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Inhibition by the Branched-Chain 2-Oxo Acids of the 2-Oxoglutarate Dehydrogenase Complex in Developing Rat and Human Brain

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1. The effect of the branched-chain amino acids, namely leucine, isoleucine and valine and their corresponding 2-oxo acids on the metabolism of 2-oxoglutarate by developing rat and human brain preparations was investigated. 2. The decarboxylation of 2-oxo[1-^{14}C]glutarate to $^{14}CO_2$ by mitochondria from adult rat brain was inhibited by the branched-chain 2-oxo acids whereas the branched-chain amino acids had no inhibitory effect on this process. 3. The activity of 2-oxoglutarate dehydrogenase complex was about 0.2 unit/g of brain from 2-day-old rats and increased by about fourfold reaching an adult value by the end of the third postnatal week. 4. The K_m value for 2-oxoglutarate of the 2-oxoglutarate dehydrogenase complex in rat and human brain was 100 and 83 μM respectively. 5. The branched-chain 2-oxo acids competitively inhibited this enzyme from suckling and adult rats brains as well as from foetal and adult human brains, whereas the branched-chain amino acids had no effect on this enzyme. 6. Approximate K_i values for the branched-chain 2-oxo acids found for this enzyme were in the range found for these 2-oxo acids in plasma from patients with maple-syrup-urine disease. 7. The possible significance of the inhibition by the branched-chain 2-oxo acids of the 2-oxoglutarate dehydrogenase complex in brains of untreated patients with maple-syrup-urine disease is discussed in relation to the energy metabolism and the biosynthesis of lipids from ketone bodies.

Maple-syrup-urine disease, an inborn error in the metabolism of the branched-chain amino acids, has been observed to cause a retardation of normal cerebral development and function (Menkes *et al.*, 1954). A genetic defect in a step of the oxidative decarboxylation of the branched-chain 2-oxo acids in the catabolic pathway of the branched-chain amino acids (Menkes, 1959; Mackenzie & Woolf, 1959; Dancis *et al.*, 1959) causes the amounts of leucine, isoleucine and valine and their corresponding 2-oxo acids, namely 2-oxo-4-methylvalerate, 2-oxo-3-methylvalerate and 2-oxoisovalerate to increase markedly in plasma (Westall *et al.*, 1957; Dent & Westall, 1961) and in the brain (Dreyfus & Prensky, 1967) of untreated patients. Although an underlying cause(s) of the pathogenesis of brain dysfunction in the untreated patients with maple-syrup-urine disease is still obscure, toxic effects of these elevated amounts of the branched-chain amino acids and their 2-oxo acids on protein synthesis and on energy metabolism have been suggested (Appel, 1966; Kanzaki *et al.*, 1969).

Animals fed on a diet containing 5% (w/w) leucine had smaller brains than those of controls (Yuwiler & Geller, 1965). Silberberg (1969) observed that the branched-chain amino acids as well as 2-oxo-3-methylvalerate and 2-oxoisovalerate had no effect on myelinating cultures of rat cerebellum whereas 2-oxo-4-methylvalerate markedly delayed myelination in these cultures. The branched-chain 2-oxo acids inhibited pyruvate decarboxylation via the pyruvate dehydrogenase complex in rat and human brains (Bowden *et al.*, 1971; Patel *et al.*, 1973). These 2-oxo acids have been also shown to inhibit a highly purified 2-oxoglutarate dehydrogenase complex from pig heart (Kanzaki *et al.*, 1969) and bovine liver (Johnson & Connelly, 1972). In the present paper the inhibition by the branched-chain 2-oxo acids of the oxidation of 2-oxoglutarate by intact rat brain mitochondria, and of the 2-oxoglutarate dehydrogenase complexes from developing rat and human brains is reported. A preliminary report of this work has been presented (Patel, 1974).

Materials and Methods

Chemicals

2-Oxo[1-^{14}C]glutarate (sp. radioactivity 10–15 mCi/mmol) was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. L-Leucine, L-isoleucine, L-valine, 2-oxo-4-methylvalerate, 2-oxo-3-methylvalerate, 2-oxoisovalerate and thiamine pyrophosphate chloride were from Sigma Chemical Co., St. Louis, Mo., U.S.A. NAD^+ and CoA were purchased from P-L Biochemicals, Milwaukee,

Wis., U.S.A. All other reagents were of the highest purity commercially available.

Animals

Pregnant rats (Carworth Farms Co., New City, N.Y., U.S.A.) and adult male Sprague–Dawley rats weighing about 200g (Charles River Breeding Laboratories, Inc., Wilmington, Mass., U.S.A.) were fed on a commercial chow and water *ad libitum*. Litters, which varied from eight to twelve pups, were kept with their mothers until weaning at about 22 days *post partum*.

Methods

Isolation of mitochondria from 14-day-old and adult rats brains. Mitochondria from rat brain were isolated by the method of Clark & Nicklas (1970) with minor modifications (Patel, 1972). The mitochondria isolated from rat brain by this procedure have previously been shown to have functional integrity as judged by the P/O and the respiratory control ratios (Patel, 1972).

Decarboxylation of 2-oxo[1-^{14}C]glutarate to $^{14}CO_2$ by rat brain mitochondria. The composition of the reaction mixture used to study the decarboxylation of 2-oxo[1-^{14}C]glutarate to $^{14}CO_2$ via the 2-oxoglutarate dehydrogenase complex in intact rat brain mitochondria under optimum conditions is shown in Table 1. In preliminary experiments it was observed that the rate of decarboxylation was linear with time for 15min both in the presence and the absence of added inhibitors (results not shown). Incubations were carried out in 25ml Erlenmeyer flasks with rubber serum stoppers equipped with hanging polyethylene cups (Kontes Glass Co., Vineland, N.J., U.S.A.). The reaction was initiated by the addition of mitochondrial suspension. After 15min 0.4ml of Hyamine-10X hydroxide was injected into the polyethylene cup through the rubber cap, followed by an injection of 0.5ml of 36% (w/v) $HClO_4$, into the reaction mixture. The flasks were shaken for an additional 40min to collect $^{14}CO_2$ and then the contents of the cup were transferred into 15ml of toluene containing (per litre) 4g of 2,5-diphenyloxazole and 100mg of 1,4-bi-(5-phenyloxazol-2-yl)benzene, and the radioactivity was measured in a Packard liquid-scintillation spectrometer.

Assay of 2-oxoglutarate dehydrogenase complex. The washed mitochondria from rat brain were suspended in a phosphate buffer, pH7.4, (10mM-potassium phosphate–1mM-dithiothreitol–1mM-EDTA) and were subjected to three cycles of freezing and thawing immediately before the activity was assayed. No increase in the activity of this enzyme was observed when a freezed–thawed preparation was exposed to ultrasonic vibration for exactly 30s at 60W (model W185, Heat Systems–Ultrasonics, Inc., Plainview, L.I., N.Y., U.S.A.). The composition of the reaction mixture is described in the legend of Table 1. Incubations were carried out in duplicate in the sealed flask and $^{14}CO_2$ was collected as described above. A munit of activity is defined as 1nmol of 2-oxo[1-^{14}C]glutarate decarboxylated to $^{14}CO_2$/min at 37°C. The specific activity is expressed as either per mg of mitochondrial protein or as per g wet wt. of tissue.

For studies in which the development of 2-oxoglutarate dehydrogenase complex activity in rat brain during the postnatal period was investigated, brain homogenates (10%, w/v) in 0.3mM-sucrose were centrifuged at 100000*g* for 30min and the pellet was resuspended in the phosphate buffer, pH7.4. It was further treated as outlined for human brain preparation.

Human brain preparation. Human foetal brains obtained after therapeutic abortion (in less than 30min after death) were placed in ice-cold 0.3M-sucrose. Adult human brain cortex was obtained at autopsy (within 16h after death) and placed in ice-cold 0.3M-sucrose. Homogenates of brain (20%, w/v) in 0.3M-sucrose were prepared and centrifuged at 100000*g* for 30min. The supernatant was discarded and the pellet was resuspended in the phosphate buffer, pH7.4. The suspension was subjected to three cycles of freezing and thawing immediately before the activity of 2-oxoglutarate dehydrogenase complex was assayed as described above. To minimize dilution of the specific radioactivity of added 2-oxo[1-^{14}C]-glutarate in the reaction mixture by endogenous 2-oxoglutarate and glutamate present in the tissue, the soluble fraction of the brain was removed by centrifugation as described above.

Protein measurements. Mitochondrial protein was measured as described by Lowry *et al.* (1951), with dry bovine serum albumin as a standard protein.

Results

In studies which are not reported in detail here, mitochondria from adult rat brain when incubated in the complete reaction mixture as described in Table 1 decarboxylated 2-oxo[1-^{14}C]glutarate at a rate of 29nmol/min per mg of mitochondrial protein. The omission of phosphate and $MgCl_2$ resulted in a marked decrease in the decarboxylation by approx. 94 and 80% respectively. The omission of L-malate decreased the decarboxylation by about 50%. The observed decrease in 2-oxoglutarate decarboxylation in the absence of either phosphate or L-malate is largely due to a decrease in the transport of 2-oxoglutarate across the inner mitochondrial membrane. Similar requirements of these compounds for the transport of 2-oxoglutarate have been also observed with isolated rat liver mitochondria (Robinson & Chappell, 1967). The omission of

Table 1. *Effect of the branched-chain amino acids and 2-oxo acids on the decarboxylation of 2-oxo[1-^{14}C]glutarate to $^{14}CO_2$ by mitochondria from adult rat brain and on the activity of 2-oxoglutarate dehydrogenase complex from brain mitochondria*

2-Oxoglutarate decarboxylation by mitochondria from adult rat brain. The reaction mixture contained, in a final volume of 2ml, 6.6mM-potassium phosphate (pH7.4), 6.6mM-triethanolamine (pH7.4), 0.5mM-L-malate, 10mM-$MgCl_2$, 1mM-2-oxo[1-^{14}C]glutarate (125d.p.m./nmol) and 155mM-sucrose. Various amino acids and 2-oxo acids were added to final concentrations of 5mM. Incubations were carried out for 15min at 37°C with about 0.4mg of mitochondrial protein being added to each flask. Each determination was carried out in duplicate and $^{14}CO_2$ was collected as described in the Materials and Methods section. The results are the means ±S.E.M. of five to eight experiments. Activity of the 2-oxoglutarate dehydrogenase complex. The reaction mixture contained, in a final volume of 1ml, 11mM-potassium phosphate buffer (pH7.4), 1.1mM-dithiothreitol, 1.1mM-EDTA, 3mM-$MgCl_2$, 0.1mM-thiamine pyrophosphate, 0.2mM-CoA, 2mM-NAD^+, 1mM-2-oxo[1-^{14}C]glutarate (50d.p.m./nmol) and about 0.3mg of mitochondrial protein. Mitochondria from adult rat brain were isolated and the activity of this enzyme was assayed as described in the Materials and Methods section. Incubations were carried out in duplicate for 10min at 37°C and $^{14}CO_2$ was collected as described in the Materials and Methods section. The results are the means ±S.E.M. of five to seven experiments.

Addition	Amount of 2-oxo[1-^{14}C]glutarate decarboxylated to $^{14}CO_2$ by mitochondria		Activity of 2-oxoglutarate dehydrogenase complex	
	(nmol/min per mg of protein)	(% inhibition)	(munits/mg of protein)	(% inhibition)
None	30.0±0.7	—	50±2.5	—
Leucine	30.7±1.5	0	43±3.9	14
2-Oxo-4-methylvalerate	20.8±2.1	31	28±2.1	44
Isoleucine	32.0±1.5	0	46±4.0	8
2-Oxo-3-methylvalerate	16.7±1.7	44	11±1.0	78
Valine	30.1±1.2	0	41±4.9	18
2-Oxoisovalerate	16.7±1.0	44	10±0.3	80

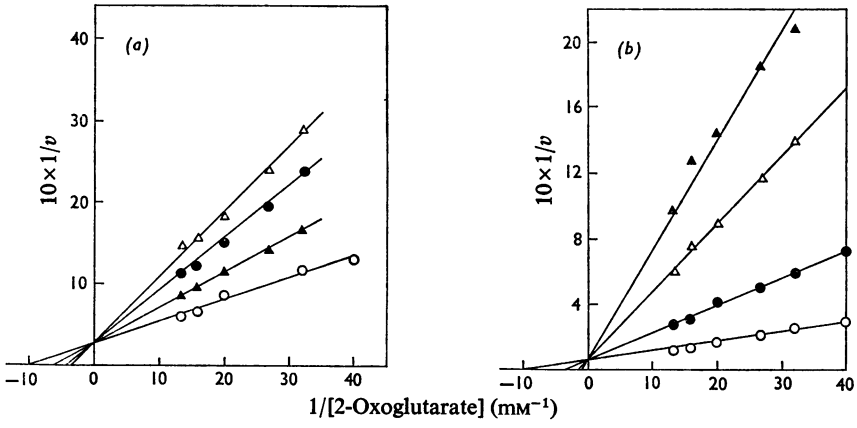

Fig. 1. *Reciprocal plots of initial velocities of the 2-oxoglutarate dehydrogenase complex in brain mitochondria from 2-week-old and adult rats against 2-oxoglutarate concentrations at a fixed inhibitor concentration*

Unless otherwise indicated, the reaction mixture was as described in Table 1. The concentration of 2-oxoglutarate was varied as indicated. Mitochondrial activity of 2-oxoglutarate dehydrogenase complex in 2-week-old (*a*) and adult (*b*) rat brain was assayed as described in the Materials and Methods section. Other additions were: ○, none; ●, 6mM-2-oxo-4-methylvalerate in both (*a*) and (*b*); △, 2-oxo-3-methylvalerate (2mM in *a* and 4mM in *b*); ▲, 2-oxoisovalerate (1mM in *a* and 4mM in *b*).

sucrose caused a small decrease (about 20%) in the decarboxylation by brain mitochondria owing to their swelling in a hypo-osmotic medium and consequently a leakage of some compounds such as CoA from mitochondria. The optimum concentration of 2-oxoglutarate in the incubation medium for a maximum rate of decarboxylation of this compound by brain mitochondria was approx. 1mM with an

apparent K_m of 0.15mM (results not shown). The addition of L-malate enhanced the rate of decarboxylation of 2-oxoglutarate by brain mitochondria; an optimum effect was observed in the range of 0.3–1mM-L-malate and in the present study 0.5mM-L-malate was used.

Because the activity of the 2-oxoglutarate dehydrogenase complex in rat brain has not been described, an assay system for this enzyme was developed by measuring the decarboxylation of 2-oxo[1-^{14}C]-glutarate to $^{14}CO_2$. In experiments not reported here it was observed that the activity of the 2-oxoglutarate dehydrogenase complex in disrupted rat brain mitochondria was linear with respect to both the amount of mitochondrial protein (at least up to 0.5mg) added and the incubation time (up to 15min). The activity of this enzyme was about 50munits/mg of mitochondrial protein in adult rat brain (Table 1). The omission of CoA, NAD^+ or $MgCl_2$ decreased markedly its activity whereas the omission of thiamine pyrophosphate caused only a 20% decrease in activity suggesting that this coenzyme is tightly bound to the enzyme. The addition of any cofactor at twice the concentration indicated in Table 1 had no effect on the activity (the results are not shown) suggesting that the maximum requirements for cofactors were met.

The effect of the branched-chain amino acids and their 2-oxo acids on the decarboxylation of 2-oxo-[1-^{14}C]glutarate by both intact mitochondria and the activity of the 2-oxoglutarate dehydrogenase complex in disrupted mitochondria was examined. As shown in Table 1, leucine, isoleucine and valine at 5mM concentration had no significant effect on the decarboxylation reaction by intact and disrupted mitochondrial preparations. The small amount of inhibition (less than 20%) of the 2-oxoglutarate dehydrogenase complex in the presence of the branched-chain amino acids was markedly decreased if aminoxyacetate (0.5mM), an inhibitor of amino acid transferases (Hopper & Segal, 1962), was also added to the mixture (results not shown). The presence of the branched-chain amino acid transferases in the mammalian brain is known (Lysiak *et al.*, 1970) and it is suggested that the observed inhibition was due to the formation of 2-oxo acids from the branched-chain amino acids in the presence of 2-oxoglutarate. The addition of 2-oxo-4-methylvalerate, 2-oxo-3-methylvalerate and 2-oxo-isovalerate at 5mM concentration inhibited the decarboxylation of 2-oxoglutarate by mitochondria by 31, 44 and 44% respectively (Table 1). In the same order of 2-oxo acids the activity of 2-oxoglutarate dehydrogenase complex was inhibited by approx. 44, 78 and 80% respectively (Table 1). The inhibition to lesser extent as observed with the intact mitochondria is possibly due to restricted permeability of the branched-chain 2-oxo acids.

Reciprocal plots for 2-oxoglutarate of the 2-oxoglutarate dehydrogenase complex in mitochondria from 2-week-old and adult rats are shown in Fig. 1. The K_m value for 2-oxoglutarate of the cerebral enzyme was 100μM which is in the range of the observed K_m values of 110 and 81μM found for highly purified enzymes from pig heart (Kanzaki *et al.*, 1969) and bovine liver (Johnson & Connelly, 1972) respectively. The activity of the 2-oxoglutarate dehydrogenase complex from brains of suckling and adult rats was competitively inhibited by three branched-chain oxo acids (Fig. 1) with apparent K_i values of 3.8, 1.3 and 1.4mM for 2-oxo-4-methylvalerate, 2-oxo-3-methylvalerate and 2-oxoisovalerate respectively (Table 2). The latter 2-oxo acids were effective inhibitors of this enzyme and this finding is consistent with experiments reported in Table 1. In an experiment not shown here using three different concentrations of 2-oxoisovalerate a competitive inhibition of the 2-oxoglutarate dehydrogenase complex from rat brain mitochondria was observed. The K_m value for 2-oxoglutarate of the 2-oxoglutarate dehydrogenase complex from foetal and adult human brains was 83μM (Fig. 2). The activity of this enzyme from foetal and adult human brains was also competitively inhibited by 2-oxo-4-methylvalerate and 2-oxoisovalerate with K_i values of 4.4 and 1.4mM respectively (Fig. 2; Table 2).

Table 2. *Comparison of K_i values of 2-oxoglutarate dehydrogenase complex from mammalian brains*

The values for human plasma are from Snyderman *et al.* (1964) and Johnson & Connelly (1972). N.D., Not determined.

Amino acid or 2-oxo acid	Human plasma (mM)		K_i values of the 2-oxoglutarate dehydrogenase complex (mM)	
	Normal	Maple-syrup-urine disease	Human brain	Rat brain
Leucine	0.1–0.4	4–6	—	—
2-Oxo-4-methylvalerate	0.1–0.4	2–4	4.5	3.8
Isoleucine	0.07–0.25	1.0–1.5	—	—
2-Oxo-3-methylvalerate	0.07–0.25	1.0–1.5	N.D.	1.3
Valine	0.24	1.85	—	—
2-Oxoisovalerate	0.24	1.85	1.4	1.4

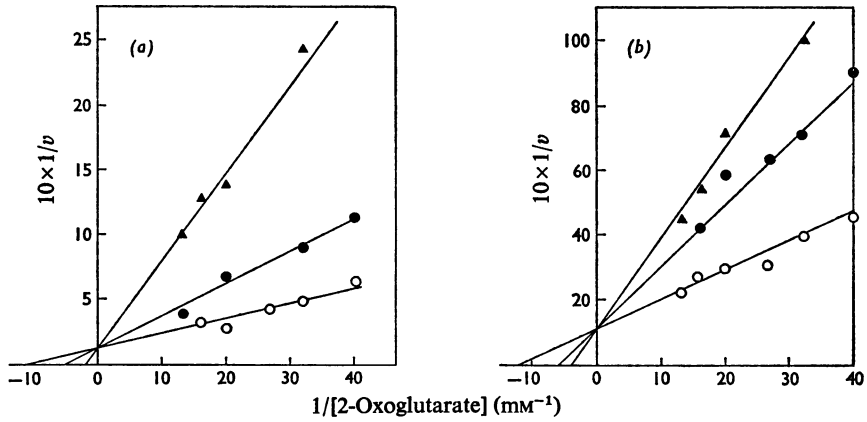

Fig. 2. *Reciprocal plots of initial velocities of the 2-oxoglutarate dehydrogenase complex from foetal and adult human brain against 2-oxoglutarate concentrations at a fixed inhibitor concentration*

Unless otherwise indicated, the reaction mixture was as described in Table 1. The concentration of 2-oxoglutarate was varied as indicated. The activity of the 2-oxoglutarate dehydrogenase complex in foetal (*a*) and adult (*b*) human brain preparations was assayed as described in the Materials and Methods section. Other additions were: ○, none; ●, 6mM-2-oxo-4-methylvalerate; ▲, 4mM-2-oxoisovalerate.

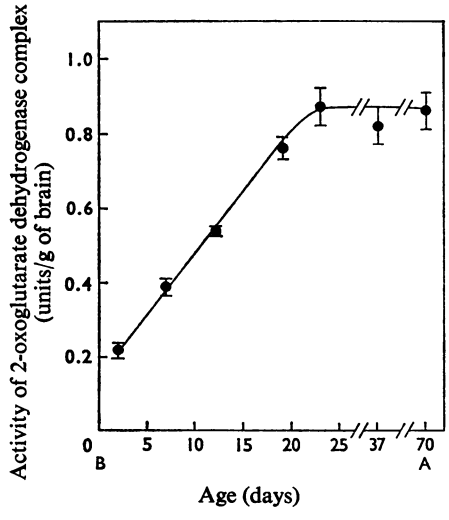

Fig. 3. *Development of the activity of 2-oxoglutarate dehydrogenase complex in rat brain during the postnatal period*

The activity of 2-oxoglutarate dehydrogenase complex in rat brain was assayed as described in the Materials and Methods section. The results are the means ±S.E.M. of six animals. A, Adults; B, birth.

During the course of our study it was observed that the activity of 2-oxoglutarate dehydrogenase complex was lower in mitochondria isolated from 2-week-old rats as compared with those from adult animals. In view of this observation the development of this enzyme complex in rat brain during the postnatal period was investigated. The activity was approx. 0.2unit/g of brain from 2-day-old rats and increased linearly by about fourfold over the next 20 days before reaching an adult value (Fig. 3). The postnatal development of this enzyme complex is similar to that of other tricarboxylic acid-cycle enzymes as reported by Wilbur & Patel (1974).

Discussion

The mechanism(s) by which brain damage is produced in untreated patients with maple-syrup-urine disease is not understood. Several theories have been advanced to suggest the toxic effects of circulating abnormally high amounts of plasma branched-chain amino acids and their 2-oxo acids on the biosynthesis of macromolecules and energy metabolism in brains of affected patients (Appel, 1966; Kanzaki *et al.*, 1969). Injection of the branched-chain amino acids in large amounts into pregnant rats has been shown to alter the free amino acid pools in brains of foetal rats by inhibiting the transport of some amino acids (Carver, 1969). Appell (1966) observed a decrease in the charging of tRNA in the presence of high concentrations of the branched-chain amino acids and suggested it as a possible mechanism by which cerebral protein synthesis is affected in these patients. A marked decrease in the amounts of proteolipids and cerebrosides in brains from patients with maple-syrup-urine disease suggests an impairment in myelination (Menkes *et al.*, 1965; Prensky *et al.*, 1968; Prensky & Moser, 1966;

Menkes & Solcher, 1967). A decrease in the biosynthesis of protein (Appel, 1966) and lipids (M. S. Patel, unpublished work as described below) is consistent with the observed decrease in the amount of myelin in brains of affected patients (Menkes *et al.*, 1965; Prensky & Moser, 1966).

O_2 uptake by rat brain slices is decreased in the presence of 2-oxoisovalerate (Howel & Lee, 1963). Pyruvate derived from glucose via glycolysis is largely metabolized via the action of the pyruvate dehydrogenase complex. The branched-chain 2-oxo acids have been shown to inhibit the decarboxylation of [1-^{14}C]pyruvate to $^{14}CO_2$ by rat (Dreyfus & Prensky, 1967; Bowden *et al.*, 1971) and human (Patel *et al.*, 1973) brain preparations. Bowden *et al.* (1971) and Johnson & Connelly (1972) observed that 2-oxo-4-methylvalerate competitively inhibited the pyruvate dehydrogenase complex from rat brain and bovine liver respectively, but Blass & Lewis (1973) reported that this enzyme complex from ox brain is not inhibited by 2-oxo-3-methylvalerate. The other two 2-oxo acids of valine and isoleucine are also found to inhibit this enzyme from bovine liver (Johnson & Connelly, 1972) and pig heart (Kanzaki *et al.*, 1969).

The branched-chain 2-oxo acids are also shown to inhibit purified 2-oxoglutarate dehydrogenase complex from pig heart (Kanzaki *et al.*, 1969) and bovine liver (Johnson & Connelly, 1972). The present paper shows that similar inhibition of the 2-oxoglutarate dehydrogenase complex also occurs in rat and human brain preparations (Figs. 1 and 2). In view of these findings it is possible to conclude that the branched-chain 2-oxo acids exert differential effects on the pyruvate dehydrogenase complex and the 2-oxoglutarate dehydrogenase complex. 2-Oxo-4-methylvalerate is shown to exert a greater inhibitory effect on the pyruvate dehydrogenase complex (Johnson & Connelly, 1972), whereas 2-oxoisovalerate and 2-oxo-3-methylvalerate exert greater inhibitory effects on the 2-oxoglutarate dehydrogenase complex (Table 2; Johnson & Connelly, 1972). The concentrations of the branched-chain 2-oxo acids in brains of patients with maple-syrup-urine disease are not known; however, an accumulation of these 2-oxo acids in a patient's brain at autopsy has been documented (Dreyfus & Prensky, 1967). It should be noted that the apparent K_i values reported here for the branched-chain 2-oxo acids of human cerebral enzyme are close to the amounts of these 2-oxo acids circulating in the plasma of untreated patients with maple-syrup-urine disease (Snyderman *et al.*, 1964; also see Table 2). An increased excretion of 2-oxoglutarate in urine of untreated patients (Patrick, 1961; Woody & Harris, 1965) is also consistent with an inhibition *in vivo* by the branched-chain 2-oxo acids of 2-oxoglutarate metabolism.

The 2-oxoglutarate dehydrogenase complex is part of the tricarboxylic acid cycle and plays an important role in the oxidation of both glucose and ketone bodies. The inhibition of the pyruvate dehydrogenase complex and the 2-oxoglutarate dehydrogenase complex by the branched-chain 2-oxo acids would significantly decrease the oxidative metabolism of glucose and ketone bodies, and hence energy metabolism. The developing brain is known to effectively utilize ketone bodies for the biosynthesis of lipids (Edmond, 1974). Succinyl-CoA is required for the formation of acetoacetyl-CoA from acetoacetate and hence a decrease in the formation of succinyl-CoA from 2-oxoglutarate in the presence of the branched-chain 2-oxo acids could impair the metabolism of ketone bodies. In fact, the branched-chain 2-oxo acids are found to inhibit the oxidation of 3-hydroxy[3-^{14}C]butyrate to $^{14}CO_2$ and its incorporation into fatty acids and cholesterol by developing rat brain (M. S. Patel, unpublished work). The decrease in energy metabolism and in the biosynthesis of lipids from glucose and ketone bodies in the developing brain may in part contribute to a poor prognosis (an early death in the first year of life or severe mental retardation) in surviving patients with maple-syrup-urine disease.

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