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The Role of Iron in the Reaction between Rabbit Transferrin and Reticulocytes*

E. Baker and E. H. Morgan

ABSTRACT: The effect of the degree of iron saturation of transferrin on the kinetics of the interaction with reticulocytes was studied using pure rabbit transferrin and rabbit reticulocytes. The increase in the association rate constant measured with iron-saturated transferrin compared with iron-free transferrin was not statistically significant. The dissociation rate constant for the reaction between reticulocytes and transferrin which was low in iron content was larger than that for iron-saturated transferrin. More iron-containing than iron-free transferrin was bound to the reticulocytes at equilibrium. This difference was largely due to the iron-containing molecules having a longer mean residence time on the reticulocyte. The efficiency of the iron transfer process was 86%, indicating that a proportion of the iron-containing transferrin molecules taken up by reticulocytes was refluxed without donating all their iron to the cells.

Each molecule of the plasma iron-binding protein transferrin is capable of binding two molecules of ferric iron (Laurell and Ingelman, 1947; Surgenor *et al.*, 1949). A specific property of the transferrin molecule is its ability to donate this bound iron to hemoglobin-synthesizing cells (Laurell, 1947; Paoletti and Durand, 1958; Jandl *et al.*, 1959) and to iron-storing cells (Mazur *et al.*, 1960). The interaction between transferrin and cells has been studied in detail using reticulocytes (Jandl and Katz, 1963; Morgan and Laurell, 1963; Morgan, 1964; Kornfeld, 1968; Baker and Morgan, 1969). Contradictory evidence has been published on the conditions of exchange of iron between the transferrin–iron complex and reticulocytes. For example, Jandl and Katz (1963) presented evidence that the rate of iron transfer was dependent upon the degree of iron saturation of the transferrin and that the reticulocyte had a greater affinity for iron-containing than iron-free transferrin. In contrast, Morgan and Laurell (1963) found that iron transfer was dependent only upon iron concentration and that iron-free and iron-containing transferrin molecules had equal affinity for the cell binding sites.

This investigation was undertaken primarily to evaluate the importance of the iron moiety in the reaction between transferrin and reticulocytes. A comparison was made of the kinetics of the association and dissociation reactions between rabbit reticulocytes and rabbit transferrin which was either iron saturated, low in iron, or iron free. An estimate of the efficiency of the iron-donating process from bound transferrin molecules to reticulocytes has been obtained.

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Methods

Unless specifically noted the preparative, analytical, and experimental procedures performed in this study were carried out as described previously (Baker and Morgan, 1969).

Removal of Iron from Transferrin. Two methods were used to remove iron from transferrin. In the first the pH of the transferrin solution was lowered to 4–5 using citric acid, and the resulting iron–citrate complex was removed by passage through a column of Amberlite-IRA 410 (A. R. Grade) prepared in the chloride form (Bothwell *et al.*, 1959). The pH was immediately readjusted to 7.5 using dilute NaOH. The iron content of transferrin treated in this way was within the range of 5–17% saturation of its iron binding capacity.

The second method of iron removal was used to render transferrin completely iron free by dialysis against a solution containing 0.01 M EDTA in 0.3 M sodium acetate (pH 5.5). The EDTA–iron complex was removed by exhaustive dialysis of the transferrin solution against deionized water and Hanks and Wallace's physiological salt solution (Hanks and Wallace, 1949) for 48 hr at 4°.

Isotope Labeling. ^{59}Fe was obtained from the Radiochemical Centre, Amersham, England, in the form of ferric chloride dissolved in 0.1 N HCl. Iron-free transferrin dissolved in Hank's solution was labeled with ^{59}Fe by incubation with the isotope solution for 30 min at 37°.

Results

Uptake of Iron-Saturated and Low-Iron Transferrin. Transferrin uptake by reticulocytes was measured in duplicate systems which differed only in the degree of iron saturation of the transferrin. As shown in Figure 1, more of the iron-rich transferrin (99% iron saturated) was bound at equilibrium than low-iron transferrin (12% iron saturated). In five similar ex-

TABLE I: The Uptake of Iron-Saturated and Low-Iron Transferrin by Reticulocytes.[a]

Mixture	Iron Saturation (%) Tr-^{131}I	Tr-^{125}I	Transferrin Uptake (μmoles/ml of reticulocytes $\times 10^3$) Tr-^{131}I	Tr-^{125}I	Total	Ratio of Tr-Fe_2/Tr
1	14	15	0.30	0.32	0.62	
2	96	15	0.45	0.30	0.75	1.50
3	14	98	0.29	0.47	0.76	1.62
4	96	98	0.40	0.39	0.79	

[a] The uptake at equilibrium of iron-saturated and low-iron transferrin by reticulocytes was measured in duplicate systems (mixtures 1 and 4) and in one system (mixtures 2 and 3). The final incubation transferrin concentration was 0.045 mM.

periments the average uptake of iron-rich transferrin at equilibrium was 10.0 ± 4.5% greater (mean ± std dev) than that of low-iron transferrin. This difference was statistically significant ($P < 0.05$). The average degree of iron saturation of the iron-rich transferrin was 99 ± 1% (mean ± std dev) and of the low-iron transferrin was 13 ± 4% (mean ± std dev).

The uptake by reticulocytes of iron-saturated and low-iron transferrin was compared in a single system using a modification of the method of Jandl and Katz (1963). Equal amounts of transferrin-^{125}I, either iron saturated or low in iron, were pipetted into a series of four sets of tubes. Equal amounts of transferrin-^{131}I, either saturated or low in iron, were then added in the order indicated in Table I. The same volume of reticulocytes was added to each mixture, and the suspensions were incubated for 30 min at 37° before washing. The uptake of iron-saturated and low-iron transferrin, and the total uptake by each suspension in a typical experiment are shown in Table I. Each value is the mean of duplicates. Comparing mixtures 1 and 4, there was a greater total uptake of iron-saturated transferrin than low-iron transferrin in duplicate systems. When unsaturated and saturated transferrins were present in equal concentration in a single system, as in mixtures 2 and 3, approximately 50% more iron-saturated transferrin was bound at equilibrium than low-iron transferrin. There should be no significant exchange of iron between saturated and unsaturated transferrin during the time involved in these incubations (Turnbull and Giblett, 1961; Katz, 1961; Aisen and Leibman, 1968).

Effect of Degree of Iron Saturation of Transferrin on the Association and Dissociation Reactions. The pseudo-first-order rate constants for the association reaction (Baker and Morgan, 1969) were determined using reticulocytes incubated in duplicate systems with transferrin which was either iron saturated or low in iron. A summary of the results is shown in Table II. In each of eight experiments the association rate constant was slightly but not significantly greater for the reaction between iron-saturated transferrin and reticulocytes than for low-iron transferrin ($P < 0.10$).

The first-order dissociation rate constant for the release of transferrin from reticulocytes was measured after equilibrating duplicate reticulocyte samples with transferrin which was

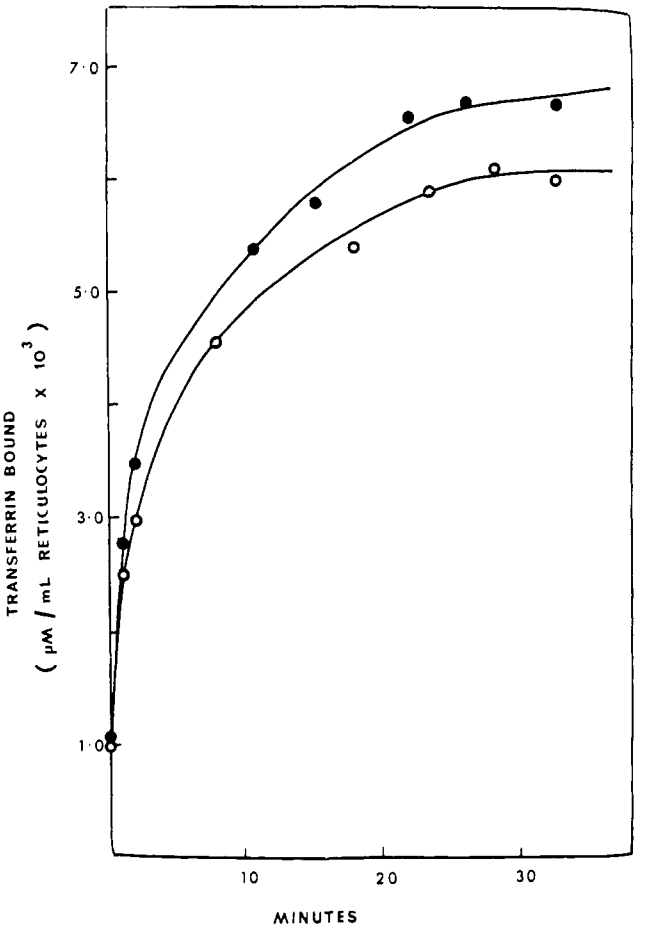

FIGURE 1: Uptake by reticulocytes of transferrin which was 99% iron saturated (●) or 12% saturated (○) during incuation at 37°. The transferrin concentration was 0.03 mM.

either iron saturated or low in iron. A summary of the results obtained in four experiments is shown in Table II. In each experiment the dissociation rate constant was greater following preincubation with transferrin which was low in iron as compared with iron-saturated transferrin. This difference was statistically significant ($P < 0.01$) in two of the four experi-

TABLE II: Kinetic Characteristics of the Reaction between Iron-Saturated Transferrin, Low-Iron Transferrin, and Reticulocytes.

	$TrFe_2$	Tr
% iron saturation ($n = 8$)[a]	100.1 ± 0.9[b]	12.1 ± 5.0
$k_1 \times 10^4$ (sec^{-1}) ($n = 8$)	0.782 ± 0.286	0.595 ± 0.272
$k_2 \times 10^4$ (sec^{-1}) ($n = 4$)	11.9 ± 5.4	17.2 ± 5.6
$t_{1/2}$ complex (min) ($n = 4$)	9.7 ± 2.5	6.7 ± 2.9

[a] Number of experiments. [b] Mean plus or minus standard deviation.

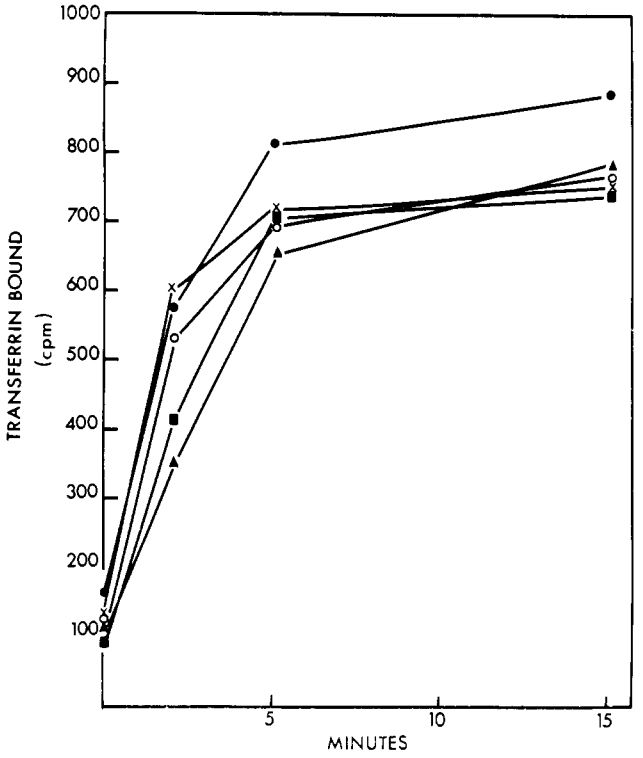

FIGURE 2: Uptake by reticulocytes of iron-free transferrin (○) or transferrin which was saturated with iron (●), copper (×), zinc (■), or manganese (▲) during incubation at 37°. The transferrin concentration was 0.02 mM.

ments. The average half-life of the transferrin–iron reticulocyte complex was correspondingly greater than that of the transferrin–reticulocyte complex.

In one experiment the activation energy for the association reaction was determined as described previously (Baker and Morgan, 1969) using iron-saturated and low-iron transferrin. There was no apparent difference in the values obtained for the activation energy of the reaction between reticulocytes and iron-saturated transferrin (8.1 kcal mole^{-1}) or transferrin which was low in iron (7.6 kcal mole^{-1}).

Effect of the Nature of the Metal Moiety on Transferrin Uptake. A comparison was made of the uptake by reticulocytes of transferrin to which iron, copper, zinc, or manganese was attached. Iron-saturated transferrin was labeled with ^{125}I, dialyzed against EDTA to remove iron, and against Hank's solution, and was then divided into five parts. A sufficient amount of each metal was added to saturate the transferrin binding sites in four of the aliquots. The salts used were $Fe(NH_4)_2(SO_4)_2$, $CuSO_4$, $MnSO_4$, and $ZnSO_4$ dissolved in saline. An equal volume of saline was added to the fifth aliquot of labeled transferrin to provide a comparison with the metal-free protein. The preparations were incubated for 30 min at 37° prior to measurement of uptake by reticulocytes. As shown in Figure 2, more iron-saturated transferrin was taken up at 0 min, and the amount of iron-saturated transferrin bound to the reticulocytes at equilibrium was approximately 13% greater than that of the iron-free transferrin or transferrin complexed to the other metals. There was no significant difference between the amount of iron-free transferrin or of the copper, manganese, and zinc complexes bound after 15-min incubation.

TABLE III: Kinetic Characteristics of the Reaction between Reticulocytes and Iron-Saturated or Iron-Free Transferrin.[a]

	$TrFe_2$	Tr
% iron saturation	99.2[b]	0.5
$k_1 \times 10^4$ (sec^{-1})	0.843	0.888
$k_2 \times 10^4$ (sec^{-1})	11.50	15.87
$t_{1/2}$ complex (min)	10.1	7.2

[a] Measurements were made using iron-free, EDTA-treated transferrin, and EDTA-treated transferrin which was resaturated with iron. [b] Each value is the mean obtained in measurements on two transferrin preparations. Each experiment was performed in duplicate.

Comparison of Uptake of EDTA-Treated Iron-Saturated and Iron-Free Transferrin. The results obtained with transferrin which was iron saturated or low in iron suggested that the presence of iron on the transferrin molecule was not essential for the uptake by reticulocytes. However, the minimum degree of iron saturation obtained using citrate to remove the iron was 5.3%. A comparison was therefore made of the reactivity of iron-saturated transferrin and transferrin rendered completely iron free by dialysis against EDTA.

Significant alterations in reactivity were observed in two out of four transferrin preparations treated with EDTA. The association rate constant for iron-free transferrin which was resaturated with iron was reduced from 0.70×10^{-4} to 0.04×10^{-4} sec^{-1}, and the dissociation rate constant was increased from 12×10^{-4} to 30×10^{-4} sec^{-1} in the affected preparations. These results suggest that EDTA can have an effect on the transferrin molecule which reduces its reactivity with reticulocyte binding sites.

Two other preparations were not affected by treatment with EDTA, and the average values obtained in kinetic measurements on these preparations are summarized in Table III. There was no significant difference in the association rate constant for iron-free and iron-saturated transferrin. The dissociation rate constant was significantly greater ($P < 0.01$) for the reaction between reticulocytes and iron-free transferrin than for iron-saturated transferrin. This difference was reflected in the half-life of the transferrin–iron–reticulocyte complex, which was considerably longer than that of the transferrin–reticulocyte complex.

Efficiency of the Iron Transfer Process. In the initial stages of the reaction between plasma transferrin and reticulocytes the rate of iron uptake represents the rate of uptake of transferrin, and hence the maximum possible rate of transfer of iron if each bound molecule donated all its iron to the cell. In later stages of the incubation the transferrin dissociation reaction is also taking place at a significant rate, and hence the rate of iron uptake here is a measure of the actual rate of iron accumulation by the cells. The efficiency of the process of iron transfer to the reticulocytes was therefore evaluated as the ratio of the rate of iron uptake measured 10–30 min after admixture of cells and transferrin, compared with that measured within the first 5

min of the reaction. Reticulocytes and fresh plasma labeled with ^{59}Fe were prewarmed to 37° prior to admixture. Cell samples were transferred to ice-cold Hank's solution after incubating at 37° for time intervals up to 30 min and washed three times before estimation of radioactivity. The uptake of iron by reticulocytes at 37° is shown in Figure 3. In six experiments, the average rate of iron uptake measured 10–30 min after the start of incubation was 86.6 ± 7.5% of the initial rate determined during the first 5 min. This decrease in rate of iron uptake with time of incubation was statistically significant ($P < 0.01$).

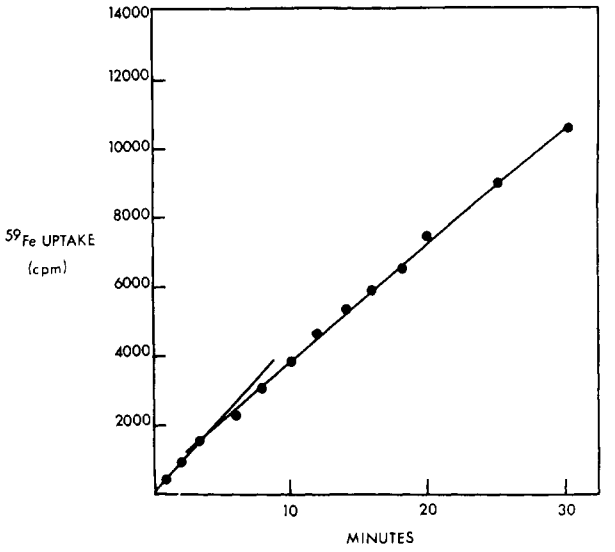

FIGURE 3: ^{59}Fe uptake by reticulocytes at 37°. Each point is the mean of duplicates. The transferrin concentration was 0.03 mM.

Discussion

The specific association rate constant was consistently larger when transferrin was iron-saturated compared with low-iron transferrin. However, the difference was small and not statistically significant, suggesting that iron had little or no effect on the association reaction between transferrin and reticulocytes. Similar results were obtained with two of the EDTA-treated iron-free transferrin preparations. In addition there was no apparent difference in the activation energies required for the reaction between reticulocytes and iron-saturated or low-iron transferrin. These results indicate that the iron moiety is not essentially or directly involved in bond formation between transferrin and reticulocyte binding sites. Any slight differences in the reactivity of iron-free and iron-saturated transferrin are probably related to slight changes in molecular configuration. The transferrin molecule is more elongated and has a lower degree of hydration when iron is attached (Bezkorovainy, 1966).

The specific rate constant for the dissociation from reticulocytes of transferrin which was initially iron saturated was lower than that of transferrin which was initially iron free or low in iron content. Hence the calculated half-life for the transferrin–iron–reticulocyte complex was larger than that of the transferrin–reticulocyte complex. The longer time of attachment of the iron-containing molecules to the reticulocyte was probably related to rate-limiting reactions involved in transferring iron to the cell.

More iron-containing than iron-free transferrin was bound to the reticulocyte at equilibrium. The results of the present study indicate that this difference was largely due to the iron-containing molecules having a larger mean residence time on the reticulocyte, rather than to them having a greater affinity for the reticulocyte binding sites. Evidence that the longer time of attachment is related to rate-limiting iron transfer reactions lies in the observation that the amounts of manganese, copper, and zinc transferrins taken up at equilibrium were the same as that of apotransferrin and significantly less than that of iron-containing transferrin. Such differences would occur if these metal–transferrin complexes had a lower binding affinity for reticulocyte binding sites or a shorter time of attachment to the reticulocyte, compared with iron-saturated transferrin. The latter is more probable because the time curves for the uptake of the metal–transferrin complexes and apotransferrin were similar, and the equilibrium positions were the same. Hence the difference in uptake at equilibrium is probably related to rate-limiting iron transfer reactions in the case of iron-containing transferrin, as there is no evidence that the reticulocyte has specific mechanisms available for the uptake of copper, zinc, or manganese from transferrin.

When equal amounts of iron-saturated and low-iron transferrin were mixed in a single system, the proportion of iron-saturated to iron-free transferrin bound at equilibrium was larger than obtained in separate incubation systems. This difference is evidence that there is a true but small difference in binding affinity of reticulocytes for iron-free and iron-containing transferrin. In a similar study on human reticulocytes (Jandl and Katz, 1963), the amount of iron-saturated transferrin bound was 400% greater than that of low-iron transferrin incubated in the same system. This difference may be related to a species difference between human and rabbit reticulocytes, to their use of EDTA to remove iron from transferrin, or to the different methods used in calculating the relative proportion of iron-saturated and iron-free transferrin bound.

The efficiency of the iron transfer process as evaluated from a comparison of the initial rate of iron uptake by reticulocytes with that measured after 10-min incubation was significantly less than 100%, indicating that a proportion of the iron-containing transferrin molecules taken up by the reticulocytes was released into the medium without donating all their iron to the cells. There is evidence that a small fraction of the iron taken up by erythroid precursor cells from transferrin may be later released from the cells and taken up by the transferrin of the surrounding medium, but most of the iron refluxed is attached to transferrin molecules which have been released from the cells after donating none or only one of their iron atoms (Morgan *et al.*, 1966). Kornfeld (1968) has evidence that some of the transferrin binding sites are not iron-uptake sites. Transferrin uptake at these sites would produce the observed inefficiency in the iron transfer process.

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2-Keto-4-hydroxybutyrate. Synthesis, Chemical Properties, and as a Substrate for Lactate Dehydrogenase of Rabbit Muscle*

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ABSTRACT: 2-Keto-4-hydroxybutyric acid, obtained by reaction of DL-homoserine with pyridoxal in the presence of Cu^{2+} ions and isolated by ion-exchange column chromatography, has been crystallized as the γ-lactone and also as its 2,4-dinitrophenylhydrazone derivative. The physicochemical properties of these crystalline compounds have been examined. Crystalline lactate dehydrogenase from rabbit muscle catalyzes the reduction of 2-keto-4-hydroxybutyrate in the presence of reduced diphosphopyridine nucleotide. Maximal rates of reduction relative to pyruvate are 61, 43, and 25% for glyoxylate, 2-ketobutyrate, and 2-keto-4-hydroxybutyrate as substrates, respectively. The apparent K_m for 2-keto-4-hydroxybutyrate is 4.6×10^{-3} M in 33 mM potassium phosphate buffer at pH 7.4 and 25° compared with values of 3.3×10^{-4} M for pyruvate, 1.8×10^{-2} M for glyoxylate, and 4.8×10^{-3} M for 2-ketobutyrate. Oxamate competitively inhibits 2-keto-4-hydroxybutyrate reduction; in contrast, oxalate is a noncompetitive inhibitor. The dissociation constants for the enzyme–inhibitor complexes (K_i values), measured in 33 mM Tris-Cl buffer at pH 7.4 and 25°, are 8.0×10^{-5} and 8.1×10^{-4} M for oxamate and oxalate, respectively. Competitive inhibition by oxamate also occurs with pyruvate, glyoxylate, and 2-ketobutyrate (K_i values: 8.3×10^{-5}, 7.6×10^{-5}, and 7.4×10^{-5} M, respectively) whereas oxalate is noncompetitive (K_i values: 8.0×10^{-4}, 8.4×10^{-4}, and 8.0×10^{-4} M, respectively). The equilibrium for the reaction of 2-keto-4-hydroxybutyrate with reduced diphosphopyridine nucleotide in the presence of lactate dehydrogenase favors reduction of the ketohydroxy acid; the K_{equil} is 1.2×10^{-10} M determined in 0.30 M Tris-Cl buffer at 25°. Reduced diphosphopyridine nucleotide oxidation is also favored with pyruvate, glyoxylate, and 2-ketobutyrate as substrates (K_{equil} values: 4.8×10^{-12}, 1.0×10^{-9}, and 3.0×10^{-11} M, respectively). One mole of reduced diphosphopyridine nucleotide is oxidized for every mole of 2-keto-4-hydroxybutyrate reduced; 2,4-dihydroxybutyric acid has been identified as the product of the reaction. The metabolic significance of this enzymic reduction of 2-keto-4-hydroxybutyrate is discussed.

2-Keto-4-hydroxybutyric acid has been identified as a normal constituent in extracts of certain plants (Virtanen and Alfthan, 1955); its role in plant biochemistry, however, is unknown. This same compound has been suggested as a possible intermediate in the metabolism of homoserine by mammals (Meister, 1957).

In earlier studies, Hift and Mahler (1952) detected an enzyme in beef liver extracts that catalyzed the condensation of pyruvate with formaldehyde yielding 2-keto-4-hydroxybutyrate. Meister (1956) noted, in a review article, that 2-keto-4-hydroxybutyrate participates in the glutamine transaminase system; data supporting this statement have not been published. In 1966, Finkelstein *et al.* reported that crude extracts of human liver catalyze the formation of 2-keto-4-hydroxybutyrate from L-homoserine. Liver extracts of a patient with cystathionuria were found by these authors to be abnormally deficient in homoserine dehydratase activity but catalyzed the formation of 2-keto-4-hydroxybutyrate at a rate comparable with that observed with control extracts.

Studies carried out recently in our laboratories have pro-

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