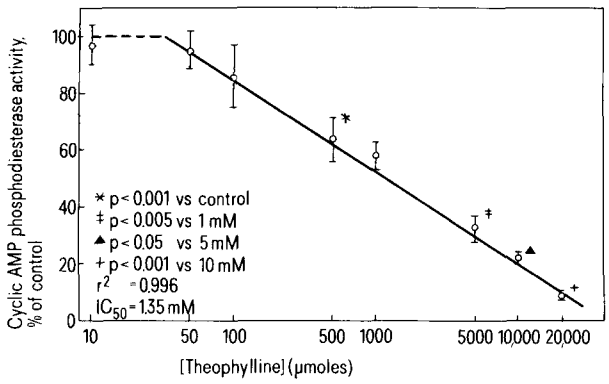
fly, 57.5 μM [^{3}H] cyclic AMP was hydrolyzed by enzyme preparations in a buffer containing 50 mM Hepes-KOH, pH 7.5, 152 mM NaCl, 4.7 mM KCl, 2.8 mM $CaCl_2$, 100 μM ascorbic acid. Additional agents were incorporated as noted, the mixture was incubated at 30 °C for 15 min, and hydrolysis terminated by boiling for 5 min. Reaction products were separated by ascending paper chromatography, visualized by UV, and counted by liquid scintillation. Protein was assayed by the method of Lowry et al.[12] using bovine serum albumin as standard.

Results and discussion. Theophylline is the most potent methyl xanthine inhibitor of *M. sexta* nerve cord PDE[7]. The dose-response of PDE to varying concentrations of theophylline was studied as a model of PDE inhibition. Such inhibition in activity becomes significant at 0.5 mM theophylline and is half-maximal at 1.35 mM (IC_{50}, figure).

Of 11 putative neurotransmitters tested, only dopamine had any significant effect on PDE activity ($p < 0.001$, table). During the course of the dopamine incubation, it was noted that a black precipitate was formed in the tube. Because insect hemolymph may contain high levels of phenol oxidase activity which may convert dopamine to a reactive quinone, resulting in protein cross-linking and subsequent enzyme inhibition, the experiment was repeated employing a phenol oxidase inhibitor, phenyl thiourea[13]. In the presence of this inhibitor, dopamine has no significant effect on PDE activity.

Serotonin elevates cyclic AMP levels in intact nerve cords[4], in neuronal-enriched cellular fractions[5], and stimulates adenylate cyclase activity in crude nerve cord homogenates[6]. Acetylcholine, aspartic and glutamic acids, glycine, and gamma-aminobutyric acid elevate cyclic guanosine 3′,5′-monophosphate levels in intact nerve cords[4]. The work presented here is consistent with the hypothesis that the action of several putative neurotransmitters known to occur in the *M. sexta* CNS[4] may be mediated by the activation of appropriate nucleotide cyclases resulting in elevations of cyclic nucleotide levels.



Inhibition of cyclic AMP phosphodiesterase activity by various concentrations of theophylline. Points represent the means ± SE of 6 different determinations. The line was drawn by linear regression analysis (coefficient of variation = 0.996). Statistical significance was assessed using Student's t-test.

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Selective and reversible modification of essential thiol groups of D-glyceraldehyde-3-phosphate dehydrogenase by isothiocyanates

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Summary. Aralkyl and alkyl isothiocyanates, like aryl isothiocyanates, undergo a selective and reversible reaction with essential thiol groups of D-glyceraldehyde-3-phosphate dehydrogenase; the former 2 substances require for a reversible reaction course a more alkaline medium and presence of a thiol.

Thiol enzymes are of special interest in many biochemical processes, and therefore they are suitable model substances in molecular enzymology[2,3]. To study these substances by chemical modification methods, selective thiol reagents are often used. An especially favourable feature of these reagents is the reversibility of reactions with thiol groups. So far, there are many selective thiol reagents; nevertheless, only a few of them satisfy the 2nd criterion[4,5].

These features have been stressed in our preceding model experiments with isothiocyanates (ITC)[6–8], which are believed to be the natural regulators of activity of some plant thiol enzymes[8]. This paper is aimed to verify the afore-mentioned favourable features of these substances with D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a notable thiol enzyme. Rabbit muscle GAPDH is a well characterized tetramer with 4 essential thiol groups ascribed Cys-149[9].

Material and methods. Activity of rabbit muscle D-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) (Biochemica Boehringer GmbH, Mannheim) was determined ac-

cording to Furfine and Velick[10] using values $A^{1\%}_{1\,cm} = 8.15$ at 280 nm[10] and mol. wt = 144,000[11]. Isothiocyanates were synthesized by the thiophosgene procedure[12], 4-bromophenyl [^{35}S] isothiocyanate (3.4 Ci/mole) by an isotope exchange reaction with elemental sulphur [^{35}S] (252 Ci/mole, ÚVVVR Prague)[13]. The degree of modification of the protein was determined by paper radiochromatography[7].

Inhibitory power of the thiol reagents was expressed in I_{50} values i.e. in log of the inhibitor concentration bringing about 50% inhibition. The I_{50} values were estimated from activity measurements of the enzyme after a 60-min incubation with ITC or a 10-min incubation with other thiol reagents. Samples (5 µl) for activity measurements were withdrawn from incubation mixtures containing 100 mM triethylamine buffer (TEA) pH 7.6 solution, 5 µM GAPDH, ITC or a thiol reagent in 6 concentrations and 1% (v/v) ethanol and kept at 25°C. Kinetic measurements of reactions with glutathione were carried out at 25°C according to Drobnica et al.[12].

Results. Although ITC are not as reactive towards thiols as e.g. 5,5′-dithio-bis-(2-nitrobenzoic acid) (VI) or 4-hydroxymercuribenzoic acid (VII), the inhibitor effect of some of them (IV) is quite close to them (table). A lower effect of some further ITC is associated with the reaction reversibility with the essential thiol groups of the enzyme. This is the case for aromatic ITC, namely phenyl (I) and 4-bromophenyl isothiocyanate (II) which should, considering their higher reactivity, even overcome aralkyl (III, IV) and alkyl isothiocyanates (V).

The selectivity of reaction of ITC with thiol groups of GAPDH was checked at pH 7 and ITC/GAPDH concentration ratio 1/10. Thus, at a concentration ratio 10, 2.3 amino acid residues were modified at the end of the reaction; a 55% inhibition of the enzyme was attained. Extrapolation of the relationship shows that modification of 4.4 amino acid residues would result in a 100% inhibition (figure 1). Since each subunit identical in primary structure has 1 essential thiol group (Cys-149), extrapolation to 4 SH-groups indicates equivalents of the subunits.

It was possible to verify, respecting the direct relation between the degree of modification of essential thiol groups and catalytic activity of GAPDH, the reversibility of modification by examination the enzyme activity. The fully inhibited enzyme by ITC V at pH 7.6 could be reactivated even after 60 min incubation with 10 mM dithiothreitol at pH 10.5 (figure 2). The reactivation degree depends not only on the analytical concentration of the thiol, but also on the concentration of its dissociated form (RS^-) at a chosen pH. Whereas the 0.1 mM thiol at pH 10.5 was not associated with reactivation of the enzyme, 1 mM thiol at the same pH resulted in a 15% reactivation.

Discussion. The influence of concentration of the incorporated aryl [^{35}S] ITC on the GAPDH activity was investigated at pH 7. About 2% of the analytical concentration of the product, i.e. N-(4-bromophenyl) [^{35}S] dithiocarbamyl GAPDH should be at this pH in a dissociated i.e. a decayed form. This follows from both determination of the pK_a value (~9) of N-phenyldithiocarbamyl glutathione and kinetics of decomposition of this and other S-esters of N-monosubstituted dithiocarbamic acids[6]. Therefore, the equilibrium shift of the reversible reaction towards the product required higher ratios of ITC/GAPDH concentration. Even at the concentration ratio ITC/GAPDH 10, the concentration of 4-bromophenyl isothiocyanate (71.5 µM) was, however, higher than its water-solubility ($S_{H_2O} = 53.7$ µM) (Drobnica and Gemeiner[7]) and consequently, further increase of the ITC concentration did not result in the required equilibrium shift of the reversible reaction. On the other hand, N-alkyl or N-aralkyl S-esters of dithiocarbamic acids as reaction products of alkyl or aralkyl ITC with aliphatic thiols or thiol groups of enzymes are still not dissociated in the given pH region (~9) ($11.5 < pK_a < 12$) and consequently, are stable enough. The pH at reactivation of the inhibited enzyme by alkyl ITC V at pH 7.6 should be shifted to a more alkaline region (pH 10.5) and thiol of equimolar concentration of ITC has to be used. Incubation of GAPDH with aryl ITC II at pH 5.5 results, after a period of rapid inhibition, in a spontaneous reactivation without addition of a thiol.

At the time being, the view is held that GAPDH involves 4 identical polypeptide chains forming an asymmetric dimer (aa_1) in which the conformation of the 2 subunits is not identical and the tetramer is a dimer of asymmetric dimers $(aa_1)_2$ (Harris and Waters[9]). The apparent catalytic equivalence of subunits at rabbit muscle holo- and apo-GAPDH was indicated by the preceding results with iodoacetic acid,

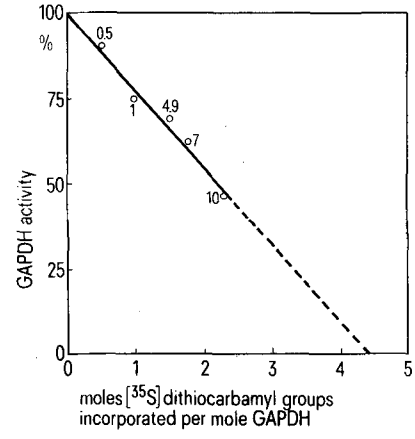

Fig. 1. Relationship between catalytic activity of D-glyceraldehyde-3-phosphate dehydrogenase and concentration of 4-bromophenyl [^{35}S] isothiocyanate incorporated into the protein. Numerical values in the figure relate to the concentration ratio isothiocyanate/enzyme. 100 mM phosphate buffer pH 7, 7.15 µM GAPDH, 3.6–71.5 µM [^{35}S] ITC (5.45 µCi) and ethanol (1% v/v), 15–120 min at 25°C.

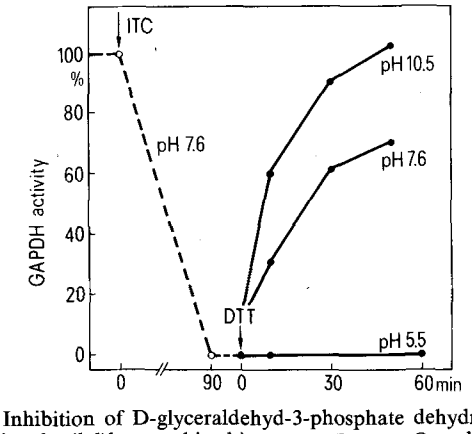

Fig. 2. Inhibition of D-glyceraldehyd-3-phosphate dehydrogenase by 4-(trimethysilyl)butanoyl isothiocyanate O – – – O and its reactivation with dithiothreitol ●——●. To reactivate GAPDH in the reaction mixture containing 100 mM TEA pH 7.6, 5 µM GAPDH, 10 mM 4-(trimethylsilyl)butanoyl isothiocyanate, 5 mM EDTA, 1% (v/v) ethanol and being kept at 25°C, the same volume of 100 mM buffer solution containing 0.1–10.0 mM dithiothreitol (DTT) was added after a complete inhibition. For this operation citrate-phosphate pH 5.5, TEA pH 7.6 and phosphate pH 10.5 buffers were employed.

iodoacetamide, N-ethylmaleimide, N-(4-dimethylamino-3,5-dinitrophenyl)maleimide, 5,5′-dithio-bis(2-nitrobenzoic acid)[14–16] and methyl methanethiosulfonate[17]. The all-of-the-sites reactivity of some of the reagents mentioned (iodoacetic acid, iodoacetamide) remained both for holo- and apo-GAPDH[14–16]. As indicated by our results, 4-bromophenyl isothiocyanate should belong to this group of reagent involving a non-cooperative fashion. The site of action of this group of reagents is localized at the nicotinamide subsite, whereas reagents showing the anticipated half-of-the-sites reactivity (cooperative fashion) towards apo-GAPDH have their site of action localized to the adenine subsite[15].

The above-mentioned properties of ITC are kept also by water-insoluble polyisothiocyanates, this being evaluated not only for immobilization of thiol enzymes[18] but also in covalent chromatography of thiols[19] and thiol enzymes[20].

Inhibitory effect of isothiocyanates and some further thiol reagents on D-glyceraldehyde-3-phosphate dehydrogenase and their reactivity towards glutathione

Number	Compound	I_{50} (μM)	$\log k^*_{SH}$	Type of reaction***
I	C_6H_5NCS	300	2.98	Ad_N
II	BrC_6H_4NCS	180	3.14	Ad_N
III	$C_6H_5CH_2NCS$	150	2.52	Ad_N
IV	$BrC_6H_4CH_2NCS$	20	2.55	Ad_N
V	$(CH_3)_3SiO_2C(CH_2)_3NCS$	90	2.95	Ad_N
VI	$[HOOCC_6H_3(NO_2)S]_2$	10	4.82**	Red-ox
VII	$HOHgC_6H_4COONa$	20	8.60**	S_R
VIII	ICH_2COOH	40	−0.01**	S_N

* Rate constants $k_{SH}(M^{-1}s^{-1})$ of reactions with glutathione; ** As reported in Jocelyn[2] and Friedman[3]; *** Ad_N nucleophilic addition, Red-ox: reduction-oxidation, S_R: radical substitution, S_N: nucleophilic substitution.

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Effect of clofibrate on the phospholipid biosynthesis in rat liver

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Summary. The effect of clofibrate on rat liver phospholipid biosynthesis was studied using ^{32}P as a precursor. Phospholipid classes, levels and specific radioactivity were evaluated. Significant increases in levels of phosphatidylethanolamine and phosphatidylcholine were found and could account for the observed increase in total phospholipids. Specific activity of phosphatidylserine increased and that of phosphatidylethanolamine decreased. This fact suggests that clofibrate seems to alter the systems engaged in the transformation occurring within the different classes of phospholipids but not the de novo biosynthesis.

The biochemical mechanism(s) of ethyl-p-chlorophenoxy-isobutyrate (CPIB) in lipid metabolism is not fully understood, in spite of the numerous investigations on this subject since the drug was applied as a hypolipidemic agent in mammals[2]. Among the several hypotheses put forward to explain the biochemical mechanism underlying the action of the hypolipidemic drugs, alterations in the synthesis of lipoproteins have been postulated[3].

Because phospholipids are important constituents of lipoproteins and the liver is the main site of formation of lipids, an analysis of the effect of CPIB on the synthesis of liver phospholipids seems to be interesting in order to obtain more data on the alteration of liver mobilization caused by this drug.

Materials and methods. Male Wistar rats (weight 150 g) were kept on a standard laboratory diet. Experimental animals received 150 mg/kg of CPIB by a stomach tube once a day for 2 weeks. Controls were treated similarly