'Tetrazolium Oxidase' Identified with the Heme-Oxidases of Human Tissues

Light (negative) protein bands appear under tetrazolium reduction staining techniques after starch gel electrophoresis of human hemolysates (Figures 1 and 2). Their identity is unknown, although some have been correlated tentatively with the cytochrome system[1]. I have observed such 'tetrazolium oxidases', including genetic variants, in tissue extracts from many vertebrate species. I find that the band of greatest intensity in human hemolysates represents the major hemoglobin component and several minor bands represent the minor hemoglobins A_2, A_3, and F (Figures 1 and 2). As the gel is incubated the hemoglobin prevents reduction of the tetrazolium compound and then diffuses out of the gel, leaving white bands. When electrophoretic variants of human hemoglobin are examined, the mobility of the major tetrazolium oxidase is shifted correspondingly; furthermore, the anodal subband of hemoglobin, HbA_3, thought to be a denaturation product of HbA[2], is represented by a tetrazolium oxidase whose mobility also shifts correspondingly with electrophoretic variants of HbA.

When electrophoresis of human hemolysates[3] is performed at pH varying from 4.5–8.6 the major tetrazolium oxidase and hemoglobin bands maintain identical mobilities, as they do when the density of the starch is varied from 10–20%; this precise parallelism of mobility renders their identity most likely. Electrophoresis of human liver and serum extracts reveals several closely spaced bands of tetrazolium oxidase which mark the site of the hemoglobin-haptoglobin complex; their pattern corresponds precisely to the haptoglobin phenotype of the individual examined[4]. There are several additional tetrazolium oxidases seen in human tissue which I have tentatively identified with methemalbumin, hemopexin, and hemoperoxidase on the basis of electrophoretic mobility[5].

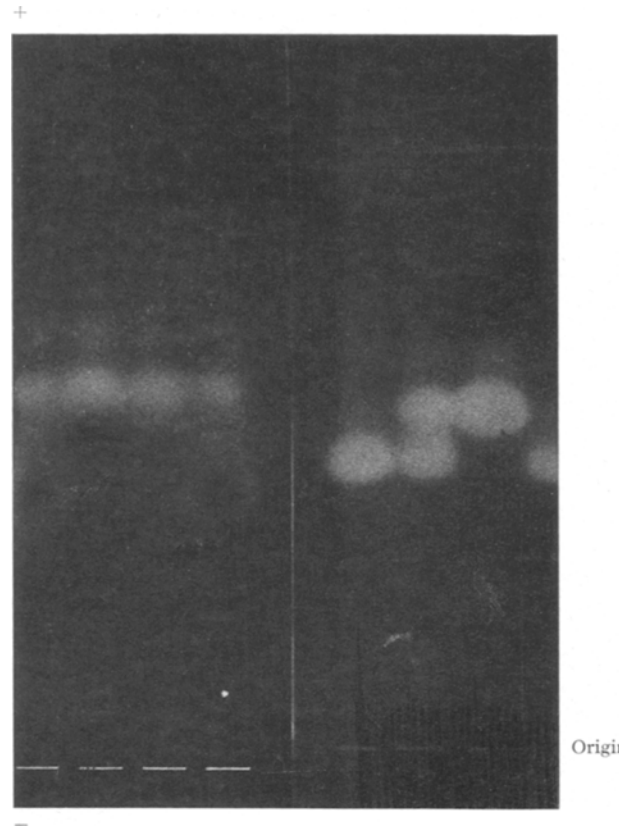

Fig. 1. Starch gel electrophoresis and tetrazolium staining[1] of human hemolysates. Right: 10 min after tetrazolium staining, all the hemoglobin was eluted with 2 l of 0.25 *M* $Na\text{-}PO_4$ buffer, pH 7, in 2 changes, at 20 °C, for 8 h. The gels were then fixed in Smithies' solution[3,4]. Left: 10 min after tetrazolium staining, the gels were fixed in Smithies' solution[3,4]. Some hemoglobin has eluted already, but most remains on the gel obscuring tetrazolium oxidase bands. The fetal hemoglobin sample is from cord blood containing small amounts of hemoglobin A. The variant hemoglobins were identified by classic techniques[5]. Photography was performed at 20 °C, 24 h after staining and 16 h after elution was complete. All white bands coincide with hemoglobin or its denaturation product, immediately anodal to the main band. Note HbA_2 is also seen (cathodal) as a white band.

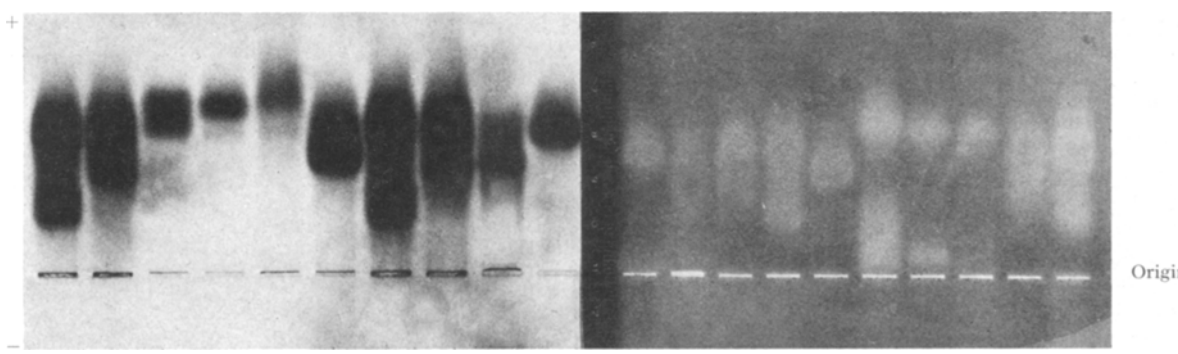

Fig. 2. Starch gel electrophoresis at pH 8.6 of various human hemolysates. Right: stained for tetrazolium oxidase and eluted as in Figure 1. Left: stained with benzidine for heme-oxidases[5], and fixed within 30 min of staining in 50% glycerine, 0.005 *M* Na Acetate, pH 5. The left and right sides are mirror images (opposite halves of the same gel). Photography at 20 °C, 10 h after staining and 2 h after elution was complete. The benzidine-positive and the white bands coincide precisely; however, there are some extra white bands, cathodally placed, of unknown identity.

[1] G. J. Brewer, American Journal of Human Genetics *19*, 674 (1967).
[2] H. G. Kunkel and A. G. Bearn, Fedn Proc. *16*, 760 (1957). – H. Water, Nature *198*, 189 (1963).
[3] O. Smithies, Biochemical Journal *61*, 629 (1955).
[4] O. Smithies, Nature *178*, 694 (1956).
[5] S. H. Lawrence, *The Zymogram in Clinical Practice* (C. Thomas, Springfield 1964), p. 73. – H. Lehman and R. G. Huntsman, *Man's Hemoglobins* (Lippincott Co., Philadelphia 1966), chapter 20.

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

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

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