BBA 3909

GLUCOSAMINE-6-PHOSPHATE DEAMINASE ACTIVITY DURING CONNECTIVE TISSUE GROWTH

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(Received August 6th, 1962)

SUMMARY

The presence of glucosamine-6-phosphate deaminase, which reversibly degrades glucosamine 6-phosphate into fructose 6-phosphate and ammonia, has been established in the connective tissue developed by the subcutaneous implantation of cotton pellets. Some of the properties of this enzyme are described. It is observed that carrageenin is without any effect on the induction of the enzyme during the development of the granulomata. Pharmacologically high doses of cortisone, however, inhibit the glucosamine-6-phosphate deaminase activity both in ordinary granulomata and in those induced by carrageenin.

INTRODUCTION

Two of the important constituents of granulation tissue are collagen and mucopolysaccharides. In view of the recent suggestion by WOOD[1] that mucopolysaccharides are concerned with the nucleation and growth of collagen, investigations have been undertaken to study the biosynthesis of mucopolysaccharides during the development of connective tissue.

In earlier reports[2,3] from this laboratory evidence was presented showing that the rate of synthesis of mucopolysaccharides in granulation tissue of rats was most active 48–72 h after subcutaneous implantation of cotton pellets. Preliminary studies indicated the presence of the enzymes involved in the synthesis of "active sulphate", glucosamine 6-phosphate, uridine diphosphoglucose, uridine diphospho *N*-acetyl glucosamine and uridine diphosphoglucuronic acid. Evidence of a synthesis *in vitro* of chondroitin sulphate and hyaluronic acid was also presented. Subsequently, the presence of the "active sulphate" synthesizing enzyme system was confirmed and its properties studied[4]. BOLLET AND SHUSTER[5] demonstrated the presence of fructose-6-phosphate glutamine transamidase, which irreversibly synthesizes glucosamine 6-phosphate from fructose 6-phosphate and glutamine, in the cultivated connective tissue.

The present paper presents the data which establish the presence of glucosamine-6-phosphate deaminase in the developing connective tissue. A study has also been made of the effects of carrageenin and of administration *in vivo* of cortisone on this enzyme.

MATERIALS AND METHODS

Glucosamine 6-phosphate and *N*-acetyl glucosamine 6-phosphate were prepared according to the methods described in an earlier paper[6].

Cortisone acetate for injection was obtained from Glaxo Laboratories (India) and carrageenin was the product of Seaplant Chemical Corporation (U.S.A.).

The subcutaneous cotton pellet implantation technique employed for the induction of connective tissue in rats was the same as that described in an earlier communication[4].

Protein was measured by the method of WARBURG AND CHRISTIAN[7].

Fructose 6-phosphate was determined by ROE's resorcinol method[8] and glucosamine 6-phosphate by the method of LEVY AND MCALLAN[9] as modified by PATTABIRAMAN AND BACHHAWAT[6].

Preparation of the enzyme

Proteins were usually extracted from the connective tissue by grinding in a mortar at 0° with an equal weight of analytical grade sand and with twice the volume of 0.05 M Tris buffer (pH 7.4) containing 0.5 M KCl. The mixture was then centrifuged at 10 000 × *g* at 0° for 20 min. The supernatant fluid was used as the source of enzyme.

Enzyme assay was performed as follows: The reaction mixture for the routine estimation of glucosamine-6-phosphate deaminase activity contained the following in a volume of 0.2 ml: 0.2 μmole of glucosamine 6-phosphate, 0.02 μmole of *N*-acetylglucosamine 6-phosphate, 10 μmoles of Tris buffer (pH 8.0) 0.1 μmole of Mn^{2+} and the enzyme preparation. After incubation at 37° for 1 h, the amount of glucosamine 6-phosphate left undegraded was determined. A reagent blank was run in all cases in which the enzyme was added after the incubation.

Enzymic formation of glucosamine 6-phosphate was determined as follows: The reaction mixture contained the following in a volume of 0.2 ml: 5 μmoles of fructose 6-phosphate, 20 μmoles of $(NH_4)_2CO_3$, 0.02 μmole of N-acetyl glucosamine 6-phosphate, 50 μmoles of Tris buffer (pH 8.0) and the enzyme preparation. After incubation at 37° for 1 h, the reaction was stopped by the addition of sodium tetraborate solution and the glucosamine 6-phosphate formed was assayed. A blank was run in all cases in which the enzyme was added after the incubation.

When the formation of fructose 6-phosphate was measured, the amounts of various components of the assay mixture were increased fivefold to increase the accuracy of the determination.

RESULTS

Properties

Effect of enzyme concentration: As can be seen from Fig. 1, the rate of degradation of glucosamine 6-phosphate by the enzyme was linear up to a protein concentration of about 600 μg.

Effect of pH

Fig. 2 shows the reaction velocity as a function of pH. It was found that the enzyme was active over a broad pH range with maximum activity at pH 8.0. The enzyme from other sources[6,10] studied showed a pH optimum of 8.5.

Effect of metal ions

It was shown in an earlier report[6] that the brain glucosamine-6-phosphate deaminase was activated by divalent metal ions, Mn^{2+} and Hg^{2+} being the most effective. Hence a study was undertaken of the effect of metal ions on the connective tissue glucosamine-6-phosphate deaminase and the results are presented in Table I.

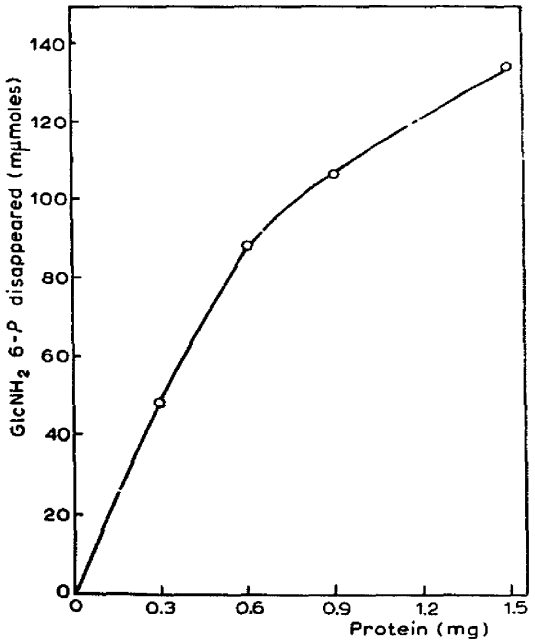

Fig. 1. Effect of enzyme concentration on the degradation of glucosamine 6-phosphate. For assay conditions see text.

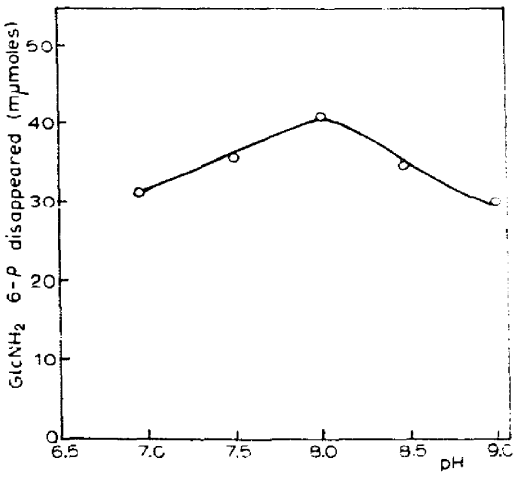

Fig. 2. Effect of H^+ ion concentration on the enzyme reaction. For assay conditions see text. 390 μg of enzyme was used in this assay.

TABLE I

EFFECT OF METAL IONS ON GLUCOSAMINE-6-PHOSPHATE DEAMINASE ACTIVITY

For assay see text. 0.1 μmole of each metal ion was added as indicated.

Metal ion added	*Glucosamine 6-phosphate disappeared (mμmoles)*
None	31.0
Mn^{2+}	45.0
Co^{2+}	41.0
Hg^{2+}	34.0
Mg^{2+}	28.0

It can be seen from the table that although the enzyme was active even in the absence of added metal ions, Mn^{2+} and Co^{2+} considerably stimulated the enzyme activity. It was, however, observed that the activation by Hg^{2+} was not as pronounced as with the brain glucosamine-6-phosphate deaminase.

Activation by N-acetyl glucosamine 6-phosphate

The activity of connective tissue glucosamine-6-phosphate deaminase was markedly diminished in the absence of *N*-acetyl glucosamine 6-phosphate. Similar observations have been made with hog kidney[10] and brain deaminases[6].

Effect of substrate concentration

Fig. 3 shows the reaction velocity as a function of glucosamine 6-phosphate concentration with and without *N*-acetyl glucosamine 6-phosphate. The K_m value for glucosamine 6-phosphate was found to be $5.75 \cdot 10^{-4}$ M. It was also found that the affinity of the enzyme for glucosamine 6-phosphate was considerably decreased in the absence of *N*-acetyl glucosamine 6-phosphate as was evident from the higher K_m ($1.8 \cdot 10^{-3}$ M) in the absence of N-acetyl glucosamine 6-phosphate. The K_m value for glucosamine 6-phosphate was found to be higher than those reported for brain and hog-kidney deaminase[6,10].

Reversibility of the reaction

As in the case of hog kidney and brain deaminase[6,10] the glucosamine 6-phosphate deaminase reaction was found to be readily reversible. The optimum conditions for maximum synthesis of glucosamine 6-phosphate are illustrated in Table II. It was found that there was no deaminase activity when $(NH_4)_2CO_3$ was omitted from the assay mixture and that glutamine did not replace $(NH_4)_2CO_3$ as the amino donor. It was also noted that *N*-acetyl glucosamine 6-phosphate powerfully stimulated the enzyme activity. It was further observed that Mn^{2+} and Co^{2+} stimulated the enzyme activity appreciably and that Mg^{2+} did not replace either Co^{2+} or Mn^{2+}.

TABLE II

CONDITIONS FOR MAXIMUM SYNTHESIS OF GLUCOSAMINE 6-PHOSPHATE

For complete assay system see text.

Components	*Glucosamine 6-phosphate formed (mμmoles)*
Complete system	20.0
Minus *N*-acetylglucosamine 6-phosphate	4.0
Minus ammonium carbonate	0.0
Minus Mn^{2+}	12.0
Minus ammonium carbonate plus glutamine	0.0
Minus Mn^{2+} plus Mg^{2+}	12.8
Minus Mn^{2+} plus Co^{2+}	14.4

Formation of fructose 6-phosphate

The effect of increasing the protein concentration on the formation of fructose 6-phosphate is shown in Table III. The rate of formation of fructose 6-phosphate was proportional to the enzyme concentration.

Effect of carrageenin on the deaminase activity

Fig. 4 illustrates a comparative study of the enzymic activities in granulomata induced by ordinary cotton pellets and those induced by pellets containing carrageenin.

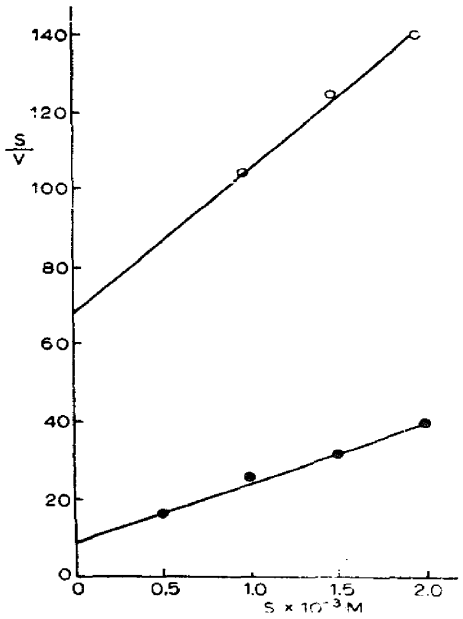

Fig. 3. The K_m value for glucosamine 6-phosphate. The assay system contained varying concentrations of glucosamine 6-phosphate, 10 μmoles of Tris buffer (pH 8.0), 0.1 μmole of Mn^{2+} and the enzyme preparation. For K_m determination in the presence of *N*-acetyl glucosamine 6-phosphate, 0.02 μmole of *N*-acetylglucosamine 6-phosphate was included. ● — ●, in the presence of *N*-acetylglucosamine 6-phosphate; O — O, without *N*-acetylglucosamine 6 phosphate.

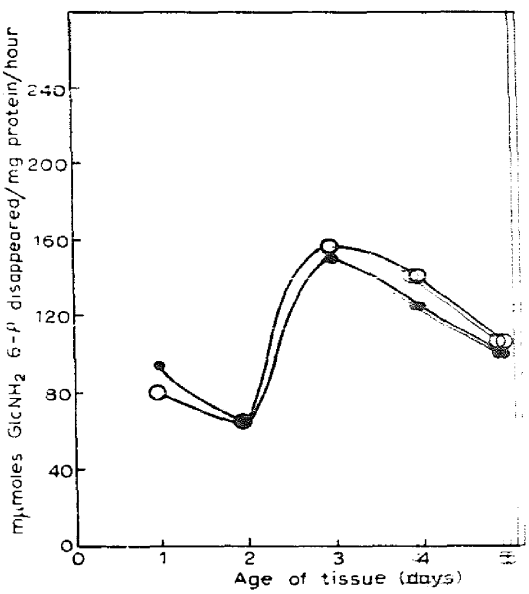

Fig. 4. The activity of glucosamine-6-phosphate deaminase in the granuloma produced by ordinary cotton pellets and by carrageenin-containing cotton pellets at various stages of the development of the granuloma. O — O, granuloma produced by ordinary cotton pellets; ● — ●, granuloma produced by cotton pellets containing carrageenin. The values expressed represent an average of four experiments.

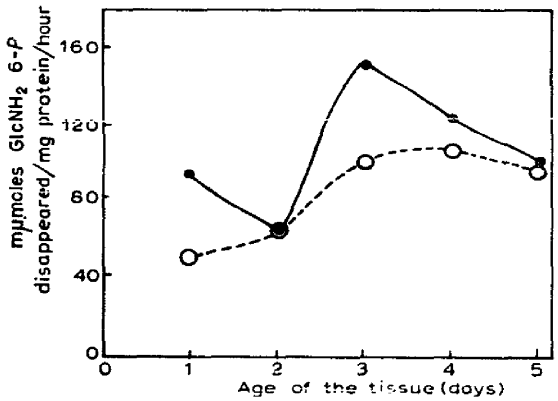

Fig. 5. The inhibition of glucosamine-6-phosphate deaminase by administration *in vivo* of cortisone at various stages of the development of granuloma induced by ordinary cotton pellets. ● — ●, normal rat granuloma; O — O, granuloma treated with cortisone. The values expressed represent an average of four experiments.

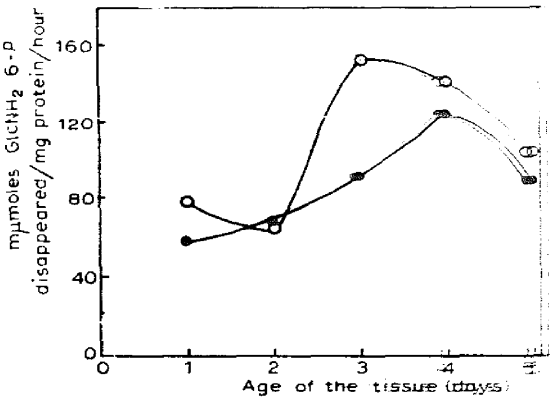

Fig. 6. The inhibition of glucosamine-6-phosphate deaminase by administration *in vivo* of cortisone at various stages of the development of granuloma induced by carrageenin. ○ — ○, normal rat granuloma; ● — ●, granuloma treated with cortisone. The values expressed represent an average of four experiments.

The results obtained showed that the enzyme activity was maximal 72 h after the implantation of either carrageenin-containing or ordinary cotton pellets. Apparently, carrageenin did not induce any accelerated formation of glucosamine-6-phosphate deaminase.

TABLE III

FORMATION OF FRUCTOSE 6-PHOSPHATE

The assay system consisted of 1 μmole of glucosamine 6-phosphate, 0.1 μmole of N-acetylglucosamine 6-phosphate, 10 μmoles of Tris buffer (pH 8.0), 0.5 μmole of Mn^{2+} and enzyme added as mentioned in table in a total volume of 0.5 ml.

Protein (μg)	*Fructose 6-phosphate formed (mμmoles)*
675	250
1350	540
2700	850

Effect of cortisone

To study the effect of cortisone on the deaminase activity in the granulomata, a pharmacologically high dose (200 mg/kg body weight per day) was administered intramuscularly to rats. The activity of the deaminase in the granulation tissue was measured at various intervals and Figs. 5 and 6 show the results obtained. These results indicated that the cortisone inhibited the deaminase activity in both ordinary granulomata and those induced by carrageenin.

DISCUSSION

This study has established the presence of glucosamine-6-phosphate deaminase, which reversibly cleaves glucosamine 6-phosphate into fructose 6-phosphate and NH_3, in the granuloma produced by the subcutaneous implantation of cotton pellets. The presence of fructose-6-phosphate transamidase, which irreversibly synthesizes glucosamine 6-phosphate from fructose 6-phosphate and glutamine, had already been demonstrated in the connective tissue by BOLLET AND SHUSTER[5]. An earlier report from this laboratory[6], along with that of COMB AND ROSEMAN[10], suggested that the deaminase plays an important role in the synthesis of glucosamine 6-phosphate. It may be of interest to note that evidence has already been presented from this laboratory showing that the rate of incorporation of labelled metabolites into mucopolysaccharides[2] is rapid during the early stages of the development of connective tissue. Studies on the "active sulphate" synthesizing enzyme system also indicate that the activity of this enzyme is very high during the initial stages of connective tissue growth. These observations, coupled with the fact that the deaminase activity also increases initially, strongly suggest a synthetic role for this enzyme. However, LOWTHER AND ROGERS[11-13] have observed that the nitrogen atom of glucosamine is derived from

the amide group of glutamine. The synthetic function of deaminase, therefore, still remains to be ascertained.

In view of the reported observation that carrageenin induces the formation of connective tissue with "explosive" violence[14], the present study on the influence of carrageenin on the development of the deaminase activity is of particular interest. It has been shown[4] that cotton pellets containing carrageenin induce a maximal formation of the "active sulphate" synthesizing enzyme system at an earlier stage after implantation than that induced by the ordinary cotton pellet. The present study, however, does not show any effect of carrageenin on the deaminase activity. It is possible that the influence of carrageenin on the development of connective tissue is restricted to its effect on the accelerated synthesis of the "active sulphate" synthesizing enzyme system.

It has been shown that the anti-inflammatory drug, cortisone, inhibits the formation of granulomata[15,16] and it has been suggested[17] that it also inhibits the synthesis of chondroitin sulphate. WHITEHOUSE AND LASH[18] have shown that the incorporation of [^{35}S]sulphate into chondroitin sulphate by cartilage grown in tissue culture is considerably inhibited by cortisone. BALASUBRAMANIAN AND BACHHAWAT[4] have further shown that the administration *in vivo* of pharmacologically high doses of cortisone has a marked inhibitory effect on the "active sulphate" synthesizing enzyme system in granulation tissue. The inhibitory effect of cortisone on the deaminase activity is thus consistent with the observation on the "active sulphate" synthesizing enzyme system. A similar observation was made by BOLLET AND SHUSTER[5] during their studies on the effect *in vitro* of cortisone on fructose-6-phosphate glutamine transamidase in connective tissue. It seems, therefore, that the inhibitory effect of administration *in vivo* of pharmacologically high doses of cortisone on the formation of granulomata is not due to its specific inhibitory effect on any single enzyme studied.

ACKNOWLEDGEMENTS

The keen interest and encouragement of Professor J. CHANDY is deeply appreciated. The investigation was supported by grants from the National Multiple Sclerosis Society, U.S.A., Grant No. 214-3 and the Council of Scientific and Industrial Research (India).

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