Lipid Peroxidation in Rat Tissue Particulates Separated by Zonal Centrifugation [1]

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SUMMARY

The distribution of protein and lipid peroxidation activity in homogenate fractions of rat liver, brain, kidney, and testis has been determined following zonal centrifugation of these homogenates in sucrose gradients. Lipid peroxidation occurred in nearly all particulate fractions. Differences in the amount of lipid peroxidation between fractions were presumed due to specific differences in their lipid and iron content. The particle profile of brain and testis homogenates differed from that of liver and kidney. Brain had a major particulate fraction with a sedimentation coefficient between those of liver microsomes and mitochondria. Brain and testis had only small amounts of material corresponding to the mitochondrial zone of liver and kidney. The procedures for the differential centrifugation developed primarily for liver, therefore, are not applicable to all tissue fractionation. The sedimentation characteristics of each tissue particulate fraction must be determined before appropriate procedures for differential centrifugation can be established. Zonal centrifugation, with continuous monitoring of the collected gradient, provides a rapid and efficient method for determining these characteristics.—Nat Cancer Inst Monogr 21: 333–344, 1966.

A NONENZYMIC peroxidation of unsaturated lipids occurs when certain rat tissue homogenates are aerobically incubated (*1*). This reaction does not normally occur *in vivo*. Homogenization and dilution change the tissue antioxidant characteristics sufficiently to allow oxygen attack of the unsaturated lipids.

Preliminary studies, using differential centrifugation, established the subcellular distribution of the components required for lipid peroxidation (*2*). Lipid peroxidation occurred in all particulate fractions (nuclei, mitochondria, and microsomes) isolated from liver homogenates when incubated with the 105,000 × *g* supernatant or with ascorbic acid, but not when incubated alone. Ascorbic acid appears to be the only essential

[1] This research performed under the Joint National Institutes of Health-Atomic Energy Commission Zonal Centrifuge Development Program which is supported by the National Cancer Institute, the National Institute of Allergy and Infectious Diseases, and the U.S. Atomic Energy Commission.

[2] Department of Zoology, University of California, Los Angeles, Calif. Aided by AEC Contract No. AT(11-1)-34 Project 49.

[3] Operated for the U.S. Atomic Energy Commission by Union Carbide Corporation.

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component contributed by the supernatant, whereas the particulate fraction contributes both the unsaturated lipids and the iron required for the reaction (*2*).

The lipid peroxidation reaction has been studied in mitochondrial suspensions (*3*, *4*), microsomal suspensions (*2*, *5*), as well as in pure unsaturated lipid suspensions (*6*). However, microsomal contamination of mitochondria is difficult to avoid when mitochondria are prepared by differential centrifugation and the contribution by microsomes to lipid peroxidation in mitochondrial fractions has not been established. The peroxidation reaction in isolated mitochondria has been correlated with mitochondrial swelling and lysis (*7*). Therefore, preparations of mitochondria free of microsomal contamination are obviously necessary for establishing peroxidation as the mechanism involved in swelling and lysis.

The development of zonal centrifugation has made it possible to separate tissue particulate fractions with increased resolution (*8*). Rate-zonal sedimentation of tissue homogenates in the B-IV rotor system (*9*), followed by high-speed centrifugation of the collected samples by conventional means, results in the separation of particulate materials on the basis of their sedimentation properties. The present study was designed to separate tissue fractions in a sucrose gradient and to determine the extent of lipid peroxidation in all fractions by aerobically incubating similar volumes of each with ascorbic acid. The membrane and lipid peroxidation profiles from rat liver, brain, kidney, and testis homogenates are compared.

MATERIALS AND METHODS

Liver, brain, kidney, and testis obtained from exsanguinated Sprague-Dawley male rats were homogenized in 0.25 M sucrose. Twenty ml of homogenate was placed in the B-IV rotor system containing a gradient volume of 1200 ml extending from 10 to 30 percent sucrose. About 300 ml of a 55 percent sucrose solution was used as the outboard cushion with 200 ml of buffer placed inboard of the sample layer. Separations were carried out at 10,000 rpm for 15 minutes ($G_c = 1100 \times 10^6$, including acceleration and deceleration) in all cases but one, which was at 20,000 rpm for 60 minutes ($G_c = 17{,}000 \times 10^6$). The rotor contents were emptied by displacement with 55 percent sucrose and the eluant was monitored continuously at 260 mμ. Forty-two fractions containing 40 ml each were collected in tubes maintained in ice. The percent sucrose was measured with an American Optical Company refractometer calibrated to read w/w percent sucrose directly and proteins were measured colorimetrically by the method of Lowry *et al.* with bovine serum albumin as the standard (*10*).

Twenty-five ml of each collected sample was placed in polycarbonate centrifuge tubes and 3 ml of water added. All samples were centrifuged for 90 minutes at 30,000 rpm in a Spinco Model L ultracentrifuge. Pellets were rinsed twice with 0.01 M phosphate buffer (containing 0.15 M NaCl)

at *p*H 7.0 and resuspended to the original volume by homogenizing in buffer. Proteins were analyzed in all resuspended pellets and an aliquot of each was used to measure lipid peroxidation.

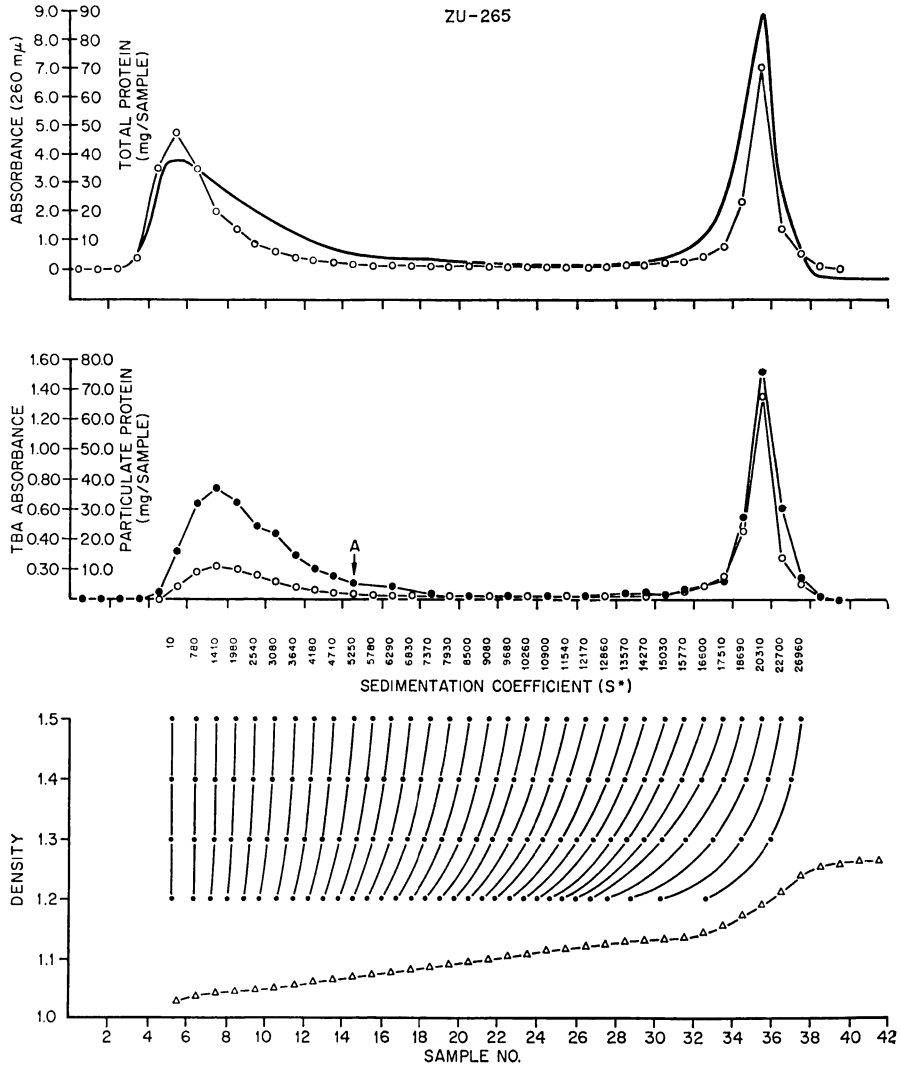
Lipid peroxidation was measured by placing equal portions of resuspended pellets in 20 ml beakers; 30 μg of ascorbic acid was added to each beaker, and the final volume was adjusted to 4.0 ml with buffer. Beakers were incubated for 90 minutes at 37° C in a Dubnoff Metabolic Shaker oscillating at 100 cycles per minute. The thiobarbituric acid (TBA) test was used to measure lipid peroxidation (*11*). Results were expressed directly as the TBA absorbance measured at 530 mμ on a Beckman Model DB Spectrophotometer rather than as units of malonaldehyde, since the relationship between the TBA color and malonaldehyde continues to be questioned (*12*).

RESULTS

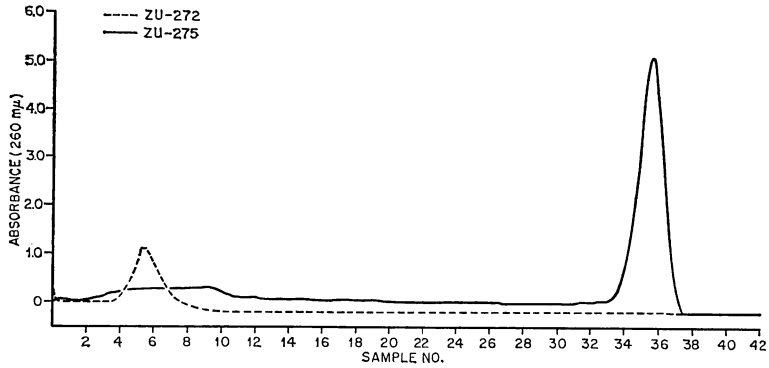
Sedimentation of a 10 percent rat liver homogenate in the zonal centrifuge for 15 minutes at 10,000 rpm resulted in the separation of two major ultraviolet (UV) absorbing fractions (text-fig. 1, top diagram). The soluble protein represented 41 percent of the homogenate protein and was recovered from the starting zone (sample 6). The microsomal fraction sedimented slightly from the starting zone (text-fig. 1, middle diagram). The mitochondria and larger particles sedimented to their isopycnic level at the outboard edge of the rotor (sample 36). Some particulate material was distributed between the microsomal and mitochondrial fractions. Assuming similar particle densities, a continuous spectrum of sizes was indicated.

The distribution of lipid peroxidation activity in liver fractions was similar to the distribution of particulate protein (text-fig. 1, middle diagram). The specific activity (TBA absorbance/100 μg protein) of the microsomal fraction (samples 6–15) was greater than that of the larger fragments (samples 33–36) and was also slightly higher than the activity of microsomes isolated by differential centrifugation (table 1). This difference in specific activity was not due to the particle size differences since sonication of large fragments from liver did not change the specific activity of lipid peroxidation (0.051 TBA absorbance/100 μg protein) but did disrupt the larger fragments to particles which centrifuged in the microsomal zone (text-fig. 2).

The microsomal pellet prepared by centrifuging a 10,000 rpm-15-minute supernatant (No. 30 rotor of a Spinco Model L ultracentrifuge) at 40,000 rpm for 60 minutes contained 14 percent of the protein of the homogenate. In the zonal centrifuge diagram shown in text-figure 1, 14 percent of the particulate protein is found to the left of point A. Particles at this experimentally determined point have an equivalent sedimentation coefficient of approximately 6300 *S** assuming a density of 1.2. The particle fraction collected between the starting boundary



TEXT-FIGURE 1.—Distribution of ultraviolet absorbance, total protein, particulate protein, and lipid peroxidation activity in a rat liver homogenate subjected to 10,000 rpm for 15 minutes in the zonal centrifuge; 20 ml of homogenate (1:10 w/v in 0.25 M sucrose) placed in B-IV rotor and overlayed with 200 ml of buffer. Starting zone at sample 6; 40 ml collected in each tube; 25 ml of each sample, diluted with 3 ml of water, was centrifuged for 90 minutes at 30,000 rpm. Pellets were rinsed twice and resuspended in phosphate buffer (0.01 M, *p*H 7.0) containing 0.15 M NaCl. Lipid peroxidation was measured in 20 ml beakers by using 2 ml of resuspended pellet and 30 μg ascorbic acid brought to a final volume of 4 ml. Incubation was for 90 minutes at 37° C. TBA reaction run was previously described (*1*). *See* text for explanation of A. Please *note* that ordinate units differ in subsequent figures. These changes were necessitated by the large differences in the amount of material present in each tissue: ○—protein (total in *upper* and particulate in *middle* diagram); ●—TBA absorbance at 530 mμ; △—sucrose gradient density.



TEXT-FIGURE 2.—Sedimentation of normal and sonicated mitochondria at 10,000 rpm for 15 minutes in the zonal centrifuge. To obtain the isopycnic rebanding of normal mitochondria, 38 ml of a mitochondrial suspension obtained from zonal centrifugation of a liver homogenate was diluted to 150 ml with buffer and reloaded into the B-IV rotor. For examining the effects of sonication, 40 ml of a pelleted sample was resuspended in buffer, sonicated for 2 minutes, and 20 ml was placed into B-IV rotor.

_____Normal _ _ _ _ _ _Sonicated

TABLE 1.—Comparison of lipid peroxidation activity in particulate fractions of rat tissue homogenates. Values represent the TBA absorbance/100 μg protein developed during 90 minutes of aerobic incubation at 37° C with 30 μg of ascorbic acid in each flask. Final volume of each flask was 4.0 ml

Tissue	Microsomes*	Microsomes†	Large fragments‡ (including mitochondria)
Liver	0.115	0.136	0.051
Brain	0.100	0.086	0.050
Kidney	0.060	0.071	0.044
Testis	0.043	0.056	0.025

*Microsomes prepared by differential centrifugation. A 10,000 rpm-15-minute supernatant solution was centrifuged at 40,000 rpm for 60 minutes. Pellets were resuspended in 0.01 M phosphate buffer, *p*H 7.0, containing 0.15 M NaCl.

†The combined specific activities of all particles between tubes 6 and 15 collected after zonal centrifugation, as described in the legend of text-figure 1. This total fraction contained approximately the same amount of protein as in microsomes collected by differential centrifugation.

‡The combined specific activities of the particulates between zonal samples 33 and 36, except for brain where it represents the material between samples 18 and 22.

and point A of text-figure 1 is therefore roughly comparable to the particles retained in the supernatant fluid following differential centrifugation at 10,000 rpm for 15 minutes in Spinco No. 30 rotors. It should be recalled that the pellet in differential centrifugation contains *all* particle species present but in different amounts. The supernatant also contains different amounts of each species excepting those completely sedimented. If microsomes and mitochondria represented two particle populations whose sedimentation coefficient distribution curves did not overlap, then clean separations could be made in the zonal centrifuge but not in single step differential centrifugation. The distribution between pellet and

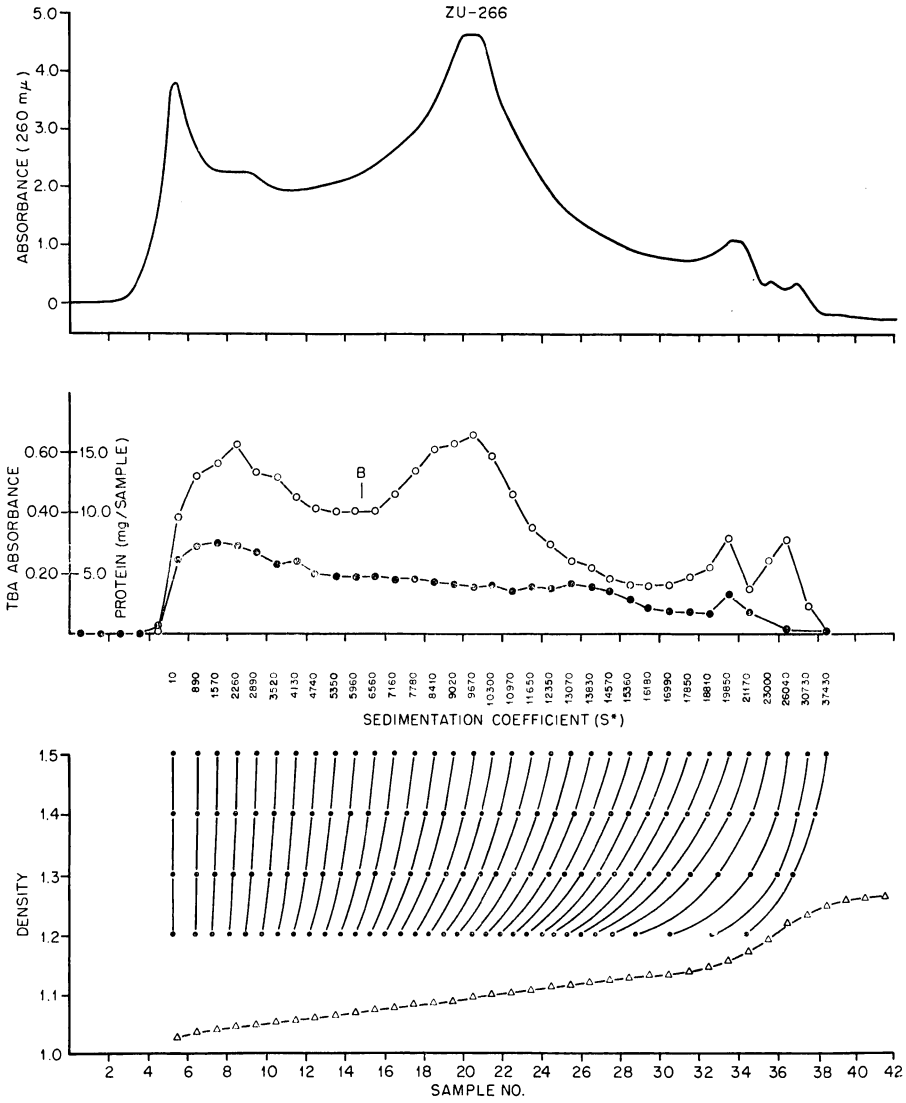
supernatant fluid in differential centrifugation cannot, therefore, be indicated by a single point in zonal centrifuge run diagrams. The particle fraction collected between the starting boundary and point A is therefore only roughly comparable to the particles retained in the supernatant fluid following differential centrifugation at 10,000 rpm for 15 minutes.

Zonal centrifugation of a 1:5 (w/v) brain homogenate at 10,000 rpm for 15 minutes separated six UV-absorbing components (text-fig. 3). The soluble protein fraction represented approximately 18 percent of the total homogenate protein, compared to 41 percent in liver. The microsomal fraction was similar to that of liver but the characteristic mitochondrial fraction was a smaller fraction of the total. The major particulate fraction of brain sedimented with an S^* of approximately 12,000 (assuming a density of 1.2) and constituted nearly half of the particulate protein. The last UV-absorbing peak (sample 37) was made up primarily of red blood cells. The particulates to the left of point B in text-figure 3 correspond roughly to the microsomal fraction remaining in the supernatant of brain homogenates centrifuged at 10,000 rpm for 15 minutes in a Spinco No. 30 rotor.

Lipid peroxidation occurred in brain particulate samples throughout the rotor (text-fig. 3). The microsomal fraction had the highest specific activity, which was only slightly less than that of microsomes collected by differential centrifugation (table 1). The lack of an increase in the peroxidation reaction in samples 15 to 25, as compared with the samples preceding and following, suggests that the particles responsible for this reaction may be uniformly distributed through this region. An additional population of particles may be present, which accounts for the peak in tube 21 observed in the absorbance monitor but which does not contribute to the peroxidation reaction. No peroxidation occurred in the particulates from tube 37, the fraction containing primarily red blood cells. Zonal centrifugation at higher speeds for longer times resulted in isopycnic banding of the 12,000 S^* fractions in 38 percent sucrose.

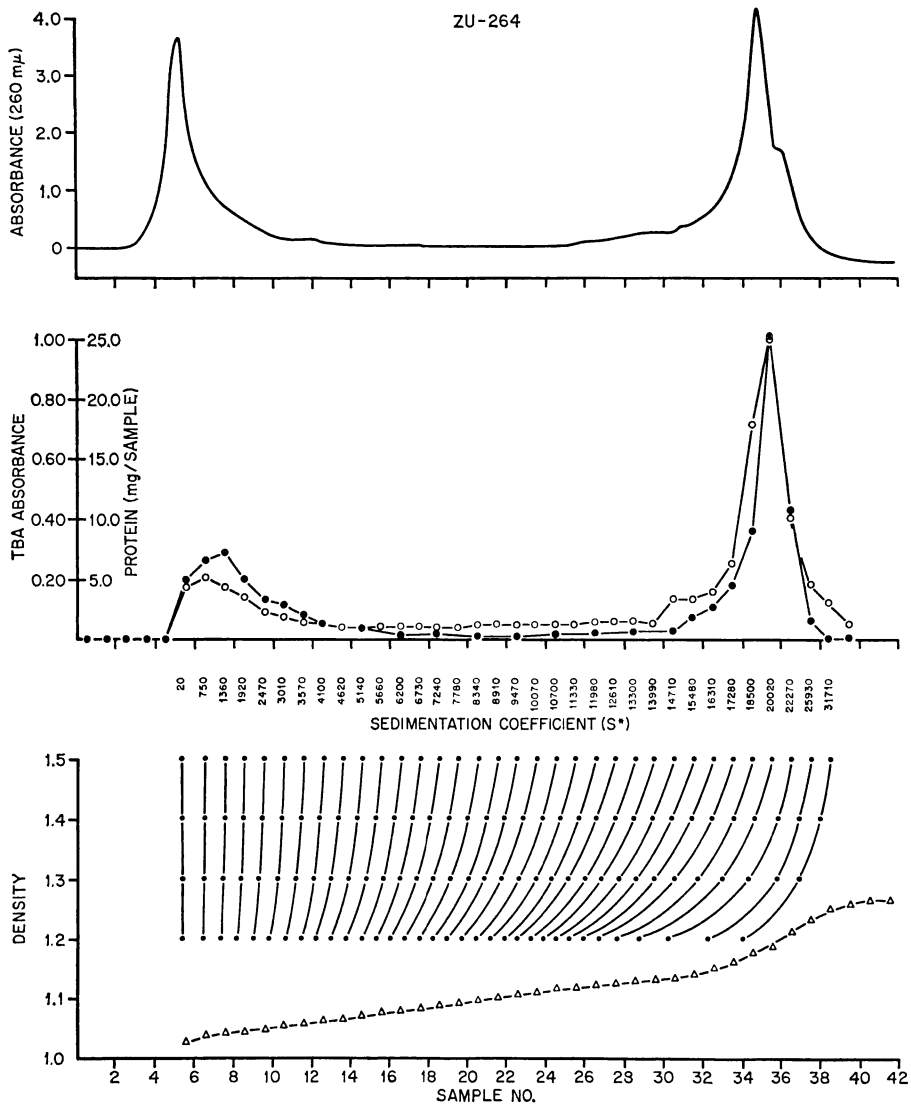
Zonal sedimentation of a 1:5 (w/v) kidney homogenate at 10,000 rpm for 15 minutes resulted in a distribution of UV-absorbing material and total protein similar to liver (text-fig. 4). The soluble protein represented 41 percent of the homogenate protein and the microsomal fraction was again well separated from the larger particulates. Small amounts of particulate material were again sedimented throughout the gradient. Lipid peroxidation occurred in all particulate fractions with the highest specific activity material associated with the microsomal particles. The specific activity of the kidney microsomal fraction was slightly more than that of a similar fraction prepared by differential centrifugation but was only about half that of similar fractions prepared from liver (table 1). The specific activity of the larger fragments was only slightly less than that of similar fragments from liver.

The profile of particulate materials obtained by the zonal centrifugation of a 1:5 (w/v) testis homogenate was different from liver, brain, or kidney



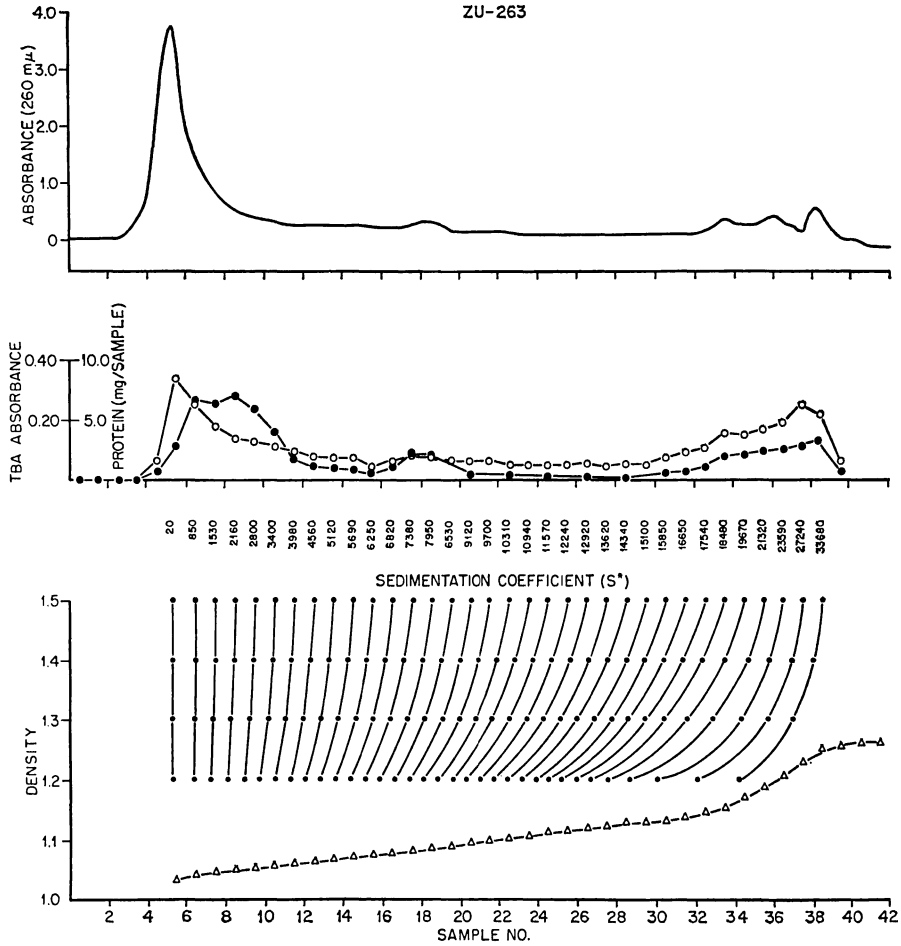
TEXT-FIGURE 3.—Distribution of ultraviolet-absorbing material, particulate protein, and lipid peroxidation activity in a rat brain homogenate (1: 5 w/v) centrifuged at 10,000 rpm for 15 minutes in the B-IV rotor. Conditions similar to those described in the legend for text-figure 1 except that lipid peroxidation was tested by using 1 ml of resuspended pellet: ○—particulate protein; ●—TBA absorbance at 530 mμ; △—sucrose gradient density.

(text-fig. 5). The soluble protein constituted approximately 40 percent of the total homogenate protein and the microsomal fraction (samples 6–15) represented the largest single particulate fraction. No large mitochondrial fraction was noted, but several small distinct peaks were noted between samples 33 and 39. One small peak of particulate material was also noted at sample number 19 (~11,000 S^* assuming a density of 1.2).



TEXT-FIGURE 4.—Distribution of ultraviolet-absorbing material, particulate protein, and lipid peroxidation in a rat kidney homogenate (1: 5 w/v) subjected to 10,000 rpm for 15 minutes in the zonal centrifuge. Conditions similar to those described in text-figure 1, except that lipid peroxidation was tested by using 2.0 ml of resuspended pellet: ○—particulate protein; ●—TBA absorbance at 530 mμ; △—sucrose gradient density.

Lipid peroxidation occurred in nearly all samples. The specific activity of the small particulate fraction was lower than in other tissues (table 1), but was probably due to the presence of a large amount of particulate material in sample 6, which was very low in activity. The particles distributed in samples 8 to 10 were comparable in activity to similar samples obtained from other tissues. The nature of this material in sample



TEXT-FIGURE 5.—Distribution of ultraviolet-absorbing material, particulate protein, and lipid peroxidation activity in a rat testis homogenate (1: 5 w/v) subjected to 10,000 rpm for 15 minutes. Conditions similar to those described in legend for text-figure 1 except that lipid peroxidation was tested by using 3.0 ml of resuspended pellet: ○—particulate protein; ●—TBA absorbance at 530 mμ; △—sucrose gradient density.

6 is unknown. The specific activity of the larger fragments is also approximately half that noted for similar fragments obtained from other tissues.

DISCUSSION

The thiobarbituric acid test for lipid peroxidation was used in the present studies since it is a very sensitive measure of the oxidation of polyunsaturated fatty acids (*13*). Linolenic and arachidonic acids are the important biological fatty acids measured by this test. The peroxidation reaction in homogenates, in isolated particulates, and in pure fatty acid emulsions can use iron in various forms, such as cytochrome, hematin,

or ferrous ions as a catalyst (*2, 6, 14*). Ascorbic acid is utilized in the reaction to maintain iron in its catalytically active reduced form (*3*). Although iron interferes with the TBA reaction, the amount of catalytic iron required in the presence of ascorbic acid is considerably less than that which causes interference with the TBA test (*14, 15*).

All particulate fractions of liver and kidney, which include mitochondria and microsomes, underwent lipid peroxidation in the presence of ascorbic acid. The mitochondrial preparations were well separated from the microsomal fractions and peroxidation in mitochondria was not due to contamination by this fraction. Swelling and lysis of mitochondria accompanied by lipid peroxidation are, therefore, not necessarily due to microsomal contamination (*7*). Brain and testis each had one major particulate fraction with low peroxidation activity. In brain this component centrifuged to the center of the rotor and had an S^* of approximately 12,000 assuming a density of 1.2. The testis component was a small particle which did not migrate appreciably from the starting boundary. The lack of peroxidation activity has not been explained for either component. The presence of unsaturated lipids as structural components in biological membranes suggests that most particulate fractions would undergo peroxidation and is compatible with these results. However, the extent of lipid peroxidation differed greatly between different fractions of the same tissue, as well as between fractions with similar sedimentation properties obtained from several tissues. These differences in peroxidation appear to depend on the lipid and iron constituents of the membrane fraction and not on the membrane size, since sonication of large membranes into smaller fragments did not change their specific activity. Several iron forms are associated with membrane fractions (*16–18*), and the binding of cytochromes by membrane structural protein has been demonstrated (*19*). The catalytic activity of various membrane fractions might be different, therefore, depending on the specific iron form available. The specificity of the TBA test itself for the higher polyunsaturated fatty acids could also explain differences in the extent of peroxidation in various membranes. Membranes with large amounts of linolenic and arachidonic acids would undergo more measurable peroxidation with the TBA test (*13*).

The successful use of differential centrifugation for liver fractionation is due, in part, to the large differences in sedimentation coefficients for the microsomal and mitochondrial fractions and the conspicuous absence of intermediate materials, as shown in the present studies. Microsomes and mitochondria can, therefore, be prepared without excessive cross contamination of particles. Brain, however, has a typical microsomal fraction as well as a major particle fraction of approximately 12,000 S^*. Preliminary observations indicated that this fraction contained the synaptosomes described by Whittaker *et al.* (*20*). Minor variations in differential centrifugation would result in differences in the amounts of this fraction remaining in the supernatant fluid. The specific activity of this fraction, therefore, would change markedly as a result of the slight variations in the centrifugal force used in this range. These results

emphasize the necessity of establishing the centrifugal distribution of the particles of homogenates from all tissues before specific differential centrifugation methods for the preparation of their fractions are used (*21*, *22*). Zonal centrifugation with continuous monitoring of the collected gradient provides a rapid and efficient method for establishing membrane profiles.

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