Probably prior findings[1] and mine complement each other: As heme-oxidases, some of the cytochromes could prevent reduction of tetrazolium, as has been proposed, as does hemoglobin. Possibly any hem^{e-} oxidase can do so.

When 2 IU/ml of the enzyme lactate dehydrogenase (Worthington Biochemicals) is incubated with our tetrazolium spot assay[6] a purple color ordinarily develops within 30 min; when human hemoglobin is added, the time for color development increases in parallel with the concentration of hemoglobin and is increased almost 5-fold at 14 gm% Hb. When LDH is assayed spectrophotometrically[7] no difference in enzyme activity is observed in the presence of hemoglobin; thus tetrazolium oxidase acts only on the dye to prevent reduction. Hemoglobins of certain species, for example some of the fish, will not prevent reduction of tetrazolium dyes; thus the protein moiety plays an essential role in this activity.

It is curious that some proteins with otherwise physiological functions should have this ability to oxidize a dye they must rarely encounter in vivo. One important consequence of the presence of these oxidases on the gel is that they can interfere with detection of enzymes which would otherwise catalyze reduction of the dye but are present in low concentration. A false medical diagnosis of enzyme deficiency might readily be made in such cases, unless spectrophotometric enzyme assays were performed. In electrophoresis of human hemolysates at pH 8.6, the 'tetrazolium oxidase' of Hb_3 can cut across the G6PD band so as to yield 2 bands which mimic heterozygosity for G6PD type A/B[5]. Masking of other enzymes is especially treacherous when the tetrazolium oxidase stain fades after exposure to light or storage; in such cases one has not a clue to the disappearance of the masked enzyme[8].

Résumé. Des bandes blanches jusqu'ici mystérieuse, apparaissent quand on teint des traces électrophorétiques avec des réactions de tétrazolium pour faire apparaître diverses enzymes. Ces bandes se révèlent comme étant de l'hémoglobine et d'autres hémo-protéines se trouvant dans le sérum et dans des parties du tissue qui arrêtent la réduction du tétrazolium. Ces bandes peuvent donc troubler le dessin électrophorétique.

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[6] C. L. MARKERT has developed this system: *Tris*, 2*M*, pH8-5 parts; lithium lactate, 15*M*-4.5 parts; nitro blue tetrazolium, 1 mg/ml 2.5 parts; phenazine methosulfonate, 1.6 mg/ml 2.5 parts.

[7] H. U. BERGMEYER, *Methods of Enzymatic Analysis* (Academic Press, NewYork 1965), p. 736.

[8] This work was supported by NSF grant No. GB 5440X to C. L. MARKERT, Professor and Chairman of Biology, Yale University, and by NSF postdoctoral fellowship No. 48080, to M. ROSENBERG.

The Intestinal Absorption of Methionine in Chickens Provided with Permanent Thiry-Vella Fistulas

The in vitro intestinal absorptions of D- and L-methionine in the chicken as well as the inhibition of these absorptions by other amino acids were reported to occur at a common L-preferring site[1]. In the same study evidence was obtained which ruled out separate binding sites with overlapping specificities for these isomers. Furthermore, both D- and L-accumulations at steady state were markedly depressed by DNP[2], and examination of L-efflux revealed that this process was accelerated by the metabolic inhibitor[3]. Presumably, metabolic energy is required to reduce affinity for exit of accumulated substrates[4]. In addition, both enantiomorphs were transported against concentration gradients in chicken intestine, though the L-form developed the larger gradient[5]. In vitro work has also shown the methionine transport system to have high specificity for neutral amino acids but low specificity for polar ones[1]. In another study, PAINE et al.[6] noted L-methionine absorption to be greater than D- in chickens with permanent fistulas. They also observed that DNP could inhibit L-absorption, but not D-, and that either isomer could impair L-histidine transport.

Methods. Mature chickens were provided with permanent Thiry-Vella fistulas as described by NEWMAN and TAYLOR[7]. Usually 3 animals were used in rotation for a given set of experiments. Amino acid solutions were perfused through the fistula and aliquots of perfusate analyzed for disappearance of administered amino acid. The isolated intestinal loop was tested for intactness by circulation of sorbose, which was assayed by the method of ROE[8]. Solutions were circulated by a Sigmamotor pump (Middleport, N.Y.) at rates of either approximately 0.6 or 1.1 ml/min, and flow rates were adjusted by a Revco speed control (Minneapolis, Minn.). The temperature of the perfusate was maintained at 41 °C. The loop was cleared of intestinal secretions by rinsing with warm saline. The latter solution was used as a control on the possible excretion of methionine-positive substances from the intestine. Such readings were always negligible. Excess saline was removed from the fistula by a small stream of air. Amino acid solutions were prepared in 0.9% saline. Methionine and its derivatives were determined by the method of ROUDRA and CHOUDHURY[9].

Results and discussion. Table I summarizes data on D- and L-methionine absorption velocities as a function of perfusion time. At all concentrations, velocities measured for the first 10 min interval were about 60–70% of the values found thereafter. Readings taken for later time

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[2] J. LERNER, Thesis, Rutgers University, New Brunswick, N.J. (1967).

[3] J. LERNER, V. MARTIN, C. R. EDDY and M. W. TAYLOR, Experientia *24*, 1103 (1968).

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[7] H. J. NEWMAN and M. W. TAYLOR, Am. J. vet. Res. *19*, 473 (1958).

[8] J. H. ROE, J. biol. Chem. *107*, 15 (1934).

[9] M. N. ROUDRA and L. M. CHOUDHURY, Analyst *76*, 432 (1951).

Table I. Time course of methionine absorption

Concentration[a] (mM)	Absorption velocity[b] (μmoles/min) min 0–10	11–20	21–30	31–40	41–50
L-methionine					
0.67	0.17	0.27	0.25	0.27	0.27
1.34	0.33	0.53	0.53	0.53	0.53
2.68	0.60	1.01	1.07	1.05	1.03
5.36	1.13	1.57	1.61	1.58	1.52
10.72	1.50	2.52	2.52	2.47	2.38
D-methionine					
0.67	0.12	0.19	0.19	0.20	0.18
1.34	0.18	0.28	0.28	0.29	0.28
2.68	0.36	0.52	0.53	0.53	0.53
5.36	0.71	0.84	0.84	0.84	0.83

[a] Concentration of methionine in perfusate. [b] Mean absorption over perfusion period; value measured at the end of the perfusion period. Average rate of perfusion: 0.6 ml/min. Each value represents a single measurement.

Table II. Effect of concentration on methionine absorption

Concentration and numerical designation (mM)	Perfusion period (min)	Absorption velocity[a] (μmoles/min) ± S.E.	p[b]
L-methionine			
0.67 (I)	50 (5)[c]	0.28 ± 0.02	I vs V, 0.01
1.34 (II)	55 (5)	0.52 ± 0.01	II vs VI, 0.001
2.68 (III)	47 (5)	1.01 ± 0.01	III vs VII, 0.001
5.36 (IV)	52 (4)	1.58 ± 0.16	IV vs VIII, 0.01
10.72	51 (4)	2.46 ± 0.23	
D-methionine			
0.67 (V)	50 (5)	0.19 ± 0.02	
1.34 (VI)	52 (5)	0.27 ± 0.04	
2.68 (VII)	56 (5)	0.52 ± 0.04	VII vs VIII, 0.05
5.36 (VIII)	46 (4)	0.88 ± 0.14	

[a] Rate found by averaging four or more samples in each experiment over the perfusion period. [b] Probability based on Student's t-test for paired data. [c] Number of experiments. Average rate of perfusion: 0.6 ml/min.

intervals were constant except for a slight decrease in the higher concentrations of the L-isomer after 20 min. The low initial readings perhaps reflect time required to establish equilibrium between lumen substrate and the transport system. They were not a reflection of water distribution since very little water absorption occurred; nor could any evidence be found for efflux of endogenous methionine. By contrast influx of the enantiomorphs in the isolated tissue procedure[1] was most rapid at the onset of incubation; the uptake rate then continued to fall off with time in proportion to increasing efflux until a steady state was reached, after which time both fluxes remained equal. Furthermore, CRANE[10] noted that substrates probably do not accumulate strongly in intact epithelial cells as they do in the static situation in vitro because of the effective diffusion gradient for their removal into the bloodstream.

Data on the effect of concentration on D- and L-methionine absorption rates are reported in Table II. While rates for the L-form at the lower concentrations appeared fairly linear with increasing perfusate methionine, the 2 higher levels gave proportionately much lower values. These results seem indicative of saturability of the transport system. The mean values for the D-data appeared to follow a curvilinear function, although no statement could be made about saturability. In comparing rates of L- with those of D-absorption, statistically significant differences were observed at each concentration. Table III shows a comparison between the ratio of mean rates of L- and D-methionine absorption v_L/v_D (data from Table II) and estimates of this ratio calculated from the following equation which employed kinetics of the in vitro system as well as constants found in that system[1,2]: $v_L/v_D = (Y_L\, k_L/Y_D\, k_D)\,(S+K_D)/(S+K_L)$, where Y_L and Y_D = maximal accumulations of L- and D-methionine found in vitro at steady state under conditions of saturating external substrate concentration (both values = 0.8 μmoles/ml tissue extract); k_L and k_D = first order rate constants for L- and D-efflux in vitro (both values = 0.076 min^{-1}); K_L and K_D = apparent Michaelis constants for L- and D-transport in vitro (K_L and K_D = 4.1 and 10.0 mM, respectively); S = methionine concentration in the perfusate. The equation thus simplifies to $v_L/v_D = (S+K_D)/(S+K_L)$. The ability to predict the experimental ratios indicated that the in vitro constants and kinetic treatment were an adequate description of transport for methionine in the intestinal loop.

Table III. Comparison of the ratio of absorption rates (v_L/v_D) found experimentally with calculated values

Methionine concentration (mM)	v_L/v_D experimental	v_L/v_D calculated[a]
0.67	1.5	2.2
1.34	1.9	2.1
2.68	1.9	1.9
5.36	1.8	1.6

[a] $v_L/v_D = (S + K_D)/(S + K_L)$, where S is the concentration of methionine in the perfusate, K_D = 10.0 and K_L = 4.1 mM (found in in vitro studies).

Table IV. Absorption of methionine analogs

Compound	Concentration (mM)	Absorption velocity (μmoles/min) ± S.E.
L-methionine	2.68 (5)	1.09 ± 0.14
D-methionine	2.68 (5)	0.59 ± 0.03
DL-methionine	2.68 (5)	0.66 ± 0.09
DL-methionine OH analog	2.68 (3)	0.30 ± 0.02
N-acetyl DL-methionine	2.68 (2)	0.24 ± 0.02
DL-ethionine	2.68 (3)	1.06 ± 0.24

Perfusion period 50 min. Average rate of perfusion 1.1 ml/min. For other explanations, see Table II.

[10] R. K. CRANE, in *Handbook of Physiology* (Ed. C. F. CODE; Williams and Wilkins Co., Baltimore 1968), Section 6, p. 1323.

Table IV summarizes data on the absorption of methionine analogs. Substitution of the α-amino group on methionine by an hydroxyl caused a reduction in transport velocity. In isolated tissue studies, WILSON[11] found no active absorption of lactic acid and others have reported β-alanine to be poorly transported[12]. The minimal absorption of the hydroxy as well as the N-acetylated derivative may have been due to diffusion, though apparent transfer of the latter compound may result from enzymatic conversion to methionine. The absorption of DL-ethionine appeared to be faster than DL-methionine but quantitative comparison was impossible because of high variability. In vitro work showed L-ethionine to have about the same affinity for the methionine transport system as L-methionine, while D-ethionine had much less affinity than D-methionine[1].

Zusammenfassung. Die Darmresorption von Methionin und seine Analoge bei Hühnern wurde in vivo untersucht. Das Verhältnis der mittleren Resorption von L- und D-Methionin stimmt befriedigend überein mit aus In-vitro-Versuchen erhaltenen Aussagen über Kinetik und Konstanten. Es wurden verhältnismässig niedrige Transportgeschwindigkeiten gemessen für die N-azetylierten und Hydroxyanalogen von Methionin.

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Intracellular Distribution of Folic Acid in Mouse Liver

WRIGHT and ANDERSON[1,2] found a pterin reductase in *Clostridium stricklandii* which can be coupled to the oxidation of pyruvate. They also noted that under anaerobic conditions the conversion of serine to glycine in the same organism required NAD Mn^{+2}, inorganic phosphate, pyridoxal phosphate and any of a number of polyglutamyl pteridines. On oxidation of pyruvate, a compound identified as dihydroteropterin was formed. These experiments suggested to us the possible importance of folate in mitochondrial oxidation of pyruvate. Further, SCRIMGEOUR et al.[3] have investigated the reduction of folate by ferredoxin. MACLEAN et al.[4] showed the importance of folate in photophosphorylation by sonicated chloroplasts. The initiation of protein synthesis through the use of N-formylmethionine and the role of folic acid in the metabolism of C_1 units, may suggest a role for folate in protein synthesis as well. With this in mind we studied the relative distribution of folate in the mitochondrial and microsomal fractions of mouse liver. We also investigated the uptake and distribution of radioactive folate in the subcellular fractions of mouse liver homogenates. These results show higher concentration of folate in mitochondria than may be expected from the known role of folate in the metabolism of C_1 units, and in increasing the levels of RNA in kidneys[5].

Materials and methods. In studies on the subcellular distribution of folate, the different cell fractions were obtained by differential centrifugation of mouse liver homogenates according to standard methods, all work having been done at 2–4 °C. The sucrose homogenate was centrifuged at 600 *g* to obtain nuclei and cellular debri. The supernatant was centrifuged at 10,000 *g* for 30 min to obtain mitochondria. The mitochondrial fraction was resuspended in 0.25 *M* sucrose and recentrifuged to obtain purer mitochondria. The first mitochondrial supernatant was centrifuged at 100,000 *g* for 60 min to obtain the microsomes.

Folic acid was assayed using *Lactobacillus casei* ATCC 7469. The cell fractions were frozen and thawed and then subjected to ultrasonication followed by dilution before they were assayed for folic acid.

In the studies on uptake from blood and distribution of radioactive folic acid-2-C^{14}, 1 μC of the folate was administered per 30 g of body weight to each of 6–9 mice i.v. The distribution over different periods of time was studied. At the end of the stated length of time after i.v. administration, the organs were isolated, homogenized and were fractioned at 2 °C as per the method described above. The different fractions were counted for radioactivity in a planchet type counter, making the necessary radiochemical corrections. The radioactive folic acid-2-C^{14} was obtained as potassium salt from Amersham-Searle and has a specific activity of 31.4 μc/μ*M*.

Results and discussion. The levels of folic acid in the different subcellular fractions of mouse liver are shown in Table I. Out of a total of 14.23 μg of folic acid per gram of mouse liver, 2.73 μg or approximately 19% is found

Table I. Folic acid content of subcellular fractions of mouse liver (assayed with *Lactobacillus casei*)

Cell fraction	Folic acid (μg/g fresh total tissue)
Mitochondria	2.73
Microsomes	0.75
Cell sap (100,000 *g* supernatant)	10.75

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