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The activity of deoxyribose-phosphate aldolase was determined in chick embryo brains, hearts and livers during the course of development. Assays were made in the direction of synthesis of deoxyribose-5-phosphate. Low levels of activity in the brain and heart remained constant throughout the course of development. Activity in the liver expressed per mg of protein increases up to 14 days, increases more rapidly between 16 and 18 days, after which it levels off until several days after hatching. All activity in the liver is contained in the supernatant after centrifugation at 30,000 x g.

Certain conditions for the assay of deoxyribose-phosphate aldolase were evaluated. Although the enzyme seems to be inactivated by ammonium sulfate precipitation, it is stable for several hours at $4^{\circ}C$, but gradually loses activity during prolonged storage at either $4^{\circ}C$ or $-20^{\circ}C$. Assay of activity of the enzyme is not affected

by the action of phosphatase on the product, since deoxyribose and deoxyribose-5-phosphate produce very nearly the same extinction coefficient. Activation by added citrate was shown for the enzyme in heart homogenate, but not in liver homogenate. It is suggested that endogenous levels of citrate in liver tissue are sufficient to provide for maximum enzymatic activity.

Inhibition of deoxyribose-phosphate aldolase by diphenylsuccinate reported for rat liver was confirmed for the chick embryo enzyme. Similar inhibition occurred in all three organ homogenates. Complete inhibition was obtained at 10 mM diphenylsuccinate.

Hydroxyurea appeared to effect a slight inhibition of the enzyme activity, although an increase in inhibitory effect was not consistent with increased hydroxyurea concentration. Another effect of hydroxyurea is inhibition of the diphenylamine reaction with deoxyribose-5-phosphate, which requires correction of assays made in the presence of hydroxyurea. A third effect of hydroxyurea is a reaction with diphenylamine which produces a blue chromogen with absorption peaks at 430 mμ and 630 mμ (instead of the single peak at 600 mμ observed in the reaction of deoxyribose-5-phosphate with the diphenylamine reagent). This reaction was obtained when fructose-1, 6-diphosphate was incubated with liver supernatant and the products were treated with diphenylamine. The development of color depends on time of enzymatic incubation, on the concentrations of fructose-1,6-diphosphate

and supernatant present in the incubation mixture, and on the concentration of hydroxyurea added either before or after enzymatic incubation. The identity of the product(s) was not determined.

The incorporation of label from 1-^{14}C-deoxyribose into DNA was studied. A maximum of about 0.2% of injected radioactivity was recovered in the nucleic acid extracts from embryonic brains, hearts and livers. Greater incorporation was obtained by injection of deoxyribose into the air space than by injection into the yolk sac. Radioactive DNA was degraded to determine the extent of incorporation of label into carbon-1 of deoxyribose from DNA. Deoxyribose was converted to levulinic acid and levulinic acid into valeric acid. Valerate was then subjected to Schmidt degradation to remove carbon-1 as carbon dioxide. Up to 75% of deoxyribose was obtained as levulinic acid and up to 50% of levulinic as valeric acid. Specific radioactivity of valerate from DNA samples was lower than that of levulinate, indicating contamination of levulinate with radioactive material that was not carried over into valerate.

Schmidt degradation did not result in the trapping of all of the ^{14}C from valerate samples. However, no radioactivity was detected in most of the residual Schmidt degradation mixtures. There is loss of radioactive material, apparently by evaporation. The lack of radioactivity in the residual solution indicates that the four carbon

atoms of valerate left behind as butylammonium ions after decarboxylation are devoid of radioactivity, suggesting that 1-^{14}C-deoxyribose is incorporated intact into DNA.

DEOXYRIBOSE-PHOSPHATE ALDOLASE AND THE BIOSYNTHESIS OF DNA IN THE CHICK EMBRYO

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DEOXYRIBOSE-PHOSPHATE ALDOLASE AND THE BIOSYNTHESIS OF DNA IN THE CHICK EMBRYO

INTRODUCTION

Since the discovery of deoxyribose-phosphate aldolase by Racker in 1951, the possibility that it is involved in the synthesis of deoxyribose for DNA has been considered. Racker indeed proposed this in his original paper. However, already at that time, evidence had begun to accumulate (Hammarsten et al., 1950) in favor of a reduction of ribose to deoxyribose while bound to a pyrimidine base. This latter reaction has now been well established and extensively studied in various animal tissues as well as in several strains of bacteria (Reichard, 1968).

In many cases the reductive route seems to be the only mechanism by which deoxyribose arises. For instance, in experiments with regenerating liver, ascites tumor and HeLa cells, Horecker et al. (1958) found that when certain specifically labeled precursors were administered, the pattern of label in deoxyribose carbon atoms was similar to that in ribose. However, in experiments with carbon-1 labeled glucose, carbon-5 of deoxyribose was heavily labeled. This was interpreted as an indication of a possible triose phosphate precursor of deoxyribose. More recently, in response to Groth and Jiang's finding (1966) that an inhibitor of deoxyribose-phosphate

aldolase also inhibits incorporation of orotate and uracil into DNA pyrimidines, Larsson and Neilands (1966) injected rats with ^{32}P and ^{14}C-cytidine and determined the ratio of ^{32}P:^{14}C in CMP and dCMP isolated from RNA and DNA. The ratios were found to be identical, and this was interpreted as further evidence that direct reduction is the sole source of deoxyribose. Recently, Foung and Norman (1968) reported work on chick tissue which indicates a pathway for the formation of purine deoxyribonucleotides other than the reduction of ribonucleotides. They administered radioactive adenosine and guanosine with a known ratio of ^{14}C in the sugar to ^{14}C in the purine base. After growth for 48 hours, DNA and RNA were isolated from liver and intestine and the ratio was similarly determined for the purine ribonucleosides and deoxyribonucleosides obtained from the nucleic acids. The ratios were significantly different. This was interpreted as indicating that the glycosidic bond of the purine ribonucleoside was cleaved prior to incorporation of radioactive precursors into DNA.

Several workers have found different labeling patterns in ribose and deoxyribose obtained from the nucleic acids. Ghosh and Bernstein (1963) supplied ^{14}C-sodium bicarbonate to rats of various ages and conditions and found that in normal young adults, skin and liver deoxyribose from DNA did not have the same pattern of label as ribose from RNA. Tissues from tumor-bearing young adults showed

similar labeling patterns in ribose and deoxyribose. Coffey, Morse and Newburgh (1966) also found different labeling patterns in ribose and deoxyribose from nucleic acids of chick embryo explants. When 1-^{14}C-glucose was administered, ribose carried 63% of the radioactivity in carbon-1 and 27% in carbon-5. Deoxyribose had less than 1% of the label in carbon-1, but 29% in carbon-2 and 60% in carbon-5.

Reviews of the biosynthesis of deoxyribose have appeared in 1963 (Glaser) and 1966 (Sable). In both cases, the authors state that no conclusive evidence has been presented to show the involvement of deoxyribose-5-phosphate in DNA biosynthesis, although Sable suggests that there may be a de novo pathway which allows the incorporation of deoxyribose-5-phosphate into deoxyribonucleotides.

The fact that not all experiments show the same pattern of label in the pentose carbon atoms may be explained by any one of at least three hypotheses: (a) The pools of nucleotides from which RNA and DNA are synthesized are compartmentalized such that the pool of ribonucleotides from which RNA arises is not the same as the pool from which DNA is synthesized via ribonucleotide reductase. (b) There is a separation in time of RNA and DNA synthesis such that even though the locus of the pool is the same, the actual pool involved at the time of synthesis is different for the two nucleic acids. (c) There is an alternate pathway for deoxyribose synthesis. One alternate pathway consists of the sequence of reactions:

(1) Acetaldehyde + Glyceraldehyde-3-phosphate $\rightleftarrows$ Deoxyribose-5-phosphate

(2) Deoxyribose-5-phosphate $\rightleftarrows$ Deoxyribose-1-phosphate

(3) Deoxyribose-1-phosphate + Purine or pyrimidine base $\rightleftarrows$ Deoxyribonucleoside + Pi

(4) Deoxyribonucleoside $\rightleftarrows$ Deoxyribonucleotide

This series of reactions permits a de novo synthesis of deoxyribose different from that involving direct reduction of ribonucleotides.

The present study was undertaken to determine whether deoxyribose-phosphate aldolase, which catalyzes reaction (1), is present in chick embryonic tissues, whether its occurrence can be correlated with the synthesis of DNA, and whether deoxyribose is incorporated directly into DNA. Certain characteristics of the enzyme are also presented, as well as methods for the degradation of deoxyribose isolated from DNA.

PART I. DEOXYRIBOSE-PHOSPHATE ADOLASE EXPERIMENTS

MATERIALS AND METHODS

Chick Embryos

Hy-Line 950-A fertile eggs were obtained from Jenk's Hatchery, Tangent, Oregon. The eggs were incubated in a Jamesway Model 252B incubator at wet bulb temperature 86°F and dry bulb temperature 99°F, and were turned every two hours.

Chemicals

Deoxyribose, fructose-1,6-diphosphate (tetrasodium salt), glyceraldehyde-3-phosphate, and rabbit muscle aldolase were products of Sigma Chemical Company. Hydroxyurea was obtained from Aldrich Chemical Company and Sigma. Sodium fluoride, acetaldehyde and diphenylamine were obtained from Matheson, Coleman and Bell. Acetaldehyde was distilled and stored in dilute aqueous solutions in the freezer or, for short periods of time, in the refrigerator. Diphenylsuccinic acid was obtained from Aldrich Chemical Company.

Deoxyribose-5-phosphate was prepared from the di(cyclohexylammonium) salt of deoxyribose dimethyl acetal 5-phosphate, which was the kind gift of Dr. D. L. MacDonald (1959).

Assay of Deoxyribose-phosphate Aldolase Activity

Chick embryonic brains, hearts and livers were homogenized in a Potter-Elvehejm tissue grinder in cold 0.08 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4. Appropriate numbers of organs and volume of buffer were selected to give conveniently measurable activity. Aliquots from such homogenates were centrifuged at 30,000 x g in a Servall refrigerated centrifuge for 30 minutes. Both homogenate and the supernatant from the centrifugation were used for assay of deoxyribose-phosphate aldolase. The enzymatic assay was conducted at 37°C over a period of time up to twenty minutes or up to eighty minutes, depending on the activity of the preparation. In most cases, linear reaction rates were observed. The assay mixtures consisted of fructose-1,6-diphosphate, 10.0 mM; acetaldehyde, 100 mM; Tris buffer (pH 7.4) 40.0 mM and sufficient homogenate or supernatant for measurable activity. Fructose-1,6-diphosphate served as the source of glyceraldehyde-3-phosphate via the action of endogenous fructose-1,6-diphosphate aldolase. Aliquots were withdrawn at selected time intervals and diluted with an equal volume of 10% (w/v) trichloroacetic acid. Deoxyribose-5-phosphate content was then determined by the diphenylamine method of Dische (1930) as modified by Burton (1956). The method consists of adding one volume of solution

containing deoxyribose or deoxyribose derivative to two volumes of diphenylamine solution. The latter solution is composed of 1.5 g of diphenylamine, 1.5 ml of concentrated sulfuric acid, made to 100 ml with glacial acetic acid. On the day in which it is used, 0.10 ml of aqueous acetaldehyde solution (16 mg/ml) is added for each 20 ml of diphenylamine solution. The assay mixture is placed in a dark room at 35°C for 16-20 hours after which the absorbance at 600 mμ is read against a blank made up of one volume of water and two volumes of diphenylamine reagent solution similarly incubated in the dark.

Determination of Protein

The protein content of homogenates was determined by the method of Lowry, et al. (1951) or by the biuret method according to Cleland and Slater (1953), with the modifications noted by Layne (1957). Prior to the determination of protein, lipids and acid-soluble materials were extracted twice with equal volumes of 5% trichloroacetic acid, with 50% acidified ethanol and with 95% ethanol. Insoluble material was sedimented at 2,000 x g after each extraction. In the case of livers from 16 to 20 day embryos, an extraction with chloroform-methanol (2:1 v/v) was inserted into the extraction sequence after 50% ethanol, in order to remove the bulk of the lipid material.

Determination of Phosphorus

Total and free phosphate determinations were made by the Fiske-SubbaRow method as modified by Bartlett (1959). Samples were ashed for total phosphate assay by the method given by Ames (1966).

EXPERIMENTAL RESULTS

Conditions for Deoxyribose-phosphate Aldolase Assay

Preliminary attempts to purify deoxyribose-phosphate aldolase by ammonium sulfate precipitation following the method of Roscoe and Nelson (1964) used for the rat liver enzyme resulted in an apparent total loss of activity, even when mercaptoethanol (ME) and ethylene-diaminetetraacetate (EDTA) were added.

In order to determine whether this loss of activity occurs during storage of the enzyme, assays of deoxyribose-phosphate aldolase activity were made on fresh homogenates and supernatants obtained from livers of eleven day old embryos. Assays were performed as noted in the methods section, both in the presence of and in the absence of ME (5 mM) and EDTA (1 mM).

From Table 1 it is clear that virtually all of the deoxyribose-phosphate aldolase activity present in the homogenate is retained in the supernatant after centrifugation at 30,000 x g. About half the activity is lost when ME and EDTA are added. Control experiments showed that neither of the latter compounds has an inhibitory effect on the diphenylamine assay. In fact, the addition of ME and EDTA at the concentrations used in the assay to solutions of deoxyribose at various concentrations slightly enhanced color development in the diphenylamine reaction. Absorbance of about 0.470 for deoxyribose

Table 1. Activity of deoxyribose-phosphate aldolase in liver preparations stored at 4°C and -20°C

	Percentage of maximum rate observed							
	Fresh Preparation		Stored 4.5 hours at 4°C		Stored 14 hours at 4°C		Stored 14 hours at -20°C	
	-ME, EDTA	+ME, EDTA	-ME, EDTA	+ME, EDTA	-ME, EDTA	+ME, EDTA	-ME, EDTA	+ME, EDTA
Homogenate	99	52	100	47	79	30	79	39
Supernatant	99	47	--	--	62	32	77	35

Assay conditions are given in the text; 0.5 ml of homogenate or supernate were added.

at 0.100 mM was increased to 0.495 in the presence of ME and EDTA. Approximately one-fourth to one-third of the activity is lost upon prolonged storage at either 4°C or -20°C in either the whole homogenate or the supernatant fraction.

It has been reported by Boxer and Shonk (1958) that deoxyribose reacts in the diphenylamine assay to produce only about one-third as intense color as does deoxyribose-5-phosphate. If this were true, then the possibility would exist that a phosphatase present in tissues may remove phosphate from deoxyribose-5-phosphate as it is synthesized. This would result in an apparent low rate of synthesis. Added fluoride should inhibit this dephosphorylation and insure an observed rate equal to the actual rate of deoxyribose-5-phosphate synthesis. For this reason, Marinello et al. (1963) have added fluoride to reaction mixtures assayed for deoxyribose-phosphate aldolase activity.

Standard curves were prepared using deoxyribose, deoxyribose-5-phosphate and the di(cyclohexylammonium) salt of deoxyribose dimethyl acetal 5-phosphate. All produced very nearly the same slope, as shown in Figure 1. Calculated extinction coefficients agreed within about 10%. The extinction coefficients obtained in this assay vary somewhat with the diphenylamine reagent solution used, so it was found advisable to run a standard curve whenever it was desired to compare rate data of one experiment with those of another.

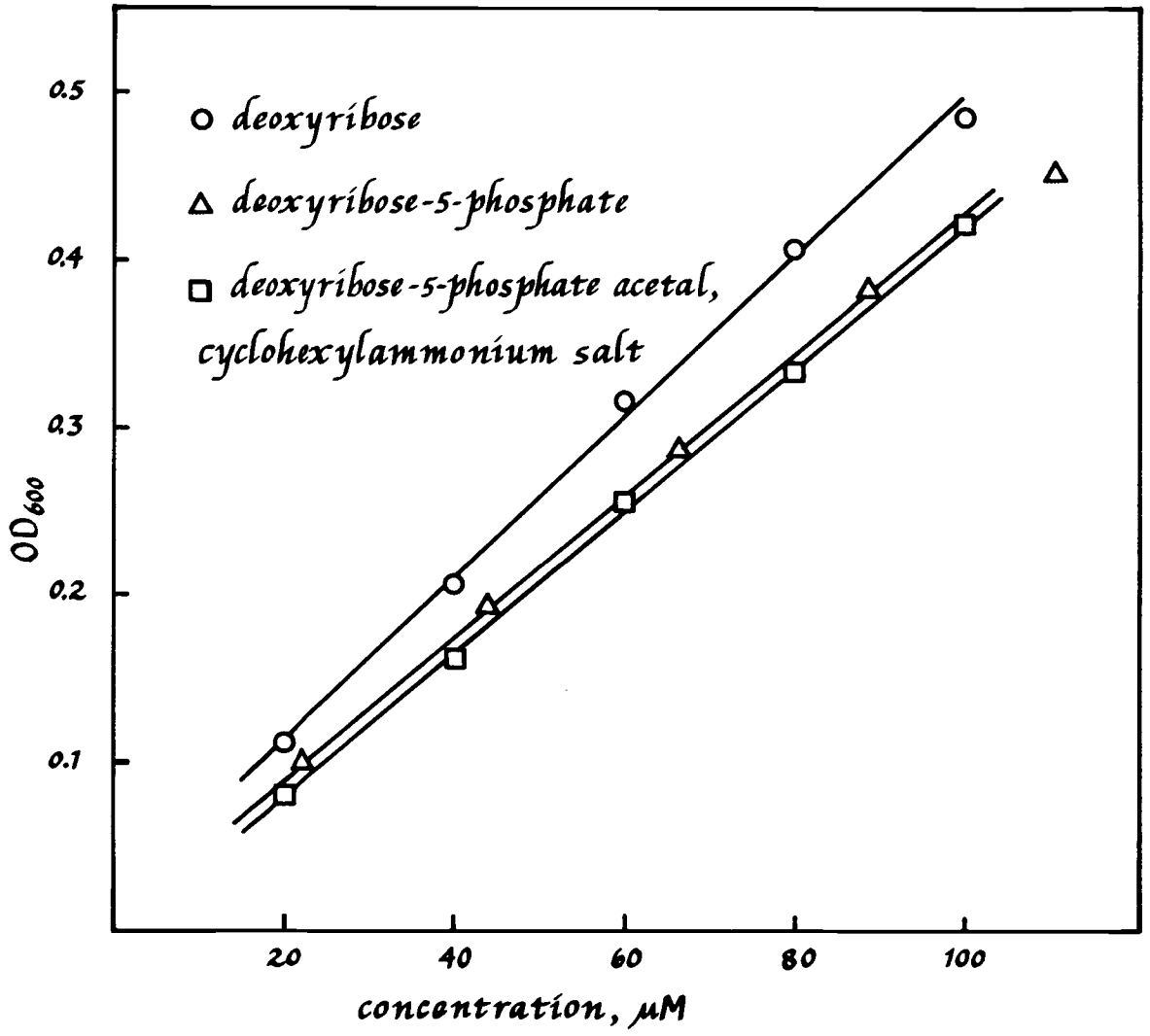

Figure 1. Standard curves for determination of deoxyribose and its derivatives by the diphenylamine reaction.

However, in every instance the extinction coefficient of deoxyribose was 1.13 $\pm$ .05 times that of deoxyribose-5-phosphate. Representative optical densities at 600 mμ obtained were 0.470 and 0.475 for deoxyribose-5-phosphate and deoxyribose respectively, when solutions containing 0.100μ mole/ml were added to twice the volume of diphenylamine reagent solution. The di(cyclohexylammonium) salt of the dimethyl acetal of deoxyribose-5-phosphate exhibited the same extinction as deoxyribose-5-phosphate. The spectra of the colored products are identical and correspond to the curve for deoxyribose-5-phosphate shown in Figure 12.

The addition of sodium fluoride, up to 50 mM in the reaction mixture in assays using liver from 12-day embryos failed to bring about any change in the apparent rate of deoxyribose-5-phosphate synthesis.

Another method of comparing deoxyribose and deoxyribose-5-phosphate involved the cleavage of the cleavage of the phosphate bond and analysis by the diphenylamine reaction before and after cleavage. Alkaline phosphatase was used for the cleavage and free phosphate determinations indicated that at least 66% and up to 90% of the phosphate was liberated in replicate trials. Results are reported in Table 2 in terms of mM deoxyribose-phosphate equivalent before and after phosphatase treatment.

Finally, an attempt was made to repeat the experiment of Boxer

Table 2. Comparison of reactions of deoxyribose-5-phosphate and deoxyribose with diphenylamine in the Burton reaction.

Experiment	mM phosphate liberated	mM Deoxyribose-phosphate equivalent	
		Before phosphatase treatment	After phosphatase treatment
I	1.95 (66%)[1]	2.92 ±0.09	2.95 ±0.26
II	2.36 (90%)[1]	2.62 ±0.24	2.61 ±0.32

Reaction mixtures consisted of deoxyribose-5-phosphate, 4.14 μmoles; glycyl glycine, 100 μmoles; $MgSO_4$, 16 μmoles and 1.0 mg of alkaline phosphatase at pH 9.3 in a total volume of 1.5 ml.

[1] Percent of bound phosphate liberated as inorganic phosphate.

and Shonk, but replacing rat liver with chick embryo liver. The supernatant after centrifugation at 30,000 x g of 18-day liver homogenates was employed as the enzyme source. Deoxyribose-5-phosphate or deoxyribose was used as the substrate. Aliquots were removed at 10-minute intervals from the incubation mixtures for determination of pentose remaining by means of the diphenylamine reaction. Results are shown in Figure 2, where it can readily be seen that deoxyribose is not attacked, whereas there is a progressive decrease in absorbance in the reaction mixture containing deoxyribose-5-phosphate. Extrapolation of absorbance to initial time gave values of 0.530 and 0.460 for deoxyribose and deoxyribose-5-phosphate, respectively. These correspond well with the extinction values obtained for 0.100 mM solutions of the compounds incubated in the absence of enzyme.

Jiang and Groth (1962) have shown that deoxyribose-phosphate aldolase activity in rat liver is stimulated by citrate and other di- and tri-carboxylic acids. To determine whether citrate affects the activity of this enzyme from chick embryo liver, the assay of activity in homogenates of 8-day or 18-day livers was carried out in the presence of 0.5 mM sodium citrate. No increase in activity was observed over the controls lacking exogenously supplied citrate in the liver homogenate. In the case of 18-day hearts, however, some stimulation was observed, as noted in Figure 3. With added citrate,

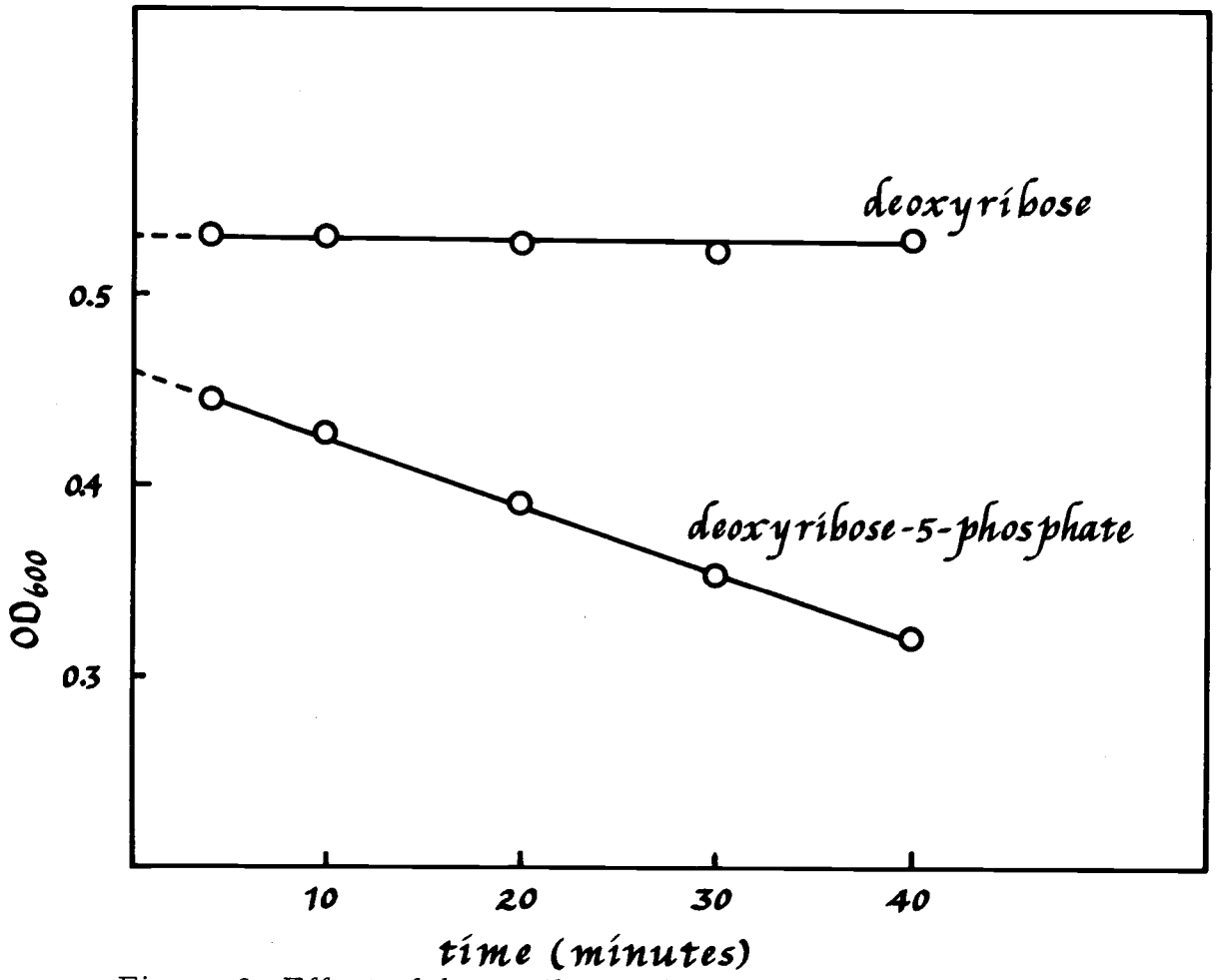

Figure 2. Effect of deoxyribose-phosphate aldolase on deoxyribose and deoxyribose-5-phosphate. Incubation mixtures consisted of 6 μmoles of deoxyribose or deoxyribose-5-phosphate, 120 μmoles of Tris buffer, pH 7.4 and 0.25 ml of 18-day liver supernatant incubated at 37°C.

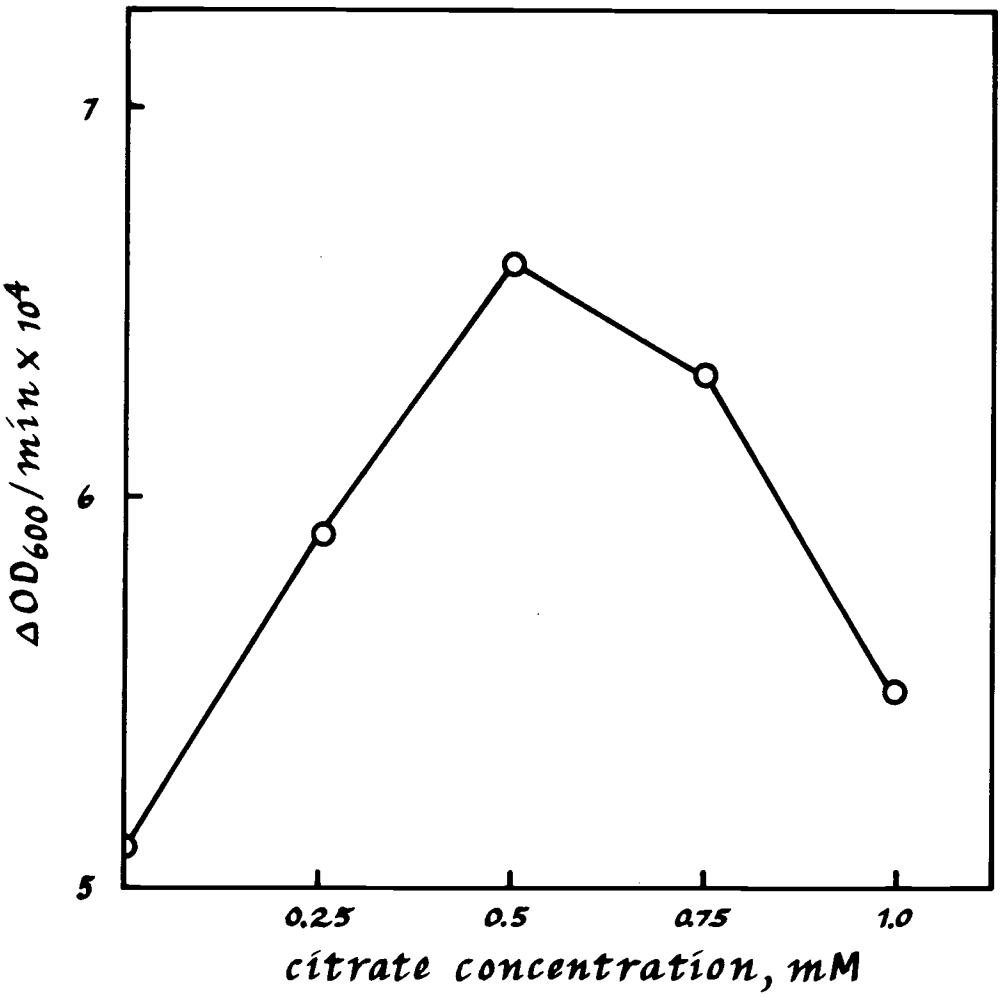

Figure 3. Stimulation of deoxyribose-phosphate aldolase in chick embryo heart by citrate. Citrate concentrations shown were present in incubation mixtures of 18-day heart homogenate. Reaction mixtures contained fructose-1, 6-diphosphate, 10 mM; acetaldehyde, 100 mM; Tris buffer, pH 7.4, 40 mM and 1.0 ml of homogenate in a final volume of 2.5 ml.

the rate of deoxyribose-5-phosphate synthesis showed a maximum at 0.5 mM citrate, and above this concentration the stimulatory effect was less.

The use of fructose-1, 6-diphosphate to provide the substrate glyceraldehyde-3-phosphate for deoxyribose-phosphate aldolase presupposes that there is sufficient fructose-1, 6-diphosphate aldolase present to convert fructose-1, 6-diphosphate to glyceraldehyde-3-phosphate at a rate rapid enough to supply saturating levels of glyceraldehyde-3-phosphate. Assays made with added fructose-1, 6-diphosphate aldolase using 8-day and 18-day liver homogenates showed no increase over control rates. As noted in the discussion section, this is expected from the known activity of aldolase in chick embryo liver.

Change in Activity of Deoxyribose-phosphate Aldolase During Development

The pattern of activity of deoxyribose-phosphate aldolase in chick embryo tissues is shown in Figures 4 and 5. No activity was detectable in whole embryos at two days' incubation, although a low level was measurable in the yolk sac at that age. Activity in the whole embryo was measured after four, six and eight days' incubation. At six days, brain and heart assays were begun, and at eight days, liver assays were started. Assays were made every two days until

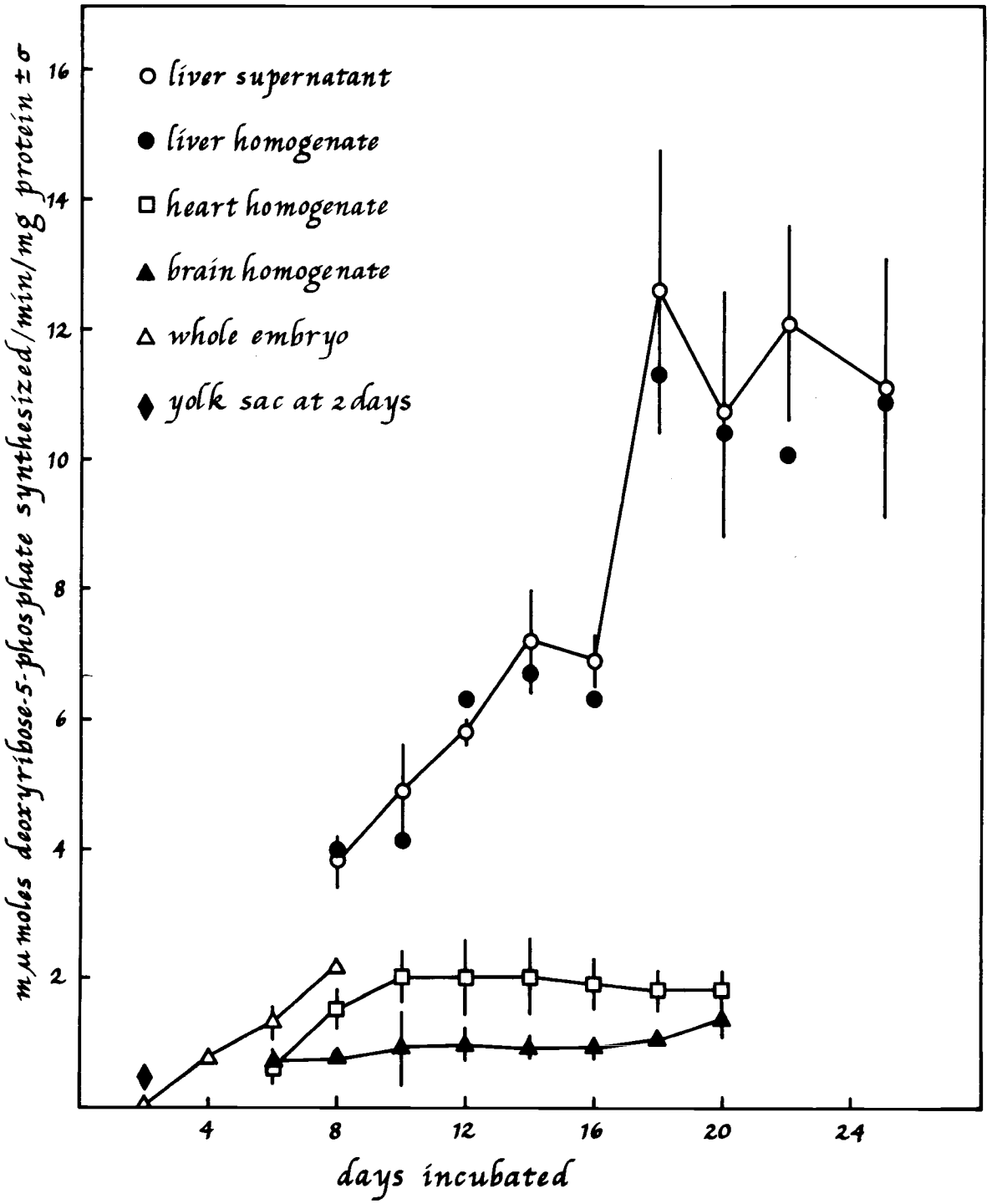

Figure 4. Deoxyribose-phosphate aldolase activity in whole chick embryos and chick embryo organs. Assays were made by the procedure given on page 6. Values of specific activity are given $\pm \sigma$ for four or more determinations.

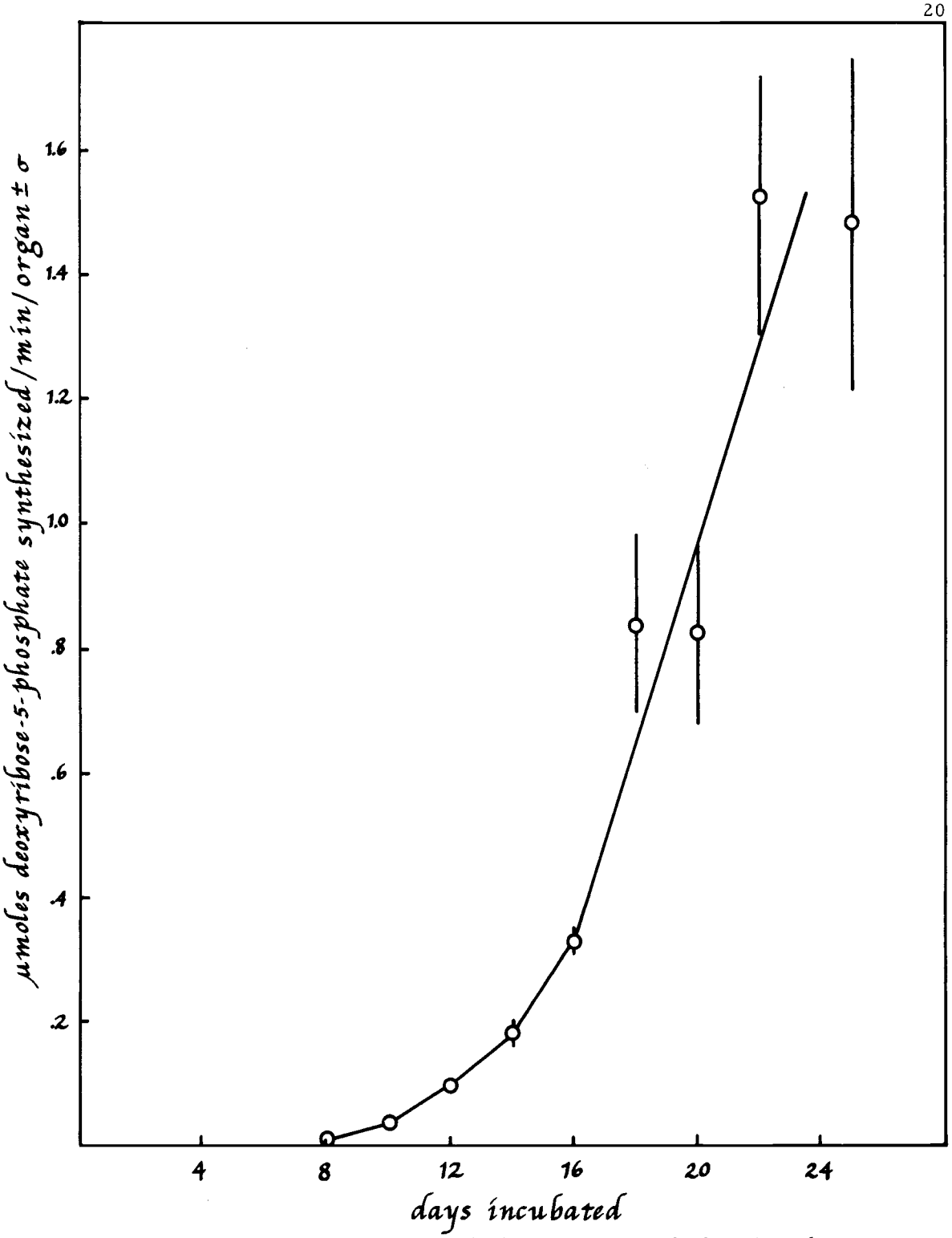

Figure 5. Deoxyribose-phosphate aldolase activity in chick embryo liver. Assays were made by the procedure given on page 6. Values of specific activity are given $\pm \sigma$ for four or more determinations.

hatch, and at one and four days after hatch. Specific activity is shown as a function of incubation time. The results indicate that the level of activity in brain and heart homogenates remains at relatively low levels throughout the term of development, whereas activity in liver increases somewhat until 14 days, increases rapidly between 16 and 18 days, then levels off after 18 days. All enzyme activity in the liver appears to be contained in the supernatant after centrifugation at 30,000 x g. The values given for supernatant were not corrected for the small decrease in volume upon discard of the pellet, and so values for supernatant are consistently slightly higher than for whole homogenate. At ages of 18 days and older, there was a large variation among individuals.

Inhibition of Deoxyribose-phosphate Aldolase by Diphenylsuccinate

Groth and Jiang (1966) reported that meso-α, β-diphenylsuccinic acid interferes with citrate activation of highly purified rat liver deoxyribose-phosphate aldolase. Since preliminary experiments indicated that diphenylsuccinate also inhibited the chick liver enzyme, experiments were done with homogenate of 12-day embryo brain, heart and liver.

The results are shown in Table 3, where it is evident that 10 mM diphenylsuccinate completely inhibits deoxyribose-phosphate

Table 3. Inhibition of deoxyribose-phosphate aldolase activity in brain, heart and liver homogenates by diphenylsuccinate.

	Percent of control rate		
	0.1 mM DPS	1.0 mM DPS	10 mM DPS
Brain	74	18	0
Heart	64	29	0
Liver	76	20	0

All assay mixtures contained fructose-1, 6-diphosphate, 10 mM; acetaldehyde, 100 mM; Tris buffer, pH 7.4, 40 mM and 33% (w/v) of tissue homogenate.

aldolase in all three tissues and partial inhibition occurs at 0.1 mM and 1.0 mM.

Effects of Hydroxyurea in Deoxyribose-phosphate Aldolase Assay

Hydroxyurea is known to inhibit DNA synthesis, and its action in inhibiting ribonucleotide reductase has been well established (Turner, et al., 1966). However, other influences on DNA synthesis have been suggested, such as interference with DNA polymerase (Brachet, 1967) and possibly other actions not yet investigated (Yarbro, 1968). It was therefore of interest to determine whether hydroxyurea inhibits deoxyribose-phosphate aldolase.

Preliminary experiments indicated that under certain conditions there was an apparent inhibition, and under other conditions an apparent stimulation of deoxyribose-phosphate aldolase activity in the presence of hydroxyurea.

To determine whether hydroxyurea in the absence of enzyme gives rise to color in the diphenylamine reaction, control reactions were run, in which hydroxyurea solutions were incubated with the diphenylamine reagent in the absence of any deoxyribose derivative. No color was noted, and no increase in absorbance at 600 mμ occurred.

In another experiment, the effect of hydroxyurea on the reaction

between diphenylamine and deoxyribose-5-phosphate was assessed, again in the absence of enzyme. Hydroxyurea was added to solutions containing deoxyribose-5-phosphate and the diphenylamine reagent was added to this mixture. The effect of increasing concentrations of hydroxyurea on color development from a single concentration of deoxyribose-5-phosphate is shown in Figure 6.

When the effects of hydroxyurea on different concentrations of deoxyribose-5-phosphate were measured, the standard curves were linear in the presence of 2.5 mM and 25 mM hydroxyurea, but the slopes of the lines were decreased considerably, as shown in Figure 7. Both in the presence and in the absence of hydroxyurea, only one peak appeared in the visible absorption spectrum, which was at about 600 mμ (Figure 12).

Finally, an experiment was performed to determine whether there is any effect of hydroxyurea on the action of deoxyribose-phosphate aldolase itself. Hydroxyurea at various concentrations was added to reaction mixtures containing 0.25 ml of the supernatant of 30,000 x g centrifugation of liver homogenate from 18-day embryos. Also present in each of the reaction mixtures were Tris buffer, 40 mM; fructose-1,6-diphosphate, 10 mM; and various concentrations of acetaldehyde in a final volume of 3.0 ml at pH 7.4. Assays of deoxyribose-5-phosphate synthesis were carried out as given on page 6.

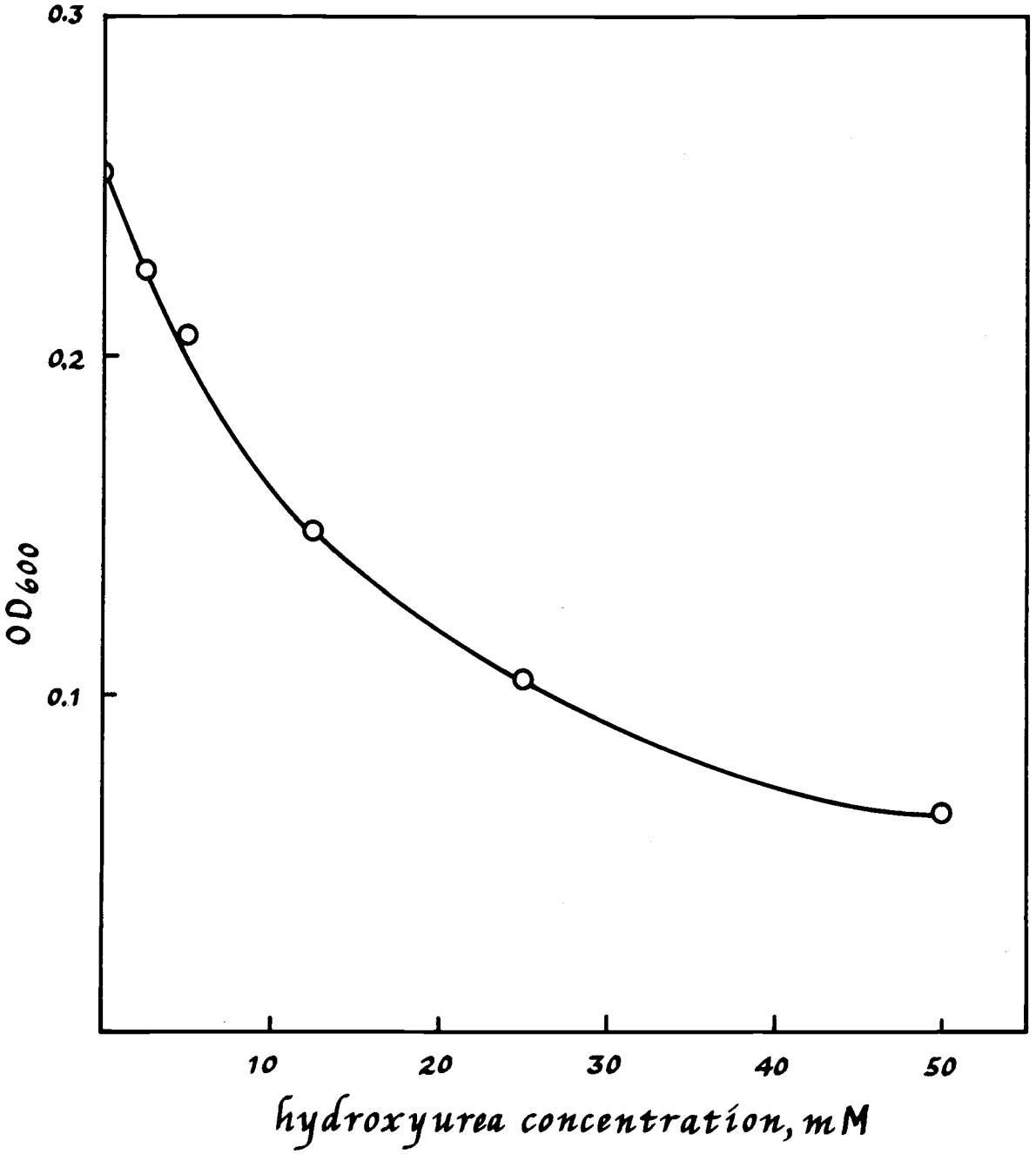

Figure 6. Effect of hydroxyurea at various concentrations on Burton diphenylamine assay at a single deoxyribose-5-phosphate concentration.

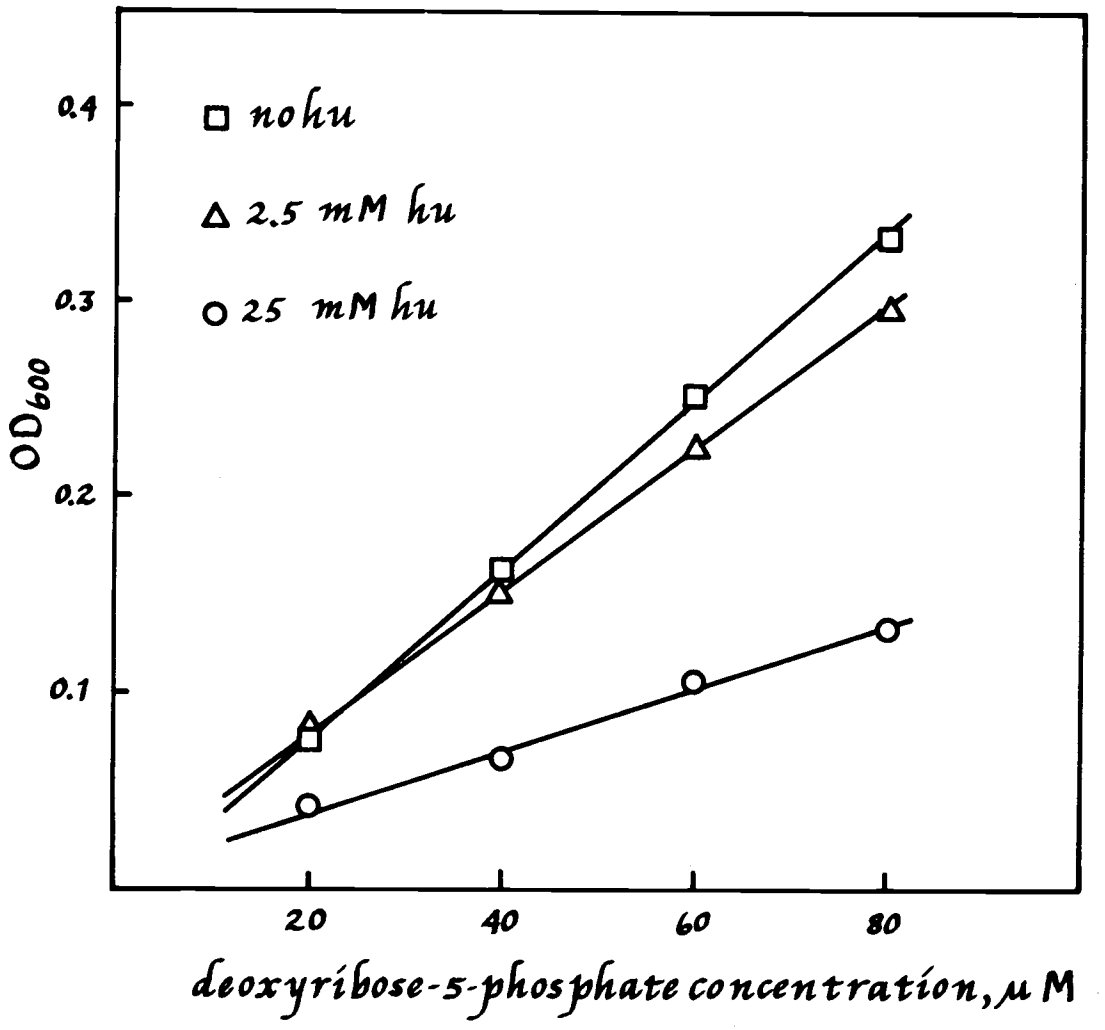

Figure 7. Effect of hydroxyurea on deoxyribose-5-phosphate standard curve.

The influence of hydroxyurea on the enzyme is shown in Figure 8. It was necessary to make a correction due to the influence of hydroxyurea on the diphenylamine assay. For this correction, a control reaction mixture containing everything except hydroxyurea was run along with each of the above reaction mixtures. To these, hydroxyurea was added after enzyme action had been terminated, so that the only difference between test samples and controls is that, in the former, hydroxyurea was present both during enzyme action and during the diphenylamine assay, whereas in the controls, hydroxyurea was present only during the diphenylamine assay. The correction for the effect of hydroxyurea was made by subtracting the apparent rate (ΔOD/min x 10^4) attained in each test sample from the apparent rate for the corresponding control sample. This gives the amount by which the rate of the enzyme reaction is depressed by each hydroxyurea concentration at each acetaldehyde concentration. Figure 9 was then obtained by subtracting the amount by which the enzyme reaction was depressed from the reaction rate in the absence of any hydroxyurea. This allows the construction of a curve for each hydroxyurea concentration showing the effect of hydroxyurea on the substrate saturation curve. There is a progressive lowering of the curve when the hydroxyurea concentration is increased up to 45 mM, but this effect is not greater at 60 mM hydroxyurea. Furthermore, at the higher acetaldehyde concentrations used, there is no decrease

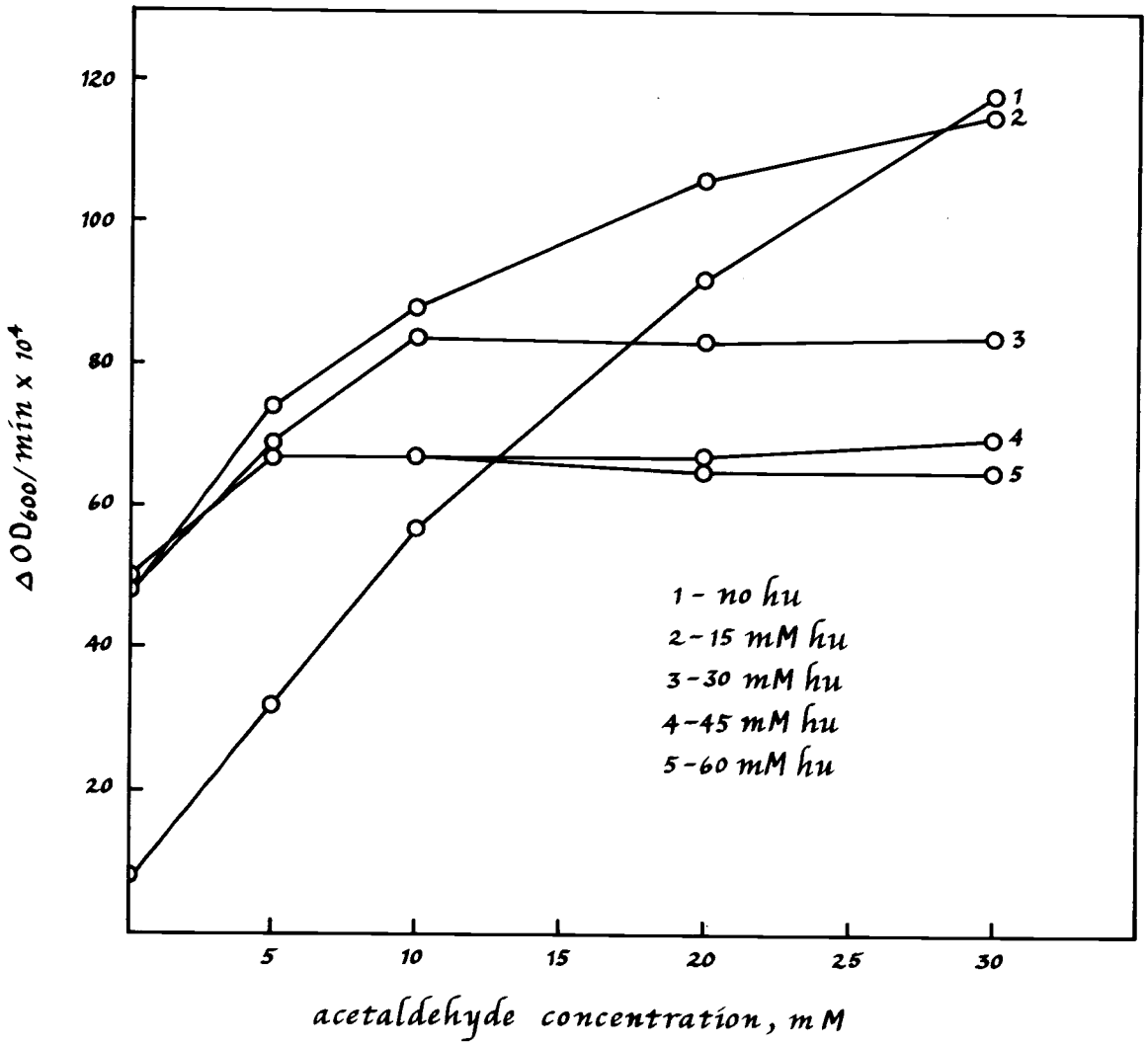

Figure 8. Effect of hydroxyurea on substrate saturation curve for deoxyribose-phosphate aldolase.

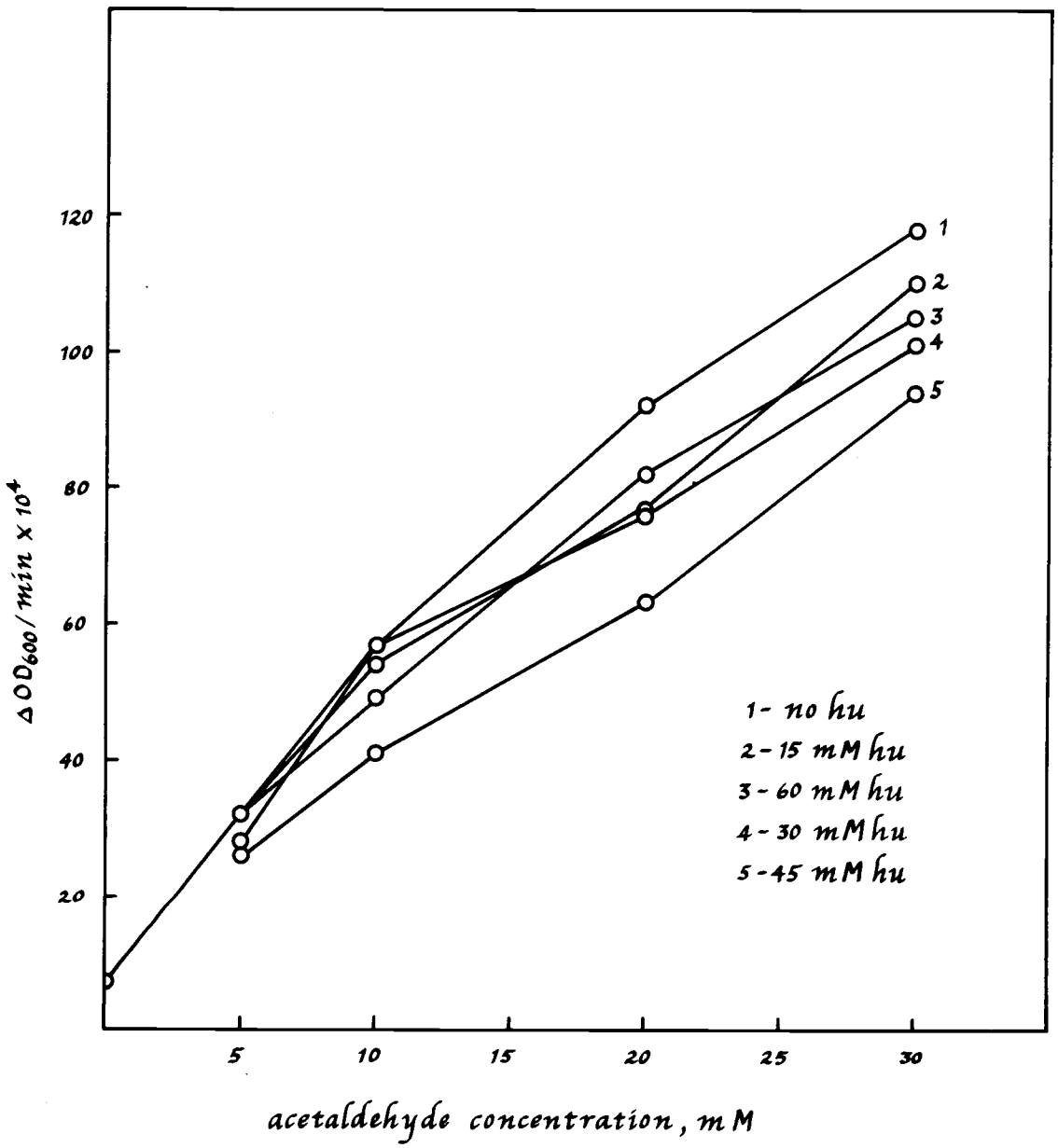

Figure 9. Inhibition of deoxyribose-phosphate aldolase by hydroxyurea.

in the slopes of the curves for the various hydroxyurea concentrations.

A surprising feature of Figure 8 is that there is an apparent synthesis of deoxyribose-5-phosphate (ΔOD at 600 mμ/min x 10^4=50), even in the absence of acetaldehyde. This did not occur when hydroxyurea was omitted, and was independent of hydroxyurea concentration above 15 mM. Further studies on this reaction showed that the rate of color development is dependent on the concentration of hydroxyurea, fructose-1, 6-diphosphate and liver supernatant present in the reaction mixture as shown in Figure 10. Heat-denatured supernatant was ineffective in producing this color increase. Fructose-1, 6-diphosphate and hydroxyurea together in the diphenylamine reaction produced no color. Furthermore, it made little difference whether hydroxyurea was present during incubation of fructose-1, 6-diphosphate with supernatant, or whether it was added after incubation was terminated by the addition of the trichloroacetic acid.

The blue color that developed in the presence of hydroxyurea, but in the absence of acetaldehyde cannot be due to the synthesis of deoxyribose-5-phosphate, since acetaldehyde is a necessary substrate for deoxyribose-phosphate aldolase. Thus another reaction, which involves hydroxyurea, must be taking place. One possibility is that a component of the liver supernatant acts upon fructose-1,

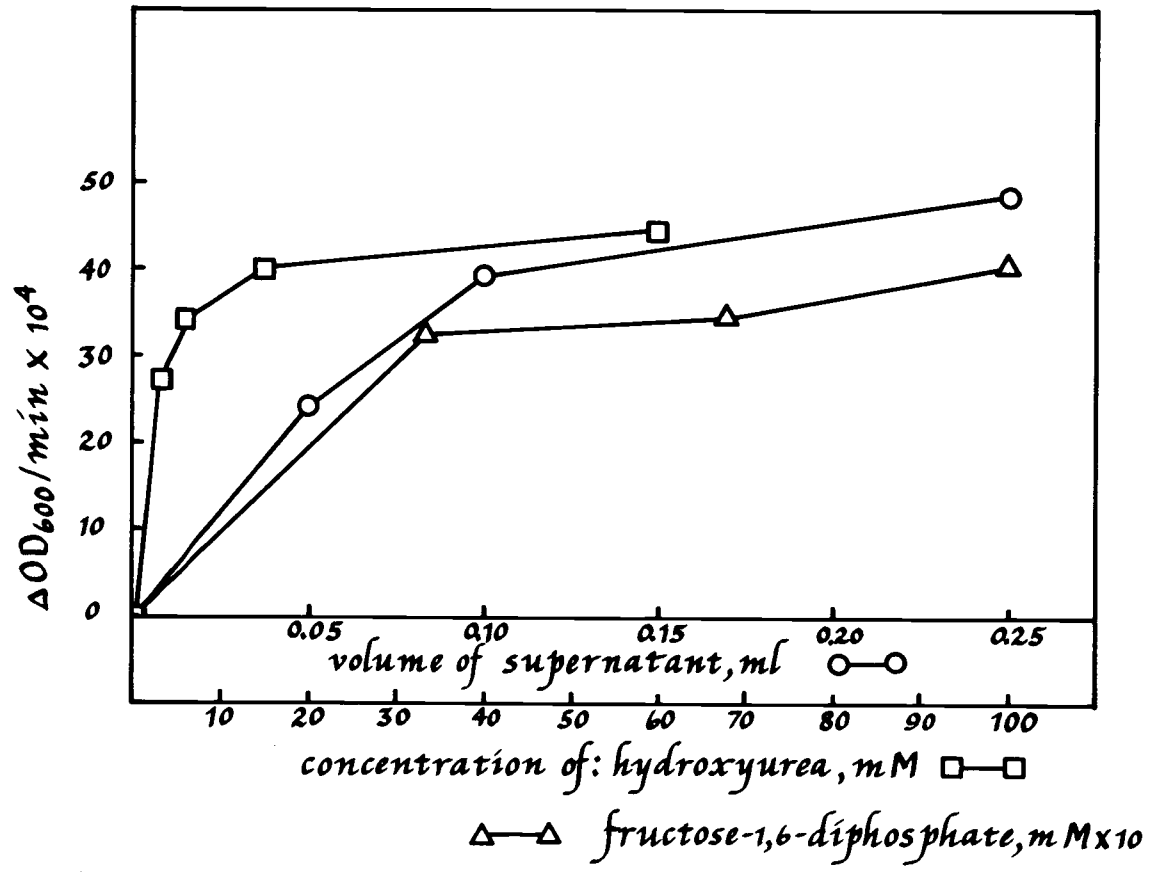

Figure 10. Dependence of hydroxyurea-mediated reaction on concentrations of hydroxyurea, liver supernatant and fructose-1, 6-diphosphate. In addition to the constituents noted on the abscissa, the reaction mixtures contained 40 mM Tris buffer, pH 7.4 plus: for the hydroxyurea curve (□—□), 10 mM fructose-1, 6-diphosphate and 0.25 ml of 18-day liver supernatant; for the supernatant curve (o—o), 10 mM fructose-1, 6-diphosphate and 60 mM hydroxyurea; for the fructose-1, 6-diphosphate curve (Δ—Δ), 0.25 ml of liver supernatant and 60 mM hydroxyurea. Final volumes were 3.0 ml.

6-diphosphate to produce a compound which in the presence of hydroxyurea, nonenzymatically gives rise to color in the diphenylamine reaction. For instance, aldolase may split fructose-1, 6-diphosphate to produce glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate. To determine whether either of these is capable of producing a color with hydroxyurea and diphenylamine, each compound was tested at various concentrations. In Figure 11, the amount of color development resulting from each of these reactions is compared with that resulting from similar concentrations of fructose-1, 6-diphosphate. The absorbance was measured at 630 mμ for these reactions rather than at 600 mμ since, as shown below, the peak for the hydroxyurea-mediated reaction occurs at 630 mμ. Figure 11 also shows the calculated sum of the absorbances obtained from dihydroxyacetone and glyceraldehyde-3-phosphate in order to compare with that obtained from fructose-1, 6-diphosphate at each concentration. At each concentration studied, the intensity of color developed from the fructose-1, 6-diphosphate incubation mixture was greater than the sum of the intensities from dihydroxyacetone and glyceraldehyde-3-phosphate (with the exception of 0.5 mM).

The visible spectra of the hydroxyurea-dihydroxyacetone or hydroxyurea-glyceraldehyde-3-phosphate or hydroxyurea-fructose-1, 6-diphosphate reactions differ significantly from that of deoxyribose-5-phosphate in the diphenylamine reaction. The spectra are

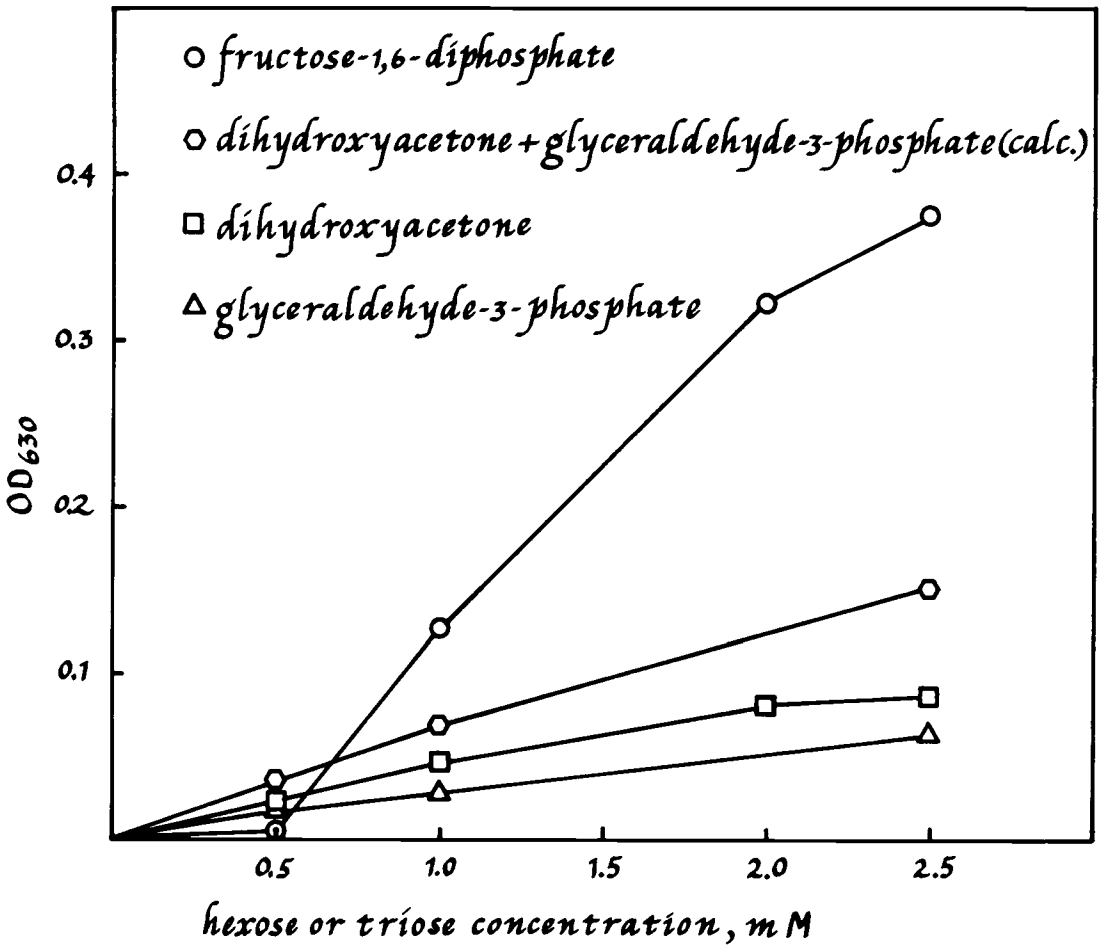

Figure 11. Color development in diphenylamine reaction in presence of hydroxyurea. Solutions of trioses at concentrations noted on abscissa also contained 30 mM hydroxyurea and 5% trichloroacetic acid. Two volumes of diphenylamine reagent solution (page 7) were added to each of the triose solutions. The resulting solutions were incubated 16 to 20 hours at 35°C, after which absorbance was read at 630 mμ. Fructose-1,6-diphosphate solutions at twice the concentrations shown were incubated with 30 mM hydroxyurea, 40 mM Tris buffer, pH 7.4, and 0.25 ml of 19-day liver supernatant in 3.0 ml total volume at 37°C for three hours. Aliquots were removed and diluted 1:1 with 10% trichloroacetic acid. Two volumes of diphenylamine reagent were added and assay completed as above.

compared in Figure 12. None of the hydroxyurea-produced peaks corresponds to the single peak at 600 mμ given by deoxyribose-5-phosphate. Spectra resulting from glyceraldehyde-3-phosphate and dihydroxyacetone correspond very closely to that given by fructose-1, 6-diphosphate products.

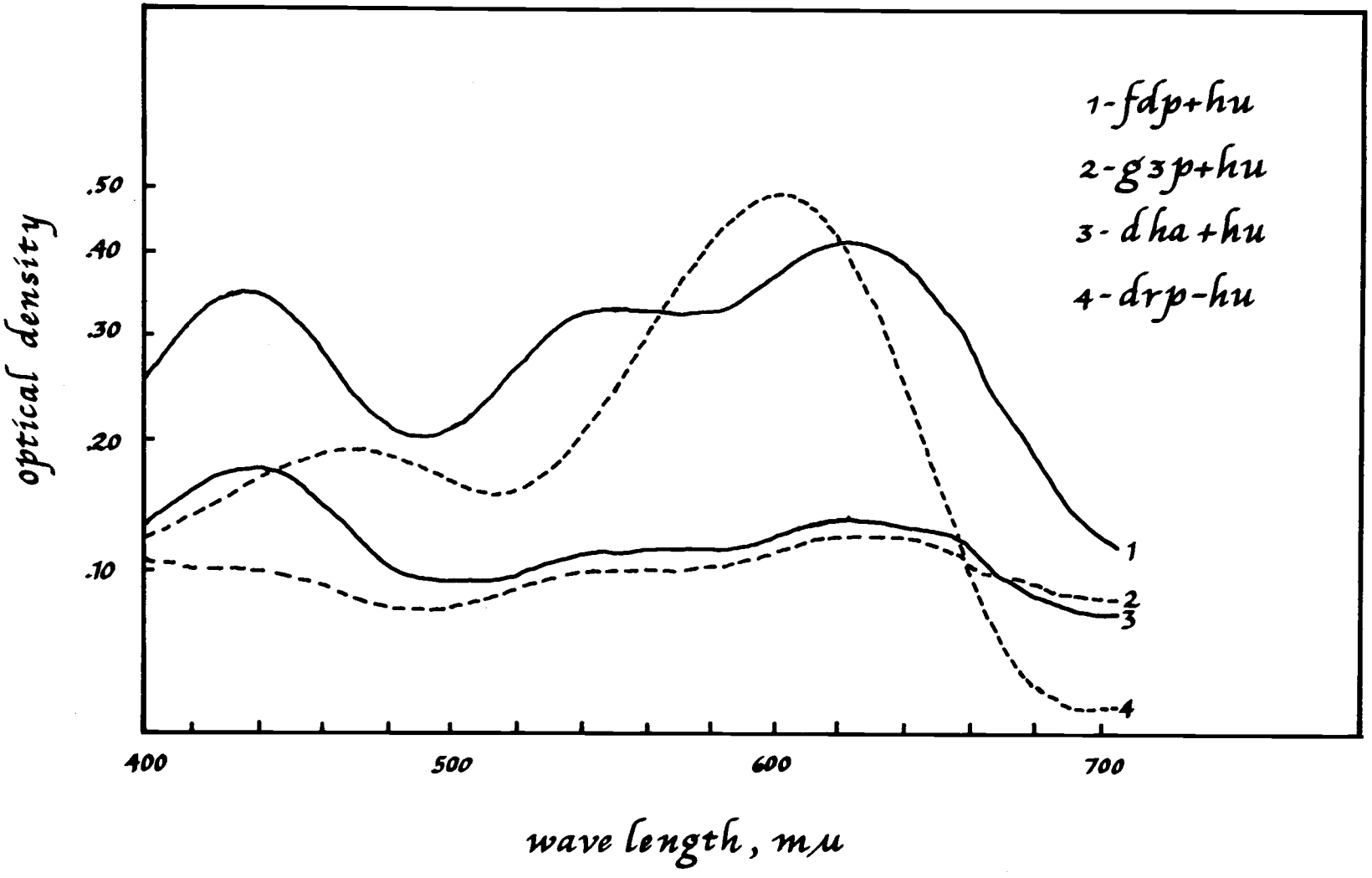

Figure 12. Absorption spectra of colored products in diphenylamine reaction.

DISCUSSION

Marinello et al. (1967) have evaluated various methods for the assay of deoxyribose-phosphate aldolase in animal tissues. In their work, two methods were used for determining the rate of breakdown of deoxyribose-5-phosphate and one method for following the synthesis of deoxyribose-5-phosphate. Coupled enzymes which reduce NAD or oxidize NADH were used to follow the production of glyceraldehyde-3-phosphate (glyceraldehyde-3-phosphate dehydrogenase) or acetaldehyde (alcohol dehydrogenase). The diphenylamine reaction of Dische (1930) or as modified by Burton (1956) was employed for assay in the direction of synthesis. They conclude that most reliable results are obtained when reaction is assessed in the direction of synthesis using the conditions of Burton for the diphenylamine reaction. All enzyme assays reported here (with the exception of the Boxer-Shonk experiment) follow the reaction in the direction of synthesis of deoxyribose-5-phosphate, and determination of product concentration by the Burton reaction.

Preliminary attempts to partially purify the enzyme from chick embryo liver failed, although it was found that all activity resided in the supernatant after sedimentation at 30,000 x g. The latter result had also been obtained for the rat liver enzyme (Groth, 1965). When ammonium sulfate fractionation of the supernatant was

attempted, however, all activity was lost, even though mercaptoethanol and EDTA were present and the pH of the solution was maintained near 7.4. Neither mercaptoethanol nor EDTA has any inhibitory effect on the diphenylamine assay for deoxyribose-5-phosphate. Both Groth (1965) and Roscoe and Nelson (1964) employed ammonium sulfate precipitation as the initial stage of their purification procedures. The reason for the apparent inactivation of the chick embryo enzyme is now thought to be absence of citrate."

Since it has been reported (Jiang and Groth, 1962) that the rat liver enzyme is activated by citrate and other di- and tri-carboxylic acids, it was of interest to determine whether this is also true of the chick embryo enzyme. Spot checks using early and late age embryo liver homogenates indicated that if there exists such a dependency, citrate is supplied endogenously in sufficient concentrations to give maximal or near-maximal rates. With heart homogenate at 18 days incubation, however, some stimulation of deoxyribose-phosphate aldolase was observed with added citrate, with a peak stimulation at 5 mM.

Another factor that must be considered in the assay system used here is the availability of fructose-1, 6-diphosphate aldolase. Glyceraldehyde-3-phosphate is required as substrate for deoxyribose-phosphate aldolase, and is supplied by the action of fructose-1, 6-diphosphate aldolase. Therefore, when fructose-1, 6-diphosphate is

added to supply substrate, it is necessary that there be sufficient fructose-1, 6-diphosphate aldolase activity to supply glyceraldehyde-3-phosphate at a concentration in excess of that required for saturation of deoxyribose-phosphate aldolase. Data presented by Romanoff (1967, p. 71) indicate that at the embryonic age at which there is the least activity of fructose-1, 6-diphosphate aldolase in liver tissue, the activity is high enough to produce glyceraldehyde-3-phosphate at about three times the rate at which it is used up by deoxyribose-phosphate aldolase as indicated by the experiments reported here. Once again, spot checks indicated that sufficient endogenous fructose-1, 6-diphosphate aldolase activity was contained in homogenate and supernatant preparations so that added aldolase did not increase observed rates of deoxyribose-5-phosphate synthesis.

The observation made by Boxer and Shonk (1958) that deoxyribose-5-phosphate reacts with diphenylamine to produce three times the absorbance at 600 mμ that deoxyribose produces was not substantiated. In fact, several lines of evidence support the contention that both react similarly. The possibility that the addition of fluoride ion, a phosphatase inhibitor, would prevent an apparent low rate of deoxyribose-5-phosphate synthesis was eliminated by the lack of any change in rate due to the addition of fluoride. This was done with chick embryo liver at an advanced stage of development, at which time both alkaline and acid phosphatase are known to be present (Romanoff,

1967, p. 74).

A direct determination of extinction coefficients for solutions of deoxyribose and deoxyribose-5-phosphate submitted to the diphenylamine reaction shows that they are within about 10% of each other. Furthermore, enzymatic dephosphorylation of deoxyribose-5-phosphate with added alkaline phosphatase produced a solution which gave the same reaction with diphenylamine after dephosphorylation as it had before phosphatase treatment.

These results all argue against greater reactivity of deoxyribose-5-phosphate in the diphenylamine reaction and suggest that the observation of Boxer and Shonk was in error. This conclusion is supported by the findings of Domagk and Horecker (1958) that deoxyribose gives 0.93 times as intense a color as deoxyribose-5-phosphate, and of Marinello et al., (1967) who also state that they have not observed the large difference noted by Boxer and Shonk.

The pattern of activity of deoxyribose-phosphate aldolase shown in Figure 4 reveals that the enzyme is present as a fraction of the total protein at very low levels in the young embryo and increases from the second to the eighth day by threefold in the whole embryo. Activity in the brain remains nearly constant at a very low level. Activity in the heart increases from the sixth to the tenth day by a factor of about three, then remains nearly constant until hatch. In sharp contrast to the other organs, activity in the liver increases

steadily between eight and sixteen days after incubation. A rapid rise occurs between sixteen and eighteen days, after which the activity levels off and remains constant until four days after hatch.

Virtually all activity in the liver at all ages is present in the supernatant fluid after centrifugation at 30,000 x g. At ages eighteen days and older, a wide variation among individuals is evident.

The correlation of the pattern of activity in the liver with DNA synthesis is problematic. Leslie and Davidson (1951) determined the DNA content of chick embryo liver and expressed values in terms of the percent increase which takes place during each day of incubation. This gives a measure of the rate of cell multiplication, assuming a constant amount of DNA per cell. They found that the greatest rate of DNA synthesis after eleven days occurs during the 13th to the 15th days, after which there is a slow decline in rate. There is no obvious relationship between this pattern of synthesis and that of deoxyribose-phosphate aldolase activity.

Inhibition of deoxyribose-phosphate aldolase by diphenylsuccinate indicates that the chick embryo liver enzyme is similar to the rat liver enzyme. This finding also provides confirmation that the assay method does indeed measure deoxyribose-5-phosphate synthesis, since the formation of another deoxypentose (which could conceivably be the compound reactive in the diphenylamine assay) would possibly not be inhibited by diphenylsuccinate, a specific

inhibitor of deoxyribose-phosphate aldolase (Groth and Jiang, 1966).

Hydroxyurea was found to have at least three different actions on the system under study. It has a direct effect on the development of color in the diphenylamine reaction with deoxyribose-5-phosphate, as shown in Figures 6 and 7. This reaction does not involve the production of another colored product, since the only change in the visible absorption spectrum is a diminution in the height of the usual peak at 600 mμ.

A second effect, and the only one pertaining to action on the enzyme system, is a small decrease in the rate of deoxyribose-5-phosphate production with increasing hydroxyurea concentrations. This effect, shown in Figure 9, is not great, and is not consistent, since increasing hydroxyurea concentration from 45 to 60 mM did not result in a further decrease in rate, but rather an increase. It is not known why this reversal takes place. Indeed, the concentrations of hydroxyurea required for measurable effect appear too high to represent specific inhibition. The small effect obtained is more likely due to non-enzyme-specific change in the characteristics of the enzymatic incubation solution, such as change in polarity or hydrogen ion activity of the solution.

The third effect of hydroxyurea seems to involve direct participation of the compound in a chemical reaction, rather than inhibition of another reaction. This effect consists of the appearance of a blue

color in the diphenylamine reaction under conditions in which deoxyribose-5-phosphate is not synthesized. It occurs when hydroxyurea is added to the products resulting from incubation of fructose-1, 6-diphosphate with liver supernatant, and the resulting solution is tested with diphenylamine reagent. The formation of the blue color cannot be due to the synthesis of deoxyribose-5-phosphate, since no acetaldehyde is supplied as substrate in the enzymatic reaction mixture. Furthermore, the spectrum of the products of the hydroxyurea reaction is quite different from that obtained with deoxyribose-5-phosphate. As shown in Figure 12, deoxyribose-5-phosphate produces a single absorbance peak at 600 mμ. The hydroxyurea-mediated reaction produces peaks at about 430 mμ and 630 mμ, with a broad shoulder on the higher peak, beginning at about 540 mμ.

The chromophore having the 600 mμ peak, according to Overend and coworkers (1950), is produced by the conversion of 2-deoxypentose to 4-keto-5-hydroxypentanal under the conditions of the strongly acidic solution and subsequent reaction of the pentanal with diphenylamine.

One possibility for the production of the colored product from hydroxyurea is that hydroxyurea reacts with a compound produced from fructose-1, 6-diphosphate upon incubation of the latter with liver supernatant. The product of this reaction then could react with diphenylamine to produce the blue-colored compound. The reaction

involving fructose-1, 6-diphosphate might be its cleavage to glyceraldehyde-3-phosphate and dihydroxyacetone-phosphate. One of the trioses could then react with hydroxyurea and the resulting product could in turn react with diphenylamine to form the colored compound. To test this hypothesis, glyceraldehyde-3-phosphate and dihydroxyacetone were individually treated with hydroxyurea, and the resulting solutions were allowed to react with diphenylamine. In both cases, some color development occurred, and the spectra (Figure 12) correspond roughly to that obtained from the fructose-1, 6-diphosphate incubation mixture. Notably, the peak at 430 mμ is very pronounced in the dihydroxyacetone reaction. Yet when the absorbance values at 630 mμ produced by the two trioses are added together, the sum does not equal the absorbance attained in the fructose-1, 6-diphosphate reaction. The facts that the spectra of the triose reactions correspond closely to that of the fructose-1, 6-diphosphate reaction, and that the development of color at 630 mμ increases with concentration of triose, indicate that there is some similarity between the reaction of the trioses and of the products from fructose-1, 6-diphosphate. The spectrum given by fructose-1, 6-diphosphate products may be a composite of spectra given by more than one colored product. This suggestion is supported by the relative heights of the peaks in the hydroxyacetone spectrum. The 430 mμ peak in the latter case is more pronounced than the 630 mμ peak, whereas the opposite is true

in the case of fructose-1, 6-diphosphate. The lower-wavelength peak is virtually absent from the glyceraldehyde-3-phosphate spectrum. Perhaps in the enzymatic incubation with fructose-1, 6-diphosphate there is a further conversion of one or both triose phosphates to compounds of similar structure which then react with hydroxyurea to produce the spectrum observed with fructose-1, 6-diphosphate. Many compounds are known to produce color with diphenylamine after acid treatment. Deriaz and coworkers (1949) list several sugars and related compounds together with the absorption peaks produced in the Dische reaction. Conditions employed there involved heating at 100°C, whereas in the Burton reaction employed here, color development was conducted at 35°C for 16 to 20 hours. According to Deriaz et al., an intense blue color with maxima at 520 and 630 mμ was obtained with (hydroxymethyl) furfural, bis(furfurylmethyl)ether, fructose, sucrose, sorbose and tetraacetoxyglucose. An alternative explanation in light of these results is that fructose-1, 6-diphosphate undergoes a reaction in the incubation with liver supernatant in which it becomes more susceptible to reaction with diphenylamine in a reaction potentiated by hydroxyurea. Or perhaps there is reaction both of a triose to give the 430 mμ peak and of a hexose to give the 630 mμ peak.

PART II. EXPERIMENTS ON INCORPORATION OF 1-^{14}C-DEOXYRIBOSE INTO CHICK EMBRYO DNA

MATERIALS

Some materials used in this part were listed in Part I. Sperm DNA was obtained from Nutritional Biochemicals and type III highly polymerized DNA from Sigma. Yeast RNA was the product of Schwartz Bioresearch. Deoxycytidine, thymidine and alkaline phosphatase were obtained from Sigma. Naja naja venom was purchased from Ross Allen's Reptile Institute, Silver Springs, Florida. 1-^{14}C-deoxyribose and 1-^{14}C-sodium acetate were obtained from Nuclear-Chicago (now Amersham/Searle).

Three fluor solutions were employed for scintillation counting. Solution I (Bray, 1960) consisted of 4 g of 2, 5-diphenyloxazole (PPO), 0.2 g of 1, 4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP), 60 g of naphthalene, 100 ml of methanol and 20 ml of ethylene glycol made to one liter with toluene. Solution II was composed of 0.4% PPO and 0.02% POPOP in toluene. Solution III was prepared by mixing 400 ml of methoxyethanol with 600 ml of Solution II.

Ion-exchange resins were products of Bio-Rad Laboratories. Dowex 2-X8 was converted to the bicarbonate form with sodium bicarbonate; Dowex 50W-X8 was used in the H^+ form, and was washed with ethanol and water before use. "Mixed Dowex" was a mixture of

Dowex 2 (HCO_3^-) and Dowex 50 (H^+) and was treated by bubbling CO_2 through the suspension for one-half hour before use (Itzhaki, 1964).

METHODS AND RESULTS

Injection of 1-^{14}C-deoxyribose

Fertile eggs incubated for an appropriate number of days were injected with 1.0 μc of 1-^{14}C-deoxyribose into the air space or into the yolk sac.

DNA Extraction

The DNA extraction procedure was adapted from that of Ellem (1966). After incubation for 24 hours with radioactive substrate, brains, hearts and livers were homogenized in convenient small volumes (3 to 7 ml) of solution containing NaCl (0.01 M), Tris (0.05 M) and EDTA (0.001 M), and the homogenate was made up to 10 ml per gram of tissue, using the same solution. One ml of sodium dodecyl sulfate (20% w/v) was added per gram of tissue, and the resulting mixture stirred 15 minutes in an ice bath. An equal volume of analytical grade 90% (v/v) phenol was then added with stirring over a period of 15 minutes in the ice bath. Phases were separated by centrifugation for 10 minutes at 2,000 x g in a refrigerated centrifuge. The phenol layer was removed by suction, leaving the white interphase with the aqueous layer. To the latter was added an equal volume of phenol and after mixing, centrifugation and removal of the phenol layer, two volumes of absolute ethanol were added. Nucleic acids were allowed to

precipitate in the freezer overnight. The precipitate was collected by winding the fibrous material onto a glass rod and was washed twice with 95% ethanol. The dried extract was then taken up in a small volume of water containing a trace of sodium acetate. Aliquots were taken for DNA determination by the Burton method and for scintillation counting in Solution I.

Results of DNA extractions are shown in Table 4, together with values for DNA content of organs published by Romanoff (1967, p. 80, 91 and 77). DNA in 8-day brain was not available. Very low recovery was obtained in some extractions from 8-day heart and 18-day brain and in all cases from 8-day liver. In all other cases, from 25 to 70% of DNA was extracted.

Table 5 gives the radioactivity of the extracts, expressed as disintegrations per minute and as disintegrations per minute per mg of DNA. It should be noted that some RNA was present in these extracts. Table 6 gives both DNA and RNA contents for the liver extracts only, along with the radioactivity in terms of disintegrations per minute per ml.

Since the amount of DNA and the total radioactivity in the samples were so small, all samples corresponding to the same age of embryo were pooled. The RNA content was reduced by hydrolysis with 1 ml of 1 N KOH at 37°C overnight, followed by precipitation with 2.5 ml of absolute ethanol and 0.45 ml of glacial acetic acid

Table 4. Amounts of DNA contained[1] in and extracted from chick embryo organs.

	Brain			Heart			Liver		
	DNA, μg		%	DNA, μg		%	DNA, μg		%
	Contained	Extracted	Recovery	Contained	Extracted	Recovery	Contained	Extracted	Recovery
8-Day Embryos 25 Organs	2	3,960	2	500	63	13	1,100	5	0.4
	2	4,130	2	500	84	18	1,100	47	4
	2	2,140	2	500	129	26	1,100	51	5
	2	1,970	2	500	185	37	1,100	16	1
14-Day Embryos 9 Organs	4,370	2,040	47	1,320	1,660	>100	5,400	1,300	24
	4,370	3,120	71	1,320	1,050	80	5,400	3,300	61
	4,370	2,180	50	1,320	690	52	5,400	2,780	52
	4,370	3,140	72				5,400	4,030	75
18-Day Embryos 5 Organs	4,650	490	10	1,750	810	46	7,900	2,160	27
	4,650	380	8	1,750	1,100	63	7,900	1,990	25
	4,650	540	12	1,750	580	33	7,900	2,190	28
	4,650	1,250	27	1,750	700	40	7,900	2,580	33
	4,650	1,550	33						

[1] Based on DNA content of organs given by Romanoff (1967).

[2] Unknown.

Table 5. Radioactivity and specific activity[1] of DNA extracted from various organs and different sites of injection.

Age of embryo	Number of organs	Brain		Heart		Liver	
		dpm	dpm/mg	dpm	dpm/mg	dpm	dpm/mg
8-Day	25						
Air Space		8,560	2,160	693	11,000	91	18,600
		9,700	2,350	546	6,500	752	16,000
Yolk sac		1,430	668	274	2,120	408	8,000
		9,500	4,820			584	3,650
14-Day	9						
Air Space		1,670	820	327	197	96,000	73,700
		378	121			116,000	35,100
Yolk Sac		910	417	1,730	1,650	809	291
		418	133	70	101	1,110	276
18-Day	5						
Air Space		211	430	391	483	96,100	44,500
		110	290	473	430	76,000	38,200
		306	567				
Yolk Sac		360	288	223	385	4,800	2,190
		1,920	1,240	176	252	5,760	2,230

[1] Calculated on basis that all radioactivity is in DNA.

Table 6. DNA and RNA content and radioactivity in liver nucleic acid extracts.

	mgRNA/ml	mgDNA/ml	dpm/ml
8-day			
Air Space	1.26	0.082	1,790
	1.80	0.54	3,280
Yolk Sac	1.72	0.075	770
	1.12	0.15	580
14-day			
Air Space	3.36	0.32	25,200
	2.86	0.52	23,400
Yolk Sac	2.36	0.63	158
	3.30	0.92	67
18-day			
Air Space	2.60	0.36	16,200
	3.54	0.51	20,800
Yolk Sac	2.74	0.43	940
	3.90	0.58	1,340

added while stirring. After cooling in ice for 90 minutes, the DNA precipitate was collected by centrifugation, washed once with cold 70% ethanol at pH 4, and allowed to dry in air. The precipitate was then dissolved in a small volume of water containing a trace of sodium acetate. Aliquots were taken for DNA and RNA determinations and for scintillation counting. The amounts of DNA and RNA obtained in the redissolved precipitate are given in Table 7. The RNA content was considerably decreased, but about 1/6 to 1/3 of the extracts still consisted of RNA as determined by the orcinol reaction. In Table 7 there also appear values for specific radioactivity of DNA and RNA. These values were obtained as follows. The supernatant and washings from the last ethanol precipitation were concentrated and DNA and RNA content and radioactivity were determined. DNA was virtually absent from the supernatant solutions, so it was possible to obtain the dpm/mg RNA in the supernatant. The specific activity of the RNA in the precipitate was assumed to be the same as that of the RNA in the supernatant. Knowing this value, it was possible to subtract the dpm's contributed by RNA from the total dpm'sand the difference was assumed to be a measure of the dpm's in the DNA. The specific activity of the DNA was then calculated using this value and the measured value for DNA content.

Table 7. DNA and RNA contents and specific activities of each in pooled samples after alkaline hydrolysis and precipitation of nucleic acids.

Age of Embryo	DNA, mg	RNA, mg	dpm	dpm/mgDNA	dpm/mgRNA
8-day	11.5	2.3	7,550	475	905
14-day	21.0	5.4	12,400	540	191
18-day	11.7	4.7	20,200	1,450	677

DNA Degradation

These extracts were then submitted to degradation by two methods. One involved the separation and purification of deoxyribose for the purpose of cleaving the deoxyribose to acetaldehyde and glyceraldehyde-phosphate, in order to obtain acetate from carbon atoms 1 and 2 and eventually CO_2 from carbon-1. The other method consisted of a hot acid treatment of DNA (also applied to deoxyribose itself) to convert deoxyribose to levulinic acid, reduction of levulinic acid to valeric acid, again to release carbon-1 as CO_2.

For the separation of deoxyribose, carrier DNA was added to DNA extracts to a total of 20 mg. Then 0.5 ml of a buffer solution (glycyl glycine, 200 mM; $MgSO_4$, 32 mM; pH 7.4) and 300 μg DNase were added. The incubation mixture was made to 2.0 ml and held at 37°C for two hours. The pH was then adjusted to 9.3 and 0.8 mg of Naja naja venom and 1.0 mg of alkaline phosphatase were added. The solution was incubated at 37°C for an hour. Another 0.8 mg of venom and 1.0 mg of phosphatase were added, a preservative layer of toluene applied and incubation continued overnight. The pH was then adjusted to 2.4 with formic acid, denatured protein was removed by centrifugation and purine deoxyribonucleosides were hydrolyzed at 100°C for 10 minutes.

The mixture of purine deoxyribose and pyrimidine

deoxynucleosides was placed on a column containing 0.5 g of acid-washed activated charcoal. Deoxyribose was eluted with 25 ml of water. Pyrimidine deoxynucleosides were eluted with 25 ml of 95% ethanol-ammonia (specific gravity 0.90)-water (5:2:3 v/v).

For the determination of recovery of purine-bound deoxyribose and pyrimidine deoxynucleosides from the charcoal column, appreliminary separation was conducted with a mixture of deoxyribose (18.6 μmoles), deoxycytidine (17.6 μmoles) and thymidine (20.2 μmoles). Recoveries in three trials were 102 $\pm$ 3% for deoxyribose as detected by the diphenylamine reaction and 95 $\pm$ 2% for the mixture of pyrimidine deoxynucleosides as detected by the absorbance of the ethanol-ammonia eluate at 260 mμ.

Deoxyribose-containing samples were evaporated to a small volume and $KClO_4$ was filtered off in the cold. The solutions were de-ionized by passage through a small column of mixed Dowex and again evaporated to small volumes for streaking on paper for chromatography.

For the hydrolysis of pyrimidine deoxynucleosides, the method of Itzhaki (1964) was used. Solutions containing pyrimidine deoxynucleosides were concentrated to about 1 ml, 8.4 mg of $NaHCO_3$ and 0.5 ml of a saturated solution of bromine in water were added and the mixture held at room temperature for 15 minutes. Excess bromine was then expelled with a stream of nitrogen and 0.2 ml of

0.5 M NaOH and 0.3 ml of water were added. The solution was held at room temperature for one hour. It was then diluted to 10 ml with water, acidified with 0.1 ml of 2 M HCl and held at 100°C for 15 minutes. After the solution had cooled to room temperature, it was neutralized with a thick suspension of Dowex 2 (HCO_3^-), which was then filtered out and washed. The filtrate was passed through a column of mixed Dowex for deionization. The deoxyribose effluent was concentrated by evaporation at reduced pressure to a small volume for paper chromatography.

Preliminary experiments with solutions of deoxyribose (10.0 μmoles), deoxycytidine (9.0 μmoles) and thymidine (10.3 μmoles) were performed to determine recoveries after bromine, and acid and base treatments (with the exception that bromine was omitted when deoxyribose was tested). After each step, analysis of deoxyribose was made by the diphenylamine reaction. Results are reported in Table 8 as the percentage of deoxyribose present in the initial sample which is detected at each step.

Deoxyribose samples were purified by paper chromatography on strips of water-washed Whatman 41 paper. Ribose and deoxyribose standards were spotted at the origin next to the unknown samples which were applied in a streak. Descending elution was carried out using n-butanol-95% ethanol-water (40:11:19 v/v) as the developing solvent.

Table 8. Recovery as diphenylamine-reacting material of deoxyribose present in solutions treated with bromine, base and acid.

Sample	After Bromine	After Base	After Acid
deoxyribose	96 ±4	97.5 ±0.5	100 ±0.5
deoxycytidine	37 ±5	23.5 ±0.5	69.5 ±0.5
thymidine	25 ±10	59 ±16	58 ±15

After development, areas containing deoxyribose were identified by comparing the location of the unknown in relation to deoxyribose spots along the edge strips. The positions of sugars present on the strips were determined by use of the periodate-benzidine method of Gordon et al. (1956). In this system, Rf's for ribose and deoxyribose were found to be about 0.45 and 0.55 respectively. Complete separation was achieved by allowing solvent to drip off the serrated lower edge and continuing elution for 15 hours. Areas containing deoxyribose were cut out and the sugar was eluted with water. Eluted samples were made to 10 ml and aliquots were taken for deoxyribose assay by the diphenylamine reaction and for scintillation counting. In preliminary trials, $76 \pm 7\%$ of deoxyribose placed on the paper was recovered.

When the entire procedure was applied to sperm DNA, approximately 38-48% of the purine-bound deoxyribose and 30-50% of the pyrimidine-bound deoxyribose were recovered.

Initially, it was planned to degrade deoxyribose by fermentation with Lactobacillus plantarum, following the method of Horecker, Domagk and Hiatt (1958). The acetaldehyde produced was to be oxidized to acetic acid and sodium acetate submitted to Schmidt degradation by the method published by Sakami (1955).

In several trials, with several different cultures of L. plantarum under various growth conditions, no acetaldehyde was

observed to be produced from deoxyribose. This method was then discontinued and it was decided to limit the degradation of deoxyribose to the removal of carbon 1 as CO_2, as follows.

The second general method employed for DNA degradation and that also used for the degradation of purified deoxyribose obtained from DNA was adapted from that of David, et al. (1960 and 1967). Deoxyribose, either free or bound in DNA, was converted to levulinic acid. The semicarbazone of levulinic acid was prepared and reduced to valeric acid. The Schmidt degradation was finally performed on valerate to release carbon 1 (carbon 1 of the original deoxyribose) as CO_2.

From one to ten mg of DNA (or a corresponding amount of deoxyribose) were sealed in a hydrolysis tube with 15 to 20 mg of levulinic acid and 1.3 ml of 2 N H_2SO_4 and held at 110°C for 7 to 8 hours. Levulinic acid was extracted with ethyl ether for 48 hours. The solution was made basic by the addition of a slight excess of sodium hydroxide solution, and ether was allowed to evaporate. Sodium ion was removed by the addition of Dowex 50 (H^+). Levulinic acid was titrated with standard 0.1 N NaOH to pH 7.0 and the salt solution was evaporated to a volume less than 0.3 ml.

To the sodium levulinate was added 0.5 ml of a solution of semicarbazide (1.1 g of semicarbazide-HCl in 5 ml of water). The mixture was heated in a boiling water bath for about 3 minutes,

then cooled in ice. If crystallization did not occur spontaneously, it was induced by scratching the inside wall of the tube with a glass rod. The mixture was allowed to stand in the ice bath for about 30 minutes. The product was collected on a small fritted glass funnel and washed with ice cold water.

The semicarbazone was transferred to a hydrolysis tube and 0.5 ml of a reducing mixture (KOH, 400 mg; hydrazine hydrate, 0.3 ml; ethylene glycol, 3.8 ml) was added. The tube was sealed and warmed at 180°C for 4 hours. After cooling, the solution was acidified with H_2SO_4 and transferred to a Markham still for steam distillation. At least 100 ml of condensate were collected and valeric acid was titrated with standard 0.1 N NaOH to pH 7.0. An aliquot was counted in the scintillation counter for specific activity determination. The remainder was evaporated to dryness and subjected to Schmidt degradation by the method of Katz, et al. (1955). The reaction was conducted in a 50 ml Erlenmeyer flask fitted with a side arm adaptable to a screw cap or a scintillation vial (Saba and DiLuzio, 1966; Kontes Glass Co., K-88235).

The sodium valerate sample was placed in a 1-dram vial and sample and vial were dried thoroughly by gentle heating over a flame. Sodium azide (30 mg) was added and the vial was placed inside the flask, with the screw cap in position on the side arm. A rubber serum bottle stopper was fitted onto the top of the flask and the flask

was evacuated for a few seconds through a hypodermic needle attached to a vacuum line. Reaction was then initiated by injection of 0.4 ml of "100%" H_2SO_4 (one volume of fuming H_2SO_4 and three volumes of concentrated H_2SO_4).

The flask was heated at 80°C for one hour, then cooled. Two ml of 2 N NaOH were injected into the main compartment of the flask. Carbon dioxide was collected in this trap at room temperature for one hour. The screw cap was removed and replaced by a scintillation vial containing a cylinder of Whatman 40 paper 1.5 by 4.0 inches, soaked with 0.6 ml of 10% KOH.

Four ml of 2 N H_2SO_4 containing 2% H_2O_2 (to oxidize SO_2 to SO_3) were injected into the NaOH trap. After several hours, the scintillation vial was removed and dried in a vacuum desiccator over P_2O_5 overnight. Twenty ml of fluor solution II were added and the vial was counted in a scintillation counter. The residual Schmidt degradation mixture was diluted to 5.0 ml with water and an aliquot was taken for scintillation counting in fluor solution III.

In preliminary experiments with non-radioactive deoxyribose and DNA, recoveries of titratable levulinic and valeric acids were determined as shown in Table 9. When 1-^{14}C-deoxyribose and radioactive DNA extracts were degraded, the results shown in Table 10 were obtained.

Upon Schmidt degradation of 1-^{14}C-sodium acetate, the

Table 9. Recovery of deoxyribose as levulinic acid and as valeric acid.

Sample	Percent Recovery as levulinic	Percent Recovery as valeric
Deoxyribose	57 ±9	21 ±9
DNA	75 ±14	42 ±5

Recoveries determined by titration of acids.

Table 10. Recovery of titratable acid and of radioactivity in conversion of levulinic acid to valeric acid.

	Age of Embryo, days	Levulinic		Valeric		% Recovery	
		meq	dpm	meq	dpm	meq	dpm
1-^{14}C-Deoxyribose		0.33	1,920	0.127	700	38	36
		0.18	1,870	0.087	930	48	50
		--	2,150	--	730	--	34
DNA samples	8	0.36	1,010	--	--	--	--
		0.25	1,660	0.097	400	38	24
		0.26	1,230	0.081	300	31	24
	14	> 0.31	490	0.126	2,060	41	100
		0.19	1,420	0.095	134	49	9.4
		0.27	460	0.083	126	49	27
	18	0.30	3,760	0.129	241	42	6.4
		0.19	680	0.079	110	41	16.1
		--	1,000	0.041	137	--	13.7

recovery of radioactivity in carbon 1 as CO_2 was 87-93% of that in acetate, as shown in Table 11. Table 12 presents the recovery of ^{14}C in CO_2 for radioactive DNA samples and for 1-^{14}C-deoxyribose samples. The radioactivity present in valerate in some samples was very low. Very little activity remained in the residual Schmidt degradation mixture. When 1-^{14}C-deoxyribose was degraded, about 45-58% of the radioactivity was trapped as CO_2. The remainder could not be detected in the residual solution. When DNA samples were degraded, most yielded from 20 to 40% of their radioactivity in valerate as CO_2.

Table 11. Recovery of radioactivity from 1-^{14}C-acetate in Schmidt degradation.

Experiment	Dpm		% Recovery
	Acetate	CO_2	
I	1,810	1,680	93
II	1,810	1,650	91
III	1,810	1,580	87

Table 12. Recovery of radioactivity from ^{14}C-valerate in Schmidt degradation.

	Age of Embryo, days	Disintegrations/min			% of dpm in CO_2
		Valerate	CO_2	Residual Solution[1]	
1-^{14}C-Deoxyribose		660	380		58
		830	440		53
		666	300		45
DNA Samples	8	347	136		39
		230	85		37
	14	1,810	69	150	3.8
		118	40		34
		106	30		28
	18	223	25		11
		87	27	27	31
		121	26		21

[1] Blank spaces indicate no detectible radioactivity.

DISCUSSION

In most cases, the DNA extraction procedure used allowed the removal of at least one-fourth of the DNA present in the organs (Table 4). Notable exceptions were 8-day liver and in certain extractions, 8-day heart and 18-day brain. The wide variation in amounts obtained is probably due to the difficulty of removing the same amount of interphase material between the aqueous layer and the phenol layer in each extraction. In the case of the small 8-day livers, the volume of solution from which the extraction was made was so small that the loss was proportionately very great. The same is true of 8-day heart tissue, but this explanation does not hold for 18-day brain tissue.

Radioactivity incorporated from 1-^{14}C-deoxyribose was determined in the DNA extracts. Assuming that all ^{14}C resides in DNA, and not in some other constituent of the extracts, the specific activities are shown in Table 5 for DNA from various organs of embryos injected at different stages of development and at different locations. In general, very little incorporation of radioactivity into DNA extracts was obtained. The maximum incorporation was obtained with 13-day liver, in which about 0.2% of injected radioactivity was recovered in the nucleic acid extract.

Usually, greater incorporation occurred when injection was

made into the air space than when made into the yolk sac. This is probably due to more intimate contact with the vascular system, which ramifies extensively into the region inside the shell membrane and beneath the air space. On the other hand, diffusion through yolk material is probably relatively slow.

Since the extracts also include RNA, determinations of RNA in the liver extracts were made (Table 6) which show they actually contain more RNA than DNA.

At this point, it was decided to pool all extracts of the same developmental age, due to low levels of radioactivity in the samples and to the necessity of removing RNA. After pooling, alkaline hydrolysis and precipitation by ethanol, the samples were enriched in DNA as shown by comparing Table 6 and Table 7. The specific activities of RNA and DNA were based upon determinations of specific activities of RNA in the ethanol-soluble hydrolyzate, which was devoid of DNA. This same specific activity was assumed for the RNA remaining in the DNA precipitate. Appropriate subtraction was made from the total radioactivity, and the remainder attributed to DNA. Table 7 shows these specific activities, which indicate greater incorporation into RNA at 7 days, but more into DNA at later ages. Most of this effect in the pooled extracts is due to liver tissue, which contained the predominating amounts of radioactivity and is a reflection of the fact that the amount of DNA synthesized during the

13th and also during the 17th day is about ten times greater than the amount synthesized during the 7th day.

Two methods for the degradation of deoxyribose were employed to determine whether, when 1-^{14}C-deoxyribose was injected, all ^{14}C in DNA is to be found in carbon-1 of DNA-deoxyribose. If it is, there would be strong evidence for intact incorporation of deoxyribose and support for a mechanism of deoxyribonucleoside synthesis via a deoxyribose compound not obtained by the reduction of a ribonucleotide.

For the determination of the amount of radioactivity of deoxyribose residing in carbon-1, it is sufficient to isolate carbon-1 as a compound free from the rest of the molecule, and to compare the specific activity of this compound with that of the deoxyribose.

In the method of Domagk and Horecker (1958), deoxyribose is cleaved by fermentation in an incubation with Lactobacillus plantarum. Carbon atoms 1 and 2 are removed as acetaldehyde, which is then oxidized to acetic acid, and the acetate is degraded by the Schmidt procedure to give carbon-1 as carbon dioxide. The carbon dioxide is trapped, assayed quantitatively and counted for radioactivity. Intitially, it was planned to employ this method for degradation of deoxyribose from DNA. However, none of the attempts to obtain a culture of Lactobacillus plantarum that would produce acetaldehyde was successful, although some strains did metabolize deoxyribose.

Deoxyribose isolated from DNA was thus not degraded.

Some information was obtained, however, concerning recoveries of deoxyribose that can be expected. The separation of the deoxyribose, once bound to purines, from pyrimidine deoxyribonucleosides was found to be quite satisfactory on charcoal columns. A relatively large loss of pyrimidine-bound deoxyribose occurred during the bromine treatment necessary to achieve hydrolysis of the base-sugar bond. About 30% of cytosine-bound and 40% of thymidine-bound deoxyribose was lost at this step alone (Table 8). Over all, the procedure employed provides about 30-50% of the deoxyribose from both purine and pyrimidine deoxynucleotides of DNA.

When the DNA degradation method of David et al. (1967) became available, it was utilized in preference to the lengthy procedure involving isolation of deoxyribose. Before applying the method to radioactive DNA samples, recoveries of the deoxyribose carbon chain in the form of levulinic acid were determined. When deoxyribose itself or DNA was treated with 2 N sulfuric acid and levulinic acid removed by extraction with ether and titrated, about one-half to three-fourths of the deoxyribose was recovered as levulinic acid (Table 9). Although recoveries were highly variable, greater recovery was usually obtained from DNA, perhaps due to some protection afforded to deoxyribose from excessive acid exposure by its incorporation in the DNA molecule.

Recoveries of valerate from levulinate were generally about 40% to 50% (Table 9), based upon the amount of each titrated by standard base. A large part of the loss involved here was undoubtedly due to incomplete precipitation and recovery of the semicarbazone of levulinic acid. When the recovery of radioactivity in levulinic and valeric acids was determined, however, the apparent recovery was considerably lower (Table 10). This indicates a lower specific radioactivity of valeric acid and suggests that in some samples, the levulinic acid solution contained radioactive material which was not carried over into valeric acid. It is not surprising that there could be other ether-extractable acids or other products present in the solutions in which DNA samples were treated with hot sulfuric acid for several hours. When commercially procured 1-^{14}C-deoxyribose was submitted to the same treatment, however, the specific activity of valeric acid was very nearly the same as that of levulinic acid.

The Schmidt degradation procedure was tested using 1-^{14}C-sodium acetate, with results shown in Table 11. About 90% of the radioactivity in acetate is recovered in carbon dioxide. This corresponds well with the 92% reported for 1-^{14}C-acetate by Katz and coworkers (1955).

However, when the Schmidt degradation was applied to valerate obtained from commercially available 1-^{14} C-deoxyribose,

only 45-58% of the radioactivity was detected in the carbon dioxide (Table 12). No significant amount of radioactivity was detected in the residual Schmidt degradation solution, even though more than 300 dpm remained not accounted for by $^{14}CO_2$. The fate of the remaining radioactive carbon is not known. Even less of the valerate radiocarbon was found in carbon dioxide in the case of DNA samples. Except for two very low samples, between 20 and 40% recovery was observed. Again, in most cases, no radioactivity was detected in the residual solutions. If there were ^{14}C present in carbon atoms other than carbon-1 of valerate, this should remain in the acidic residual solution as butylammonium ion, and thus be detectible upon dilution and scintillation counting of the solution. The amounts of radioactivity not counted in CO_2 should be readily detected.

The fate of the remaining radioactivity has not been determined. Since the degradation of 1-^{14}C-acetate yields about 90% of its radioactivity as carbon dioxide, one would expect a similar yield from valerate. At least, this recovery indicates high efficiency of trapping and counting of $^{14}CO_2$. Perhaps the decarboxylation of valerate is not complete. In such a case, one would expect that the radioactivity of the undegraded valerate would be counted in the residual solution. However, it is possible that undegraded valerate was converted to valeric acid in the acidified solution and subsequently lost by evaporation during the heating period and escape to

the atmosphere when the flask was opened for positioning of the scintillation vial. In some cases, there was a slight odor of valeric acid detectible when the flask was opened.

The percentage of radioactivity present in valerate preparations that was recovered in CO_2 was even smaller in the case of the DNA samples than for 1-^{14}C-deoxyribose samples. It is possible that not all radioactivity attributed to valerate was indeed in that compound in the case of the DNA samples. Perhaps another compound produced from deoxyribose or from a purine or pyrimidine base upon hot acid treatment was carried through the ether extraction, semicarbazone precipitation and steam distillation and eventually lost by evaporation in the Schmidt degradation step.

The results of the experiments reported here do not allow a definitive conclusion to the question of the intact incorporation of deoxyribose into DNA. Failure to find in CO_2 all the radioactivity once present in valerate indicates that the method used is not fully satisfactory, since the chemical form in which loss occurred is not known. However, it seems probable that no or very little radioactivity was present in carbon atoms other than carbon-1, since the other product, butylammonium ion, should remain dissolved in the residual solution and any radioactivity in this compound should be readily detected. If 1-^{14}C-deoxyribose were degraded prior to incorporation of radioactive carbon atoms into the deoxyribose of

DNA, then one would expect to find the label scattered throughout the deoxyribose carbon atoms. Since this does not appear to occur, it seems probable that the administered 1-^{14}C-deoxyribose was incorporated intact into DNA.

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