Deoxyribose-5-Phosphate Metabolism by Normal Liver and Malignant Hepatoma

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The requirement for net synthesis of deoxyribonucleic acid in a growing tissue, normal or malignant, is well established. The synthetic pathways of the purine and pyrimidine moieties of nucleic acids have been largely elucidated, but most of the available data are concerned with ribonucleic acid synthesis. The metabolic origin of the deoxyribose moiety of DNA is, however, not clearly established. Direct conversion in vivo of cytidine to deoxycytidine without splitting of the ribosidic linkage has been observed to occur in rats (1). On the other hand, Racker (2) has described an enzymatic mechanism for the synthesis of deoxyribose-5-phosphate by aldol condensation of glyceraldehyde-3-phosphate and acetalddehyde. This enzyme has been studied in detail in bacterial extracts and its occurrence in animal tissues such as mouse liver and thymus has been noted. McGeown and Malpress (3) have demonstrated the presence of the enzyme in guinea pig liver. These investigators have identified deoxyribose-5-phosphate as the product formed when either triose phosphate or fructose-1,6-diphosphate (hexose diphosphate) were incubated with acetalddehyde and suitable enzyme preparations. Isotopic evidence that synthesis in vivo of the deoxyribose moiety of DNA does occur from 2 and 3 carbon precursors has recently been described (4-6).

The data presented compare the deoxyribose phosphate aldolase activity of normal tissues to that of malignant tissue and report observations on the fate of deoxyribose phosphate and deoxyribo-nucleosides and -nucleotides added to these tissues. A preliminary account of this work has been published (7).

EXPERIMENTAL

For measurement of deoxyribose phosphate synthesis, tissues were homogenized with 4 volumes of ice-cold isotonic potassium chloride in a Potter Elvehjem homogenizer with a Teflon pestle. Aliquots (0.1 to 0.4 ml.) of the homogenates or their centrifugal fractions containing equal wet weights of tissue were incubated in 0.04 M Tris buffer of pH 7.4 with 5 μmoles of hexose diphosphate and 10 μmoles of acetalddehyde in a total volume of 1 ml. After incubation at 37°C for the indicated times, the reaction was stopped by the addition of an equal volume of 10 per cent trichloroacetic acid. Deoxyribose phosphate was determined calorimetrically with diphenylamine using the modification of the Dische procedure described by Burton (8) for DNA. This modification is 3.5 times more sensitive for deoxyribose than the original Dische procedure and even more sensitive for deoxyribose phosphate and deoxyribose purine derivatives (see Table II). More important is the fact that the substrates in the concentrations used do not interfere with the color reaction. In order to improve the specificity of the color reaction, we have used the dichromatic spectrophotometry recommended by Racker (2) that involves subtracting the absorbance at 650 μm from the absorbance at 590 μm. This reduces the sensitivity by about 30 per cent but avoids interferences encountered with some tissue fractions. All the figures in this paper have as their ordinate, ΔA × 10^6, the absorbance at 590 μm minus the absorbance at 650 μm. Under these experimental conditions, a ΔA × 10^6 of 400 is equivalent to 0.1 μmoles of deoxyribose phosphate (see Table II). Stability and purity of color are greatly improved by keeping the tubes in the dark during color development. It has been observed that the color development is much less sensitive to light in perchloric acid filtrates than in trichloroacetic acid filtrates. Deoxyguanosine was used to establish adequacy of color development in tissue extracts. The only disadvantage of this method is the fact that at least 16 hours are required for optimal color development.

The modification of the cysteine-sulfuric acid reaction for deoxy sugars described by Stumpf (9) and Dische (10) was frequently employed to confirm the results with the diphenylamine reaction. Lack of sensitivity restricted the routine use of this method.

Deoxyribose phosphate was prepared from deoxyadenylic acid as described by Racker (2) or from deoxyguanylic acid according to Lamport (11). It was identified by the following criteria: paper strip chromatography; positive diphenylamine and cysteine-sulfuric acid reaction; liberation of inorganic phosphate on acid hydrolysis at 100°C in 20 minutes; absence of free inorganic phosphate; absence of absorption in the region of 260 to 280 μm.

Deoxyribo-nucleotides and -nucleosides were obtained from the California Foundation for Biochemical Research, crystalline aldolase and glyceraldehyde phosphate dehydrogenase from Sigma Chemical Company and C. F. Boehringer and...
Soehne, G. m.b. H., Mannheim, and hexose diphosphate (Mg salt) from Schwarz Laboratories, Inc.

The Novikoff hepatoma and the Murphy lymphosarcoma were grown by sterile inoculation into 150 to 200 gm. Holtzman strain rats, intraperitoneally and subcutaneously, respectively. The Morris hepatoma was transplanted subcutaneously into AXC strain rats. The butter-yellow hepatoma was induced in Holtzman strain rats according to Miller et al. (12) on a high fat, normal riboflavin diet. All tumors were carefully inspected for necrosis and only non-necrotic tissue was used for the homogenates.

RESULTS

Fig. 1 illustrates the comparison of the deoxyribose phosphate aldolase activities of a supernatant fraction obtained at 1800 × g of homogenates from normal rat liver, regenerating liver, and a hepatoma (Novikoff) growing as a solid tumor in the peritoneum of the rat.

In normal liver, the amount of deoxyribose phosphate formed increases to a peak and gradually disappears. The peak occurs in about 10 to 40 minutes, being reached earlier with higher concentrations of tissue extract. Nearly complete disappearance occurs after 60 to 90 minutes.

In the regenerating liver, the synthesis and disappearance of deoxyribose phosphate is increased 2- to 3-fold. Each animal is its own control since enzyme activity of the portion of liver removed at the time of the operation was compared with activity of the remaining regenerating liver. Maximal increase of synthetic capacity (represented by the curve in Fig. 1) for deoxyribose phosphate occurred about 72 hours after hepatectomy and the return to control values in about 7 days. This lags somewhat behind the peaks of DNA synthesis and mitotic activity after hepatectomy which occur at about 24 to 48 hours (13). The age of the animal has some bearing on the deoxyribose phosphate aldolase activity of the liver since younger animals have greater activity and the rise after hepatectomy is more pronounced in the older animals.

In the hepatoma homogenate, the synthetic reaction proceeds at a rate 2 to 3 times faster than in normal liver homogenate and there is no indication of disappearance of deoxyribose phosphate even when incubation is extended for 3 to 6 hours. The actual rate of formation decreases as shown by the asymptotic nature of the curve, but deoxyribose phosphate formed does not disappear. The decrease in rate of synthesis may be due to loss of volatile substrate, acetaldehyde, or to denaturation of enzyme protein. In one instance more acetaldehyde was added after 40 minute incubation and the rate of formation of deoxyribose phosphate was found to increase again. The identity of the chromogenic material formed in liver and tumor with deoxyribose phosphate was established by paper chromatographic comparison with authentic samples of deoxyribose phosphate.

Fig. 2. Deoxyribose phosphate aldolase pattern in tumors. The conditions used were the same as in Fig. 1.
The curves in the experiment of Fig. 1 are reported on the basis of equal wet weight of tissues. On dry weight or nitrogen basis the difference between the normal and malignant tissue becomes more pronounced because of the somewhat higher water content of the tumor. On the basis of DNA content, essentially the same curves are obtained as in Fig. 1, but this affords a doubtful standard of comparison since the constancy of DNA content per cell does not hold for the tumor.

The characteristic pattern of deoxyribose phosphate aldolase activity is demonstrated in six different tumors in Fig. 2. Synthetic activity is rapid and even after prolonged incubation there is no indication of disappearance of deoxyribose phosphate.

The curves represent three different rat hepatomas; the Novikoff tumor growing intraperitoneally, the Morris tumor growing subcutaneously in AXC rats, and a hepatoma, induced by butter-yellow feeding, growing in the liver of the rat. Direct comparison with normal and regenerating tissue is available for these tumors (Fig. 1). The Murphy lymphosarcoma was growing subcutaneously in the rat and the spontaneous tumor had been found growing retroperitoneally in an untreated rat. It consists of large epitheloid cells, but its origin cannot be established histologically. The tumor proved to be readily transplantable. The HeLa cells, a human cancer growing in tissue culture, were obtained from 3 week-old cultures. It is difficult to be certain of quantitative differences in enzyme activity between these tumors since considerable variations in specific activity were found on individual specimens of each tumor. This is in good part due to the difficulty in including, even after thorough gross inspection, necrotic areas from the material used to prepare the homogenate.

The deoxyribose phosphate aldolase activity of centrifugal fractions of normal liver and Novikoff hepatoma homogenates are represented in Fig. 3.

In normal liver homogenates, most of the deoxyribose phosphate aldolase is found in the material settling with centrifugation at 600 × g. Precipitates obtained at 1,500, 8,500, 20,000 and 80,000 × g are completely inert. The supernatant fraction obtained at 80,000 × g still possesses a small amount of synthetic activity. In the hepatoma homogenate, the precipitate obtained at 600 × g shows very little activity, whereas most of the increased activity is found in the supernatant fraction obtained at 80,000 × g. Intermediate centrifugal fractions were again inactive. The possibility that aldolase becomes the rate limiting reaction with hexose diphosphate as one of the substrates has been excluded by determining aldolase activity (14). In all instances, aldolase activity in excess of that required for the conversion of the added hexose phosphate to triose phosphate was present. Crystalline aldolase was added in some of the experiments as an added precaution but did not alter the picture presented in Figs. 1–3.

Quantitatively, the most striking difference is the fact that deoxyribose phosphate disappears from the normal liver but not from the tumor in the presence of substrates for synthesis. However, if deoxyribose phosphate or deoxyguanosine alone are added to certain centrifugal fractions from liver or hepatoma, material reacting as deoxy sugar in the color test disappears (Fig. 4).

Most of this activity is found in the supernatant fraction from high speed centrifugation, derived from normal liver, with a small residual activity in the precipitate obtained at 600 × g. The intermediate centrifugal fractions are again completely inactive. In the supernatant fraction from high speed centrifugation, derived from hepatoma, a small activity can be demonstrated but only in the complete absence of substrates for deoxyribose phosphate synthesis. This is in contrast to the fraction from normal liver where it disappears rapidly and readily even in the presence of deoxyribose phosphate aldolase substrates (Figs. 1 and 3).

Reversal of the deoxyribose phosphate aldolase reaction would be a simple explanation for the disappearance of di-

![Fig. 3. Deoxyribose phosphate aldolase activity of various centrifugal fractions of normal liver and Novikoff hepatoma homogenates. Particulate fractions were resuspended in original volume of isotonic KCl; otherwise, conditions were the same as in Fig. 1.](http://www.jbc.org/)
Fig. 4. Disappearance of deoxyribose reacting material from normal liver and hepatoma. Reaction mixture contained 0.2 ml. of enzyme fraction and 0.125 μmoles of deoxyguanosine in 1 ml. of 0.04 M Tris buffer, pH 7.4. It was incubated at 37° for the indicated times.

Table I
Incubation of deoxyribose phosphate and deoxyguanosine with dialyzed liver fractions

The enzyme was obtained from a supernatant fluid obtained at 30,000 × g of normal rat liver by fractionation with ammonium sulfate between 40 to 60 per cent saturation, followed by dialysis. Incubation at 37° in 1 ml. of 0.04 M Tris buffer, pH 7.4, containing 0.2 μmoles of substrate. Additions as indicated. Boiled or ashed liver corresponded to 100 mg. wet weight of fresh liver.

<table>
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<th>Substrate</th>
<th>Supplement</th>
<th>ΔA after incubation for:</th>
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<td></td>
<td></td>
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<tr>
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<td>810</td>
</tr>
<tr>
<td>Deoxyribose phosphate</td>
<td>Boiled liver</td>
<td>800</td>
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<tr>
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phenylamine reactive material when either deoxyribose phosphate or purine deoxyribose derivatives are added to liver supernatant fractions. Such an interpretation is inconsistent with the following observations. First, the biphasic curve (Fig. 1) observed does not indicate the establishment of an equilibrium state. Secondly, deoxyribose phosphate aldolase and the enzymes involved in the metabolism of deoxyribose phosphate are prevalent in different centrifugal fractions of liver as well as tumor homogenates. In liver, most of the deoxyribose phosphate aldolase is in the fraction settling at 600 × g but the reactions leading to the disappearance of deoxyribose phosphate are absent (Figs. 3 and 4). The supernatant fraction from high speed centrifugation, derived from normal liver, is the best source for the enzymes involved in the metabolism of deoxyribose phosphate, but a poor source of deoxyribose phosphate aldolase. This relation is reversed in tumor supernatant fractions. Separation of deoxyribose phosphate aldolase from the enzymes involved in deoxyribose phosphate metabolism can be obtained by ammonium sulfate fractionation of liver supernatant fluid. All of the deoxyribose phosphate aldolase is precipitated by 40 per cent saturation with ammonium sulfate, whereas the bulk of the enzymes of deoxyribose phosphate metabolism precipitate at between 40 and 60 per cent saturation. Finally, Racker (2) originally demonstrated the reversibility of the reaction with a deoxyribose phosphate aldolase derived from Escherichia coli by measuring the formation of glyceraldehyde phosphate from deoxyribose phosphate with glyceraldehyde phosphate dehydrogenase. With the enzyme present in liver or tumor fractions and deoxyribose phosphate or the purine deoxyribonucleosides as substrates, formation of glyceraldehyde-3-phosphate could not be demonstrated by enzymatic methods (2) or the colorimetric method (14), in spite of the rapid disappearance of diphenylamine-reacting material.

The complex nature of the reactions involved in the disappearance of diphenylamine-reacting material is indicated in Table I. Whereas deoxyribose phosphate aldolase from bacteria, liver, thymus (2), and tumor supernatant fractions is not inactivated by extensive dialysis, deoxyribose phosphate metab-
The physiological significance of deoxyribose phosphate aldolase has been somewhat doubtful in the past (2) since one of the substrates, acetaldehyde, had to be supplied in high concentration. However acetaldehyde, in concentrations equivalent to the hexose diphosphate, sufficed in the tumor systems to obtain sustained deoxyribose phosphate synthesis. Although acetaldehyde or an activated derivative has to be postulated in the metabolic pathways of pyruvic acid, its existence in free form had not been demonstrated in mammalian metabolism. A pyridoxal phosphate-dependent aldolase that reversibly forms glycine and acetaldehyde from threonine was first described by Brunsström and Vilenkina (17) and recently studied in detail by Karnes and Greenberg (18) and Gilbert (19). The enzyme was found to be present in tumor supernatant fractions, and deoxyribose phosphate synthesis comparable in rates to those obtained with acetaldehyde has been obtained if acetaldehyde was replaced by equimolar concentrations of threonine in the presence of pyridoxal phosphate. Thus, deoxyribose phosphate synthesis occurred by the interaction of three aldolases with hexose diphosphate and threonine as the sole substrates. The observations that deoxyribose phosphate aldolase can be coupled to the threonine aldolase system and that it responds to the stimulus of liver regeneration, strengthen the case for a physiological role for deoxyribose phosphate aldolase.
indicated by isotope experiments (4-6) do not indicate the actual pathway of these precursors, but the results obtained are not in conflict with a role for deoxyribose phosphate aldolase for this incorporation.

Decreased ability to metabolize deoxyribose phosphate has been found in all the tumors investigated. Although in the presence of substrates for synthesis deoxyribose phosphate accumulates in the tumor system, this does not indicate a qualitative difference between normal and malignant tissue, since in the absence of substrates for synthesis deoxyribose phosphate can be metabolized by tumor systems to some extent. Since a complex of probably simultaneous reactions is involved in the metabolism of deoxyribose phosphate, the individual reactions will have to be clarified, before the over-all changes observed in tumors can be classified as quantitatively or qualitatively different.

**SUMMARY**

The synthesis of deoxyribose phosphate from glyceraldehyde-3-phosphate and acetaldehyde (deoxyribose phosphate aldolase) has been measured in normal and regenerating liver and in malignant tissue. In normal liver, deoxyribose phosphate reaches a peak in about 30 minutes but subsequently disappears within 60 minutes. The physiological growth stimulus of liver regeneration increases both synthesis and disappearance of deoxyribose phosphate 2- to 3-fold. In contrast, the synthetic reaction, in transplanted and induced hepatoma and in three other tumors, proceeds at a rate 2 to 3 times faster without any indication of disappearance of deoxyribose phosphate even after incubation for 3 hours.

With normal liver, the synthetic capacity is found primarily in the nuclear fraction (precipitated at 600 X g), whereas the disappearance of deoxyribose phosphate occurs primarily in the supernatant fraction obtained at 80,000 X g. With the hepatoma, most of the increased synthetic activity is found in the fraction not precipitated at 80,000 X g whereas the activity for deoxyribose phosphate disappearance is of such low order that it can be demonstrated only in the absence of substrates for deoxyribose phosphate synthesis.

In supernatant fractions of normal liver, the reactions leading to the disappearance of deoxyribose phosphate can be separated from deoxyribose phosphate aldolase. This activity is lost on dialysis but can be reactivated by the addition of boiled liver.

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**REFERENCES**

Deoxyribose-5-Phosphate Metabolism by Normal Liver and Malignant Hepatoma
George E. Boxer and Carl E. Shonk