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GEROGENIC FRACTIONS IN THE TRITIATED RAT

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ABSTRACT: **A rat that had received tritiated acetate perinatally was killed at the age of 609 days, and was found to have retained substantial quantities of tritium in all organs examined. This study was focussed on the liver, which—after a succession of extractions with a series of various solvents followed by catalytic hydrolysis at body temperature—yielded a residue that was insoluble in a wide range of common solubilizing media. Treatment with hot mineral acid partially dissolved this residue and electrophoretic fractionation further led to 4 fractions of which a single fraction contained most of the tritium in the insoluble residue.**

Our analyses showed that the insoluble residue contained a variety of common amino acids and a considerable amount of phosphorus. The solubilized fractions derived from the insoluble residue all contained substantial concentrations of pentose, deoxypentose, and phosphorus. They showed ultraviolet absorption spectra qualitatively similar to those of nucleic acids. From their chromatographic behavior on crosslinked dextran columns, all 4 solubilized fractions showed molecular weights greater than 5000. In addition, these fractions showed substantially greater resistance to hydrolytic degradation than do authentic RNA and DNA. Taken together, this is interpreted as evidence that the gerogenic insoluble residue is composed of a highly crosslinked network of at least RNA, DNA and protein, which is stabilized by covalent crosslinkages of unusual stability. Formation of these crosslinked structures could easily interfere with the function of certain critical molecules of RNA, DNA or other polymers, leading to impaired cell function and death.

In a preceding paper (1) from this laboratory, it was reported that

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nitrogenous molecules were found in the liver of a rat whose mother received tritium-labeled tyrosine at the time of giving birth.

In the present study a pregnant Sprague-Dawley rat received 40 mc of 3H-acetate, administered orally with drinking water as follows: 10 mc seven days prepartum, 10 mc postpartum, 10 mc two days postpartum, 5 mc five days postpartum, and 5 mc six days postpartum.

The litter consisted of 8 rats, all apparently healthy. A male of this litter was sacrificed 609 days after birth. The only radioactivity this animal received was that from its mother. Its liver was examined first because this is a large organ with an active metabolism.

MATERIALS AND METHODS

1. Substances that could be extracted from the wet liver with water, acetone, chloroform:methanol, and which could be catalytically hydrolyzed (about body temperature), were further resolved into various subfractions by preparative electrophoresis on Whatman 3 MM paper at 2500 V, 150 m A, for forty-five minutes in a buffer system of pH 6.5 containing pyridine, acetic acid, and water in the proportions (v/v/v) 300:12:2700 [Michl (2)].

2. Individual amino acids from the foregoing substances were obtained by 6 *M* HCl hydrolysis at 110°C for twenty-four hours, and were detected and isolated both by preparative paper electrophoresis under the above conditions and by preparative paper chromatography with use of a solvent system n-butanol:formic acid:H_2O; 245:45:30.

For measuring the color that each of these separated amino acids formed with ninhydrin, a corresponding authentic amino acid was taken as standard. The quantitative ninhydrin reaction (3) was determined on 10λ of the 1-ml solution of the sample.

3. Tritium activity of each amino acid was determined in a Packard Tricarb (Model 3365) scintillation counter on the 0.99-ml of the sample remaining from operation 2, after mixing with 15 ml of BBOT-dioxane.

Radioactivity in various extractable and catalytically hydrolyzable fractions of the liver was, however, determined from aliquots of these fractions in scintillation medium "Diotol" (Berdick & Jackman Laboratories)—except the insoluble fraction, which was combusted in oxygen to water which was then counted in the same manner (4).

4. Ribose was determined according to the method of Dische (5) after a mild hydrolysis of the samples with 0.1 *N* HCl in the amount of 0.1 ml/1 mg at 110°C for one hour and passing over a Dowex-50 column.

5. *Isolation of soluble fractions from the insoluble residue of liver* was carried out as follows: 50 mg of the insoluble residue was hydrolyzed with 10 ml of 1 *M* H_2SO_4 at 100°C for one hour. Insoluble material was removed by filtration. The filtrate and several washings were made up to 17 ml. The pH of the solution was brought to 1.0 by addition of 11 *N* KOH. Then 3-ml aliquots were heated to 90°C, and 0.3 ml of 20% $AgNO_3$ was added to each. Silver salts of the bases were precipitated. After standing overnight, the samples were centrifuged, and the precipitate washed with 1-ml portions of 0.1 *N* H_2SO_4; the original supernatants and the washings were filtered and combined. These solutions and the precipitates were

separately subjected to HCl treatment to free the bases. The insoluble silver salts of the bases were heated four times with 3 ml of 0.1 *N* HCl in boiling water each time for five minutes; the solids sedimented by centrifugation and the supernatants were combined. The combined supernatants after filtration were made up to 17 ml each. After suitable dilution, their ultraviolet absorption spectra were measured with a Gilford spectrophotometer (6).

The soluble silver salts of the bases were warmed to 70°C, 1 ml of 1 *N* HCl added in each, and the precipitate washed three times by suspension with 1-ml portions of 0.1 *N* HCl. The combined filtrates were diluted to 15 ml. The pH of these solutions was brought to 4.7 with NaOH and the volumes brought to 17 ml each. They were then incubated with 3 ml of acid phosphatase (wheat germ, Sigma) solution (containing 1 mg/1 ml) at 38°C for three hours to bring about dephosphorylation.

The solutions from the three assay samples were mixed, the pH brought to 8.3 with NaOH, and the solution filtered through Dowex-1 (bicarbonate) column 1 cm × 40 cm. The column was finally washed with 2% $NaHCO_3$.

The effluent was suitably concentrated, brought to pH 1 and put on Whatman 3 MM paper for electrophoresis in a pyridine acetate buffer of 3.5 pH (H_2O:pyr:HOAC; 287:1:10) [Michl (2)] at 2500 V, 80 m.a., for forty-five minutes. Zones were detected under ultraviolet radiation in the preparative procedure. They were eluted with 0.1 *N* HCl and each fraction so eluted was subjected to a second dephosphorylation treatment and passage over Dowex-1 (bicarbonate) columns as described above.

Throughout the procedure a control was run with authentic RNA (core-pancreatic, Sigma) (7).

6. *Characterization of the soluble fractions* was performed in three stages: (a) Analysis and determination of pentose, deoxypentose, and phosphorus. Pentose was determined according to Dische (5) technique, deoxypentose according to the method of Burton (8), and phosphorus according to the method of Bartlett (9).

(b) Estimation of the molecular size of these complexes and their sub-units obtained after perchloric-acid treatment in relation to those of authentic RNA-core and DNA (Calbiochem) respectively which have undergone similar experimental procedures. The perchloric-acid digestion was performed according to the method of Wyatt (10). The size estimation was made by elution with water. The Sephadex we used has an exclusion limit of 5000 m.w. The bed volumes of columns ranged from 30 ml to 42 ml and their void volumes from 13 to 18 ml.

Molar quantities of ultraviolet-absorbing bases in these substances were estimated by dividing their absorbancies at 260 nm by a mean molar extinction coefficient of 10,000.

(c) For purposes of comparison of these 4 nucleic acid complexes with RNA (core), DNA, and a physical mixture of RNA and DNA, ultraviolet spectra of these substances were also obtained at pH 1 and pH 11, on Beckman scanning spectrophotometer Model 10 within the region 320 nm to 210 nm.

THE INSOLUBLE RESIDUE

The insoluble residue, which weighed 195 mg, proved to be insoluble in all solvents that are usually employed to dissolve proteins, e.g., dilute acids and alkalies, methyl formamide, acetamide, peracetic acid, dimethyl sulfox-

ide, 8 *N* urea, 6 *N* guanidine with and without the addition of detergents, anhydrous hydrofluoric acid, and the like.

Preliminary fractionation

The liver weighed 21.75 grams wet. It was comminuted with 135 ml of water in a Virtis disintegrator and exhaustively extracted with a series of solvent systems. The results are shown in Table 1.

From the hydrolysate (6 *N* HCl, 110°C, twenty-four hours) of a portion of the insoluble residue, we identified and estimated a number of amino acids. The results are shown in Table 2.

Amino Acid analyses of hydrolysate of 1.3 mg of insoluble residue from liver

The neutral amino acids were calculated on the basis of the equivalent weight and color yield of isoleucine. Separations were made by paper electrophoresis; quantitative estimation was performed photometrically on the color produced with ninhydrin. Standard solutions of the same amino

TABLE 1

	Dry Weight (mg.)	Total Radioactivity in Fraction (DPM)
1. Water extract	1590	3120
2. Acetone	200	3060
3. $CHCl_3$:MeOH (2:1) Soxhlet extraction, 64 hrs.	131	4280
4. Hydrolysis, room temp., analogous to Andrews et al. (11)	250+	2670
5. Insoluble residue	195	4350

TABLE 2
Amino Acid Analyses of Liver Insoluble Residue

Amino Acid	O.D. at 570 mμ	μg	mμM	Molar Ratio
Standards				
Ile	0.142	4.9		
His	0.138	3.2		
Arg	0.290	10.0		
Lys	0.220	2.5		
Glu	0.098	3.7		
Asp	0.098	3.2		
Samples				
Neutral a.a's	1.354	5.2	370	2
His	0.150	3.5	23	0.12
Arg	0.420	14.0	80	0.42
Lys	0.238	2.7	185	1
Glu	0.458	1.7	115	0.64
Asp	0.186	6	46	0.24
Total		33		

acids were carried through the whole separation and estimation procedure as controls.

In a separate experiment the neutral amino acids were resolved chromatographically, showing predominantly phenyl alanine, isoleucine and valine. Quantitation of these was not attempted. We found no evidence of either proline or oxyproline in the neutral amino acid fraction. The total of the amino acids measured (Table 2) was about 2 per cent of the total weight of the insoluble residue. Material available was insufficient to make adequate tests of the specific activity of the amino acids.

SEPARATION AND CHARACTERIZATION OF SOLUBLE FRACTIONS FROM THE INSOLUBLE RESIDUE

A 50-mg aliquot of the insoluble residue of the liver was subjected to hydrolysis with 10 ml of 1 *N* H_2SO_4 at 100°C for one hour. A large portion of (presumably) the peptides remained insoluble and was filtered off. Subsequent steps such as reaction with silver nitrate, dephosphorylation by acid phosphatase and chromatography on Dowex-1 (bicarbonate) column were carried out as described in the Methods section. The effluent of the Dowex-1 (bicarbonate) column was concentrated and the pH adjusted to 1.0 with H_2SO_4 and put on Whatman 3 MM paper for electrophoresis. Four dark zones were detected under ultraviolet radiation, three moving to cathode and one to anode (Fig. 1).

After paper electrophoresis in a preparative system, the separated zones were eluted with 0.1 *N* HCl and lyophilized. Each fraction was subjected to a second dephosphorylation treatment and again passed over Dowex-1 (bicarbonate) columns (1 cm × 40 cm) and precipitated with acetone. Aliquots of each fraction were taken for the determination of pentose, deoxypentose, and phosphorus and for the measurements of their absorption spectra.

Table 3 gives the results of these analyses. As may be seen, the molar quantities of pentose and deoxypentose together exceed the molar quantities

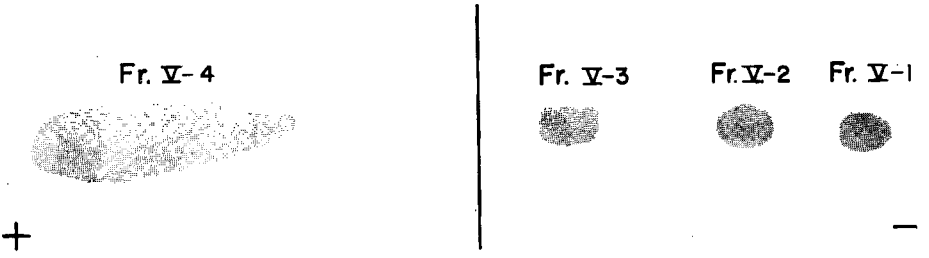


FIG. 1. Electrophoretic separation of soluble fractions of the insoluble residue of liver obtained by hydrolysis with 1 *N* H_2SO_4 at 100°C for one hour, on Whatman No. 3 paper. The electrophoresis was carried out for 45 minutes at 2500 V, 80 m.a. in a buffer containing H_2O:pyr:HoAC:: 287:1:10, and of pH 3.5. Fr.V-4 moved to the anode. Dark zones were detected under ultraviolet light.

TABLE 3
Composition of Electrophoretically Separated Fractions Isolated from 50 mg of Insoluble Residue of Liver (mμ moles)

Sample	Pentose	Deoxypentose	Phosphorus	Bases
Fr. V-1	61	55	108	74
Fr. V-2	109	126	142	41
Fr. V-3	320	133	440	198
Fr. V-4	1100	46	315	146

of bases in all the fractions. The values of phosphorus are also greater than those of the bases.

Figures 2 and 3 show the ultraviolet absorption spectra obtained for these 4 fractions. The strong similarity between these spectra and the spectra of authentic nucleic acids (12) is apparent. However, since these electrophoretic fractions had already been subjected to several steps (beginning with 1 *N* H_2SO_4 hydrolysis, 100°C for one hour) in which fractionation in all likelihood occurs, no particular significance can be attached to the stoichiometry. What is surprising, however, is the finding of nucleic-acid components in these materials after the initial strong hydrolysis, in spite of the known acid lability of deoxyribose and (to a lesser degree) its phosphate ester linkages. Indeed, both RNA and DNA are hydrolyzed to the level of monomers by 1.5 *N* HCl at 100°C for one hour (13). Table 3 also shows that substantial amounts of phosphorus remained after acid hydrolysis and two cycles of acid-phosphatase treatment.

GEL FILTRATION CHROMATOGRAPHY OF THE 4 ELECTROPHORETIC COMPONENTS

Figures 4, 5 and 6 Show the elution patterns of these 4 components on Sephadex G-25 columns. In these figures we see that whereas the uridine and cytidine bases obtained as soluble silver salts from standard RNA (core) are eluted well beyond the void volumes of their respective columns, our samples (even after 1 *N* H_2SO_4 treatment at 100°C for one hour) invariably come within the void volumes. Since the exclusion limit of the Sephadex G-25 is generally accepted to be m.w. = 5000, these results show that the nucleic-acid complexes we have in hand are macromolecules having molecular weights greater than 5000. When samples of these 3 electrophoretic components (the fourth was not available in sufficient quantity) were hydrolyzed with 60% perchloric acid at 100°C for one and a half hours, gel filtration chromatography showed *only partial* degradation to units of lower molecular weight.

The results of these experiments are shown in Figures 4, 5 and 6. It is seen that this very strong acid hydrolysis results in breakdown to the point where the ultraviolet-absorbing products are retarded beyond the void volume, but not to the extent shown by the products from the control in which DNA was treated in an identical manner.

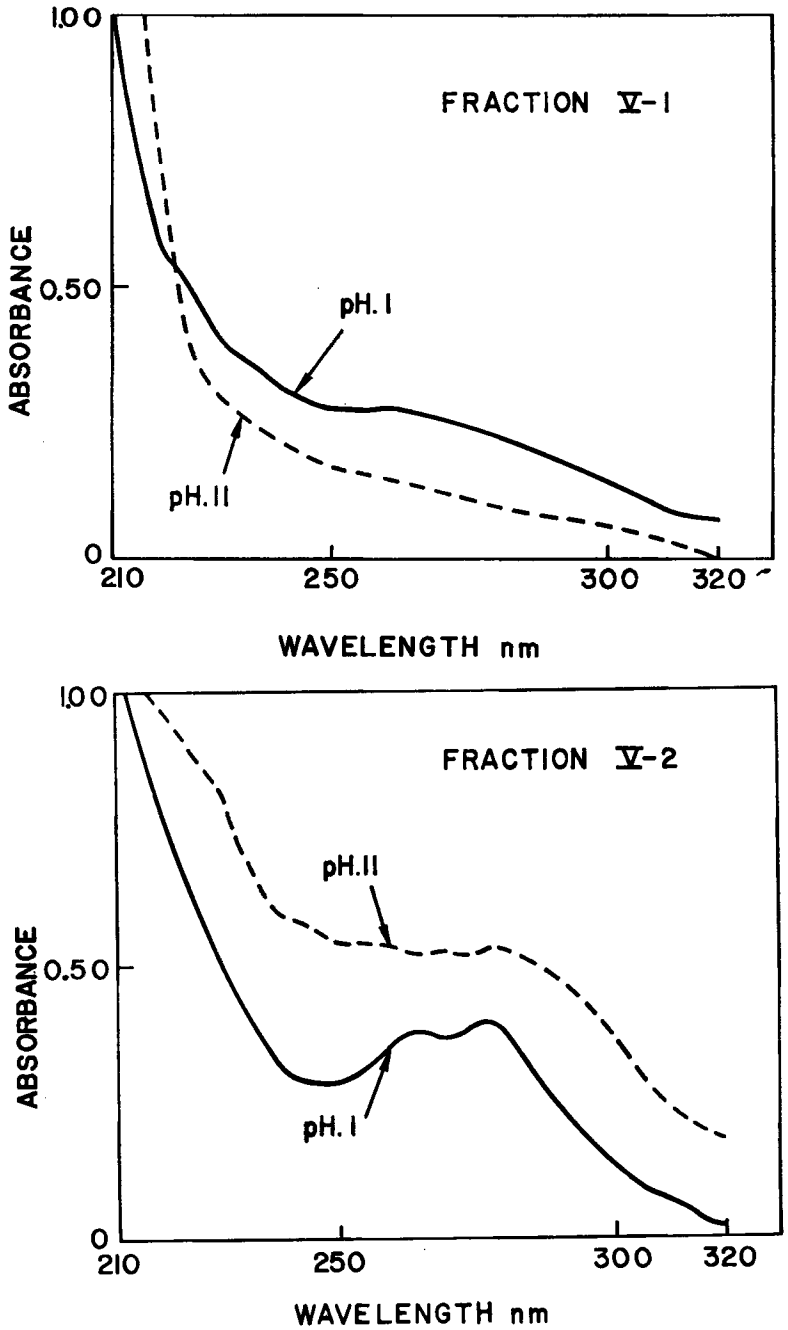
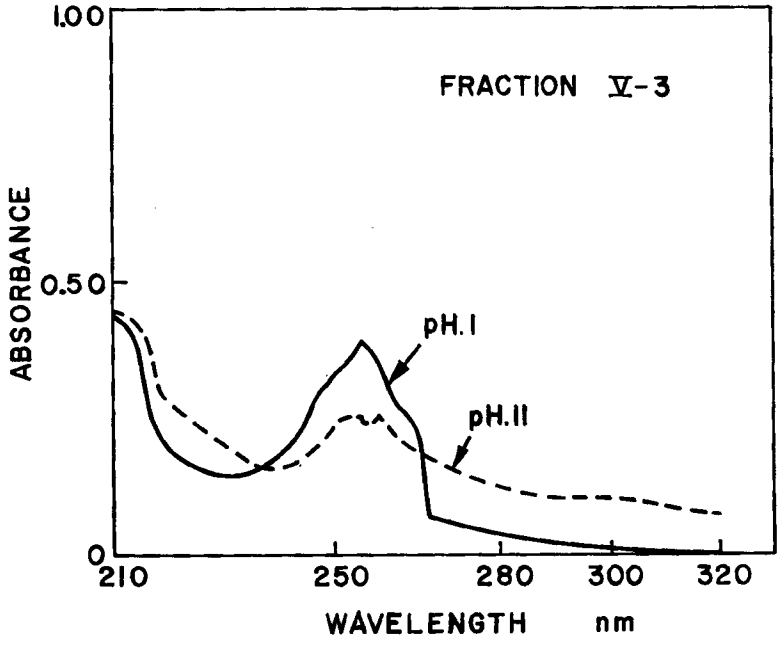


FIG. 2. Ultraviolet absorption spectrum of electrophoretically separated fractions of insoluble residue of rat liver, after hydrolysis with 1 *N* H_2SO_4, 100°C, one hour. Above, Fr.V-1; below, Fr.V-2. The spectra were taken on Beckman Model 10. The solid line shows the spectrum at pH 1, and the broken line shows the spectrum at pH 11. 0-1A is the measure of absorbance selected.



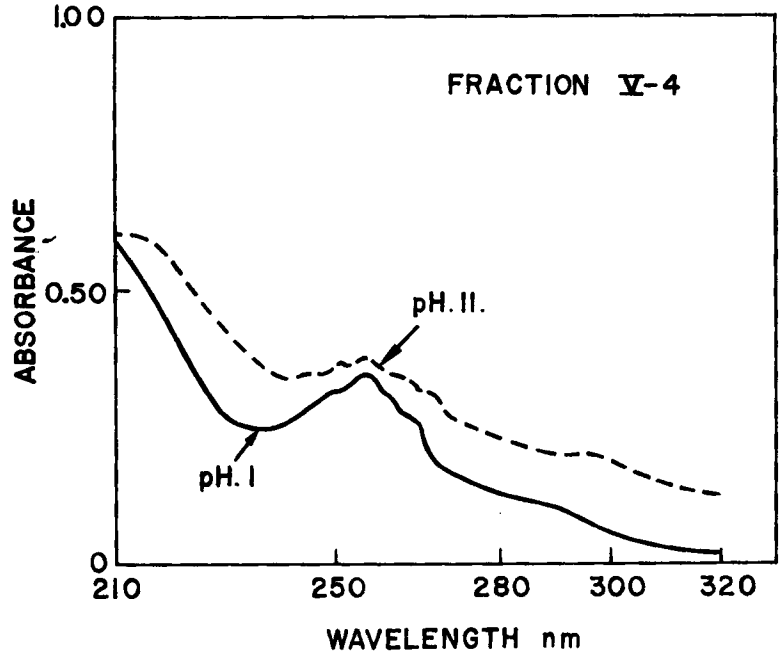


FIG. 3. Ultraviolet absorption spectrum of electrophoretically separated fractions of insoluble residue of rat liver after hydrolysis with 1 *N* H_2SO_4, 100°C, one hour. Above, Fr.V-3; and below, FR.V-4. The spectra were taken on Beckman Model 10. The solid line shows the spectrum at pH 1 and the broken line shows the spectrum at pH 11. 0-3A is the measure of absorbance selected. This means with 0-3A, three anodes of the ultraviolet-absorbing material give as much absorption as 1 mole of the same material with the selector at 0-1A.

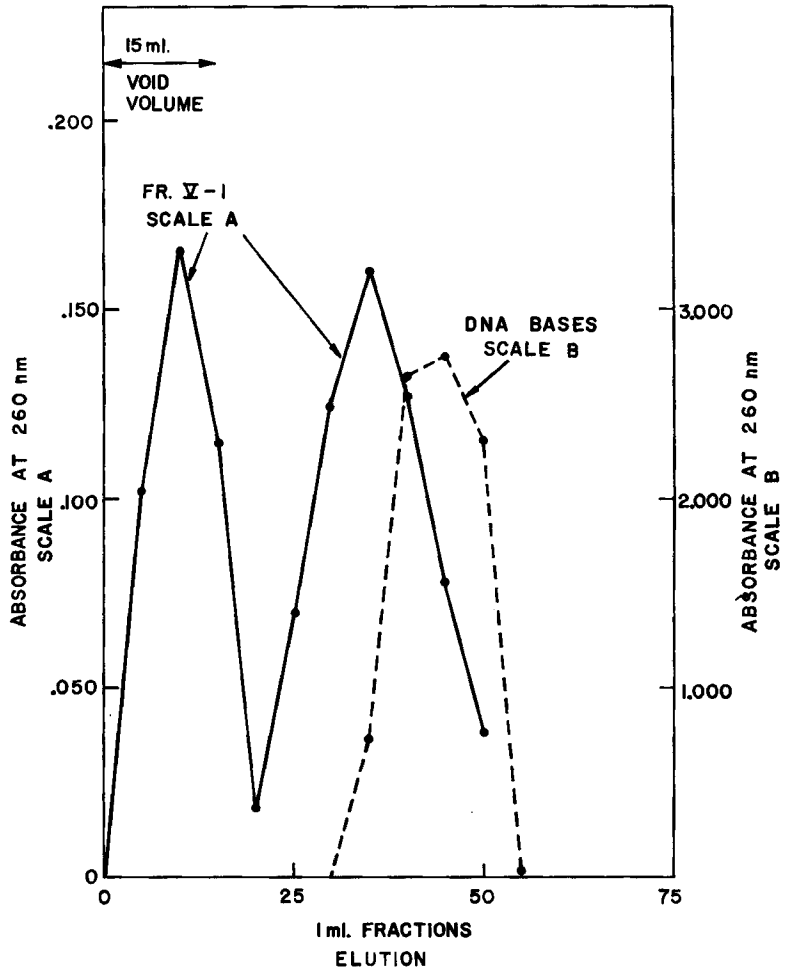


FIG. 4. Elution chromatogram of Fraction V-1 treated with 60% $HClO_4$, 100°C, ninety minutes, on a Sephadex G-25 column, along with that of DNA bases liberated by treatment of DNA with perchloric acid under similar conditions. Fraction V-1 gives two peaks, one substantially within the void volume, and the other outside the void volume but substantially before that of the DNA bases.

TRITIUM ACTIVITY IN THE ELECTROPHORETICALLY SEPARATED FRACTIONS

Tritium activity was examined only in the 4 soluble electrophoretic complexes, the silver salts of which were soluble in acid (0.1 *N* HCl) medium, and which were eluted within the void volumes of their respective Sephadex G-25 columns. Their composition and ultraviolet spectra have already been discussed.

Table 4 gives the tritium activity of the 0.1-ml portions of 1-ml solutions of the 4 substances. The counting was done for ten minutes. DPM was calculated by taking the efficiency of the Packard scintillation counter at 60

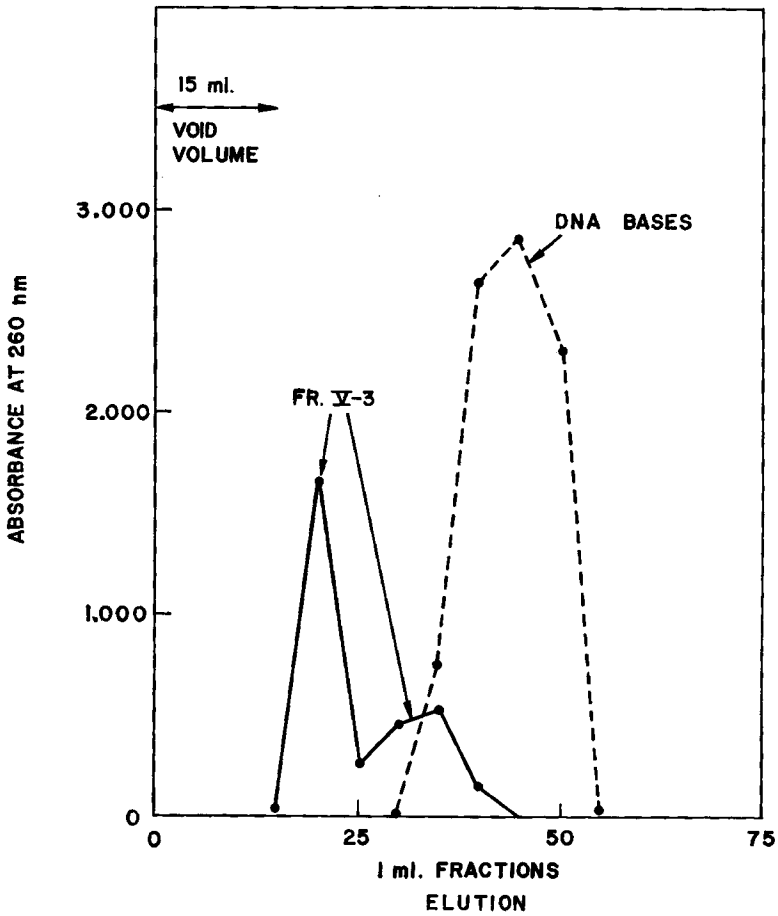


FIG. 5. Elution chromatogram of Fraction V-3 treated with 60% $HClO_4$, 100°C, ninety minutes, on a Sephadex G-25 column, along with that of DNA bases liberated by treatment of DNA with perchloric acid under similar conditions. Fraction V-3 gives two peaks, one at the end of the void volume, the other far beyond but substantially before the peak given by DNA bases.

per cent. It may be seen in Table 4 that even though small amounts of activity were present in Fractions V-1 and V-2, almost the entire activity originally found in the liver-insoluble material was in the negatively charged Fraction V-4.

The apparent recovery of 110 per cent of the starting radioactivity (cf. Table 1) is considered to be within the experimental error for 100 per cent, on a conservative estimate of sampling and counting errors.

DISCUSSION

Radioactivity was found in soluble fractions prepared by solvent extraction under mild conditions, with a succession of solvents. Radioactivity

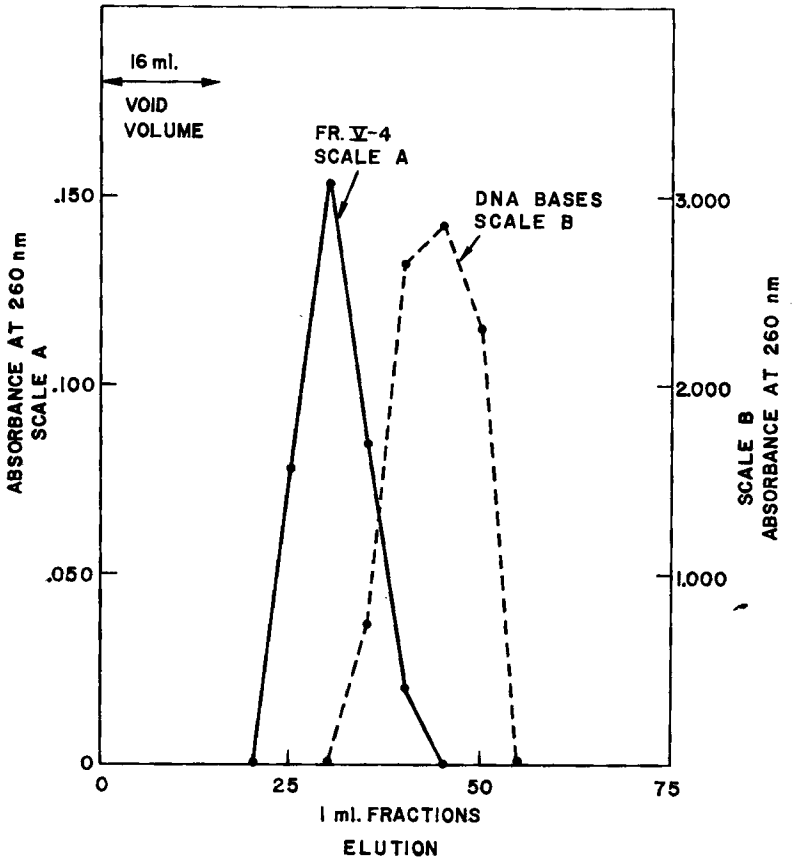


FIG. 6. Elution chromatogram of perchloric-acid treated (60% $HClO_4$, 100°C, ninety minutes) Fr.V-4 of Sephadex G-25 column along with that of DNA bases liberated by treatment of DNA with perchloric acid under similar conditions. Fr.V-4 gives one peak coming beyond the void volume but substantially before that of the DNA bases.

TABLE 4
Tritium Activity in the Nucleic Acid Complexes

Substance	CPM in 0.1 ml/1 ml Solution (calculated from 10 min. counting)	Total DPM Accounting for 195 mg of Liver Hard Core
145 mg of liver hard core	—	4350 (Table 1)
Fraction V-1	19	210
Fraction V-2	18	140
Fraction V-3	15.7	0
Fraction V-4	85.3	4500
Background	15.7	—

also was found in the insoluble residue remaining after the successive extractions. The specific activity was greatest in the chloroform-methanol extract, and next greatest in the insoluble residue. The other 3 fractions showed substantially lower specific activities. We first concentrated our attention on the insoluble residue, for the obvious connection it may have with the theory from this laboratory about the molecular mechanisms of aging (14–16).

Our analyses, although not yet extensive, show that the insoluble residue contains amino acids and the components of both DNA and RNA (Table 2 and Figs. 2 and 3). We found about 2 per cent of the total weight of the insoluble residue to be identifiable amino acids, although the nitrogen content of such insoluble residues in our experience corresponds to about 40–60 per cent protein. Appreciable quantities of pentose and deoxypentose were found in the soluble fractions V-1 to V-4, prepared by acid hydrolysis of the insoluble residue.

The absence of proline and oxyproline can be taken as evidence that there was no appreciable collagen component in the insoluble residue.

The second and third lines of evidence depend on the high molecular weight of the electrophoretic fractions, and on their resistance to strong hydrolytic procedures. The behavior of these soluble fractions on Sephadex columns before concentrated $HClO_4$ hydrolysis indicates a molecular weight greater than 5000 for all 4 electrophoretic fractions. The behavior after $HClO_4$ hydrolysis shows the persistence of some ultraviolet-absorbing fragments, of molecular weight greater than that of DNA monomer. Taken together, these two lines of evidence indicate that the solubilized fragments of the insoluble residue are of high molecular weight and that they are bound together by linkages almost certainly covalent, which have a stability beyond the ordinary for nucleic acids. The evidence as it presently stands provides substantial support that the insoluble residue is constituted from natural polymers of various kinds, held together by covalent crosslinks of extraordinary stability.

The findings on the composition of soluble Fraction V-4, which contains most of the radioactivity of the insoluble residue, strongly suggest that RNA is the component carrying the radioactivity. Although the evidence is not yet compelling, we cannot but note that various species of RNA are involved in very active metabolism and are more exposed to a variety of potential crosslinking agents than is DNA. DNA, in contrast, is most of the time protected from potential crosslinking agents by its intimate association with nucleohistones, commensurate with its function as a stable information-storage molecule (17).

Various kinds of crosslinkages between RNA, DNA and protein are conceivable and numerous possibilities of this sort have been presented [Bjorksten (15, 16)]. Of the possible crosslinking combinations between these 3 polymers, a covalent crosslink between the two strands of a DNA

molecule would appear to result in irreparable damage to the DNA. Repair mechanisms are known by which an organism can excise the damaged portion in a single strand of DNA and use the intact companion strand as a template in reconstructing the original structure (18). But a crosslinkage joining both strands at the same point is irreparable because neither strand can then serve as template for repair. The chromosome then cannot complete its replication because the DNA strands cannot separate. The cell will die in the next mitosis, or in some rare instances, mutate. This appears a highly probable mechanism among all those suggested so far, for the progressive cell death observed in aging [Bjorksten (16, 19)].

Fraction V-4, containing most of the radioactivity of the insoluble residue, is clearly of great interest and will provide one of the foci of our continuing investigations.

This investigation provides molecular evidence of crosslinkage as an important factor in the gerogenic insoluble fraction, because:

1. The insoluble gerogenic fraction contains elements of RNA, DNA and proteins in substantial quantities.
2. Even after hydrolytic solubilization with strong acids at high temperatures, the soluble fractions are of high molecular weight.
3. The linkages in the insoluble gerogenic fraction appear to be more resistant to acid hydrolysis than do most of the linkages in normal biological polymers.

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