Changes in the phospholipid catabolism of mitochondria and microsomes during the development of rat liver

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The lipolytic activities of mitochondrial and microsomal fractions ('microsomes') isolated from foetal, suckling and adult rat liver were compared. The catabolism of endogenous phospholipids was followed by measuring the loss of phospholipids and the appearance of non-esterified fatty acids and lysophosphatides. The rate of mitochondrial phospholipid catabolism does not change significantly during development, but the rate of lipolysis of microsomal phospholipids increases 3-fold during development. Balance studies showed that, in mitochondria and microsomes of foetal, suckling and adult rat liver, fatty acid formation is greatly in excess of the fatty acids that can be accounted for by measuring phospholipid disappearance and lysophosphatide appearance. The hypothesis that this excess fatty acid formation resulted from the lipolysis of mitochondrial and microsomal triacylglycerols were tested and confirmed by preliminary experiments. Mitochondria and microsomes isolated from all developmental ages investigated had phospholipases with A₁ and A₂ activities. The degree of unsaturation of the fatty acids derived from the phospholipids of mitochondria did not vary significantly during development.

In a preliminary study it was shown that the apparent turnover of phospholipids as measured by the incorporation and decay of ¹⁴C-labelled glycerol was considerably faster in foetal than in adult rat liver mitochondria and microsomal fraction ('microsomes') (Pollak, 1978). These results were unexpected since during development there is a significant increase of phospholipid/g of liver (Weinhold & Vilee, 1965; Dallner *et al.*, 1966; Pollak & Munn, 1970), as well as a significant daily increase in liver weight. Hence a greater turnover coupled with an increase of phospholipid/g of liver make considerable energy demands on the growing foetus. As far as the degradation of phospholipids in foetal liver is concerned, it has been found that secondary lysosomes are absent in foetal rat liver and even primary lysosomes are significantly less abundant in foetal rat liver than at later stages of development (Kotoulas & Phillips, 1971). It therefore seemed relevant to investigate and compare changes in phospholipid degradation in late-foetal, suckling and adult rat liver mitochondria and microsomes. Phospholipid catabolism by mitochondria and microsomes of rat liver has been investigated separately in other laboratories (Bjørnstad, 1966a,b; Waite *et al.*, 1969; Nachbaur *et al.*, 1972), but no information is so far available on the developmental changes of phospholipase

activities in hepatic mitochondria and microsomes. Since the present investigation is particularly concerned with the changes in lipolysis in mitochondria and microsomes during differentiation, lipolysis of endogenous phospholipids of liver mitochondria and microsomes during development from 3 days before birth to the adult rat was studied.

Experimental

Animals

Random-bred Wistar rats from the University Animal House were used throughout this investigation. For experiments involving adult rat liver mitochondria, female Wistar rats weighing between 150 and 200 g were used. Foetal and suckling rats of known age were obtained from overnight (16 h) matings.

Isolation of mitochondria and microsomes

Mitochondria were isolated as described previously (Pollak & Sutton, 1980). The isolation medium contained 250 mM-sucrose, 1 mM-Hepes [4-(2-hydroxyethyl)-1-piperazineethanesulphonate], pH 7.4, 1 mM-EDTA, adjusted to pH 7.4 with NaOH, and 0.2% bovine serum albumin.

Microsomes were isolated by centrifuging the

supernatant obtained from the first mitochondrial sedimentation for 65 min at 105 000 *g* in a 42.1 rotor of a Beckman L2-65 centrifuge. The pellets were drained and unless otherwise stated the mitochondrial and microsomal pellets were resuspended in 0.1 M-glycine buffer, pH 9.0.

Measurement of lipolysis

All incubations were performed in flat-bottomed tubes shaken at 100 cycles/min at 37°C. The total incubation volume was 0.5 ml and contained 40 μ mol of glycine, adjusted to pH 9.0, except where indicated otherwise; 4 μ mol of $\text{Ca}(\text{NO}_3)_2$ (unless otherwise stated) and 0.1 ml of mitochondrial or microsomal suspension, containing between 1 and 2 mg of protein.

The reaction was stopped by withdrawing 100 μ l from the incubation mixture and adding it to 3.0 ml of 0.1 M-glycine adjusted with H_2SO_4 to pH 2.7 for the assay of non-esterified fatty acids by the method of Bowyer *et al.* (1978). The method was modified only to the extent that it was converted from an autoanalyser method into a manual method. After the removal of the 100 μ l of the incubation mixture for the fatty acid analysis, 8 ml of chloroform/methanol (2:1) was added to the remaining 0.4 ml to extract phospholipids by the method of Folch *et al.* (1957).

After Folch washing, a portion of the lipid extract was dried on a rotary evaporator at room temperature and made up to 100 μ l with chloroform/methanol (2:1) and samples were applied to a t.l.c. plate. The tube was washed with an additional 50 μ l of chloroform/methanol (2:1), which was also applied on the same t.l.c. spot (2.5 cm long). Any residual phospholipid phosphorus left within the tube was assayed and the phospholipid values were corrected accordingly. Between 85 and 95% of lipid phosphorus was usually recovered from the plates.

The t.l.c. plates were 500 μ m thick, made from a slurry of Kieselgel H (type 60; Merck) and 1 mm- NaCO_3 . The chromatograms were developed with a solvent mixture consisting of chloroform/methanol/acetic acid/water (85:50:10:3, by vol.). After development with solvent and subsequent air drying the spots were made visible by standing the plates in I_2 vapour. All spots were identified by running standards in separate lanes, as well as by co-chromatography. Separation was achieved (in ascending order) for lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, lysophosphatidylethanolamine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol and diphosphatidylglycerol. The individual spots were scraped off the plate into a test-tube and after combustion in the presence of $\text{Mg}(\text{NO}_3)_2$ total phosphate phosphorus was determined (Ames, 1966; Kuiper *et al.*, 1971).

The protein concentration of mitochondria and microsomes was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (fraction V; Sigma) as standard.

G.l.c. of fatty acid methyl esters

Lipid extracts were rotary evaporated (temperature below 50°C) and dissolved in a mixture of 0.8 ml of chloroform, 7.5 ml of methanol, 0.65 ml of water and 0.25 ml of 1 M-NaOH. The lipids dissolved in this mixture were exposed to methanolysis, as described by Dawson (1967), giving rise to the methyl esters of fatty acids derived only from the phospholipids in the lipid extract. After drying by rotary evaporation the fatty acid methyl esters were dissolved in acetone and analysed on a Pye-Unicam 104 gas chromatograph using a 1.5 m column of 10% diethylene glycol succinate adsorbed on to Chromosorb W with a column temperature of 160°C. The fatty acid methyl esters were identified by comparison with the retention time of standard fatty acid methyl esters for each of the fatty acids, as well as by co-chromatography. The methyl esters were prepared by methylating standard fatty acids from Sigma Chemical Co., St. Louis, MO, U.S.A. and P.-L. Biochemicals, Milwaukee, WI, U.S.A., by the method of Nicolaides & Apon (1977). Areas under the peaks were measured by triangulation and the resulting mass-percentage values were converted into mole percentage values. An unsaturation index was obtained by the summation of the number of double bonds $\times$ percentage contribution of the unsaturated fatty acids; the resulting number was divided by 100. This gives the average number of double bonds per fatty acid residue.

Results and discussion

Characterization of phospholipases

The initial experiments were concerned with establishing the optimal conditions for the assay of lipolysis. In Fig. 1 the pH curves for foetal, suckling and adult rat liver mitochondria and microsomes are shown, measuring the unesterified fatty acids derived from the degeneration of endogenous lipids. In all instances optimal production of unesterified fatty acids occurred at about pH 9.0. The pH optima of adult rat liver mitochondria and microsomes with respect to free fatty acid formation, using both endogenous (Bjørnstad, 1966a) and exogenous phospholipid (Nachbaur *et al.*, 1972; Waite *et al.*, 1969) had previously also been shown to be pH 9.0.

By measuring the disappearance of mitochondrial phosphatidylcholine and phosphatidylethanolamine, which amount to 76% of the total phospholipid phosphorus in foetal rat liver (Pollak & Munn, 1970), it was ascertained that pH 9.0 was also the optimal pH when phospholipase activity

Fig. 1. *pH-curves of endogenous fatty acid production by mitochondria and microsomes*

Rat liver mitochondria and microsomes were incubated with shaking at 37°C for 60 min in glycine buffer adjusted to a pH as indicated. Ca^{2+} ($4\mu\text{mol}$) was present and incubation was stopped and fatty acids assayed as described in the Experimental section. Symbols in (a): ■, 21-day-foetal rat liver mitochondria; ○, 3-day-suckling rat liver mitochondria; ●, adult rat liver mitochondria. Symbols in (b): ■, 21-day-foetal rat liver microsomes; ○, 3-day-suckling rat liver microsomes; ●, adult rat liver microsomes.

was measured in this manner (Fig. 2). Microsomal phospholipase activity as measured by phospholipid loss also had an optimal pH of 9.0 (J. K. Pollak, unpublished work).

In all these experiments $4\mu\text{M-Ca}^{2+}$ was included routinely, as it had been shown by other workers, using adult rat liver mitochondria, that Ca^{2+} enhances mitochondrial phospholipase activity when either exogenous or endogenous substrate was used to carry out the assays (Bjørnstad, 1966b; Nachbaur *et al.*, 1972). In Fig. 3 results are presented that show clearly the activation effect of Ca^{2+} on both unesterified fatty acid formation and the disappearance of phospholipids.

Thyroxine and the mercurial mersalyl also activated lipolytic activity of mitochondria, but not to

Fig. 2. *pH curves of phospholipid loss from 21-day-foetal rat liver mitochondria*

Mitochondria isolated from 21-day-foetal rat liver were incubated at 37°C for 60 min in glycine buffer as for experiments described in Fig. 1. The loss of phosphatidylethanolamine and phosphatidylcholine was determined as described in the Experimental section. Symbols: ●, phosphatidylethanolamine; ○, phosphatidylcholine.

Fig. 3. *Effect of Ca^{2+} on endogenous phospholipase action of 21-day-foetal rat liver mitochondria*

Mitochondria isolated from 21-day-foetal rat liver were incubated at 37°C for 60 min as described in the Experimental section, except that Ca^{2+} concentration was varied as indicated. Phospholipid loss and unesterified fatty acid formation was determined as described in the Experimental section. Symbols: ○, phospholipid lost; ●, unesterified fatty acids formed.

the same extent as Ca^{2+} (Table 1). On the other hand, ADP and the analogue of ATP, adenosine 5'-[β,γ -imido]triphosphate, inhibited both mitochondrial and microsomal phospholipase activity, whereas AMP had no effect on the mitochondrial phospholipase and ATP did not inhibit microsomal phospholipase activity (Table 1).

The snake venom phospholipase inhibitor dimethyl DL-2,3-distearoyloxypropyl-2-hydroxyethylammonium acetate (Rosenthal & Geyer, 1960) was found to enhance the release of fatty acids from mitochondria (Table 1). It was subsequently shown by g.l.c. (J. K. Pollak, unpublished work) that the reason for this apparent activation of phospholipase activity was due not only to the fact that this compound did not inhibit mitochondrial and microsomal phospholipase activity, but that the inhibitor served as substrate. Therefore the resulting free stearic acid derived from the inhibitor made up a significant contribution to the liberated unesterified fatty acids.

Comparison of phospholipase A activities at different developmental ages

It was at first considered that the unesterified fatty acids released were derived either by phospholipase A_1 or phospholipase A_2 activities, as it had been established that in adult rat liver neutral lipids amounted to only 9% and 7% of the total mitochondrial and microsomal lipids (Rouser *et al.*, 1968; Glauman & Dallner, 1968). In addition it had also been shown that adult rat liver mitochondria have only marginal triacylglycerol lipase (EC 3.1.1.3) activity (Nachbaur *et al.*, 1972).

The comparison of time curves of fatty acid production from mitochondria isolated from 19-day- and 21-day-foetal, 3-day and 5-day suckling and adult rats showed that unesterified fatty acid formation was most rapid in foetal mitochondria and

least active in mitochondria isolated from adult rat liver (Fig. 4). On the other hand fatty acid liberation in microsomes increased with developmental age (Fig. 5). It should be noted here that in the present experiments no lag period was observed (Figs. 4 and 5); in this respect the results of the present investigation differ from those obtained by Nachbaur *et al.* (1972). These workers used exogenous phospholipids as substrate to assay mitochondrial phospholipase A activity, and found that a 20 min lag period occurred before lipolysis became apparent.

It is difficult to speculate on the role of the greater rate of lipolysis in foetal rat liver mitochondria. Glycogen metabolism in late-foetal rat liver is geared towards glycogen synthesis (Devos & Hers, 1974); therefore the fatty acids released by lipolysis could serve as substrate for oxidative phosphorylation. On the other hand lipolysis may serve to deacylate phospholipids for subsequent reacylation with different fatty acids. This may be of particular importance during foetal and neonatal development when rapid mitochondrial biogenesis takes place. The inhibitory action of ATP on lipolysis may be related to such reacylation.

The increase of microsomal lipolysis that occurs during development may be interpreted in terms of the significant increase of the endoplasmic reticulum within hepatocytes after birth (Dallner *et al.*, 1966).

Balance studies of fatty acid formation and phospholipid loss

In the experiments that indicated that mitochondrial fatty acid liberation decreases with developmental age (Fig. 4), it was found that phospholipid (phosphatidylethanolamine + phosphatidylcholine) loss did not mirror this trend (Table 2). The microsomal lipolytic activity increased with developmental age, whether measured by un-

Table 1. *Activators and inhibitors of lipolysis*

Lipolysis was measured in terms of unesterified fatty acid formation during 60 min incubation as described in the Experimental section.

Compound added	Lipolysis (% of control value)	
	Mitochondria	Microsomes
Control	100	100
$\text{Ca}(\text{NO}_3)_2$ (4 μM)	561	767
Thyroxine (4 μg)	185	—
Mersalyl (25 μg)	185	—
AMP (10 mM)	100	26
ADP (10 mM)	58	23
ATP (10 mM)	47	97
Adenosine 5'-[β,γ -imido]triphosphate (10 mM)	40	39
Snake venom lecithinase A inhibitor (0.3 mg)	221	119

Fig. 4. Time curves of fatty acid formation by mitochondria isolated from different developmental ages

Details of incubation and assay conditions are given in the Experimental section. Results presented are means of at least three experiments. Bars represent $\pm$ S.E.M. (a) Mitochondria from 19-day-foetal rat liver; (b) mitochondria from 21-day-foetal rat liver; (c) mitochondria from 3-day-suckling rat liver; (d) mitochondria from 5-day-suckling rat liver; (e) mitochondria from adult-rat liver.

Fig. 5. Time curves of fatty acid formation by microsomes isolated from different developmental ages

Details of incubation and assay conditions are given in the Experimental section. Results presented are means of at least three experiments. Bars represent $\pm$ S.E.M. Symbols: $\circ$, microsomes from 19-day-foetal rat liver; $\bullet$, microsomes from 21-day-foetal rat liver; $\square$, microsomes from 3-day-suckling rat liver; $\blacksquare$, microsomes from 5-day-suckling rat liver; $\triangle$, microsomes from adult-rat liver.

esterified fatty acid release or phospholipid disappearance (Fig. 5 and Table 3).

When the differences between fatty acid formation and phospholipid loss were more thoroughly investigated, taking into account not only the loss of phospholipid phosphorus, but also allowing for the formation of lysophosphatidylcholine and lysophosphatidylethanolamine, it was found that in 19-day-foetal rat liver mitochondria only 36% of the fatty acids liberated could be accounted for in terms of fatty acids derived from phospholipids (Table 2). The loss of phosphatidylethanolamine + phosphatidylcholine approximated in many instances the total phospholipid loss (J. K. Pollak & W. Harsas, unpublished work), particularly in the microsomal fractions, where there was only marginal formation of lysophosphatides (Table 3). Phosphatidylethanolamine was more readily catabolized than phosphatidylcholine in rat liver mitochondria and microsomes isolated from all developmental ages (results not shown). This has also been shown before for adult rat liver mitochondria (Bjørnstad, 1966b). The only lysophospholipids that were observed in the present investigation were derived from phosphatidylcholine and phosphatidylethanolamine.

The excess of unesterified fatty acids formed in mitochondria decreased with developmental age; in adult rat liver mitochondria the unesterified fatty

Table 2. *Excess of unesterified fatty acid formation over fatty acid loss from mitochondrial phospholipids*

The data represent means (when appropriate $\pm$ S.E.M.). All values represent changes in lipid concentration after 60 min incubation as described in the Experimental section. Columns (3), (5) and (6) are derivative data. Column (3) represents the apparent total fatty acid disappearance as calculated from phospholipid (phosphatidylethanolamine + phosphatidylcholine) loss. Column (6) represents the excess fatty acid formed over loss of fatty acid from phospholipids. Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine.

Source of hepatic mitochondria	Number of experiments	Lipid concn. (nmol/mg of protein)					
		(1) Fatty acid formed	(2) Phospholipid (PE + PC) loss	(3) Column (2) $\times$ 2	(4) Total lysophospholipid formed	(5) Column (3) - (4) (fatty acid from phospholipid)	(6) Excess fatty acid
Foetal rats (19 days)	4	151 $\pm$ 29	36 $\pm$ 4	72	17 $\pm$ 4	55	96
Foetal rats (21 days)	5	115 $\pm$ 12	49 $\pm$ 9	98	16 $\pm$ 3	82	33
Suckling rats (3 days)	2	75	24	48	11	37	38
Suckling rats (5 days)	6	98 $\pm$ 23	37 $\pm$ 4	74	19 $\pm$ 7	55	43
Adult rats	5	73 $\pm$ 13	47 $\pm$ 10	94	24 $\pm$ 4	70	3

Table 3. *Excess of unesterified fatty acid formation over fatty acid loss from microsomal phospholipids*

The data represent means (when appropriate $\pm$ S.E.M.). All values represent changes in lipid concentration after 60 min incubation as described in the Experimental section. Columns (3), (5) and (6) are derivative data. Column (3) represents the apparent total fatty acid disappearance as calculated from phospholipid (phosphatidylcholine + phosphatidylethanolamine) loss. Column (6) represents fatty acids formed in excess of loss of fatty acids from phospholipids. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Source of hepatic mitochondria	Number of experiments	Lipid concn. (nmol/mg of protein)					
		(1) Fatty acid formed	(2) Phospholipid (PE + PC) loss	(3) Column (2) $\times$ 2	(4) Total lysophospholipid formed	(5) Column (3) - (4)	(6) Excess fatty acid
Foetal rats (19 days)	5	91 $\pm$ 13	26 $\pm$ 6	52	4 $\pm$ 1	48	43
Foetal rats (21 days)	5	120 $\pm$ 22	33 $\pm$ 6	66	0	66	54
Suckling rats (3 days)	3	161 $\pm$ 27	52 $\pm$ 17	104	0	104	57
Suckling rats (5 days)	5	149 $\pm$ 37	48 $\pm$ 13	96	0	96	53
Adult rats	4	191 $\pm$ 36	94 $\pm$ 5	188	2	186	5

acid formation and the phospholipid loss virtually balanced (Table 2). This trend was also observed in the microsomes isolated from livers of different developmental ages (Table 3).

The additional fatty acids formed in foetal-rat liver mitochondria and microsomes implies that a significantly greater percentage of neutral lipids, such as triacylglycerols, may be present in these organelles than in the same organelles isolated from adult rat liver. Such neutral lipids may then serve as

substrates to a triacylglycerol lipase present in mitochondria and also present to a lesser extent in microsomes isolated from foetal and suckling rats. Preliminary assays of the triacylglycerol content and the rate of lipolysis of mitochondria and microsomes obtained from foetal and suckling rat liver confirm this hypothesis (J. K. Pollak & W. Harsas, unpublished work).

It has been suggested that rat liver mitochondria possess only phospholipase A₂ (EC 3.1.1.4) activity

Table 4. *The changing fatty acid composition of the phospholipids of rat liver mitochondria during development*
 Conditions of extraction, methanolysis and gas chromatography were as described in the Experimental section. All results are means for four experiments $\pm$ S.E.M. Fatty acid compositions are expressed as mol%. Unsaturation index = average number of double bonds/fatty acid.

Fatty acid	Fatty acid composition (mol%)				
	3 days before birth (foetal)	1 day before birth (foetal)	3-day suckling rats	5-day suckling rats	Adult rats
C _{14:0}	1.2 $\pm$ 0.7	1.1 $\pm$ 0.2	0.5 $\pm$ 0.1	0.6 $\pm$ 0.1	0.6 $\pm$ 0.1
C _{16:0}	22.5 $\pm$ 1.0	20.7 $\pm$ 0.8	23.0 $\pm$ 2.4	25.3 $\pm$ 0.7	17.9 $\pm$ 1.9
C _{16:1}	5.3 $\pm$ 0.4	6.3 $\pm$ 0.7	2.3 $\pm$ 0.2	2.8 $\pm$ 0.7	2.7 $\pm$ 0.6
C _{18:0}	14.6 $\pm$ 1.0	15.1 $\pm$ 0.5	19.4 $\pm$ 2.0	16.3 $\pm$ 1.1	24.0 $\pm$ 2.1
C _{18:1}	18.5 $\pm$ 0.3	18.8 $\pm$ 0.8	8.7 $\pm$ 0.6	7.7 $\pm$ 0.8	11.5 $\pm$ 2.4
C _{18:2}	10.1 $\pm$ 0.4	12.0 $\pm$ 0.7	10.3 $\pm$ 1.2	12.4 $\pm$ 0.3	15.6 $\pm$ 1.9
C _{20:4}	18.1 $\pm$ 0.7	13.2 $\pm$ 0.5	19.1 $\pm$ 0.5	19.2 $\pm$ 0.9	21.1 $\pm$ 1.7
C _{22:6}	9.7 $\pm$ 0.9	12.9 $\pm$ 0.6	16.8 $\pm$ 0.9	15.8 $\pm$ 1.4	6.7 $\pm$ 0.9
Unsaturation index	1.75 $\pm$ 0.08	1.79 $\pm$ 0.03	2.09 $\pm$ 0.08	2.07 $\pm$ 0.06	1.70 $\pm$ 0.10

(Nachbaur *et al.*, 1972). In the present study it has been clearly shown that at all developmental ages phospholipid loss is significantly greater than lysophospholipid formation, and therefore rat liver mitochondria possess either phospholipase(s) with both A₁ and A₂ specificity or alternatively phospholipase A₁ or A₂ plus lysophospholipase activity.

It has been claimed that the phospholipid compositions of foetal-rat liver mitochondria and microsomes have a lower unsaturation index than those isolated from adult liver (Luit *et al.*, 1975); in our experience this is not the case. In the study by Luit *et al.* (1975) docosahexaenoic acid was not included in the comparison between foetal and adult rat liver mitochondria, and since this very-unsaturated fatty acid is present in somewhat greater amounts in foetal- than in adult-rat liver mitochondria it brings the unsaturation index of foetal mitochondria up to the level of that of adult rat liver mitochondria (Table 4). The results from the present study are not only in agreement with a previous study (Dallner *et al.*, 1966), in which docosahexaenoic acid was also shown to be present in foetal rat liver mitochondria and microsomes, but are also compatible with the observation that in foetal rat liver mitochondria and microsomes, lipid peroxidation occurs more extensively than at later developmental ages (Yoshioka *et al.*, 1977). For lipid peroxidation to occur, the presence of unsaturated fatty acids would be a prerequisite, which together with the lower activity of superoxide dimutase in foetal-rat liver (Yoshioka *et al.*, 1977) provides a climate for lipid peroxidation.

In summary it may be stated that the catabolism of mitochondrial phospholipids as measured by the loss of phospholipid phosphorus does not change significantly during development, but lipolysis of microsomal phospholipids increases about 3-fold

during development and differentiation. In foetal rat liver mitochondria and microsomes, the triacylglycerols make a bigger contribution to the lipid composition than at later developmental stages (J. K. Pollak & W. Harsas, unpublished work). The excess of unesterified fatty acid release over the disappearance of phospholipid phosphorus implies that foetal-rat liver mitochondria and microsomes contain an active triacylglycerol lipase as well as triacylglycerols that serve as substrate for this enzyme. The catabolism of the endogenous triacylglycerols becomes less marked as development progresses.

The changes in lipid catabolism that occur in mitochondria and microsomes as described in the present investigation provide an excellent example of the continuous changes and modifications that cellular organelles do undergo during differentiation.

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