed the presence of a single labeled compound with an R_f value almost identical with that of the *766 tet utilized in the various studies. In feces, the autoradiograms indicated the presence of minute amounts of ^{131}I-activity other than that identical to the *766 tet standard. Even though the film had been exposed to the chromatogram for 4 weeks the image produced on the film by radioactivity other than that associated with the *766 tet was barely detectable. The *766 tet utilized in these studies was recoverable in the excreta of the animals in essentially unaltered form. This strongly signifies that ^{131}I-activity remaining in the animal tissue is associated with tetracycline, but does not provide unequivocal proof of this assumption. It would appear that the *766 tet utilized in the various studies was essentially unmetabolized.

Aware that iodination of tetracycline might result in a compound that was not biologically representative of the tetracycline family, attention was devoted to assembling *in vivo* parameters of retention, excretion, and distribution of the iodinated tetracycline. In Table V, biological observations for the iodinated tetracycline are compared to results obtained by other investigators using several commercially available tetracycline antibiotics. There is good correlation between the radioiodinated tetracycline prepared in this laboratory and the other tetracyclines for whole body retention, skeleton and bone uptake, and metabolism. Examination of the percent of the dose eliminated in the feces shows that the radioiodinated tetracycline is excreted in the feces in far greater quantities than the other tetracyclines. Since in most cases the radioiodinated tetracycline compared favorably to the other tetracyclines, the radioiodinated tetracycline is considered to be representative of the tetracycline family of compounds.

SUMMARY AND CONCLUSIONS

Investigators have reported that tetracyclines are rapidly excreted within 7 days of administration and that the majority of tetracycline retained after 7 days remains bound to the skeleton (15, 16). Also, the quantity of tetracycline fixed and retained by the skeleton of animals is proportional to the age of the animal and/or skeletal turnover rate (2, 6, 17). Based upon these reports, an attempt was made to assess skeletal burdens of tetracycline in rats by measurements of whole body radioactivity following administration of radioiodinated tetracycline.

When the whole body retention of labeled tetracycline was studied in young and mature rats, statistically greater body burdens were observed in the younger group. Distribution experiments showed that the higher body burdens of the younger animals were related to their increased skeletal uptake of tetracycline. In subsequent experiments, skeletons of several test rats from each age group were surgically cleansed of soft tissue. Tetracycline skeletal burdens were determined and found to be very uniform within each group, and statistically higher levels of skeletal tetracycline were found in the younger animals when compared to the older. Tetracycline levels within soft tissue were obtained for the same animals. While skeletal levels were very consistent within each experimental group, the soft tissue values showed relatively large variations among members of each group. The younger rats retained larger amounts of tetracycline in soft tissues, but differences in soft tissue values between age groups could not be statistically verified. The data from the various experiments indicate that age differences in whole body retention of labeled tetracycline did reflect age differences of skeletal tetracycline fixation; however, persistent localization of tetracycline in soft tissue compartments, and the animal-to-animal variation of soft tissue tetracycline residue, reduced the accuracy of predicting skeletal levels by whole body measurements. The proposed technique may be of extreme value in the study of skeletal metabolic disorders provided a rapid, complete, or uniform clearance of tetracycline from soft tissue compartments could be obtained.

In conclusion, the results of this investigation indicate:

1. The labeled tetracycline synthesized in this laboratory was representative of the tetracycline antibiotics.
2. Whole body retention of iodinated tetracycline in young growing rats was greater than in mature rats, and resulted from enhanced skeletal deposition of tetracycline in the young rats as contrasted to the older animals.
3. Residual and variable amounts of tetracycline persisted in soft tissues for prolonged intervals after injection, but the skeleton showed the greatest uptake and consistent binding of the drug.
4. Skeletal fixation of tetracycline can be estimated by whole body measurements of radioiodinated tetracycline, but the accuracy of the estimates obtained is dependent upon a uniform soft tissue clearance and this has been shown to be variable. The technique may be of value for assessment of skeletal metabolic activity, especially when comparing normal to pathological states, providing a uniform clearance of tetracycline from soft tissues can be achieved.

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Effect of Probenecid on Renal Clearance of Riboflavin in Man

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Abstract □ The renal clearance of riboflavin was determined in three human subjects at various serum concentrations of the vitamin with and without prior administration of probenecid. Renal clearances of riboflavin exceeded (up to three times) the endogenous creatinine clearances, which indicates that riboflavin excretion involves renal tubular secretion. The clearance of riboflavin was less at low than at high serum concentrations of the vitamin, characteristic of a saturable tubular reabsorption process. Probenecid decreased the renal clearance of riboflavin, and this effect was directly related to the serum concentration of the inhibitor. The serum protein binding of the vitamin was essentially constant (60%) over the concentration range encountered and was unaffected by the presence of probenecid.

Keyphrases □ Riboflavin, renal clearance, man—probenecid effect □ Probenecid, effects—riboflavin, renal clearance, plasma protein binding □ Spectrophotometry—analysis

A pharmacokinetic analysis of literature data by Levy and Jusko (1) yielded renal clearance values for riboflavin in a human subject which were appreciably higher than the normal glomerular filtration rate. Subsequently, it was found that probenecid, an inhibitor of certain specialized renal transport processes (2), decreased the initial rates of urinary excretion of oral and parenteral doses of the vitamin in human subjects (3). These studies suggested that the renal excretion of riboflavin in man involves tubular secretion. The existence of such a mechanism for riboflavin has already been demonstrated in the chicken by Rennick (4) who also noted an inhibitory effect of probenecid on this process.

The purposes of the study to be described were to determine directly the renal clearance of riboflavin in man and to assess quantitatively the effect of probenecid on this process as well as on the plasma protein binding of the vitamin.

EXPERIMENTAL

The studies were carried out in three healthy human subjects: an adult male (Subject J), age 26 years, and two female children (Subjects A and C), ages 8 and 11 years, respectively. The adult subject received a single intravenous dose of riboflavin-5′-phosphate (FMN)[1] equivalent to about 30 mg. of riboflavin (FR) with and without 1 g. probenecid[2] given orally in suspension 1 hr. prior to FMN injection. Urine was collected at appropriate intervals for a total of 48 hr. Blood samples were drawn from the antecubital vein at −0.5, 0.25, 0.75, 1.25, 2.25, 3.25, 4.5, 6.5, 8.5, 13.0, and 25.0 hr. relative to the time of FMN injection. These times were midpoints of urine collection periods.

The two younger subjects received an initial intramuscular dose of FMN[3] equivalent to 16 mg. FR followed by three hourly oral doses of 6 mg. FR as FMN in solution. Urine was collected at hourly intervals and blood was drawn from the antecubital vein at the midpoints of the three urine-collection periods. A 0.5-g. dose of probenecid in tablet form was administered in crossover fashion to the two children 1 hr. prior to the initial dose of FMN. There was an interval of 7 days or more between the control and probenecid studies.

Protein-Binding Determinations—The ultrafiltration technique described in an earlier publication (5) was used to determine the extent of protein binding of the flavins and probenecid in the serum samples.

FMN Stability Study—A series of samples, each containing 1 ml. of FMN in pH 7.4 isotonic Sorensen's buffer and 1 ml. of freshly drawn whole blood from Subject J, were incubated at 37° in the dark. The concentrations of FMN and FR in the samples were determined as a function of time. Control solutions, without blood, were similarly analyzed.

Analytical Method—Riboflavin and FMN were determined fluorometrically by methods previously described (5, 6). Endogenous creatinine levels in urine and serum were determined colorimetrically by the alkaline picrate method (7). Probenecid in serum was assayed by the spectrophotometric method of Dayton *et al.* (8). Initial serum samples were assayed for albumin content as described previously (5).

Data for flavins and probenecid were corrected for blank readings of urine and serum samples obtained prior to administration of the compounds to the test subjects. There was no interference in the assay of any of the compounds due to the presence of the others.

RESULTS

Renal Clearances of Riboflavin and Effect of Probenecid—Riboflavin clearances in Subjects A and C (Table I) were determined

[1] Sodium riboflavin-5′-phosphate, Hoffmann-LaRoche, Nutley, N. J.
[2] Benemid tablets, Merck Sharpe and Dohme, West Point, Pa.
[3] Hyrye injection, S. F. Durst and Co., Inc., Philadelphia, Pa.