Bile Acid Composition of Rainbow Trout, *Salmo gairdneri*

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ABSTRACT

The bile acid composition and metabolism of rainbow trout *Salmo gairdneri* has been investigated by thin layer chromatography, gas liquid chromatography, and radio gas liquid chromatography methods. For this purpose gallbladder bile was collected from fed fish at 6 and 13 months and from starved fish at 12 months of age. Cholic acid was found to be the main component and constituted over 85% of total. Chenodeoxycholic acid accounted for 14% or less and the 3α,12α-7-keto- and 7α,12α-3-keto-5β-cholanoates for 1% or less of total. The bile acids were conjugated mainly with taurine, only small amounts of glycocholic acid being detected. Ca. 5% of the taurocholate was sulfated, as were trace amounts of cholic and glycocholic acids. The size of the bile acid pool was found to increase in the older fish and to decrease in starved fish. Unlike mammalian livers, the livers of the trout converted radioactive chenodeoxycholic acid into cholic acid.

INTRODUCTION

In the past, bile acid composition has been studied primarily in mammalian species. Studies on nonmammalian vertebrates have been limited. Haslewood (1) has summarized the types of bile salts found in nature as alcohol sulfates, taurine conjugates of 27-carbon acids, taurine conjugates of 24-carbon acids, and glycine conjugates and has claimed that their distribution bears a close relationship to the evolutionary position of the animal. This idea is interesting and can be developed further as data on bile acid composition of a wider range of vertebrates becomes available.

In the following study, we have examined the composition of the bile acids of the gallbladder bile of the rainbow trout, *Salmo gairdneri*, under normal conditions and as influenced by age and starvation.

MATERIALS AND METHODS

Standard cholic (CA), deoxycholic, chenodeoxycholic, and lithocholic acids of 99% purity were obtained from Supelco (Bellefonte, Pa.) and standard hyodeoxycholic acid of 99% purity from Applied Science Laboratories (State College, Pa.). The glycine and taurine conjugates of the common bile acids of 99% purity were purchased from Supelco. The 3-sulphate esters of cholic, deoxycholic, chenodeoxycholic, and lithocholic acids and of their glycine and taurine conjugates were prepared in the laboratory using sulfur trioxide by method 3 of Jenkins and Sandberg (2). Cholic acid-24-^{14}C (50 mCi/mMole) was obtained from New England Nuclear, Boston, Mass., and chenodeoxycholic-24-^{14}C, deoxycholic-24-^{14}C, and lithocholic-24-^{14}C (35.8 mCi/mMole) were purchased from Tracerlab, Waltham, Mass.

Rainbow trout were obtained from Willow Beach National Fish Hatchery, Willow Beach, Ariz. Three groups of fish were used in the study: group 1 included 6 fish which were 6 months old and fed ad libitum; group 2 had 6 fish which were 13 months old and fed ad libitum; and group 3 had 9 fish which were 12 months old and were fasted for 45 days. The gallbladder of each fish was dissected, removed, immediately frozen, and kept at -20 C until analyzed.

Extraction of Bile Acids

The bile (0.2-0.5 ml) was extracted by the addition of 10 ml hot ethanol-methanol (95:5 v/v) and shaking (3). The precipitated protein was removed by centrifugation at 2000 g at 4 C for 15 min. The alcoholic extract was decanted off, the precipitate washed with 2 ml hot methanol and filtered off. The extracts were combined, diluted to 40% alcohol with distilled water, and delipidated with petroleum ether. The aqueous alcohol solution then was evaporated to dryness under nitrogen in vacuo. The residue was dissolved in 1 ml methanol, and 0.5 ml was used for analysis of the conjugated bile acids, while the other 0.5 ml was saved for total bile acid determination and identification.

TLC of Conjugated Bile Acids

An aliquot of the bile acid extract was applied as a band to a thin layer chromatographic (TLC) plate (20 x 20 cm) coated with Silica Gel G (Merck, Darmstadt, Germany) in a

0.25 mm thick layer. The plates were developed in n-butanol-glacial acetic acid-water (10:1:1, v/v/v) for 4 hr (4). After evaporation of the solvents, the bile acids were located by iodine vapor and the relative R_f values of any bands compared to those of authentic standards of CA and CDCA, their taurine and glycine conjugates, and the conjugate sulfates for identification. Each band was scraped off and the bile acid conjugates extracted with 0.05 N HCl in 75% ethanol. Each conjugate group was subjected to further analysis, as described below. The recovery of the bile salts from the TLC plates was 95 ± 2%, as indicated by analysis of radioactive glycocholic acid.

Solvolysis of Sulfate Esters

The TLC fractions corresponding to sulfated bile acid standards were subjected to solvolysis (5). The sulfate esters (1 mg or less) were hydrolyzed at room temperature (24 hrs) with acetone-ethanol (9:1, v/v) adjusted to pH 1 with 2 N HCl. The solvents then were evaporated and residue subjected to TLC, as described above. Any free bile acids or their taurine and glycine conjugates were recovered and analyzed further.

Hydrolysis of Taurine and Glycine Conjugates

The TLC fractions (1 mg or less) corresponding to standard taurine and glycine conjugates of bile acids were subjected to saponification with 2.5 N NaOH (2.5 ml) at 115 C for 12-15 hr in a sealed glass tube (3). After cooling, the reaction mixture was acidified with dilute HCl and the bile acids extracted with diethyl ether. The recovery of radioactive bile acids from the taurine conjugates under the above conditions averaged 90 ± 5%.

Gas Liquid Chromatography (GLC) and Radio-GLC

For this purpose, the bile acids were converted into the methyl esters by methanolic 2,2-dimethoxypropane (Aldrich Chemical Co., Milwaukee, Wisc.) and concentrated HCl (6). Prior to GLC, the methyl esters were converted into the trifluoroacetates by reaction with 0.3 ml trifluoroacetic anhydride at 40 C for 40 min (3). The GLC analyses were performed on a Packard 7401 gas chromatograph system equipped with dual glass columns (4 ft x 2 mm inside diameter) containing a mixture of 3% QF-1 and 3% OV-17 (5:1, w/w) both on 100-120 mesh Gas Chrom Q (Applied Science Laboratories, State College, Pa.). The separations were made at 225 C isothermally using helium (30 ml/min) as the carrier gas. The GLC system was calibrated (3) by means of a standard mixture made up of equal wt proportions of lithocholic, deoxycholic, chenodeoxycholic, hyodeoxycholic, cholic, and 7-ketolithocholic acids. Unknown bile acids were quantitated using 5β-cholanoic acid as internal standard. The radio-GLC system was similar to that described by Swell (7).

The column effluent was passed through a combustion furnace containing copper oxide where it was converted into CO_2 and any radioactivity monitored in the proportional radioactive gas counter. The efficiency of the latter system was ca. 80% for ^{14}C, and any acids yielding more than 1000 dpm were measured with a relative error of 10% or less.

The quantitative GLC data were subjected to t-test between groups, and $P>0.05$ was considered not significant. The estimates from total bile acid analyses were compared to those obtained by combined TLC-GLC analysis following summation and normalization of the data (8).

TLC of Bile Acid Methyl Esters

The bile acid methyl esters were separated according to the number of hydroxyl and keto groups by TLC on Silica Gel G using chloroform-acetone-methanol (70:25:5, v/v/v) as developing solvent (9). The bands of the bile acids were located by briefly exposing the plates to iodine vapor. The areas corresponding to standard mono-, di-, and tri-hydroxy and keto bile acids were cleared of silica gel, and the scrapings were extracted with 10-20 ml methanol-acetone (1:9, v/v).

Part of the eluate was evaporated to dryness on the direct probe attachment of the mass spectrometer and the spectrum obtained. The rest of the sample was evaporated to dryness and the residue trifluoracetylated or trimethylsilylated prior to gas liquid chromatography-mass spectrometry (GLC-MS), as described below.

GLC-MS of Bile Acids

Combined GLC-MS analysis was performed with a Varian Mat CH-5 single focusing mass spectrometer coupled to a Varian Mat computer (10). For this purpose, the bile acid methyl esters were converted into the trimethylsilyl ethers using trimethylsilyl-chloride, hexamethyldisilazane and dry pyridine as described by Elliott, et al. (11). The GLC separations were obtained on a Varian model 2700 moduline gas chromatograph equipped with a 180 cm x 2 mm inside diameter glass column containing 3% OV-210 on 100-120 mesh Gas Chrom Q. The bile acids were resolved isothermally at 250 C, passed through a transfer line and a Watson-Bieman separator into an ion source, all oper-

ated at 270 C. The ionization voltage was 70 ev, the accelerating voltage 3000 volts, and the electron emission energy 100 μA. Scanning was done at 5 sec/decade at a resolution of 800-1000. All spectra taken over the GLC peaks were corrected for total ion current variation.

Incubations

The biochemical conversion of CDCA into CA was demonstrated by incubating radioactive CDCA with the liver homogenates (12). Liver tissue (1 g) was homogenized in 0.1 M phosphate buffer (pH 7.6) containing 0.25 M sucrose, 0.01 M $MgCl_2$, and 0.03 M nicotinamide. Aliquots of the homogenate were incubated with a 0.1% lecithin emulsion of 100 nmoles CDCA containing 0.1 μCi CDCA-24-^{14}C for 1 hr at 37 C. The purity of labeled CDCA was determined by TLC and radio-GLC to be better than 99%. At the end of the incubation, 19 ml ethanol containing 0.01% ammonium hydroxide was added along with 100 μg unlabeled CDCA and CA as carriers. The contents of the tubes were mixed thoroughly, heated at 60 C for 30 min, centrifuged, and the supernatant removed and evaporated to dryness under nitrogen. The bile acids were recovered following saponification of the extracts, as described above. After methylation, the bile acids were separated by TLC (13) using isooctane-isopropyl ether-acetic acid (50:25:40, v/v/v) and the bands corresponding to standard CA and CDCA recovered and the radioactivity measured by radio-GLC, as described above, and by scintillation counting. For the latter purpose, 15 ml Aquasol (New England Nuclear, Boston, Mass.) was added and the radioactivity determined in a Packard liquid scintillation spectrometer equipped with an automatic external standard. Each fraction was counted for 10 min in triplicate.

RESULTS

Identification of Bile Acids

Figure 1 shows the TLC separation of the bile acid samples. The various fractions were identified by reference to standards and the relative R_f values recorded in the literature. In most cases, strong spots were seen corresponding to taurocholic, sulfated taurocholic, sulfated cholic, glycocholic, and sulfated glycocholic acids. The various R_f values of the standards corresponded closely to those reported by Palmer (5,14) in a similar TLC system. Table I gives the mole percentages of each conjugate in the total bile acid mixture of each group of fish as determined by GLC following hydrolysis of the conjugates. It is evident that all groups are similar in their conjugation patterns and that differences in age

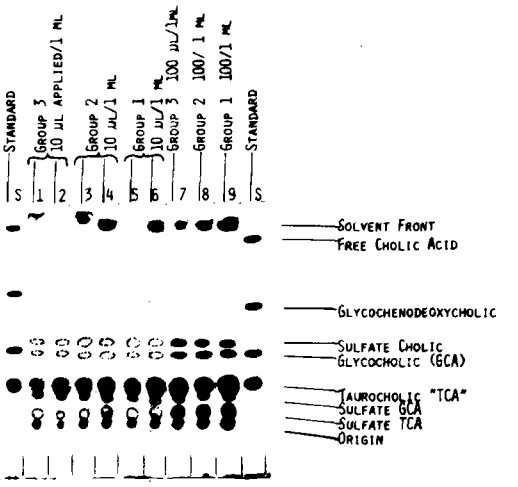

FIG. 1. Thin layer chromatogram of biliary bile acid conjugates of rainbow trout. Chromatography conditions: Silica Gel G; butanol-acetic acid-water (10:1:1, v/v/v) as developing solvent; phosphomolybdic acid spray. Lanes S, standard bile acids and conjugates as shown on the right of the figure. Lanes 1 and 2, 10 μliter applications of 1 ml solutions of total bile salts from two fish in group 3; lanes 3 and 4, 10 μliter applications of 1 ml solutions of total bile salts from two fish in group 2; lanes 5 and 6, 10 μliter applications of 1 ml solutions of total bile salts from two fish in group 1; lanes 7, 8, and 9, 100 μliter applications of 1 ml solutions of total bile salts from one fish each in group 3, 2, and 1, respectively.

TABLE I

Mole Percentage of Various Conjugates in Total Bile Acids of Rainbow Trout[a]

Type of conjugate	6 Months old	13 Months old	Starved fish 12 Months old
Sulfated tauro cholic acid	4 ± 0.4	3 ± 0.2	4 ± 0.2
Sulfated glyco cholic acid	3 ± 0.2	3 ± 0.2	5 ± 0.4
Sulfated cholic acid	2 ± 0.2	3 ± 0.3	1 ± 0.01
Tauro cholic + chenodeoxycholic acid	89 ± 2.1	89 ± 3.4	88 ± 1.5
Glyco cholic acid	2 ± 0.1	2 ± 0.2	2 ± 0.2

[a]Corrected gas liquid chromatographic estimates ± standard error (see text).

or starvation had no significant effect upon the conjugation type. In all instances, the bulk of the bile acid was conjugated with taurine, although sulfated bile acids occasionally made up as much as 10% of the total bile acid pool.

TLC of the bile acid methyl esters derived from the various conjugate classes revealed the presence of large amounts of CA and smaller amounts of CDCA, along with traces of keto bile acids. GLC of the trifluoroacetates of the bile acid methyl esters confirmed that, in all groups of fish, the peak corresponding to CA was the major component. The peak corresponding to CDCA was much smaller, especially in the adult fish (groups B and C). In addition, a minor peak of variable size emerged in the region corresponding to the trifluoroacetyl ester of methyl deoxycholate.

A GLC-MS examination of the mixed bile acid methyl esters, as the trifluoroacetyl derivatives using a QF-1 column, revealed that the major peak was indeed due to CA (15). The mass spectrum of this derivative showed a molecular ion at m/e 710 and a base peak at 367, which corresponded to a loss of 2 trifluoroacetyl groups and the side chain, M − (2 x 114 + 115). A major fragment also was seen at m/e 253, which was due to the steroid nucleus of a trihydroxy bile acid. A correct mass spectrum also was obtained for the smaller peak identified as CDCA (15). It had a molecular ion at 598 and a base peak at 369, which corresponded to M − (114 + 115). It also had major fragments at m/e 255 and at m/e 484. The fragment at m/e 255 corresponded to that anticipated for the steroid nucleus of a dihydroxy bile acid.

The GLC peak corresponding ca. to the trifluoroacetyl ester of methyl deoxycholate was identified as a degradation product of the trifluoroacetyl ester of methyl cholate (10). It had a molecular ion at 596, corresponding to the di-trifluoroacetate of the methyl ester of a monounsaturated dihydroxy bile acid. The base peak was at m/e 367, which corresponded to M − (114 + 115). Other major fragments were seen at m/e 482, which corresponded to M − 114, and at m/e 253, which was due to the steroid nucleus of a trihydroxy bile acid. Since the retention time of the peak was slightly lower than that of deoxycholic acid, it was concluded that the loss of the trifluoroacetyl ester group had occurred at carbon 7, resulting in the formation of a monounsaturated deoxycholic acid. The presumed origin of this bile acid was confirmed by GLC-MS analysis of the high temperature degradation products of the trifluoroacetate of pure methyl cholate. Likewise, two other minor GLC peaks eluted in the monohydroxy bile acid region were identified as the degradation products of the trifluoroacetate of methyl cholate. Their mass spectra corresponded to that of lithocholic acid, except for a discrepancy of 4 hydrogens in the fragment corresponding to the steroid nucleus. The identification of unsaturated bile acids as artifacts of GLC of trifluoroacetates has been described elsewhere (10). No evidence of the occurrence of unsaturated bile acids in the trout was obtained when the analyses were made with the methyl esters or the methyl ester trimethylsilyl ethers.

The identification of the bile acids was completed by a direct probe MS of the TLC fractions of the bile acid methyl esters. The most polar band corresponded to trihydroxy bile acids and gave a correct spectrum for methyl cholate (16) with a base peak at m/e 386 and a large fragment at m/e 253, which is characteristic of trihydroxy bile acid. The next most polar TLC band gave no recognizable spectra when examined as the methyl ester, but, after trimethylsilyaltion, it was possible to identify it as the 3α,12α-dihydroxy-7-keto-5β-cholanoate, which is a known compound with a published spectrum (15). This trimethylsilyl ether showed a molecular ion at m/e 564, and a base peak at m/e 341, corresponding to the loss of the side chain and one trimethylsilyl ether group and one molecule of water. Other major peaks were seen at m/e 251, corresponding to the steroid nucleus of a dihydroxy ketone; at m/e 269, which represents the steroid nucleus of a dihydroxy bile acid plus an oxygen; at m/e 366, corresponding to M − (2 x 90 + 18); at m/e 474, corresponding to M − 90; and at m/e 549, corresponding to M − 15, as well as other smaller fragments characteristic of the overall structure of this acid. The third TLC band corresponded to CDCA, and, on direct probe mass spectrometry, the appropriate spectrum was obtained (15). It had a molecular ion at m/e 406, a base peak at m/e 370, as well as a large fragment at m/e 255, corresponding to the steroid nucleus of a dihydroxy bile acid. The fourth TLC band corresponded to 3-keto,7α, 12α-dihydroxy bile acid, which could be identified without trimethylsilylation. It is a known bile acid with a published mass spectrum (14). This methyl ester had a molecular ion at m/e 420, a base peak at m/e 269, and a large fragment at m/e 251 corresponding to the steroid nucleus of a dihydroxy monoketo bile acid. The solvent front contained small amounts of a steroid material of low polarity which could not be immediately identified when analyzed as the methyl ester by direct probe.

Table II gives the mole percentages of the individiual bile acids in the total bile acid mixtures from the various groups of fish. Ca. 14% of the total bile acid in the 6 month old fish was CDCA and 80% CA. In 13 month old fish, the percentage of CDCA had decreased to ca. 6% and in starving 12 months old fish to ca. 1%, with corresponding increases in the proportion of CA. Table II also shows that the pool size of the bile acids was ca. 20 times as large in the 12 month old as in the 6 month old fed fish ($P>0.001$), and twice as large as in the starved 12 month old fish ($P>0.002$). The bile acid pool size measured by direct GLC and by combined TLC-GLC methods showed good agreement for all groups.

Metabolism of Bile Acids

Figure 2 shows the elution patterns of mass and radioactivity as obtained by radio-GLC of the trimethylsilylethers of the bile acid methyl esters recovered from the incubation of chenodeoxycholic-24-^{14}C with the liver homogenate of 12 month old fish. Ca. two-thirds of the added chenodeoxycholate has been converted into cholate. Table III gives the distribution of radioactivity among the bile acids of the trout liver following incubation of radioactive chenodeoxycholate with the various trout liver homogenates. It is seen that the livers of both 6 and 12 month old fish were capable of converting CDCA into CA, but that the older livers accomplished it ca. 3 times more rapidly. No conversion was observed when boiled homogenates were employed. This interconversion of the bile acids may explain the lower molar percentage of chenodeoxycholate in the older group of fish. There was no significant radioactivity found in any other bile acid band recovered by TLC of the bile acid methyl esters.

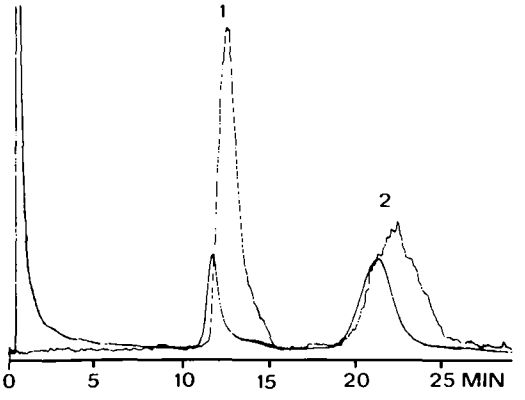

FIG. 2. Radio-gas liquid chromatography of bile acids of fish liver homogenates following incubation with chenodeoxycholic acid-24-^{14}C. Upper tracing, radioactivity; lower tracing, mass. Peaks 1 and 2 represent cholic and chenodeoxycholic acids, respectively. Instrument: Packard model 7401 gas chromatograph equipped with model 1894 proportional radioactivity monitor and a copper oxide furnace. Column: 120 cm x 2 mm inside diameter glass tube packed with 19:1 (w/w) mixture of 3% HI-EFF-8BP and 3% OV-210 on 100-120 mesh Gas Chrom Q, respectively. Carrier gas, argon, 55 ml/min. Temperatures: column, 225 C; injector, 225 C; detector, 240 C. Proportional counter conditions: range, 3000 cpm; time constant, 10 sec.; high voltage, 1650 v; quench gas:propane, 5 ml/min. Combustion furnace, 750 C. Flame ionization mass detector with 10:1 stream splitter. Sample: 2 μliter 1% solution of the bile acid methyl ester trimethylsilylethers in silylation mixture. Total radioactivity, 9000 dpm.

DISCUSSION

In general, our findings on the rainbow trout agree with the overall evolutionary pattern proposed by Haslewood (1). The bile salts of many fishes, including some bony fishes of fresh water contain chiefly either sulfated or taurine conjugated bile acids (17). Since the sulfates constitute only 5-10% of the total bile

TABLE II

Bile Acid Composition of Rainbow Trout[a]

Bile acids	6 Months old	13 Months old	Starved fish 12 months old
Total bile acid pool (μmoles)	0.63 ± 0.13	14.17 ± 0.46	7.91 ± 1.30
Total bile acid pool (μmoles)	0.60 ± 0.13	13.99 ± 0.57	7.11 ± 1.45
Cholic acid (mole % total)	85.35 ± 0.85	93.62 ± 0.31	98.58 ± 1.19
Chenodeoxycholic acid (mole % total)	14.67 ± 0.85	5.95 ± 0.29	1.40 ± 0.63
3α,12α-Dihydroxy-7-keto-cholanoic acid (mole % total)	Trace	Trace	Trace
7α,12α-Dihydroxy-7-keto-cholanoic acid (mole % total)	Trace	Trace	Trace

[a]Corrected gas liquid chromatographic-thin layer chromatographic analyses ± standard error (see text).

TABLE III

Metabolism of Labeled Chenodeoxycholic Acid in Liver Homogenate of Rainbow Trout[a]

cpm of:	6 Months old[b] cpm x 10^3	13 Months old[b] cpm x 10^3
cpm of chenodeoxycholic acid-24-^{14}C added	125	125
cpm of chenodeoxycholic acid-24-^{14}C after incubation	95 ± 4	31 ± 4
cpm of cholic acid-24-^{14}C after incubation	23 ± 3	87 ± 6
cpm of chenodeoxycholic acid-24-^{14}C after incubation with boiled homogenate	120 ± 5	118 ± 5
Total recovery of ^{14}C	118 ± 5	118 ± 7

[a]The substrate 100 nmole chenodeoxycholic acid-24-^{14}C (125 x 10^3 dm) was incubated with an amount of homogenate equivalent to 500 mg liver at 37 C in air for 1 hr in 5 ml 0.1 M phosphate buffer (pH 7.6) containing 5 mM $MgCl_2$, 1 mM nicotinamide, 0.5 mM glutathione, and NADPH generating system (1 μmole glucose-6 phosphate, 0.5 kornberg unit glucose-6 phosphate).

[b]Number of incubations were 6/group.

salt, the rainbow trout may be ranked in an advanced position among the *Teleostei*. Although sulfated bile acids are theoretically characteristic of primitive vertebrates (1), recent evidence indicates that higher vertebrates, i.e. man and laboratory rat, also can form sulfated bile acids under certain conditions (5,14,18). The chief bile acid in rainbow trout is CA, but CDCA is present in small amounts. Both of these acids are conjugated mainly with taurine.

Unlike that of the mammals (19), rainbow trout liver appears to convert efficiently CDCA into CA. A hydroxylation of CDCA to CA also is accomplished in other nonmammalian species, such as python (20), eel (21), and chicken (22). The higher ratio of CA to CDCA in the year old, as compared to the 6 month old fish may be related to hydroxylation of the CDCA to CA. The year old trout could be shown to affect this conversion at a considerably higher rate than the 6 month old trout.

Starvation of trout resulted in a reduction in the size of the bile acid pool and an increase in the ratio of CA to CDCA acid. It is difficult to explain the reduction in the pool size, since no information is available on the number of enterohepatic circulations of the bile acid pool of rainbow trout. However, in Rhesus monkey fasting is known to decrease the enterohepatic circulation and the volume of bile (23). The reduced secretion of the bile salts in starvation also would lead to decreased synthesis of bile acids as a result of a feed-back inhibition (24) which also could contribute to reduced bile acid pool.

ACKNOWLEDGMENTS

This work was supported by National Science Foundation Grant GB-35281 to Desert Research Institute, University of Nevada, and Grants MT-1046 and MA-4865 provided by the Medical Research Council of Canada.

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[Revised manuscript
received August 5, 1974]