the data presented here, it is suggested that such viruses be tested for hemagglutinating capacity at 4°C and 37°C with human cord erythrocytes in diluents of both high and low pH as an integral part of their characterization.

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Deoxyribonucleic Acid Polymerase Activity During Azo Dye Carcinogenesis.* (28962)

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Many investigations have been made of the carcinogenesis which occurs in the livers of rats fed azo dyes. Changes in tissue and cell morphology(1), alterations in subcellular components(2), and variations in certain proteins(3,4) have been studied. During the period of azo dye carcinogenesis, some enzymes of liver tissues have been examined both histochemically(5), and in extracts(6). Intense cellular proliferation has been observed to characterize several stages of this carcinogenesis. This observation prompted an

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investigation of DNA† polymerase activity in extracts of liver tissue from rats receiving azo dye. DNA polymerase‡ catalyzes the transfer of deoxynucleotides, derived from the deoxynucleoside triphosphates, to form a polymer in the pattern of a "primer" DNA which is present in the reaction mixture(7). This enzyme has been studied in extracts from bacteria(8), in phage-infected bacteria(7), in regenerating rat liver(9), in calf thymus (10), and in tumors(11,12,13). In comparison to similar extracts of regenerating rat liver(9), or of Novikoff hepatoma(12), which was originally derived from azo dye-induced tumor, extracts of normal rat liver have been shown to contain lower levels of DNA polymerase. The present paper reports the results of measurements of this enzymatic activity in extracts of liver tissue from rats fed azo dye; experiments designed to detect changes correlated with the carcinogenic transformation were utilized for these measurements.

Methods. Animals and diet. Male rats of the Holtzman strain, 150 to 200 g in weight, were fed a basal diet similar to that described by Griffin *et al*(3); USP Salt Mixture XIV replaced the individually added salts. After 7 days of this diet, test animals were fed the same diet augmented with 3'MeDAB (0.06% by weight). Test rats were sacrificed at 6, 13, 20, 27, 34, 41, 50, and 60 days of dye-feeding, and controls at 0, 13, 34, and 50 days. Three to 5 rats were processed at each time point.

Preparation of extracts. Rats were sacrificed by exsanguination following cervical dislocation. The livers were homogenized in a Potter-Elvehjem homogenizer with 10 passes of a motor-driven teflon pestle. The livers were perfused with ice cold isotonic saline, weighed, and homogenized in 9 volumes (by weight) of .25 M sucrose, 0.1 M in pH 8.0 tris. Homogenates were centrifuged for 60 minutes at 40,000 rpm in a Spinco model L centrifuge with the temperature maintained at 2°C. The ultracentrifugal supernate (S-3) was siphoned off, frozen rapidly at —80°C, and stored at —4°C. Proteins were determined by a biuret procedure (14).

DNA polymerase assay procedure. A modification of the disc method was developed, which allows the use of a tritiated substrate. In a volume of 225 μl, the assay medium contains: 10 μmoles of phosphate buffer pH 7.3, 2 μmoles of $MgCl_2$, 0.25 μmole of mercaptoethanol, 63 μg of DNA primer, .025 μmole each of dATP, dGTP, and TTP, and .025 μmole of H^3-dCTP (150,000 counts/.025 μmole). A volume of 25 μl of S-3 containing approximately .13 mg protein/assay was placed in the assay mixture at ice water temperature. The contents were mixed, the tubes were stoppered and placed at 35°C. At 0, 15, and 45 minutes after the incubation was started aliquots of 25 μl were removed and placed on 12.5 × 20 mm pieces of cellulose acetate film (Oxoid electrophoresis strip). After the sample had been absorbed (usually about 1 minute), these cellulose acetate pieces were washed in a beaker of ice cold 5% TCA (10 ml TCA per sample) for 10 minutes. A second wash in ice cold 5% TCA, two 5 minute washes in ice cold isopropyl alcohol (5 ml per sample), and a final rinse in absolute ether (2 ml per sample) followed. The pieces were dried in air and placed in 2 ml of ethyl acetate in a scintillation vial. The ethyl acetate dissolved the cellulose acetate; a clear solution was obtained. (The clear plastic backing was not dissolved.) Then, 8 ml of a solution composed of .5% POP and .01% POPOP in toluene was added and mixed. Tritium activity was counted in a Packard Tri-Carb model 314-DC liquid scintillation spectrometer at

† Abbreviations used in this paper are as follows: DNA, deoxyribonucleic acid; dCMP, dCDP, dCTP, deoxycytidine, mono-, di-, and triphosphate; TTP, dATP, ATP, dGTP, the triphosphates of thymidine, deoxyadenosine, adenosine, and deoxyguanosine; 3'-Me-DAB, 3'-methyl-4-dimethylaminoazobenzene; tris, tris (hydroxymethyl) amino methane hydrochloride; S-3, supernate from centrifugation at 100,000 × *g* for 1 hour; POP, 2,5 diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazol)-benzene, KPGA, potassium phosphoglycerate.

‡ Although the name "DNA nucleotidyltransferase," more accurately describes the function of this enzyme, the more commonly used "DNA polymerase" is used throughout this paper.

9-10% efficiency; this percentage efficiency is based on a H^3-dCMP standard prepared on cellulose acetate.

Preparation of H^3-dCTP. Tritiated dCMP of specific activity .93 counts/mmole (obtained from Schwarz BioResearch) was diluted in a molar ratio of 1:24 with non isotopic dCMP, and phosphorylated in a manner similar to that described by Bessman(8). Of this H^3-dCMP mixture 2.5 μmoles were placed in 1.4 ml of a medium containing 50 μmoles tris buffer (pH 8.1), 12.5 μmoles of $McCl_2$, 1.1 μmoles of mercaptoethanol, 15 μmoles of KPGA, and 12.5 μmoles of ATP. To this solution was added 0.5 ml of normal rat liver S-3 prepared as described for the kinase assay procedure; the medium was then incubated at 36°C for 20 minutes. Formation of polyphosphate was followed by the method of Furlong(15). Proteins were coagulated by heating the medium at 100°C for 2 minutes; the tubes were than chilled on ice, and centrifuged at approximately 400 $\times$ *g* for 10 minutes. The supernate was siphoned off, and the residue was washed twice in 0.5 ml H_2O. The combined volume of the supernatant solutions was 2.5 ml. The percentage of the conversion specifically to dCTP was determined by chromatography of 25 μl of the supernatant solution on a 5 $\times$ 30 cm DEAE paper strip. An ascending elution with .4 M ammonium bicarbonate was used. Following elution and drying of the sheet, segments along the sample trace which corresponded to dCMP, dCDP, and dCTP were placed in scintillation vials. Radioactivity on these spots was measured after adding 10 ml of the scintillation solution described above. The triphosphate accounted for 90% of the total radioactivity present and the solution was not purified further for these experiments.

Protein determination and enzyme stability. The protein concentrations of the S-3's were within the range 4.2 to 6.4 mg/ml and were not adjusted further before use in the polymerase assay. When these enzymatic activities are prepared as described and are stored at —4°C, their stability is indicated by the fact that analyses on the same extracts made 5 weeks apart yielded results which were within the error of multiple assays performed on the same day.

TABLE I. DNA Polymerase Activity as a Function of Protein Concentration.

Protein (mg)	Conversion (mμmoles) 15′	60′
.075	.013	.080
.150	.028	.120
.300	.045	.180
.525	.075	.255
.750	.105	.300

Incorporation of H^3-dCTP into DNA was measured as described under *Methods*. The protein source was normal liver S-3.

Results. DNA polymerase assays. The polymerase assay method was standardized by using an S-3 fraction from normal liver. Incorporation of dCTP in 15 minutes is seen in Table I to be a linear function of the amount of protein present up to .50 mg/assay; by 60 minutes, the reaction is no longer linear.

The data of Table II show average DNA polymerase activities from assays on liver S-3's prepared on the days indicated during the period of azo dye feeding. Enzyme activity values are expressed in terms of the activity per milligram protein in the extract. DNA polymerase activity detectable by this assay in the liver S-3's of the azo dye-fed rats shows a significant increase at 6 days, the earliest time at which activity was tested.

TABLE II. DNA Polymerase Activity of Liver S-3 Fraction During Azo Dye Carcinogenesis. Units of activity are expressed as mμmoles H^3-dCTP incorporated into DNA/mg protein/hr based on the linear value at 15′. Total c/m/assay were 15,000. Protein amount/assay ranged from .121 to .145 mg.

Group	Days dye fed	No. of animals	No. of assays	Avg Enzyme activity*	Stand dev
Dye	6	4	8	1.408	.185
"	13	4	8	1.451	.183
"	20	5	10	1.825	.129
"	27	5	10	1.260	.387
"	34	5	10	1.250	.226
"	41	4	8	1.065	.086
"	50	3	6	.954	.081
"	60	4	8	.938	.147
Control	0	4	4	.765	.075
"	13	3	3	.763	.067
"	34	3	3	.847	.109
"	50	3	3	.814	.031

* Determined as described under *Methods*.

This level remains elevated during the entire early carcinogenic period and in the period before visible tumors have developed, falls gradually toward the normal range.

Discussion. Only the S-3 extracts of liver tissue were assayed for DNA polymerase activity, for overwhelming evidence indicates that DNA polymerase activity is found almost exclusively in this fraction when the liver is homogenized in aqueous media(9,11, 12,13).

The results of these assays indicate that DNA polymerase activity is increased in the liver of azo dye-fed rats. The nature, sequence, and timing of the major morphological changes which accompany azo dye carcinogenesis(2) are sufficiently reproducible to permit a gross correlation to be made with the present data. In this regard, it is interesting that the increase in observed DNA polymerase occurs as early as 6 days. This time precedes even the earliest proliferative response to azo dye feeding, which is that of parenchymal cells, and characteristically occurs late in the second week of dye-feeding. Since there is no major alteration of the amount of liver protein in this period, the enzyme specific activity as given in the Table indicates an actual increase in amount of enzyme present, doubling in amount in the first week and tripling in amount by 3 weeks. On an extended time scale, the response of the DNA polymerase level to dye feeding resembles the response evoked in the liver by partial hepatectomy. Perhaps in the initial stages of azo dye carcinogenesis, injury to liver cells stimulates production of DNA polymerase. The maximum activity, 20 to 27 days, occurs at a time in the carcinogenic process that is associated with extensive bile duct proliferation. Except for this period, there is little correlation between the gross mitotic index of the liver and the specific activity of DNA polymerase. There are several possible explanations for this lack of correlation. If the dye interfered with cell division or changed the timing of DNA biosynthesis in some types of liver cells, the enzyme might accumulate in these cells. Alternatively, the dye could alter the formation or action of nucleases, pyrophosphatases or other enzymes that would appear in the crude enzyme extract and affect the assay without actually changing the DNA polymerase. This latter possibility is made less plausible, however, by the observation that the polymerase catalyzed incorporations measured at 15 and 45 minutes give about the same ratio for all of the extracts.

Observations on the titer of enzymatic activities in an extract of whole liver are not sufficient to permit a cytological analysis of the events taking place. Chang(5) has reveloped methods for measurement of certain enzymatic activities in tissue sections prepared from the livers of azo dye-fed rats. Methods similar to these are being developed for histochemical demonstration of DNA polymerase, of DNA that is competent as a primer, and of various kinases. These methods should provide further insight into the cellular alterations brought about by azo dyes.

Summary. DNA polymerase activity was measured in extracts of liver prepared from rats fed a carcinogenic azo dye. Groups of rats were sacrificed during the period of carcinogenic transformation in the liver, *i.e.,* the first 8 weeks of dye-feeding, and the DNA polymerase activities in the soluble ultracentrifugal supernatant fraction of liver homogenates were assayed. A doubling of the rather low enzyme activity of normal rat liver was found at 6 days, the earliest time point, after initiation of dye feeding. Activity continued to increase through the third week of dye feeding to almost 3 times the normal value, then it gradually dropped to values slightly above normal at the end of the eighth week.

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Inhibition of Alcohol Dehydrogenase by Folic Acid and Several of Its Analogs.* (28963)

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The biochemical function of folic acid as a necessary cofactor in one-carbon metabolism has been well documented. While studying the oxidation of ethanol in homogenates of rat liver, it was observed that folic acid arrested ethanol metabolism. This finding led to the discovery that folic acid inhibited alcohol dehydrogenase. Since 4-amino analogs of folic acid exert actions antagonistic to the vitamin, the effect of these substances on alcohol dehydrogenase was studied. It was found that aminopterin and amethopterin share with folic acid the property of inhibiting alcohol dehydrogenase. The common inhibition of alcohol dehydrogenase by folic acid and analogs cannot be explained on the basis of the known chelating properties of folic acid with zinc.

To determine whether alcohol dehydrogenase could be inhibited in intact animals, the influence of folic acid was studied (a) on oxidation of ethanol and acetate in the rat, and (b) on rate of ethanol disappearance from the plasma of dogs. Large amounts of folic acid decreased ethanol but not acetate metabolism which is evidence that alcohol dehydrogenase was inhibited *in vivo* by folic acid.

Materials and methods. Folic acid and aminopterin were obtained from the California Corp. for Biochemical Research. 1,10-Phenanthroline was purchased from Eastman Organic Chemicals. Folvite, calcium leucovorin, amethopterin and 4-dimethylamino-4-deoxy-N^{10}-methylpteroylglutamic acid (4-dimethylamethopterin) were kindly supplied by Lederle Labs. Radioactive ethanol, acetaldehyde and acetate were obtained from Volk Radiochemical Corp. Crystalline alcohol dehydrogenase derived from horse liver was purchased from Worthington Biochemical Corp.

Studies with rat liver homogenates. Each incubation vessel contained 0.01 M K phosphate (pH 7.4), 0.0032 M $MgCl_2$, 0.088 M KCl, 0.0015 M K_2 ATP, 8.8 mg cytochrome c, 1 g of rat liver (Sprague-Dawley strain), 100 μmoles of ethanol-1-C^{14} or 50 μmoles of acetaldehyde-1-C^{14} plus inhibitors (Table I) in a total volume of 40 ml. Each vessel was incubated for 2 hours in air at 37°C before the reaction was stopped with $CuSO_4$ and

TABLE I. Effect of Folic Acid or Analogs (3×10^{-5}M) on Ethanol Metabolism in Homogenates of Rat Liver.

Inhibitor	μmoles per vessel: Ethanol metabolized	μmoles per vessel: Acetaldehyde present
None	27	15.5
Folic acid	0	0
Aminopterin	0	0
Amethopterin	2	.6
Leucovorin	24	12.5

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