solution contained an amount of uronic acid corresponding to all the PP-L initially present.

The inhibition by PP-L of sedimentation at low g values of calcium phosphate and of BaPSS, and the removal of PP-L from solution by sedimenting BaPSS is of obvious concern in considering properties of connective tissues and the behavior of macromolecular polyelectrolytes. The results may be due to the diffuse nature of the PP-L molecule in solution. Hyaluronate chains, spread through several liters per gram (*12*), can inhibit sedimentation of large particles (*2*). In the same way, PP-L, but not chondroitin sulfate, could inhibit sedimentation of BaPSS chains, or the coming together of microcrystals of calcium phosphate. Sedimentation of BaPSS would be expected to remove PP-L by mutual entanglement of their chains, but sedimentation of microcrystals of calcium phosphate might not.

In connective tissues the presence of diffuse proteinpolysaccharides may inhibit or control calcification. In tissues where different proteinpolysaccharides exist, their mutual entanglement could modify each other's properties, as PP-L and PSS modify each other's properties in the experiments described (*13*).

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30 August 1963

Lactic Dehydrogenase: Genetic Control in Man

Abstract. *A genetically determined variant of lactic dehydrogenase has been observed in the red cells of four members of two generations of a Brazilian family. The appearance of the variant after starch-gel electrophoresis of the hemolysates supports the concept that the lactic dehydrogenase isozymes are determined by the interaction of two subunits which are under separate genetic control. The products of the mutant and normal allele do not, however, appear to associate randomly to form isozymes. The similarity of the relative retardation of normal and mutant isozymes in gels made with increasing concentrations of starch suggests that they do not differ significantly in size.*

In 1961 Markert proposed that the isozymes of lactic dehydrogenase arise from the five possible combinations of two different subunits, A and B, taken four at a time to form enzymatically active tetramers (*1*). Recently, Markert has presented compelling evidence in favor of this hypothesis by the demonstration that a mixture of all five isozymes, including the three hybrid varieties, A_1B_3, A_2B_2, and A_3B_1, can be formed in vitro by the random reassociation of a mixture of dissociated A_4 and B_4 subunits (*2*).

During the course of a genetic investigation of a Brazilian population (*3*), we encountered a family with a variant form of lactic dehydrogenase. As shown in Fig. 1, only three of the five isozymes, LDH-1, LDH-2, and LDH-3 are clearly apparent in hemolysates prepared from erythrocytes of normal adults (*4*), in agreement with the observations of others (*5*); in the new phenotype, upon electrophoresis, isozyme bands 2 and 3 are doubled, but not band 1. Identical electrophoretic patterns were observed in the propositus and three of his six children, as shown by the pedigree in Fig. 2. Autosomal inheritance is suggested by the existence of two products attributable to allelic genes in the father. The occurrence of consanguinity in this family is probably not relevant to the observed LDH variant.

The new phenotype is readily explained by the hypothesis of Markert if it is assumed that the observed mutation involved the polypeptide chain of subunit A. Since LDH-1 is a tetramer of B subunits, doubling of the LDH-1 band would not be anticipated. Two LDH-2 bands would be expected, corresponding to the formulas A_1B_3 and $A^*_1B_3$, where A* indicates the mutant subunit. These two bands are, in fact, observed. If completely random association of the mutant and normal A subunits with each other and with the B subunit is assumed, and if the mutant and normal A alleles are equally "efficient," as judged by the enzyme activity of their products, then three LDH-3 bands would be expected, corresponding to the following formulas in these proportions: 1 A_2B_2 : 2 $A^*_1A_1B_2$: 1 A^*B_2. In case the association of the products of the mutant and the normal alleles is not random, however, fewer than three LDH-3 bands might be seen. For example, two equally prominent bands (A_2B_2 and $A^*_2B_2$) would be observed if like chains of A subunits dimerize prior to association with the B chains. In our best preparations we consistently observed only two LDH-3 bands. Furthermore, the ratio of the distances of migration of the mutant and normal LDH-2 bands is 1.07, while that of the two LDH-3 bands is 1.15. Since the displacement of the mutant LDH-3 band relative to its homolog is very nearly twice that of the LDH-2 variant, it seems reasonable to assume that the observed LDH-3 variant has twice as many A* subunits per molecule

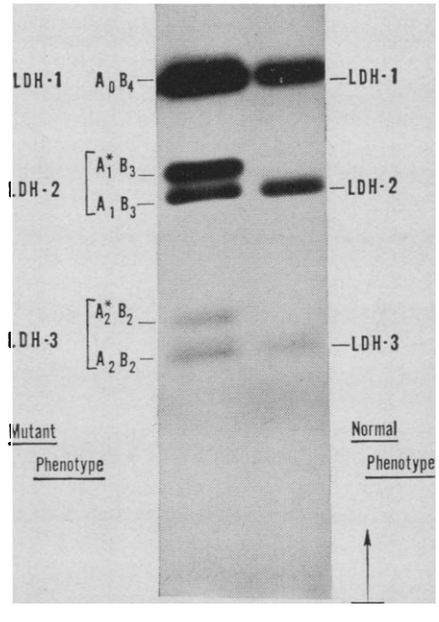

Fig. 1. Zymogram illustrating mutant and normal phenotypes. The probable composition of the isozymes in terms of the mutant (A*) and normal (A and B) subunits (*1*) is indicated.

as has the LDH-2 variant, and therefore to assign to it the formula $A^*_2B_2$. If the hypothetical $A^*_1A_1B_2$ isozyme, expected on the basis of random association, were to occur, it should migrate between the two observed bands and have twice the intensity of either. Under the electrophoretic conditions we have used we have not observed a band of this description. We therefore conclude that the mutant and normal A subunits, for some unexplained reason, are not randomly associated in the erythrocytes of our subjects. This phenomenon appears to be in some ways analogous to the nonrandom association of hemoglobin polypeptide chains.

Although hemoglobin differs from lactic dehydrogenase in that the α and β chains are not randomly associated, even the products of mutant and normal β chain alleles are not randomly associated. Thus in sickle-cell heterozygotes, for example, three species of hemoglobin, $\alpha_2\beta_2$, $\alpha_2\beta^S{}_2$, and $\alpha_2\beta\beta^S$, would be expected with random association of mutant and normal β chains; only the first two are actually found, just as only two LDH-3 isozyme bands are observed in zymograms showing the new phenotype. Using buffers of low ionic strength, we observed in our initial studies inconstant extra bands in both the mutant and normal patterns, but they were not in the position in which $A^*_1A_1B_2$ would be expected to occur. Prompted by the observations of Ressler, Schultz, and Joseph on the effect of ionic strength on isozyme separations (*6*), we now use higher ionic strength buffers and, as indicated previously, consistently obtain patterns showing only two LDH-3 bands in the mutant phenotype, and single bands in the usual phenotype.

In order to investigate the nature of the mutant form of LDH, we subjected a suitable hemolysate to simultaneous electrophoresis in gels containing increasing concentrations of starch (Fig. 3). Using the reciprocal of the starch concentration as the independent variable and the distance of migration of each band divided by its mean value over the range of starch concentrations as the dependent variable, we calculated retardation coefficients, as defined by Smithies (*7*), for each band, and performed an analysis of covariance. This analysis showed no significant heterogeneity among the estimates of the retardation coefficient of lactic dehydrogenase for all five enzyme bands ($F_{(4,34)} = 0.24$), nor did the retardation

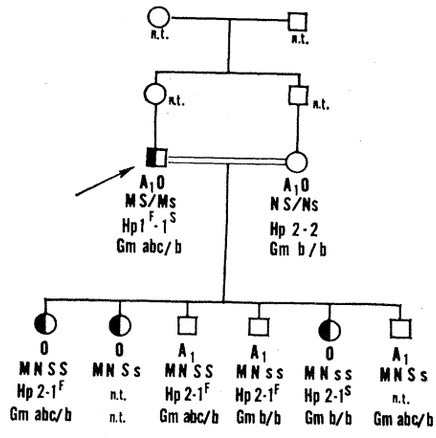

Fig. 2. Pedigree of the family showing segregation of the LDH variant (darkened square and circles). The propositus is indicated by an arrow. Segregation of the four other genetic markers shown was also observed in this family.

coefficient of each mutant isozyme band differ significantly from that of its homolog ($F_{(1,32)} = 0.09$ and 0.07). The retardation coefficient of hemoglobin, on the other hand, was significantly smaller than that of lactic dehydrogenase ($F_{(1,48)} = 11.62$). We conclude that the isozymes LDH-1, LDH-2, and LDH-3 do not differ markedly in size, and that the observed variant may tentatively be regarded as the result of a point mutation leading to a difference in charge.

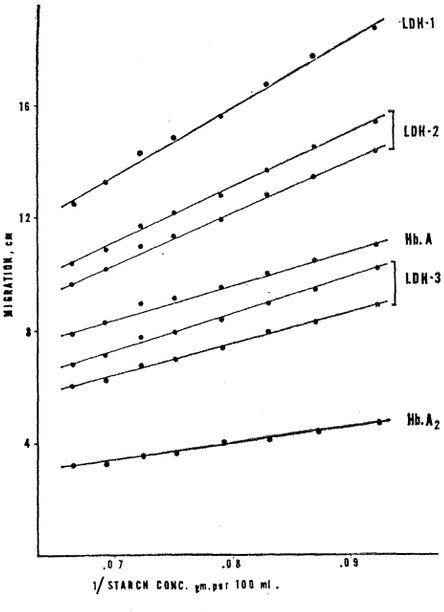

Fig. 3. A graph of the distance of migration of the LDH isozymes and hemoglobins A and A_2 against the reciprocal of the starch concentration, obtained by measurements in eight starch gels. Retardation coefficients were determined by dividing the calculated slopes by the corresponding mean distance of migration as described by Smithies (*7*).

The family was also tested for the following blood and serum group systems: ABO, MNS, Rh, P, Kell, Lewis, Lutheran, Sutter, Duffy, Diago, haptoglobin, transferrin, hemoglobin, catalase, Gm, and Inv. Of these, the father was also heterozygous only at the ABO, MNS, haptoglobin, and Gm loci, as shown in Fig. 2, and the pedigree excludes close linkage of the locus controlling the LDH subunit to all of these four loci except the ABO locus.

A genetically transmitted variant of lactic dehydrogenase has recently been reported in *Peromyscus* (*8*), and an isolated case of an LDH-B chain variant has been found in man (*9*), both of which also support Markert's hypothesis concerning the nature of LDH isozymes. The patterns observed in these cases were interpreted as showing random association between the products of the mutant and normal alleles. This interpretation is at variance with our observations, but could reflect species differences, differences in the behavior of the A and B subunits, or, more likely, differences in technique, as discussed earlier. The functional homology of the A and B subunits suggests the possibility that their genetic determinants had a common origin, and, as in the case of the determinants of the β and δ chains of human hemoglobin, the subsequent demonstration of close linkage between the genes controlling the LDH subunits would favor a duplicational origin of the loci. The absence of linkage, however, would not exclude the possibility. Markert has shown (*10*) that the A and B subunits differ extensively in their amino acid composition and peptide patterns after tryptic digestion. Such differences, however, may not be incompatible with gene evolution by duplication (*11*). Thus the α and β chains of human hemoglobin have striking structural homologies, and, although their genetic determinants are not in this case closely linked, they are thought to have arisen from a common ancestral gene by duplication (*12*). Nevertheless, after tryptic digestion the proteins give almost completely different electrophoretic and chromatographic peptide patterns.

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4. Hemolysates were prepared from erythrocytes, washed in 0.9 percent saline and preserved in glycerol at —60°C. until use. Starch gels were prepared with a tenfold dilution of a solution containing 0.9*M* tris (primary standard), 0.5*M* boric acid, and 0.02*M* ethylene diamine tetraacetic acid (EDTA). Two milliliters of 0.005*M* diphosphopyridine nucleotide (NAD were added to 500 ml of gel just prior to degassing. A solution of 0.11*M* tris, 0.06*M* boric acid, 0.0024*M* EDTA was used as a bridge solution and 1 ml of 0.005*M* NAD was added to the 500 ml of solution in the anodic bridge vessel. Vertical electrophoresis was carried out at 4°C for 10 to 15 hours with a voltage gradient of 4 to 5 volts per centimeter. Zymograms were developed as described by Vesell and Bearn (see 5).
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Trachoma Agent: Glucose Utilization by Purified Suspensions

Abstract. *Strain TE-55 of trachoma "virus" was extracted from infected chick-embryo yolk sacs, purified with the aid of diethylaminoethyl cellulose, and incubated with C^{14}-glucose. Highly significant amounts of $C^{14}O_2$ were produced from carbon-1, but not from carbon-6 of glucose. This demonstrates independent carbohydrate metabolism in an extracellular environment by a microorganism of this group.*

The agent of trachoma, which is currently placed in the large and poorly-defined group of microorganisms known as the psittacosis-lymphogranuloma-trachoma (PLT) group (*1*) has been successfully cultivated in the yolk sac of the chick embryo (*2*). This intracellular agent has been of interest not only because of the disease it causes but also because of its complexity, as evidenced by its developmental cycle within the host cell (*3*), the formation of conspicuous glycogen-like granules within the intracellular inclusion (*4*), and the susceptibility both to sulfonamides (*5*) and penicillin (*6*), which suggests the possibility of independent enzymatic activity. The closely related agents of the psittacosis group oxidize reduced nicotinamide adenine dinucleotide (NAD) in the presence of cytochrome *c* (*7*), synthesize folic acid (*8*), and decarboxylate diaminopimelic acid to lysine (*9*). Independent carbohydrate metabolism, however, has not been demonstrated. Conclusive evidence for such an activity by the agent of trachoma is presented in this report.

Strain TE-55 (*10*) of the agent of trachoma was selected because of its luxuriant growth in the yolk sac of chick embryos (*11*). For each experiment, approximately 60 yolk sacs were harvested from infected moribund embryos and suspended in an equal volume of solution X (0.1*M* KCl, 0.2*M* sucrose, 0.02*M* phosphate buffer) at *p*H 7.2 and stored overnight at 6°C. The procedure of purification differed principally from that recently developed for psittacosis (*12*) in that one anion exchanger, diethylaminoethyl cellulose [DEAE, (*13*)], was used instead of another, ECTEOLA, because the former possesses a higher absorptive capacity. The yolk sacs suspended in solution X were ground in a Waring blender for 1 minute and centrifuged at 30,000*g* for 30 minutes. The pellet was resuspended in solution X and treated with 0.5 percent trypsin (Difco Bacto) for 45 minutes at room temperature. The suspension was then again centrifuged at 30,000*g* and the pellet resuspended in 20 ml of solution X at *p*H 6.9. To this was added DEAE in a concentration of 1 g per 50 g of original yolk sac. After it had been mixed gently for 5 minutes, the suspension was centrifuged at 800*g* for 5 minutes and the supernatant was again subjected to the cycle of DEAE treatment. The resulting suspension was then centrifuged at 30,000*g* for 30 minutes; the supernatant and upper, brownish portion of the pellet were scraped off and discarded, leaving only the lower whitish layer. This was again resuspended in solution X at *p*H 7.2 and treated with DEAE. The resulting suspension was concentrated by centrifugation which produced a pellet almost homogeneous in appearance and almost entirely white.

Table 1. The production of $C^{14}O_2$ from C^{14}-labeled glucose by purified suspensions of the trachoma agent, strain TE-55. The three preparations of labeled glucose were diluted with unlabeled glucose to a concentration of 1 μmole per flask and a specific activity of 4 mc/mmole, or 1.2×10^6 count/min per flask. Each flask also contained 18 μmoles of ATP, 9 μmoles of NAD, 7.5 μmoles of Mg^{++}, 1.5 μmoles of Mn^{++} and 7.5 mg of bovine plasma albumin. The trachoma and control preparations contributed 3.5 mg and 13.2 mg of protein per flask, respectively. The control preparation consisted of the discard from one of the final steps of purification of *C. burneti* from chick embryo yolk sacs. The total volume in each flask was 3.0 ml. All flasks were incubated for 2 hours at 34.4°C. The CO_2 was trapped by 0.2 ml of 40 percent KOH placed in the center well.

Substrate	$C^{14}O_2$ recovered (count/min per mg of protein)	
	Trachoma suspension	Control preparation
Glucose-*R*-C^{14}*	1990	76
Glucose-1-C^{14}	5240	101
Glucose-6-C^{14}	232	93

* Randomly labeled glucose.

Examinations by light and electron microscopy of suspensions purified by this method revealed typical small and large forms of the agent of trachoma, relatively free from contaminating host-cell debris. Enumeration of the microorganisms by electron microscopy after sedimentation onto collodion-coated grids (*14*) indicated that typical preparations contained 4 to 6×10^9 particles per milliliter. Titrations in yolk sacs of chick embryos yielded a ratio of total particles to infectious particles of approximately 200:1. The yield of purified trachoma particles in terms of total protein (*15*) ranged from 0.2 to 0.5 mg per gram of yolk sac in different preparations.

Concentrated suspensions of the trachoma agent were immediately tested for metabolic activity by conventional manometric techniques in the Warburg respirometer. To each vessel were added the suspension of microorganisms, adenosine triphosphate (ATP), nicotinamide adenine dinucleotide, and bovine plasma albumin (crystalline, Calbiochem). Small but steady uptake of oxygen was observed (1 to 2 μl/mg of protein per hour) under these conditions but oxygen consumption was not increased by the addition of either pyruvate or glucose. However, when C^{14}-labeled glucose was added and the radioactivity in the CO_2 was tested, it became apparent that this substrate was metabolized. The data of a representative experiment are presented in Table 1. Glucose was labeled