The Activities of the Citric Acid-Cycle Enzymes in Rat Bone-Marrow Cells

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The activities of the eight citric acid-cycle enzymes of rat bone-marrow cells were determined along with several other mitochondrial and non-mitochondrial enzymes. Four of the citric acid-cycle enzymes (aconitase, succinyl-CoA thiokinase, α -oxoglutarate dehydrogenase and succinate dehydrogenase) have closely similar low activities; two [isocitrate dehydrogenase (NAD) and citrate synthase] have intermediate activities; the remaining two (malate dehydrogenase and fumarase) have high activities. The other enzymes surveyed also exhibited a spread of three orders of magnitude, the mitochondrial enzymes showing no less variation than the others.

The course of development of mature mammalian erythrocytes from undifferentiated precursor cells is characterized by several striking biochemical changes. In addition to the acquisition of new functions such as haemoglobin synthesis there is the virtually complete loss of oxidative metabolism mediated via the citric acid cycle and associated reactions (Schweiger, 1962). As a preliminary step to the study of possible molecular mechanisms operating in this aspect of cytodifferentiation, a survey of the activities of the citric acid-cycle enzymes in bone-marrow cells was carried out. These studies were not designed to investigate the regulation of the operation of the citric acid cycle, but to enable the eventual study of the regulation of synthesis of the enzymes concerned.

METHODS AND MATERIALS

Bone-marrow cells from the femora and tibiae of adult Wistar rats were flushed out of the bones with cold 0.15M-NaCl in 4mM-sodium phosphate buffer, pH 7.4, filtered through glass cloth to remove bits of connective tissue and bone fragments, and collected by centrifugation at 1° for 5 min. at 500g. After washing with phosphate-buffered saline, the packed volume of the cells was measured; it usually amounted to about 0.2 ml. per rat. Except where indicated otherwise, the cells were then suspended in 0.1M-sodium phosphate buffer, pH 7.4, and disrupted ultrasonically in an ice bath for 2 min. with an MSE 100W ultrasonic disintegrator at an amplitude of 6 μ . The suspension was then centrifuged in the cold for 20 min. at 1000g and the clear pink supernatant removed for assay of enzymic activity after suitable dilution. For some enzymes, as noted below, the ultrasonically treated suspension was used without prior centrifugation. Each assay

was done on a freshly prepared extract of marrow cells unless there were data showing no loss on storage.

All assays using the 340 m μ absorption band of NADH were done at 25° on a thermostatically controlled Gilford recording spectrophotometer. For other spectrophotometry a Zeiss PMQ II instrument was used. All assays were done under conditions where the rate of the measured reaction was proportional to the amount of marrow-cell extract used.

Where mitochondria were prepared, the marrow cells, in 0.25M-sucrose, were disrupted ultrasonically for 10 sec. and centrifuged for 10 min. at 750g; the pellet was discarded and the supernatant centrifuged at 13000g for 10 min. The pelleted mitochondria were taken up in 0.1M-sodium phosphate buffer, pH 7.4.

Citrate synthase (EC 4.1.3.7) was determined by method b described by Ochoa (1955a). The rate of NADH formation was proportional to the amount of extract (made in 0.1M-tris-HCl buffer, pH 7.4) only when the change in E_{340} was 0.015/min. or less. Aconitase (EC 4.2.1.3) (in 0.1M-sodium phosphate buffer containing 1mM-cysteine) was determined by linking the reaction to that of NADP-linked isocitrate dehydrogenase (EC 1.1.1.42) by using the method of Ochoa (1951). With this method there was a marked lag in increase in E_{340} unless the citrate concentration was kept very low, about 0.5 mM. With fresh marrow-cell extracts added iron salts had no effect, and extracts stored at -15° overnight were devoid of aconitase activity that could not be restored by addition of iron salts and cysteine. For the determination of NAD-linked isocitrate dehydrogenase (EC 1.1.1.41), the extraction medium and method of Goebel & Klingenberg (1964) were used. The method of Kaufman (1955) was used for α -oxoglutarate dehydrogenase (EC 1.2.4.2) with the medium of Goebel & Klingenberg (1964) for the extraction. Succinate dehydrogenase (EC 1.3.99.1) was determined by using the method of Clark & Porteous (1964) originally described for succinate estimation. Succinyl-CoA thiokinase (EC 6.2.1.4) was determined by using an excess of washed pigeon breast-muscle succinate dehydrogenase with succinyl-CoA as

substrate, and determining the rate of GDP (0.5mm)-dependent succinate formation. The marrow-cell preparation used for this determination was treated ultrasonically for 30sec. and used without centrifugation. The rate of GDP-independent succinate formation (non-specific deacylation) was about the same as that of the GDP-dependent process. The indicator succinate dehydrogenase preparation did not contain any GDP-dependent deacylase activity. At the same time a determination of marrow-cell succinate dehydrogenase was done on the same preparation and the observed value was within the range of values found with other preparations.

Fumarase (EC 4.2.1.2) was determined by the method of Racker (1950) with the extraction buffer containing 13mm-cysteine. Malate dehydrogenase (EC 1.1.1.37) was determined by the method of Ochoa (1955b). The mitochondrial component of malate dehydrogenase was found to be 28% of the total, and 72% of the total was indicated as non-mitochondrial malate dehydrogenase. Cytochrome oxidase (EC 1.9.3.1) was determined as described by Smith (1955), lactate dehydrogenase (EC 1.1.1.27) as described by Bergmeyer, Bernt & Hess (1963) and glutamate dehydrogenase (EC 1.4.1.2) as described by Williamson, Lund & Krebs (1967). For the determination of β -hydroxybutyrate dehydrogenase (EC 1.1.1.30) the marrow cells were treated ultrasonically in the medium described by Lehninger, Sudduth & Wise (1960) and the extract was assayed by the method of the same authors.

Acetyl-CoA was prepared as described by Ochoa (1955a), succinyl-CoA was a gift from Dr D. H. Williamson, indicator enzymes and coenzymes were bought from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany) and all other reagents were A.R. grade wherever possible.

All results are expressed as units (μ moles of substrate used or product formed/min.)/g. wet wt. of marrow cells, assuming that the density of the cell pellet was unity.

RESULTS AND DISCUSSION

Fourteen enzymes were surveyed; the eight of the citric acid cycle, three other mitochondrial enzymes (cytochrome oxidase, glutamate dehydrogenase and β -hydroxybutyrate dehydrogenase) and three non-mitochondrial enzymes [malate dehydrogenase, isocitrate dehydrogenase (NADP) and lactate dehydrogenase], the last-named as a representative of the glycolytic system that is retained by the mature erythrocyte. The results of these assays are summarized in Table 1. In addition to units/g., the enzyme activities are also expressed as activities relative to the value for aconitase, which was the lowest of the citric acid-cycle enzymes, as unity. The enzymic activities were not expressed with relation to cytochrome oxidase as suggested by Pette (1966), since it is one of those enzymes concerned with oxidative metabolism that is lost during erythrocyte development (Schweiger, 1962). Lactate dehydrogenase activities were determined for each marrow-cell preparation, but these were not used to normalize the activities of various preparations since simple

Table 1. *Activities of citric acid-cycle and other enzymes in rat bone-marrow cells*

One activity unit is equivalent to 1 μ mole of substrate used or product formed/min. at 25°. Relative activities are based on the amount of aconitase activity taken as unity.

Enzyme	Activity (units/g.)	Relative activity
Aconitase	0.16	1.0
Succinyl-CoA thiokinase	0.17	1.1
α -Oxoglutarate dehydrogenase	0.19	1.2
Succinate dehydrogenase	0.20	1.2
NAD-linked isocitrate dehydrogenase	0.36 (1.4*)	2.2 (8.8*)
Citrate synthase	1.62	10.1
Malate dehydrogenase (mitochondrial)	12.5	78
Fumarase	25.2	158
β -Hydroxybutyrate dehydrogenase	0.11	0.7
NADP-linked isocitrate dehydrogenase	1.53	9.6
Glutamate dehydrogenase	2.40	15
Cytochrome oxidase	20.7	130
Malate dehydrogenase (non-mitochondrial)	32	200
Lactate dehydrogenase	49	306

* Activity and relative activity of NAD-linked isocitrate dehydrogenase when the fourfold activation by 6.6mm-citrate is taken into account.

statistical analysis showed just as much variation in lactate dehydrogenase activity as, e.g., in aconitase activity. Generally the standard deviation of the mean for those enzyme activities from several different marrow preparations was about 30% of the mean.

The relative activities found fell into three fairly distinct groups based on order of magnitude. The lowest group (range 0.7–2.2) includes five of the citric acid-cycle enzymes, namely aconitase, isocitrate dehydrogenase (in the absence of citrate), α -oxoglutarate dehydrogenase, succinyl-CoA thiokinase and succinate dehydrogenase, in addition to β -hydroxybutyrate dehydrogenase. The second group (range 9.6–15) includes one citric acid-cycle enzyme, citrate synthase, as well as glutamate dehydrogenase and the NADP-linked isocitrate dehydrogenase. The group with the highest activity (range 78–306) contains the remaining two citric acid-cycle enzymes (malate dehydrogenase and fumarase), in addition to cytochrome oxidase, the non-mitochondrial malate dehydrogenase and lactate dehydrogenase.

Because bone marrow was the only tissue studied in detail, no direct comparisons can be made with the data on 'constant-proportion' enzymes of the citric acid cycle reported by Pette (1966). There

are some evident differences, however, between bone marrow and the other tissues. In marrow, citrate synthase activity is an order of magnitude greater than those of the five members of the citric acid cycle with low activity, and fumarase activity is still another order of magnitude greater, whereas in rat heart, liver and kidney cortex activities of fumarase, citrate synthase and succinate dehydrogenase are all about the same, with that of NAD-linked isocitrate dehydrogenase being an order of magnitude lower. In brain cortex NAD-linked isocitrate dehydrogenase activity is somewhat higher than in the other three tissues. The observations on marrow cells do not materially weaken the concept of 'constant-proportion' enzymes involved in the citric acid cycle, but neither do they offer any support.

The properties of some of these marrow-cell enzymes deserve further comment. In agreement with the finding with *Neurospora* NAD-linked isocitrate dehydrogenase (Sanwal, Zink & Stachow, 1963), there is appreciable activation by added citrate, the magnitude of the citrate effect being greatest at low isocitrate concentration and disappearing at higher concentration. For the purpose of this paper I have chosen the concentration of isocitrate that shows a citrate effect amounting to a fourfold stimulation when the citrate concentration is 6.6mM. This yields a minimal estimate of the total potential activity of NAD-linked isocitrate dehydrogenase. When marrow-cell preparations have been stored at -15° for 3 weeks there appears to be no effect of citrate discernible, other than a slight, non-competitive, inhibition.

Succinate dehydrogenase was determined on a mitochondrial preparation obtained from cells after a very short period (10sec.) of ultrasonic treatment. If the cells in phosphate buffer were treated ultrasonically for 2 min. and the suspension was centrifuged for 30min. at 750g, a clear supernatant was obtained that had about 80% of the original activity in a soluble form. In contrast, when isolated mitochondria were subjected to 1 min. of ultrasonic treatment, about 70% of the succinate dehydrogenase activity was lost. At present I have no explanation for these contradictory findings.

Marrow-cell and erythrocyte lactate dehydrogenase are very closely similar with respect to the two isoenzymic forms of this enzyme. I find that the ratio of activity with 0.3mM-pyruvate to that with 10mM-pyruvate for erythrocytes is 1.20 whereas for marrow it is 1.11; both are therefore of the skeletal-muscle type. Undoubtedly an appre-

ciable fraction of the marrow-cell lactate dehydrogenase derives from the accompanying erythrocytes. The question whether marrow-cell lactate dehydrogenase might be significantly different from the erythrocyte enzyme with respect to the proportion of the isoenzymes could be approached by using methods that would remove selectively either the erythrocytes or the nucleated cells of the heterogeneous marrow-cell populations.

Though it is obvious that analyses of enzyme activities cannot, and in this case were not intended to, give any definitive information about the regulation of the citric acid cycle, these data do provide a start into the investigation of how synthesis of these enzymes may be regulated. This latter goal is the continuing purpose of these studies.

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