CANCER RESEARCH

VOLUME 11 JANUARY 1951 NUMBER 1

Cytochemical Studies of Mammalian Tissues: The Isolation of Cell Components by Differential Centrifugation: *A Review**

WALTER C. SCHNEIDER AND GEORGE H. HOGEBOOM

(From the National Cancer Institute, National Institutes of Health, Bethesda, Md.)

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001001.pdf by guest on 24 August 2022

Cytologists have appreciated for many years that the cell is not a homogeneous mass of protoplasm but that it contains several discrete structures that can be seen and identified in all cells of the plant and animal kingdom. The function and the chemical composition of these structures have been the source of considerable speculation and the inspiration for much experimental work, mainly of an indirect nature. In the present review, an attempt will be made to examine critically one of the methods that have been devised to determine directly the chemical composition and the function, in terms of enzymatic activity, of the structural components of the cell. This method involves the mechanical rupture of tissue cells in a suitable medium followed by the isolation of the cell components by means of differential centrifugation.

The method of differential centrifugation of broken cell suspensions was introduced in 1934 by Bensley and Hoerr (11), who described the isolation of mitochondria from guinea pig liver. Since the paper of Bensley and Hoerr, numerous publications have appeared dealing with the isolation of individual cellular components. Many have dealt with the isolation of nuclei (9, 34). Others have described improvements in the procedures for the isolation of mitochondria (19–21, 23–25, 60), while still others have dealt with the isolation of chromosomes (28, 90–92) and submicroscopic particles (19–21, 23–25, 60, 77), including glycogen particles (24, 76, 77) in a submicroscopic form. More recent papers have described procedures for the complete fractionation of a tissue into nuclei, mitochondria, submicroscopic particles, and soluble material (60, 122, 125, 129).

The fact that particulate components of the cell can be isolated from broken cell suspensions in almost unlimited yields makes it apparent that this technic is capable of utilizing to the fullest extent modern biochemical methods for the study of cell composition and function. In this respect, the procedure of cell fractionation is more versatile than either of two other methods at present available in the field of cytochemistry; namely, the histochemical technics (44) and the submicro technics of Linderstrom-Lang, Holter, and their associates (82). The former, aside from the possible artifacts introduced by the necessary procedures of freezing or fixation, are severely limited by their dependence on microscopic visualization for the localization of an enzymatic reaction or a chemical compound and as a result usually employ indirect methods of analysis which cannot readily be quantitated and generally entail the possibility of still further artifacts. The latter technic, which most nearly approaches the ideal cytochemical tool in that it is aimed at the study of single cells and portions thereof, is apparently not as yet sufficiently sensitive to be applied to

* For the sake of brevity and convenience the following abbreviations will be employed in this paper: DNA = desoxypentosenucleic acid; PNA = pentosenucleic acid; AMP = adenosine-5-phosphate; AMP-ase = AMP phosphatase; ATP = adenosinetriphosphate; ATP-ase = adenosinetriphosphatase; DPN = diphosphopyridine nucleotide; TPN = triphosphopyridine nucleotide; and DAB = 4-dimethylaminoazobenzene.

Received for publication October 19, 1950.

single mammalian cells. Although it is hoped that the results obtained with the cell fractionation technic will be checked by some other method, it will be extremely difficult for other methods to keep pace, owing to the versatility and wide applicability of the former. Accordingly, it is necessary to understand clearly the limitations of the cell fractionation technic and to define the criteria that identify an adequate isolation procedure.

Some investigators (14, 16, 33) have taken the view that the procedure of cell fractionation can be dismissed *a priori* on the grounds that the very act of cell rupture necessarily produces artifacts such as redistribution, adsorption, morphological alterations, etc. Although such artifacts are obvious problems, the dismissal of the procedure as an experimental tool, without concrete evidence of its uselessness and without the submission of an adequate alternative, constitutes a negative approach that is difficult to defend. The reviewers prefer to adhere to the *positive* approach of conducting cell fractionation experiments in such a way that possible artifacts may be tested experimentally.

In a consideration of artifacts likely to be encountered in the procedure of cell fractionation, it must first be realized that when the cell is disrupted, its structural components are released into distinctly abnormal surroundings. Since cytological studies have demonstrated that at least two cellular components, nuclei (141) and mitochondria (26, 32, 66, 95, 143, 144), possess well defined membranes, it appears entirely possible that these membranes may be so damaged in the process of cell disruption as to allow the escape of soluble substances. Although experiments providing direct evidence for the integrity of the membranes have not as yet been devised, strong indirect evidence can be offered by certain lines of approach. Thus, it seems likely that the isolation of a cell component, in morphological and cytologically unaltered form, will maintain the integrity of the membrane. In this respect, it has been shown (60) that the cytological and morphological properties of liver mitochondria are profoundly affected by the composition of the medium in which the liver cells are disintegrated. Only when the cells were disrupted in hypertonic solutions of nonelectrolytes was the morphology and cytology of the mitochondria maintained. Additional and more direct evidence for the integrity of the mitochondrial membranes has also been provided by the recent demonstration that the mitochondria contain large amounts of soluble proteins that can be released only by disruption of the mitochondrial membranes (57, 59). Another method that might be suggested to test the integrity of the membranes of particulate structures is that of equilibrating the isolated structure with a solution of the isotopically labeled material in question. Labeled material should appear within the structure if its membrane is permeable. A method peculiar to certain enzymes can also be used to test the integrity of particulate structures. Thus, in the case of cytochrome c it was found (128) that liver mitochondria isolated from water homogenates contained high concentrations of cytochrome c but that it was biologically inactive in the succinoxidase system present in the mitochondria. On the other hand, cytochrome c present in mitochondria isolated from isotonic saline or sucrose or hypertonic sucrose (128, 129) was highly active in the oxidation of succinate by the mitochondria.

A second important problem, namely, the possibility of adsorption of soluble material on cellular particles, can perhaps best be approached experimentally by washing the isolated particulate structure in various media. If the material is tightly bound to the structure and not adsorbed, repeated washing should fail to reduce either its total amount or its concentration. This has been shown to be the case for certain of the respiratory enzymes associated with liver mitochondria and microsomes (55, 56, 125). On the other hand, cytochrome c has been shown to be adsorbed on liver microsomes when these were isolated from water homogenates but not when the microsomes were isolated from saline or sucrose homogenates (128, 129). Furthermore, the cytochrome adsorbed on the microsomes was completely removed when the particles were washed with isotonic saline (128).

A consideration of the quantitative aspects of the intracellular distribution of the substance in question also has a decided bearing on the question of adsorption. Thus, if a large percentage of the substance present in the whole tissue can be shown to be localized in a single fraction, then the probability of adsorption would appear to be minimal. Some of the enzymes referred to in the preceding paragraph fulfill this condition. On the other hand, the finding of a *small* percentage of a substance in a fraction must be seriously considered as an adsorption phenomenon. In the case of isocitric dehydrogenase, for example, about 82 per cent of the enzyme activity was recovered in the soluble fraction and 12 per cent in the mitochondria (61). The significance of the latter seems rather doubtful and can probably be considered as the result of adsorption. Dounce (36) has argued that, for results to be significant, the proportion of the total enzyme in a particulate structure need not be

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

greater than the proportion of the total cell volume occupied by the particulate structure. Although this may be true, the actual proof that it is the case can come only from measurements on single cells.

The need for establishing balance sheets in which the summation of the activities of the tissue fractions is compared with that of the whole tissue has been emphasized repeatedly (56, 62, 122, 127). Such balance sheets serve several purposes, one of which has already been mentioned in the preceding paragraph. In addition, tabulations of this type test the validity of the analytical methods and of the assay procedures employed, and in the case of enzymes indicate whether inhibitors or activators are present. By judicious recombination of fractions it is possible to determine which fraction contains the inhibitor or activator and thus to learn how the activities of the various cell fractions are integrated to reproduce the activity of the cell as a whole.

METHODS FOR THE ISOLATION OF PARTICULATE CELL STRUCTURES

An adequate method for the preparation of a particulate component of the cell should provide that component in pure form, morphologically and cytologically unaltered, and in high yield. Because of the possibility of heterogeneity of cells and of cell fractions and because many of the present applications of the cell fractionation technic involve comparative studies on different tissues, the need for large yields is immediately obvious.

One of the factors affecting yield in cell fractionation studies is the method used for cell disruption. This was discussed in a previous publication (127), and it was concluded that the best available method involved the use of the Potter-Elvehjem homogenizer (107), because it produced maximum degrees of cell rupture without causing breakage of subcellular elements. In the experience of the reviewers, the Waring Blendor is entirely unsatisfactory for the preparation of homogenates suitable for cell fractionation studies. The subjection of liver, for example, to the action of the Waring Blendor for periods sufficient to disrupt most of the cells results in the breakage of many nuclei and apparently even of some mitochondria. Excessive frothing occurs, and the temperature is controlled with difficulty. A recent report (75) also indicates that the Waring Blendor is capable of inactivating some enzymes and related compounds.

Another factor affecting the yield is that of aggregation of particles. Thus, in the case of liver homogenates it was demonstrated that electrolytes caused pronounced aggregation of mitochondria and that this aggregation was largely prevented by the use of nonelectrolytes (60). More recently, it has been reported that agglutination of cytoplasmic particles in electrolyte solutions can be prevented by the use of heparin (63).

Another point to be considered is the tissue chosen for cell fractionation studies. The ideal tissue would contain only one cell type. In practice, however, very few tissues can even approach this ideal. Among those that might be mentioned are liver, epidermis, and certain tumors. It is to be hoped that the technics of tissue culture may some day provide cells of all types in amounts sufficient for fractionation studies.

Isolation of nuclei.—Several methods have been employed for the isolation of mammalian nuclei. The first method involves the use of citric acid, and, in the procedure of Dounce (34) for the isolation of liver nuclei, only enough citric acid is added to attain a pH of 6. The yield of nuclei with this method has not been established, but, judging from the description of the procedure, the yield is probably low. A photomicrograph of nuclei isolated by this procedure shows quite clearly that the nuclei are altered morphologically, as shown by the presence of precipitated chromatin as well as well as by deviations of some of the nuclei from a characteristic spherical form. Unaltered nuclei are completely homogeneous under the microscope and, in the case of liver, are generally spherical in shape (73, 118, 137). It should be mentioned that nuclei of liver homogenates in either isotonic or hypertonic sucrose fulfill these cytological criteria (60, 73, 101, 102, 118, 137). The isolation of nuclei from homogenates of spleen in isotonic sucrose containing citric acid (4) has been described, but photomicrographs of the isolated nuclei, by means of which the morphological properties might be judged, were not presented. The addition of citric acid was stated to be necessary to prevent the loss of nucleoprotein from the nuclei during the isolation. However, the experiment which was stated to prove that DNA was lost from nuclei in sucrose proved that DNA could be extracted with molar NaCl from nuclei in sucrose but not from nuclei in sucrose containing citric acid. The latter is at variance with the findings of Dounce (34, 35).

Another method, devised by Behrens (9), consisted of lyophilization of the tissue, grinding of the dry tissue to disrupt the cells, suspension of the powder in an organic solvent mixture of suitable density, and centrifugation of the resulting suspension at a suitable speed. Dounce and his associates (37) have recently applied this method to the study of amino acid distribution in nuclei and have published the first photomicrograph of such nuclei. These nuclei show morphological al-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001001.pdf by guest on 24 August 2022

terations similar to those obtained with citric acid. The adequacy of the Behrens' method for enzyme investigations might also be questioned, since it is well known that some enzyme systems are inactivated by freezing and drying. However, the method does possess the important advantage that artifacts produced by the redistribution of materials should be absent. From the latter standpoint, the Behrens' method may prove valuable for testing the results of fractionations conducted in aqueous solutions.

Another preparation that has proved to be of considerable use in comparative studies is the nuclear fraction obtained as a first step in the complete fractionation of tissues (60, 122). This fraction contains all the nuclei of the tissue, in addition to some unbroken cells and mitochondria. Despite these impurities, however, much useful information has been supplied by a study of this fraction. Barnum *et al.* (8) have recently subjected the nuclear fraction to further treatment with citric acid in order to obtain nuclei sufficiently pure for studies on uptake of radioactive phosphorus. Nuclei so treated appear to have lost a large amount of their original protein, however.

Recent cytological studies by Pollister and Leuchtenberger (105) have far-reaching implications in regard to methods for the isolation of nuclei and perhaps for the isolation of other particulate components of the cell as well. These authors have found that the ratio of protein to DNA in nuclei in tissue sections was several-fold greater than the ratios reported by various authors for nuclei isolated with citric acid. It is not clear at present, however, whether the cytologically determined ratios are correct, because of the difficulties involved in assigning absolute values to the extinction values determined on the nuclei in tissue sections (119, 137). However, these authors performed an experiment which would appear to be independent of the above objection. They incubated rat liver slices in isotonic saline prior to fixation and noted a marked loss of nonhistone-type protein from the nuclei. This experiment raises several important questions, e.g., whether protein is lost from nuclei at the moment of cell rupture or whether a medium can be devised to prevent loss of protein from nuclei. Recent experiments by Dounce *et al.* (37), with the technic of Behrens for the isolation of nuclei, appear to confirm the cytological results. Nuclei isolated by this method had much higher protein-DNA ratios than did those isolated in dilute citric acid (although the ratio in the latter instance was also much higher than that for nuclei isolated by citric acid technics at a lower pH than that employed by Dounce). However, the nuclei isolated by the Behrens' method were peculiar in that their *PNA* content was greater than their DNA content. If correct, this finding would mean that about one-third of the PNA present in liver is localized in the nucleus and would imply that isolation of nuclei in aqueous media results in loss of not only protein from nuclei but also considerable amounts of PNA. It should be possible to check the latter findings cytologically.

In regard to improved media for the isolation of nuclei, it might be mentioned that several lines of evidence indicate that hypertonic solutions of nonelectrolytes may be necessary. The fact that nuclei appear normal morphologically in such media has already been mentioned above. In addition, it should be noted that the isolation of liver mitochondria in a morphologically and cytologically unaltered form was accomplished in such solutions (60). Furthermore, Opie (100) has found that tissue slices take up water when immersed in isotonic solutions but not when immersed in hypertonic solutions. There is thus apparently an increasing body of evidence that the osmotic pressure inside the liver cell is considerably greater than in blood serum.

Isolation of chromosomes.—Chromatin threads were isolated independently by Claude and Potter (28) and by Mirsky and Pollister (90). Subsequently, Mirsky and Ris (91) presented evidence that the chromatin threads possessed the morphological characteristics of chromosomes.

The isolation of chromosomes is accomplished by means of differential centrifugation after rupture of nuclear membranes by one of the following methods: grinding with sand, prolonged mixing in a Waring Blendor, or passage through a colloid mill. The yield of chromosomes has not been reported, although this can be estimated from the amount of DNA recovered in the chromosomes as compared to the DNA content of the whole tissue. The purity of the chromosomes was tested by applying immunological tests for serum proteins as well as cytological tests for cytoplasmic proteins. According to Mirsky (89), these tests were essentially negative. Ris and Mirsky (118) also point out that isolated chromosomes are derived from resting nuclei, in which the chromosome structures cannot be seen. They consider the isolation of structures possessing the morphological characteristics of chromosomes as evidence for the preexistence within the nuclei of chromosomal structures, even though invisible in the living cell. However, the possibility that chromosomes are *not* present as discrete structures within resting nuclei but are formed from submicroscopic or soluble ma-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110010001.pdf by guest on 24 August 2022

terial at the moment of nuclear rupture must also be considered, inasmuch as Ris and Mirsky (118) have demonstrated that the nuclei from which the chromosomes were isolated had been altered morphologically by the electrolyte medium in which they were suspended.[1]

In experiments with isolated chromosomes, it must also be remembered that the chromosome preparation contains nucleoli, some of which apparently stick to the chromosomes and cannot be removed (28, 91). It is not clear whether all nucleoli are so trapped or whether only a few are. In any event, any property ascribed to chromosome preparations must be considered in the light of nucleolar contamination.

In regard to the method of isolation, Mirsky (89) dismisses the isolation of nuclei as a desirable step preliminary to rupture of nuclear membranes because of the undesirable properties of nuclei prepared in citric acid. It would appear desirable, however, to wash the minced tissue as completely as possible or to isolate the washed nuclear fraction in order to remove as much cytoplasm as possible prior to the rupture of the nuclear membranes.

Isolation of mitochondria.[2]—The isolation of mitochondria was first reported by Bensley and Hoerr (11). The details of their procedure, however, were not reported until a few years ago (54, 77). Meanwhile, Claude (19, 20) described the isolation from various tissues of large granules, which were at first considered to be secretory granules (19, 20). Subsequently, however, Claude stated that the large granule preparations were either mitochondria or mixtures of mitochondria and secretory granules, depending upon the tissue from which they were obtained (23, 24). Thus, in the case of liver and pancreas the large granules were considered to be mixtures, because of the secretory function of these tissues, while in the case of a lymphosarcoma the large granule preparation was considered to consist of mitochondria. Hogeboom, Schneider, and Palade (60) found, however, that the large granule fraction isolated from homogenates of rat liver in 0.88 M sucrose consisted entirely of morphologically and cytologically unaltered mitochondria. This was shown by the fact that many of the isolated granules were elongated in form, a characteristic of intracellular mitochondria, and also by the fact that all the granules in the preparation were stained vitally by Janus Green B, the generally accepted stain for intracellular mitochondria. Furthermore, none of the isolated granules was stained with neutral red, a stain widely used for the demonstration of secretory granules, although the latter could readily be demonstrated in unbroken liver cells present in the homogenate.

The procedure for the isolation of mitochondria consists of the following steps: (*a*) disruption of cell membranes (Potter-Elvehjem homogenizer [107]) in a large volume of a suitable medium, (*b*) sedimentation of nuclei and unbroken cells at low speed, and (*c*) sedimentation of mitochondria at higher speed. The medium in which the homogenization is made has a profound effect on the morphological, cytological, and biochemical properties of the isolated mitochondria, as well as on their yield. If distilled water is used, the mitochondria swell to enormous size and eventually burst if maintained in water for a sufficient length of time (21, 24). The latter finding is not of great importance to the isolation of mitochondria, however, because the isolation can be completed long before appreciable lysis occurs. Of more importance is the fact that the biochemical properties of the mitochondria have been altered. Thus, liver mitochondria isolated from water homogenates contain high concentrations of cytochrome c, but the cytochrome c is unable to function in the oxidation of succinic acid by the mitochondria (128). Mitochondria isolated from water homogenates are also unable to oxidize octanoic acid (71). In isotonic solutions, both intracellular mitochondria and mitochondria released from ruptured cells are

[1] Experiments in progress at this writing have demonstrated conclusively that the distribution of DNA and of structures resembling chromosomes in liver nuclei was much different in sucrose solution than in isotonic saline solution. In sucrose, the nuclei were homogeneous in appearance under the microscope, while in saline the nuclei showed the typical pattern of precipitated chromatin (cf. 118). When the nuclei in sucrose were disrupted, chromosome-like structures were not visible, and over 60 per cent of the DNA of the nuclei was soluble; i.e., nonsedimentable at 60,000 *g*. In saline, however, structures similar to the chromosomes described by Mirsky and Ris (91) were released from the nuclei, and 92 per cent of the DNA was sedimented at the low speed (5,000 *g*) required to segregate these structures. These experiments would appear to indicate that DNA is not associated with structures comparable to chromosomes but is colloidally dispersed within the resting nucleus.

[2] Chantrenne (18) has reported experiments which indicated that the cytoplasm of the liver cell was a heterogeneous collection of particles of varying size, enzymatic activity, and chemical composition, and he concluded that the separation of liver particles into large granules (mitochondria) and microsome fractions was a partly arbitrary procedure. Since these liver homogenates were made in electrolyte solutions, however, it seems probable that a large proportion of the large granules were lost during the initial centrifugation used to prepare the nuclei and cell-free extracts employed in the experiments (Chantrenne, personal communication). The fact that microsomes are also aggregated by electrolytes (61, 66) would also have a bearing on these experiments, and it would appear that the question of heterogeneity of cytoplasmic particles requires reinvestigation.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001001.pdf by guest on 24 August 2022

morphologically altered, as shown by the fact that the mitochondria are predominantly spherical in shape (60). In addition, in isotonic *salt* solutions, the mitochondria do not stain vitally with low concentrations of Janus Green B and are agglutinated to an extent sufficient to prevent their adequate separation from the nuclei (60). Thus, losses of mitochondria as great as 80 per cent have been reported when isotonic saline solutions were used (112, 122). In addition, the contamination of the mitochondria by aggregated microsomes has also been reported (66). The agglutination caused by electrolytes is largely avoided by the use of either hypertonic or isotonic sucrose solutions (60). In addition, the mitochondria isolated in hypertonic sucrose retain their ability to stain vitally with Janus Green B and possess the morphological characteristic of intracellular mitochondria—namely, an elongated shape (60). The use of hypertonic sucrose has the disadvantage, however, of causing inhibition of the activity of several enzyme systems (72, 78, 125). Comparative studies of the distribution of enzymes in rat liver fractions obtained from both hypertonic and isotonic sucrose homogenates have failed to demonstrate any substantial differences between the two sucrose concentrations (129), and it has been concluded that for biochemical studies the use of isotonic sucrose is preferable at the present time (61, 127, 129). The use of the latter has the added technical advantage that the fractionations can be completed in much less time and at much lower centrifugal forces than are required by the greater viscosity and density of the hypertonic sucrose homogenates.

Several modifications have been proposed for the isolation of mitochondria from hypertonic sucrose homogenates. Kennedy and Lehninger (71) have suggested the addition of KCl to the nuclei and cell-free liver extract to agglutinate the mitochondria and thus to permit their isolation at a much lower centrifugal force. The permissibility of such a procedure is questionable, since, in our experience, electrolytes produce agglutination of submicroscopic particles as well as of mitochondria (61, 66). Evidence that this procedure does not yield a mixture of the two types of particles would require data on the total nitrogen and PNA content of this fraction. Thus, the ratio of PNA to total nitrogen would permit a decision as to whether this fraction was contaminated with submicroscopic particles, since this ratio is much higher for liver submicroscopic particles than for liver mitochondria.

Similar criticisms might be made of the modifications employed by Leuthardt and Müller (80). In their procedure, the homogenate is made in isotonic KCl and centrifuged at 1,500–2,000 *g*. The sediment is then resuspended in isotonic mannitol and recentrifuged. The mitochondria remain in the supernatant and are removed. It would appear that some submicroscopic material may also be present in the supernatant fluid.

Cunningham *et al.* (31) have fractionated frozen liver in a solution containing 0.88 M sucrose, 0.14 M NaCl, and 0.01 M phosphate buffer. They state that the use of unfrozen tissue or the omission of salt from the medium did not affect the amount of nitrogen or nucleic acid in the mitochondrial fraction. An examination of their data, however, revealed several facts that appear to disagree with this statement, e.g., the large amounts of nitrogen in the nuclear fraction and of PNA in the mitochondrial fraction, the low amounts of nitrogen and PNA in the microsome fraction, and the large amount of PNA in the supernatant or soluble fraction. The fact that Kennedy and Lehninger (71) were able to add salt to 0.88 M sucrose extracts of liver to cause aggregation of mitochondria would also appear to disagree with the findings of Cunningham *et al.*

As discussed later in this review, the yield of mitochondrial material with the sucrose method represents about 25 per cent of the total nitrogen of the whole liver tissue (60, 125, 130). The yield has not been determined in terms of number of mitochondria, although this would appear to be a project of considerable importance in view of the fact that the amount of mitochondrial material may increase or decrease in various physiological and pathological conditions (116, 140). The yield of mitochondria can also be expressed in another manner, namely, in terms of enzymatic activity that appears to be associated exclusively with the mitochondria. Thus, in the case of cytochrome oxidase, which appears to be localized exclusively in the mitochondria, the yield would appear to be as high as 80 per cent (122, 130). In regard to the purity of the fraction, both chemical studies and studies with the light, dark field, phase, and electron microscopes have indicated that the fraction is free from contamination (26, 32, 60).

One point in the procedure for the isolation of mitochondria that has not been described sufficiently is the removal of submicroscopic material that sediments along with the mitochondria. Muntwyler *et al.* (97) have recently called attention to difficulties in the preparation of the mitochondrial fraction occasioned by the presence of incompletely sedimentated material above the mitochondrial pellet. These authors reached the conclusion that this material belonged to subsequent fractions, since microscopic examination re-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001001.pdf by guest on 24 August 2022

vealed the presence of relatively few mitochondria. The reviewers have also observed the presence of this partially sedimented material and have always removed it from mitochondria, since it was found to be submicroscopic in nature on the basis of both microscopic examination and biochemical properties. On only one occasion (127), however, have the reviewers mentioned the importance of separating it from mitochondria. The appearance of this material is considerably different from that of the mitochondrial pellet, since it is pink-white in color, in contrast to the tan color of the mitochondria. This difference in color, coupled with the fact that it is not firmly packed, makes its removal from the mitochondria quite simple. The separation is most easily made after the second sedimentation of mitochondria, since the differences between the mitochondria and the submicroscopic particles are best seen at this stage. Failure to remove this submicroscopic material from the mitochondrial fraction results in rather obvious redistributions, such as the presence of too much nitrogen and PNA in the mitochondria and too little nitrogen and PNA in the submicroscopic particles, and may account for the high concentrations of PNA in the mitochondrial fraction reported by other workers (31, 111–116).

Secretory granules.—The isolation of secretory granules from liver and pancreas was reported by Claude (19–21). However, Lazarow (77) and Hoerr (54) argued that the secretory granules of Claude were actually mitochondria. Furthermore, Hogeboom *et al.* (60) have found that this fraction in rat liver was composed entirely of mitochondria and that a large proportion of the secretory granules apparently disintegrated when the cells were broken. In more recent publications, Claude (23–25) states that the secretory granules or large granule fraction consists mainly of mitochondria mixed with unknown proportions of secretory granules. According to the experiments of Palade and Claude (101, 102), granules that apparently correspond to secretory granules are present in homogenates of liver in water, saline, or hypertonic sucrose. When homogenates in the latter medium are fractionated, these granules migrate centripetally and collect in a lipid layer at the top of the centrifuge tube. In addition to the secretory granules, this lipid layer also contains neutral fat droplets in large numbers. It is not clear whether the secretory granules present in the lipid layer constitute the entire complement of secretory granules present in the whole tissue. In the case of the homogenates of liver in water and in saline, the secretory granules did not appear in this lipid ayer, and the fate of the granules in these media remains to be clarified. From this discussion it would appear that adequate methods for the isolation of secretory granules remain to be developed, and, in future studies, the question of the stability of the secretory granules would appear to be a primary concern. However, the use of hypertonic sucrose would appear to provide a point of departure for further experiments. Isolation of the lipid layer from sucrose homogenates, followed by its transfer to other media of lower density, might be suggested as a means of testing both the stability of the granules and also of separating them from the fat droplets with which they are contaminated.

Isolation of melanin granules.—Melanin granules have been isolated from amphiuma liver (25), from the ciliary processes of beef eyes (52), from mouse and rat melanomas (25, 142), and from frog eggs (117) by procedures similar to those employed for the isolation of mitochondria. The size and density of the melanin granules are, in fact, similar enough to those of mitochondria to raise the question of mitochondrial contamination. Such contamination has apparently been ruled out in the case of the melanin granules isolated from amphiuma liver, since photomicrographs and electron micrographs of these particles fail to show the presence of other particulate material (25). That the mitochondria of frog eggs can be separated from melanin granules was clearly shown by Recknagel (117).

In the experiments with the other tissues, however, this had not been demonstrated as convincingly, and in the case of the melanomas conflicting reports have appeared on the question of the simultaneous presence of mitochondria and melanin granules in the cells of this tissue. Thus, evidence has been presented to show that the melanin granules possess the staining characteristics usually considered specific for mitochondria and that these melanin granules were the only granules with the cytological properties of mitochondria in the melanoma cells (142). On the other hand, particulate structures present in melanoma cells have been idéntified provisionally as mitochondria (32). These bodies are morphologically different from the melanin granules, are unpigmented, and are so small that some of them cannot be resolved in the light microscope.

Isolation of particulate glycogen.—Glycogen is present in liver homogenates in submicroscopic form and sediments at centrifugal forces intermediate between those required for mitochondria and submicroscopic particles. Particulate glycogen has been isolated by Lazarow (76, 77) and by Claude (24).

Isolation of submicroscopic particles (micro-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

somes).[2]—Microsomes were first recognized and isolated by Claude (19). These particles were at first considered to be mitochondria but were subsequently renamed microsomes, in view of the facts that they were submicroscopic in size and that the larger, microscopically visible particles had been identified as mitochondria. The isolation of microsomes is accomplished by the high speed centrifugation of extracts that have been cleared of nuclei, cells, mitochondria, and glycogen in previous centrifugations.

There has been considerable discussion whether the microsomes exist as such within the intact cell or whether they are produced during or after cell rupture. This question is difficult to answer, since these particles are too small to be seen in living cells. Claude (21), however, has presented evidence to show that the microsomes possess the same staining characteristics as the so-called ground substance of the cytoplasm. On the other hand, Lagerstedt (74), in an extensive study of livers of normal and starved animals, reached the conclusion that the microsomes obtained by cell fractionation are probably breakdown products of the basophilic inclusions present in the cytoplasm of fixed liver cells. It seems possible, however, that these inclusions may themselves be aggregates produced by the cytological procedures. Although the exact derivation of microsomes is thus not clearly established, studies with the electron microscope (27, 106) have revealed the presence of cytoplasmic structures too small to be visible in the optical microscope. The size of these structures is of the same order as that of microsomes, as estimated for the latter by their behavior in the centrifuge.

Procedure for the complete fractionation of tissues.—In the past, the tendency has been to concentrate on the isolation of a single tissue fraction and to discard the remainder of the tissue and ignore it entirely or to estimate the properties of the remainder by difference. Although such a policy may be of value in gaining an idea of the properties of a given cell fraction, from the standpoint of the cell as a whole the procedure may lead to an entirely erroneous impression of the role played by a given portion of the cell. To obtain information on the contributions made by each portion of the cell, comprehensive fractionation procedures have been devised and applied to various normal and tumor tissues (55, 58, 60, 61, 103, 122–125, 129–132). These methods permit the separation of the tissue into four fractions: a nuclear fraction, a mitochondrial fraction, a submicroscopic particulate or microsome fraction, and a supernatant or soluble fraction. The preparation and properties of the first three fractions have been already described in previous paragraphs. The soluble fraction is merely the supernatant fluid remaining after removal of nuclei, mitochondria, and microsomes and contains, in addition to soluble material, lipid droplets that have migrated centripetally.

The Results of Studies of Isolated Tissue Fractions

Nuclei.—No adequate determinations have been made of the proportions of the tissue mass that is represented by the nuclei. This is largely due to the fact that most studies on nuclei have dealt with their isolation in a "purified" state without attention to yield. On the other hand, studies in which the nuclei have been isolated quantitatively from the tissue have failed to yield a cytologically pure fraction. However, investigations of the latter fraction do permit an estimate of the nuclear content of tissues. Thus, present results indicate that less than 15 per cent of the total nitrogen of rat (129) and mouse (130) liver homogenates and 23 per cent of rabbit liver (79), rat kidney (132), or mouse hepatoma (130) nitrogen is present in this fraction. Values as low as 5 per cent of the total protein of mouse liver have been reported for nuclei isolated with citric acid (8) but from the low protein-DNA ratio reported for these nuclei, it seems clear that they had lost a considerable amount of their original protein (105). Marshak (83) has reported that the nucleus of the mouse liver cell occupies only 6 per cent of the cell volume, on the basis of cytological measurements. However, the ratio of cytoplasmic volume to nuclear volume on sections of rat liver indicated that the nuclear volume was about 15 per cent of the total cell volume (121). More recently, Mirsky and Ris (93) stated that nuclear volumes in their liver preparations ranged from 10 to 18 per cent. It would appear that the total nitrogen content of the nuclear fraction approximates that expected from the latter two groups of cytological determinations.

The distribution of DNA has been studied in rat (60, 112, 122, 125, 129), rabbit (79), and mouse liver (131); rat kidney (122, 132); normal and leukemic mouse spleen (103); primary rat liver tumors (115, 123); and mouse hepatomas (131). The results of these studies have demonstrated that essentially all the DNA present in the tissues is recovered in the nuclear fraction. These findings provide confirmation of cytological studies which have indicated that DNA is exclusively a nuclear constituent. Cases (115, 131) in which the entire tissue DNA was not recovered in the nuclear fraction can probably be explained on other grounds,

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

e.g., fragmentation of nuclei during the isolation procedure or the presence in the original whole tissue of nuclear fragments resulting from the disintegration of nuclei.

Although the nuclear fraction obtained by the systematic fractionation of tissues is admittedly a mixture of nuclei with some mitochondria and unbroken cells, the results that have been obtained with this fraction in certain comparative studies have been sufficiently striking to eliminate nonnuclear contaminants as a source of great error. Thus, it was observed several years ago that the nuclear fraction of primary rat liver tumors induced with 4-dimethylaminoazobenzene (DAB) contained much larger amounts of DNA and of dry material than did the nuclear fraction of normal liver (123). The data therefore implied that the liver tumor contained more nuclei per volume of tissue than did normal liver, since the increase in DNA in the liver tumor was paralleled by an increase in dry weight. This view was supported by determinations of the ratio of cytoplasmic volume to nuclear volume in sections of the two tissues (121). The latter studies indicated that this ratio was much greater for normal liver than for liver tumor, and the magnitude of the difference was approximately sufficient to account for the increased amounts of DNA and dry weight in the nuclear fraction of the tumor. These findings were confirmed and extended by Price *et al.* (113–116), who were able to show that the DNA and protein content of the nuclear fraction obtained from the livers of rats fed carcinogenic azo dyes increased after only short periods of feeding and long before tumors appeared. They were also able to show that the number of nuclei increased in proportion to the increase in DNA, with the result that the amount of DNA per nucleus remained approximately constant (116). Thus, it appeared from these studies that the carcinogenic process in liver and increased cellularity were intimately related. More recent work, however, indicates that this increased cellularity is apparently much greater in azo dye than in other types of carcinogenesis, because in the case of liver tumors induced with acetylaminofluorene (48) and of a transplantable mouse hepatoma (131) the DNA content (and presumably the degree of cellularity) was only slightly increased above that of control livers. Furthermore, in the case of normal and leukemic spleens (103), the DNA content of the leukemic spleen was somewhat lower than that of the normal spleen. A possible explanation for the findings in azo dye carcinogenesis is extensive proliferation of bile duct epithelium.

The presence of PNA in the liver nucleus appears to be definitely established, but its exact concentration remains to be determined. The strongest evidence for the presence of PNA in the nucleus comes from studies with radioactive phosphorus, which have demonstrated that the PNA present in isolated nuclei has a much higher turnover rate than the PNA associated with other fractions of the liver cell (68, 84). Recent analyses by Dounce *et al.* (37) on nuclei isolated from rat liver by means of the Behrens' procedure (9) indicate that the PNA concentration in nuclei may be much greater than was previously supposed. PNA concentrations 1.27 and 1.78 times as great as the DNA concentrations were obtained in two separate experiments. On the assumption that all the DNA was present in the nuclei and that the DNA and PNA contents of the liver were 250 and 1,000 mg. per cent, respectively (121), this finding would mean that 32–45 per cent of the total PNA was present in the nucleus. This would appear to be much too high in view of other studies in which it was found that about 80–90 per cent of the total liver PNA was present in the mitochondrial, submicroscopic particulate and supernatant fractions (60, 125, 131, 132). The possibility that the nuclei isolated by the Behrens' procedure could be contaminated by submicroscopic material was considered to be improbable by these authors (37) in view of the method used for the isolation of the nuclei. However, another possibility that should be considered is that the submicroscopic particles or the PNA they contain are actually localized in the liver nucleus and are released during the isolation procedures in aqueous media. If such is the case, it is certainly remarkable that the presence of such large amounts of PNA in the nucleus has escaped the attention of the cytologist.

The lipid content of rat liver nuclei has been studied by Dounce (35), who found that the amount of lipid present depended upon the pH at which the nuclei were prepared. Thus, at pH 6 the nuclei contained as much as 10.8 per cent lipid, while at pH 4 they contained only 3.2 per cent. The low values were considered to be more nearly correct, because, according to Dounce (35), nuclei prepared at this pH do not lose either DNA, lipid, or protein. The data do not entirely support this statement, however, because in several instances the DNA contents of nuclei prepared at the two pH's did not differ markedly. Further work will be required to clarify this point. In regard to the nature of the lipid associated with nuclei, it would appear that phospholipid was absent, because Dounce *et al.* (37) have reported that all the phosphorus of isolated nuclei was accounted for by acid-soluble and nucleic acid phosphorus.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

The occurrence of free amino acids in nuclei isolated by the Behrens' technic was studied by the use of paper chromatographic procedures (37). The distribution of amino acids in isolated nuclei was similar to that obtained with whole liver cells.

The association of enzymes with isolated nuclei has been studied by Dounce and his associates and reviewed previously (36). Most of these studies have been made with nuclei isolated in dilute citric acid, although some have employed the nuclei isolated with the Behrens' technic. Interestingly enough, the enzymatic activity of the latter nuclei was as great as that of the former, in the case of two enzymes studied (37). The enzymes that have been found to be associated with nuclei include aldolase, D-amino acid oxidase, arginase, catalase, cytochrome oxidase, enolase, esterase, acid and alkaline phosphatases, phosphorylase, lactic dehydrogenase, and uricase. With the exception of alkaline phosphatase, the concentration of the enzymes in the isolated nuclei was the same as or lower than the concentration in the whole tissue. The concentration of alkaline phosphatase in the isolated nuclei was about twice as great as in the whole tissue. It is not clear what significance can be attached to the association of these enzymes with nuclei, inasmuch as the total enzymatic activity present in the nuclei usually represented only a small fraction of the total tissue activity. Thus, in the case of cytochrome oxidase, studies with the nuclear fraction have shown that as little as 5 per cent of the total liver activity is present in this fraction (122). In view of the fact that 75–80 per cent of the total liver activity has been found to be associated with the mitochondria and that the latter are contaminants of the nuclear fraction (122, 130), it would appear that the total activity of the nucleus may well be much less. Thus, as has been pointed out in earlier paragraphs, the possibility that enzymes were adsorbed on the nuclei or that the nuclei were contaminated must be considered in view of the small amount of enzymatic activity present in the nuclei and the fact that the enzyme concentrations in the nuclei were less than, or the same as, those in the whole tissue.

Studies on other enzymatic properties of the nuclear fraction have indicated that nuclei may contain high concentrations of certain phosphatases. Thus, high concentrations of ATP-ase and AMP-ase have been reported for the nuclear fraction isolated from rat liver (98, 122), rat liver tumor (123), mouse liver, and mouse hepatomas (131). In the case of AMP-ase, the nuclear fraction accounted for as much as 45 per cent of the total activity of rat liver (98), while, in the case of ATP-ase, somewhat lower percentages were present in the nuclear fraction.

Chromosomes.—Chromosomes have been isolated from a number of tissues, including thymus (91–93), a rat lymphosarcoma (28), normal and leukemic mouse spleen (104), normal and hyperplastic epidermis, and a squamous-cell carcinoma (45). One of the major constituents of the chromosomes is DNA. In the case of the first four tissues mentioned, the DNA content of the chromosomes was 37 per cent (28, 92, 104). On the other hand, the DNA content of the chromosomes obtained from normal and hyperplastic epidermis was only about 12 per cent, while that of the squamous-cell carcinoma was approximately 21 per cent (45). No data have been reported which permit an estimate of the proportion of the total tissue DNA that is associated with the chromosomes. Thus, although it is apparently established that the entire DNA of the tissue is present in the nuclei, it is not clear whether DNA can exist in the nucleus apart from the chromosomes.[1] The latter problem would appear to be of considerable importance from the standpoint of the postulated genic properties of DNA.

PNA has also been found to be present in isolated chromosomes but in considerably lower concentrations than DNA (104). The association of PNA with isolated chromosomes must be considered in light of the fact that the chromosomes contain adhering nucleoli which are known to contain high concentrations of PNA. The amount of PNA accounted for by the nucleoli remains to be established, and the low concentrations of PNA in the chromosomes also need to be assessed in regard to the high concentration of PNA found by Dounce *et al.* (37) in nuclei isolated by the Behrens' procedure.

Proteins represent another major component of isolated chromosomes and are of two types in the case of mammalian chromosomes (85, 92). The main protein is of the histone type, although a smaller amount of an acidic protein is also present. Little is known concerning possible enzymatic functions of chromosomal proteins.

Chromosomes have been fractionated by means of 1 M NaCl into a soluble portion and a residue possessing the morphological properties of the original chromosomes (91–93). The soluble fraction contains most of the DNA and histone, while the residual chromosome contains PNA, some DNA, and the nonhistone protein. In the case of the residual chromosomes obtained from leukemic spleen, the PNA content was about twice as great as that of the residual chromosomes of normal spleen (104). It has also been stated that the entire

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110010001.pdf by guest on 24 August 2022

alkaline phosphatase activity of thymus chromosomes is localized in the residual chromosome (89).

Mitochondria.—The use of data obtained by the cell fractionation technic in estimating the proportion of the cell mass represented by mitochondria is somewhat complicated by the fact that none of the procedures used in the homogenization of tissues allows disruption of all cells. A variable proportion of the mitochondria is thus present in the intact cells of the nuclear fraction. Another factor influencing the yield of mitochondria is that complete separation of free mitochondria from nuclei is not readily obtained. The data obtained from cell fractionations involving cell disruption with the Potter-Elvehjem homogenizer and carried out in solutions of nonelectrolytes (to avoid aggregation of particles) probably provide a basis for the most reliable estimates of the mitochondrial content of tissues.

In experiments carried out with sucrose solutions (either 0.25 M or 0.88 M) as media, the mitochondrial fraction isolated from rat liver accounted for 23–26 per cent of the total nitrogen (60, 125, 130, 132) and 30–33 per cent of the total protein (112, 115) of the original homogenate. The mitochondria of C3H mouse liver contained 24 per cent of the original total nitrogen (130). A much lower value (11 per cent of the total nitrogen) was obtained with rabbit liver (79). Somewhat lower values have also been obtained with such media as water (122, 123, 128) and isotonic NaCl (6, 66, 99, 128).

In view of evidence that mitochondria probably contain all the succinoxidase activity of the liver cell (60), it is possible to determine roughly the amount of mitochondrial material in the nuclear fraction from the succinoxidase activity of the latter. On this basis it can be estimated that mitochondria account for 30–35 per cent of the total nitrogen of rat or mouse liver.

A finding of interest is that rat and mouse hepatomas contain considerably less mitochondrial material than does normal liver (115, 123, 130). In this respect, Price *et al.* have noted a decline in the mitochondrial protein of livers of rats fed 4-dimethylaminoazobenzene (DAB) and a number of derivatives of DAB for periods insufficient to produce tumors (114, 116). Furthermore, the extent of this decline was roughly proportional to the carcinogenicity of the compound fed. A toxic but noncarcinogenic derivative, 2-methyl-4-dimethylaminoazobenzene (2-MeDAB) had the remarkable effect of producing a considerable rise in the protein of the mitochondrial fraction (109, 116).

Relatively few data are available showing the mitochondrial content of other tissues. Kidney mitochondria accounted for 17 per cent of the dry weight and 20 per cent of the total nitrogen of the whole tissue (122, 132). The mitochondria of the Flexner-Jobling rat carcinoma contained 8 per cent of the total nitrogen of the tumor (79). Values of 2.5 and 2.2 per cent were found by Petermann *et al.* (3, 103) for the mitochondrial nitrogen of normal and leukemic mouse spleen. These extremely low values are probably a reflection of the small number of mitochondria in the cells of lymphoid tissue and of the fact that a considerable proportion of the cells were not disrupted by homogenization (3, 103).

The PNA content of isolated mitochondria has been determined by a number of investigators. Most of the results obtained with rat and mouse liver indicate that the concentration of PNA in mitochondria is somewhat lower than in whole liver and much lower than in submicroscopic particles. It has been shown that the PNA content of mitochondria declines on repeated sedimentation, as a result of the removal of submicroscopic particles, and eventually reaches a constant level after three or four sedimentations (60). Thus, the PNA content of mitochondria can be used as an index of the degree of contamination of this fraction with submicroscopic particles.

A number of determinations (55, 60, 97, 122, 125, 132) have yielded an average value of 11 (range: 7 to 13) μg. of PNA phosphorus per milligram of total nitrogen for rat liver mitochondria isolated in sucrose solutions, as compared with a value of 27 (range: 23 to 30) for the original whole tissue and 63 (range: 46 to 77) for submicroscopic particles. The proportion of the total PNA of whole rat liver present in mitochondria appears to be approximately 15 per cent.

Results obtained with the mitochondria of mouse liver (131), rabbit liver (79), and rat kidney (122, 132) for the most part indicate a concentration of PNA in the same general range as that obtained with rat liver. Barnum and Huseby (6) found a somewhat high value for mouse liver mitochondria; later, however, these investigators pointed out that considerable amounts of submicroscopic particulate material contaminated their mitochondrial preparation, presumably because of the use of 0.85 per cent NaCl as the medium for fractionation (66). Ada (2) reported a very high value for rabbit liver mitochondria and did not confirm the unequal PNA distribution between mitochondria and submicroscopic particles previously noted by others. Ada's results are open to question, however, because they were not obtained by direct PNA determinations but on the assumption that only PNA phosphorus remained

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001001.pdf by guest on 24 August 2022

after extraction of the preparations with ethanol-ether and ether.

The concentration of PNA in the mitochondria of tumors derived from the livers of rats and mice is, in general, higher than the concentration of PNA in normal liver mitochondria (115, 123, 131). The proportion of the PNA of whole tissue recovered in the mitochondrial fraction is, however, less in hepatomas than in normal liver because of the great decrease in the amount of mitochondrial material in the tumors. In this respect, Price *et al.* (114, 116) found that the decline in the total amount of PNA recovered in the mitochondrial fraction obtained from rats fed DAB and related compounds generally followed the decline in the total protein of the fraction. Of additional interest in these experiments was the finding that 2-MeDAB, which caused an increase in mitochondrial protein, produced a considerable decrease in mitochondrial PNA. The PNA content of mitochondria isolated from leukemic spleen was not greatly different from that of normal spleen mitochondria (3, 103).

It should be pointed out that the relatively low PNA concentration in mitochondria brings up the question whether the PNA found in the fraction may be due entirely to submicroscopic particles not completely removed by repeated sedimentation of mitochondria. This is a difficult question to answer in view of the established principle that the presence of a substance in a cell fraction in a lower concentration than in the original whole tissue should be interpreted with caution. Certain other observations, however, including electron microscopical evidence for the homogeneity of the mitochondrial fraction (60), the effect of ribonuclease on mitochondria (144), and their staining properties (60), indicate that mitochondria contain PNA.

A few studies have been made of the lipides of mitochondria (2, 6, 24, 122, 123). In general, the results indicate that 25–30 per cent of the dry weight of liver mitochondria consists of lipides, of which approximately two-thirds is present in the form of phospholipide. It may be mentioned that the concentration of lipide, like that of PNA, is considerably lower in mitochondria than in submicroscopic particles.

The association of enzyme activity with mitochondria has been the subject of extensive investigations in the last few years. As indicated in a previous review (126), many of the earlier data were obtained from experiments involving the isolation of a mixture of mitochondria and submicroscopic particles (13, 17, 46, 135). In addition, both these and many later results are open to the serious objection that not all fractions of the tissue were analyzed, and it is therefore not possible to draw up balance sheets or to compare the enzymatic activity of mitochondria with that of the original whole tissue. In short, so many enzymatic functions have been ascribed to the mitochondrion that the remainder of the cell would almost seem to be excess baggage.

The most striking and the earliest discovered enzymatic property of mitochondria is their content of cytochrome oxidase and certain related respiratory enzyme systems. It has been clearly demonstrated, for example, that by far the majority of the cytochrome oxidase and succinoxidase of rat (56, 60, 122, 123, 128) and mouse (130) liver and of rat kidney (122) is recovered in the mitochondrial fraction (cf. 15), and it seems entirely likely that the small amount of activity shown by other fractions is the result of contamination with mitochondria (60). Other respiratory enzyme systems concentrated in liver mitochondria are octanoic acid oxidase (70, 71, 125) and oxalacetic acid oxidase (132). In both of these systems, however, the activity of mitochondria is enhanced by the addition of other fractions which alone have little or no activity. The enhancement is slight in the case of octanoic acid oxidase and pronounced in the case of oxalacetic oxidase, both submicroscopic particles and final supernatant having the effect. The fact that mitochondria are capable of oxidizing oxalacetate is of particular interest, since it is an indication, in addition to their content of cytochrome oxidase and succinoxidase, of their participation in the Krebs cycle reactions. The role played by other cell fractions in the oxidation of oxalacetate, together with recent data on the oxidation of *d*-isocitrate (61), makes it clear, however, that mitochondria are not *solely* responsible for the Krebs cycle reactions.

Additional enzyme systems associated with liver mitochondria are DPN-cytochrome c reductase (55, 58) and TPN-cytochrome c reductase (61). It may be noted that both these systems are also present in submicroscopic particles, DPN-cytochrome c reductase being concentrated to a greater extent in the latter fraction than in mitochondria. The other system is more concentrated in mitochondria. Several phosphatases are also concentrated in the mitochondrial fraction of liver; these include acid phosphatase (98), AMP-ase (98), and ATP-ase (98, 122, 131). A large proportion of the uricase activity of whole liver was also recovered in the mitochondria (120).

The system capable of synthesizing *p*-aminohippuric acid (PAH) was recovered almost in its entirety in the mitochondrial fraction (72). In

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

these and other experiments (110) it was also found that mitochondria effectively maintained high levels of ATP under conditions optimal for the synthesis of PAH. More recently, it has been shown that the particles, in the presence of an oxidizable substrate such as glutamate or α-ketoglutarate, are capable of synthesizing ATP from AMP at a rapid rate.[3] The presence of respiratory enzymes in mitochondria and their ability to carry out aerobic phosphorylation are, of course, indicative of an enzyme organization capable of supplying energy for other synthetic reactions, including the synthesis of peptides and proteins. Although recent work has indicated that mitochondria can incorporate glycine and lysine into proteins (12), the significance of such findings with respect to the participation of mitochondria in the synthesis of proteins is not as yet clear.

Cytochrome c (128, 129), vitamin B_6 (113), and riboflavin (112) are concentrated in mitochondria to a considerable extent, the latter finding indicating the probable presence of flavoproteins not as yet studied. Catalase was found in mitochondria but not in appreciably greater concentration than in whole liver (42). Most of the catalase activity was recovered in a supernatant containing submicroscopic particles and soluble material.

A number of other enzymes and related compounds have been detected in suspensions of liver mitochondria. Further work will be necessary to clarify the situation with respect to these findings, however, because complete recovery data are not available. Among the enzymes found are D-amino acid oxidase (22), α-glycerophosphate dehydrogenase (22), ribonuclease (22), citrullin synthesis (96), "myokinase,"[3] and the oxidation of citrate (71), α-ketoglutarate (71), and glutamate (72). Recently, Hird and Rowsell (53) have reported that the insoluble particle fraction of rat liver homogenates contained all the glutamate-phenylpyruvate transaminase activity of the whole tissue and was also able to catalyze the formation of tyrosine, alanine, and aspartate from the corresponding keto-acids. The supernatant catalyzed transamination between glutamate, aspartate, and alanine only. According to these investigators (53), mitochondria isolated in 0.88 M sucrose were found to be active in promoting the reactions found in the insoluble particle fraction. Other workers have also detected vitamin A in preparations of mitochondria (41, 43).

The enzyme content of the mitochondria of liver tumors is markedly different in several respects from that of normal liver mitochondria. Thus, the specific succinoxidase, cytochrome oxidase, and ATP-ase activities of rat liver tumor mitochondria were 3–5 times lower than the corresponding specific activities of normal rat liver mitochondria (123). A similar picture for these three enzyme systems has been obtained in studies of C3H mouse liver and C3H mouse hepatoma 98/15 (130, 131). DPN-cytochrome c reductase, on the other hand, was found in the latter studies to be present in much higher concentration in hepatoma mitochondria than in liver mitochondria (58). The synthesis of PAH, a function of normal liver mitochondria, is carried out by hepatoma 98/15 homogenates and mitochondria at a negligible rate (72). Whether the latter finding is a reflection of the absence of the PAH-synthesizing enzyme itself or the result of the inability of the tumor mitochondria to maintain ATP levels (a condition necessary for PAH synthesis) is an interesting question. The extremely low activity of tumors in the oxidation of oxalacetate (108) and octanoate (5) is probably also a reflection, at least in part, of a defect in the mitochondria. Recent studies of the amino acid composition of the mitochondria of a number of tissues, including several tumors, revealed no qualitative differences (81). The amino acid composition of the *total proteins* of the nuclear, mitochondrial, microsomal, and supernatant fractions has been studied by Schweigert *et al.* (133). These workers observed several differences in the amino acid composition of the proteins obtained from the fractions of normal liver, the livers of animals fed the carcinogen, DAB, and the liver tumors induced by DAB.

Green *et al.* (47) have described an enzyme suspension made by preparing homogenates (by means of the Waring Blendor) of various tissues (e.g., liver, kidney, heart) in isotonic KCl, centrifuging the preparation at 2,000 *g*, and washing the sediment several times in the same medium. This sediment (cf. the washed liver residue of Lehninger and Kennedy [78] and Cohen and McGilvery [29]) has been shown to catalyze the complete oxidation of pyruvic acid, fatty acids, and amino acids to CO_2 and H_2O through the citric acid cycle, and has been given the name "cyclophorase" to indicate that it is an integrated enzyme complex and thus, presumably, a cellular entity. The mode of preparation of cyclophorase is such as to indicate the presence of whole cells, nuclei, nuclear fragments, mitochondria, mitochondrial fragments, and submicroscopic particles, the latter probably being present as a result of aggregation in the presence of KCl (61, 66). Harman (49) has recently reported experiments designed to prove that cyclophorase is associated

[3] R. K. Kielley and W. W. Kielley, unpublished experiments.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

with the mitochondria present in the sediment, and the terms cyclophorase and mitochondria are now used interchangeably (50, 64, 136). Since this conclusion by Harman has important cytochemical implications, it is felt that the evidence supporting it should be discussed in some detail.

A correlation of cyclophorase activity with mitochondria (49) was based on the following findings: (*a*) When nuclear fragments were separated from cell-free cyclophorase preparations, a portion of the activity (21–61 per cent), as indicated by oxygen uptake in the presence of α-ketoglutarate, remained. (*b*) It was stated that nuclear and "microsomal" elements of cyclophorase were incapable of oxidizing α-ketoglutarate. (*c*) Prolonged subjection of cyclophorase to the action of the Waring Blendor and of certain "transforming agents" resulted in disintegration of or damage to mitochondria and at the same time inactivated α-ketoglutarate oxidation. (*d*) An association of oxidative phosphorylation with the mitochondria of cyclophorase was demonstrated.

In the opinion of the reviewers, these findings do not constitute decisive proof that the cyclophorase activity of cyclophorase is associated with the mitochondria of the preparation. As mentioned previously, both α-ketoglutarate oxidation and oxidative phosphorylation have been demonstrated to occur in suspensions of mitochondria that have been freed from other cell constituents by differential centrifugation of liver homogenates (71, 72). These reactions can therefore be considered tests for mitochondria; they are not necessarily tests for cyclophorase, as *originally* defined, since complete oxidation of pyruvate to CO_2 and H_2O was not shown to occur. Thus, their use as tests for cyclophorase only leads to confusion concerning the definition of the term cyclophorase.

Another and perhaps more decisive indication of the association of cyclophorase activity with mitochondria would be the demonstration that the majority of the cyclophorase activity of *whole tissue* is recovered and concentrated in the mitochondrial fraction and that other cell fractions do not contribute to the activity of the whole tissue. That such is probably not the case has already been shown, however, by experiments involving the oxidation of the citric acid cycle substrates, oxalacetate (132) and *d*-isocitrate (61). An important point, which was demonstrated in the latter experiments with *d*-isocitrate (61) and which may have some bearing on the interpretation of the finding of a large number of oxidases (64) in cyclophorase, is based on the fact that cytochrome oxidase is localized, probably exclusively, in mitochondria (56, 122, 130). Thus, in the study of the intracellular distribution of any system ultimately dependent on cytochrome oxidase for oxygen uptake, the only fractions that could be expected to take up oxygen would be the mitochondria and the nuclei, the latter because of contamination by mitochondria. If the mitochondrial fraction contained only a small proportion of the total activity of a given dehydrogenase of the whole tissue (e.g., the isocitric dehydrogenase of liver [61]), the addition of the appropriate substrate would result in oxygen uptake, provided a large enough quantity of mitochondria was used. Results of this type can be very misleading from the cytochemical standpoint unless all fractions of the tissue are analyzed, a complete balance sheet drawn up, and unless the method of enzyme assay is a direct measure of the activity of the enzyme in question.

The finding that some enzymes (e.g., "lactic oxidase") are very readily removed from cyclophorase or occur largely in the supernatant after the first sedimentation of cyclophorase has been explained by Still and Kaplan (136) as the result of damage to mitochondria. It is further suggested that methods have not been devised for isolating mitochondria in their original state. In the reviewers' opinion, these findings can also be interpreted in another way, namely, that the enzymes in question are not associated with mitochondria, either within or outside the cell.

The gel-like consistency of cyclophorase has been considered a striking property of the preparation (64, 65, 136). In this respect, the reviewers have never observed gel-formation by mitochondria that have been washed numerous times with a variety of media. On the other hand, repeated homogenization and sedimentation of the nuclear fraction cause this pellet to swell and to assume a gel-like consistency. Thus, it seems possible that the gel-like properties of cyclophorase are due to the presence of nuclei and nuclear fragments rather than to mitochondria.

Recently, Harman (50) has questioned the evidence offered by a number of investigators for the existence of a mitochondrial membrane. Utilizing cyclophorase as a source of material, Harman has concluded that the behavior of mitochondria is compatible with a gel-like structure and does not require the presence of a semipermeable membrane. This conclusion is mainly based on an apparent lack of lysis of mitochondria in deionized water and a nonselective penetration of sodium and potassium ions. It may be pointed out that Harman's observations with respect to the lysis of mitochondria are directly contradictory to the observations of a number of other investigators (23, 24, 32, 56, 101). In addition, it appears doubt-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001001.pdf by guest on 24 August 2022

ful that a study of selective penetration of sodium and potassium ions is a crucial test for the presence of a membrane other than that of the erythrocyte. Finally, no explanation is offered for the fact that a structure having the morphological characteristics of a membrane has been seen repeatedly (26, 32, 66, 95) in electron micrographs of isolated mitochondria. Available evidence would seem, therefore, to be in favor of the existence of a mitochondrial membrane. It should be pointed out, however, that the mitochondrial membrane, if existent, is certainly unusual in its elasticity, for the particles will increase greatly in volume before lysing in hypotonic media (23, 24).

The probable existence of a mitochondrial membrane has brought up the question whether this membrane remains sufficiently intact after isolation of the particles to retain soluble compounds. Evidence for the integrity of the membrane was first indicated by the experiments of Claude (24), who found that after prolonged contact with distilled water the large granules of guinea pig liver underwent lysis, yielding particulate material of small size and a soluble fraction that included proteins and dialyzable compounds. More recently, it has been found that the disruption by means of sonic vibrations of the membranes of isolated rat liver mitochondria results in the release into solution of approximately 60 per cent of the total nitrogen (57). Furthermore, most of the nitrogen represents proteins that can be characterized on the basis of sedimentation constants in the analytical centrifuge. Similar results (59) have been obtained by disintegration of mouse liver mitochondria in an apparatus similar to that described by Milner *et al.* (88). Studies with the analytical centrifuge (59) also showed that three components of the mixture of proteins obtained from the mitochondria of C3H mouse hepatoma 98/15 corresponded in sedimentation constants to the proteins of normal liver mitochondria. A fourth component, present in the latter preparation, could not be detected in the hepatoma preparation.

In these experiments it was found that disintegration of mitochondria was accompanied by partial to complete inactivation of several complex enzyme systems (e.g., succinoxidase, octanoic acid oxidase) (57). Less complex enzymes were affected only slightly (57). These findings suggest that the functioning of a complicated enzyme system is dependent on a definite, spatial arrangement of its individual components and thus on the structural integrity of the mitochondrion.

Melanin granules.—The biochemical properties of melanin granules isolated from mammalian tissues have been the object of several studies. Hermann and Boss (52), utilizing the ciliary body as a source of material, found dopa (3,4-dihydroxyphenylalanine) oxidase, cytochrome oxidase, and succinoxidase activity in preparations of isolated pigment granules. No tyrosinase activity was detected. DuBuy *et al.* (38) made similar studies of the granules isolated from both melanotic and "amelanotic" melanomas of the mouse. Pigment-producing enzymes, both dopa oxidase and tyrosinase in one case and only dopa oxidase in another, were associated with the granules isolated from two pigmented melanomas. The colorless granules obtained from an amelanotic tumor did not possess dopa oxidase or tyrosinase activity. All preparations, however, contained cytochrome oxidase and succinoxidase.

The results of these two studies bring up the interesting question of a possible relationship between mitochondria and melanin granules (cf. the relationship between mitochondria and plastids [39]), since preparations of the latter showed respiratory enzyme activity characteristic of mitochondria. The possibility that the respiratory enzymes were present in the suspensions of melanin granules as a result of contamination by mitochondria was considered by duBuy *et al.* (38) and apparently ruled out by microscopic studies of the preparations obtained from the highly pigmented Harding-Passey melanoma. It was concluded that the data were consistent with the view that the melanoma granules represent a modified form of mitochondria. The possible presence of mitochondria too small to be seen in the optical microscope, however, arises from the results of studies by Dalton *et al.* (32).

The recent studies of Recknagel (117) may have some bearing on the problem. Utilizing mature eggs of the frog as a source of material, Recknagel was able to separate mitochondria from melanin granules and demonstrated that the cytochrome oxidase of the whole cells was associated with the former type of particle. Since these results were obtained from amphibian rather than mammalian tissue, however, they are not strictly comparable to the results reported by duBuy *et al.* (38).

Glycogen.—Particulate glycogen has been isolated from guinea pig livers by Lazarow (76, 77) and Claude (24). The actual yield of glycogen was 6.5 per cent of the total dry weight of the liver, but it was estimated that the probable concentration of particulate glycogen in the liver was 10–15 per cent (24). Particulate glycogen contains 92–93.5 per cent glycogen and 1 per cent protein according to Lazarow (76, 77). The nitrogen values reported by Claude (24) for particulate glycogen are in agreement with the latter. Claude also reported

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

that particulate glycogen contained sulfur in the same concentration as nitrogen. The significance of the presence of protein and sulfur in particulate glycogen is not known, but Lazarow (77) suggests that the protein may serve as a framework for maintaining glycogen in particulate form, because agents which disperse the glycogen markedly alter proteins.

Submicroscopic particles.—It is of interest that the submicroscopic particle (or microsome) content of several tissues approaches the mitochondrial content. In studies of rat liver fractions (60, 125, 132), 18–20 per cent of the total nitrogen was accounted for by submicroscopic particulate material. The microsomes of mouse liver accounted for 23 per cent of the total nitrogen (130), and the corresponding fraction from rabbit liver contained 15 per cent (79). A lower recovery of submicroscopic particles from mouse liver, reported by Barnum and Huseby (6), was later explained (66) on the basis of the use of isotonic NaCl as the medium, the electrolyte apparently having caused aggregation of the particles and difficulty in separating them from mitochondria (cf. [61]). The microsome content of rat kidney (132), on the basis of total nitrogen, was 16 per cent. The amount of nitrogen recovered in the submicroscopic particles of C3H mouse hepatoma 98/15 (130) was somewhat lower than the corresponding value obtained with normal C3H mouse liver. This finding is of some interest, since the mitochondrial content of the tumor was much lower than that of normal liver (130). Low, and approximately equal, amounts of nitrogen were recovered, however, in the mitochondria and microsomes of the Flexner-Jobling rat carcinoma (79).

One of the most striking properties of submicroscopic particles, first suggested by the experiments of Claude (23, 24), is a high concentration of PNA. In later studies (60, 125, 131, 132), it has been shown that approximately 50 per cent of the PNA of whole rat or mouse liver is present in this fraction. Furthermore, as indicated earlier in this review, microsomes comprise the only liver fraction in which PNA is concentrated in terms of total nitrogen (6, 55, 60, 66, 125, 131, 132), the average PNA/N ratio (micrograms PNA phosphorus per milligram nitrogen) being 63 (55, 60, 125, 132), and 64 (115, 131) for rat and mouse liver microsomes, as compared to 27 and 28 for the corresponding whole tissues. Although PNA was also found to be concentrated in the microsomes of rat kidney (132), the recovery of the nucleic acid in this fraction of kidney was considerably lower than that in the microsomes of liver. A high concentration of PNA was noted in the microsomes of hepatomas (115, 131) and a rat carcinoma (79) and to a lesser extent in the microsomes of both normal and leukemic spleen (3, 103). A decrease in the PNA content of liver microsomes of rats fed DAB and related compounds was noted by Price *et al.* (114, 116). Barnum and Huseby (7) have recently studied the relationship between the mammary tumor agent and the microsomes obtained from lactating mammary gland. It was found that a large percentage of microsome PNA could be removed without loss of mammary tumor agent activity. It was concluded that supposedly purified preparations of the agent consisted largely of microsomes.

Several studies have been made of the lipide content of liver submicroscopic particles (2, 6, 24, 66). The results are generally in agreement, in that a relatively high concentration of lipide, mostly in the form of phospholipide, is present in this fraction. The data indicate that about 40 per cent of the dry weight of the particles is composed of lipide, a value considerably higher than that reported for mitochondria. Some further information on the composition of the microsome fraction was presented by Barnum and Huseby (6), who separated the fraction by means of differential centrifugation into two components. The faster sedimenting microsomes were rich in both lipide and PNA, whereas the slower sedimenting particles were relatively poor in lipide but rich in PNA.

Another striking characteristic of the microsome fraction is the pronounced red color of the particles when packed by centrifugation. Bensley (10) succeeded in extracting this pigment and studied some of its properties. The conclusion was reached that the pigment represented products of the oxidation of unsaturated fats and particularly of phospholipides.

As mentioned previously, Lagerstedt (74) concluded that isolated microsomes are probably breakdown products of cytoplasmic basophilic inclusions. In a cytological study of the effect of fasting and protein depletion, Lagerstedt reported that under these conditions the basophilic inclusions completely disappeared. Some doubt as to the correctness of Lagerstedt's conclusion regarding the relation of microsomes and the inclusion bodies arises from the experiments of Muntwyler *et al.* (97). The latter investigators found that severe protein depletion resulted in some decline in the amount of submicroscopic particulate material but that the fraction by no means disappeared.

Relatively little is known concerning the enzymatic properties of the microsomes. The only

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

enzymes that have been shown thus far to occur in the fraction in a concentration exceeding that in whole tissue are an esterase (methyl butyrase) studied by Omachi *et al.* (99), DPN-cytochrome c reductase (55, 58), and TPN-cytochrome reductase (61). Heller and Bargoni (51) also studied the distribution of esterase in liver fractions and recovered most of the activity in a supernatant containing microsomes and soluble material. This supernatant was not further fractionated. The specific enzyme activities reported by the latter investigators were considerably lower than those reported by Omachi *et al.* (99). In this respect, in a recent study of the determination of esterase in homogenates, Copenhaver *et al.* (30) obtained specific activities similar to those reported by Omachi *et al.*

It may be noted that all three of the above enzymes are also present in the other particulate fractions of liver. Both esterase and DPN-cytochrome c reductase are concentrated to a much greater extent in the microsomes than in any other fraction; TPN-cytochrome c reductase, however, is slightly more concentrated in mitochondria than in microsomes. A relatively small proportion of the total ATP-ase activity of normal mouse liver is recovered in the submicroscopic particles (131). In the case of a mouse hepatoma, however, much more ATP-ase is recovered in this fraction (131). DPN-cytochrome c reductase is also more concentrated in hepatoma microsomes than in normal liver microsomes (58).

Submicroscopic particles also play a role in certain other enzyme reactions, such as anaerobic glycolysis (79), the oxidation of oxalacetate (132), and the reductive cleavage of DAB (94). In the case of the latter reaction, TPN was reduced by the final supernatant in the presence of glucose-6-phosphate, and the reduced TPN was utilized by the particulate fractions in cleaving DAB, the microsome fraction being more active than either nuclei or mitochondria. Tagnon and associates (138, 139) found that microsomes isolated from lung activated serum proplasmin. Claude (24) has stated that submicroscopic particles contain thromboplastic activity.

As mentioned previously, the question of the origin of the microsomes is at present unsettled. Recently (49, 50, 64), use has been made of the term microsomes, indicating that they may be products of disintegrated mitochondria. In this respect, although it has been shown that disintegration of mitochondria does yield particles of smaller size, it has also been clearly shown that the latter particles, in their content of respiratory enzymes (57), are qualitatively different from the microsomes isolated from liver homogenates by differential centrifugation.

Soluble or supernatant fraction.—This fraction contains the nonsedimentable or soluble material present in the cell, as well as any material, such as lipide droplets, which would migrate centripetally because of its low density. It is to be noted, however, that the preparation of this fraction is usually an arbitrary one dependent upon the limiting centrifugal force available to the investigator. The conditions that we have employed for the isolating of microsomes (129) were chosen to sediment particles as small as 50 mμ in diameter. Thus, the supernatant fluid or soluble fraction would contain all particles *smaller* than 50 mμ or, in terms of molecular weight, all particles having molecular weights less than about 100,000,000.

The soluble fraction comprises a considerable proportion of the tissue in terms of total nitrogen or protein. Thus, 29–42 per cent of the total nitrogen of mouse liver has been recovered in this fraction (6, 66, 130), while values of 32–44 per cent have been reported for rat liver (55, 60, 113, 125, 129, 132) and 49 per cent for rabbit liver (79). The proportions recovered in the supernatant fraction of rat kidney (132), primary rat liver tumors (115), mouse hepatoma (130), and Flexner-Jobling carcinoma (79) were 47, 46, and 50 per cent, respectively, while in the case of normal and leukemic mouse spleen considerably lower proportions were recovered in this fraction (27 and 23 per cent [103]).

PNA is also present in the soluble fraction, although in most tissues its concentration in this fraction is lower than in the whole tissue. However, in the case of normal and leukemic mouse spleen (103) and of rat kidney (132), the concentration of PNA in the supernatant is greater than in the whole tissue. Furthermore, the total amount of PNA in the soluble fraction of leukemic spleen is much greater than that in the same fraction obtained from normal spleen (103). Similar increases in the PNA content of the soluble fraction have been reported for primary rat liver tumors (115), mouse hepatoma (130), the livers of rats fed the carcinogen 3′-methyl-DAB (116), and regenerating rat liver (111). These findings, together with the observations of Brachet and Jeener (13, 67) on the high PNA content of the soluble fraction of rapidly growing cells suggest that the PNA associated with this fraction may be involved in the carcinogenic process or at least the process of growth.

The main component of the soluble fraction appears to be protein in nature. Thus, Price *et al.* (112) reported that 39 per cent of the total protein

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001001.pdf by guest on 24 August 2022

of rat liver was recovered in the soluble fraction. A preliminary report of the electrophoretic properties of the soluble fraction of rabbit liver has been made by Sorof and Cohen (134), who found that four components were present. However, electrophoretic studies with calf thymus and human lymphoid tissue (1) indicate that the soluble fractions of these tissues were considerably more complex. Considering the enzymatic complexity of the supernatant it would appear unlikely that there could be as few as four components, unless the components were inhomogeneous or the enzymatic activities were bound to other proteins. Thus, in the case of rabbit liver and Flexner-Jobling carcinoma (79), all the enzymes involved in the glycolysis of glucose to lactic acid were found to be present in the soluble fraction, and the total activity of this fraction was sufficient to account for over 50 per cent of the total activity of the whole tissue. The mitochondria and microsomes of these tissues possessed essentially no glycolytic activity by themselves but produced pronounced stimulation of the supernatant, indicating that these fractions were deficient in some of the enzymes of the glycolytic cycle or that they contained necessary enzymes or cofactors required by the supernatant.

Other enzymes and related substances that occur in the supernatant of rat and mouse liver include cytochrome c (128, 129), isocitric dehydrogenase (61), and acid and alkaline phosphatase (98). The amount of cytochrome c present in the rat liver supernatant accounts for 35–40 per cent of the total liver cytochrome c, while in the case of isocitric dehydrogenase 82 per cent of the total liver activity was recovered in the supernatant. The distribution of acid and alkaline phosphatase in rat liver was considerably different from that of other phosphatases, such as ATP-ase (cf. above). Thus, in the case of the latter, most of the total liver activity was recovered in the nuclei and mitochondria, while 35–50 per cent of the total acid phosphatase and 55–70 per cent of the total alkaline phosphatase was recovered in the soluble fraction (98).

Miller and Miller (86) recently discovered that feeding azo dyes to rats resulted in the formation in the liver of dye-protein complexes in which the dye was tightly bound to the protein. The dye-protein complex was not found in other tissues of the rat, in the primary liver tumors produced by the dye, or in the tissues of other species in which these dyes are noncarcinogenic. The rate of formation of the dye-protein complexes in rat liver was found to be proportional to the carcinogenicity of the dye fed (87). In more recent studies (113–116), the intracellular distribution of the dye-protein has been studied, and it has been found that over 50 per cent of the protein-bound dye was always recovered in the supernatant fraction. The significance of this dye-protein complex and of its presence in the soluble fraction will require further study.

Julen, Snellman, and Sylven (69) have recently reported the results of cytological and fractionation studies made with mast cells in an attempt to determine the intracellular localization of heparin. The cytological studies indicated that the heparin was not bound to microscopically visible particles but was localized in the intergranular spaces. This was confirmed by fractionating mast cell extracts made by grinding ox liver capsules in a mortar with isotonic phosphate buffer and centrifuging at low speed to remove unbroken cells. The extract so obtained was further fractionated into large granules, microsomes, and a final supernatant remaining after 5–6 hours at 60,000 *g*. The latter contained 82 per cent of the heparin present in the cell-free extract. Electron microscopic examination of the supernatant showed the presence of particles with an estimated diameter less than 10 mμ. Electrophoretic and ultracentrifugal studies indicated that the heparin was bound to these particles in the form of a protein complex.

REFERENCES

1. Abrams, A., and Cohen, P. P. Electrophoretic and Chemical Characterization of Human Lymphoid Tissue and Calf Thymus. J. Biol. Chem., **177:**439–49, 1949.
2. Ada, G. L. Phospholipin Metabolism in Rabbit Liver Cytoplasm. Biochem. J., **45:**422–28, 1949.
3. Alfin-Slater, M. B.; Larack, A. M.; and Petermann, M. L. The Preparation and Properties of the Mitochondria and Submicroscopic Particles of Normal and of Leukemic Mouse Organs. Cancer Research, **9:**215–16, 1949.
4. Arnesen, K.; Goldsmith, Y.; and Dulaney, A. D. Antigenic Properties of Nuclei Segregated from Spleens of Normal and Leukemic Mice. Cancer Research, **9:**669–71, 1949.
5. Baker, C. G., and Meister, A. Studies on Fatty Acid Oxidation by Normal and Neoplastic Liver. J. Nat. Cancer Inst., **10:**1191–98, 1950.
6. Barnum, C. P., and Huseby, R. A. Some Quantitative Analyses of the Particulate Fractions from Mouse Liver Cytoplasm. Arch. Biochem., **19:**17–23, 1948.
7. ———. The Chemical and Physical Characteristics of Preparations Containing the Milk Agent Virus: *A Review.* Cancer Research, **10:**523–29, 1950.
8. Barnum, C. P.; Nash, C. W.; Jennings, E.; Nygaard, O.; and Vermund, H. The Separation of Pentose and Desoxypentose Nucleic Acids from Isolated Mouse Liver Cell Nuclei. Arch. Biochem., **25:**376–83, 1950.
9. Behrens, M. Untersuchungen an isolierten Zell- und Gewebsbestandteilen. I. Isolierung von Zellkernen des Kalbsherzmuskels. Ztschr. f. physiol. Chem., **209:**59, 1932.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

10. BENSLEY, R. R. On the Nature of the Pigment of Mitochondria and of Submicroscopic Particles in the Hepatic Cell of the Guinea Pig. Anat. Rec., **98:**609–20, 1947.
11. BENSLEY, R. R., and HOERR, N. The Preparation and Properties of Mitochondria. Anat. Rec., **60:**449–55, 1934.
12. BORSOOK, H. Protein Turnover and Incorporation of Labeled Amino Acids into Tissue Proteins *in vivo* and *in vitro*. Physiol. Rev., **30:**206–19, 1950.
13. BRACHET, J., and JEENER, R. Recherches sur des particules cytoplasmiques de dimensions macromoléculaires riches en acide pentosenucléique. I. Propriétés générales, relations avec les hormones, les protéines de structure. Enzymologia, **11:**196–212, 1944.
14. BRADFIELD, J. R. G. The Localization of Enzymes in Cells. Biol. Rev. (Cambridge), **25:**113–57, 1950.
15. BRENNER, S. The Demonstration by Supravital Dyes of Oxidation-Reduction Systems on the Mitochondria of the Intact Rat Lymphocyte. S. Afr. J. M. Sc., **14:**13–19, 1949.
16. CHAMBERS, R. The Isolation of Visible Protoplasmic Structures. Cancer Research, **10:**210, 1950.
17. CHANTRENNE, H. Recherches sur des particules cytoplasmiques de dimensions macromoléculaires riches en acide pentosenucléique. II. Relations avec les ferments respiratoires. Enzymologia, **11:**213–21, 1944.
18. ———. Hétérogénéité des granules cytoplasmiques du foie de souris. Biochem et Biophys. Acta, **1:**437–48, 1947.
19. CLAUDE, A. Particulate Components of Cytoplasm. Cold Spring Harbor Symp. Quant. Biol., **9:**263–70, 1941.
20. ———. The Constitution of Protoplasm. Science, **97:** 451–56, 1943.
21. ———. Distribution of Nucleic Acids in the Cell and the Morphological Constitution of Cytoplasm. Biol. Symp., **10:**111–29, 1943.
22. ———. Distribution of Enzymatic Activities in Fractions of Mammalian Liver. A.A.A.S. Research Conference on Cancer, pp. 223–26, 1944.
23. ———. The Constitution of Mitochondria and Microsomes and the Distribution of Nucleic Acid in the Cytoplasm of a Leukemic Cell. J. Exper. Med., **80:**19–29, 1944.
24. ———. Fractionation of Mammalian Liver Cells by Differential Centrifugation. I. Problems, Method, and Preparation of Extract. II. Experimental Procedures and Results. *Ibid.*, **84:**51–98, 1946.
25. ———. Studies on Cells: Morphology, Chemical Constitution, and Distribution of Biochemical Functions. Harvey Lectures, **43:**121–64, 1947–48.
26. CLAUDE, A., and FULLAM, E. F. An Electron Microscope Study of Isolated Mitochondria. J. Exper. Med., **81:** 51–62, 1945.
27. ———. The Preparation of Sections of Guinea Pig Liver for Electron Microscopy. *Ibid.*, **83:**499–504, 1946.
28. CLAUDE, A., and POTTER, J. S. Isolation of Chromatin Threads from the Resting Nucleus of Leukemic Cells. J. Exper. Med., **77:**345–54, 1943.
29. COHEN, P. P., and MCGILVERY, R. W. Peptide Bond Synthesis. III. On the Mechanism of *p*-Aminohippuric Acid Synthesis. J. Biol. Chem., **171:**121–33, 1947.
30. COPENHAVER, J. H.; STAFFORD, R. O.; and MCSHAN, W. H. The Determination of Esterase in Animal Tissue Homogenates. Arch. Biochem., **26:**260–68, 1950.
31. CUNNINGHAM, L.; GRIFFIN, A. C.; and LUCK, J. M. Effect of a Carcinogenic Azo Dye on Liver Cell Structure. Isolation of Nuclei and Cytoplasmic Granules. Cancer Research, **10:**194–99, 1950.
32. DALTON, A. J.; KAHLER, H.; KELLY, M. G.; LLOYD, B. J.; and STRIEBICH, M. J. Some Observations on the Mitochondria of Normal and Neoplastic Cells with the Electron Microscope. J. Nat. Cancer Inst., **9:**439–49, 1949.
33. DANIELLI, J. F. Establishment of Cytochemical Techniques. Nature, **157:**755–57, 1946.
34. DOUNCE, A. L. Enzyme Studies on Isolated Cell Nuclei of Rat Liver. J. Biol. Chem., **147:**685–98, 1943.
35. ———. Further Studies on Isolated Cell Nuclei of Normal Rat Liver. *Ibid.*, **151:**221–33, 1943.
36. ———. Enzyme Systems of Isolated Cell Nuclei. Ann. N.Y. Acad. Sc., **50:**982–99, 1950.
37. DOUNCE, A. L.; TISHKOFF, G. H.; BARNETT, S. R.; and FREER, R. M. Free Amino Acids and Nucleic Acid Content of Cell Nuclei Isolated by a Modification of the Behrens' Technique. J. Gen. Physiol., **33:**629–42, 1950.
38. DUBUY, H. G.; WOODS, M. W.; BURK, D.; and LACKEY, M. D. Enzymatic Activities of Isolated Amelanotic and Melanotic Granules of Mouse Melanomas and a Suggested Relationship to Mitochondria. J. Nat. Cancer Inst., **9:**325–36, 1949.
39. DUBUY, H. G.; WOODS, M. W.; and LACKEY, M. D. Enzymatic Activities of Isolated Normal and Mutant Mitochondria and Plastids of Higher Plants. Science, **111:** 572–74, 1950.
40. DULANEY, A. D.; GOLDSMITH, Y.; ARNESEN, K.; and BUXTON, L. A Serological Study of Cytoplasmic Fractions from the Spleens of Normal and Leukemic Mice. Cancer Research, **9:**217–21, 1949.
41. ERNSTER, L.; ZETTERSTRÖM, R.; and LINDBERG, O. Vitamin A as a Component in the Mechanism of Aerobic Energy Transport. Exper. Cell. Research, **1:**494–96, 1950.
42. EULER, H. VON, and HELLER, L. Katalaseaktivität in Leberfraktionen normaler und sarkomtragender Ratten. Ztschr. Krebsforsch., **56:**393–406, 1949.
43 GOERNER, A., and GOERNER, M. M. Vitamin A and Tumor Mitochondria. J. Biol. Chem., **123:**57–59, 1938.
44. GOMORI, G. The Microchemical Demonstration of Sites of Lipase Activity. Proc. Soc. Exper. Biol. & Med., **58:** 362–64, 1945.
45. GOPAL-AYENGAR, A. R., and COWDRY, E. V. Desoxyribose Nucleic Acid from Isolated Chromosome Threads in Experimental Epidermal Methylcholanthrene Carcinogenesis in Mice. Cancer Research, **7:**1–8, 1947.
46. GRAFFI, A., and JUNKMAN, K. Beitrag zum chemischen Aufbau normaler und maligner Zellen. Klin. Wchnschr., **24:**78–81, 1946.
47. GREEN, D. E.; LOOMIS, W. F.; and AUERBACH, V. H. Studies on the Cyclophorase System. I. The Complete Oxidation of Pyruvic Acid to Carbon Dioxide and Water. J. Biol. Chem., **172:**389–403, 1948.
48. GRIFFIN, A. C.; COOK, H.; and CUNNINGHAM, L. Tissue Proteins and Carcinogenesis. III. Precancerous Changes in the Liver and Serum Protein of Rats Fed Acetylaminofluorene. Arch. Biochem., **24:**190–98, 1949.
49. HARMAN, J. W. Studies on Mitochondria. I. The Association of Mitochondria with Cyclophorase. Exper. Cell. Research, **1:**382–93, 1950.
50. ———. Studies on Mitochondria. II. The Structure of Mitochondria in Relationship to Enzymatic Activity. *Ibid.*, pp. 394–402, 1950.
51. HELLER, L., and BORGONI, N. Studien über die intrazellulare Verteilung der Enzyme. III. Die intrazellulare Verteilung der Monobutyrase in der Leber normaler und sarkomatöser Ratten. Arkiv Kemi, **1:**447–54, 1950.
52. HERMANN, H., and BOSS, M. B. Dopa Oxidase Activity in Extracts from Ciliary Body and in Isolated Pigment Granules. J. Cell. & Comp. Physiol., **26:**131–38, 1945.
53. HIRD, F. J. R., and ROWSELL, E. V. Additional Trans-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

amination by Insoluble Particle Preparations of Rat Liver. Nature, **166**:517–18, 1950.

54. HOERR, N. L. Methods of Isolation of Morphological Constituents of the Liver Cell. Biol. Symp., **10**:185–231, 1943.
55. HOGEBOOM, G. H. Cytochemical Studies of Mammalian Tissues. II. The Distribution of Diphosphopyridine-nucleotide-Cytochrome c Reductase in Rat Liver Fractions. J. Biol. Chem., **177**:847–58, 1949.
56. HOGEBOOM, G. H.; CLAUDE, A.; and HOTCHKISS, R. D. The Distribution of Cytochrome Oxidase and Succinoxidase in the Cytoplasm of the Mammalian Liver Cell. J. Biol. Chem., **165**:615–29, 1946.
57. HOGEBOOM, G. H., and SCHNEIDER, W. C. Sonic Disintegration of Isolated Liver Mitochondria. Nature, **166**: 302–303, 1950.
58. ———. Intracellular Distribution of Enzymes. VIII. The Distribution of Diphosphopyridinenucleotide-Cytochrome c Reductase in Normal Mouse Liver and Mouse Hepatoma. J. Nat. Cancer Inst., **10**:983–87, 1950.
59. ———. Proteins of Liver and Hepatoma Mitochondria. Science (in press).
60. HOGEBOOM, G. H.; SCHNEIDER, W. C.; and PALLADE, G. E. Cytochemical Studies of Mammalian Tissues. I. Isolation of Intact Mitochondria from Rat Liver; Some Biochemical Properties of Mitochondria and Submicroscopic Particulate Material. J. Biol. Chem., **172**:619–35, 1948.
61. HOGEBOOM, G. H., and SCHNEIDER, W. C. Cytochemical Studies of Mammalian Tissues. III. Isocitric Dehydrogenase and Triphosphopyridine Nucleotide-Cytochrome c Reductase in Mouse Liver. J. Biol. Chem., **186**:417–27, 1950.
62. HOLTER, H. Establishment of Cytochemical Techniques. Nature, **158**:917, 1946.
63. HOSTER, M. S.; McBEE, B. J.; ROLNICK, H. A.; VAN WINKLE, Q.; and HOSTER, H. A. Macromolecular Particles Obtained from Human Neoplastic and Non-neoplastic Lymph Nodes. I. Procedure and Preliminary Results. Cancer Research, **10**:530–38, 1950.
64. HUENNEKENS, F. M., and GREEN, D. E. Studies on the Cyclophorase System. X. The Requirement for Pyridine Nucleotide. Arch. Biochem., **27**:418–27, 1950.
65. ———. Studies on the Cyclophorase System. XI. The Effect of Various Treatments on the Requirement for Pyridine Nucleotide. *Ibid.*, pp. 428–40, 1950.
66. HUSEBY, R. A., and BARNUM, C. P. Investigation of the Phosphorus-containing Constituents of Centrifugally Prepared Fractions from Mouse Liver Cell Cytoplasm. Arch. Biochem., **26**:187–98, 1950.
67. JEENER, R., and BRACHET, J. Recherches sur l'acide ribonucléique des levures. Enzymologia, **11**:222–34, 1944.
68. JEENER, R., and SZAFORZ, D. Relation between the Rate of Renewal and the Intracellular Localization of Ribonucleic Acid. Arch. Biochem., **26**:54–67, 1950.
69. JULEN, C.; SNELLMAN, O.; and SYLVEN, B. Cytological and Fractionation Studies on the Cytoplasmic Constituents of Tissue Mast Cells. Acta Physiol. Scandinav., **19**:289–305, 1950.
70. KENNEDY, E. P., and LEHNINGER, A. L. Intracellular Structures and the Fatty Acid Oxidase System of Rat Liver. J. Biol. Chem., **172**:847–48, 1948.
71. ———. Oxidation of Fatty Acids and Tricarboxylic Acid Cycle Intermediates by Isolated Rat Liver Mitochondria. *Ibid.*, **179**:957–72, 1949.
72. KIELLEY, R. K., and SCHNEIDER, W. C. Synthesis of *p*-Aminohippuric Acid by Mitochondria of Mouse Liver Homogenates. J. Biol. Chem., **185**:869–80, 1950.
73. KURNICK, N. B. The Quantitative Estimation of Desoxyribose Nucleic Acid Based on Methyl Green Staining. Exper. Cell. Research, **1**:151–58, 1950.
74. LAGERSTEDT, S. Cytological Studies on the Protein Metabolism of the Liver in Rat. Acta Anatomica, Suppl. IX, 1949.
75. LAMBDEN, M. P. Considerations on Indiscriminate Use of the Waring Blendor: Copper Contamination and Ascorbic Acid Loss. Fed. Proc., **9**:193, 1950.
76. LAZAROW, A. Particulate Glycogen: A Submicroscopic Component of the Guinea Pig Liver Cell; Its Significance in Glycogen Storage and the Regulation of Blood Sugar. Anat. Rec., **84**:31–50, 1942.
77. ———. The Chemical Structure of Cytoplasm as Investigated in Professor Bensley's Laboratory during the Past 10 Years. Biol. Symp., **10**:9–26, 1943.
78. LEHNINGER, A. L., and KENNEDY, E. P. The Requirements of the Fatty Acid Oxidase Complex of Rat Liver. J. Biol. Chem., **173**:753–71, 1948.
79. LEPAGE, G. A., and SCHNEIDER, W. C. Centrifugal Fractionation of Glycolytic Enzymes in Tissue Homogenates. J. Biol. Chem., **176**:1021–27, 1948.
80. LEUTHARDT, F., and MÜLLER, A. F. Mitochondrien und Citrullensynthese in der Leber. Experientia, **4**: 478, 1948.
81. LI, C., and ROBERTS, E. Amino Acids in the Mitochondrial Fractions of Tissues as Determined by Paper Partition Chromatography. Science, **110**:559–60, 1949.
82. LINDERSTROM-LANG, K. Distribution of Enzymes in Tissues and Cells. Harvey Lectures, **34**:214–45, 1938–39.
83. MARSHAK, A. P^{32} Uptake by Nuclei. J. Gen. Physiol., **25**:275–91, 1941.
84. MARSHAK, A., and CALVET, F. Specific Activity of P^{32} in Cell Constituents of Rabbit Liver. J. Cell. & Comp. Physiol., **34**:451–56, 1949.
85. MAYER, D. T., and GULICK, A. The Nature of the Proteins of Cellular Nuclei. J. Biol. Chem., **146**:433–40, 1942.
86. MILLER, E. C., and MILLER, J. A. The Presence and Significance of Bound Amino-Azo Dyes in the Livers of Rats Fed *p*-Dimethyl Aminoazobenzene. Cancer Research, **7**:468–80, 1947.
87. MILLER, E. C.; MILLER, J. A.; SAPP, R. W.; and WEBER, G. M. Studies on the Protein-bound Amino-Azo Dyes Formed *in vivo* from 4-Dimethylamino-azobenzene and Its c-Monomethyl Derivatives. Cancer Research, **9**:336–43, 1949.
88. MILNER, H. W.; LAWRENCE, N. S.; and FRENCH, C. S. Colloidal Dispersion of Chloroplast Material. Science, **111**:633–34, 1950.
89. MIRSKY, A. E. Chemical Properties of Isolated Chromosomes. Cold Spring Harbor Symp. Quant. Biol., **12**:143–46, 1947.
90. MIRSKY, A. E., and POLLISTER, A. W. Fibrous Nucleoproteins of Chromatin. Biol. Symp., **10**:247–60, 1943.
91. MIRSKY, A. E., and RIS, H. Isolated Chromosomes. J. Gen. Physiol., **31**:1–6, 1947.
92. ———. The Chemical Composition of Isolated Chromosomes. *Ibid.*, pp. 7–18, 1947.
93. ———. Variable and Constant Components of Chromosomes. Nature, **163**:666–67, 1949.
94. MUELLER, G. C., and MILLER, J. A. The Reductive Cleavage of 4-Dimethylamino-azobenzene by Rat Liver: The Intracellular Distribution of the Enzyme System and Its Requirement for Triphosphopyridine Nucleotide. J. Biol. Chem., **180**:1125–36, 1949.
95. MÜHLETHALER, K.; MÜLLER, A. F.; and ZOLLINGER, H. U. Zur Morphologie der Mitochondrien. Experientia, **6**:16, 1950.
96. MÜLLER, A. F., and LEUTHARDT, F. Oxydative Phos-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110001.pdf by guest on 24 August 2022

phorylierung und Citrullinsynthese in den Leber Mitochondrien. Helvet. chem. acta, **32**:2349–56, 1949.

97. MUNTWYLER, E.; SEIFTER, S.; and HARKNESS, D. M. Some Effects of Restriction of Dietary Protein on the Intracellular Components of Liver. J. Biol. Chem., **184**: 181–90, 1950.
98. NOVIKOFF, A. B.; PODBER, E.; and RYAN, J. Intracellular Distribution of Phosphatase Activity in Rat Liver. Fed. Proc., **9**:210, 1950.
99. OMACHI, A.; BARNUM, C. P.; and GLICK, D. Quantitative Distribution of an Esterase among Cytoplasmic Components of Mouse Liver Cells. Proc. Soc. Exper. Biol. & Med., **67**:133–36, 1948.
100. OPIE, E. L. The Movement of Water in Tissues Removed from the Body and Its Relation to Movement of Water during Life. J. Exper. Med., **89**:185–208, 1949.
101. PALADE, G. E., and CLAUDE, A. The Nature of the Golgi Apparatus. I. Parallelism between Golgi Apparatus and Intracellular Myelin Figures. J. Morphol., **85**:35–70, 1949.
102. ———. The Nature of the Golgi Apparatus. II. Identification of the Golgi Apparatus with a Complex of Myelin Figures. *Ibid.*, pp. 71–112, 1949.
103. PETERMANN, M. L.; ALFIN-SLATER, R. B.; and LARACK, A. M. The Nucleic Acid Distribution in Normal and Leukemic Mouse Spleen. Cancer, **2**:510–15, 1949.
104. PETERMANN, M. L., and MASON, E. J. Nucleic Acid Content of Chromosomes of Normal and Leukemic Mouse Spleen. Proc. Soc. Exper. Biol. & Med., **69**:542–44, 1948.
105. POLLISTER, A. W., and LEUCHTENBERGER, C. The Nucleoprotein Content of Whole Nuclei. Proc. Nat. Acad. Sc., **35**:66–71, 1949.
106. PORTER, K. R.; CLAUDE, A.; and FULLAM, E. F. A Study of Tissue Culture Cells by Electron Microscopy. J. Exper. Med., **81**:233–46, 1945.
107. POTTER, V. R., and ELVEHJEM, C. A. A Modified Method for the Study of Tissue Oxidations. J. Biol. Chem., **114**: 495–504, 1936.
108. POTTER, V. R., and LE PAGE, G. A. Metabolism of Oxalacetate in Glycolyzing Tumor Homogenates. J. Biol. Chem., **177**:237–45, 1949.
109. POTTER, V. R.; PRICE, J. M.; MILLER, E. C.; and MILLER, J. A. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. III. Effects on Succinoxidase and Oxalacetic Acid Oxidase. Cancer Research, **10**:28–35, 1950.
110. POTTER, V. R., and SCHNEIDER, W. C. Oxidative Phosphorylation without Deamination in Mitochondrial Preparations. Fed Proc., **8**:237, 1949.
111. PRICE, J. M., and LAIRD, A. K. A Comparison of the Intracellular Composition of Regenerating Liver and Induced Liver Tumors. Cancer Research, **10**:650–58, 1950.
112. PRICE, J. M.; MILLER, E. C.; and MILLER, J. A. The Intracellular Distribution of Protein, Nucleic Acids, Riboflavin, and Protein-bound Aminoazo Dye in the Livers of Rats Fed *p*-Dimethylaminoazobenzene. J. Biol. Chem., **173**:345–53, 1948.
113. ———. Intracellular Distribution of Vitamin B_6 in Rat and Mouse Livers and Induced Rat Liver Tumors. Proc. Soc. Exper. Biol. & Med., **71**:575–78.
114. PRICE, J. M.; MILLER, E. C; MILLER, J. A.; and WEBER, G. M. Studies on the Intracellular Composition of Livers from Rats Fed Various Aminoazo Dyes. I. Cancer Research, **9**:398–402, 1949.
115. ———. Studies on the Intracellular Composition of Liver and Liver Tumor from Rats Fed 4-Dimethylaminoazobenzene. *Ibid.*, pp. 96–102.
116. ———. Studies on the Intracellular Composition of Liver from Rats Fed Various Aminoazo Dyes. II. 3'Methyl-, 2' Methyl-, and 2 Methyl-4-Dimethylaminoazobenzene, 3-Methyl-4-Dimethylaminoazobenzene, and 4'-Fluoro-4-Dimethylaminoazobenzene. *Ibid.*, **10**:18–27, 1950.
117. RECKNAGEL, R. O. Localization of Cytochrome Oxidase on the Mitochondria of the Frog Egg. J. Cell. & Comp. Physiol., **35**:111–30, 1950.
118. RIS, H., and MIRSKY, A. E. The State of the Chromosomes in the Interphase Nucleus. J. Gen. Physiol., **32**: 489–502, 1949.
119. ———. Quantitative Cytochemical Determination of Desoxyribosenucleic Acid with the Feulgen Nucleal Reaction. *Ibid.*, **33**:125–45, 1950.
120. SCHEIN, A. H.; PODBER, E.; and NOVIKOFF, A. B. Intracellular Localization of Uricase Activity. Fed. Proc., **9**: 224, 1950.
121. SCHNEIDER, W. C. Phosphorus Compounds in Animal Tissues. II. The Nucleic Acid Content of Homologous Normal and Cancer Tissues. Cancer Research, **5**:717–21, 1945.
122. ———. Intracellular Distribution of Enzymes. I. The Distribution of Succinic Dehydrogenase, Cytochrome Oxidase, Adenosinetriphosphatase, and Phosphorus Compounds in Normal Rat Tissues. J. Biol. Chem., **165**:585–93, 1946.
123. ———. Intracellular Distribution of Enzymes. II. The Distribution of Succinic Dehydrogenase, Cytochrome Oxidase, Adenosinetriphosphatase and Phosphorus Compounds in Normal Rat Liver and in Rat Hepatomas. Cancer Research, **6**:685–90, 1946.
124. ———. Nucleic Acids in Normal and Neoplastic Tissues. Cold Spring Harbor Symp. Quant. Biol., **12**:169–77, 1947.
125. ———. Intracellular Distribution of Enzymes. III. The Oxidation of Octanoic Acid by Rat Liver Fractions. J. Biol. Chem., **176**:259–66, 1948.
126. ———. The Distribution of Enzymes within the Cell, p. 273. In: Respiratory Enzymes. Minneapolis: Burgess Publishing Co., 1949.
127. ———. Methods for the Isolation of Particulate Components of the Cell, p. 148. In: Manometric Techniques and Tissue Metabolism. Minneapolis: Burgess Publishing Co., 1949.
128. SCHNEIDER, W. C.; CLAUDE, A.; and HOGEBOOM, G. H. The Distribution of Cytochrome c and Succinoxidase Activity in Rat Liver Fractions. J. Biol. Chem., **172**: 451–58, 1948.
129. SCHNEIDER, W. C., and HOGEBOOM, G. H. Intracellular Distribution of Enzymes. V. Further Studies on the Distribution of Cytochrome c in Rat Liver Homogenates. J. Biol. Chem., **183**:123–28, 1950.
130. ———. Intracellular Distribution of Enzymes. IV. The Distribution of Succinoxidase and Cytochrome Oxidase Activities in Normal Mouse Liver and in Mouse Hepatoma. J. Nat. Cancer Inst., **10**:969–75, 1950.
131. SCHNEIDER, W. C.; HOGEBOOM, G. H.; and ROSS, H. E. Intracellular Distribution of Enzymes. VII. The Distribution of Nucleic Acids and Adenosinetriphosphatase in Normal Mouse Liver and Mouse Hepatoma. J. Nat. Cancer Inst., **10**:977–82, 1950.
132. SCHNEIDER, W. C., and POTTER, V. R. Intracellular Distribution of Enzymes. IV. The Distribution of Oxalacetic Oxidase Activity in Rat Liver and Rat Kidney Fractions. J. Biol. Chem., **177**:893–903, 1949.
133. SCHWEIGERT, B. S.; GUTHNECK, B. T.; PRICE, J. M.; MILLER, J. A.; and MILLER, E. C. Amino Acid Composition of Morphological Fractions of Rat Livers and

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110010001.pdf by guest on 24 August 2022

Induced Liver Tumors. Proc. Soc. Exper. Biol. & Med., **72**:495–501, 1949.

134. Sorof, S., and Cohen, P. P. Electrophoretic Studies of the Soluble Protein of Rabbit Liver. Fed. Proc., **8**:254, 1949.
135. Steinbach, H. B., and Moog, F. The Localization of Adenylpyrophosphatase in Cytoplasmic Granules. J. Cell. & Comp. Physiol., **26**:175–83, 1945.
136. Still, J. E., and Kaplan, E. H. Localization of Oxidases. Exper. Cell. Research, **1**:403–9, 1950.
137. Swift, H. H. The Desoxyribose Nucleic Acid Content of Animal Nuclei. Physiol. Zoöl., **23**:169–98, 1950.
138. Tagnon, H. J., and Palade, G. E. Activation of protoplasmin by a Factor from Mammalian Tissue. J. Clin. Investigation, **29**:317–24, 1950.
139. Tagnon, H. J., and Petermann, M. L. Activation of Proplasmin by a Tissue Fraction. Proc. Soc. Exper. Biol. & Med., **70**:359–60, 1949.
140. Tipton, S. R.; Leath, M. J.; Tipton, I. H.; and Nixon, W. L. The Effects of Feeding Thyroid Substance and of Adrenalectomy on the Activities of Succinoxidase and Cytochrome Oxidase in the Liver Tissue of Rats. Am. J. Physiol., **145**:693–98, 1946.
141. Wilson, E. B. The Cell in Development and Heredity, p. 85. New York: Macmillan Co., 1928.
142. Woods, M. W.; DuBuy, H. B.; Burk, D.; and Hesselbach, M. L. Cytological Studies on the Nature of the Cytoplasmic Particulates in the Cloudman S 91 Mouse Melanoma, the Derived Algire S 91A Partially Amelanotic Melanoma and the Harding-Passey Mouse Melanoma. J. Nat. Cancer Inst., **9**:311–24, 1949.
143. Zollinger, H. U. Cytologic Studies with the Phase Microscope. II. The Mitochondria and Other Cytoplasmic Constituents under Various Experimental Conditions. Am. J. Path., **24**:569–88, 1948.
144. ———. Zur qualitativen Nucleoproteingehalt und zur Morphologie der Mitochondrien. Experientia, **6**:14–19, 1950.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/11/1/1/2369383/cr0110010001.pdf by guest on 24 August 2022