The metabolism of L-phenylalanine and L-tyrosine by liver cells isolated from adrenalectomized rats and from streptozotocin-diabetic rats

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1. Flux through, and maximal activities of, key enzymes of phenylalanine and tyrosine degradation were measured in liver cells prepared from adrenalectomized rats and from streptozotocin-diabetic rats. 2. Adrenalectomy decreased the phenylalanine hydroxylase flux/activity ratio; this was restored by steroid treatment *in vivo*. Changes in the phosphorylation state of the hydroxylase may mediate these effects; there was no significant change in the maximal activity of the hydroxylase. 3. Tyrosine metabolism was enhanced by adrenalectomy; this was not related to any change in maximal activity of the aminotransferase. Steroid treatment increased the maximal activity of the aminotransferase. 4. Both acute (3 days) and chronic (10 days) diabetes were associated with increased metabolism of phenylalanine; insulin treatment *in vivo* did not reverse these changes. Although elevated hydroxylase protein concentration was a major factor, changes in the enzyme phosphorylation state may contribute to differences in phenylalanine degradation in the acute and chronic diabetic states. 5. Tyrosine metabolism, increased by diabetes, was partially restored to normal by insulin treatment *in vivo*. These changes can, to a large extent, be interpreted in terms of changes in the maximal activity of the aminotransferase.

Phenylalanine and tyrosine share a common degradative pathway in the liver. The key enzymes of this pathway are influenced by the hormonal status of the animal.

The first step of phenylalanine breakdown is catalysed by phenylalanine hydroxylase [L-phenylalanine:tetrahydropteridine: oxygen oxidoreductase (4-hydroxylating), EC 1.14.16.1]. In the short term, hydroxylase activity is increased by phenylalanine itself (Shiman, 1980; Shiman & Gray, 1980) and by phosphorylation (Donlon & Kaufman, 1978; Fisher & Pogson, 1984). Glucocorticoids have a role in the long-term control of hydroxylase activity. Using cultured hepatoma cells, Haggerty *et al.* (1973) and Sorimachi *et al.* (1981) have observed increased hydroxylase activity after exposure to corticosteroids. This results from enhanced protein synthesis (Baker & Shiman, 1979). Haggerty *et al.* (1974) estimated that

maximally stimulated activity in cells is similar to that normally seen *in vivo*, suggesting that corticosteroids maintain hydroxylase activity *in vivo*. This may explain the conflicting data obtained from studies *in vivo*, which indicate an increase of (Freedland, 1963; Haggerty *et al.*, 1983), decrease of (Koller *et al.*, 1974) or no effect on (McGee *et al.*, 1972) hydroxylase activity after corticosteroid treatment of rats. Experimental diabetes is also associated with increased hydroxylase activity (Donlon & Beirne, 1982) and phenylalanine degradation (Carr & Pogson, 1981). This appears to result from an increase in hydroxylase concentration (Howard & Donlon, 1985).

Phenylalanine aminotransferase [L-phenylalanine (L-histidine):pyruvate aminotransferase, EC 2.6.1.58] normally plays only a minor role in the degradation of phenylalanine (Carr & Pogson, 1981). This activity is, however, elevated by steroid treatment or in diabetes (Morris *et al.*, 1973). Glucagon administration also increases the concentration of the enzyme (Brand & Harper, 1974a; Shih & Chan, 1979); this may not, however, be reflected in increased transamination *in vivo* (Brand & Harper, 1974b).

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Tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5), which catalyses the initial step of tyrosine disposal, is particularly sensitive to hormonal influences. Glucagon (Holten & Kenney, 1967; McNamara & Webb, 1974) and corticosteroids (Kenney, 1962; Granner *et al.*, 1968) induce enzyme activity *in vivo* and in isolated liver cells (Marston *et al.*, 1981). Insulin increases activity by decreasing the rate of aminotransferase degradation in hepatoma cells (Spencer *et al.*, 1978). In contrast, using primary culture liver cells, Nakamura *et al.* (1981) have shown that insulin inhibits corticosteroid-mediated enzyme induction. Alloxan-diabetes (Holten & Kenney, 1967) and streptozotocin-diabetes (Shimura *et al.*, 1983) both increase aminotransferase activity.

Despite the considerable data available concerning the long-term control of these enzymes of aromatic amino acid metabolism, the importance of these changes in the control of metabolic fluxes *in vivo* is less understood. In this paper we report the effects of adrenalectomy and experimental diabetes (acute and chronic) on the phenylalanine and tyrosine metabolism of isolated liver cells. The effects are related to the activities of key enzymes measured simultaneously.

Materials and methods

Animals

Male Sprague-Dawley rats (180–220 g) were used throughout. Animals were given a standard laboratory diet (CRM diet; Labsure, Poole, Dorset, U.K.).

Adrenal glands were removed by means of bilateral incisions under diethyl ether anaesthesia. Adrenalectomized animals were given 1% (w/v) NaCl in place of water and were left for 5–8 days before use. Where indicated, adrenalectomized animals were treated with dexamethasone phosphate (2.5 mg/kg body wt., given subcutaneously) each day at noon.

Diabetes was induced by a single injection of streptozotocin (60 mg/kg body wt., given intravenously). Glucosuria was confirmed with Clinistix (Ames Co., Stoke Poges, Slough, Bucks., U.K.). Animals were used 3 days (acute) or 10 days (chronic) after injection. Where indicated, diabetic animals were treated with insulin (10 insulin units/kg body wt. for 2 days, then 15–20 insulin units/kg body wt. per day given intraperitoneally). In all cases, solutions for injection were prepared in 0.9% (w/v) NaCl.

Chemicals

All radiolabelled compounds were from Amersham International, Amersham, Bucks., U.K. Each was purified by chromatography on Aminex

Q-15S (Bio-Rad Laboratories, Watford, Herts., U.K.) as described in Salter *et al.* (1984). The sources of other reagents were as given previously (Smith & Pogson, 1980; Smith *et al.*, 1980). Dexamethasone phosphate was from Sigma (Poole, Dorset, U.K.). Insulin (Neusulin) was from the Wellcome Foundation, Wellcome Research Laboratories, Beckenham, U.K., and streptozotocin was given by Dr. C. N. Earl, I.C.I. Pharmaceuticals Division, Macclesfield, Cheshire, U.K.

Preparation and incubation of cells

The procedures were as described previously (Smith & Pogson, 1980; Smith *et al.*, 1980), incorporating the modifications outlined by Salter *et al.* (1984). The incubation medium consisted of Krebs-Henseleit (1932) bicarbonate buffer containing 2.5 mM-CaCl₂ and 2% (w/v) bovine serum albumin (defatted as described by Chen, 1967) and supplemented with 5 mM-D-glucose, 5 mM-L-glutamine, 4.5 mM-L-lactate and 0.5 mM-pyruvate. Glucose was used to maintain glycogen stores, lactate and pyruvate were provided as a gluconeogenic substrate in their physiological ratio, and glutamine provided a nitrogen-containing substrate with input into the citric acid cycle. Cells were pre-incubated for 25 min before addition of amino acid substrate.

Flux from 50 μ M-phenylalanine through phenylalanine hydroxylase and homogentisate oxidase [homogentisate: oxygen 1,2-oxidoreductase (de-cyclizing), EC 1.13.11.5] was assessed as described by Fisher & Pogson (1984) with L-[4-³H]phenylalanine as substrate (final concn. 50 μ M; sp. radioactivity 1.5 Ci/mol). Fluxes through hydroxyphenylpyruvate oxidase [4-hydroxyphenylpyruvate: oxygen oxidoreductase (hydroxylating, decarboxylating), EC 1.13.11.27] and phenylalanine aminotransferase were measured as described by Carr & Pogson (1981) with L-[1-¹⁴C]phenylalanine as substrate (final concn. 50 μ M; sp. radioactivity 0.15 Ci/mol).

Flux from 100 μ M-tyrosine through tyrosine aminotransferase and hydroxyphenylpyruvate oxidase was assessed as described by Dickson *et al.* (1981), with L-[2,3-³H]tyrosine (final concn. 100 μ M; sp. radioactivity 0.75 Ci/mol) and L-[1-¹⁴C]tyrosine (final concn. 100 μ M; sp. radioactivity 0.15 Ci/mol) as respective substrates. Flux through homogentisate oxidase (from tyrosine as substrate) was measured as described by Salter *et al.* (1984).

The general principles of the flux assays, together with a diagram of the fate of radiolabelled phenylalanine and tyrosine, have been published previously (Salter *et al.*, 1984).

Other assays

Phenylalanine hydroxylase was extracted and

assayed at 25°C as described by Shiman *et al.* (1979), except that cells were disrupted by three cycles of freezing (liquid N₂) and thawing (25°C). Tyrosine aminotransferase was measured as described by Marston & Pogson (1977) after disruption of cells as above.

Cell viability was checked by measurement of ATP content (Stanley & Williams, 1969). Under the incubation conditions described above, cellular ATP was maintained above 7.5 nmol/mg dry wt. of cells throughout the incubation period. DNA was measured as described by Burton (1956).

Expression of results

Rates of metabolism of L-phenylalanine and L-tyrosine are expressed as nmol/h per mg dry wt. of cells. There was no significant difference in dry-weight content (expressed on a cell DNA basis) of cells prepared from normally fed, adrenalectomized, steroid-treated, adrenalectomized or acutely diabetic rats. Chronic diabetes was associ-

ated with a slight (13%) decrease in liver cell dry weight when expressed in terms of DNA. This, however, did not affect the outcome of statistical analyses. Rates of metabolism were estimated from the initial linear portions of time courses. A two-tailed Student's *t* test was used to assess differences between means throughout. All results are expressed as means $\pm$ S.E.M. for three different cell preparations.

Results and discussion

Adrenalectomized animals

Rates of metabolism of physiological concentrations of phenylalanine and tyrosine by liver cells isolated from adrenalectomized and steroid-treated adrenalectomized rats are shown in Tables 1 and 2. Flux data obtained under identical incubation conditions from normal fed animals have been reported previously (Salter *et al.*, 1984). Adrenalectomy had no significant effect on phenylalanine metabolism; in particular, flux rates through

Table 1. *Effect of adrenalectomy and steroid administration on the metabolism of 50 μ M-L-phenylalanine by isolated rat liver cells*

Conditions for the treatment of animals and for the preparation and incubation of liver cells were as described in the Materials and methods section. Results are the means $\pm$ S.E.M. for three different cell preparations; rates are expressed as nmol/h per mg dry wt. of cells. Differences between means were assessed by a two-tailed Student's *t* test: *P* (versus adrenalectomy only) * <0.05 ; ** <0.01 . Other differences not significant.

Flux	Adrenalectomized	Adrenalectomized + dexamethasone treatment
Through phenylalanine hydroxylase	3.73 $\pm$ 0.56	5.93 $\pm$ 0.33*
Through hydroxyphenylpyruvate oxidase	1.49 $\pm$ 0.12	1.60 $\pm$ 0.11
Through homogentisate oxidase	1.60 $\pm$ 0.23	1.67 $\pm$ 0.29
Through phenylalanine aminotransferase	0.038 $\pm$ 0.008	0.070 $\pm$ 0.011*
Activity of phenylalanine hydroxylase in cell extracts	298 $\pm$ 9	268 $\pm$ 14
Flux through phenylalanine hydroxylase (% of enzyme maximal activity)	1.2 $\pm$ 0.2	2.3 $\pm$ 0.1**

Table 2. *Effect of adrenalectomy and steroid administration on the metabolism of 100 μ M-L-tyrosine by isolated rat liver cells*

Conditions for the treatment of animals and for the preparation and incubation of liver cells were as described in the Materials and methods section. Results are the means $\pm$ S.E.M. for three different cell preparations; rates are expressed as nmol/h per mg dry wt. of cells. Differences between means were assessed by a two-tailed Student's *t* test: *P* (versus adrenalectomy only) * <0.05 ; ** <0.01 . Other differences not significant.

Flux	Adrenalectomized	Adrenalectomized + dexamethasone treatment
Through tyrosine aminotransferase	4.50 $\pm$ 0.33	9.70 $\pm$ 0.41**
Through hydroxyphenylpyruvate oxidase	3.85 $\pm$ 0.19	6.90 $\pm$ 1.07*
Through homogentisate oxidase	3.43 $\pm$ 0.24	5.39 $\pm$ 0.74
Activity of tyrosine aminotransferase in cell extracts	114 $\pm$ 5	223 $\pm$ 16**
Flux through tyrosine aminotransferase (% of enzyme maximal activity)	3.95 $\pm$ 0.25	4.38 $\pm$ 0.18

phenylalanine hydroxylase and phenylalanine aminotransferase were not significantly different from those recorded for liver cells from intact rats (normal fed, 5.07 ± 0.27 ; adrenalectomized, 3.73 ± 0.56 nmol of phenylalanine hydroxylated/h per mg; normal fed, 0.033 ± 0.004 , adrenalectomized, 0.038 ± 0.008 nmol of phenylalanine transaminated/h per mg). There is therefore no absolute dependence on the presence of steroid for the degradation of phenylalanine. It is noteworthy that the hydroxylase flux/activity ratio was significantly decreased by adrenalectomy (normal fed, 0.022 ± 0.001 ; adrenalectomized, 0.012 ± 0.002 ; $P < 0.05$).

Adrenalectomy resulted in enhanced degradation of tyrosine. Fluxes from exogenous tyrosine through tyrosine aminotransferase and hydroxyphenylpyruvate oxidase were significantly increased in liver cells from adrenalectomized animals [normal fed, 3.32 ± 0.20 , adrenalectomized, 4.50 ± 0.33 (nmol of tyrosine transaminated/h per mg); $P < 0.05$; and normal fed, 2.88 ± 0.16 , adrenalectomized, 3.85 ± 0.19 (nmol of homogentisate formed/h per mg); $P < 0.05$]. These changes in flux clearly did not arise from increased tyrosine aminotransferase activity (normal fed, 127 ± 6 ; adrenalectomized, 114 ± 5 nmol/h per mg).

The factor(s) responsible for these observations remain to be identified. We have previously highlighted the significance of the [2-oxoglutarate]/[glutamate] ratio as a regulator of tyrosine metabolism (Salter *et al.*, 1984). This results from the similarity between the K_m value of tyrosine aminotransferase (Hayashi *et al.*, 1967) and the cytosolic [2-oxoglutarate] under normal conditions (Siess *et al.*, 1978). Although an increase in this ratio would account for the present observations, such a change remains to be demonstrated under these conditions.

Liver cells from steroid-treated adrenalectomized animals metabolized phenylalanine through the hydroxylase and phenylalanine aminotransferase steps at significantly higher rates than for cells from untreated adrenalectomized animals. Phenylalanine transamination, however, still accounted for less than 2% of the overall phenylalanine degradation. Interestingly, enhanced flux through the hydroxylase was not accompanied by a significant change in maximal enzyme activity. The hydroxylase flux/activity ratio was returned to normal by steroid treatment (steroid-treated, adrenalectomized, 0.023 ± 0.001 ; normal fed, 0.022 ± 0.001). It therefore appears that steroid treatment influences phenylalanine metabolism by altering factors other than the hydroxylase protein concentration. One possibility is that the pattern of hydroxylase isoenzymes is steroid-sensitive (Miller

& Shiman, 1976). Differential enzyme phosphorylation probably represents the molecular basis of these isoenzymic forms (Donlon & Kaufman, 1980). Changes in phosphorylation state resulting from steroid treatment would not be detected by the assay used in the present studies (Milstien *et al.*, 1976). It is noteworthy that the basal extent of phosphorylation of pyruvate kinase (ATP:pyruvate 2-*O*-phosphotransferase, EC 2.7.1.40) has been reported to be decreased in liver cells isolated from adrenalectomized rats (Postle & Bloxham, 1982).

In contrast with the insensitivity of the assayable phenylalanine hydroxylase activity to glucocorticoid administration, tyrosine aminotransferase activity was very sensitive to such treatment (see Table 2). An approx. 2-fold increase in flux through the aminotransferase and subsequent steps of the catabolic pathway was observed in response to steroid treatment. This resulted from a doubling of maximal aminotransferase activity, as confirmed by the unchanged flux/activity ratio. The steroid-sensitivity of the aminotransferase reported here is consistent with previous observations (Marston *et al.*, 1981). Because treatment did not normalize the flux/activity ratio for tyrosine transamination (normal fed, 0.026 ± 0.001 ; steroid-treated adrenalectomized, 0.044 ± 0.002 ; $P < 0.01$), it must be assumed that treatment did not reverse any effect of adrenalectomy on factors, other than maximal enzyme activity, that affect tyrosine metabolism (see above).

Acute- and chronic-diabetic animals

Metabolism of phenylalanine and tyrosine by liver cells isolated from acute (3 days) and chronic (10 days) diabetic rats is shown in Tables 3 and 4. Both states produced significantly higher rates of phenylalanine degradation than that measured in liver cells from normal fed rats [normal fed, 5.07 ± 0.27 , acute-diabetic, 8.53 ± 0.74 ($P < 0.05$), chronic-diabetic 14.93 ± 1.57 ($P < 0.01$) nmol of phenylalanine hydroxylated/h per mg]. The flux rates associated with the chronic-diabetic state were similar to those reported previously (Carr & Pogson, 1981). The lower phenylalanine transamination rate recorded in the present study may reflect the presence of glutamine (an alternative to phenylalanine as an amino donor for the aminotransferase: Cooper, 1977; Noguchi *et al.*, 1978) in the incubation medium. This is the only apparent effect of glutamine on either phenylalanine or tyrosine metabolism that we have observed in these studies. Phenylalanine metabolism in liver cells from insulin-treated diabetic animals was not significantly different from that in cells from untreated diabetic animals (chronic-diabetic, 14.93 ± 1.57 , insulin-treated chronic-diabetic,

Table 3. *Effect of acute and chronic streptozotocin-diabetes on the metabolism of 50 μ M-phenylalanine by isolated rat liver cells*

Conditions for the treatment of animals and for the preparation and incubation of liver cells were as described in the Materials and methods section. Results are the means $\pm$ S.E.M. for three different cell preparations; rates are expressed as nmol/h per mg dry wt. of cells. Differences between means were assessed by a two-tailed Student's *t* test: *P* (versus acute diabetic) * <0.05 ; ** <0.01 . Other differences not significant.

Flux	Acute diabetic	Chronic diabetic
Through phenylalanine hydroxylase	8.53 $\pm$ 0.74	14.93 $\pm$ 1.57*
Through hydroxyphenylpyruvate oxidase	2.31 $\pm$ 0.10	2.53 $\pm$ 0.03
Through phenylalanine aminotransferase	0.150 $\pm$ 0.017	0.159 $\pm$ 0.032
Activity of phenylalanine hydroxylase in cell extracts	617 $\pm$ 16	464 $\pm$ 37*
Flux through phenylalanine hydroxylase (% of enzyme maximal activity)	1.3 $\pm$ 0.1	3.2 $\pm$ 0.2**

Table 4. *Effect of acute and chronic streptozotocin-diabetes on the metabolism of 100 μ M-L-tyrosine by isolated rat liver cells*

Conditions for the treatment of animals and for the preparation and incubation of liver cells were as described in the Materials and methods section. Results are the means $\pm$ S.E.M. for three different cell preparations; rates are expressed as nmol/h per mg dry wt. of cells. Differences between means were assessed by a two-tailed Student's *t* test: *P* (versus acute diabetic) * <0.05 ; ** <0.01 . Other differences not significant.

Flux	Acute diabetic	Chronic diabetic
Through tyrosine aminotransferase	8.42 $\pm$ 1.22	11.80 $\pm$ 0.12*
Through hydroxyphenylpyruvate oxidase	5.91 $\pm$ 0.78	8.16 $\pm$ 0.37
Through homogentisate oxidase	5.42 $\pm$ 0.35	8.74 $\pm$ 0.21**
Activity of tyrosine aminotransferase in cell extracts	159 $\pm$ 4	294 $\pm$ 17**
Flux through tyrosine aminotransferase (% of enzyme maximal activity)	5.25 $\pm$ 0.64	4.04 $\pm$ 0.20

13.60 $\pm$ 2.10 nmol of phenylalanine hydroxylated/h per mg). This is consistent with factors other than insulin deficiency being important in changes of phenylalanine metabolism in the chronic-diabetic state. We have confirmed the findings of Donlon & Beirne (1982), who reported that there was no significant decrease in maximal hydroxylase activity of liver extracts from insulin-treated diabetic animals (chronic-diabetic, 464 $\pm$ 37, insulin-treated chronic-diabetic, 445 $\pm$ 64 nmol/h per mg).

Phenylalanine hydroxylation was significantly higher in the chronic-diabetic than in the acute-diabetic state. Fluxes through hydroxyphenylpyruvate oxidase and phenylalanine aminotransferase, however, were not significantly different in the two states. Both diabetic states were associated with an increase in maximal hydroxylase activity compared with the normal fed rat (normal fed, 232 $\pm$ 14, acute diabetic, 617 $\pm$ 16, chronic diabetic, 464 $\pm$ 37 nmol/h per mg; *P* <0.01). Interestingly, the maximal hydroxylase activity in the chronic diabetic state was significantly less than that observed in the acute state (see Table 3). This may represent an adaptive response to the diabetic state

as reflected in the altered protein-turnover rates associated with this condition (Garlick, 1980).

The combination of decreased activity and unchanged flux through phenylalanine hydroxylase in the chronic-diabetic state results in a significant increase in the hydroxylase flux/activity ratio compared with the acute-diabetic state (see Table 3). This may be related to alterations in the phosphorylation state of the hydroxylase reported to occur during diabetes (Donlon & Beirne, 1982). An additional factor may be an altered amount of dihydrobiopterin reductase (NADH:6,7-dihydropteridine oxidoreductase, EC 1.6.99.10), the enzyme responsible for maintaining cofactor in a reduced state. The reductase is induced by glucagon treatment *in vivo* (Brand & Harper, 1974a) and may therefore be sensitive to the changed [glucagon]/[insulin] ratio associated with the diabetic state.

Comparison of tyrosine metabolism in liver cells isolated from normal fed rats and diabetic rats indicates that the diabetic state (acute or chronic) was associated with enhanced tyrosine metabolism [normal fed, 3.32 $\pm$ 0.20, acute-diabetic, 8.42 $\pm$ 1.22 (*P* <0.05), chronic-diabetic, 11.80 $\pm$ 0.12

($P < 0.001$) nmol of tyrosine transaminated/h per mg] and increased maximal aminotransferase activity [normal fed, 127 ± 6 , acute-diabetic, 159 ± 4 ($P < 0.05$), chronic-diabetic, 294 ± 17 ($P < 0.001$) nmol of tyrosine transaminated/h per mg]. Insulin treatment of diabetic animals *in vivo* resulted in decreased tyrosine metabolism (insulin-treated chronic-diabetic, 6.33 ± 1.10 , chronic-diabetic, 11.80 ± 0.12 nmol of tyrosine transaminated/h per mg; $P < 0.001$). This is consistent with the previously observed inhibitory effect of insulin on aminotransferase synthesis (Nakamura *et al.*, 1981). It should be pointed out, however, that other workers have reported that insulin may also decrease the rate of aminotransferase degradation (Spencer *et al.*, 1978).

Comparison of tyrosine metabolism in the acute- and chronic-diabetic states shows that chronic diabetes was associated with higher flux rates (see Table 4). Indeed, chronic diabetes was associated with the highest rates of tyrosine metabolism brought about by any of the treatments used in these studies. The aminotransferase activity was much increased in the chronic-diabetic state compared with the acute-diabetic state; the flux-activity ratio was, however, similar in each case. This contrasts sharply with the phenylalanine hydroxylase flux/activity ratio in the two diabetic states (see above). The fact that the aminotransferase flux/activity ratio of the diabetic states was higher than that of the normal fed state, and was decreased by insulin treatment of diabetic animals, *in vivo*, suggests that other factors, in addition to changed enzyme concentration, may contribute to altered metabolic rates.

General comments

The data presented here emphasize the key roles of phenylalanine hydroxylase and tyrosine aminotransferase in controlling metabolism of their respective substrates. This supports and extends the conclusions of our previous work (Salter *et al.*, 1984). What clearly emerges is that measurement of maximal enzyme activity alone does not necessarily reflect the capacity of intact cells to degrade phenylalanine or tyrosine. A variety of factors, other than maximal enzyme activity, influence the rates of metabolism of these aromatic amino acids in states of extreme hormonal imbalance. The net result is an increased ability of liver cells to dispose of these amino acids. This may be of significance in view of the altered rate of muscle proteolysis associated with such states *in vivo* (Brosnan *et al.*, 1983).

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