A Method for Measuring DNA Damage and Repair in the Liver in Vivo

Ray Cox, Ivan Damjanov, S. E. Abanobi, and D. S. R. Sarma

Fels Research Institute [R. C., I. D., S. E. A., D. S. R. S.,] and the Department of Pathology [D. S. R. S.], Temple University School of Medicine, Philadelphia, Pennsylvania 19140

SUMMARY

Alkaline sucrose gradients were used to study the induction of single-strand breaks in and repair of rat liver DNA in vivo. Liver DNA was labeled during regeneration after partial hepatectomy. DNA was isolated from the organ in such a way as to minimize the induction of breaks due to handling or enzymatic activity. Different methods of preparing high-molecular-weight liver DNA were compared. Best results were obtained by squashing the liver in an ethylenediaminetetraacetic acid-sodium chloride buffer. Using this squash technique and alkaline gradients, high-molecular-weight DNA was regularly obtained. This DNA behaved like single-stranded DNA on hydroxyapatite columns and contained no detectable contamination of protein or RNA. It was demonstrated by the method described that hepatic DNA damage was produced within four hr by the administration of methyl methanesulfonate and that the rat can repair such hepatic DNA damage in vivo within 48 hr.

INTRODUCTION

It is now clear that DNA is one of the common target macromolecules with which chemical carcinogens interact in susceptible cells or tissues (14, 18, 29–32, 45, 47, 48). However, neither the chemical nor the biological consequences of this interaction are understood, no doubt due in part to the unavailability of sufficiently versatile methods for their study in the intact animals. Although the repair of altered DNA has been implicated often in speculations concerning mechanisms of carcinogenesis, little detailed knowledge based on experimental data is available.

The only relevant studies correlating DNA repair with carcinogenesis are those with fibroblasts in culture from patients with xeroderma pigmentosum (7–9, 38, 40). Cleaver (7) first reported that such cells cannot repair the damage induced in their DNA by UV. Since patients with this disease frequently develop skin cancer, apparently related to exposure to sunlight (7, 8), it was postulated that the absence or inadequacy of repair might be an important component in predisposing them to cancer.

It was of interest, therefore, to investigate the nature and consequences of DNA damage and repair in the carcinogenic process initiated with chemicals or viruses as well as with UV or other physical agents. Methods must be developed or refined for such studies not only in cells in culture but also in the intact organism under conditions in which the various stages of neoplastic development induced by chemicals or by other carcinogenic stimuli are taking place.

At least 4 basic approaches are now available to monitor damage and repair of DNA in eukaryotic cells: (a) the induction or removal of the chemical alterations in DNA, be they pyrimidine dimer (37), bound forms of a chemical (18, 45, 47, 48), or some chemical consequences of the latter (21); (b) the availability of new groups, such as phosphate groups, to enzymatic attack upon rupture of a DNA strand (5, 46); (c) the incorporation of deoxynucleotides or analogs such as bromodeoxyuridine into DNA not actively involved in its own replication ("unscheduled DNA synthesis") (26, 35); and (d) measurement of the average size of the DNA, either as a double-stranded (neutral sucrose gradient) or single-stranded (alkaline sucrose gradient) macromolecule (1, 31).

To date, few studies have been reported on any aspect of DNA repair in the intact organism. Skin in mice (12) and in man (13) was found to show unscheduled DNA synthesis following exposure to UV. Patients with xeroderma pigmentosum were deficient in this response. The loss of radioactive carcinogens such as methylyating or ethylating agents (41, 43, 44), azo dye (47), or 2-fluorenylacetamide (18, 45, 48) bound to the liver DNA in vivo was followed with time. In none of these studies was actual repair of DNA demonstrated by reformation of broken strands.

This report is concerned with the development of a method for obtaining large pieces of liver DNA, modeled after that of McGrath and Williams (31). In this method, the 2 strands of DNA are separated by treatment with alkali and are centrifuged in an alkaline sucrose gradient to separate fragments of different sizes. By this method, DNA breaks as well as fragments rejoining during the repair process can be monitored. This technique has been used for several years in the study of DNA damage in mammalian cell cultures, following irradiation with X-rays and treatment with chemicals (1, 10, 22, 23), but has not been de-
Damage and Repair of Hepatic DNA in Vivo

Liver DNA was labeled by injecting thymidine-methyl-3H during the peak of DNA synthesis following partial hepatectomy. Radioactive thymidine was given i.p. at a dose of 50 μCi animal every 4 hr beginning at 16 to 17 hr after the operation until each animal received a total of 450 μCi. The schedule of injection of labeled thymidine being started at 7 a.m. continued through 11 a.m., 3 p.m., 7 p.m., and 11 p.m. and on the following day at 7 a.m., 11 a.m., 3 p.m., and 7 p.m. The animals were used after a minimum recovery period of 2 weeks, at which time the liver has returned to a quiescent state (4).

Labeling of Liver Protein and RNA

Liver proteins were labeled 2 weeks after partial hepatectomy by administering i.p. 14C-labeled amino acid mixture at a dose of 100 μCi/animal every hr for 4 hr. The animals were killed 1 hr after the last injection. Hepatic DNA of these animals was labeled by 3H-labeled thymidine as described above. In 2 separate experiments lysine-2-14C or uniformly labeled leucine-14C were given 15 hr after partial hepatectomy to label the histones and other proteins that are synthesized during liver regeneration. These labeled amino acids were given i.p. at a dose of 100 μCi/animal every hr for 4 hr. The rats were killed 1 hr after the last injection. Liver radioactive protein was isolated, and the specific activity was determined as described previously (28).

Fifteen hr after partial hepatectomy, orotic acid-3H was given i.p. to rats at a dose of 100 μCi/animal every hr for 4 hr. The animals were killed 30 min after the last injection. Hepatic RNA was isolated, and the specific activity was determined as described previously (28).

Preparation of Rat Liver DNA for Alkaline Sucrose Gradient Analysis

Phenol Method. Rat liver DNA labeled as described above was isolated from approximately 5 g of liver by the method of Kidson et al. (19) as modified by Swann and Magee (43). Carbohydrates were removed by the use of methoxynethanol as described by Kirby (20). The final DNA was essentially free of protein and RNA as measured by Lowry et al. (27) and orcinol (3) methods, respectively.

CsCl Method. In these experiments rat liver DNA was labeled with 3H-labeled thymidine as detailed above. Approximately 1 g of rat liver was homogenized in 5.0 ml of 0.001 M Tris (pH 8.5) and 1% sodium dodecyl sulfate. CsCl was added to give an initial density of 1.715 g cm−3 (15). After an initial centrifugation at 3000 rpm for 10 min at room temperature, the clear solution was removed with a syringe. Five ml of this solution were taken and placed in a cellulose nitrate tube. The rest of the tube was filled with mineral oil and centrifuged at 38,000 rpm at 20° for 24 hr in a Spincso 50 Ti rotor using a Spincso Model L2-65B ultracentrifuge. The rotor was decelerated to a stop without using the brake. The bottom of the tube was punctured and 20-drop fractions were collected. An aliquot from each fraction was assayed for the DNA radioactivity as described below. Fractions containing DNA were pooled and dialyzed against distilled water at 5° to remove CsCl.

From Rat Liver Homogenate, Nuclei, and Cell Suspension. Liver tissue was prepared by 3 methods: homogenization, isolation of nuclei, and a squash technique which produces a suspension of liver cells. One g of liver was homogenized in 10 ml of buffer containing 0.024 M EDTA-0.075 M NaCl (pH 7.5), or 0.9% NaCl solution, using a Potter-Elvehjem

### Materials and Methods

#### Animals

White male Wistar rats weighing approximately 100 g were obtained from Carworth Farms, Rockland County, N. Y. All animals were maintained on Purina rat chow and water. Partial hepatectomy was performed by the method of Higgins and Anderson (17). Throughout the recovery period all animals had free access to food and water. All animals were killed by a sharp blow to the head followed by jugular vein exsanguination.

#### Chemicals

- Thymidine-methyl-3H (specific activity, 20 Ci/mmol), orotic acid-5-3H (specific activity, 12.2 Ci/mmol), uniformly labeled L-leucine-14C (specific activity, 291 mCi/mmol), Dl-lysine-2-14C (specific activity, 3.87 mCi/mmol), and an 14C-labeled L-amino acid mixture (specific activity, 83 to 396 mCi/mmol) were purchased from New England Nuclear, Boston, Mass. Protease type VI (repurified) was obtained from Sigma Chemical Co., St. Louis, Mo., RNase (bovine pancreas) was obtained from P-L Biochemicals, Inc., Milwaukee, Wis.; MMS was purchased from Eastman Kodak, Rochester, N. Y. Cesium chloride (optical grade) and hydroxyapatite (DNA grade) were obtained from the Harshaw Chemical Co., Solon, Ohio, and Bio-Rad Laboratories, Richmond, Calif., respectively. NCS solubilizer was obtained from Amersham/Searle, Arlington Heights, Ill. Omnifluor and liquifluor were obtained from Eastman Kodak, Rochester, N. Y. All animals were maintained on Purina rat chow and water. Partial hepatectomy was performed by the method of Higgins and Anderson (17). Throughout the recovery period all animals had free access to food and water. All animals were killed by a sharp blow to the head followed by jugular vein exsanguination.

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### References

The abbreviations used are: MMS, methyl methanesulfonate; TCA, trichloroacetic acid.
homogenizer with a clearance of 0.006 to 0.009 inch between Teflon pestle and glass chamber. The livers were homogenized with 5 strokes (1 up and 1 down movement per stroke). Nuclei were isolated according to the method of Pogo et al. (34). A suspension of liver cells was prepared by placing about 2 g of liver in a Petri dish with about 2 ml of cold 0.024 M EDTA-0.075 M NaCl buffer (pH 7.5). The tissue was held with a pair of forceps and squashed gently with the blunt end of spatula. To sediment the pieces of liver tissue, the resulting suspension was centrifuged for 1 min at 200 rpm at 4°C in an International Model PR-6 refrigerated centrifuge. The supernatant containing the suspension of liver cells was drawn with a Pasteur pipet and used for the analysis of DNA sedimentation. Liver microscopic examination of this suspension revealed good nuclei with very little cytoplasm. In this communication, this preparation will be referred to as liver cell suspension. The liver cells were counted in a hemocytometer chamber. DNA was determined by diphenylamine method of Burton (6).

Gradients

Alkaline sucrose gradients were prepared in cellulose nitrate tubes (diameter 9/16 inch and length 3-3/4 inches). Five-ml linear alkaline sucrose gradients (5 to 20%) containing 0.9 M NaCl and 0.3 M NaOH were prepared over a l-ml shelf of 2.3 M sucrose (22, 33). A lysing solution (0.3 ml) of 0.30 M NaCl, 0.03 M EDTA, 0.1 M Tris-HCl buffer and 0.5% sodium dodecyl sulfate, pH 10 (10), was carefully layered on top of the gradient. To this was added 1 volume of homogenate, nuclei, or liver cells prepared by squash technique ranging from 0.01 to 0.3 ml and consisting of approximately 1 μg of DNA that contained about 300 to 700 cpm. Another aliquot (0.1 ml) of lysing agent was added on the top of the cells to ensure complete lysis, and the rest of the tube was filled with mineral oil. Lysing of the cells under these conditions was immediate as judged by light microscope. This confirms the observation reported by Coyle and Strauss (10). Extended lysing periods of 1 hr or more resulted in fragmentation of DNA (J. Zubroff and D. S. R. Sarma, unpublished data). The gradients were centrifuged at 25,000 rpm for 30 min at 20°C using the Beckman SW40 rotor in a Spinco model L2-65B ultracentrifuge. The rotor was decelerated to a stop without the brake. Fractions of 20 drops each were collected from the bottom of pierced tubes by pumping mineral oil from the top. The 1st 2 fractions comprise the 2.3 M sucrose shelf. Bovine serum albumin (20 μg) was added to each fraction before precipitating with 4 ml of cold 5% TCA.

Chromatography of Rat Liver DNA on Hydroxyapatite Columns

Rat liver DNA for fractionation on a hydroxyapatite column was prepared as follows. The liver cell suspension was prepared by squash technique as described above and was lysed on the top of 5 ml of 5 to 20% alkaline sucrose gradient. The preparation and centrifugation of the gradients were essentially the same as detailed above except that the pH of the 2.3 M sucrose shelf used in this experiment was 12.5 and the sucrose contained 5% formaldehyde. Twenty-drop fractions containing DNA were pooled and diluted to 6 times the original volume with water containing 5% formaldehyde (pH 7.5). An aliquot of 15 ml was loaded directly on the hydroxyapatite columns, and another aliquot of 15 ml was sonically disrupted using a Branson Sonifier to give small fragments of an average size of 14 S. These were then loaded on hydroxyapatite columns.

Fractionation of rat liver DNA on a hydroxyapatite column was carried out essentially as described by Bernardi (2). One g of hydroxyapatite was used in columns 1 cm in diameter. The gel was equilibrated with 0.005 M potassium phosphate buffer (pH 6.8). Sonically treated or untreated DNA (2 to 3 μg) was loaded onto the gel. The column was washed with 20 ml of 0.005 M potassium phosphate buffer (pH 6.8), and the DNA was eluted with increasing concentrations of potassium phosphate buffer. A constant flow rate (3 ml/10 min) was maintained by pumping the buffer with a polystaltic pump. Both discontinuous and continuous linear gradients were used. Three-ml fractions were collected. An aliquot of the eluted solution was used to measure the refractive index and another aliquot was used for determining radioactivity. Refractive index was measured using a Bausch and Lomb refractometer. The molarity of phosphate buffer was calculated from the refractive index using a calibration curve obtained by determining the refractive indices of phosphate buffers of known molarity.

Determination of Radioactivity

The amount of radioactivity in each preparation (homogenate, nuclei, or cells) was determined by precipitating an aliquot with cold 5% TCA. The precipitate was collected on a glass fiber filter and washed 6 times with 4 ml each of cold 5% TCA. The filters were then placed in liquid scintillation vials and dissolved in 1 ml of NCS solubilizer. Fifteen ml of a scintillation fluid (Omnifluor, 4 g; toluene, 975 ml; NCS solubilizer, 25 ml) were added, and the radioactivity was determined.

After precipitation with TCA, fractions from the gradients were collected on Millipore nitrocellulose filters and washed 6 times with 4 ml each of cold 5% TCA. The filters were dissolved in 1 ml of ethyl Cellulose. Ten ml of a scintillation fluid (toluene, 2365 ml; ethyl Cellulose, 788 ml; and Liquifluor, 132 ml) were added, and the radioactivity was determined using an Intertechnique SL30 liquid scintillation spectrometer.

Treatment of Animals with MMS

MMS was used immediately after diluting with 0.9% NaCl solution to a final concentration of 129 mg/ml (1.27 M). The appropriate volume of this solution was injected i.p. at a dose of 120 mg/kg body weight, and the animals were killed at 4, 24, and 48 hr and 6 days after the administration of either 0.9% NaCl solution or MMS. Rat liver cell suspension was prepared and the DNA was analyzed on alkaline sucrose gradients as described above. A
portion of the liver was taken for histological examination in order to observe the presence or absence of necrosis.

RESULTS

Methods of Preparing Rat Liver DNA for Alkaline Sucrose Gradient Analysis. The data presented in Chart 1 clearly show that rat liver DNA prepared by the conventional methods such as with phenol (19, 20, 43) or CsCl (15) yielded fragmented DNA which banded near the top of the alkaline sucrose gradient under the centrifugation methods used. Therefore, aliquots of whole-liver homogenate, nuclei, or liver cell suspension lysed directly on the top of the alkaline sucrose gradient were used.

Liver tissue DNA was obtained from 3 different types of preparations: whole homogenates, isolated nuclei, or liver cell suspensions obtained by squash technique. The alkaline sucrose gradient profiles of DNA prepared by the 3 different methods are shown in Chart 2. The homogenate and the nuclei were prepared in the absence of EDTA, whereas the cell suspension was prepared by squashing the liver in 24 mM EDTA-75 mM NaCl, pH 7.5. DNA from the homogenate and the nuclei band near the top of the gradient, whereas DNA from the liver cell suspension bands as a sharp peak at the bottom of the 5 to 20% alkaline sucrose gradient in the 2.3 M sucrose shelf. The necessity of EDTA to prevent nicking of the DNA is further indicated in Chart 3. When EDTA was added to the homogenizing solution, the majority of DNA banded as a sharp peak near the bottom of the gradient. The peak of acid-precipitable radioactivity obtained near the bottom of the gradient, when cell suspension was used, was resistant to hydrolysis by 0.5 N KOH for 30 min at 37° but was readily hydrolyzed by DNase.

We have routinely squashed the liver in EDTA-NaCl as a method of preparing liver DNA for sucrose gradient analysis for the following reasons. The recovery of radioactivity added to the gradient was greater with the cellular suspension prepared by squash technique when compared to that obtained with whole homogenate (Table I). The cells may be counted and a known quantity of cells can be added to the gradient. In addition, a disadvantage of the use of homogenate was the presence of pieces of connective tissue which may interfere with the banding of DNA when layered on the gradient.

To prevent the loss of the sharp radioactive peak, the 5 to 20% sucrose gradient was layered over 1 ml of 2.3 M sucrose shelf. The absence of this shelf resulted in the complete loss of radioactivity from the gradient within a 30-min spin. Overloading of the gradient also resulted in a loss of radioactivity (Table I). With approximately 1 x 10⁴ cells or less than 1 µg of DNA, the radioactivity rapidly sedimented through the 5 to 20% sucrose gradient but did not pass completely through the 2.3 M sucrose shelf during the 30-min period of centrifugation and yielded good recoveries.

Contamination of DNA with Protein or RNA. A contamination of DNA with protein or RNA could seriously affect the pattern of radioactivity observed on the alkaline sucrose gradient. Cell suspensions from livers labeled in protein or in RNA were prepared, and alkaline sucrose gradients were run to see whether the labeled protein or RNA would band with the DNA peak. The total liver protein labeled with a ¹³C-labeled amino acid mixture had a specific activity of 71,000 dpm/mg. There was no detectable ¹³C radioactivity in the DNA peak, but a peak was observed at the top of the gradient (Chart 4). Similar patterns were obtained in 2 separate experiments when liver proteins were labeled with leucine or lysine during liver regeneration, in order to label
the histone and non-histone proteins. There was no detectable radioactivity in the DNA peak. Total RNA was labeled with orotate-\(^{3}H\) to a specific activity of about 18,000 cpm/mg of RNA. During the 30-min period of centrifugation in the alkaline sucrose gradient, the majority (70%) of the counts added to the gradient was lost probably due to alkaline hydrolysis of the RNA. The remaining 30% banded near the top of the gradient and gave a pattern similar to the protein in Chart 4.

The addition of Pronase or RNase to the lysing agent had no effect on the DNA profile. Pronase (10 mg/ml water) was autodigested for 24 to 30 hr at 37° and pancreatic RNase (10 mg/ml of 0.05 M Tris, pH 8.0) was heated for 10 min in a boiling water bath to inactivate any DNase. Optimum conditions for Pronase and RNase activities were determined using rat liver labeled proteins and nucleic acids, respectively, as substrates in the presence of lysing agent. In the studies reported here, autodigested Pronase (2 mg/ml of lysing agent) and boiled RNase (100 µg/ml of lysing agent) were used.

**Single-Strand Nature of DNA.** The DNA that traveled through 5.0 ml of the 5 to 20% alkaline sucrose and that banded in the 2.3 M sucrose layer was analyzed for the extent of single strandedness. In this experiment, the 2.3 M sucrose shelf was adjusted to pH 12.5 and contained 5% formaldehyde to maintain the DNA strands as single strands. DNA so prepared was eluted from the hydroxyapatite column with 0.25 M or higher molar potassium phosphate buffer, pH 6.8. However, after sonic disruption when long pieces of DNA were sonically disrupted to smaller pieces of about 14 S, they chromatographed like single-stranded DNA and the DNA was eluted from the column by 0.05 M or less molar potassium phosphate buffer, pH 6.8. It is the experience of others, as is ours, that long pieces of single-stranded DNA elute from the hydroxyapatite columns like native DNA, probably because of aggregation (2). Thus the

![Chart 3: Effect of the presence of EDTA in the homogenate on the sedimentation of DNA in alkaline sucrose gradients.](image-url)

![Chart 4: Alkaline sucrose gradient profiles of DNA-\(^{3}H\) (○) and protein-\(^{14}C\) (●).](image-url)

![Table 1: Recovery of DNA from alkaline sucrose gradients](table)

<table>
<thead>
<tr>
<th>DNA preparation</th>
<th>DNA (µg)</th>
<th>cpm added to gradient</th>
<th>cpm recovered/gradient</th>
<th>% recovery of cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate in 0.9% NaCl solution</td>
<td>1.00</td>
<td>530</td>
<td>278</td>
<td>52</td>
</tr>
<tr>
<td>Homogenate in EDTA-0.075 M NaCl solution</td>
<td>0.92</td>
<td>466</td>
<td>245</td>
<td>53</td>
</tr>
<tr>
<td>Cell suspension</td>
<td>0.96</td>
<td>708</td>
<td>600</td>
<td>85</td>
</tr>
<tr>
<td>Cell suspension</td>
<td>1.92</td>
<td>1416</td>
<td>534</td>
<td>38</td>
</tr>
<tr>
<td>Cell suspension</td>
<td>2.89</td>
<td>2124</td>
<td>678</td>
<td>32</td>
</tr>
</tbody>
</table>
acid-precipitable radioactivity sedimenting near the cushion of
2.3 M sucrose in the alkaline sucrose gradient is (a) alkali
stable and (b) RNase and Pronase stable and readily hydro-
lyzable by DNase; hence by definition the acid-precipitable
radioactive peak behaves like DNA. Further, this material
on sonic disruption behaves like single-stranded DNA on
hydroyapatite columns.

Induction of Single-Strand Breaks. In this paper, the
terms single-strand and double-strand breaks are defined for
operational purposes. Fragmentation of DNA seen only on
alkaline sucrose gradients is referred to as single-strand
breaks and fragmentation of DNA seen on both neutral and
alkaline sucrose gradients is termed as double-strand breaks.
Since MMS induced single-stranded breaks in the DNA of
cultured mammalian cells (10, 16, 36), it was used as the
agent for testing the method of studying breaks and repair of
DNA in vivo. Four hr after treatment with MMS the he-
ptic DNA was shifted toward the lighter side of the gradi-
ent and exhibited a wide band rather than the sharp peak
near the bottom of the alkaline sucrose gradient (Chart 5).
Variable results were obtained at 24 hr, with some animals
exhibiting partial recovery from MMS damage and some
complete recovery. However, by 48 hr the DNA sedimenta-
tion profile in all animals had returned to normal, indicating
repair of the damage induced by MMS. Administration
of MMS to rats did not induce double-strand breaks in
hepatic DNA (R. O. Michael, and D. S. R. Sarma, un-
published data).

Histological examination of the liver showed scattered
areas of some morphological changes characterized by in-
creased basophilia of the nucleus and more pronounced
staining of the cytoplasm with both eosin and hematoxylin.
The majority of the altered cells were in the periportal re-
gions of the liver lobule. These changes were seen at 4 hr
were less at 24 and virtually absent at 48 hr and at 6 days.
No obvious zonal and focal necrosis was seen at any time.
However, a few widely scattered isolated cells in mitosis
were evident at 24 and 48 hr and at 6 days. Thus, some iso-
lated single-cell necrosis probably was induced by the treat-
ment with MMS.

DISCUSSION

The results of this study clearly show that very large pieces
of DNA, essentially free of any major contamination with
protein or RNA and sedimenting rapidly in a 5 to 20% alka-
line sucrose gradient, can be reproducibly obtained from
liver labeled in vivo with 3H-labeled thymidine. The size of
the DNA is not known and no attempt was made to arrive at
an estimate of its molecular weight. Many authors have
stressed how inaccurate this calculation is when DNA is
handled under the conditions used in this study (e.g., see
Refs. 22 to 24 and 30, 33, and 42). However, regardless of the
uncertainties concerning the size distribution of the sedi-
mnting material, there is almost no doubt that it consists
predominantly if not exclusively of DNA. The sensitivity to
hydrolysis by DNase and the resistance to RNase, Pronase,
and alkali offers strong support to this conclusion. Also, it is
evident that the method is useful especially in the study of
the damage and repair of liver DNA induced in vivo by a
well-known methylating agent, MMS. Rejoining of frag-
mented DNA seen in Chart 5 is considered as repair rather
than MMS-induced de novo DNA synthesis. As pointed
earlier, on histological examination, mitotic cells were
not obvious in MMS-treated rat liver. Further, in these rats,
there is a decreased incorporation of exogenous thymidine
into hepatic DNA (R. Cox and D. S. R. Sarma, unpublished
observations). By definition, rejoining of DNA chains in the
absence of semiconservative replication is termed as repair
of DNA.

Among several factors known to influence the success of
this modified McGrath-Williams (31) technique are the na-
ture of the suspending medium, the type of tissue prepara-
tion, and the concentration of DNA. Regardless of the type
of tissue preparation used, EDTA was essential to prevent
rapid fragmentation of DNA. The mechanism for this is
presumably the removal of Mg++ and other ions essential
for DNase action (39).

A whole-liver cell preparation, made mechanically by
hand, proved to be the most satisfactory form in which to
add the DNA to the sucrose gradients. Although a whole
homogenate was reasonably good, some fragmentation of
DNA was often found with this preparation. Isolated nuclei
were found to be entirely unsatisfactory because of the
breakup of the DNA. DNA isolated by conventional meth-
ods such as with phenol, which has been shown to be a de-
naturating agent (25), or CsCl gave a highly fragmented size
range. The squash technique used in this study with liver
can also be used with kidney and spleen to yield DNA of
high molecular weight when analyzed on alkaline sucrose
gradients.

Since the velocity sedimentation of DNA in alkaline
sucrose gradients is concentration dependent (10, 42), the

![Chart 5. Effect of MMS on the sedimentation of DNA in alkaline sucrose gradients. MMS was given i.p. at 120 mg/kg body weight. ○, controls; ×, MMS, 4 hr; O, MMS, 48 hr. Lysis of the cells and centrifugation of the gradients were the same as in Chart 2. Each gradient represents 1 animal. The experiment was repeated 5 times, and similar patterns of results were obtained.](chart5.png)
quantity of DNA to be loaded on the sucrose gradient becomes critical. In our study, 1 μg or less was optimal in that the recovery was consistently above 80% and often over 90%. With 2 μg or more, the recovery rapidly fell off to 50% or less (Table 1). This requirement for a low concentration almost necessitates the use of highly labeled DNA until a very sensitive quantitative method, valid for single-stranded DNA, is developed for the 0.05- to 1-μg range.

It is also evident from this study that the time scale for the induction of both DNA damage and DNA repair in liver is quite different than that in various cells in culture (10, 16, 36). Both the rate of induction of damage to DNA and the recovery from such damage occurs in minutes and hours in cells in culture rather than hours and days as has been found in this study with liver. This aspect is intimately related to the effects of other methylating agents and will be discussed more fully in the following paper (11).

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A Method for Measuring DNA Damage and Repair in the Liver *in Vivo*

Ray Cox, Ivan Damjanov, S. E. Abanobi, et al.

*Cancer Res* 1973;33:2114-2121.

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