METABOLISM OF MUCOPOLYSACCHARIDES IN CONNECTIVE TISSUE. II. SYNTHESIS OF GLUCOSAMINE–6–PHOSPHATE *

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New methods of studying metabolic processes in connective tissue provide opportunity to obtain information concerning enzymatic steps in the synthesis of mucopolysaccharides in that tissue. These techniques supplement studies done in microorganisms and in other tissues (1–3), and allow investigation of phenomena of interest in relation to the diseases of connective tissue at an enzymatic level. Glucosamine and galactosamine are present in the disaccharide repeating units of the mucopolysaccharides of connective tissue. Uridine diphosphoacetylglucosamine has been shown to be the donor of acetylglucosamine in hyaluronate synthesis and the precursor of uridine diphosphoacetylgalactosamine which serves a similar function in chondroitin sulfate synthesis (1). An initial step in the synthesis of uridine diphosphoacetylglucosamine is the synthesis of glucosamine-6-phosphate.

The present report concerns the activity of the enzyme involved in the synthesis of glucosamine-6-phosphate in connective tissue and liver of rats and in human synovial tissues, as well as investigation of the influence of cortisol and gold compounds on the activity of this enzyme. A comparison of the effects of cortisol on several aspects of connective tissue metabolism has also been made.

METHODS

Polyvinyl (Ivalon) sponges were inserted under the dorsal skin of male Wistar albino rats weighing 200 ± 50 g, anesthetized with pentobarbital (50 mg per kg). The animals were sacrificed 2 weeks later; the sponges and liver were removed, weighed and homogenized separately. Human synovial tissue obtained at the time of arthrotomy for orthopedic surgery was homogenized within a few minutes after removal.

Fructose-6-phosphate-glutamine transamidase. The activity of this enzyme was determined by a modification of the procedure of Pogell and Gryder (4). Homogenates were made in 0.15 M KCl, with 0.001 M disodium ethylenediamine tetraacetate (EDTA), 0.065 per cent mercaptoethanol, and 0.024 M glutamine, pH 7.0. Assays were done in a volume of 2 ml containing 20 μmoles of fructose-6-phosphate, 25 μmoles of glutamine and 50 μmoles of phosphate buffer, pH 7.7. After 15 to 60 minutes at 37° C, the incubation mixture was heated at 100° for 2 minutes, centrifuged, and the hexosamine content of an aliquot of the supernatant solution was determined following conversion to acetylhexosamine by the procedure of Roseman and Daffner (5), using the method of Reissig, Strominger and Leloir (6). Glucosamine, subjected to the same procedure, was used as reference standard. The absorption spectrum of the product of the enzymatic reaction matched that of the reference standard.

Glutamic-oxaloacetic transaminase and glutamic-pyruvic transaminase. Activity of these enzymes was determined using homogenates made in 0.1 M phosphate buffer, pH 7.5. Glutamic-oxaloacetic transaminase was determined using 0.1 mmole of aspartate, 0.02 mmole of α-ketoglutarate and 0.2 mmole of phosphate buffer, pH 7.5, in a total volume of 2.0 ml. After 20 minutes at 37° C, the solutions were heat inactivated at 100° for 2 minutes, centrifuged, and 0.2 ml of supernatant was then added to 0.2 mg reduced diphosphopyridine nucleotide (DPNH), 400 units of malic dehydrogenase (Sigma), and 60 mmoles of phosphate buffer, pH 7.5, in a total volume of 2.0 ml. After incubation for 20 minutes, DPNH disappearance was determined fluorometrically, following the procedure of Lowry, Roberts and Kapphahn (7). Glutamic-pyruvic transaminase was assayed in a similar fashion, using 50 μmoles of alanine and 400 units of lactic dehydrogenase (Sigma) in place of aspartate and malic dehydrogenase. The amount of homogenate used in each assay was kept within the range which would give zero order reaction and a straight line relationship between the amount of homogenate and the amount of DPNH oxidized. Protein determinations were done by ultraviolet absorption (8).

RESULTS

Activity of the transamidase which synthesizes glucosamine-6-phosphate from fructose-6-phosphate and glutamine was demonstrated in liver and in the connective tissue which entered polyvinyl sponges in rats, and in human synovial tissue samples. Expressed as micromoles of hexosamine formed per milligram of protein in the crude homogenate, connective tissue homogenates usually had a slightly greater activity than did liver homogenates from each animal (Table IV). The findings in human synovial tissue are given in Table I. No correlation was found between the level of enzymatic activity and the diagnosis, age of the patient or histological appearance of the

TABLE I

Fructose-6-phosphate-glutamine transamidase activity of human synovial tissue obtained surgically

Site	Age	Sex	Diagnosis	Glucosamine synthesis
				$\mu moles \times 10^{-4}/ hr/mg protein$
Knee	67	M	Traumatic arthritis with effusion	97
Knee	46	M	Osteochondritis desiccans	61
Hip	40	F	Aseptic necrosis of femoral head	56
Elbow	58	M	Traumatic bursitis	49
Tendon	33	F	Ganglion	47
Knee	31	M	Fractured patella	42
Knee	58	M	Fractured patella	37
Knee	30	M	Fractured patella	22
Elbow	37	M	Traumatic bursitis	22
Knee	54	F	Traumatic bursitis	19
Hip	25	F	Aseptic necrosis of femoral head	14
Hip	56	M	Ankylosing spondylitis	6

tissue in a limited group of individuals studied. The lowest value was found in a man with ankylosing spondylitis who was noted to have marked softening of the femoral head at the time of surgery.

Synthesis of hexosamine occurred in connective tissue homogenates with either fructose-6-phosphate or glucose-6-phosphate, but little or no synthesis occurred when other sugars were substituted (Table II). Hexosamine synthesis occurred in liver homogenates with a variety of sugars, although the greatest activity was found with fructose-6-phosphate or mannose-6-phosphate. Ammonium chloride and glutamine were compared as

TABLE II

*Comparison of the synthesis of hexosamine by connective tissue homogenates with equimolar amounts of various substrates**

Substrate	Homogenate used	
	Connective tissue	Liver
Fructose-6-phosphate	100	100
Glucose-6-phosphate	99	97
Galactose-6-phosphate	12	70
Mannose-6-phosphate	15	106
Fructose	0	71
Glucose	0	48
Galactose	0	68
Mannose	0	56

* Figures are percentage of synthesis with fructose-6-phosphate.

donors of the amino groups in both systems; these results were summarized in Table III.

Effect of cortisol (Table IV). Beginning the seventh day after the insertion of the polyvinyl sponges, rats were injected daily with 5 mg of cortisol subcutaneously for one week before sacrifice. Cortisol administration caused a decrease in the weight and protein content of the granuloma. A fall in fructose-6-phosphate-glutamine transamidase activity occurred, but this change was not statistically significant. The glutamic-oxaloacetic transaminase activity in the connective tissue rose to a significant extent, but there was no change in the glutamic-pyruvic transaminase activity.

The fall in fructose-6-phosphate-glutamine transamidase activity in the liver was of the same magnitude as that in connective tissue. This dosage of cortisol produced very definite increases in the hepatic glutamic-oxaloacetic and glutamic-pyruvic transaminase activities. Cortisol was not found to influence the activity of these enzymes

TABLE III

Assay of relative effectiveness of glutamine and NH₄Cl as donors of amine group in glucosamine-6-phosphate synthesis

Glucosamine synthesis ($\mu moles \times 10^{-4}/hr/mg protein$)		
	Homogenate used	
Donor added	Connective tissue	Liver
Glutamine, 12.5 $mmoles$	55.6	23.8
NH₄Cl, 12.5 $mmoles$	17.2	15.3
Neither	11.8	19.8

TABLE IV

*Effect of cortisol on sponge connective tissue and liver of rats**

		Connective tissue			Liver		
		Mean	SEM†	No.	Mean	SEM†	No.
Weight of granuloma (g)	Treated	1.48	± 0.07	12			
	Control	1.93	± 0.133	17			
			p = < 0.01				
Protein content of granuloma (mg)	Treated	142	±26.9	12			
	Control	297	±41.4	17			
			p = < 0.01				
Fructose-6-phosphate-glutamine transamidase	Treated	29.4	± 6.74	12	21.6	±5.55	11
	Control	41.9	± 5.03	17	32.4	±4.16	17
			p = < 0.1			p = < 0.1	
Glutamic-oxaloacetic transaminase	Treated	9.54	± 1.35	11	73.9	±6.50	12
	Control	6.45	± 0.83	14	44.5	±4.95	14
			p = < 0.05			p = < 0.01	
Glutamic-pyruvic transaminase	Treated	9.47	± 1.45	11	15.0	±1.92	12
	Control	8.88	± 1.13	14	5.1	±0.80	14
			p = < 0.3			p = < 0.001	

* Data for enzyme activities are in the following units: fructose-6-phosphate transamidase, μmoles $\times 10^{-4}$/hr/mg protein; glutamic-oxaloacetic transaminase, μmoles/hr/mg protein; glutamic-pyruvic transaminase, μmoles/hr/mg protein for liver and μmoles $\times 10^{-2}$/hr/mg protein for connective tissue.

† Standard error of the mean.

in connective tissue or in liver homogenates when added *in vitro*.

Effect of gold chloride in vitro. Since EDTA and mercaptoethanol were necessary to protect the fructose-6-phosphate-glutamine transamidase in tissue homogenates, it seemed possible that gold salts might influence the activity of the enzyme. Homogenates made in medium containing EDTA, but no mercaptoethanol, were incubated with and without a series of potential inhibitors. Gold chloride inhibited the activity of this enzyme in connective tissue homogenates in a lower concentration than in liver homogenates (Table V). Other sulfhydryl reactants such as iodoacetamide, iodobenzoic and *p*-chloromecuribenzoate were also found to inhibit the activity of this enzyme in connective tissue homogenates, although the concentration of these substances needed to give 50 per cent inhibition was ten times that found for gold chloride.

The effect of gold sodium thiomalate in vivo. One week after insertion of sponges, 10 mg of gold sodium thiomalate (Myochrysine) was administered subcutaneously. Seven to ten days later enzyme assays were performed. A marked decrease in the activity of the fructose-6-phosphate-glutamine transamidase was found in the connective tissue homogenates, but no effect was noted on the activity of the enzyme in liver ho-

mogenates obtained from the same animals (Table VI).

The acid mucopolysaccharide concentration of the sponge connective tissue in three of the animals injected with gold sodium thiomalate was de-

TABLE V

Effect of gold chloride and other inhibitors on the activity of the fructose-6-phosphate-glutamine transamidase in rat tissue homogenates

		Per cent inhibition	
	Molarity ($\times 10^{-5}$)	Connective tissue	Liver
Gold chloride	500	100	100
	50	100	89
	5	84	27
	3.75	38	
	2.5	24	5
	1.25	16	6
	0.5	0	
Iodoacetamide	500	100	81
	50	56	9
	37.5	48	
	25	33	
	12.5	7	12
	5	0	6
	2.5		0
Iodobenzoate	50	54	
	25	31	
	5	6	
p-Chloromercuri-benzoate	50	52	
	25	42	
	5	26	

TABLE VI

Effect of gold sodium thiomalate administration on fructose-6-phosphate-glutamine transamidase in rats*

	Connective tissue			Liver		
	Mean	SEM†	No.	Mean	SEM	No.
Treated	12.9	±3.04	11	31.5	±3.48	11
Control	41.9	±5.03	17	32.4	±4.16	17
	p =	< 0.001				

* Activity is expressed as μmoles $\times$ 10^{-4}/hr/mg protein.
† Standard error of the mean.

termined by a method previously described (9). Expressed as uronic acid content of the acid mucopolysaccharide fraction, a decrease from an average of 85 mg per 100 g of dry connective tissue in control animals to 52 mg per 100 g in the treated animals was noted.

DISCUSSION

Many of the characteristics of the enzyme involved in the synthesis of glucosamine-6-phosphate were demonstrated comparatively easily in homogenates of the granulation tissue which formed in polyvinyl sponges. Similar homogenates of liver tissue contained a lower activity of the fructose-6-phosphate-glutamine transamidase and a relatively greater concentration of other enzymes which interfered with the characterization of the substrates of the transamidase.

Cortisone and cortisol have been shown to inhibit connective tissue growth, fibril formation, sulfate fixation and oxygen consumption (10). An objective of this study was to determine if adrenal steroids uniformly inhibit all metabolic activities of connective tissue, or whether selective effects on certain metabolic functions occur. The glutamic-pyruvic and glutamic-oxaloacetic transaminases were studied in connective tissue since an increase in the activities of these enzymes in the liver of rats given cortisol has been reported (11, 12). Our finding of an increase in glutamic-oxaloacetic transaminase activity in connective tissue after cortisol administration indicates that this agent does not uniformly inhibit all metabolic activities in that tissue. Cortisol and gold sodium thiomalate were found to have different effects on liver than on connective tissue metabolism; the

former increased glutamic-pyruvic transaminase activity only in the liver, while the latter depressed fructose-6-phosphate-glutamine transamidase activity only in connective tissue. These findings indicate the need for direct study of connective tissue metabolism.

It has been assumed that gold compounds exert their effects on patients with rheumatoid arthritis through inhibition of enzymes with labile sulfhydryl groups (13). Evidence has been obtained in animals that the effects of gold compounds used therapeutically are due to the gold content of these agents and not to the rest of the molecule, regardless of the type of linkage involved (14, 15). The need to protect the enzyme which synthesizes glucosamine-6-phosphate from sulfhydryl inhibitors *in vitro* led to assay of gold compounds on the activity of this enzyme. Concentrations of gold which caused inhibition in connective tissue homogenates were of the order of magnitude found in the serum of patients receiving gold compounds for therapy of rheumatoid arthritis (16).

SUMMARY

The transamidase, which synthesizes glucosamine-6-phosphate, and the glutamic-oxaloacetic and glutamic-pyruvic transaminases were studied in connective tissue and liver of rats, and in human synovial tissue. Cortisol was found to cause a moderate diminution in the activity of the fructose-6-phosphate-glutamine transamidase, but caused an increase in the glutamic-oxaloacetic transaminase activity of connective tissue homogenates. No change in the glutamic-pyruvic transaminase activity occurred. In the liver, cortisol administration caused a moderate decrease in fructose-6-phosphate-glutamine transamidase activity and marked increase in glutamic-oxaloacetic and glutamic-pyruvic transaminase activities. Gold chloride inhibited the activity of the transamidase which synthesizes glucosamine-6-phosphate in connective tissue homogenates *in vitro* in concentrations which are found in the serum of patients receiving gold compounds therapeutically. Gold sodium thiomalate administration *in vivo* resulted in decrease in the activity of this enzyme in connective tissue but not in liver.

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