JOURNAL OF THE AMERICAN GERIATRICS SOCIETY Vol. 18, No. 2
 Printed in U.S.A.

NITROGENOUS COMPOUNDS IMMOBILIZED IN AN AGED RAT

JOHAN BJORKSTEN, Ph.D.* AND STEPHEN ASHMAN (Technician)

Bjorksten Research Foundation, Madison, Wisconsin

ABSTRACT: **A pregnant rat received 8 mc of tritiated tyrosine at the time of giving birth (from seven days before, to six days after). No radioisotopes were ever given directly to the litter born. A male from this litter died from pneumonia at age 809 days. After removal of water and acetone solubles and of phospholipids, hydrolysis of the residue released the following radioactive amino acids, parts of molecules fixed until death and containing tritium present at birth: lysine, arginine, aspartic acid, glutamic acid, serine, listed in order of decreasing radioactivity, with lysine carrying 29 per cent of the total tritium present.**

Still (1) has reported that radioisotopes administered to mice at birth remain in their brain, heart and skeletal muscles to a very measurable degree after 164 days, but that kidney and liver within this time are free from radioisotopes.

The work by Thompson and Ballou (2) with Sprague-Dawley rats given tritium for six months (dosage, 5 microcuries per milliliter of their drinking water supply) and analyzed after nine months, is not directly comparable with ours because of the long feeding period.

Still's report that the liver was cleared of isotopes in 164 days appears irreconcilable with the finding of Bjorksten, Andrews, Bailey and Trenk (3) who reported that an enzymatically nonhydrolyzable nitrogenous substance accumulated in the liver as a result of exposure of the animal to ionizing radiation; and the further finding (4) that a comparable quantity of similar material accumulated in the control animals from this experiment, when they reached senility about three years later.

It seemed important to clarify these observations, because the turnover in the liver is so much more rapid than in any other major organ that the liver presents a highly accelerated condition compared with the organism as a whole.

Therefore we repeated Still's experiment using a higher dosage of radioactivity and instrumentation not available in 1957.

While we essentially confirmed Still's important findings regarding retention of radioactivity in brain, heart and muscle, we also found appreciable quantities of radioisotope retained in the liver. These were further resolved.

* *Address:* Bjorksten Research Foundation, P. O. Box 775, Madison, Wisconsin 53701.

EXPERIMENTAL DETAILS AND RESULTS

A pregnant Sprague-Dawley female rat received 2 mc of tyrosine-H^3 orally seven days before giving birth to a litter of six. The first and second days after giving birth she received 2 mc of tyrosine-H^3 daily in her drinking water, and the fifth and sixth days she received 1 mc daily. Thus the total dose (before and after the birth) was 8 mc.

The animals lived a normal laboratory life, being fed with Purina Lab. Chow and tap water ad lib. Eight hundred and nine days after birth one of the male offspring died of pneumonia. Its excised liver weighed 14.56 gm. This liver was homogenized for twenty-five minutes in a Virtis homogenizer, at half speed, to facilitate temperature control. A few large pieces had been wrapped around a blade. They were detached and chopped with a scalpel; the homogenization was resumed for an additional twenty-five minutes. A few larger particles still required manual chopping, whereupon the material was again blended in the Virtis homogenizer for thirty minutes. Then 1-ml samples were taken out, dried in air in dialyzing bags, and combusted in oxygen to water, according to the Thomas-Ogg procedure (5). The resultant water which contained all the tritium of the sample free from all organic substance was mixed with 5 ml of scintillation fluid and counted (0.5 ml of sample to 15 ml of scintillator fluid).

The remaining homogenate was washed ten times with water and centrifuged at 17,000 rpm between washings. Acetone (40 ml) was added to the residue and the mixture was blended fifteen minutes with the Virtis homogenizer. The slurry was centrifuged, washed with additional acetone, and vacuum dried; it yielded a residue that weighed 691 mg. This material was suspended in 40 ml of 2:1 chloroform:methanol. The mixture was blended for fifteen minutes at high speed in the Virtis homogenizer, and centrifuged at 17,000 rpm. This procedure was repeated three times, after which no radioactivity was apparent in the wash liquid.

The residual solid was vacuum dried to yield a residue which weighed 675 mg.

This material was hydrolyzed at 37°C and pH 9.3, in 25 ml of phosphate buffer. Two 0.5-ml aliquots were added to the scintillation liquid and counted:

Sample 1	86 CPM (above background)	101 DPM
Sample 2	87 CPM (above background)	102 DPM

Taking the average of these samples, the total radioactivity of all the material processed was 4,975 disintegrations per minute (DPM). An insoluble residue weighing 84 mg remained; this contained a total of 360 DPM.

The liquid sample was dialyzed for removal of buffer, and was subjected to further study as follows:

A part of the original hydrolysate was used for probing, before the detailed procedure was evolved. This investigation is confined to the nitro-

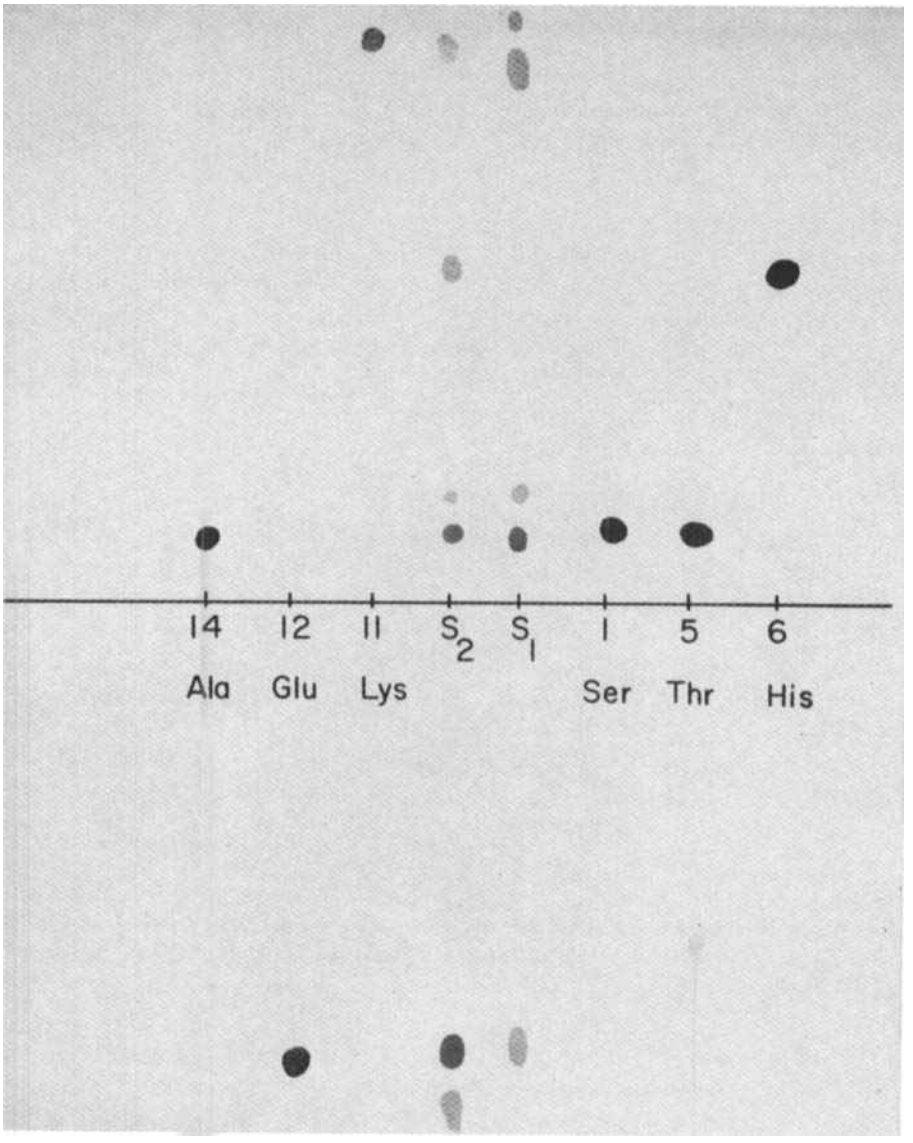

FIG. 1. Electrophoresis of peptides.

S_1 = primary hydrolysate fractions separated into 5 parts not corresponding to any amino acids, at pH 3.7.

S_2 = amino acids obtained after further hydrolysis of the primary hydrolysate.

The numbers represent amino-acid standards.

genous compounds. A smaller radioactive part, tentatively considered phospholipids because of solubility in chloroform:methanol, may become the subject of a separate study.

The material remaining after the foregoing dialysis was lyophilized and subjected to electrophoresis on 3-mm Whatman paper at 2,500 v and 150 amp. for forty-five minutes at pH 6.5 (Fig. 1) and at pH 3.5 (Fig. 2). At the higher pH we obtained 4 components; at the lower pH, 5 compo-

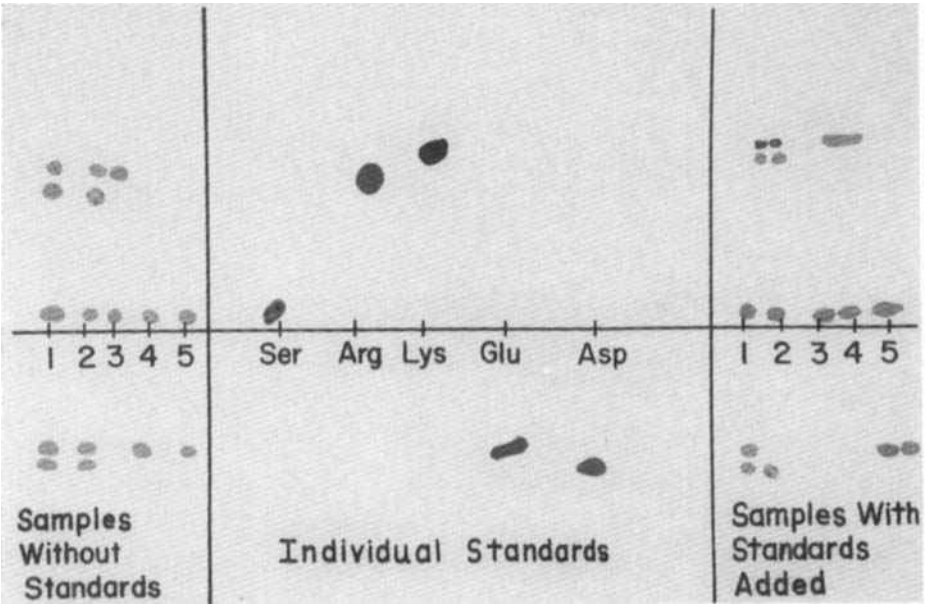

FIG. 2. Electrophoresis of amino acids of the five fractions of Sample S. On the left, amino acids in the hydrolysate; on the right, amino acids in the hydrolysates plus standard amino acids.

The following standard amino acids were added to the fractions on the right of the electropherogram: Fractions 1 and 2, ser, lys, arg, glu and asp; Fraction 3, ser and lys; Fraction 4, ser and glu; Fraction 5, arg and glu.

nents. These showed the smear characteristic of peptides. The buffers employed were made up in the following proportions by weight: 1) pyridine 2.4; acetic acid 60; water 540; and 2) pyridine 1; acetic acid 10; water 287.

Preparative electrophoresis was applied, followed by acid hydrolysis of the peptides at 110°C for twenty-four hours with 6 M HCl, the amount of acid being 0.1 ml per mg. Chromatography of the hydrolysates was then carried out on Whatman grade 1 paper previously washed with isopropanol and water (2:1) made slightly alkaline with ammonia. Two different solvent systems were used: 1) butanol:formic acid:water—225:45:30, and 2) butanol, pyridine, water—1:1:1; each system for eighteen hours.

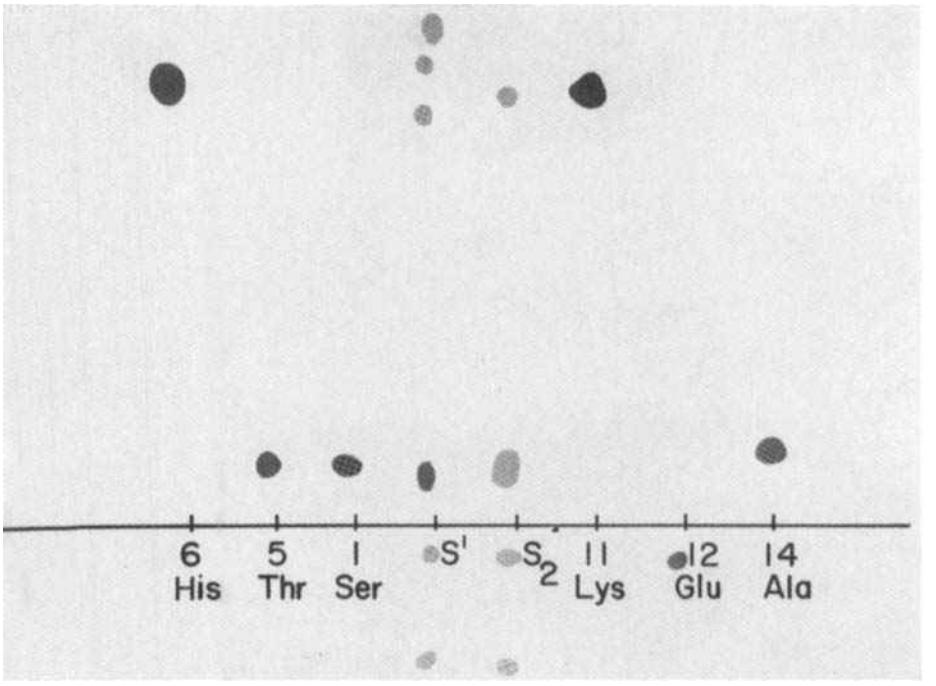

FIG. 3. Electrophoresis.
S_1 = the same as for Figure 1, at pH 6.7.

The peptides were separated and resolved as follows:

Peptide No.	Weight Separated (mg)	Amino Acids
1	1.3	Ser, Glu, Asp, Lys, Arg
2	1.2	Ser, Glu, Asp, Lys, Arg
3	13.0	Ser, Lys
4	12.0	Ser, Glu
5	0.6 (some lost, quantity estimated)	Ser, Glu

To quantitate the amino acids, we combined the hydrolysates of the peptides, because the quantities at hand seemed too small for individual resolution.

	CPM above Background	% of Total Radioactivity
Lysine	425	29.2
Arginine	293	20.1
Aspartic acid	291	20.1
Serine	141	9.7
Glutamic acid	258	17.7
Ether-soluble substance not observed before strong acid hydrolysis	46	3.2
	1454	100.0

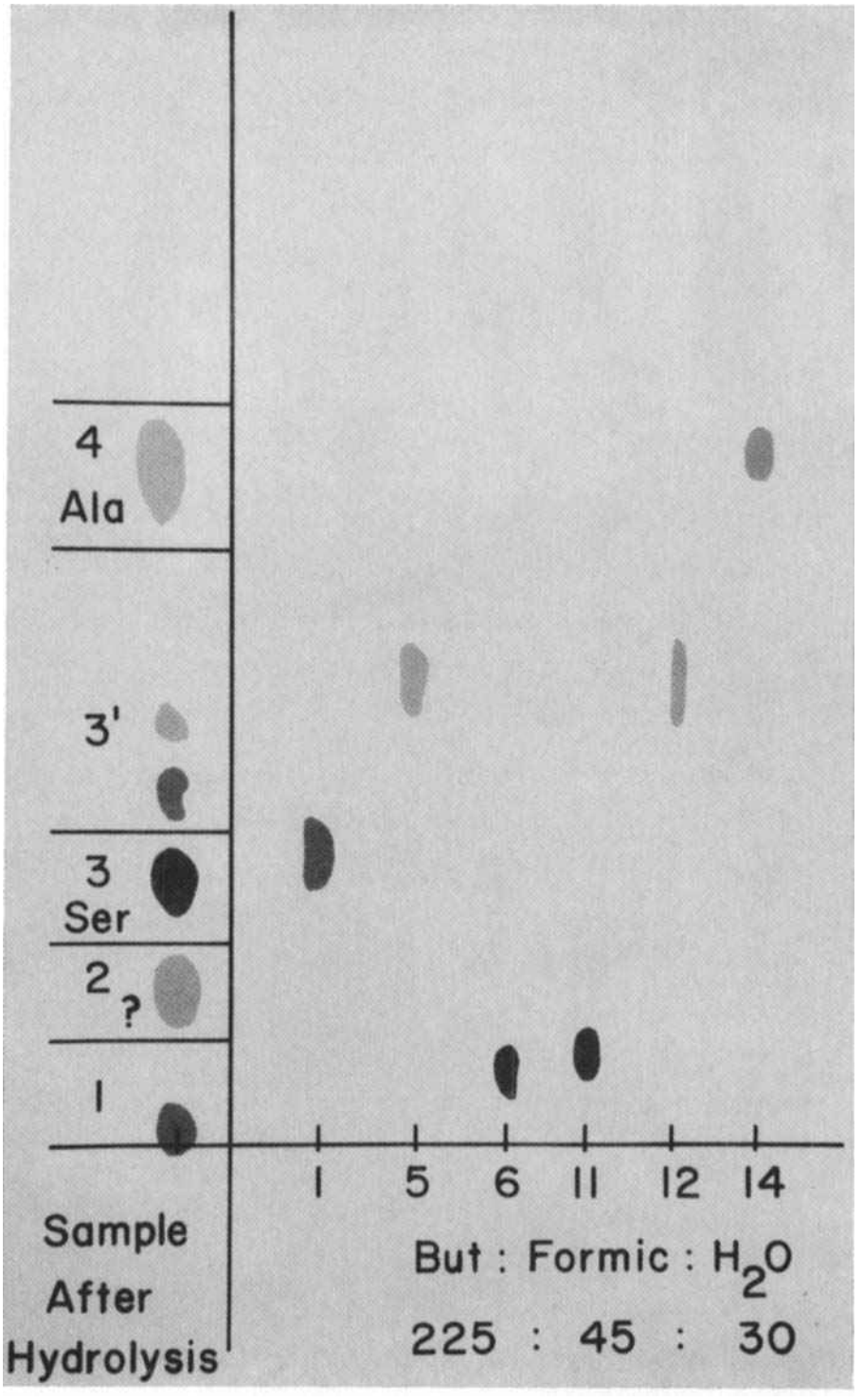

FIG. 4. Paper chromatography of amino acids of mixture S, with butanol:formic acid:water (225:45:30).

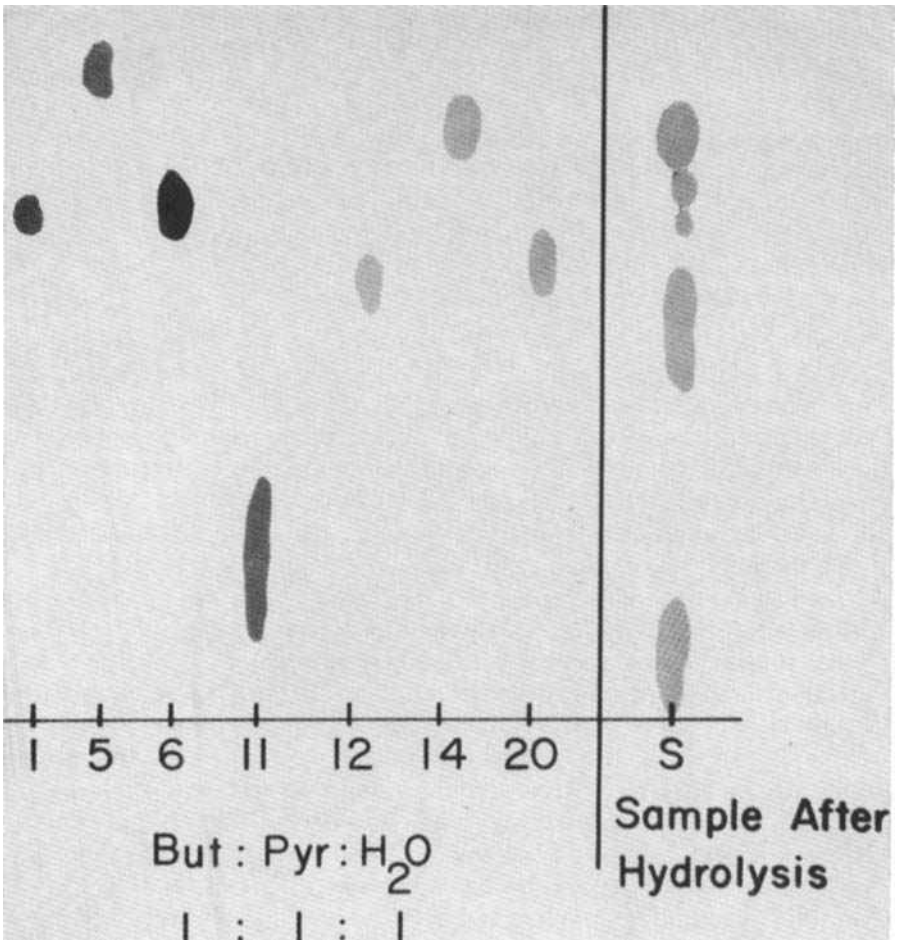

FIG. 5. Paper chromatography of amino acids of mixture S, with butanol:pyridine: water (1:1:1).

The identity of the amino acids was confirmed by co-electrophoresis with the known amino acids at 1,000 v and 150 amp. for two hours.

An attempt was made to identify the ether-soluble fraction, but the quantity at hand (less than 1 mg) proved insufficient. Gas-liquid chromatography was performed on a diethyleneglycol isophosphate column after clarification of lipids with CH_2N_2 and temperature programming from 110° to 270°C. There was no known fatty acid in the range C_{10}–C_{26}.

The infra-red spectrum showed a strong band at 1,725 cm^{-1}, suggesting the presence of carbonyl groups. A band between 1,430 and 1,500 cm^{-1} indicated aromatic structures. A strong band between 750 and 800 cm^{-1} indicated steroids. The CH_2 stretching band at 3,000 cm^{-1} was slight.

Nuclear magnetic resonance (NMR) (on a 60 MHz spectrometer) gives only rough approximations when application to sub-milligram quantities is attempted. Peaks at 48, 72, 84 and 108 cps could be attributed to steroid structures.

From these data it is apparent that the radioactive ether-soluble material was not a single simple fatty acid, but multiple, or complex, or both. Larger quantities and a substantial effort will be necessary for positive identification. Although the extraction and washing procedures used were thorough, it is not entirely certain whether the radioactive ether-soluble substance was chemically bound prior to hydrolysis, or only strongly adsorbed.

DISCUSSION

The radioactive substances present in the liver of a rat 809 days after the administration of tritium to its mother can be regarded as stabilized in the organism in a manner not susceptible to normal in vivo removal. They are representative of the substances which have accumulated irreversibly in the liver, on aging of this organ.

From the analysis of these substances, the following negative and positive conclusions can be drawn:

The amino acids whose R groups could participate in hydrophobic bonding of a protein are alanine, valine, leucine, isoleucine, phenyl alanine, tryptophan, methionine, tyrosine and proline. None of these was present. Therefore hydrophobic bonding was not involved.

Of the 20 amino acids present in collagen, 15 (including oxyproline) were missing in the radioactive peptides obtained, and those present were in proportions different from their proportions in collagen. Thus there was no indication that collagen or a collagen derivative might be among the irreversibly accumulating substances in the liver of the rat.

The sulfur-containing amino acids, cysteine and methionine, were absent. It is inferred that in the case of the rat liver, the enzymes present are capable of resolving these amino acids, including disulfide cross-linkages.

The following 4 amino acids are regarded as playing the most significant role in the hydrogen bonding of tertiary or quaternary structures: lysine, histidine, aspartic acid, and serine (6). Three of these were among the 5 amino acids found in our peptides. Therefore, hydrogen bonding could be significantly involved.

The following 5 amino acids can bear a charge at physiological pH: lysine, arginine, aspartic acid, glutamic acid, histidine (6). Four of these amino acids were among the 5 found. Accordingly, it is indicated that cross-linkages based on co-valent bond reactions of the charge-carrying side groups of these amino acids are involved in the lifetime immobilization of radioactivity administered at birth.

The 5 amino acids found are those which have a reactive group positioned at the end of an extended side chain. The most abundantly present,

lysine, is the one which has the most reactive group on the most extended side chain.

These findings are consistent with the cross-linkage theory of aging (7) and have no apparent relation to any of the other theories advanced (8).

Acknowledgments

We are indebted to The Upjohn Company for the donation of a liquid hydrolysate of a radioactive liver of known history, a by-product of Upjohn's project with Bjorksten Research Laboratories, Inc.; and to the Marcus and Bertha Coler Foundation for a donation to defray part of the expenses of this work. We acknowledge with thanks the valuable help of Dr. Roswell Boutwell in preparing animals, of Dr. P. V. N. Acharya in analysis, and of Mr. Raymond Kandler in preparing some of the samples.

REFERENCES

1. STILL, J. W.: Amino acid turnover in brain compared with turnover in other tissues, *J. Heredity 48:* 204 (Sept.–Oct.) 1957.
2. THOMPSON, R. C., AND BALLOU, J. E.: Studies of metabolic turnover with tritium as a tracer. V. The predominantly non-dynamic state of body constituents in the rat, *J. Biol. Chem. 223* (2): 795 (Dec.) 1956.
3. BJORKSTEN, J.; ANDREWS, F.; BAILEY, J., AND TRENK, B.: Fundamentals of aging: immobilization of proteins in whole-body irradiated white rats, *J. Am. Geriatrics Soc. 8:* 37 (Jan.) 1960.
4. BJORKSTEN, J., AND ANDREWS, F.: Chemical mechanisms underlying the biological mechanisms of the aging process, *J. Am. Geriatrics Soc. 12:* 628 (July) 1964.
5. OGG, C. L.: Proc. International Symposium on Microchemical Techniques. Microchem. J. Symposium Series, Vol. II. New York, Interscience, 1961.
6. STEPHENSON, W. K.: Concepts in Biochemistry. Great Lakes Colleges Association. New York, John Wiley & Sons, 1967, pp. 124, 125.
7. BJORKSTEN, J.: The crosslinkage theory of aging, *J. Am. Geriatrics Soc. 16:* 408 (Apr.) 1968.
8. BJORKSTEN, J.: *In* Bakerman, S.: Aging Life Processes. Springfield, Ill., Charles C Thomas, Publisher, 1969, pp. 147–179.