artifact of the acid isolation procedure, a simple alkaline extraction method was next used. A small piece of the intact integument was spotted directly on a thin layer plate and chromatographed with a solvent system of n-propanol/2% ammonium acetate (1:1, v/v). The purple fluorescent substance on the thin layer plate with R_f-value 0.52 was dissolved in 0.1% ammonia water and centrifuged at $3000 \times g$ for 15 min. The supernatant solution was then rechromatographed in various solvent systems. The R_f-values of the compound coincided with those of authentic 7-hydroxybiopterin. This indicates that 7-hydroxybiopterin originates in scorpion fly integument and is not an isolation artifact.

According to Matsumoto[19], 7-hydroxybiopterin is assumed to be a characteristic compound for Cyprinidae. With the report of its occurrence in Salmonidae and with this report of its presence in the scorpion fly, 7-hydroxybiopterin is thus more extensively distributed in nature than previously thought.

Forrest et al.[20] demonstrated that xanthine oxidase/dehydrogenase catalyzes the oxidation of 2-amino-4-hydroxypteridine into isoxanthopterin. However, it was found by means of paper chromatographic analysis that incubations of biopterin and dihydrobiopterin with xanthine oxidase (from cow's milk, Boehringer Mannheim) at pH 7.9 did not result in the formation of either 7-hydroxybiopterin or dihydro-7-hydroxybiopterin. The elucidation of the biosynthetic pathway for this compound and its biological significance in the scorpion fly will be the subject of a future paper.

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Effect of controlled atmospheres on the sorbitol pathway in *Ephestia cautella* (Walker) pupae[1]

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Summary. Exposure of *Ephestia cautella* pupae to hypercarbic atmospheres causes an accumulation of sorbitol in the tissues. This accumulation is maximal at 80% CO_2 and decreases at higher concentrations. The reason for this paradoxical behavior is the inhibition by carbon dioxide of the reduction of glyceraldehyde catalyzed by aldose reductase. This inhibition is competitive and is overcome by accumulation of the substrate. It is suggested that the sorbitol pathway might be a bypass of phosphofructokinase, although its efficiency is questionable.

The enzymes of the sorbitol pathway by which glucose is transformed into fructose via sorbitol have been found in seminal vesicles and placenta of sheep[3,4], in the eye lens[5,6], and in human brain[7]. Various authors have dealt with this pathway in insects: Faulkner[8] found a NADP-dependent polyoldehydrogenase in silkworm hemolymph, Chino[9,10] investigated polyol formation in the silkworm egg during diapause and Kageyama and Ohnishi[11] described the effect of anaerobiosis on the formation of polyols in *Bombyx mori* eggs. This and other stress conditions and their effect on polyol formation in *Callitroga macellaria* eggs have been the subject of investigations by Meyer[12].

We have been investigating the effects of controlled, especially hypoxic and hypercarbic, atmospheres on stored product insects for some time[13,14] in connection with the storage of durable agricultural products under carbon dioxide[15]. The present report deals with studies of these effects on polyol production and the enzymes involved.

Materials and methods. Ephestia cautella pupae were reared according to Navarro and Gonen[16] and treated as described previously[14]. Tissues for metabolite determinations were prepared as described before but were extracted twice with ice-cold perchloric acid (8%), each time at a ratio of 1:5 (tissue weight: acid volume). Glucose and fructose were determined according to Bergmeyer et al.[17] and Bernt and Bergmeyer[18], respectively. Sorbitol was determined according to Bergmeyer et al.[19].

Soluble protein preparations for the determination of enzyme activities were made as follows: Pupae were homogenized in double distilled water (at a ratio of 1:4, w/v) in the

Table 1. Levels of glucose, fructose and sorbitol in *Ephestia cautella* pupal tissue (μM/g insect ± SE)

Gas composition N_2	O_2	CO_2	Glucose	Fructose	Sorbitol
80	20	-	0.348 ± 0.073	0.100 ± 0.030	0
90	10	-	0.233 ± 0.083	0.186 ± 0.038	0.097 ± 0.064
99	1	-	1.273 ± 0.215	0.260 ± 0.054	0.464 ± 0.088
-	20	80	0.535 ± 0.020	0.248 ± 0.130	1.543 ± 0.101
-	10	90	0.908 ± 0.093	0.268 ± 0.060	0.730 ± 0.140

Values given are the mean of 4 determinations.

Table 2. Rates of reduction of sugars with NADPH in the presence or absence of HCO_3^-; in Tris buffer pH 7.5; (0.02 M; 0.004 M Mg^{++})

Substrate	Concentration	HCO_3^- concentration	Relative rate
D-Glyceraldehyde	5 mM	-	100
D-Glyceraldehyde	5 mM	100 mM	67
Dihydroxyacetone	5 mM	-	83
Dihydroxyacetone	5 mM	100 mM	72
D-Glucose	50 mM	-	16
D-Glucose	50 mM	100 mM	17

Table 3. Michaelis constants and maximal velocities of key enzymes of the polyol pathway

Enzyme	Substrate	K_m (M)	V_{max} (μM/min/mg protein)
Aldose reductase	Glyceraldehyde	1.07×10^{-4}	2.42×10^{-3}
Aldose reductase	Glyceraldehyde (0.1 M HCO_3^-)	1.98×10^{-4}	2.30×10^{-3}
Aldose reductase	Glucose	8.24×10^{-2}	1.06×10^{-3}
Aldose reductase	Dihydroxyacetone	5.52×10^{-4}	1.99×10^{-3}
Hexokinase	Glucose	2.66×10^{-4}	3.02×10^{-2}
Hexokinase	Fructose	1.79×10^{-3}	6.33×10^{-3}
Phosphofructoaldolase	Fructose-1-phosphate	-	1.22×10^{-3}
Sorbitol dehydrogenase	Sorbitol	5.59×10^{-2}	5.06×10^{-3}

presence of a crystal of phenylthiourea. The homogenate was centrifuged for 10 min at 0 °C at 2600 × g. The solid was discarded and the supernatant dialyzed for 18 h against distilled water. The dialysate was filtered through Whatman No. 1 and stored at −20 °C until used.

Aldose reductase was determined according to Chino[9], hexokinase according to Bergmeyer[20], phosphofructoaldolase according to Leuthardt and Wolf[21], phosphofructokinase according to Ling et al.[22], and sorbitol dehydrogenase according to King and Mann[23]. Apparent maximal velocities and Michaelis constants were calculated by linear regression using Lineweaver-Burk plots.

Proteins were determined by the method of Lowry et al.[24]. Chemicals and biochemicals were products of Sigma and BDH.

Results. Glucose, fructose and sorbitol levels are given in table 1. There was a significant increase in glucose with increasing hypercarbia and hypoxia, and a sharp increase in sorbitol at 80% CO_2 but a subsequent decrease when CO_2 was raised to 90%.

Table 2 shows the reaction rates, relative to the reduction of glyceraldehyde (= 100) in the presence and absence of bicarbonate. Glyceraldehyde reduction was significantly reduced in the presence of bicarbonate (0.1 M). Dihydroxyacetone reduction was affected less and glucose reduction not at all. From table 3, giving V_{max} and K_m, it can be seen that in the case of glyceraldehyde, the Michaelis constant, but not the maximal velocity, was affected, indicating competitive inhibition by the gas[25] or by bicarbonate ion.

Discussion. The sorbitol pathway can be activated in a number of stress conditions such as heat, cold, anaerobiosis, inhibitors[12], as well as in diapause[11,26]. In the present report we show that hypercarbia is equally able to activate the pathway.

As shown by Chino[10], activation occurs whenever there is a defect in the electron transport system. We have demonstrated such a defect[14] by showing a decrease in the ATP/ADP ratio, which is an indication of this effect[27].

The role of the polyol pathway is obscure[6,28]. Van Heyningen discussed the possibility that it was used to bypass hexokinase. She believed that there was an enzyme producing fructose-6-phosphate which could then enter the glycolysis cycle. However, no such enzyme has ever been described. Fructose is a poor substrate for hexokinase, judging from the Michaelis constant determined (table 3) and is preferentially phosphorylated at carbon 1 by ketohexokinase (EC 2.7.1.3), the K_m of which for fructose is reported to be less than 4×10^{-4} M (Parks et al.[29]). Fructose-1-phosphate, however, is not a substrate for phosphofructokinase and will enter the glycolysis cycle only after it is split to dihydroxyacetone phosphate and glyceraldehyde by means of phosphofructoaldolase. The sorbitol pathway could therefore be an means of by-passing hexokinase and phosphofructokinase to some extent. The K_m of sorbitol dehydrogenase for sorbitol is large enough to make it a regulatory enzyme.

Both hexokinase and phosphofructokinase are subject to reduced activity because of hypercarbia. Hexokinase is blocked by an excess of glucose-6-phosphate[30,31] formed by one of the oxidative enzymes of the pentose-phosphate shunt[32], and phosphofructokinase activity reduced by a lack of ATP[12] occurring in pupae exposed to hypercarbic atmospheres[14]. Assuming a bypass of these 2 enzymes is thus legitimate, although its efficiency is probably low[33].

The maximum of sorbitol at 80% carbon dioxide and its decrease with higher hypercarbia can be explained by the blocking of the aldose reductase-catalyzed reduction of glyceraldehyde by carbon dioxide. Since this inhibition is apparently competitive (see above), it is overcome by an accumulation of substrate. Indeed, the production of glycerol is maximal at 90% CO_2 (Friedlander and Navarro[14]).

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Subcellular distribution and binding of heavy metals in the untreated liver of the squid; comparison with data from the livers of cadmium and silver-exposed rats[1]

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Summary. In natural squid liver, about 30% of the total Cd present was found in the cytosolic fraction. A large portion of this Cd was bound to high molecular weight species (mol. wt > 70,000). In contrast to Cd, about 60% of the total Ag occurred in the cytosolic fraction; Ag was bound mainly to low molecular weight species (mol. wt < 20,000).

It is well known that in mammals a unique Cd-binding protein of low molecular weight, named 'metallothionein'[2,3], occurs in the cytosolic fractions of the livers or kidneys of animals exposed to high concentrations of Cd. Recent studies have indicated that metallothionein-like proteins are also found in marine invertebrates, such as the mussel, *Mytilus edulis*[4,5], and the crab, *Scyllus serratus*[6], exposed to Cd. In our laboratory, high concentrations of heavy metals including Cd have been observed in the liver of the normal squid, *Todarodes pacificus*[7]. Similar observations have been reported by Martin et al.[8] on 3 other species of squid, *Loligo opalescens, Ommastrephes bartrami* and *Symplectoteuthis oualaniensis.* However, to our knowledge, there are no reports on the subcellular distribution and the molecular association of heavy metals in squid liver. In the present study we determined first the natural subcellular distribution of heavy metals in livers from the squid, *Todarodes pacificus.* Subsequently we compared the gel filtration behavior of the heavy metal-containing components of the liver cytosolic fraction of the squid and of the livers of rats exposed to Cd and Ag. The data on heavy metal-binding substances in squid liver are of practical significance from the view point of food sanitation because it is used in preparing 'Ika-no-shiokara', a food which is very popular in Japan.

Materials and methods. Samples. Mature squids, *Todarodes pacificus,* were collected in the Sea of Japan near the Oki Islands. They were frozen immediately after capture and stored at −20 °C or below until used. Female Wistar rats (2–3 months old) weighing 180–220 g were used. The rats were maintained on a commercial laboratory diet but were given a solution of $Cd(NO_3)_2$ and $AgNO_3$ (about 100 µg/ml each of Cd and Ag) as their sole source of drinking water for 7 months prior to killing. Livers of these Cd and Ag-exposed rats were removed immediately after killing and stored frozen at −20 °C or below until used.

Subcellular fractionation of squid liver. After the frozen squid sample was thawed, the liver was removed and immediately homogenized in 4 vols of ice-cold 0.02 M Tris-HCl, pH 8.6, 0.25 M sucrose. In order to isolate subcellular fractions, the liver homogenate was subjected to differential centrifugation at 600×g for 10 min, 10,000×g for 10 min, and 100,000×g for 60 min at 0–4 °C[9]. Each fraction was digested with concentrated HNO_3 and monitored for Cd, Zn, Cu, Fe and Ag in a Nippon Jarrel-Ash Model AA-8500 atomic absorption spectrophotometer, using an air-acetylene flame or a heated graphite furnace.

Gel filtration analysis of the cytosolic fractions of the liver of squid and of Cd and Ag-exposed rats. The cytosolic fractions were prepared from squid liver and Cd and Ag-exposed rat livers by centrifuging at 100,000×g for 60 min as mentioned above. A sample of the cytosolic fraction (3 ml) was applied to a column of Sephadex G-75 (2.6×62 cm), equilibrated with 0.02 M Tris-HCl buffer, pH 8.6, contain-