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Effect of Anti-inflammatory Drugs on Glucosamine-6-phosphate Synthetase from Inflamed Tissue of Rats[1)]

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1) The *in vitro* synthesis of glucosamine-6-phosphate (Gm-6-P) was inhibited effectively by adding aurothiomalate and acid non-steroidal anti-inflammatory drugs to the incubation mixtures. The drug-action was more sensitive in the reaction with the supernatant fluid of the croton oil-induced granulation tissue homogenate using as a source of Gm-6-P synthetase, as compared to the case of the liver homogenate. Potency of each compound was in the following descending order; aurothiomalate, flufenamic and mefenamic acids, phenylbutazone and salicylic acid. Chloroquine and aminopyrine had no effect on Gm-6-P synthesis with the enzymes from both sources at 10^{-3}M.

2) A markedly high activity of Gm-6-P synthetase was found in the swollen hind paws of the rats which were suffered from adjuvant disease. pH-difference was seen in the maximum production of Gm-6-P between the inflamed tissue and liver enzymes. The optimum pH was 6.8 for the reaction with the homogenates of the swollen paws and croton oil-induced granulation tissue, while that for the liver enzyme was in the range from 7.7 to 8.3.

3) Good correlation was seen between increases of the enzyme activity and the swelling in the hind paws as adjuvant disease appeared and developed in rats. On the other hand, the activity of the liver enzyme was not changed significantly by the development of the typical inflammatory lesions.

4) The drug-action to Gm-6-P synthesis with the paw enzyme was much clearer in the reaction at pH 6.8 than pH 7.7.

Synthesis of glucosamine-6-phosphate (Gm-6-P) is an initial step of biosynthesis of the mucopolysaccharides containing glucosamine and galactosamine. The reaction is starting with D-fructose-6-phosphate (D-F-6-P) and L-glutamine. Gosh, *et al.*[3)] stated that this transamination reaction is catalyzed irreversibly by an enzyme, L-glutamine-D-F-6-P aminotransferase EC.2.6.1.16 (Gm-6-P- synthetase).

A high activity of Gm-6-P synthetase is distributed in mammalian livers as well as in several kinds of microorganisms.[3,4)] The activity of the enzyme is also noted in the connective tissues, such as mucous-secreting epithelium,[5)] epiphyseal cartilage,[6)] aortic and pulmonary valves[7)] and human synovial tissue.[8)]

Bollet and Shuster[8)] showed that a sponge-induced granulation tissue of rats had a high activity of the enzyme as much as the liver, and suggested that the enzyme from the inflamed tissue might be more sensitive to some anti-inflammatory drugs than that from the liver. Thereafter, Schönhöfer and Anspach[9)] obtained a similar data using a carrageenin-induced granulation tissue of rats.

The present study confirmed the above authors' finding[8,9)] using the intact liver and croton oil-induced granulation tissue of rats. The distribution study showed a highest activity of

1) The 89th Annual Meeting of the Pharmaceutical Society of Japan, Nagoya, April 1968.
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Gm-6-P synthetase in the hind paw of the rats suffered from so-called adjuvant disease. Thus, determination of the optimum pH of the enzyme, progressive change of the enzyme activities as the elapsing time after the adjuvant injection and effects of some anti-inflammatory drugs on the enzyme activity *in vitro* were investigated.

Material and Method

Materials——Flufenamic and mefenamic acids were the same as those used in the previous work.[10] Salicylic acid, aminopyrine and chloroquine were of J.P. grade. They were used after re-crystallization. Phenylbutazone, aurothiomalate and prednisolone hemisuccinate were of commercial products. Barium D-F-6-P was purchased from Sigma Chemicals. The sodium salt was obtained by stoichiometric addition of sodium sulfate to the barium salt solution and removal of the resulting precipitate before use. L-Glutamine and disodium ethylenediamine tetraacetate (EDTA) were purchased from Wako Pure Chemical Industries. *p*-Dimethylaminobenzaldehyde (Kokusan Chemical Works) was re-crystallized by ethanol and stored in the dark.

Animal Experiments——The granulation tissue was induced in male Wistar rats weighing 150—200 g by the method of Selye[11]: 0.5 ml of the olive oil solution containing 1% croton oil (Tokyo Kasei Co.) were injected into the air sac which was formed on the back of animals by subcutaneously injecting 25 ml of air. Seven days after the injection, every rat was sacrificed by decapitation and the resulting granulation tissue was taken out for enzyme assay. Adjuvant disease[12] was induced in female Sprague Dawley rats weighing 130—150 g by subcutaneously injecting 0.1 ml of the mineral oil suspension containing 0.6 mg of dead mycobacteria (human Aoyama B strain) in the tail. The adjuvant composition and route of injection were the same as described previously.[13] The animals which developed fully swelling in the hind paw were chosen and killed by decapitation 21 days after the adjuvant injection. The paws were cut off from the tibia at the point of the ankle. The skin and fingers were removed and the remainder was used for the subsequent homogenization.

Tissue Homogenization——All the following operation were kept in the cold (0—4°). After shortly rinsed, one volume of the tissue sample was homogenized with two volumes of the solution containing 0.154M KCl, 0.024M L-glutamine, 0.001M EDTA and 0.065% mercaptoethanol. The croton oil–induced granulation tissue and other organs, liver, lung, kidney, small intestine, heart, diaphragm and femoral muscle, were homogenized by a glass homogenizer with teflon or loosely fitting glass pestle at low speed for 3 min. On the other hand, the hind paws were cut into pieces and homogenized by a Vir Tis 45 Macrohomogenizer at high speed for 4 min. The supernatant was obtained from each tissue homogenate by a 30000 g centrifugation for 30 min, using a Hitachi Ultracentrifuge.

Enzyme Assay——According to the procedure of Pogell and Gryder,[14] 0.5 ml of the supernatant, as the enzyme solution, was added to 2 ml of the solution containing 25 μ moles of D-F-6-P, 30 μ moles of L-glutamine, 2.5 μ moles of EDTA and 125 μ moles of phosphate buffer. For inhibition test, anti–inflammatory drugs were added to this reaction mixture to make final concentrations of 10^{-3} and 10^{-4}M, unless otherwise indicated. The mixture was then incubated at 37° for 60 min. The reaction was stopped by heating the mixture in a boiling water bath for 2 min. After cooling and centrifuging, the resulting clear supernatant was used for Gm-6-P assay slightly modifying the method of Gosh, *et al.*[9] The zero time sample was carried out in every experiment. N content of the enzyme solution was determined by the Kjeldhal's micro–method.

Result

Effect of Some Compounds on Liver and Granulation Tissue Enzymes

In this test, the enzyme reaction was carried out at pH 7.7, the optimum pH for Gm-6-P synthetase action which was described in the literature.[3,8,9,14] The typical results are shown in Table I.

A distinguished inhibition of Gm-6-P synthetase activity was seen by adding aurothiomalate to the reaction mixtures containing both liver and granulation tissue homogenates. A complete inhibition was obtained at the 10^{-4}M concentration of this compound. Flufenamic

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Table I. Effect of Anti-inflammatory Drugs on Gm-6-P Synthetase from Rat Liver and Croton Oil-induced Granulation Tissue

Drug	Final concentration (M)	Gm-6-P formed	
		Liver (%)	Granulation tissue (%)
Aurothiomalate	10^{-4}	0	0
	10^{-5}	76	60
Chloroquine	10^{-3}	105	135
Aminopyrine	10^{-3}	105	100
Salicylate	5×10^{-2}	34	0
	10^{-2}	113	93
	10^{-3}	113	123
Flufenamic acid	10^{-3}	13	0
	10^{-4}	100	80
Mefenamic acid	10^{-3}	80	26
Phenylbutazone	10^{-3}	96	65
Prednisolone	10^{-3}	91	88

The control values were fixed arbitrarily at 100% and the individual averages were obtained from three determinations. Incubation pH was fixed at pH 7.7 in both instances and the reaction was performed at 37° for 60 min.

acid was mostly enough for the complete inhibition at 10^{-3}M. A difference of the inhibition against the enzyme activity was seen at 10^{-4}M; the liver enzyme was not inhibited at this concentration of flufenamic acid, while the granulation tissue sample was inhibited slightly. At 10^{-3}M, mefenamic acid and phenylbutazone showed larger inhibition on the enzyme extracted from the granulation tissue than from the liver. Salicylic acid inhibited largely both enzyme samples only at a considerably high concentration. No inhibition was obtained by chloroquine and aminopyrine at 10^{-3}M. Prednisolone inhibited slightly the activity of Gm-6-P synthetase in both tissue samples at 10^{-3}M.

Distribution of the Enzyme Activity in Several Organs and Tissues of Rats

Gm-6-P synthetase activity was determined in the homogenates of several rat organs and tissues. As seen in Table II, high activity of the enzyme was found in liver following lung and small intestine. The other organs and tissues, kidney and muscular tissues, showed no enzyme activity. Croton oil–induced granulation tissue showed a high enzyme activity similar to that of liver tissue. However, the highest specific activity of Gm-6-P synthetase, 3.5 times higher than that of liver, was found in the swollen paws of rats suffered from adjuvant disease.

Effect of pH on Gm-6-P Synthesis

Fig. 1 shows a difference of the enzyme activity between liver and inflamed tissues amples by pH-variation.

The optimum pH of Gm-6-P synthesis was found for the liver enzyme in the range from pH 7.7 to 8.3, while that was shown at pH 6.8 for both inflamed tissue samples, granulaion tissue and swollen paw.

Change of the Enzyme Activity in the Liver and Hind Paw of Rats as the Elapsing Time after Adjuvant Injection

Adjuvant disease has characteristic of the development of inflammatory lesions in the joints, ears, eyes or skin of rats approximately 2 weeks after the injection of a mycobacterial suspension in mineral oil.[12] The most striking feature of the disease is intense swelling of the hind paws.

Gm-6-P synthetase activity in the hind paw, as compared with the change in paw volume, was determined at arbitrary intervals after the injection of adjuvant. Good correlation

Table II. Localization of Gm-6-P Synthetase Activity in Rat Organs and Tissues

Organ and tissue	Gm-6-P formed[a] (μmoles/h/mg N ±S.E.)	
Liver	0.063±0.005	(9)[b]
Lung	0.049	(3)
Intestine	0.041	(3)
Kidney	0.003	(3)
Diaphragm	0.004	(3)
Heart	0	(3)
Muscle[c]	0	(3)
Granulation tissue[d]	0.066±0.009	(6)
Swollen paw[e]	0.239±0.011	(5)

a) by incubation at 37° for 90 min (pH 7.7)
b) number of determinations
c) femoral
d) produced on the back of rats by injecting 0.5 ml of 0.1% croton oil in olive oil 7 days before.
e) from the rats given adjuvant 21 days before

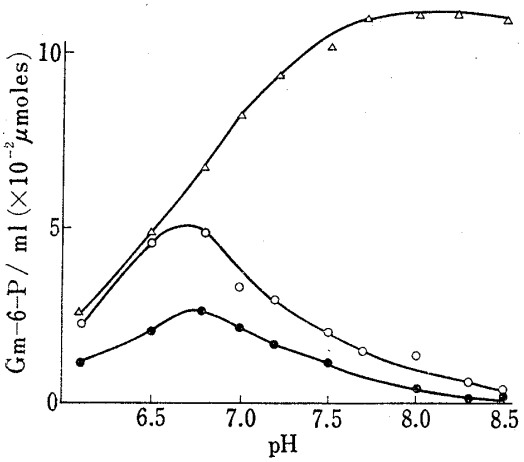

Fig. 1. Effect of Incubation pH on Gm-6-P Synthesis in Rat Liver and Inflamed Tissue Homogenates

Incubation was carried out at 37° for 60 min. Blank was treated with each zero time incubation mixture.
intact liver (△), swollen paw (○), granulation tissue (●)

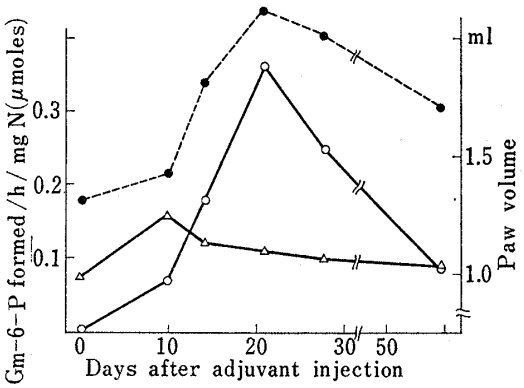

Fig. 2. Activity of Gm-6-P Synthetase in the Liver and Hind Paw, and Swelling in the Paw of Rats after Adjuvant Injection

Paw enzyme was incubated at pH 6.8, and liver enzyme at pH 7.7. Gm-6-P was determined after the incubation at 37° for 60 min. Each point was obtained from three to five determinations. Average paw volume was that of the rats used for enzyme assay.
paw volume (●), liver enzyme (△), paw enzyme (○)

was seen between increases of the enzyme activity and the swelling in the hind paws. The maximum activity of Gm-6-P synthetase was shown at 21 days after the injection of adjuvant, following its relatively rapid fall.

In the livers of these rats, on the other hand, no characteristic was seen regarding to Gm-6-P synthetase activity as the elapsing time after the treatment.

Effect of Some Compounds on Paw Enzyme *in Vitro*

The swollen paws 21 days after the injection of adjuvant were used for evaluating effects of some anti-inflammatory and anti-rheumatic chemicals on Gm-6-P synthetase activity at pH 7.7 or 6.8. Data are shown in Table III.

Inhibition of the enzyme activity with drugs was more sensitive in the incubation mixture at pH 6.8 rather than pH 7.7. Aurothiomalate showed markedly high inhibition to the enzyme activity. Chloroquine and aminopyrine did not inhibit but promoted actually Gm-6-P

Table III. Effect of Anti-inflammatory Drugs on Gm-6-P Synthetase from Swollen Paw of Rats Affected with Adjuvant Disease

Drug	Final concentration (M)	Gm-6-P formed at[a] pH 6.8	pH 7.7
Aurothimalate	10^{-4}	2±1.4	8±3.5
	10^{-5}	53±1.7	68±1.9
Chloroquine	10^{-3}	141±4.4	172±6.2
	10^{-4}	110±5.2	131±5.0
Aminopyrine	10^{-3}	92±1.7	106±5.5
Flufenamic	10^{-3}	—[b]	6±4.0
acid	10^{-4}	—	73±2.6
Phenylbutazone	10^{-3}	—	62±7.0
Salicylic acid	10^{-3}	96±1.9	95±6.7
Prednisolone	10^{-3}	80±3.4	82±7.7

a) Values were percentages of synthetizing Gm-6-P containing drug with control; five determinations (±S.E.).
b) deleted because of the insolubility at this pH
Swollen hind paws were obtained from rats given adjuvant 21 days before and homogenates were prepared as indicated in the text.

synthesis at 10^{-3}M, at which concentration the presence of these compounds showed no interference with the color reaction of Gm-6-P. If the reaction mixture was adjusted at pH 6.8, instead of pH 7.7, so much promotion of Gm-6-P production was not found by chloroquine and aminopyrine. As to the mode of action of salicylic acid and prednisolone, it seems to be no correlation of a pH-variation to the produced amounts of Gm-6-P. Flufenamic acid and phenylbutazone gave clear inhibition against Gm-6-P synthesis in the incubation mixture at pH 7.7. However, a difficulty was seen to make a homogeneous solution of these compounds at pH 6.8, and their data shoud be, then, deleted.

Discussion

In general, Gm-6-P synthetase activity is determined readily on the supernatant fluid of tissue homogenates, mainly due to the extreme instability of the enzyme. Kornfeld[15] succeeded to concentrate approximately 100 times the enzyme activity from rat liver homogenate by using DEAE-cellulose chromatography, with a 50% loss of the initial activity during the purification procedure. The liver and connective tissue show a high activity of Gm-6-P synthetase and the optimum pH of the enzyme is believed to be pH 7.7.

The present study demonstrates that inflammation causes a marked increase in activity of Gm-6-P synthetase in the connective tissue, but not in the liver, and makes clear that the optimum pH of the connective tissue enzyme is actually more acidic than that of the liver. The latter finding is of much interest regarding to the fact that a progressive and dynamic phenomenon of inflammatory reaction proceeds in its initial stage at an alkaline pH but subsequently becomes acid in character.[16] Granuloma formation is pathologically in the late phase of inflammatory processes.

The acidic drugs tested here, flufenamic and mefenamic acids, and phenylbutazone, inhibited more largely the enzyme activity in the inflamed connective tissue than in the liver. Winter[17] demonstrated that all these compounds were effective in the cotton pellet-granuloma

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test in rats. The acidic drugs seem to exist in the inflamed tissue as a molecular from which is probably more suitable for drug–action than an ionic one.

On the other hand, the basic drugs, chloroquine and aminopyrine, had no effect on the activity of the enzymes from both the inflamed tissue and liver at 10^{-3}M. Jacobsen and Boström[7] reported that a large quantity of chloroquine inhibited the enzymes from the aortic and pulmonary–valves of calves and cows, and thus made an assumption that this may perhaps be correlated with the slow clinical action of this compound. However, the concentration of chloroquine used in their work is practically too high. It is said that the inhibitory effect of chloroquine on granuloma formation is doubtfull.[18] The *in vivo* action of chloroquine may be due to the suppression of overall cellular activities in inflamed tissue.[19]

Bollet and Shuster[8] reported that gold chloride inhibited considerably Gm-6-P synthesis *in vitro*. The present study showed that aurothiomalate, an organic compound of gold, had a marked inhibitory effect on the enzyme activity *in vitro*. These results appears to support the view that the effect of gold compounds used therapeutically is mainly due to the gold content of these agents and not to the rest of the molecules, regardless of the type of linkage involved.[20]

Although suppression of granuloma formation is the most well-known activity of anti–inflammatory steroids,[21] prednisolone hemisuccinate had only a slight inhibitory effect on Gm-6-P synthesis *in vitro*, even at a relatively high concentration. Bollet and Shuster[8] described that the repeated administration of hydrocortisone caused a marked inhibition of Gm-6-P synthetase in the sponge-induced granulation tissue of rats. Steroids may act possibly at early stages of inflammation, plasma exudation and cell infiltration.

Since Gm-6-P synthesis is a limiting step of the biosynthesis of mucopolysaccharides, the inhibition of Gm-6-P synthetase with drugs appears of very much importance to connective tissue metabolism in inflammation.

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