Organ Specificity and Lactate-Dehydrogenase Activity

SOME PROPERTIES OF HUMAN SPERMATOZOAL LACTATE DEHYDROGENASE

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1. The presence of a characteristic lactate-dehydrogenase isoenzyme (LD_x) in human, mouse and dog testis and in human spermatozoa has been confirmed by electrophoresis on cellulose acetate and on polyacrylamide gel. 2. The human spermatozoal isoenzyme exhibits a much higher affinity for 2-oxobutyrate than any of the five isoenzymes found in other tissues. K_m values of 0·05mM for pyruvate and 0·18mM for 2-oxobutyrate were obtained. 3. LD_x differs from other lactate-dehydrogenase isoenzymes in that its properties cannot be correlated with its electrophoretic mobility. It resembles LD_1 in being strongly inhibited by 0·2mM-oxalate and relatively resistant to 2M-urea, and in being relatively stable to heat. 4. The surprisingly high activity of LD_x with 2-oxobutyrate suggests that this substance or 2-hydroxybutyrate may play a part in spermatozoal metabolism.

The presence of five lactate-dehydrogenase isoenzymes (LD_{1-5}) in most human and animal tissues is well established and can be demonstrated by a variety of electrophoretic techniques (Wieland & Pfleiderer, 1957; Wieme, 1959; Plagemann, Gregory & Wróblewski, 1960*a*). The electrophoretically fastest component (LD_1) migrates with the mobility of an α_1-globulin, whereas the slowest fraction (LD_5) is associated with γ-globulin. An unusual lactate-dehydrogenase isoenzyme (LD_x) has recently been detected in post-pubertal human testis and spermatozoa by Blanco & Zinkham (1963) and by Goldberg (1963).

According to Zinkham, Blanco & Kupchyk (1963) LD_x accounts for about 80% of the lactate-dehydrogenase activity of human spermatozoa. These workers also detected LD_x components in the testes of other species. The relative electrophoretic mobility of the extra isoenzyme differs from species to species and in some it occurs in multiple forms, but in man the single LD_x has an electrophoretic mobility intermediate between those of LD_3 and LD_4. The LD_{1-5} isoenzymes exhibit a gradation in their properties in parallel with their mobilities. Thus Blanco & Zinkham (1963) showed that LD_x of human testis is intermediate between LD_3 and LD_4 in terms of electrophoretic mobility, heat stability and kinetic behaviour with NAD analogues.

Among the properties that can be used to differentiate between LD_{1-5} isoenzymes are thermal stabilities (Plagemann, Gregory & Wróblewski, 1960*b*), substrate affinities (Rosalki & Wilkinson, 1960; Plummer, Elliott, Cooke & Wilkinson, 1963) and the effects of inhibitors (Wieland, Pfleiderer & Ortanderl, 1959; Plummer & Wilkinson, 1961, 1963; Richterich & Burger, 1963; Plummer, Wilkinson & Withycombe, 1963; Emerson, Wilkinson & Withycombe, 1964; Withycombe, Plummer & Wilkinson, 1965). We have therefore examined the effect of preincubation at different temperatures on the dehydrogenase activities of spermatozoal extracts, and determined the relative rates of reduction of pyruvate and 2-oxobutyrate by sperm and testes extracts and by electrophoretically purified LD_x, as well as the effects of the inhibitors, oxalate and urea. These properties of LD_x have been compared with those of the five usual LD_{1-5} isoenzymes from human tissues.

A preliminary account of these results has been presented at a meeting of The Biochemical Society (Withycombe & Wilkinson, 1964).

MATERIALS AND METHODS

Extracts of human spermatozoa. Specimens of normal semen, provided by volunteers, were centrifuged at 2000*g* for 10min. at 4°. The seminal plasma was removed and the spermatozoal pellet was washed twice with a volume of 0·067M-Sörensen phosphate buffer, pH 7·4, equal to that of the seminal plasma. The washings were discarded, and the spermatozoa were suspended in phosphate buffer (1ml.) and ultrasonically disintegrated for 1min. in an MSE ultrasonic disintegrator (18000–20000cyc./sec.; 1·5–1·8A), with the probe just under the surface of the suspension contained in an

ice-cooled polythene centrifuge tube. The homogenate was then centrifuged at 2000*g* for 10 min. at 4°, and the supernatant fraction was used in the work described below. The protein content of the extract was determined by measuring the extinction of a 1:100 dilution at 280 mμ and comparing with a serum of known protein content (determined by the micro-Kjeldahl method).

Extracts of testis. Human, cat, mouse and dog testes, obtained at autopsy, were cut into small pieces and washed free from blood with 0·9% NaCl. The tissues were then homogenized with 5 vol. of 0·067 M-Sörensen phosphate buffer, pH 7·4, in a Potter–Elvehjem homogenizer. The homogenates were centrifuged at 6000*g* for 20 min. at 4°, after which the precipitates were discarded. The protein contents of the supernatants were determined as described above.

Extracts of other tissues. Human heart, kidney, skeletal muscle and liver tissues were obtained at autopsy within 24 hr. of death and extracts were prepared as described for testis.

Determination of dehydrogenase activities. (*a*) With pyruvate as substrate. The method of Kubowitz & Ott (1943) as described by Kornberg (1955) was used. A suitable dilution of the enzyme preparation in Sörensen phosphate buffer, pH 7·4 (2·7 ml.), was equilibrated with 0·35 μmole of $NADH_2$ in phosphate buffer (0·1 ml.) at 25 ± 0·5° for 15 min. Then 0·02 M-sodium pyruvate was added and the extinction at 340 mμ was measured at 30 sec. intervals for 3–5 min. in a Unicam SP. 500 spectrophotometer.

(*b*) With DL-lactate as substrate. A modification of the method of Wacker & Dorfman (1962) was used. The enzyme solution was diluted with M-tris–HCl buffer, pH 8·8, to produce a total volume of 1·2 ml. Then 0·05 M-NAD (0·3 ml.) was added and the mixture allowed to equilibrate at 25 ± 0·5° for 15 min. Finally 0·2 M-sodium DL-lactate (1·5 ml.) was added and the extinction at 340 mμ was measured at 1 min. intervals for 5–10 min.

(*c*) With 2-oxobutyrate as substrate. The procedure described for pyruvate was employed except that 0·1 M-sodium 2-oxobutyrate (0·1 ml.) replaced sodium pyruvate (Rosalki & Wilkinson, 1960).

(*d*) With DL-2-hydroxybutyrate as substrate. The method used was identical with that for lactate except that 0·2 M-sodium DL-2-hydroxybutyrate (1·5 ml.) replaced sodium lactate.

All enzyme activities are expressed as μmoles of NAD reduced or $NADH_2$ oxidized/min./mg. of protein at 25°.

Electrophoretic techniques. (*a*) On cellulose acetate. The procedure of Kohn (1960), with the Shandon Universal apparatus, was adopted. Lactate-dehydrogenase isoenzymes were located by the tetrazolium staining technique of Barnett (1962). In some experiments DL-2-hydroxybutyrate replaced DL-lactate. Alternatively, the cellulose acetate strips were cut into 3 mm. sections, each of which was eluted with Sörensen phosphate buffer, pH 7·4, and the activity of the eluate was determined as described above.

(*b*) On polyacrylamide gel. The method of Raymond & Weintraub (1959) with the vertical-electrophoresis apparatus of Raymond (1962) (E. C. Apparatus Corp., Philadelphia, Pa., U.S.A.) was used. The 0·1 M-tris–borate buffer, pH 9·2, was prepared by dissolving 121g. of tris, 9·3g. of EDTA (disodium salt) and 4·8g. of boric acid in deionized water and adjusting the volume to 10 l. A 6% (w/v) solution of Cyanogum 41 (acrylamide monomer containing 5% of *NN*-methylenebisacrylamide; American Cyanamid Co.) in 0·1 M-tris–borate buffer, pH 9·2, was treated with 0·2% dimethylaminopropionitrile and 0·2% ammonium persulphate. Immediately after mixing, the solution (150 ml.) was transferred to the electrophoresis apparatus in the horizontal position and after 30 min., when gel formation was complete, the apparatus was restored to the vertical position and both electrode compartments were filled with 0·1 M-tris–borate buffer, pH 9·2. Water at 0–5° was circulated through the cooling coils.

The enzyme preparations were treated with approx. 2% bovine albumin and a trace of bromophenol blue, and 20 μl. samples were applied to the sample slots. A potential of 20 v/cm. was applied and electrophoresis was continued until the albumin–bromophenol blue marker had almost reached the lower end of the gel. The buffer was discarded and the gel immersed in a staining solution consisting of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (20 mg.), NAD (50 mg.), M-sodium lactate (25 ml.) and *N*-methylphenazonium methosulphate (1 mg.) in 0·1 M-tris–borate buffer, pH 9·2 (50 ml.). The solution was protected from light and after about 45 min. the stained gel was rinsed several times with distilled water and fixed by immersion in aq. 50% (v/v) methanolic 1·5 N-acetic acid.

RESULTS

Electrophoretic separation of isoenzymes. The lactate-dehydrogenase isoenzyme patterns of cat, dog and mouse testis, separated electrophoretically on cellulose acetate, are illustrated in Fig. 1. Though cat testis shows the five fractions charac-

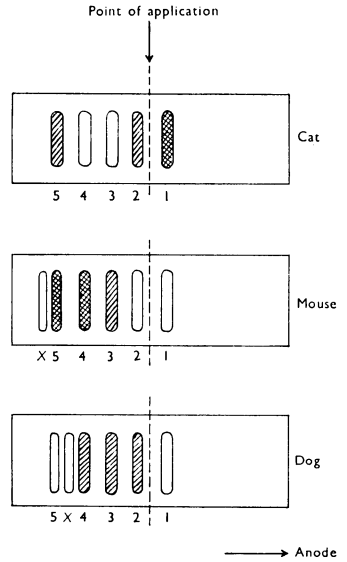

Fig. 1. Lactate-dehydrogenase isoenzyme patterns of cat, mouse and dog testis extracts separated by electrophoresis on cellulose acetate. □, Light staining; ▩, medium staining; ▩, intense staining.

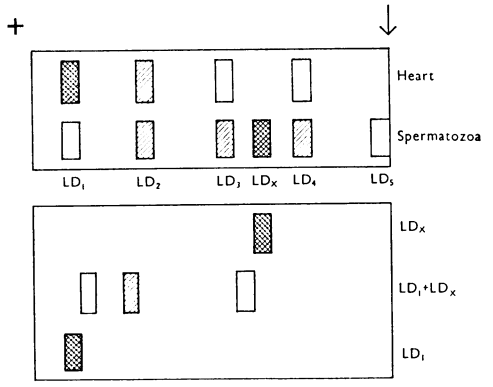

Fig. 2. Lactate-dehydrogenase isoenzyme patterns of human heart and spermatozoal extracts separated by polyacrylamide-gel electrophoresis, and also of the pattern obtained after freezing and thawing, in M-NaCl, a mixture containing equal activities of LD_1 and LD_x. □, Light staining; ▩, medium staining; ▩, intense staining.

teristic of other tissues, mouse and dog testes each contain an additional zone of activity, LD_x. The latter fraction is cathodic to LD_5 in the mouse and between LD_4 and LD_5 in the dog.

The LD_x zone of post-pubertal human testis appears between LD_3 and LD_4, but it is present in much greater proportions in spermatozoal extracts. Fig. 2 shows the lactate-dehydrogenase isoenzymes of human spermatozoal and heart extracts separated by polyacrylamide-gel electrophoresis. In the spermatozoal extract most activity is associated with the LD_x zone, but there is significant activity in the LD_3 and LD_4 zones and minor activity in the LD_2 and LD_1 regions. No LD_5 activity was detected. When 2-hydroxybutyrate was used as substrate, the LD_x fraction exhibited very high activity. Seminal plasma contains all of the usual isoenzymes (LD_{1-5}) as well as LD_x, which is present in relatively small amounts.

Dehydrogenase activities of extracts from testes and spermatozoa. The dehydrogenase activities of extracts of human, rabbit, cat, dog and mouse testis and of human spermatozoa and seminal plasma are shown in Table 1. By comparison with other human tissues, the ratio of activity with 2-oxobutyrate to activity with pyruvate is high and this is especially so with human spermatozoal extracts. In no other tissue has such a high value (1·17) for this ratio been obtained. The ratios for the oxidation of lactate and 2-hydroxybutyrate parallel those for the reverse reactions, the highest ratio of activity with 2-hydroxybutyrate to activity with lactate again being shown by the human spermatozoal extract. The results obtained with crude human tissue extracts were confirmed with

Table 1. *Dehydrogenase activities of extracts of testes and spermatozoa*

Activities are expressed as μmoles of $NADH_2$ oxidized/min./mg. of protein. The substrate concentrations used are optimum for human sera.

Material	Dehydrogenase activity: Substrate...0·7 mM-pyruvate	Dehydrogenase activity: 3·3mM-2-Oxobutyrate	Ratio of dehydrogenase activities (2-oxobutyrate/pyruvate)
Human testis	2·72	1·96	0·72
Cat testis	2·48	2·04	0·82
Dog testis	2·06	1·38	0·67
Mouse testis	2·05	1·20	0·59
Rabbit testis	2·44	1·95	0·80
Human spermatozoa	0·168	0·197	1·17
Human seminal plasma	0·167	0·214	0·78
Human heart	3·39	3·08	0·91
Human liver	3·26	1·04	0·32

Table 2. *Ratio of activity with 3·3 mM-2-oxobutyrate to that with 0·7 mM-pyruvate of lactate-dehydrogenase isoenzymes prepared from human tissue extracts*

All values are the means of duplicate determinations on three separate specimens. Variations between specimens were negligible.

Isoenzyme	Source	Ratio of dehydrogenase activities (2-oxobutyrate/pyruvate)
LD_1	Heart, kidney	0·90
LD_2	Heart, kidney, testis	0·77
LD_3	Kidney, skeletal muscle, testis	0·68
LD_x	Spermatozoa	1·17
LD_4	Liver, skeletal muscle	0·57
LD_5	Liver, skeletal muscle	0·32

human isoenzymes partly purified by electrophoresis on cellulose acetate. The 2-hydroxybutyrate dehydrogenase/lactate dehydrogenase activity ratios for the five isoenzymes found in human tissues are compared with the ratio for LD_x in Table 2. The activity ratio for each individual isoenzyme was the same regardless of its tissue source; for example, the ratio for LD_2 from human heart, kidney and testis tissue was 0·77. The isoenzymes are listed in order of electrophoretic mobility towards the anode at pH 8·6, and it is evident that there is no correlation between the mobility of LD_x and its relative activities with these substrates.

Relationships between reaction velocities and substrate concentrations for LD_x are shown in Fig. 3. This isoenzyme is slightly inhibited by excess of pyruvate and the optimum concentration of this substrate is the same as that for LD_3 (Table 3). Excess of 2-oxobutyrate is a powerful inhibitor (Fig. 3) of LD_x and the optimum concentration of this substrate is half of that for LD_1 and only about one-eighth of that for LD_3 (Table 3). The Michaelis constants (Table 3) determined by the method of Lineweaver & Burk (1934) show that LD_x has a high affinity for both substrates, and a greater affinity for 2-oxobutyrate than any of the other isoenzymes. The affinity of LD_x for 2-oxobutyrate is ten times that of LD_3 and nearly five times that of LD_1.

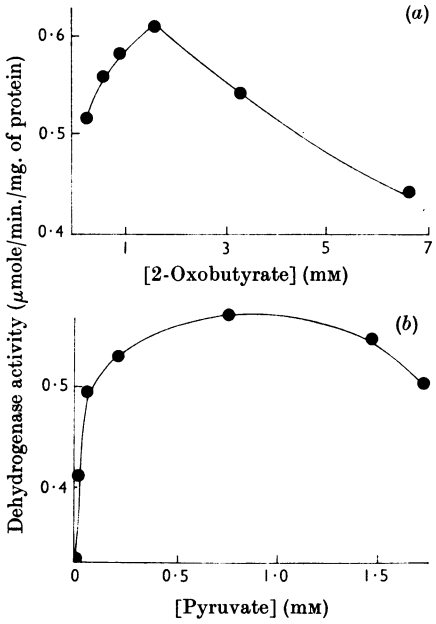

Fig. 3. Relationship between substrate concentration and dehydrogenase activity of a human spermatozoal extract with 2-oxobutyrate (*a*) and pyruvate (*b*) as substrates.

Effects of oxalate and urea on the reduction of pyruvate and 2-oxobutyrate. Lactate-dehydrogenase isoenzymes are differentially inhibited by 0·2mM-oxalate (Emerson *et al.* 1964) and 2M-urea (Withycombe *et al.* 1965). The effects of these substances on the enzymic activity of human spermatozoal, heart and liver extracts are shown in Table 4, together with the effects on human LD_{1-5} and human LD_x. The spermatozoal lactate dehydrogenase resembles that of heart in being strongly inhibited by oxalate and relatively insensitive to urea. LD_x was more strongly inhibited than LD_1 by oxalate and affected to a smaller extent by urea.

Thermal stability of the isoenzymes. The effects of previous heating at various temperatures for 30min. on the dehydrogenase activities, determined at 25°, of LD_1, LD_x and LD_5 are shown in Fig. 4. Like LD_1, LD_x is a relatively thermostable isoenzyme. Relative losses of enzyme activity were the same irrespective of whether pyruvate or 2-oxobutyrate was the substrate.

Dissociation of lactate dehydrogenase into subunits. Markert (1963) has shown that lactate-dehydrogenase isoenzymes prepared from ox tissues are tetramers that may be resolved into monomeric units by freezing in M-sodium chloride. Recombination occurs on subsequent thawing to produce active tetramers. We subjected electrophoretically purified LD_x to this freezing-and-thawing procedure but found no change in its electrophoretic mobility. However, when a mixture containing equal activities of LD_x and LD_1 was similarly treated, new hybrid enzymes were produced. Most activity was found in a zone migrating somewhat faster than LD_2, but there were also smaller amounts of other new bands: one migrating between LD_1 and LD_2 and a second close behind LD_3 (Fig. 2). Bands of LD_2 and LD_3 were not detectable.

DISCUSSION

Our results confirm the observation of Zinkham *et al.* (1963) that about 80% of the lactate-dehydrogenase activity of human spermatozoa resides in

Table 3. *Optimum substrate concentrations and Michaelis constants (K_m) for lactate-dehydrogenase isoenzymes derived from human tissues*

		Optimum concentration (mM)		K_m (mM)	
Isoenzyme	Source	Substrate...Pyruvate	2-Oxobutyrate	Pyruvate	2-Oxobutyrate
LD_1	Heart	0·40	3·3	0·08	0·84
LD_2	Heart	0·48	5·7	0·11	1·24
LD_3	Kidney	0·62	12·5	0·17	1·90
LD_x	Spermatozoa	0·62	1·65	0·05	0·18
LD_4	Liver	0·77	50	0·18	3·47
LD_5	Liver	1·00	90	0·83	10·00

Table 4. *Effect of final concentrations of* 0·2 mM-*oxalate and* 2 M-*urea on the dehydrogenase activities of human spermatozoa, heart and liver extracts and purified isoenzyme fractions of human tissues*

Enzyme–inhibitor mixtures were set aside for 30 min. before enzyme activity was determined. Final substrate concentrations were 0·7 mM-pyruvate and 3·3 mM-2-oxobutyrate.

		Percentage inhibition produced			
		By 0·2 mM-oxalate		By 2 M-urea	
Material	Substrate...	Pyruvate	2-Oxobutyrate	Pyruvate	2-Oxobutyrate
Spermatozoa					
Mean of 5 extracts		73	76	16	41
Range		63–90	73–83	6–24	36–55
Heart extract		70	70	20	80
Liver extract		30	26	100	100
LD_1		68	71	15	55
LD_2		66	63	76	90
LD_3		61	52	88	95
LD_x		72	75	12	45
LD_4		47	45	90	96
LD_5		31	32	95	98

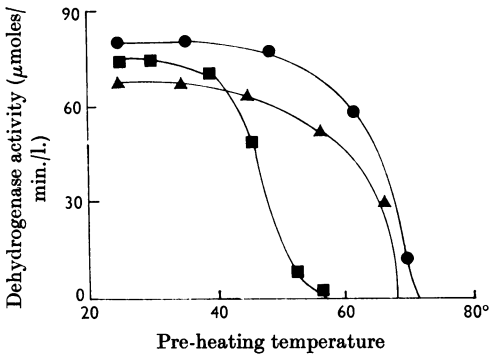

Fig. 4. Effect of preincubation at different temperatures on the activity of LD_1 (●), LD_5 (■) and LD_x (▲), with 0·7 mM-pyruvate as substrate.

isoenzyme LD_x, which has an electrophoretic mobility between those of LD_3 and LD_4. The same authors have shown that LD_x can utilize coenzyme analogues in a manner different from LD_3 or LD_4, and we have now demonstrated that it has several other properties that do not correlate with its electrophoretic mobility.

It has been reported that mouse testis contains an isoenzyme which exhibits relatively high activity with DL-2-hydroxybutyrate and DL-2-hydroxyvalerate (Allen, 1961), an observation that has been confirmed by Zinkham *et al.* (1963). Our preparations of human LD_x oxidized DL-2-hydroxybutyrate and DL-lactate, although the rates of reaction were much lower than the corresponding reverse reactions. This demonstration is at variance with the finding of Zinkham *et al.* (1963) that LD_x of man and certain other species does not react with DL-2-hydroxy acids other than lactate. Human LD_x has an unexpectedly high affinity for 2-oxobutyrate, the K_m value being 0·18 mM, which compares with 0·84 mM for human LD_1 and 10·0 mM for human LD_5. In exhibiting high activity with 2-oxobutyrate and 2-hydroxybutyrate and having a high ratio of activity with 2-oxobutyrate to activity with pyruvate, LD_x behaves rather like LD_1. It also resembles LD_1 in being relatively little affected by 2 M-urea, in being strongly inhibited by 0·2 mM-oxalate and in being relatively stable to heat.

LD_x is inhibited by excess of pyruvate and maximum activity is obtained with a substrate concentration of 0·6 mM (Fig. 3). This is somewhat higher than the concentration producing maximum activity of LD_1 (0·4 mM) but much lower than that for LD_5 (1·0 mM). LD_1 is strongly inhibited by excess of pyruvate and hence does not allow rapid accumulation of lactate. Tissues such as heart muscle, which are rich in this isoenzyme, require aerobic conditions, but LD_5, the major isoenzyme of skeletal muscle, is not much inhibited by excess of pyruvate and consequently is well adapted to anaerobic glycolysis and the accumulation of lactate (Cahn, Kaplan, Levine & Zwilling, 1962). In this respect LD_x has properties intermediate between those of LD_1 and LD_5, and therefore appears to be capable of functioning under either aerobic or anaerobic conditions, a conclusion in accord with present views on the energy sources available to spermatozoa (Mann, 1964). Fructose is the chief source of energy in semen (Mann, 1964) and under anaerobic conditions lactic acid is the chief breakdown product.

The regular gradation in properties in parallel with the electrophoretic mobilities of LD_{1-5} is in accord with present views of the tetrameric structure of the enzyme (Appella & Markert, 1961; Cahn *et al.* 1962; Markert & Apella, 1963). The lactate-dehydrogenase molecule comprises four monomeric sub-units, each with mol.wt. about 32000. LD_5 appears to consist of four identical A sub-units, whereas LD_1 consists of four identical B sub-units. LD_{2-4} are regarded as hybrids containing both A and B sub-units. Studies on dissociation and reassociation, however, have indicated that LD_x is also a tetramer but that it is composed of another type of sub-unit (C), which is distinct from the A and B monomers (Zinkham *et al.* 1963). Our results are consistent with this theory. Dissociation and reassociation of LD_x produced only one isoenzyme, LD_x, whereas the same treatment of a mixture of LD_x and LD_1 produced new isoenzymes, thus confirming that LD_x is homogeneous and composed of monomers that can combine with B monomers from LD_1 to produce a new series of hybrid isoenzymes.

Since LD_x is not found in pre-pubertal testes it has been suggested that the production of C sub-units is under the control of a gene that becomes operative at puberty. This gene is distinct from those governing the synthesis of the A and B sub-units (Zinkham *et al.* 1963). The new isoenzyme may be connected in some way with the metabolism of the haploid generation.

The surprisingly high activity of LD_x with 2-oxobutyrate poses the question whether this substance or 2-hydroxybutyrate plays a part in spermatozoal metabolism. At present 2-oxobutyrate is known to be converted into 'active amyl alcohol' by yeast extracts (Yoshizawa, 1963), and it is produced from threonine in animal tissues and micro-organisms by the action of threonine dehydratase (Yanofsky & Reissig, 1953; Sayre & Greenberg, 1955; Stadtman, 1963), a stage in the metabolic conversion of threonine into isoleucine. Although appreciable concentrations of threonine and isoleucine are present in human seminal plasma (Krampitz & Doepfmer, 1962), it is not at present known whether their interconversion and hence the occurrence of 2-oxobutyrate is of physiological significance.

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