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Equilibria in Transamination Systems

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Preliminary determinations of the equilibrium constants in the glutamic-aspartic and the glutamic-alanine system by Cohen (1940) gave the following values at 38°:

$$\frac{[\alpha\text{-ketoglutarate}] \times [\text{L-aspartate}]}{[\text{L-glutamate}] \times [\text{oxaloacetate}]} = 3{\cdot}5, \quad (1)$$

$$\frac{[\alpha\text{-ketoglutarate}] \times [\text{L-alanine}]}{[\text{L-glutamate}] \times [\text{pyruvate}]} = 1. \quad (2)$$

Improved techniques employed by Darling (1945, 1947) gave 5·44 for (1) and 2·25 for (2) whilst Lénárd & Straub (1942) reported a value of 1·43 for (2). In the present work the two constants were redetermined with the help of the newer more accurate manometric methods for the determinations of glutamic, α-ketoglutaric, aspartic, pyruvic and oxaloacetic acids. Under optimum conditions the probable error of a single estimation of any of these substances is not greater than 1%, but a complication arises in the determination of the equilibrium constant of (1) from the instability of oxaloacetate; its slow conversion into pyruvate and bicarbonate is unavoidable and no perfect equilibrium can be reached. However, any error due to this side reaction is negligible if the rate of the side reaction is slow in relation to that of transamination. The presence of additional substances formed by side reactions does not cause errors if the concentrations of all four reactants in the transamination systems are determined directly.

EXPERIMENTAL AND RESULTS

Measurements in the glutamic-aspartic system

Enzyme preparation. Finely minced horse heart muscle was extracted with 2 vol. 0·5% NaCl solution for 60 min. and then centrifuged. The supernatant was dialysed for at least 4 hr. against running tap water to remove preformed substrates. The dialysed extract was stored in the refrigerator after addition of, and thorough mixing with, *sec.*-octanol. The extract contained a powerful glutamic-aspartic transaminase and showed weak alanine-glutamic transaminase and oxaloacetic decarboxylase activity. That the rate of side reactions under the test conditions was very small can be judged from data given in Table 1. In the absence of the side reactions the sum of aspartic and glutamic acids should remain constant and should equal the amount of glutamate added, any deficit being due to the formation of alanine. Furthermore, the sum of oxaloacetate and aspartate should be constant and equal the amount of aspartate added, any loss being due to the decarboxylation of oxaloacetate.

Reaction mixture and sampling for analysis. Equal volumes (10–15 ml.) of about 0·08 M-α-ketoglutarate, about 0·08 M-L-aspartate, enzyme solution and 0·1 M-phosphate buffer, pH 7·4, and a few drops of *sec.*-octanol were mixed and placed in a water bath at 25·0°. The actual initial concentrations of the α-ketoglutarate and L-aspartate solutions were determined in each experiment. A control mixture contained buffer and enzyme but no substrate. At specified times samples were removed for analysis. For the determination of oxaloacetic acid 3 ml. were placed in the main compartment of an ice-cooled conical manometer vessel containing 1 ml. 2 N-HCl. For the determination of the three other reactants 9 ml. were pipetted into a graduated

test tube containing 1 ml. 2N-NaOH, to stop the enzyme activity; these samples were then placed for 30 min. in a boiling-water bath to inactivate the enzyme and to decompose the oxaloacetate. Care was taken to avoid loss of water by evaporation. Under these conditions there was no loss of glutamate, aspartate or α-ketoglutarate, but losses of glutamic acid up to 10% occurred when NaOH was replaced by HCl. After cooling, glutamate was determined in 3 ml., α-ketoglutarate and aspartate in 0·5 ml. All analyses were carried out in duplicate and specially calibrated pipettes were used. The amounts of gas measured were of the order of 300 μl. in all four cases.

Analytical procedures. Oxaloacetate was determined manometrically according to Ostern (1933). The side arm of the vessel containing the acidified solution of oxaloacetic acid was charged with 0·5 ml. aniline. HCl was used for acidification rather than citric or acetic acids because the spontaneous decomposition of oxaloacetic acid is slower in strongly acid medium. The manometric determination was carried out at 3–5° in a cold room. Under these conditions loss owing to the spontaneous decomposition of oxaloacetic in the equilibration period was negligible. The solubility of CO_2 in the reaction mixture was found to be the same as in pure water. This was determined by adding a known amount of bicarbonate to the solution and using the equation for the vessel constant for the calculation of α_{CO_2}.

Glutamic acid was determined with the specific decarboxylase of *Clostridium welchii* S.R. 12 (Krebs, 1948, 1950). The 3 ml. sample of the alkaline solution was neutralized in the manometer vessel by 0·3 ml. 2N-HCl. In addition, 0·5 ml. M-acetate buffer, pH 4·5, 0·5 ml. 2% Cetavlon (cetyltrimethylammonium bromide) and 0·05 ml. aniline were added. Under these conditions the presence of aspartic and pyruvic acids in the test solution did not interfere with the determination of glutamic acid. Meister, Sober & Tice (1951) have found that suspensions of *Cl. welchii* can form CO_2 and α-alanine in the presence of aspartic acid provided that small (catalytic) quantities of an α-ketonic acid are present. This reaction may interfere with the determination of glutamic acid under some conditions, but it does not occur in the presence of Cetavlon. The bacterial suspension did not decarboxylate pyruvic acid (see Boulanger & Osteux, 1949; Krebs, Boulanger & Osteux, 1950).

L-Aspartate and α-ketoglutarate were determined in 0·5 ml. samples of the alkaline solutions according to the method of Krebs (1950). Special care was taken to ensure the quantitative liberation of CO_2, by adjusting the pH of the solutions to a value below 5·0. They were brought to approximately this pH with acid or alkali before the addition of Cetavlon, bromocresol green serving as external indicator, and 0·5 ml. 0·2M-acetate buffer of pH 4·5 was added to the main compartment, 0·2 ml. to the side arm.

Results. Preliminary experiments showed that under the conditions of the experiments equilibrium was established within about 20 min. In the main experiments, the solutions were analysed after 30, 40 and 50 min. incubation. The results are given in Table 1. As will be seen the value of the equilibrium constant K remained virtually constant during incubation although, owing to the two side reactions mentioned, the concentration of the reactants changed slightly. The concentrations of oxaloacetate, aspartate and α-ketoglutarate fell, that of glutamate rose very slightly. The eighteen recorded values of K vary between 6·45 and 7·06, the mean being 6·74. Darling (1945) gives four measurements in support of his average value of 5·44, the range being 4·1–6·3. Only oxaloacetate was determined (colorimetrically) and the concentration of the other components was calculated from their initial quantity and the oxaloacetate value, a procedure which is likely to be less reliable than that used in the present paper.

Table 1. *Equilibrium in the aspartic-glutamic-transaminase system*

(Temp., 25°; pH 7·4; concentrations are expressed in μl. substrate/ml. solution.)

Exp. no.	Period of incubation (min.)	Concentrations found				K
		Oxaloacetate	Glutamate	α-Ketoglutarate	Aspartate	
1	30	111	121	298	307	6·81
		111	122	297	306	6·71
	40	106	120	289	298	6·78
		104	123	289	284	6·78
	50	108	123	297	305	6·82
		100	123	292	297	7·06
2	30	114	122	315	300	6·78
		115	122	316	302	6·82
	40	111	122	313	294	6·78
		111	124	313	293	6·68
	50	108	127	319	296	6·72
		109	127	314	289	6·45
3	30	119	123·5	324	309	6·82
		118	123	324	314	7·01
	40	117	124	318	302	6·62
		113	123	315	306	6·65
	50	115	127·5	311	300	6·60
		114	126	315	298	6·48

Measurements in the glutamic-alanine system

Enzyme preparation. Sheep heart, removed from the animal within a few minutes after death, was carried in ice from the abattoir to the laboratory and on arrival the muscle tissue, freed from fat, was minced in the Latapie mill. 1 part of mince was stirred into 2 parts of 0·9% NaCl solution. After standing at room temperature for 4 hr., the suspension was filtered through muslin, and to each 100 ml. filtrate 35 g. $(NH_4)_2SO_4$ were added. The precipitate containing the enzyme was allowed to form overnight in the refrigerator and was centrifuged off; the inactive supernanant was discarded. The precipitate was stirred into a small volume of water to form a liquid paste which was dialysed for 16 hr. against running tap water. Insoluble matter in the dialysed material which proved inactive was separated by centrifugation. The active supernatant was dried *in vacuo* over H_2SO_4. About 1 g. dry powder was obtained from 1 kg. fresh heart muscle. It was pink coloured and readily soluble in water. To test the activity, 0·25 ml. 0·5M-phosphate buffer, pH 7·4, 0·25 ml. 0·4M-sodium pyruvate, 0·25 ml. 0·4M-sodium glutamate and 4·25 ml. enzyme solution were incubated at 25°. After 30 min. the reaction was stopped by the addition of 0·5 ml. 2N-HCl and heating to 100° for 10 min. α-Ketoglutarate was determined manometrically in 1 ml. of this solution; 1 mg. of the dry powder produced about 1·5 mg. α-ketoglutaric acid under these conditions.

As precipitation with $(NH_4)_2SO_4$ always caused considerable losses (see O'Kane & Gunsalus, 1947) a concentrated, dialysed tissue extract was used in some experiments. The tissue extract prepared from minced tissue as described above was reduced *in vacuo* to about one-fifth of the original volume and the concentrate was dialysed against running tap water. The solution obtained after the removal of insoluble particles by centrifugation was stored in the frozen state. Horse heart gave less active enzyme preparations. Heating of the tissue suspension to 55° for 50 min. (see Lénárd & Straub, 1942) did not increase the yield.

Reaction mixture and sampling. 2 ml. 0·4M-L-glutamate (or α-ketoglutarate), 2 ml. 0·4M-pyruvate (or 0·8M-DL-alanine), 4 ml. 0·5M-phosphate buffer, pH 7·4 and 32 ml. enzyme solution were incubated at 25°. Controls contained buffer and enzyme, but no substrates. Three types of special controls containing either all constituents except glutamate, or all constituents except pyruvate, or all constituents except the enzyme were set up in the first experiments to test the disappearance of substrates by reactions other than

Table 2. *Equilibrium in the glutamate-alanine-transaminase system*

(Initial substrates: L-glutamate and pyruvate. Temp., 25°, pH 7·4. Concentrations expressed as μl. substrate/ml. solution. Enzyme preparation 1:20 ml. dialysed heart extract in total volume of 40 ml.; enzyme preparation 2:70 mg. dry powder in total volume of 40 ml.)

Exp. no.	Period of incubation (hr.)	Enzyme preparation	Initial substrate concentration		Final substrate concentration			Equilibrium constant $\frac{[\alpha\text{-Ketoglutarate}]\times[\text{L-alanine}]}{[\text{L-glutamate}]\times[\text{pyruvate}]}$
			L-Glutamate	Pyruvate	L-Glutamate	α-Ketoglutarate and alanine	Pyruvate	
1	4	1	432	428	196	235	193	1·48
	6				197	238	190	1·51
					197	238	190	1·51
	24				197	238	190	1·51
					198	231	197	1·37
2	3	1	426	431	196	236	195	1·46
					196	235	196	1·44
	4				200	232	199	1·38
					200	236	200	1·39
	6				194	239	192	1·54
					196	235	196	1·44
	24				191	230	201	1·38
					193	229	202	1·38
3	6	2	432	433	193	239	194	1·53
					193	241	192	1·57
	24				193	241	192	1·57
					191	241	192	1·58
4	3	2	454	470	194	252	208	1·57
					196	250	210	1·52
	4				195	251	209	1·43
					196	250	210	1·52
	6				194	252	208	1·47
					194	252	208	1·47
	24				191	255	205	1·66
					194	252	208	1·57
							Av	**1·50**

Table 3. *Equilibrium in the glutamate-alanine-transaminase system*

(Initial substrates: α-ketoglutarate and DL-alanine. For other particulars see Table 2.)

Exp. no.	Period of incubation (hr.)	Enzyme preparation	Initial substrate concentrations: α-Keto-glutarate	Initial substrate concentrations: DL-Alanine	Final substrate concentrations: L-Glutamate and pyruvate	Final substrate concentrations: α-Keto-glutarate	Final substrate concentrations: L-Alanine	Equilibrium constant: [α-Ketoglutarate] × [L-alanine] / [L-glutamate] × [pyruvate]
1	3	1	463	896	203	257	245	1·53
					205	256	243	1·48
	4				201	250	249	1·54
					200	252	248	1·56
	6				201	246	247	1·50
					202	245	246	1·47
	24				201	246	247	1·50
					200	247	248	1·51
2	3	2	461	496	197	259	251	1·67
					198	260	250	1·66
	4				202	261	246	1·57
					202	260	246	1·57
	6				193	258	249	1·62
					203	256	245	1·52
	24				201	257	247	1·57
					200	259	248	1·61
							Av.	1·56

transamination. These tests were negative. The sample for the determination of glutamic acid was treated with alkali and that for the determination of α-ketoglutarate with acid as described above.

Analytical procedure. The procedures for the determination of glutamate and α-ketoglutarate have already been described. In addition the initial concentrations of the reactants was known. The concentration of pyruvate in the stock solutions (from which initial pyruvate concentration in the reaction mixture was obtained) was determined by the carboxylase method and the ceric sulphate methods (Krebs & Johnson, 1937) which gave identical results with pure solutions. In the carboxylase method the pH of the acetate buffer was 4·5 to ensure that the CO_2 evolution was quantitative, 0·02 vol. 3M buffer being used to acidify the yeast extract and 0·5 ml. 0·2M buffer to acidify the solution in the main compartment. The direct determination of pyruvate in the equilibrium mixture was not attempted because α-ketoglutarate interfered with the method of analysis.

Reagents. As the concentrations of alanine and pyruvate were determined by difference, the purity of these substances was of importance. Solutions of aqueous pyruvic acid or sodium pyruvate were found to change on storage in the refrigerator, forming substances (presumably condensation products) which were decarboxylated by yeast carboxylase but did not react in the transaminase system. Pure pyruvic acid solutions were obtained as follows. Freshly prepared pyruvic acid was subjected to fractional distillation *in vacuo* and weighed portions of the middle fractions were immediately diluted with about 4 vol. of water. The strength of this stock solution was determined by the carboxylase and ceric sulphate methods and was found to agree with the calculated value. On storage of frozen solutions at −10° no changes occurred within 3 months. For use, a sample of stock solution was freshly neutralized with an equivalent amount of $NaHCO_3$ immediately before the start of the experiment. Commercial L-alanine (Roche Products Ltd.) was found by paper chromatography to contain an impurity (serine or threonine) and also traces of D-amino-acid when tested by D-amino-acid oxidase. DL-Alanine was therefore used. Its assay with D-amino-acid oxidase showed that its purity was above 99·5%. D-Alanine did not react with the transaminase preparation used.

In all experiments, irrespective of whether glutamate, pyruvate or α-ketoglutarate and alanine were the substrates, both α-ketoglutarate and glutamate were determined in the incubated solution. When L-glutamate and pyruvate were the substrates, the equilibrium concentration of L-alanine was taken to be the same as that of α-ketoglutarate and that of pyruvate to be the difference between its initial concentration and the final concentration of α-ketoglutarate. When α-ketoglutarate and DL-alanine were the substrates, the equilibrium concentration of pyruvate was taken to be the same as that of glutamate and that of L-alanine to be the difference between its initial concentration and that of the equilibrium concentration of glutamate. The assumptions made would become invalid if side reactions occurred, but there is no evidence suggesting that this was the case. The sum of the two C_5 compounds recovered agreed with the amounts added, and once equilibrium was established the concentrations of the two C_5 compounds remained constant (Tables 2 and 3). These findings ruled out significant side reactions of the two C_5 compounds.

Results. The results of the measurements carried out after the perfection of the methods are given in Tables 2 and 3. The same equilibrium position was reached from both sides, the average value for the constant being 1·52. This is in fair agreement with the value of 1·43 of Lénárd & Straub (1942).

SUMMARY

The equilibrium constants of two transamination systems have been measured at 25°. The equilibrium constants, as defined in equations (1) and (2) were found to be, on the average, 6·74 in the glutamic-aspartic system and 1·52 in glutamic-alanine system.

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The Free-energy Changes for the Reduction of Diphosphopyridine Nucleotide and the Dehydrogenation of L-Malate and L-Glycerol 1-Phosphate

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The free energy of reduction of diphosphopyridine nucleotide (DPN) may be evaluated by adding the free-energy change calculated from the equilibrium of a suitable DPN dehydrogenase system (1) to the free-energy change for the reduction of the substrate (2)

$$AH_2 + DPN^+ \rightarrow A + DPNH + H^+, \quad (1)$$

$$A + H_2 \rightarrow AH_2, \quad (2)$$

$$DPN^+ + H_2 \rightarrow DPNH + H^+. \quad (3)$$

The standard free-energy change (ΔG^0) of reaction (3) is related to the oxidation-reduction potential (E_0')

$$E_0' = -\frac{\Delta G^0}{2F} - \frac{RT\,\mathrm{pH}}{2F \log e},$$

where F is the Faraday (23068 cal.), R the gas constant and T the absolute temperature. By this procedure, several workers have obtained values for the oxidation-reduction potential of DPN using data for different hydrogenase systems (Green & Dewan, 1937; Clark, 1938; Schlenk, Hellström & Euler, 1938; Borsook, 1940). Several serious discrepancies appear when the available free-energy data for different dehydrogenase systems are critically examined, e.g. the generally accepted value for the DPN potential (−0·282 V. at pH 7 and 30°; Borsook, 1940) differs from that (−0·320 V. at pH 7 and 25°) which may be obtained from equilibrium and thermochemical data for the ethanol-DPN-acetaldehyde system (Dixon, 1949; Burton, 1952). A value closer to that of Borsook (namely −0·29 V. at pH 7 and 25°) is indicated by the potential measurements of Barron & Hastings (1934) and the equilibrium data of Kubowitz & Ott (1943) and Racker (1950) for the lactic dehydrogenase system. Another value of about −0·21 V. may be obtained by combining the potential measurements of Lehmann & Hoff-Jörgensen (1939) for the malate-oxaloacetate reaction with equilibrium data given by Schlenk *et al.* (1938).

The object of the work described in this paper was, first, to obtain a reliable independent value for the free-energy change (ΔG^0) for the reduction of DPN (i.e. the oxidation-reduction potential), and secondly to use this value in conjunction with equilibrium data to obtain the free-energy change of several biological dehydrogenations. To evaluate ΔG^0 for the reduction of DPN, the equilibrium constant has been measured for the reaction:

*iso*propanol + DPN^+ → acetone + DPNH + H^+, (4)

which is catalysed by crystalline preparations of yeast alcohol dehydrogenase. This reaction was

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