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Aspartate Aminotransferase

THE EFFECTS OF IONIC CONCENTRATION ON KINETIC CONSTANTS OF BOTH ISOENZYMES

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1. The Michaelis constants for both isoenzymes for both substrates depend strongly on ionic concentration, being approximately proportional to phosphate concentration over considerable ranges. This is probably an effect of anions only. 2. In the absence of added salt, K_m (2-oxoglutarate) (anionic isoenzyme) is so small as to be indeterminate. 3. K_m (L-aspartate) (anionic isoenzyme) passes through a sharp minimum at about 3·3mM-phosphate. It is not clear whether this is a specific effect of phosphate. 4. Both substrates are inhibitory at sufficiently low ionic concentrations. 5. A modified graphical procedure is described for the derivation of the kinetic constants.

Anionic and cationic isoenzymes of aspartate aminotransferase (L - aspartate – 2 - oxoglutarate aminotransferase, EC 2.6.1.1) were reported independently by Moore & Lee (1960), Fleisher, Potter & Wakim (1960) and Augustinsson, Grant, Olsson & Thafvelin (1960). Rosenthal, Thind & Conger (1960) showed that the cationic isoenzyme was associated with the mitochondria, and this was confirmed by Boyd (1961) and Borst & Peeters (1961). Fleisher *et al.* (1960) showed considerable differences in kinetic behaviour between the isoenzymes, and this too was soon confirmed.

Velick & Vavra (1962) showed that the Michaelis constants as previously determined ('apparent K_m') were each dependent on the concentration of the other substrate present. It seemed that their 'true K_m' values should prove universally valid, and yet the values obtained by other workers differed radically from theirs (Table 2). Henson & Cleland (1964) found that preparations, supposedly of anionic and cationic isoenzymes, showed virtually identical 'true K_m' values, in sharp contrast with the already accepted differentiation based on 'apparent K_m'. The present investigation was undertaken partly to account for these discrepancies and partly to explore the basis of an observed inhibition of the cationic isoenzyme by phosphate (as compared with tris–acetate buffers).

The rate equation for the mechanism exemplified by this enzyme has been derived from steady-state theory (Alberty, 1953; Velick & Vavra, 1962) and via the Michaelis–Menten approach (T. R. C. Boyde, unpublished work). If each kinetic constant is recognized explicitly as a function of the concentration, i, of an inhibitor, one may write:

$$V(i)/v = 1+K_a(i)/a+K_b(i)/b$$

where v is observed velocity, $V(i)$ is maximal velocity and $K_a(i)$ and $K_b(i)$ are the 'true K_m' values (Velick & Vavra, 1962) for the substrates A and B, whose concentrations are represented by a and b. For the purposes of this paper, A is identified as 2-oxoglutarate, B as L-aspartate, and i as the concentration of sodium phosphate buffer, pH 7·4. At a given value of i, a plot of $1/v$ versus $1/a$ yields for the slope:

$$K_a(i)/V(i) \quad (1)$$

and for the intercept on the ordinate:

$$1/V(i)+K_b(i)/bV(i) \quad (2)$$

Similarly, a plot of $1/v$ against $1/b$ gives values for:

$$K_b(i)/V(i) \quad (3)$$

and

$$1/V(i)+K_a(i)/aV(i) \quad (4)$$

Then $V(i)$ can be obtained from $1/[(2)-(3)/b]$ and from $1/[(4)-(1)/a]$, and $K_a(i)$ and $K_b(i)$ are calculated from $V(i)$, (1) and (3).

The kinetic constants set out below have for the most part been derived as indicated above, but some determinations by the methods of Velick & Vavra (1962) and Henson & Cleland (1964) are also reported. The new method has some practical advantage over that of Velick & Vavra (1962).

Fewer observations are needed, and there is not the same requirement to match substrate concentrations to the K_m being measured. This has made it possible to use a set of substrate concentrations unaltered over a wide range of phosphate concentrations, and also makes it easy to measure very low K_m values, though the method of Henson & Cleland (1964), involving substrate inhibition, is even better in this respect.

MATERIALS

Malate dehydrogenase, $NADH_2$ and 2-oxoglutaric acid were purchased from Boehringer Corporation Ltd. (London, W. 5). The malate dehydrogenase was presented in the form of a suspension in 2·0M-$(NH_4)_2SO_4$, which was removed before use by dialysis against at least 500vol. of 20mM-tris–acetate buffer, pH7·4. 2-Oxoglutarate was prepared as a 100mM stock solution neutralized (pH7·4) with NaOH, preserved with chloroform and kept at 4°.

L-Aspartic acid was purchased from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.), batches 15007 and 20111. A stock solution was kept as described for 2-oxoglutarate, but more concentrated (1·0M). Tris (Trizma base) was purchased from Sigma Chemical Co. (London, S.W. 6). All other chemicals were AnalaR grade from British Drug Houses Ltd. (Poole, Dorset).

Stock tris–acetate buffer was prepared by weighing out 0·165mole of acetic acid, diluting with water, dissolving in this solution 0·2mole of tris and making up to 1l. This is referred to as 0·2M and gives pH7·4 at the dilutions used.

METHODS

Assay. Enzyme activities quoted in the text were determined under the following standard conditions: temperature, 25°; final volume, 3·0ml., containing malate dehydrogenase (5μg.), $NADH_2$ (0·4mg.) and the following, at the final concentrations stated, L-aspartate (100mM), 2-oxoglutarate (6·7mM) and tris–acetate buffer (33·3mM).

The reaction vessel was a spectrophotometer cuvette of 1cm. path length. The reaction rate was determined by recording E_{340}. In calculating activities, the molar extinction coefficient of $NADH_2$ was taken as $6{\cdot}023 \times 10^3$. The actual cuvette temperature was checked from time to time during every session. As a routine, all the components of the reaction mixture except L-aspartate were added to the cuvette, and the volume was brought to 2·7ml. After temperature equilibration, 0·3ml. of 1·0M-aspartate was added, and mixed in with an L-shaped plastic paddle.

The substrate concentrations specified are optimum for the anionic isoenzyme, in this buffer, and give rates about 25% higher than in the original assay conditions of Karmen (1955). The optimum for cationic isoenzyme appears to be about 50mM-L-aspartate and 20mM-2-oxoglutarate, giving rates 10% higher than in the standard assay described above.

Kinetic investigations. The principle employed was the same as for the standard assay, and the same doses of malate dehydrogenase and $NADH_2$ were always used. The amounts of substrates, and the amount and nature of the buffer, were varied as the experimental situation required. For cationic isoenzyme, the 'varied substrate' concentrations were within the ranges 0·033–0·4mM-2-oxoglutarate and 0·1–1·0mM-L-aspartate. These were used for the Velick & Vavra procedure, for the Henson & Cleland procedure with inhibitory concentrations of 3·3–33mM-L-aspartate and for the new method (above) with 0·2mM-2-oxoglutarate or 0·5mM-L-aspartate.

For anionic isoenzyme, 2-oxoglutarate was employed as 'varied substrate' within the range 0·02–0·20mM, with L-aspartate concentrations of 0·4–2·0mM (Velick & Vavra procedure, 10mM-phosphate), 3·3–33mM (Velick & Vavra procedure, 33mM-phosphate), 33–333mM (Henson & Cleland procedure) or 3·3mM (new method, above). L-Aspartate was used as 'varied substrate' within the range 0·2–2·0mM with 2-oxoglutarate concentrations of 0·67–33mM (Henson & Cleland procedure) or 0·33mM (new method, above).

In kinetic studies, as for assays, the reaction was initiated by adding the required amount of aspartate and mixing in with a plastic paddle.

Preparation of isoenzyme concentrates. Human liver (60g.), obtained from autopsy, was placed in a 1l. dimple beaker, with 200ml. of 8mM-sodium phosphate buffer, pH7·0, and homogenized in a Townson and Mercer Ltd. Top-Drive Atomizer for 5min. The homogenate was strained, dialysed against running tap water overnight, centrifuged (Spinco model L, no. 30 head, 20000 rev./min. for 20min.), concentrated by ultrafiltration and dialysed against 8mM-phosphate buffer, pH7·0. A portion (20ml.) of the resulting solution, containing 0·9g. of protein (biuret method) and corresponding to 13·5g. of liver, was applied to a DEAE-cellulose column (Whatman DE11, 30g., 3cm.-diam. column) pre-equilibrated with the 8mM-phosphate buffer. Batchwise elution with sodium phosphate buffers was as follows: 250ml. of 8mM, pH7·0; 250ml. of 10mM, pH6·0; 250ml. of 20mM, pH6·0; 400ml. of 50mM, pH6·0. Activity was recovered in eluent I (200μmoles/min., cationic, fraction I) and eluent IV (50μmoles/min., anionic, fraction IV). The isoenzymes were identified by electrophoresis on polyacrylamide gels (T. R. C. Boyde, unpublished work). The recovery of activity in the chromatographic step was 95%. The cationic isoenzyme contained no detectable amount of the anionic material; 1% contamination would have been detectable. The anionic isoenzyme preparation was later shown (T. R. C. Boyde, unpublished work) to contain 0·6% of the cationic material. Both preparations were undersaturated with coenzyme (50% or less).

Each of the active fractions was concentrated by ultrafiltration to 10·0ml. and was stable for over 9 months at −20°. Kinetic investigations were done with a standard dose of 50mμmoles/min. of anionic isoenzyme or 40mμmoles/min. of cationic isoenzyme per cuvette.

RESULTS

The relationship of K_m to concentration of phosphate buffer is shown in Figs. 1–4, for both isoenzymes and for both substrates.

Table 1 contains results of determinations of the substrate inhibition constants by the procedure of Henson & Cleland (1964), also at various phosphate concentrations.

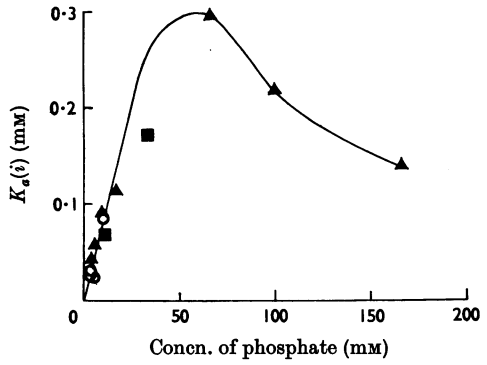

Fig. 1. Anionic isoenzyme: variation of K_m for 2-oxoglutarate with phosphate concentration. ▲, Determinations by the method described in the text, 25°; ■, method of Velick & Vavra (1962), 25°; ○, method of Henson & Cleland (1964), 25°.

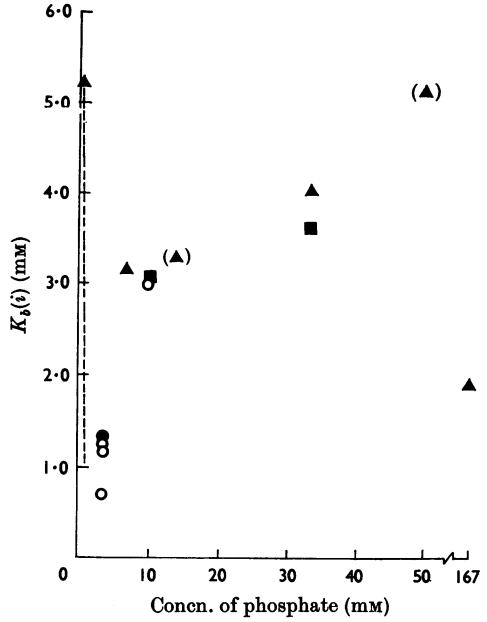

Fig. 2. Anionic isoenzyme: variation of K_m for L-aspartate with phosphate concentration. ▲, Determinations by the method described in the text, 25°; ■, method of Velick & Vavra (1962), 25°; ○, method of Henson & Cleland (1964), 25°; ●, method of Henson & Cleland (1964), 37°. Bracketing of a point, (▲), indicates that corresponding values of slope and intercept from the two sets of double-reciprocal plots were not available for this phosphate concentration, so that one of these was obtained by graphical interpolation. There is a sharp minimum near 3·3mM-phosphate. To obtain satisfactory measurements of K_m between 0 and 3·3mM-phosphate, it would be necessary to work with lower substrate concentrations. This was not possible with the apparatus available and accordingly the precise location and value of the minimum remain undetermined.

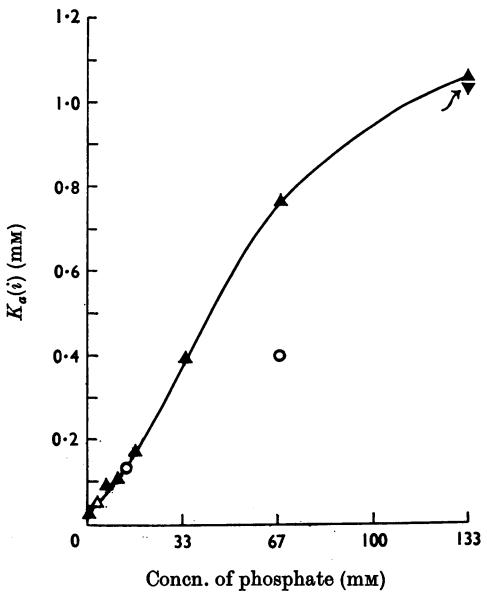

Fig. 3. Cationic isoenzyme: variation of K_m for 2-oxoglutarate with phosphate concentration. ▲, Determinations by the method described in the text, 25°; ▼, method described in the text, 233mM-phosphate; ○, method of Henson & Cleland (1964), 25°. At 3·3mM-phosphate determinations were made under all five sets of conditions. The symbol △ at $K_a(i)=0{\cdot}054$mM (mean) is used to represent the following experimental results: method described in the text, 0·065mM; method of Henson & Cleland (1964), 0·042mM (25°), 0·047mM (37°); method of Velick & Vavra (1962), 0·063mM (25°), 0·054mM (37°).

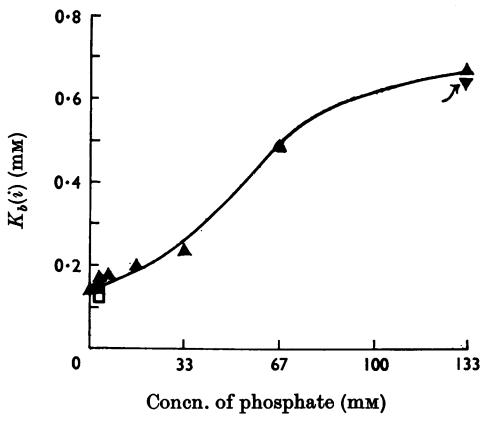

Fig. 4. Cationic isoenzyme: variation of K_m for L-aspartate with phosphate concentration. ▲, Determinations by the method described in the text; ▼, method described in the text, 233mM-phosphate; ■, method of Velick & Vavra (1962), 25°; □, method of Velick & Vavra (1962), 37°.

Table 1. *Substrate inhibition constants at* pH 7·4

The method of Henson & Cleland (1964) was used. The values listed are the results of individual determinations. Determinations of K_i (L-aspartate) in the absence of added salts and also in 3·3mM-phosphate buffer were subject to error because of very low ordinate intercepts of the secondary plots, but this source of difficulty does not apply to K_i (2-oxoglutarate) or to higher buffer concentrations.

		Inhibition constant (mM)			
		Anionic isoenzyme		Cationic isoenzyme	
Medium	Inhibitory substrate.....	L-Aspartate	2-Oxoglutarate	L-Aspartate	2-Oxoglutarate
No added salts				47	
Tris–acetate (33mM)		36	4	73, 69	13, 13
Sodium phosphate (3·3mM)		48, 107, 15*, 55, 32	1·7, 1·7, 1·8*	32, 75*	11, 14*
Sodium phosphate (10mM)		131	2·3		
Sodium phosphate (13·3mM)				405	
Sodium phosphate (67mM)				Infinite	

* Determined at 37°, otherwise at 25°.

DISCUSSION

It is shown above that the K_m values for both isoenzymes for both substrates are strongly dependent on the ionic composition of the medium. Detailed results are given here only for phosphate buffers (Figs. 1–4), but sufficient information is now available (T. R. C. Boyde, unpublished work; Turano, Fasella & Giartosio, 1962; Banks, Lawrence, Vernon & Wootton, 1963; Sizer & Jenkins, 1963; Jenkins & D'Ari, 1966) to make it certain that phosphate is not unique in this respect.

Previous reported values for K_m are listed in Table 2, together with the ionic composition of the medium in which each was determined. Henson & Cleland (1964) believed that they had prepared anionic and cationic isoenzymes, but found that their fractions were indistinguishable kinetically. Nisselbaum & Bodansky (1966) showed that the fractions obtained by Henson & Cleland (1964) probably both contained anionic isoenzyme only. The results of Velick & Vavra (1962), Wada & Morino (1964), Henson & Cleland (1964) and Nisselbaum & Bodansky (1964, 1966) on anionic isoenzyme form a series showing the increase of K_m for both substrates with increasing buffer concentration, and the results can be reconciled with Figs. 1 and 2 if the arsenate concentrations are each multiplied by an appropriate factor to give a 'phosphate-equivalent inhibitory concentration'. The factor required is about 0·5 for Fig. 1 (K_a), and about 0·2 for Fig. 2 (K_b).

The K_m values calculated from the microscopic rate constants of Hammes & Fasella (1962) are much smaller than any others in Table 2. Although these workers used 160mM-phosphate buffer, the results correspond in order of magnitude to what is reported here in the absence of added salts. The explanation may lie in their use of the temperature jump–relaxation principle, involving very rapid measurements and very high concentrations of the enzyme.

Turano *et al.* (1962) give high values for both substrates, and for this their buffer (16mM-pyrophosphate) is presumably responsible. These workers obtained evidence of the remarkable activating effect of phosphate, effectively decreasing K_b (Fig. 2), although they did not observe the phosphate inhibition that occurs at slightly higher concentrations. Turano *et al.* (1962) followed the transamination reaction by observing the formation of enoloxaloacetic acid through its ultraviolet absorption. Banks *et al.* (1963) pointed out that this procedure is invalid unless the enolization reaction is much faster than the enzymic transamination, and attributed all the salt activation reported by Turano *et al.* (1962) to catalysis of the enolization reaction. However, a re-examination of the evidence shows that this effect on the K_m for aspartate cannot be so explained. In this respect phosphate may be unique.

For cationic isoenzyme, the only other valid values for 'true K_m' are those of Nisselbaum & Bodansky (1964, 1966). The values given by these authors for the isoenzyme from pig heart (Nisselbaum & Bodansky, 1966) are close to the corresponding results of Figs. 3 and 4. The determinations reported by Nisselbaum & Bodansky (1964) are nearly threefold greater; these were done on human material. Further work is needed to determine whether there is a consistent species difference and to account for the discrepancy between the present results and those of Nisselbaum & Bodansky (1964) on human cationic isoenzyme.

Table 2. *Previously reported values for 'true' Michaelis constants of mammalian aspartate aminotransferase*

The values attributed to Hammes & Fasella (1962) were calculated from the microscopic rate constants given by these workers, by using the relationships derived by Velick & Vavra (1962). The values from Henson & Cleland (1964) and Nisselbaum & Bodansky (1966) (anionic isoenzyme) are for fractions separated by CM-cellulose chromatography (see the text).

Reference	K_m (mM) L-Aspartate	K_m (mM) 2-Oxoglutarate	Medium and conditions	Source of enzyme
Anionic isoenzyme				
Velick & Vavra (1962)	0·9	0·1	40mM-Arsenate, pH7·4, 26°	Pig heart
Turano *et al.* (1962)	5·9	12	16mM-Pyrophosphate, pH8·2, 25°	Pig heart
Hammes & Fasella (1962)	0·015	$8·7 \times 10^{-5}$	160mM-Phosphate, pH8·0, 25°	Pig heart
Henson & Cleland (1964)	4·4, 3·9	0·38, 0·43	100mM-Arsenate, pH7·4, 37°	Pig heart
Wada & Morino (1964)	2·0–2·5	0·3–0·4	60mM-Arsenate, pH7·4, 20°	Ox liver
Nisselbaum & Bodansky (1964)	5·9*	0·7*	67mM-Phosphate, pH7·4, 37°	Human heart and liver
Nisselbaum & Bodansky (1966)	3·9, 4·9	0·57, 0·61	67mM-Phosphate, pH7·4, 37°	Pig heart
Cationic isoenzyme				
Nisselbaum & Bodansky (1964)	0·9*	3·2*	67mM-Phosphate, pH7·4, 37°	Human heart and liver
Nisselbaum & Bodansky (1966)	0·35	1·1	67mM-Phosphate, pH7·4, 37°	Pig heart

* Mean of several experimental values quoted.

Wada & Morino (1964) observed non-parallel sets of double-reciprocal plots for this isoenzyme, and deduced that the reaction mechanism must be different from that of the anionic isoenzyme. However, their results were probably due to the use of inhibitory concentrations of 'non-varied' substrate.

Substrate inhibition by oxoglutarate was reported by Henson & Cleland (1964) and confirmed by Nisselbaum & Bodansky (1964), but neither of these groups could demonstrate inhibition by L-aspartate, a rather surprising lack of symmetry. In fact, such inhibition does occur (Table 1), but only in relatively dilute buffers. The interaction of a substrate with the inappropriate form of the enzyme is expected to be similar to that with the appropriate form, and it is not surprising that the substrate inhibition constant, like the Michaelis constant, is a function of the ionic composition of the medium; the two are affected simultaneously by the same interaction of enzyme with ions.

The papers of Alberty & Bock (1953) and Alberty, Massey, Frieden & Fulbrigge (1954) on fumarase appear to be the only previous publications describing a comparable dependence of K_m and inhibition constants on buffer concentration. These workers found that over a substantial range the kinetic constants involved were directly proportional to phosphate concentration.

The study by Jenkins & D'Ari (1966) became available after the present investigations were complete. These authors describe work with aspartate aminotransferase (anionic isoenzyme from pig heart) in which the reaction with dicarboxylic acid inhibitors was observed by spectrophotometric titration, by using the change in absorption spectrum involved in binding such inhibitors. They found that at pH8·2 the dissociation constant was directly proportional to the salt concentration, the proportionality factor being a property of the anion involved. Although their experiments were not kinetic, the results have a clear implication for the kinetics of the enzyme. As far as comparison is possible, their results are in accord with what is recorded here.

The major effect reported here is inhibitory in nature. In principle, it would be possible to describe this effect in terms of the product of a K_m and an inhibition function. This is not done here because of the simultaneous occurrence of activation effects, which complicate the treatment, and because with the anionic isoenzyme K_a in the absence of added salts is so small that any value allotted to it would be arbitrary.

Apart from the striking activation of anionic isoenzyme by phosphate buffer, there appears also to be a general ionic activation of both isoenzymes, probably largely an effect of the cations (T. R. C. Boyde, unpublished work). There is every reason to suppose that the substrates or their associated ions or both participate. It is therefore necessary to consider the possible effects of every ionic species in solution. In particular, determinations of the kinetic constants with substrate concentrations higher than those used here may be expected to give substantially different results, especially at low buffer concentrations.

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