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Enzymes of *myo*-Inositol and Inositol Lipid Metabolism in Rats with Streptozotocin-Induced Diabetes

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Diabetes, with only mild ketosis, was induced in male rats by a single injection of streptozotocin. After 12 weeks the specific activities of enzymes concerned with the metabolism of inositol and of inositol lipids were measured in various tissues. Inositol 1-phosphate synthase (EC 5.5.1.4) was most active in testis and the activity was significantly less in diabetic rats than in controls on a similar diet. Inositol oxygenase (EC 1.13.99.1), which converts *myo*-inositol into glucuronic acid, was also less active in kidney from diabetic animals. CDP-diacylglycerol–inositol phosphatidyltransferase (EC 2.7.8.11) and phosphatidylinositol 4-phosphate kinase (EC 2.7.1.68) showed decreased specific activities in brain and sciatic nerve of diabetic rats. By contrast the diabetic state did not affect the specific activities of phosphatidylinositol kinase (EC 2.7.1.67) or phosphatidylinositol 4,5-bisphosphate phosphatase (EC 3.1.3.36) in these tissues. The results are discussed in relation to diabetic neuropathy.

It has been known for many years that diabetics excrete more inositol in the urine than normals (Daughaday *et al.*, 1954). Furthermore, long-standing diabetes mellitus in man is sometimes associated with nerve damage. This diabetic neuropathy involves segmental demyelination and axon loss in peripheral nerves, an early sign being decreased motor-nerve-conduction velocity (Sharma & Thomas, 1974; Winegrad & Greene, 1976). Decrease in peripheral motor-nerve-conduction velocity has also been shown in experimental diabetes (Eliasson, 1964; Preston, 1967; Hildebrand *et al.*, 1968) concomitant with a decrease in nerve *myo*-inositol content (Stewart *et al.*, 1967; Greene *et al.*, 1974). A decrease in motor-nerve-conduction velocity in experimental galactosaemia was accompanied by an increase in peripheral-nerve galactitol and a decrease in inositol (Sharma *et al.*, 1976).

We have confirmed the decrease of sciatic-nerve free inositol in streptozotocin-diabetic rats, and also found a lowered concentration of total lipid inositol in sciatic nerve of acutely diabetic animals, but not in a milder chronic diabetes (Palmano *et al.*, 1977). Although the role of inositol in nerve conduction remains uncertain it seems likely that triphosphoinositide (phosphatidylinositol 4,5-bisphosphate) may be involved in the control of membrane permeability during axonal conduction (White & Larrabee, 1973; White *et al.*, 1974; Tret'jak *et al.*, 1977).

Additional findings of abnormal inositol concentrations in various tissues of diabetic animals suggest a more widespread derangement in inositol metabolism in this disease (Palmano *et al.*, 1977). It was hoped that a study of some of the enzymes involved in inositol and phosphoinositide metabolism might throw light on the pathogenesis of diabetic neuropathy and also help explain some of the abnormalities in inositol concentrations noted in diabetic tissues. To this end, various enzymes involved in inositol metabolism have been measured in nervous and other tissue from normal and diabetic animals.

Materials and Methods

Materials

All chemicals used were of analytical grade. CDP-diacylglycerol synthesized via egg phosphatidylcholine was obtained from Miles Laboratories, Stoke Poges, Bucks., U.K. and [^{3}H]inositol from The Radiochemical Centre, Amersham, Bucks., U.K. The streptozotocin was a gift from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A. Cutscum [iso-octylphenoxypoly(ethoxyethanol)] was obtained from Kodak, Kirkby, Liverpool, U.K. and Triton

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X-100 from Lennig Chemicals, Croydon, Surrey, U.K.

Animals

The male Wistar rats used and their diets have been described previously (Palmano *et al.*, 1977).

Induction of diabetes

The rats were injected intraperitoneally with streptozotocin in 50mM-sodium citrate buffer, pH4.5, at a dosage of 35mg/kg body wt. Control animals were injected with an equivalent volume (approx. 0.2ml) of the citrate buffer. The streptozotocin solution was made up immediately before use. Some of the characteristics of the chronic diabetes produced by this single dose of streptozotocin have been described by Palmano *et al.* (1977). Enzymes were determined in various tissues 12 weeks after the injection of the rats. At this stage the diabetic animals exhibited mild ketosis, hyperglycaemia, hypertriacylglycerolaemia, glycosuria and polyuria. The 31 control animals weighed 308 ± 7 (mean $\pm$ S.D.) g compared with 218 ± 19g for the 32 diabetic animals after this period.

Preparation of tissue homogenates

Diabetic and control animals were killed by a blow on the head followed by decapitation. For the enzyme assays of Table 2, brain and sciatic nerve were treated as follows. Whole brains were removed and 10% (w/v) homogenates prepared in 0.32M-sucrose containing 10mM-Tris/HCl buffer, pH7.4. For brain homogenates a rotating Teflon pestle (600rev./min) and glass homogenizer were used, ten up-and-down strokes being made. For sciatic-nerve homogenates, pooled samples (150–200mg wet wt.) were homogenized by hand in a ground-glass pestle and mortar containing 3.0ml of 0.32M-sucrose/10mM-Tris/HCl buffer, pH7.4. All homogenates were filtered and centrifuged at 200*g* for 5min to remove cell debris.

Tissues were treated differently for the inositol 1-phosphate synthase and inositol oxygenase assays. Details are given in the relevant sections that follow.

Preparation of labelled ATP

Terminally labelled ATP was prepared by the method of Glynn & Chappell (1964). The 0.25M-HCl eluate from the resin column was brought to pH7 with 1.0M-Tris and carrier disodium ATP added to give specific radioactivities of 1.7×10^7 d.p.m./μmol for phosphatidylinositol 4-phosphate kinase assays and 7.0×10^6 d.p.m./μmol for phosphatidylinositol kinase.

Preparation of phosphoinositides

Phosphoinositides were prepared from ox brain by the method of Folch (1949) and purified by chromatography on DEAE-cellulose as described by Hendrickson & Ballou (1964).

Enzyme assays

All enzyme activities were measured as far as possible under optimum conditions of pH and concentrations of cofactors and protein. Reaction rates were proportional to protein concentration under these conditions. The method of Lowry *et al.* (1951) was used for the determination of protein.

Tissue homogenates used for the assay of phosphatidylinositol kinase, phosphatidylinositol 4-phosphate kinase and CDP-diacylglycerol–inositol phosphatidyltransferase were routinely frozen and thawed twice to give maximum activities.

(*a*) *Myo-inositol 1-phosphate synthase* (*EC* 5.5.1.4). This enzyme was measured in partially purified preparations from various tissues essentially as described by Burton & Wells (1974).

Testis, whole brain, liver, kidney and sciatic nerve were removed and suspended in 5vol. of ice-cold 50mM-Tris acetate buffer, pH7.4, containing 10mM-EDTA, 14mM-ammonium acetate and 1mM-dithiothreitol. The tissues were minced with scissors and all samples other than sciatic nerve homogenized at 4°C with a motorized Teflon pestle and glass tube. Sciatic nerve (pooled samples of 150–200mg wet wt.) was homogenized by hand in a ground-glass pestle and glass tube. The crude homogenates were filtered through two layers of nylon mesh to remove cell debris and heated at 60°C for 2min (sciatic nerve) or 10min (other tissues). After cooling, the homogenates were centrifuged at $106000g_{av.}$ for 1h. The high-speed supernatants were made 40% saturated with $(NH_4)_2SO_4$ (226mg/ml) and centrifuged at $17000g_{av.}$ for 30min. The protein pellets were dissolved in 0.5–1.0ml of 50mM-Tris acetate buffer, pH7.4, containing 10mM-EDTA, 14mM-ammonium acetate and 1.5mM-2-mercaptoethanol and then dialysed for 16h at 4°C against 5 litres of the above buffer and centrifuged at 200*g* for 10min.

Sciatic-nerve supernatants were not fractionated but concentrated to small volumes in Amicon (Lexington, MA, U.S.A.) B15 concentrator cells before dialysis.

The colorimetric procedure of Barnett *et al.* (1970) was employed for assay of synthase activity directly after dialysis. All reagents were prepared in the assay buffer and final assay volume was 0.5ml. Approx. 0.5mg of protein was used in each incubation.

(*b*) *CDP-diacylglycerol–inositol phosphatidyltransferase* (*EC* 2.7.8.11). This enzyme activity was measured by the method of Salway *et al.* (1968). The specific radioactivity of the [^{3}H]inositol used was 0.2μCi/μmol.

(*c*) *Phosphatidylinositol kinase* (*EC* 2.7.1.67) was assayed as described by Lefebvre *et al.* (1976), except that the assay was for 3min at pH7.4 in the presence of 0.2% (v/v) Cutscum, with 1mM-phosphatidylinositol as substrate.

(*d*) *Phosphatidylinositol 4-phosphate kinase* (*EC* 2.7.1.68). Method A of Kai *et al.* (1968) was used with 0.2mM-phosphatidylinositol 4-phosphate as substrate. The extraction and separation of phosphatidylinositol 4,5-bisphosphate was by the method of Cooper & Hawthorne (1976). The radioactivity of labelled phosphatidylinositol 4,5-bisphosphate produced was determined as described by Lefebvre *et al.* (1976).

(*e*) *Phosphatidylinositol bisphosphate phosphatase* (*EC* 3.1.3.36). This was assayed by the method of Nijjar & Hawthorne (1977).

(*f*) *Kidney myo-inositol oxygenase* (*EC* 1.13.99.1). This activity was measured by the method of Charalampous & Lyras (1957) by using postmitochondrial supernatants.

Results

The aim of this work was to compare specific activities of various enzymes in tissues from normal and diabetic rats. Since some of the enzymes concerned with phosphoinositide metabolism occur in more than one subcellular fraction, it seemed best to measure activities in homogenates and in crude supernatant fractions. Such enzyme preparations might also retain any inhibitory factor of the diabetic environment.

Inositol 1-phosphate synthase

The biosynthesis of *myo*-inositol from D-glucose 6-phosphate is catalysed by two enzymes, *myo*-inositol 1-phosphate synthase (EC 5.5.1.4) and 1-L-*myo*-inositol 1-phosphatase (EC 3.1.3.25) (Eisenberg & Bolden, 1966). The synthase-catalysed step in which the cyclization of glucose 6-phosphate to L-*myo*-inositol 1-phosphate occurs is probably rate limiting in tissues other than testis (Eisenberg, 1967) and for this reason it was chosen for study. Initial attempts to measure the synthase activity in crude heat-treated supernatants proved unsatisfactory (P. H. Whiting & K. P. Palmano, unpublished work), as spuriously high activities were obtained in conjunction with high concentrations of phosphate in controls. Hence $(NH_4)_2SO_4$ fractionation was adopted to yield a partially purified preparation that gave more satisfactory and reproducible results. The pH optimum had to be closely adhered to, since fluctuation greater than 0.2 pH unit caused variable loss of activity. The enzyme was not stable on freezing and thawing and was therefore assayed immediately.

Table 1 shows the activity of synthase in a number of tissues of normal and diabetic rats. Testis shows as much as 10-fold higher activity compared with other tissues. Values for control activity compare favourably with those obtained for testis by Eisenberg (1967) with a radioassay. In diabetic testis, synthase activity is significantly decreased compared with the normal value ($P<0.0005$). As there is no change in testis wet weight during diabetes it can be assumed that overall activity is decreased.

The values obtained for kidney, liver, brain and

Table 1. *Specific activity of inositol 1-phosphate synthase in tissues from normal and diabetic rats*

For experimental details see the text. Results are means ± S.D. with numbers of experimental animals in parentheses.

	Inositol 1-phosphate formed (nmol/h per mg of protein)	
	Normal	Diabetic
Testis	160 ± 40 (6)	69 ± 35 (10)
Kidney	17 ± 3 (5)	14 ± 4 (6)
Liver	15 ± 9 (6)	10 ± 7 (10)
Brain	19 ± 5 (4)	15 ± 10 (5)
Sciatic nerve	13 ± 4 (5)	11 ± 5 (5)

Table 2. *The specific activities of some enzymes involved in phosphoinositide metabolism in brain and sciatic nerve from normal and diabetic animals*

For experimental methods see the text. Results are means ± S.D. The numbers of experimental animals are given in parentheses. For normal values against diabetic values: *$P<0.1$, **$P<0.025$, ***$P<0.005$.

	Activity of enzyme (nmol of substrate converted/min per g wet wt. of tissue)			
	Brain		Sciatic nerve	
	Normal	Diabetic	Normal	Diabetic
CDP-diacylglycerol–inositol phosphatidyltransferase	15.1 ± 2.1 (10)	11.5 ± 2.1*** (8)	23.5 ± 1.9 (5)	13.2 ± 2.5*** (5)
Phosphatidylinositol kinase	16.6 ± 6.6 (8)	16.6 ± 13.3 (9)	1.5 ± 1.0 (5)	1.8 ± 1.0 (5)
Phosphatidylinositol 4-phosphate kinase	4.57 ± 0.75 (5)	3.34 ± 0.74** (6)	9.21 ± 2.47 (4)	6.50 ± 2.18* (5)
Phosphatidylinositol 4,5-bisphosphate phosphatase	4430 ± 630 (5)	3970 ± 410 (6)	1640 ± 490 (4)	1620 ± 120 (5)

sciatic-nerve tissue again agree with those found by Eisenberg (1967). There were no significant differences in activity between diabetic and normal rats for any of these tissues.

Phosphoinositide enzymes

Table 2 shows the activities of some enzymes concerned with phosphoinositide metabolism. The synthesis of phosphatidylinositol from CDP-diacylglycerol in homogenates of both brain and sciatic nerve is significantly decreased ($P<0.005$) in diabetic animals. Phosphatidylinositol kinase, an enzyme difficult to measure because of its low activity, is unchanged in the diabetic state, as is phosphatidylinositol bisphosphate phosphatase. Phosphatidylinositol 4-phosphate kinase is less active in diabetic brain and though the mean activity is also lower in diabetic sciatic nerve, the change is probably not significant ($P<0.1$).

Inositol oxygenase

This kidney enzyme, which converts *myo*-inositol into glucuronic acid, was investigated, since in a chronic diabetic state rat kidney contained an increased concentration of inositol (Palmano *et al.*, 1977). A marked decrease in oxygenase activity was found in kidney from diabetic rats [25.8 ± 6.94 (mean$\pm$S.D.) nmol of glucuronic acid formed/h per mg of protein, compared with a value of 121.1 ± 48.6 for control rats]. The values are for eight determinations and differ significantly ($P<0.005$).

Discussion

It is well known that alterations in enzyme activity may occur in diabetes. Thus the activities of key enzymes in the glycolytic pathway are decreased, whereas enzymes of gluconeogenesis are increased. This may be a direct consequence of insulin deficiency. However, it is likely that other enzymes in less-central metabolic pathways are variously affected by diabetes. We have shown in the present paper that the specific activities of several enzymes concerned with inositol metabolism are altered.

The synthesis of *myo*-inositol from glucose 6-phosphate occurs in many tissues, although by far the greatest activity is seen in testis (Eisenberg, 1967; Barnett *et al.*, 1970; Naccarato *et al.*, 1974; Burton & Wells, 1977). Of the tissues examined in the present work, only testis showed a change in the activity of the inositol synthase, which in diabetic rats was decreased to half the control value. This would seem difficult to correlate with an increased testicular inositol concentration found in streptozotocin-induced diabetes [1.55 ± 0.40 (mean$\pm$S.D.) μmol/g wet wt. in seven controls, 3.68 ± 2.59 μmol/g wet wt. in six diabetic rats ($P<0.05$); K. P. Palmano, unpublished results]. However, tissue concentrations depend on several metabolic processes, transport, utilization, removal and catabolism, as well as synthesis. Also, the overall synthesis of inositol in testis may be governed by the specific phosphatase of reaction (2) rather than the synthase reaction (1).

$$\text{Glucose 6-phosphate} \xrightarrow{Mg^{2+},NAD^+} \text{inositol 1-phosphate} \quad (1)$$

$$\text{Inositol 1-phosphate} + H_2O \longrightarrow \text{inositol} + P_i \quad (2)$$

In most tissues, the synthase activity is low and the phosphatase activity 2–10-fold higher (Eisenberg, 1967). In such cases synthase will be the rate-limiting enzyme. However, in testis where synthase activity is more than twice that of phosphatase (Eisenberg, 1967), it is more likely that the latter enzyme controls overall production of inositol.

It is also possible that in diabetic tissues the synthase may have altered enzyme kinetics. Increased concentrations of free *myo*-inositol *in vivo* coincide with an increase in the concentration of glucose 6-phosphate (Burton & Wells, 1977). Glucose 6-phosphate is likely to be increased in diabetic testis as the utilization of glucose is unimpaired (Middleton, 1973) and there are increased concentrations of glucose in the testis (K. P. Palmano, unpublished results). An increase in the K_m of inositol synthase would account both for an increased rate of conversion of glucose 6-phosphate into inositol *in vivo* and the apparent decrease in enzyme activity seen *in vitro*.

The increase in the concentration of free inositol in brain and kidney in diabetes would not appear to be directly governed by inositol synthesis; nor indeed the depression in the concentration of inositol in sciatic nerve. Rather, in kidney, the increased concentration of inositol (Palmano *et al.*, 1977) might be due to a decreased rate of degradation of inositol to glucuronic acid. In diabetic kidney we have shown that the enzyme responsible for inositol degradation, *myo*-inositol oxygenase, has only 25% of the control activity. Hence a decreased rate of inositol degradation could contribute both to increased concentrations of kidney inositol and to the increased clearance of inositol by diabetic kidney. Glucose itself is likely to be the major cause of inosituria in diabetes, since it inhibits the active transport of inositol. The administration of glucose intravenously to non-diabetic patients raised the renal clearance of inositol to concentrations seen in uncontrolled diabetes (Daughaday & Larner, 1954).

It has also been shown in the present paper that the specific activities of some enzymes associated with phosphoinositide metabolism are decreased in diabetes. Specifically, the activities of the enzymes responsible for the synthesis of phosphatidylinositol and of phosphatidylinositol 4,5-bisphosphate (CDP-diacylglycerol–inositol phosphatidyltransferase and phosphatidylinositol 4-phosphate kinase respectively)

were decreased in nervous tissue of diabetic rats. We have reported decreased concentrations of lipid inositol in sciatic nerves of acutely diabetic rats and in brains of mildly diabetic animals (Palmano *et al.*, 1977), but there is no obvious relationship between these changes and the changes in CDP-diacylglycerol–inositol phosphatidyltransferase measured under optimal conditions *in vitro*. In guinea-pig brain this enzyme has a K_m for *myo*-inositol of 1.5mM (Benjamins & Agranoff, 1969). If the rat sciatic-nerve enzyme has a similar K_m, it is unlikely that a decrease of free-inositol concentration from 3.10 to 2.44 μmol/g wet wt. in chronic diabetes (Palmano *et al.*, 1977) will affect the activity of the transferase *in vivo*.

There will be no simple relationship between enzyme activities and metabolite concentrations of rat tissue homogenates and functional changes such as the decreased nerve-conduction velocity of diabetes. Nevertheless, the decreased enzyme activities of Table 2 may be pertinent to diabetic neuropathy since phosphatidylinositol and its derivatives are implicated in nerve conduction. Transmission at certain synapses involves hydrolysis and resynthesis of phosphatidylinositol (Michell, 1975), and phosphatidylinositol 4,5-bisphosphate may be involved both in axonal conduction (Hawthorne & Kai, 1970) and in synaptic responses to Ca^{2+} ions (Hawthorne & Pickard, 1979). The impaired synthesis now reported in diabetic nervous tissue may lead to the decrease in conduction velocity that is well established (Eliasson, 1964; Hildebrand *et al.*, 1968; Greene *et al.*, 1975).

Clements & Reynertson (1977) have suggested that the hyperglycaemia of diabetes leads to intracellular deficiencies of *myo*-inositol. In animal models, at least, this deficiency is well established for peripheral nerves. The present work suggests that there may also be disordered metabolism of the inositol lipids in nerve tissue. Such changes could certainly contribute to human diabetic neuropathy.

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