Identification and Partial Characterization of Inositol:NAD^+ Epimerase and Inosose:NAD(P)H Reductase from the Fat Body of the American Cockroach, *Periplaneta americana* L.†

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ABSTRACT: In a previous study (Candy, D. J. (1967), *Biochem. J. 103*, 666) a locust fat body extract was shown to convert *myo-* to *scyllo*-inositol in a reaction involving NAD^+. The same enzyme preparation reduced *scyllomyo*-inosose (2,4,6/3,5-pentahydroxycyclohexanone) to *scyllo*-inositol, however, with NADPH. This difference in cofactor requirements suggested the presence of more than one enzyme and led us to the study of these systems in the American cockroach, *Periplaneta americana* L. Dialyzed fat body homogenates were the enzyme source. Analysis of the products of the enzyme reactions was by gas chromatography and by gas chromatography–mass spectrometry of the trimethylsilyl ethers. An inositol:NAD^+ epimerase (pH optimum 7.5–8.0) was found which was capable of carrrying out the following inositol conversions: *epi-* → *myo-* ⇄ [*scyllo-* + *chiro-* + *neo-*]. The system epimerizes both (D)- and (L)*chiro*-inositol but not *muco*-inositol. The degree to which the epimerase operates on the inositol substrates correlates with the relative free energy of the inositol products formed. Deuterium-labeled inositols lose label when incubated with the epimerase in H_2O indicating both a degree of loose binding of the reduced cofactor intermediates and the formation of a tightly bound inositol intermediate, presumably an inosose. An inosose reductase (pH optimum 4.2–8.0) that utilizes both NADH and NADPH was also found. Reduction of *scyllomyo*-inosose to *myo*-inositol was favored by NADH and to *scyllo*-inositol by NADPH. Of the other inososes tested *myochiro*-inosose (3,5,6/2,4-pentahydroxycyclohexanone) was reduced with NADPH to *myo*-inositol but not to *chiro*-inositol. *myoepi*-Inosose (2,3,4,6/5-pentahydroxycyclohexanone) was reduced to both *myo-* and *epi*-inositol using NADPH and NADH. With all three inososes NADPH favored reduction to the equatorial hydroxyl product and NADH less specifically directed axial product formation.

Of the nine possible unsubstituted inositols (for structures, see Figure 1) only *myo-*, *scyllo-*, *neo-*, and the D and L forms of *chiro*-inositol have been found to occur naturally. Of these, *myo*-inositol is virtually ubiquitous in nature (Posternak, 1965) as *scyllo*-inositol may be, when sensitive enough methods are used for its detection. Recently *neo*-inositol has been recognized as occurring in mammals (Sherman *et al.*, 1971).

Evidence has been accumulating for some time that *scyllo*-inositol is formed from *myo*-inositol by an epimerization reaction thought to proceed through *scyllomyo*-inosose[1] (Helleu, 1957; Posternak *et al.*, 1963; Scholda *et al.*, 1964; Candy, 1967; Horner and Thaker, 1968). The natural occurrence of *scyllomyo*-inosose in animals has been shown by Sherman *et al.* (1968a,b).

chiro-Inositol has been shown to be formed from *myo*-inositol by *Chlorella fusca* with the intermediacy of *myochiro*-inosose (Wöber and Hoffmann-Ostenhof, 1969; Wöber *et al.*, 1971).

Until this work the only known route to *neo*-inositol was the cyclization of D-mannose 6-phosphate by enzyme preparations from testis (Sherman *et al.*, 1971).

The pyridine nucleotide cofactor requirements of the locust fat body enzymes which converted *myo-* to *scyllo*-inositol and reduced *scyllomyo*-inosose to both *myo-* and *scyllo*-inositols were studied by Candy (1967). In his paper Candy concluded that NAD(H) mediated the interconversion of *myo*-inositol and *scyllomyo*-inosose and that NADP(H) was the cofactor for the interconversion of *scyllomyo*-inosose and *scyllo*-inositol.

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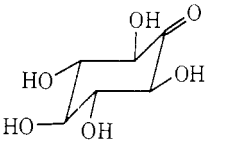
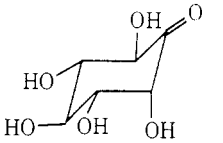
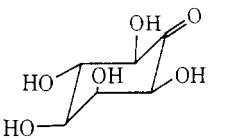
[1] Three pentahydroxycyclohexanones (inososes) are studied here. The IUPAC–IUB Tentative Cyclitol Nomenclature Rules recommend that these be designated by the Maquenne (1900) fractional notation. However, a more descriptive notation after Posternak (1965) allows the reader to more easily identify the inosose under discussion without referring to structures. The names used are given directly below the structures and, below that, the Maquenne nomenclature and one other common name for the inosose. The nomenclature refers to the inositols which would be formed by reduction of the inosose, an equatorial hydroxyl resulting from reduction giving the first-named product, an axial hydroxyl the second-named product.



scyllomyo-inosose
2,4,6/3,5-pentahydroxycyclohexanone
myo-inosose-2



myochiro-inosose
3,5,6/2,4-pentahydroxycyclohexanone
myo-inosose-3



myoepi-inosose
2,3,4,6/5-pentahydroxycyclohexanone
myo-inosose-4(6)

TABLE I: Inositol Levels in Cockroach Tissues.

Inositol	Tissue[a] Hemolymph (μmol/kg wet wt)[b]	Fat Body (μmol/kg wet wt)[b]
myo-	247, 212, 139 [250]	474, 584, 562 [810]
scyllo-[c]	29, 81, 18	355, 170, 200
chiro-	27, 27, 28 [44]	106, 9, 27 [14]
neo-	–, 119, 16 [91]	–, 38, 77 [28]

[a] Pooled tissue from three insects. Hemolymph was collected by severing the third leg at the coxa and expressing the fluid. Fat body was collected after removal of the abdominal cuticle. The pooled tissue was extracted, deproteinized, and lyophilized, and the trimethylsilyl derivatives were prepared. [b] Values for three different extracts chromatographed on 1% SE-30. The first extract was analyzed by GC-MS scanning across the peak and the other two were analyzed by MID. One sample on 3% OV-17 with MID analysis is given in brackets. [c] No data for the scyllo concentrations from GC on OV-17 are given because trimethylsilyl-*scyllo*-inositol is not chromatographically separable from trimethylsilyl-β-glucose on 3% OV-17.

We present here what we believe to be evidence that there are at least two separate enzyme systems for inositol conversions in cockroach fat body: inositol epimerase and inosose reductase. We further show that these enzymes can interconvert *myo-*, *scyllo-*, *chiro-*, and *neo*-inositols as well as catalyze the reduction of inososes.

Materials and Methods

Enzyme Preparation. Fat body was removed from 25 adult American cockroaches, *Periplaneta americana* L. (Ward's Natural Science Establishment, Rochester, N. Y.), and immediately homogenized in a Potter-Elvehjem glass homogenizing vessel containing 0.2 ml of water. After the homogenizing vessel was rinsed nine times with 0.2 ml of water, the combined homogenate and washings were centrifuged at 15,000*g* for 30 min and the inactive pellet was discarded. The supernatant was dialyzed overnight against 2 l. of water and centrifuged at 15,000*g* for 30 min, and the clear supernatant was stored frozen at −18° until used for epimerase and reductase assays. All operations were carried out at 4°. The enzyme activity did not decrease on repeated freezing and thawing. For deuterium-labeling studies the dialyzed fat body extract was lyophilized, then taken up in the original volume of D_2O. After standing in the cold for 45 min the enzyme was lyophilized and taken up again in the original volume of D_2O. The deuterated enzymes were found to have 75% of their original activity.

Enzyme Assay. Substrate (200 μl, 27.9 mM, final concentration 5.58 mM) and Tris buffer (500 μl, pH 8.6, 0.05 M) containing 1.25 mM cofactor (NAD^+, $NADP^+$, NADH, NADPH, FMN, FAD; Sigma Chemical Co., St. Louis, Mo.) were incubated with the enzyme preparation in a total volume of 1 ml for 2 hr at 24°. The reaction was linear for at least 2 hr. The enzymic reaction was stopped by placing the reaction vessel in boiling water for 10 min. Protein was precipitated by the addition of 25 μl of 1 M $ZnSO_4$ and removed by centrifugation. Aliquots (500 μl) of the clear centrifugate were lyophilized and the Me_3Si ethers of the products prepared for gas chromatography by addition of 150 μl of pyridine–*N*,*O*-bis(trimethylsilyl)trifluoroacetamide–trimethylchlorosilane (1:1:0.2, v/v). After shaking for 24 hr the samples were analyzed by gas chromatography on 6 ft × 0.25 in. glass columns packed with 1% SE-30 on 100–120 mesh silanized diatomaceous earth support, helium flow 50 cm^3/min, column temperature 160°, isothermal. Detection was by flame ionization detector except for the smallest samples, when the MID[2] (see below) was used. Peak heights and retention times were compared with weighed standards of authentic inositols gas chromatographed as their Me_3Si ethers. For retention times of the trimethylsilylinositols, see Sherman *et al.* (1971).

Confirmation of the identity of the inositols obtained from the enzyme reactions and tissue extracts was by GC-MS of the Me_3Si ethers (Sherman *et al.*, 1970).

Small quantities of inositols in tissues were determined using the MID in which the computer controlled mass spectrometer is used as a detector for the gas chromatograph. In this method the magnetic field of the mass spectrometer is held constant and the accelerating voltage is controlled by the computer so that selected masses are observed. The computer samples each of the selected masses 352 times/sec, averages these samples, and displays this average each second. By selecting masses that are unique to a particular compound and by utilizing the increased precision of the MID, it is possible to measure trimethylsilylinositols at the level of 10 pg/injected sample, or less. For a complete description of this system, see Holmes *et al.* (1973).

In this paper the trimethylsilylinositol fragments at *m/e* 432, 433, and 507 were chosen as inositol-selective chromatographic detectors because of their close structural relationship to this family of compounds (loss of two Me_3SiOH moieties, loss of Me_3SiOH and $Me_3SiO\cdot$ moieties and loss of CH_3 and Me_3SiOH moieties from the molecular ion respectively; Sherman *et al.*, 1970), and compared with a standard curve from dilutions of authentic trimethylsilylinositols. To our knowledge these ions are not present in any substance eluting with the trimethylsilylinositols in these samples.

Protein was determined by the method of Lowry *et al.* (1951).

Preparation of Substrates. epi-, *muco-*, and *scyllo*-inositols were gifts from S. J. Angyal, University of New South Wales; C. E. Ballou, University of California, Berkeley, Calif.; and L. Anderson, University of Wisconsin, Madison, Wis., respectively. *neo*-Inositol was synthesized (Angyal and Matheson, 1955). *myochiro*-Inosose was synthesized by the Pt/C-catalyzed air oxidation of *chiro*-inositol (Heyns and Paulsen, 1953). *myoepi*-Inosose was synthesized by nitric acid oxidation of *myo*-inositol (Posternak, 1962). *myo*-Inositol, *chiro*-inositol, *scyllomyo*-inosose, D_2O, and deuterium gas were commercial samples. D- and L-*chiro*-inositol were checked for purity by measurement of their optical rotation (specific rotation, literature and found, +64.5 and −64.5°, respectively). Monodeuterated inositols were obtained by Pt-catalyzed reduction of the respective inosose with deuterium gas in D_2O solution. The Me_3Si ethers of the deuterated inositols were then purified by passage through a 1% SE-30 gas chromatographic column fitted with an effluent splitter. The collected derivatives were converted to the free inositols by heating at 80° overnight in 50% methanol. Tris buffer of appropriate initial pH and inositol substrates used with D_2O in labeling studies were

[2] Abbreviations used are: GC-MS, gas chromatography–mass spectrometry; MID, multiple ion detector.

TABLE II: Effect of Different Cofactors on Inositol Epimerase.

	Substrate					
	myo-Inositol		*scyllo*-Inositol		L-*chiro*-Inositol	
	Inositol Product (%)[a]					
Cofactor	*scyllo*-	*chiro*-	*myo*-	*chiro*-	*myo*-	*scyllo*-
NAD^+	100	5	100	4	100	66
$NADP^+$	0[b]	0	0	0	0	0
NAD^+ + NADPH	90	4	87	3	88	57
FMN	0	0	NA[c]	NA	NA	NA
FAD	0	0	NA	NA	NA	NA
NONE	0	0	0	0	0	0

[a] The trimethylsilylinositol products were measured by comparison of the GC peak height (flame detection) with that of authentic weighed standards following the standard epimerase reaction (5.58 mM inositol substrate, 0.625 mM cofactor, 25 mM Tris buffer (pH 8.6), and 100 μl of dialyzed fat body extract containing 72-100 μg protein in a total volume of 1 ml, incubated for 2 hr at 24°). With each substrate the principal product from the reaction using NAD^+ as the cofactor was taken as 100% and all other products were normalized to 100%. [b] 0 is <1.3 μg of product/incubation. [c] Not assayed.

prepared by lyophilization of a solution of the samples twice from D_2O followed by dissolution in sufficient D_2O to give the original concentration.

Inosose in alkaline media was shown to enolize with incorporation of label (tritium or deuterium) (Isbell *et al.*, 1969; unpublished observation, this laboratory); therefore *scyllo-myo*-inosose was enolized in alkaline D_2O to prepare multiply deuterated inosose. The deuterated Me_3Si inosose was shown by GC-MS to be a mixture of non-, mono-, di-, tri-, and tetradeuterated inososes. The per cent deuterium enrichment and the atom per cent deuterium were calculated as described by Biemann (1962) using the silicone isotope cluster at the molecular ion (*m/e* 538–543) for inosose and using the cluster at $M\cdot^+ - 105$ (*m/e* 507–513) for inositols.

Lysozyme Injection of the Cockroach and Bacterial Staining of Fat Body. Adult cockroaches were injected intraabdominally with 0.05 ml of a solution of 2% lysozyme (Sigma Chemical Co., St. Louis, Mo.) in 0.5% NaCl (Wharton and Lola, 1969). After 27 days the cockroaches were injected again and after an additional 27 days portions of the dissected fat bodies of the injected insects were stained for bacteria with Wrights stain (Wharton, 1968).

Results

Identification and Measurement of Fat Body and Hemolymph Inositols. The identification and measurement of *chiro-*, *scyllo-*, and *myo*-inositols in extracts of fat body and hemolymph from the cockroach by GC-MS have been previously reported (Hipps *et al.*, 1972). *neo*-Inositol has now been found as a naturally occurring constituent of the cockroach fat body and hemolymph.

When extracts of fat body and hemolymph were lyophilized and the Me_3Si derivatives examined by GC-MS it was observed that trimethylsilylinositol fragment ions were present at the retention time of trimethylsilyl-*neo*-inositol. On simple gas chromatography trimethylsilyl-*neo*-inositol is obscured by a large quantity of trimethylsilylglucose and trimethylsilylmannose which elute at the same time. This is the situation in mammalian tissues, also (Sherman *et al.*, 1971). By using the MID the levels of *chiro-*, *scyllo-*, and *myo*-inositols have been reexamined and the levels of *neo*-inositol measured (Table I). Some differences were noted in the levels found as compared to those previously reported. We believe this is due to natural variation from insect pool to insect pool which, as can be seen in Table I, is considerable.

Action of NAD^+ and Fat Body Extract on myo-Inositol. Dialyzed fat body extract was incubated with NAD^+ and *myo*-inositol at pH 8.6 and the products were converted to their Me_3Si ethers. Gas chromatography and GC-MS showed that *myo*-inositol had been converted to *scyllo-*, *chiro-*, and *neo*-inositols as well as *scyllomyo*-inosose. Mass spectra as well as gas chromatographic retention times were identical with those of authentic standards. Because of the small amount of *scyllomyo*-inosose produced (rate: 0.9 ng/min, measured by MID using *m/e* 420 and 433), its presence was obscured by coeluting trimethylsilyl-α-glucose and *-neo*-inositol. The characteristic spectrum of trimethylsilyl-*scyllomyo*-inosose (Sherman *et al.*, 1968b) was found in the mixed spectrum along with that of trimethylsilylglucose and *-neo*-inositol. *muco-*, *allo-*, and *epi*-inositols were not observed as enzymic reaction products when *chiro-*, *neo-*, *epi-*, and *muco*-inositols were used as substrates.

Cofactor and Substrate Specificities of Cockroach Inositol Epimerase. Table II shows that NAD^+ is an absolute requirement for the epimerase activity. No activity is found with $NADP^+$, FMN, or FAD and the addition of NADPH to NAD^+ resulted in slight inhibition of the reaction. The addition of 5 mM dithiothreitol to the inositol epimerase reaction, with *myo*-inositol as substrate, resulted in 60% inhibition of the formation of both *chiro-* and *scyllo*-inositols. At the time the data for this table were obtained we had not yet discovered that *neo*-inositol was also formed by the epimerase.

A series of epimerase reactions was then carried out using different inositols as substrates and NAD^+ as the cofactor in order to define the substrate specificity of the epimerase system. The results in Table III suggest that although the enzyme can epimerize a wide range of inositols the rate and specificity of the epimerization are dependent upon the steric and thermodynamic (free energy) properties of both the substrate and the product. Eliel *et al.* (1965) have calculated the free energy of underivatized inositols relative to a hypothetical inositol in which there are no nonbonded interactions. These values are: *myo-*, 3.0; *scyllo-*, 3.2; D-*chiro-*, 4.0; *neo-*, 4.3; *epi-*, 4.9; *allo-*, 5.05; *muco-*, 5.1; and *cis-*, 8.05 kcal/mol at 25°. It can be seen in Table III that the products of a single-

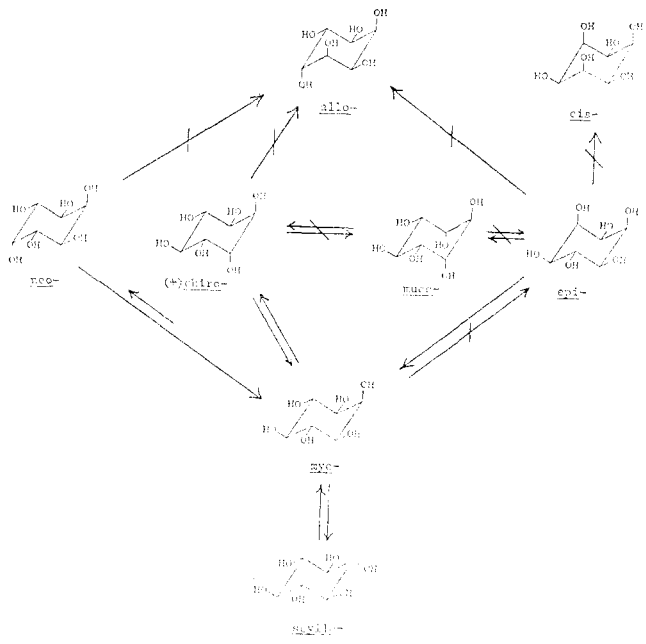


FIGURE 1: NAD^+ dependent inositol epimerization observed with cockroach fat body extract.

center epimerization reaction with *myo*-inositol as substrate follow the free energies of the products such that the formation of *scyllo-* $\gg$ *chiro-* $\simeq$ *neo*-inositol. Also although no *epi*-inositol is formed by the epimerization of the C-4(6)-OH of *myo*-inositol, *epi*-inositol does epimerize to the lower free-energy *myo*-inositol. *muco*-Inositol did not serve as a substrate, *allo-* and *cis*-inositols were not examined, and none of these triaxial hydroxylinositols were formed as products (MID). The fact that the epimerization appears to be an equilibrium reaction to the free-energy level of *epi*-inositol and that *epi*-inositol can epimerize to *myo*-inositol, but not the reverse, suggests that there is an energy barrier between 4.3 and 4.9 kcal per mol which prevents formation of the inositols with higher free energies. The rates of epimerization, however, are not solely related to the free energies (Table III) because D-*chiro*-inositol is epimerized to *myo*-inositol 1.9 times faster than the L isomer, presumably for steric reasons. Figure 1 summarizes the epimerase reactions.

On the assumption that no multiple simultaneous epimerizations are possible by the enzyme it is necessary that conversions of diaxial inositols proceed through *myo*-inositol to produce *scyllo*-inositol and *vice versa*. Thus *chiro*-inositol would be converted first to *myo*-inositol and then *via* a second epimerase reaction to *scyllo*-inositol. In the case of the epimerization of *epi-* to *myo*-inositol, the *scyllo*-inositol which is probably formed is obscured by the large amount of the *epi*-inositol substrate (Me_3Si derivative retention times on SE-30 relative to trimethylsilylglucose of 1.97 and 1.80, respectively). In the case of *neo*-inositol as substrate, the amount of *scyllo*-inositol formed would have been below the detection limit.

In a separate experiment, *trans*-1,2-cyclohexanediol, an analog of *scyllo*-inositol, was not epimerized to the cis isomer using NAD^+. Thus additional hydroxyls are necessary for activity.

Cofactor and Substrate Specificities of Cockroach Inosose Reductase Activity. Table IV shows that either NADH or NADPH are utilized by the reductase. The specificity of the reductase activity to produce an equatorial or axial hydroxyl is variable with respect to the substrate and cofactor; however, general trends are observed. For example, the reduction

TABLE III: Substrate Specificity of Inositol:NAD^+ Epimerase.

Inositol Substrate	Inositol Product (ng/min)[a]			
	myo-	*scyllo-*	*chiro-*	*neo-*
myo-[b]	NA[c]	107[d]	13	21[e]
scyllo-[f]	219	NA	14	0[g]
D-*chiro-*[b]	183[h]	65	NA	0
L-*chiro-*[b]	95[h]	29	NA	0
neo-[i]	53[j]	0	0	0
epi-[k]	130[f]	NA	0	0
muco-[i]	0[l]	0	0	0

[a] All rates in the standard assay (5.58 mM substrate, 0.625 mM cofactor, 25 mM Tris buffer (pH 8.6), and suitable enzyme dilution in a total volume of 1 ml, 2-hr incubation, 24°) are normalized to an epimerase conversion of *myo-* to *scyllo*-inositol equal to 107 ng/min. Detection was by flame except as indicated. [b] Enzyme preparation contained 1.24 mg of protein/ml of incubation. [c] Not assayed. [d] No *epi*-inositol observed to the level $\leq$900 pg/incubation (MID). [e] MID. [f] Contained 0.65 mg of protein/ml of incubation. [g] 0 $\leq$900 pg/ml of incubation (MID). [h] No *muco-* or *allo*-inositol observed to the levels $\leq$900 pg/incubation (MID). [i] Contained 0.65 mg of protein/ml of incubation. [j] Contained no *allo*-inositol to the level $\leq$900 pg/ml of incubation (MID). [k] Contained 5.4 mg of protein/ml of incubation. [l] No *epi*-inositol observed to the level $\leq$900 pg/ml of incubation (MID).

of *scyllomyo*-inosose and *myoepi*-inosose with NADH gives a larger proportion of reduction to the axial hydroxyl than to the equatorial. *myochiro*-Inosose may be an exception in that the axial hydroxyl product, *chiro*-inositol, apparently was not produced. However, it is possible that *myochiro*-inosose is not a substrate in this reaction although it is reduced with NADPH. Although *myo*-inositol was generated in the NADH incubation, the substrate was impure, containing *scyllomyo*-inosose[3] which would also be reduced to *myo*-inositol. That this is the case was shown by the formation of *scyllo*-inositol as a product (not shown in Table IV).

With NADPH as cofactor the reduction of inososes proceeds to give mainly the equatorial hydroxyl products (Table IV). Once again *myochiro*-inosose was exceptional, again producing no axial reduction product, but in this case giving the equatorial hydroxyl product *myo*-inositol at the highest rate observed for any of the reductase products.

We considered the possibility that the ratios of products obtained in the NADH reductase experiments could be affected by the epimerase activity present in the fat body extracts utilizing the NAD^+ produced in the reduction, at alkaline pH. A calculation based on the reciprocal plot of NAD^+ with *myo*-inositol (Figure 3) and the amounts of inositol formed in the reductase experiment (Table IV) showed that less than 5 ng/min of *myo*-inositol would have been epimerized in these experiments, a negligible amount.

[3] We have been unable to carry out the classical Pt/C air oxidation of either *myo-* or *chiro*-inositol to give a single product by gas chromatographic analysis. The principal product is that of the oxidation of an axial hydroxyl; however other inososes are formed in considerable amounts. Thus air oxidation of *chiro*-inositol with Pt/C gave mainly *myochiro*-inosose; however *scyllomyo*-inosose was always present. The pH of these aqueous solution reactions is neutral or acidic, not conditions that promote spontaneous enolization of inososes. This is presently under study (unpublished observation, this laboratory).

TABLE IV: Effect of Different Cofactors on Inosose Reductase Activity.

	Inosose Substrate[a]					
	scyllomyo-[b]		*myoepi-*[c]		*myochiro-*[d]	
	Inositol Product (ng/min)					
Cofactor	*scyllo-*	*myo-*	*myo-*	*epi-*	*myo-*	*chiro-*
NADH	101 (64)[e]	191 (191)[e]	3.6 (2.4)[f]	10 (15)[f]	NA[g]	0[h,i]
NADPH	350 (344)[e]	51 (43)[e]	110 (63)[f]	26 (17)[f]	396[i]	0
NAD^+	0	0	NS[j]	NS	NS	NS
$NADP^+$	0	0	NS	NS	NS	NS
None	0	0	0	0	0	0

[a] Standard 2 hr assay (24°) using the designated inosose (5.58 mM) and cofactor (0.625 mM) in Tris buffer (25 mM pH as described below). [b] Enzyme preparation contained 1.04 mg of protein/ml of incubation. [c] Contained 1.32 mg of protein/ml of incubation. [d] Contained 0.65 mg of protein/ml of incubation. [e] Assay pH 5.0, in parentheses pH 8.0. [f] pH 5.4, in parentheses pH 7.8. [g] Not assayed because the substrate contained 56% *scyllomyo-* and 44% *myochiro-*inosose. The incubation using NADPH contained ≤ 1.3 μg of product from the axial reduction of *scyllomyo-*inosose present in the substrate. [h] 0 ≤ 1.3 μg of product/incubation (flame detection). [i] pH 6.7. [j] Not studied.

In all cases NADPH was used more effectively than NADH. This is in agreement with the finding of Piña and Tatum (1967), who reported a *myoepi-*inosose reductase in *Neurospora crassa* that utilized NADPH 20 times more efficiently than NADH.

The failure of the NADH reductase system to reduce *myo-chiro-*inosose to *chiro-*inositol and the low activity associated with the *myoepi-*inosose NADH reductase system when compared with NAD^+ epimerase activity (Table III *myo-* → *chiro-*inositol and *epi-* → *myo-*inositol) suggest that the reductase activity is not the reductase of an oxidoreductase system, *i.e.*, the reductase is not an epimerase binding the inosose and then reducing the inosose with NADH.

Deuterium-labeling studies (*loc. cit.*) will support this suggestion by showing that if the intermediate is an inosose it remains enzyme bound during the epimerase reaction.

Effect of pH on Epimerase and Reductase Activity. The epimerase is active at neutral and alkaline pH with a sharp optimum pH for the conversion of *myo-*inositol to *scyllo-*inositol between pH 7.5 and 8.0 (Figure 2). At pH 7.0 62% of the epimerase activity remains and no activity was observed at pH 6.0. The conversion of *myo-* to *chiro-*inositol appears to occur maximally at somewhat higher pH. *scyllomyo-*Inosose, when reduced by the inosose reductase with both NADH and NADPH, exhibited a broad pH optimum with more than 80% of the activity remaining at pH 4.2. These curves are undoubtedly biased by the instability of the pyridine nucleotide cofactors (oxidized cofactors being unstable at alkaline pH and reduced cofactors at acidic pH). The different pH optima extend the suggestion that the epimerase and reductase are different enzyme systems.

Similar results were observed by Larner *et al.* (1956) using partially purified extracts of *Aerobacter aerogenes*. They followed *myo-*inositol oxidase activity and *scyllomyo-*inosose reductase activity spectrophotometrically (reduction of NAD^+ and oxidation of NADH respectively) and found the pH optimum for NAD^+ reduction was 9.0 and for NADH oxidation was 7.3–7.7.

There are notable effects of pH on the product ratios obtained in the NADH reductase reactions. In the reduction of *scyllomyo-*inosose the ratio of axial reduction to equatorial reduction (*myo-*inositol/*scyllo-*inositol) increases as the pH is raised: (pH followed by ratio) 4, 1.9; 5, 1.9; 6, 1.9; 7, 2.1; 8, 2.9; 9, 3.3. This is also the case with *myoepi-*inosose (Table IV) where the ratio of axial/equatorial reduction is 2.8 at pH 5.4 and 6.2 at pH 7.8. The pH effect on ratio with NADPH is unchanged within experimental error.

Effect of Lysozyme Treatment of Cockroaches on Inositol Epimerase and Inosose Reductase Activities. Cockroach fat body is known to contain intracellular symbiotic bacteria which we felt could be the source of both the *chiro-*inositol and *chiro-*inositol-forming epimerase activity (as noted earlier, simple plants are capable of producing *chiro-*inositol). Therefore lysozyme injections were used to remove the intracellular bacteria as described by Wharton and Lola (1969). Thirty-

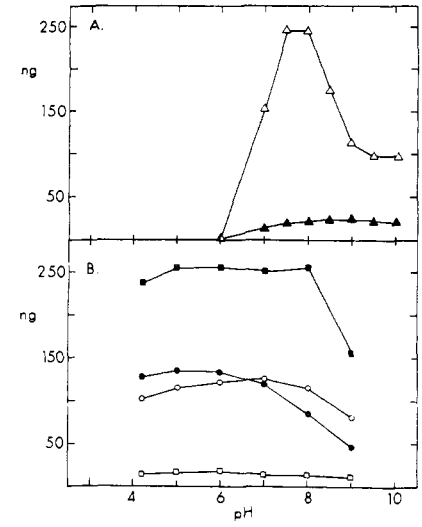


FIGURE 2: The effect of pH on epimerase activity. (A) Epimerase activity—100 ng of product represents a rate of 150 ng/min in the standard assay: 0.625 mM NAD^+ and 5.58 mM *myo-*inositol in 25 mM Tris buffer incubated with dialyzed fat body extract (1.1 mg of protein/incubation) for 2 hr at 24°: (△) *scyllo-*inositol product; (▲) *chiro-*inositol product. (B) Reductase activity—cofactor: 0.625 mM NADH. 100 ng of product represents a rate of 75 ng/min in the standard assay: 5.58 mM *scyllomyo-*inosose in 25 mM Tris buffer incubated with dialyzed fat body extract (1.0 mg of protein/incubation) for 2 hr at 24°: (■) *myo-*inositol product; (●) *scyllo-*inositol product. Cofactor: 0.625 mM NADPH. 100 ng of product represents a rate of 300 ng/min in the standard assay (see NADH): (□) *myo-*inositol product; (○) *scyllo-*inositol product.

TABLE V: K_m and V_{max} Values for Inositol Epimerase.

Variable Substrate	Product: *myo*-Inositol K_m (mM)	*myo*-Inositol V_{max} (μg of Product/min per μl of E)	*scyllo*-Inositol K_m (mM)	*scyllo*-Inositol V_{max} (μg of Product/min per μl of E)	*chiro*-Inositol K_m (mM)	*chiro*-Inositol V_{max} (μg of Product/min per μl of E)
myo-Inositol[a]	NA[b]	NA	29	83	2	8
scyllo-Inositol[a]	25	100	NA	NA	NA	NA
D-*chiro*-Inositol[a]	38	167	NA	NA	NA	NA
NAD^+ [c]	NA	NA	0.48	234	0.42	18

[a] Fixed substrate: NAD^+ (0.625 mM) in 25 mM Tris buffer, pH 8.6; incubation 2 hr at 24°; variable substrate 2.78–16.7 mM; contained 1.2 mg of protein/ml of incubation. [b] Not assayed. [c] Fixed substrate: *myo*-inositol 5.58 mM in 25 mM Tris buffer, pH 8.6, incubation 2 hr at 24°, variable substrate 0.2–5.0 mM, contained 1.08 mg of protein/ml of incubation.

four cockroaches were injected with egg-white lysozyme (mortality 4). After 27 days the surviving cockroaches were injected again (mortality 7). After a further 27 days the twice-injected cockroaches were dissected and a portion of the fat body was stained for bacteria. Twelve cockroaches showed no bacteria, three had reduced bacteria, and eight showed no significant change when compared with untreated insects. The remaining fat body was removed from those cockroaches showing no bacterial staining and from an equal number of untreated cockroaches that showed bacterial staining. Enzyme extracts were prepared from each group and assayed for epimerase activity using D-*chiro*-, L-*chiro*-, *scyllo*-, and *myo*-inositols as substrates and NAD^- as the cofactor. Reductase activity was assayed using *scyllomyo*-inosose with NADH and NADPH as cofactors. No difference in activity or products formed was observed in any case, strongly suggesting that the inositol epimerase(s) producing chiro and other inositols as well as the inosose reductase(s) are native to the cockroach and not bacterial enzymes.

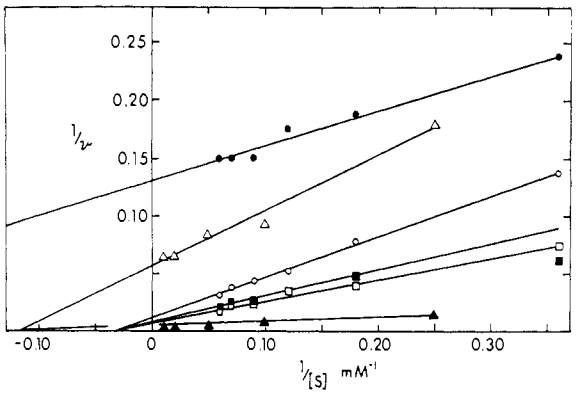


FIGURE 3: Effect of substrate concentration on epimerase activity. Velocity is ng/min. Inositol variable substrate (2.78–16.7 mM): fixed substrate, 0.625 mM NAD^+ in 25 mM Tris buffer, pH 8.6; incubation 2 hr at 24° containing 1.2 mg of protein/ml: (●) *chiro*-inositol product from *myo*-inositol:NAD^+ epimerase; (○) *scyllo*-inositol product from *myo*-inositol:NAD^+ epimerase; (■) *myo*-inositol product from *chiro*-inositol:NAD^+ epimerase; (□) myo-inositol product from *scyllo*-inositol:NAD^+ epimerase. NAD^- variable substrate (0.2–5.0 mM): fixed substrate, 5.58 mM *myo*-inositol incubated as described above but with 1.08 mg of protein/ml of incubation. The abscissa in this experiment is plotted as $mM^{-1} \times 0.05$: (△) *scyllo*-inositol product from *myo*-inositol: NAD^+ epimerase; (▲) *chiro*-inositol product from *myo*-inositol: NAD^+ epimerase.

Effect of Substrate Concentration on Inositol Epimerase Activity. The relationship between substrate concentration and reaction was studied using *myo*-, *scyllo*-, and D-*chiro*-inositols and NAD^- as the substrate and cofactor for the epimerase system. A linear relationship between the reciprocal velocity and reciprocal substrate concentration was observed (Figure 3) with each substrate (0.2–5.0 mM NAD^+, 2.78–16.7 mM inositol). The apparent K_m and V_{max} were calculated from the slope and intercept of the double-reciprocal plots (Table V). This K_m is an overall kinetic constant made up of both affinity and rate constants. Thus the low apparent K_m for the epimerization of *myo*-inositol to *chiro*-inositol is probably a reflection of a reduced rate of oxidation of the *myo*-inositol C-3-OH and/or slower reduction to *chiro*-inositol (the thermodynamically less-favored product). This follows from the fact that the affinity of the enzyme for *myo*-inositol would be the same in each case, the oxidation–reduction step controlling the relative rates. Conversely in the reaction going from *chiro*-inositol to *myo*-inositol the K_m is about the same as that for the other epimerase reactions suggesting that this is the preferred biological pathway.

Deuterium-Labeling Studies. Three experiments using deuterium labels were carried out to determine if the enzymic reaction intermediates are tightly bound to the enzyme systems or if they are capable of equilibration with the incubation medium solvent. The results of these experiments are presented in Table VI.

INOSITOL EPIMERASE. *myo*-, *scyllo*-, and *chiro*-inositols, on epimerization with D_2O-exchanged enzyme in Tris-D_2O (original pH 8.6), showed no incorporation of deuterium into the products. We have found (unpublished observation) and others have reported (Isbell *et al.*, 1969) that inososes undergo spontaneous rapid enolization at pH 8.6 and higher with incorporation of hydrogen isotopes from solvent. Since no deuterium was incorporated into the products of the epimerization at pH 8.6 in D_2O, we conclude that: (1) if the intermediate of the epimerization is an inosose it is (a) protected from the solvent, (b) converted very rapidly to product inositol, (c) not dissociated from the enzyme (or if dissociated does not recombine with the enzyme), or some combination of these, and (2) as with many pyridine nucleotide proton transfers, the transferred proton is not exchangable with the solvent, that is, there is a direct transfer to NAD^+ and thence back to the substrate molecule.

A second experiment with inositol epimerase was carried out in water using [2-^{2}H]*myo*-inositol and DL-[3-^{2}H]*chiro*-

inositol of 50% isotopic enrichment (8.3 atom %) as substrates for the enzymic epimerization. In these experiments *both substrates and products* were found to lose more than half of their deuterium (Table VI) although it was expected that the label would be retained. We have no data which can explain this intrusion of hydrogen into the deuterated inositols. It clearly supports the view that the [$^2H_1$]NADH is dissociated from the enzyme during the epimerization, and that [$^1H_1$]NADH from some other enzyme source in the preparation reduced the inosose (?) intermediate. We know from the epimerization carried out in D_2O that solvent cannot provide the hydrogen, and therefore neither could any exchangable hydrogen on proteins.

In spite of its shortcomings, the experiment provides some useful information. The fact that label was lost from both [2-^{2}H]*myo*- and [3-^{2}H]*chiro*-inositol shows that the epimerase is probably operating *via* the familiar pyridine nucleotide dependent pathway. (For a review of this subject, see Glaser, 1972). Thus, in this case, hydrogen is removed from carbon, presumably to form an inosose intermediate, and replaced on that carbon on the side opposite to that from which it was removed.

INOSOSE REDUCTASE. *scyllomyo*-Inosose was allowed to incorporate deuterium in D_2O, pH 8.7. The product obtained was a mixture of mono-, di-, tri-, and tetradeuterioinososes as assessed by GC-MS (Table VI). This inosose-d_n mixture was incubated in D_2O with D_2O-exchanged fat body enzyme at pH 7.0 using [$^1H_1$]NADH and [$^1H_1$]NADPH. The expected loss of deuterium in the products by incorporation of hydrogen from the hydrogen-reduced cofactors was *exceeded* by more than four times that calculated.

The experiment where NADPH was the cofactor may be explained by our observation that when *scyllo*-inositol is incubated as a substrate with $NADP^+$, a small amount of *scyllomyo*-inosose is formed. Thus the reductase reaction is, to a small degree, reversible. Once the all-equatorial *scyllo*-inositol is formed (having no specific site for enzyme attack) a portion can be reoxidized to *scyllomyo*-inosose-d_{n-1} and [$^2H_1$]-NADPH. The inosose would then be preferentially reduced by the larger pool of [$^1H_1$]NADPH, thus incorporating another hydrogen in the inositol product.

We do not know the degree to which NAD^+ oxidizes inositols *via* the reductase. Thus we do not know if the large loss of deuterium in the *scyllomyo*-inosose-d_n incubation with [$^1H_1$]NADH could also be explained in this way.

Even with the uncertainties in the interpretation of these reactions, it seems clear that (1) the reduced cofactors are dissociable in the epimerase reaction, (2) if inososes are intermediates in the epimerase reaction they do not dissociate to a significant extent, (3) the reductase, at least in the case with NADPH as cofactor, is slightly reversible, (4) neither epimerase nor reductase is open to proton exchange with the incubation solvent.

TABLE VI: Loss of Label on Incubating Deuterated Substrates with Cockroach Fat Body Enzymes.

Substrate before Incubation	% Loss of Deuterium[a]			
	Substrate after Incubation	Product Inositol		
		myo-	*scyllo*-	*chiro*-
[2-^{2}H]*myo*-Inositol[b]	55	NA[c]	81	58
DL-[3-^{2}H]*chiro*-Inositol[b]	50	72	77	NA
[G-^{2}H]Inosose (NADH)[d,e]	NA	83[f]	83[f]	NA
[G-^{2}H]Inosose (NADPH)[d,e]	NA	63[f,g]	71[f]	NA

[a] Per cent loss relative to initial substrate level. [b] Substrate before incubation was 8.3 atom % deuterium (50% enrichment at the specified position). [c] Not assayed. [d] G is general labeling where a molecule may have ^2H at any or all, but not necessarily all, of the 5 positions and is in contrast to uniformly labeled. [e] Substrate before incubation was 35 atom % deuterium. This is a mixture of inososes and contains *scyllomyo*-, *myoepi*-, *myochiro*-, *myoneo*-inosose (8:4:1:1) produced on deuterium exchange of *scyllomyo*-inosose at pH 8.7. They consist of 23% monodeuterioinosose, 45% dideuterioinosose, 22% trideuterioinosose, and 10% tetradeuterioinosose. [f] The per cent loss expected from the introduction of 1 hydrogen from [$^1H_1$]NAD(P)H upon reduction was 17%. [g] Formed from *myoepi*-, *myochiro*-, and *myoneo*-inososes present in the substrate.

Discussion

In 1967 Candy presented evidence which led him to propose that locust fat body contained two dehydrogenases which used NAD(H) to carry out the oxidation–reduction between *myo*-inositol and *scyllomyo*-inosose on one hand and NADP(H) as cofactor between *scyllomyo*-inosose and *scyllo*-inositol on the other. There are similarities between the cockroach fat body enzyme(s) and those of the locust, as reported by Candy. For example, NAD^+ was more effective than $NADP^+$ (factor of 8 in the Candy study) in the conversion of *myo*- to *scyllo*-inositol. Further, NADPH also inhibited the epimerization (by 14%) in Candy's work. Also, Candy found that NADH preferentially reduced *scyllomyo*-inosose to *myo*-inositol, while NADPH gave almost exclusively *scyllo*-inositol.

We feel our data support the presence in cockroach fat body of at least two enzyme systems: inositol:NAD^+ epimerase and inosose:NAD(P)H reductase. The strongest support for this is found in the pH curves (Figure 2) wherein the reductase activities are clearly broader in pH optima than the epimerase reactions and have maximal activities at acidic pH's whereas the epimerase reaction is maximally stimulated at alkaline pH. Further support for two separate systems derives from clear differences in cofactor requirements. Thus not only does NAD^+ seem to be uniquely required for epimerization of the inositols, $NADP^+$ being ineffective, but NADPH inhibits the epimerization of *myo*- to *scyllo*-inositol (as well as the other epimerizations) while it is highly selective and efficient in the conversion of *scyllomyo*-inosose to *scyllo*-inositol. Also, *myoepi*-inosose is reduced in the presence of NADPH to both *myo*- and *epi*-inositols whereas NAD^+ only effects the conversion of *epi*- to *myo*-inositol, not the reverse. The latter observation argues against the possibility that the epimerase might consist of an inositol:NAD^+ oxidase together with an inosose:NADH reductase with different pH optima. The much slower rate of reduction of *myoepi*-inosose to *myo*-inositol with NADH than the rate of epimerization of *epi*-inositol to *myo*-inositol with NAD^+ and the absence of *chiro*-inositol formation with NADH from *myochiro*-inosose in contrast to the formation of *chiro*-inositol from the NAD^+ epimerization of *myo*-inositol also preclude this possibility.

The pH curves do not show sufficient differences with respect to pyridine nucleotide cofactor and substrate requirements to evaluate the possibility that there might be separate enzymes for NAD^+, $NADP^+$, NADH, and NADPH, not to mention the separate substrates. The observation that dithiothreitol equally inhibited the epimerization of *myo*- to *scyllo*- and *myo*- to *chiro*-inositols suggests that the epimerase is a single enzyme. The same conclusion can be drawn from the fact that NAD^+ plus NADPH inhibited the epimerization of *chiro*-, *scyllo*-, and *myo*-inositol equally. Further, the K_m values for these three substrates are similar.

The activities of the epimerase can apparently all be accounted for by the epimerization of a single hydroxyl, the only inositols not accepted being those with more than two axial hydroxyls. It is possible that the epimerase is an oxidoreductase of low specificity for substrate, the function of which is to produce *myo*- and perhaps *scyllo*-inositols from dietary inositols which, for this insect, could include *chiro*- and *neo*-inositols.

As is the case with many epimerases the demonstration of the carbonyl-containing intermediate is a difficult matter. We have no direct proof that the epimerization intermediates are inososes, only evidence that hydrogen is specifically removed from the carbon we would believe becomes a carbonyl. This can be studied only after complete separation of the reductase and epimerase activities. One might then be able to show reduction of inosose by NADH using an enzyme that had been freed of native NAD^+ by charcoal treatment in the way of Barnett *et al.* (1973).

It is curious that a pathway exists for the epimerization of *neo*-inositol, an inositol which has been shown (Sherman *et al.*, 1971) to result from the cyclization of mannose 6-phosphate by enzyme preparations containing D-glucose-6-phosphate–L-*myo*-inositol-1-phosphate cyclase.

It is interesting to find, in a single animal, enzymatic systems capable of synthesizing the four inositols which are found in nature: *myo*-, *scyllo*-, *neo*-, and *chiro*-inositol.

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