Again, exposure to constant light was associated with decreased weight of the pineal gland, unchanged monoamine oxidase activity, and an increase in the incidence of estrus.

About one third of the animals kept in constant light had no detectable HIOMT activity in the pineal gland. Male rats and female guinea pigs, which were not in constant estrus, also responded to constant light exposure with decreased HIOMT activity and unchanged monoamine oxidase activity.

That exposure to light can inhibit selectively the activity of the enzyme required for the synthesis of the pineal hormone, melatonin, is of especial interest in view of the possibility that this enzyme limits the rate of melatonin synthesis in vivo. Since melatonin appears to be released from the pineal gland into the circulation, and to act on distant organs as a hormone (*10*), the relationship presented here between illumination and HIOMT activity in the pineal gland may describe a new kind of neuroendocrine regulatory mechanism: control by light of the availability of a hormone by regulating the activity of an enzyme required for its synthesis. It is suggested that one means by which light exposure may induce an increase in ovary growth and in the incidence of estrus (*15*) is by inhibiting the synthesis of melatonin, a hormone which inhibits both these functions (*10*).

Information relating to environmental lighting could be transmitted to the pineal gland by several routes: (i) retinal stimulation could be transmitted to the pineal gland through nerve pathways; (ii) light could act indirectly by influencing other circulating hormones, which in turn would affect enzyme activity; or (iii) light could impinge directly upon the mammalian pineal gland.

Previous work, in which light exposure was found to be related to the RNA content of the pineal gland (*7*), by histochemical estimations, suggests that the differences in enzyme activity described here may actually represent differences in the net rate of synthesis of enzyme protein. The fact that HIOMT activity in the pineal gland can be increased in the absence of light in adult animals, treated for a short period, as effectively as in immature animals treated from birth, suggests that cyclic variations in the activity of this enzyme may occur physiologically, perhaps even diurnally. Since light exposure and the incidence of estrus both show periodicity, it is possible that diurnal variations in light may influence the estrous cycle by influencing the synthesis of melatonin.

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Proteinpolysaccharide in Connective Tissue: Inhibition of Phase Separation

Abstract. *A macromolecule of protein and chondroitin sulfate (PP-L) inhibits sedimentation of barium-polystyrene sulfonate (BaPSS) and of calcium phosphate at low but not at high values of gravity. Sedimentation of BaPSS removes a large fraction of PP-L from solution, but sedimentation of calcium phosphate does not. The results suggest entanglement among linear polyanionic chains.*

The behavior of collagen fibrils or large particles, upon sedimentation in solutions containing hyaluronate, has led to the suggestion that the diffusely spread polyanionic hyaluronate chain may become entangled with the collagen fibrils (*1*) or mechanically inhibit the sedimentation of large particles (*2*). The anionic polysaccharides of connective tissue occur bound to protein as macromolecules called proteinpolysaccharides (PP). Their existence in solution as diffusely spread molecules may underlie some special properties of connective tissues, such as their capacity to hold water, their control of calcification, and the elasticity of cartilage (*3*).

The present report concerns phenomena which seem related to the diffuse nature in solution of a proteinpolysaccharide from cartilage called PP-L (*4*). This substance consists of about 15 percent protein, 70 percent chondroitin sulfate, and 5 percent keratan sulfate (*5*) and has a molecular weight above 10^6 (*6*). The phenomena investigated are the inhibition of precipitation of insoluble salts from water in the presence of PP-L, and the effect on PP-L of sedimenting these salts at high values of *g*.

Precipitates of two types were studied, calcium phosphate and barium-polystyrene sulfonate (BaPSS). Both precipitate readily from water and sediment completely at values below 100*g*. In the presence of 0.5 to 5.0 mg/ml of PP-L, only small amounts of the insoluble salts sediment when centrifuged at about 500*g*, leaving strongly opalescent supernatant solutions from which either calcium phosphate or BaPSS can be completely sedimented at high centrifugal speeds (30,000 to 100,000*g*). The calcium phosphate sediments contain 10 to 30 percent of the PP-L originally in solution while the BaPSS sediments contain 30 to 70 percent, depending on conditions.

A series of solutions was made up in 0.12*M* NaCl and 0.03*M* veronal buffer, each at *p*H 7.8 and containing in 11 ml, 40.5 μmole Ca^{++}, 41.4 μmole of phosphate, and the variable amount of PP-L indicated in Table 1, column 1. The phosphate was always added last. After standing 16 hours at either 25°C or 37°C, the solution containing no PP-L had a deposit of calcium phosphate with a clear supernatant solution; all others had progressively smaller deposits of calcium phosphate and more opalescent supernatant solutions. All were centrifuged at about 500*g* for 15 minutes and the residues left after drainage were called R1. The supernatant solutions were then centrifuged at 100,000*g* for 30 minutes and the residues after drainage were called R2.

All the supernatant solutions were now clear and were called S. The residues R1 and R2 and the clear supernatants, S, were analyzed for Ca (*7*), phosphate (*8*), and PP-L (*9*).

The results, in Table 1, show that with increasing amounts of PP-L the amount of calcium phosphate deposited as R1 progressively decreased while that deposited as R2 increased. All the supernatant solutions (S) contained the same amounts of Ca (0.4 mg) and phosphate (1.47 mg) and, in addition, most of the PP-L initially added, averaging 80 percent. The total amount of calcium phosphate (R1 + R2) precipitated in each of these experiments is constant. The inhibiting effect of PP-L is not due to its viscosity since viscous solutions of glycerine allow all the calcium phosphate to precipitate as R1. Nor is it due to lowering the activity coefficient of Ca (*10*), since in experiments with chondroitin sulfate substituted for PP-L all the calcium phosphate was precipitated as R1. The chondroitin sulfate was made as described elsewhere (*4*). That intact PP-L was essential to inhibiting the precipitation of all the calcium phosphate as R1 was shown in another way. The opalescent solutions left after removal of R1 in the experiments of Table 1 were stable for days and showed no tendency to deposit calcium phosphate. Destruction of the PP-L by the addition of catalytic amounts of either trypsin or of hyaluronidase to these opalescent solutions caused precipitation, within a few hours, of all the calcium phosphate held in suspension.

To study precipitation of the polystyrene sulfonate (PSS) anion with barium, solutions were made up each containing in 7 ml the weights of PP-L and of potassium PSS indicated in the first two columns of Table 2. To each solution was added $BaCl_2$ (3 ml, 0.1*M*). Results resembled those described for calcium phosphate precipitation. From the solution containing no PP-L, BaPSS was precipitated completely, leaving a clear supernatant solution. All others had a progressively smaller amount of precipitate and a progressively more opalescent supernatant solution. All were centrifuged at 800*g* for 20 minutes. The residues were washed twice with 8 ml of 50 percent aqueous ethanol, then with ethanol and ether, dried, and called R1. The supernatant solutions were centrifuged at 60,000*g* for 20 minutes. The residues were washed as before and called R2. All supernatant solutions were now clear and were called S. The weights of R1 and R2 are given in columns 3 and 4 of Table 2. Residues R1 and R2, and the supernatant solutions, S, were analyzed for hexuronate (*11*), and from the value found the weight of PP-L in each was calculated (columns 5, 6, and 7 of Table 2).

Table 2 shows that with increasing PP-L and constant PSS, R1 progressively decreases in weight while R2 increases. The amount of PP-L associated with the total precipitate (R1 + R2) increases with the amount of PP-L initially present. The amount of PP-L left in the supernatant solution (column 8) is a constant fraction (averaging 37 percent) of the amount of PP-L initially present.

With constant PP-L and increasing PSS, the weight of R1 is zero with small amounts of PSS but rises rapidly with amounts of PSS over 25 mg. As PSS is increased R2 at first rises and then decreases. Due to included PP-L, the total weight of R1 + R2 always exceeds the weight of BaPSS precipitated. The amount of PP-L left in S progressively decreases to 30 percent, showing that the PP-L brought down with the BaPSS increases.

Table 3 also shows that chondroitin sulfate (CS), in contrast to PP-L, has no effect on the precipitation of BaPSS and that no chondroitin sulfate is brought down with the BaPSS. This makes it unlikely that the divalent Ba^{++} could form crosslinks between PSS and PP-L so that when PSS is sedimented it carries some PP-L with it.

Experiments with pepsin or trypsin similar to those described for calcium phosphate showed that to inhibit the precipitation of BaPSS, the presence of intact PP-L is essential. Each of the solutions (at appropriate *p*H) to which protease was added yielded a sediment of BaPSS within an hour which could be removed completely by centrifuging at low speed, while the supernatant

Table 1. Effect of PP-L on sedimentation of calcium phosphate (in milligrams).

PP-L at start (mg)	R1		R2		S
	Ca	PO_4	Ca	PO_4	PP-L
0	1.64	2.37	0	0.02	0
3.1	1.38	2.14	0.18	0.28	2.2
6.2	0.88	1.38	0.60	1.04	4.6
12.4	0.36	0.65	1.12	1.76	11.2
24.8	0.04	0.17	1.48	2.33	22.4

Table 2. The effect of PP-L on sedimentation of barium-polystyrene sulfonate (BaPSS), and the effect of sedimentation of BaPSS on PP-L.

Amount at start (mg)		Yields (mg)		Amount of PP-L in fractions (mg)			PP-L in S (%)
PP-L	PSS	R1	R2	R1	R2	S	
0	40	42.7	0	0	0	0	
6.0	40	39.2	8.2	2.6	1.8	1.7	28
10.8	40	35.0	14.0	3.5	2.3	5.0	46
12.0	40	2.9	45.5	0.5	7.7	3.9	32
16.0	40	2.9	50.2	0.6	10.5	5.8	36
24.0	40	1.5	54.1	0.5	13.8	9.9	41
30.0	40	2.0	57.0	0.6	18.6	12.3	41
0	12	14.7	0	0	0	0	
0	25	28.1	0	0	0	0	
12	0	0	1.4	0.1	1.3	10.8	90
12	1	0	5.3	0	4.0	7.7	64
12	2	0	8.8	0.1	5.7	5.3	44
12	5	0	12.3	0.1	6.1	5.5	46
12	10	0.5	17.9	0.4	6.2	4.4	37
12	25	2.8	35.3	0.4	8.1	3.9	33
12	50	44.3	22.0	4.6	4.1	3.7	31
12	75	75.3	16.4	6.0	2.8	3.1	26
12	100	109.9	11.2	6.7	1.8	3.4	28

Table 3. The effect of chondroitin sulfate on sedimentation of BaPSS.

Amount at start (mg)		Yields (mg)		Amount of CS in fractions (mg)			CS in S (%)
CS	PSS	R1	R2	R1	R2	S	
6.0	40	42.7	0	0.1	0	5.8	96
36.0	40	42.1	0.1	0.8	0	35.2	98
18.0	0	0	0	0	0	17.8	99

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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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

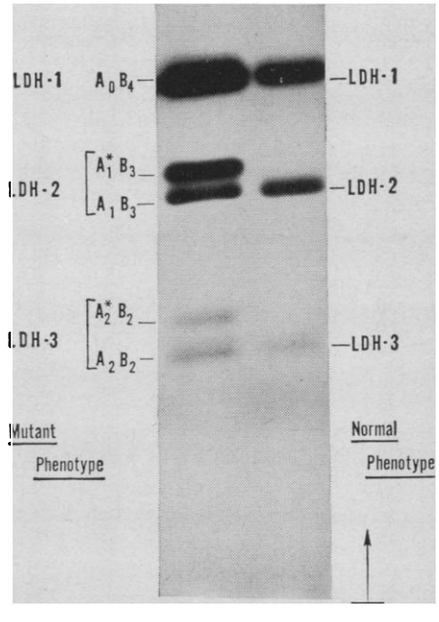

Fig. 1. Zymogram illustrating mutant and normal phenotypes. The probable composition of the isozymes in terms of the mutant (A*) and normal (A and B) subunits (*1*) is indicated.