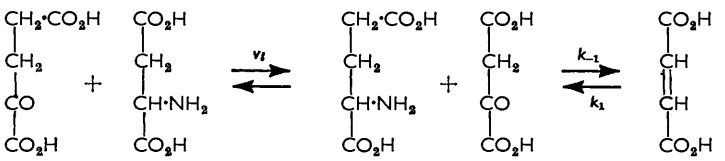
1106. *Transamination. Part III.* Spectrophotometric Methods for the Assay of Glutamic–Aspartic Transaminase.*

By Barbara E. C. Banks, A. J. Lawrence, E. M. Thain, and C. A. Vernon.

The keto-form of oxaloacetic acid has been shown to be the true substrate for glutamic–aspartic transaminase (pig cardiac muscle). Analytical methods for following the enzymic reaction based on the formation of the enol form of oxaloacetic acid are shown to be valid only under certain conditions. Other conditions lead to low values of the initial rate. Previous results are discussed in the light of these findings. The effect of anions on the enzymic reaction is briefly described.

Aqueous solutions of oxaloacetic acid absorb relatively strongly in the ultraviolet region (ε 550; λ 2800 Å) owing to the presence of the enolic form (16% at 25°).[1] This is the basis of the most convenient and commonly used analytical method for following the enzyme-catalysed reversible reaction between L-aspartic and α-oxoglutaric acid:



v_t is the overall velocity of formation of products (mole l.$^{-1}$ sec.$^{-1}$) at time t. k_1 and k_{-1} are first-order rate coefficients.

* Part II, *J.*, 1961, 4235.

[1] Banks, *J.*, 1961, 5043.

Typically, the initial rate of increase in optical density, at some suitable wavelength, of solutions containing substrates, buffer, and enzyme (glutamic–aspartic transaminase) is measured by means of a photoelectric spectrophotometer and is taken as a measure of the initial velocity (v) of the enzyme-catalysed reaction (direct spectrophotometric method).

However, a recent study by one of us [1,2] of the keto–enol transformation in oxaloacetic acid showed that under a variety of commonly used experimental conditions the rate of enolisation is relatively slow. This suggested that if the overall reaction is correctly represented by the above scheme, *i.e.*, if the enol form of oxaloacetic acid is not a substrate for the enzyme, then measurements of v obtained by the direct spectrophotometric method might be in error by an amount depending on the extent to which the enolisation step is rate-limiting. We have investigated this point, using a homogeneous preparation of glutamic–aspartic transaminase from pig-heart muscle and have shown that (*a*) oxaloacetic acid is produced in the keto-form by the enzymic reaction, (*b*) low values of v are obtained by using low buffer concentrations or by using buffers which are not effective catalysts for the enolisation reaction (*e.g.*, barbiturate or pyrophosphate), and (*c*) many of the kinetic results reported for this enzymic reaction may be seriously in error.

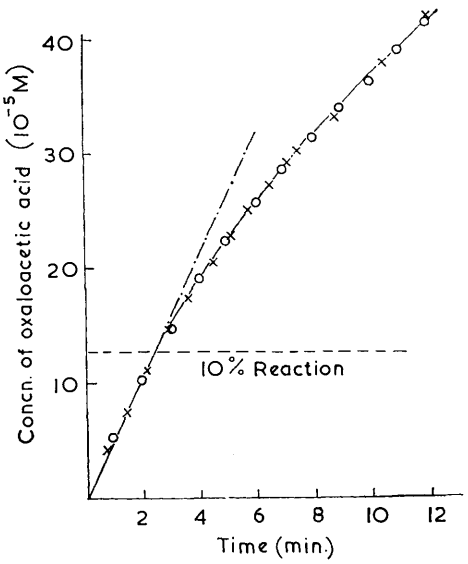


FIG. 1. Formation of oxaloacetic acid. Aliquot method.

○, × points measured in different experiments. Aspartate 0·01M, α-oxoglutarate 0·0025M; phosphate buffer 0·1M; pH 7·38; 25°.

The analytic method introduced by Greenwood and Greenbaum [3] depends, as does the direct spectrophotometric method, on enolisation of oxaloacetic acid but the possibility that this process may be rate-limiting is avoided. Essentially the procedure consists of stopping the enzymic reaction in successive aliquot parts of the reaction mixture by addition of concentrated acid. The amounts of oxaloacetic acid present in the aliquot parts are then determined spectrophotometrically under conditions where equilibrium between the keto- and the enol form is reached. The method is inconvenient and unsuitable for routine work but gives, unambiguously, the true value of v_t at any time. Fig. 1 shows typical results obtained by a modification of the orginial method. It was found that curves representing the time course of the reaction for given substrate concentrations could always be superimposed (by adjusting the time scale) irrespectively of temperature, of the concentration of the enzyme, and of the concentration and nature of the buffer. The initial velocity (v) can be found easily since the velocity at the substrate concentrations used is sensibly independent of time for at least the first 10% of the reaction. The

[2] Banks, *J.*, 1962, 63.
[3] Greenwood and Greenbaum, *Biochem. Biophys. Acta*, 1953, **10**, 623.

accuracy is *ca.* ±5% but decreases markedly as the time for 10% reaction falls below 2 minutes.

The sum of the first-order rate coefficients ($k_T = k_1 + k_{-1}$) associated with the keto–enol tautomerism of oxaloacetic acid in a given medium can be found from the rate of decrease in optical density when the enolic acid is dissolved in that medium. The individual coefficients can then be calculated from the known equilibrium constant.[1,2] Values of k_T obtained in a variety of the buffers used for the enzymic reaction are given in Table 1. Aspartic acid has a small catalytic effect: the quoted values refer, therefore, to solutions containing this substance (0·01M). The enzyme has no catalytic effect except in large concentration.

TABLE 1.

Rates of equilibration (enol ⇌ keto) of oxaloacetic acid.

Buffer	Buffer concn. (M)	pH	Temp.	$k_1 + k_{-1}$ (sec.$^{-1}$)	Buffer	Buffer concn. (M)	pH	Temp.	$k_1 + k_{-1}$ (sec.$^{-1}$)
Phosphate	0·1	7·38	25	0·043	Imidazole	0·1	6·6	25	~0·10
	0·05	7·38	25	0·018		0·1	7·38	25	~0·10
	0·02	7·38	25	0·006		0·1	8·0	25	~0·10
	0·01	7·38	25	0·0044	Arsenate	0·04	7·4	25	0·024
	0·005	7·38	25	0·00295	Pyrophosphate ...	0·1	7·4	25	0·0127
	0·1	8·0	25	0·025	β-Glycerophosphate	0·1	7·4	25	0·0194
	0·1	7·38	1·5	0·0054	Barbiturate	0·1	8·0	25	0·007
	0·02	7·38	1·5	0·0009					
Triethanolamine...	0·1	7·95	25	~0·09					
	0·06	7·95	25	0·061					
	0·04	7·95	25	0·046					
	0·02	7·95	25	0·034					

From each set of values of v_t, k_1, and k_{-1} measured as described, it is possible to calculate the optical density–time relation to be expected from the direct spectrophotometric method. Let x_e and x_k be the amounts of the enol and the keto-form, respectively, of oxaloacetic acid present at time t.

Then
$$dx_e/dt + dx_k/dt = v_t; \quad (1)$$

$$x_e + x_k = \int_0^t v_t \,.\, dt; \quad (2)$$

and
$$dx_e/dt = k_{-1}x_k - k_1x_e. \quad (3)$$

Hence
$$dx_e/dt + k_Tx_e = k_{-1}\int_0^t v_t \,.\, dt. \quad (4)$$

Let
$$x_e = ye^{-k_Tt}$$

Then
$$dx_e/dt = e^{-k_Tt} \,.\, dy/dt - k_Ty \,.\, e^{-k_Tt},$$

and
$$dy/dt = e^{-k_Tt}k_{-1}\int_0^t v_t \,.\, dt.$$

By substitution and integration,
$$x_e = e^{-k_Tt} \,.\, k_{-1}\int_0^t e^{k_Tt}\int_0^t v_t \,.\, dt \,.\, dt. \quad (5)$$

From the time course of the reaction as determined by the aliquot method the value of the function $\int_0^t v_t \,.\, dt$ can be found at successive times by using equation (2). The function $\int_0^t e^{k_Tt} \,.\, \int_0^t v_t \,.\, dt \,.\, dt$ can then be evaluated by numerical integration. For comparative purposes it is convenient to plot x_T against t, where x_T is given by

$$x_T = x_e(k_T/k_{-1}) = x_e(K_e + 1);$$

here K_e is the equilibrium constant for the keto–enol tautomeric mixture and x_T gives the total amount of oxaloacetic acid formed at time t if, and only if, the keto- and the enol form are in equilibrium. Comparison of the values of x_T obtained in this way and those obtained from the aliquot method indicates for any given conditions the extent to which this assumption is justified.

Fig. 2 shows the results obtained in conditions deliberately chosen to be extreme (1·5°, 0·02M-phosphate buffer, pH 7·38). It can be seen that (*a*) the direct method does not give the true velocity of the enzymic reaction and (*b*) the agreement between the observed and the calculated points proves conclusively that the keto-form of oxaloacetic acid is the true substrate for the enzyme. Fig. 3 shows results obtained in more usual

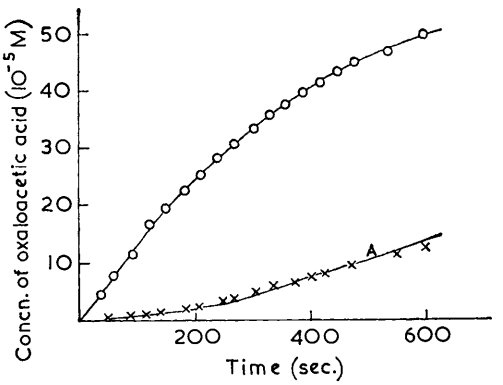


FIG. 2. Comparison of methods.

○ Aliquot method. × Direct Method. Curve (A) calculated by equation (5). Aspartate 0·01M; α-oxoglutarate 0·0025M; phosphate buffer 0·02M; pH 7·38; 1·5°.

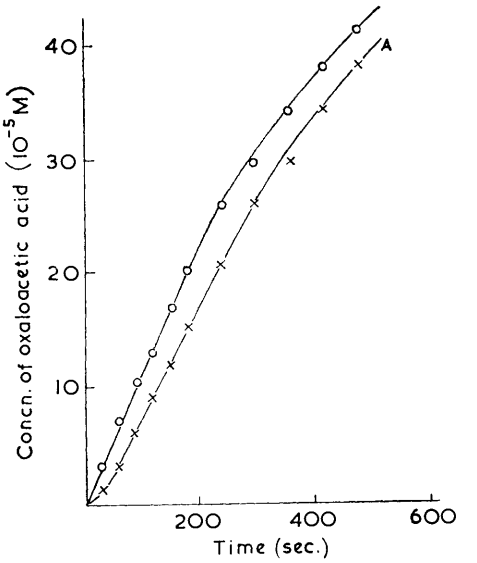


FIG. 3. Comparison of methods.

○ Aliquot method. × Direct method. Curve A calculated by equation 5.

assay conditions (25°, 0·05M phosphate, pH 7·38). The reaction velocity at a given time can be obtained from the tangent to the curve at that time; the curves in Fig. 3 lead to the following (approximate) values for v:

Time (sec.)	30	150	180	210	250	300	350	400	450
Aliquot method ...	0·113	0·113	0·113	0·090	0·081	0·070	0·065	0·059	0·051
Direct method ...	0·052	0·103	0·103	0·093	0·086	0·079	0·068	0·066	0·057

In this experiment the direct method leads to substantially correct values of the reaction velocity only when this has dropped some 10% from its initial value.

Quantitative comparison of the errors produced by using different buffer systems can most easily be made by considering conditions in which v is substantially constant over the first 10% of the reaction (*e.g.*, 0·01M-aspartate, 0·0025M-α-oxogluarate). Equation (4) becomes:

$$dx_e/dt + k_T x_e = k_{-1} v_t t,$$

and by integration

$$x_e = v_t \cdot \frac{k_{-1}}{k_T^2}\left\{k_T t + e^{-k_T t} - 1\right\},$$

whence

$$\frac{k_T}{k_{-1}} \cdot \frac{dx_e}{dt} \Big/ v_t = (1 - e^{-k_T t}); \qquad (6)$$

here (k_T/k_{-1}) . dx_e/dt, the velocity as measured by the direct method, becomes equal to v_t as $e^{-k_T t}$ approaches zero. Table 2 gives the error in the velocity at 5% reaction ($v_{0\cdot05}$) for different buffer systems and for different values of $t_{0\cdot05}$. It can be seen that the error decreases as the time ($t_{0\cdot05}$) for 5% reaction increases and where the buffer is an effective catalyst for the enolisation of oxaloacetic acid (cf. Table 1).

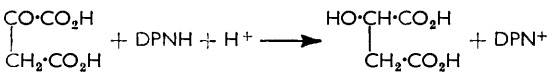
TABLE 2.

Percentage error in apparent $v_{0\cdot05}$ as measured by the direct method for various buffers (pH 7·38; 25°).

$t_{0\cdot05}$ (min.)	1	2	3	4	5
Phosphate (0·1M)	7·6	0·6	0	0	0
,, (0·05M)	33·9	9·4	3·9	1·3	0·4
,, (0·02M)	69·7	48·7	34·0	23·7	16·5
Imidazole (0·1M)	0·2	0	0	0	0
Arsenate (0·04M)	23·7	5·6	1·3	0·3	0
Pyrophosphate (0·1M)	31·3	21·9	10·1	4·7	2·1
Barbiturate (0·1M) (pH 8·0)	65·7	43·2	28·4	18·6	12·2

Kinetic studies on enzyme systems are conventionally made in terms of initial reaction rates which are estimated from the initial portions of reaction–time plots. Since it is usually desired to vary the substrate concentrations at some fixed enzyme concentration, and since long reaction times should be avoided because of possible denaturation of the enzyme,* kinetic studies generally include some runs in which the reaction time is relatively short. The results in Table 2 show that with glutamic–aspartic transaminase such kinetic studies made in buffers other than imidazole or concentrated phosphate are likely to be seriously in error.

An alternative to the direct method, introduced by Karmen,[4] involves rapid reduction of oxaloacetic acid, as it is formed, by reduced diphosphopyridine nucleotide (DPNH) under the influence of malic dehydrogenase, *viz.*:



At low concentrations of oxaloacetic acid and in the presence of an excess of DPNH the reaction can be described in terms of a first-order rate coefficient (k). Arguments similar to those leading to equation (5) show that the amount of DPNH consumed (x_{DPNH}) after time t is given by

$$x_{DPNH} = e^{-kt} \cdot k \int_0^t e^{kt} \int_0^t v \cdot dt \cdot dt. \quad (7)$$

Trial calculation with this equation shows that, if the value of k exceeds 0·1 sec.$^{-1}$, then the method correctly gives the rate of transamination for velocities whose values fall within the range given in Table 2.

The validity of the above analysis was tested by making measurements by both methods in conditions which, based on predictions made from equations (5) and (7), should lead to correct values of the rate of transamination. The small differences (Table 3) are probably due to errors in the values of the extinction coefficients used (oxaloacetic acid, ε 550, λ 2800 Å; DPNH, ε 6220, λ 3400 Å). Because of the high absorption by DPNH, the malic dehydrogenase method has the advantage of high sensitivity (*ca.* 10 times that of the direct method) but it cannot be used to follow the transamination reaction in

* In the present case long reaction times must also be avoided because of the slow spontaneous decarboxylation of oxaloacetic acid.

[4] Karmen, *J. Clin. Invest.*, 1955, **34**, 131.

TABLE 3.

Comparison of rates (v; 10^{-5} mole $l.^{-1}$ $min.^{-1}$) obtained by the direct method (D) and the malic dehydrogenase method (M).

(Imidazole 0·1M; pH 7·38; 25°; DPNH ~2 × 10^{-4}M.)

Transaminase concn. (arbitrary units)	1	2	4
Method D	0·85	1·68	3·21
Method M	0·87	1·70	3·38

reverse, *i.e.*, the reaction between glutamic and oxaloacetic acid. For this purpose the direct method must be used. The reaction is represented by equation (1), reading from right to left. If we assume that the keto- and the enol forms of oxaloacetic acid are initially present in equilibrium amounts, then, for the case where v is constant, it can be shown that the rate of change of concentration of the enol form of oxaloacetic acid is given by

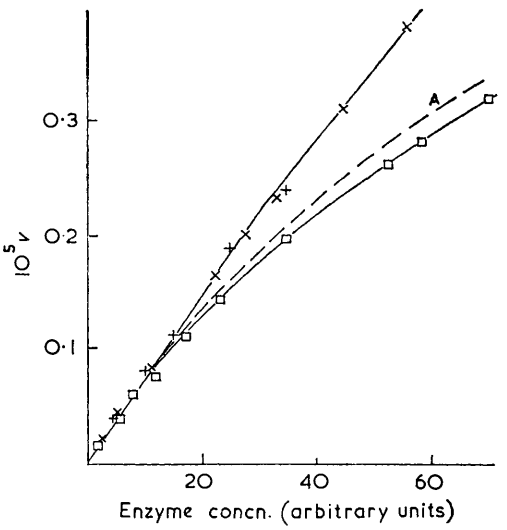


FIG. 4. Variation of rate (v, mole $l.^{-1}$ $sec.^{-1}$, oxaloacetic acid) with enzyme concentration.

Aspartate (0·1M); α-oxoglutarate 0·0025M; pH 7·38; 25°. × Aliquot method, 0·1M-phosphate. + Direct method, 0·1M-imidazole. □ Direct method, 0·1M-phosphate. Curve A calculated for direct method (0·1M-phosphate).

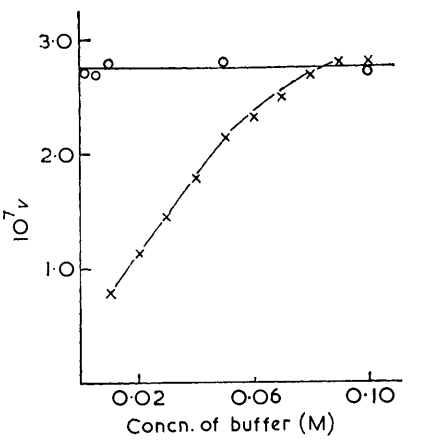


FIG. 5. Variation of rate (v, mole $l.^{-1}$ $sec.^{-1}$, oxaloacetic acid) with buffer concentration.

○ Aliquot method. × Direct method. Phosphate buffer; pH 7·38; 25°; aspartate 0·01M; α-oxoglutarate, 0·0025M.

equation (6). The limitations of the direct method, due to inefficient catalysis of the enolisation of oxaloacetic acid, are, therefore, the same, irrespective of the direction in which the transamination reaction is studied.

Variation of Rate with Enzyme Concentration.—Nisonoff and Barnes [5] reported that the initial rate of the enzyme-catalysed reaction, as measured by the direct method, did not increase proportionally with increasing enzyme concentration. Inspection of the data in Table 2 shows that this effect could arise from a failure to measure the correct rates at high enzyme concentration. The values in Fig. 4 were obtained from experiments designed to test this point. It can be seen that the rate measured by the aliquot method is proportional to enzyme concentration up to very high values ($t_{0·1}$ = ~30 sec.), whereas the direct method, under the same conditions, *i.e.*, 0·1M phosphate buffer, pH 7·38, 25°,

[5] Nisonoff and Barnes, *J. Biol. Chem.*, 1952, **199**, 713.

gives rates which are less than the true rates at high enzyme concentration, the discrepancy becoming more pronounced as the enzyme concentration increases. If we assume that at high enzyme concentrations the enolisation of oxaloacetic acid is partially rate-limiting, a curve of rate against enzyme concentration to be expected from the direct method can be constructed by using equation (5). The agreement with the experimental facts is good. Consistently, in imidazole buffer the discrepancy between the two methods is negligible. It may be concluded, therefore, that with glutamic–aspartic transaminase, the initial reaction rate is linearly dependent on enzyme concentration.* This view is consistent with experiments reported by Jenkins, Yphantis, and Sizer [6] who used Tris buffer, which, like imidazole, is an effective catalyst for the keto–enol transformation of oxaloacetic acid.

Variation of Rate with Buffer Concentration.—Experiments in which the direct method was used have been reported as showing that glutamic–aspartic transaminase is activated by phosphate ions.[7] Since the direct method is inapplicable at low buffer concentrations, it appeared that such a result might be an artefact. The results given in Fig. 5 show that, in the stated conditions, the initial velocity of the enzyme-catalysed reaction is, in fact, independent of buffer strength, whereas measurements made by the direct method indicate a pronounced catalysis by the buffer.†

Effect of Temperature on the Initial Velocity.—A further example of errors arising from the use of the direct spectrophotometric assay procedure under conditions when enolisation of oxaloacetic acid is partially rate-limiting is given by the recorded [5] activation energy of transamination. The experimental conditions used (phosphate, 0·033M, pH 7·4, aspartate and α-oxoglutarate 0·0025M) were such that, at the lower temperatures (13—25°), enolisation must be rate-limiting. The activation energy of the enolisation of oxaloacetic acid [8] is *ca.* 14,000 cal. mole^{-1}. At 13° (the lowest temperature used by Nisonoff and Barnes) the appropriate value of $(k_1 + k_{-1})$ is 0·00364 sec.$^{-1}$ and, for the substrate concentrations used, the equilibrium concentration of oxaloacetic acid is 0·000704M. The true rate of reaction in these conditions cannot be evaluated from the data but, if the apparent $t_{0\cdot05}$ (necessarily greater than the true $t_{0\cdot05}$) is used in calculating the percentage error in $v_{0\cdot05}$ (cf. Table 2) it can be shown that the rate of change of optical density at 13° is 40% too low at $t_{0\cdot05} = 4\cdot52$ min.‡

The true activation energy for the overall reaction appears to be of the order of 8000 cal. mole^{-1}.

Effect of Added Salts on the Initial Velocity.—The above considerations make it possible to carry out a complete kinetic investigation of glutamic–aspartic transaminase without introducing errors due to faulty measurements of initial reaction rates. There remains one other ambiguity. Variation of pH, or of the concentration of a substrate, or of an inhibitor might influence the rate by an ionic-strength effect. Table 4 shows the effect of added salts on the initial rate of the forward reaction at pH 8·0 in imidazole buffer ($\mu = 0\cdot01$ in absence of added salts). In each case the rate is substantially reduced. Kinetic measurements should, therefore, be carried out at constant ionic strength.

The effect of perchlorate ion is rather larger than that of other simple anions. A detailed kinetic analysis shows that the values of the so-called Michaelis constants are altered by an amount about twice as large with perchlorate ions as with chloride ions.[9] This phenomenon is not, at the moment, understood, but it is of interest since the rate of

* We have occasionally had preparations in which the curve of rate against enzyme concentration, as determined by the direct method, fall well below the predicted curve. The origin of this is not understood.

† We do not claim that there are no buffer effects with this enzyme but only that there are none under our stated conditions and that the direct method is, in general, unsuitable for investigation in this field.

‡ It is of interest that the authors [5] noted a considerable lag at the beginning of the reaction.

[6] Jenkins, Yphantis, and Sizer, *J. Biol. Chem.*, 1959, **234**, 51.
[7] Turano, Fasella, and Giartosio, *Biochem. Biophys. Acta*, 1962, **58**, 255.
[8] Banks and Lawrence, unpublished results.
[9] Lawrence, unpublished results.

TABLE 4.

Relative rates (v) of the transamination reaction in the presence of added salts.

(v in absence of added salts = 1·00; 25°, pH 8·0, aspartate 0·02M, α-oxoglutarate 0·005M.)

Concn. (M)	0·025	0·05	0·10	0·125	0·15	0·20	0·25
NaCl	1·00	0·97	0·86	—	0·64	0·48	0·35
KCl	1·00	0·98	—	0·77	—	—	0·46
Na_2SO_4	0·99	0·93	0·86	—	0·68	0·61	0·55
NaOAc	0·99	0·97	0·85	—	0·73	0·64	0·52
$NaClO_4$	0·94	0·83	—	0·51	—	—	0·28

combination of cofactor and apoenzyme is increased by the presence of perchlorate ions.[10] It is possible that the perchlorate ion partially opens the tertiary structure of the apoenzyme in such a way that combination with the cofactor, perhaps for steric reasons, occurs more easily. If, however, the normal transamination involves some degree of co-operation between two cofactor molecules,[11] such a change in tertiary structure would result, consistently with the observations, in a decreased catalytic efficiency.

Experimental.—Materials. Commercial imidazole was sublimed *in vacuo*. Sodium chloride, potassium chloride, sodium acetate, and sodium sulphate were of " AnalaR " grade. Sodium perchlorate was made from " AnalaR " sodium hydroxide and perchloric acid. Triethanolamine was purified by recrystallisation of the hydrochloride. Other buffers (Table 1) were commercial samples.

DPNH was obtained from Sigma Chemical Co. and was used without purification. Malic dehydrogenase was obtained from British Drug Houses Ltd. and was free from transaminase activity.

Other material were as previously described.[12]

Kinetic methods. (*a*) Direct method. This was essentially as described previously.[12] Greater sensitivity was achieved by using a 4-cm. cell and measuring the change in absorbency at 2600 Å (oxaloacetic acid in 0·1M-phosphate buffer, pH 7·38, 25°, ε 1090; α-oxoglutaric acid under the same conditions, ε 57). It was convenient to use a manually operated spectrophotometer (Unicam model S.P. 500) fitted with a constant-temperature cell housing. The extinction coefficient of oxoloacetic acid varies slightly with ionic strength. Each experiment must, therefore, include a determination of the extinction coefficient under those particular conditions.

(*b*) Aliquot method. This was as previously described.[12]

(*c*) Malic dehydrogenase method. Mixtures containing substrates, buffer, malic dehydrogenase, and DPNH (*ca.* 10^{-4}M) were allowed to come to thermal equilibrium in a 4-cm. cell fitted into a constant-temperature cell housing. The reaction was started by adding a solution of transaminase and followed by measuring the changes in absorbency at 3400 Å.

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[10] Wootton, unpublished results.
[11] Wootton and Vernon, 140th Meeting Amer. Chem. Soc., 1961, Abs. 10c.
[12] Banks and Vernon, *J.*, 1961, 1698.