These filters must be thin enough ($\lesssim$ 0.018 mm) to be penetrated by the bombarding particles, but filters which are several centimeters in diameter and only 0.006 mm thick are quite tough and can support a large weight of liquid without the necessity for a supporting framework. Because of the random positions of the holes, the fraction of the filter surface which is open cannot exceed about 2 percent without some overlap of holes. With increasing porosity the efficiency of a filter in separating particles of closely similar size is impaired.

Various plastics which are suitable for filters resist attack by strong acids, weak bases, and many organic solvents such as acetone and xylene. Some of them can also be used at temperatures up to approximately 175°C and, therefore, can be conveniently sterilized.

Irradiated and etched plastic filters have a number of advantages over conventional filters of fiber and metal mesh. The number and diameter of holes can be easily adjusted by varying the irradiation time and etching time. The holes are circular cylinders which have exactly the same diameter within the resolution of an optical microscope. For 6-μ holes the variation in diameter is less than 0.5 μ. Because of the ideal geometry of the holes, the filter does not become clogged and delicate particles such as blood cells can be filtered without destruction by gravitational action rather than by application of a differential pressure. Because of the transparency and chemical resistance of these sieves, materials such as cells and substrates can be collected, stained, and viewed *in situ*. Specifically, the study of the structure of blood cells is facilitated because cells are flattened onto the top surface of the filter prior to fixation and staining.

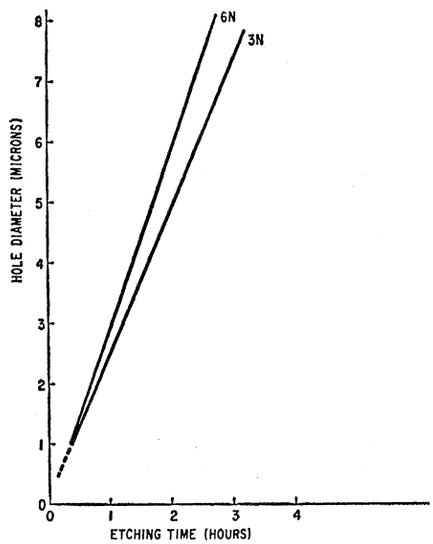

Fig. 2. Etching characteristics of polycarbonate film in unstirred aqueous solutions of 6*N* and 3*N* NaOH at 75°C. The film thickness decreases as the hole size increases, so that a film containing 6-μ holes has been reduced in thickness by about 6 μ.

Recently, with H. M. Rozendaal, we have used 6-μ plastic sieves to separate HeLa cells (diameter about 10 μ) which had been added to human blood. Experiments now in progress by S. H. Seal (Sloan-Kettering Institute) have as their goal the nondestructive filtration of free-floating cancer cells from the blood of patients harboring malignant disease (*3*). Because of the precise uniformity of hole sizes in irradiated and etched filters, it should be possible in cytological studies to separate large numbers of cells of uniform sizes to facilitate the study of cell growth and multiplication.

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References and Notes

1. P. B. Price and R. M. Walker, *J. Appl. Phys.* **33**, 3407 (1962); ———, *Phys. Letters* **3**, 113 (1962); R. L. Fleischer and P. B. Price, *J. Appl. Phys.* **34**, 2903 (1963); ———, *Science* **40**, 1221 (1963).
2. R. L. Fleischer, P. B. Price, R. M. Walker, *Rev. Sci. Instr.* **34**, 510 (1963).
3. S. H. Seal, *Cancer* **12**, 590 (1959).
4. We thank Dr. S. H. Seal for stimulating our interest in the application of these filters and Dr. H. M. Rozendaal for collaborating in the experiments.

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Precipitins in the Rabbit Produced by Protein Polysaccharide from Bovine Nasal Cartilage

Abstract. *Precipitins have been produced in the rabbit by injecting protein polysaccharide from bovine nasal cartilage in Freund's adjuvant. These antibodies did not precipitate either protein polysaccharides extracted from other species, or the separated protein and carbohydrate moieties of the antigen. It is postulated that the antibodies are directed toward determinants consisting of carbohydrate and amino acid residues linked in three-dimensional arrangements specific for different protein polysaccharides.*

In the past decade, several unsuccessful attempts have been made to demonstrate the antigenicity of acid mucopolysaccharides (*1*). The failure of hyaluronic acid and chondroitin sulfate (either injected alone or absorbed on different bacteria) to induce production of specific antibodies in the rabbit is not surprising, in view of the fact that preparations of these mucopolysaccharides obtained from different species have proved to be chemically identical.

Recent studies have demonstrated that chondroitin sulfate exists in cartilage firmly bound to a complex protein structure of which keratosulfate, neutral sugars, and sialic acid also seem to be part (*2*), and that hyaluronic acid, isolated from human synovial fluid, contains a small but firmly bound protein moiety (*3*).

On the basis of these findings, chondrotin sulfate and hyaluronic acid may be considered very large prosthetic groups of conjugated proteins whose tissue or species specificity may be determined by the structure of the protein moiety or by the secondary and tertiary structure of the complex as a whole. This report shows the production of antibodies against such complexes.

Protein polysaccharide was extracted from nasal septa of steers 11 to 14 months old. Isolation, purification, and fractionation into "light" and "heavy" fractions were achieved according to the techniques described by Schubert and co-workers (*4*).

Sixty milligrams of unfractionated complex, such as potassium salt (Table 1), were dissolved in 5 ml of saline solution, then emulsified with 15 ml of Freund's adjuvant containing 25 mg of *Mycobacterium phlei* to which merthiolate was added to insure a 1:10,000 final concentration. Intramuscular injections of portions corresponding to 1 mg of protein polysaccharide were given at weekly intervals to 2.5-kg male white albino rabbits. The total amount of antigen administered was 4 mg. Four weeks after the last injection, the rabbits were bled and the serum obtained (containing merthiolate to 1:10,000 final concentration) was absorbed with fresh calf serum in order to remove possible antibodies to fractions of bovine serum protein. It was then assayed for preci-

pitating antibodies by double diffusion in agar gel—performed as described by Kabat and Mayer (*5*)—and by the "microspot" test of Feinberg (*6*).

Sixty milligrams of the potassium salt of the light fraction (Table 1) were digested with crystalline papain (phosphate buffer *p*H 6.5, 0.20*M*, containing 0.001*M*, EDTA disodium salt, and 0.005*M* cysteine HCl monohydrate). Forty milligrams of material referred to as "papain-treated" light fraction were recovered and a portion was analyzed (Table 1). Sixty-three milligrams of the potassium salt of the light fraction were incubated with purified testicular hyaluronidase (acetate buffer *p*H 5.40, 0.20*M*, containing 0.15*M* NaCl). Seventeen milligrams of material (possibly including some of the enzyme used) referred to as "hyaluronidase-treated" light fraction were recovered and a portion was analyzed (Table 1).

The potassium salt of the light fraction (500 mg) was dissolved in 50 ml of 0.20*M* KOH and incubated at 37°C for 5 hours. After neutralization with acetic acid and exhaustive dialysis against distilled water, the nondialyzable material was applied to a column (50 by 2 cm) of modified cellulose (*7*) equilibrated with 0.05*N* HCl. The material was eluted from the column with 250 ml of 0.05*N* HCl and then with 300 ml of 4*M* NH_4Cl in 0.05*N* HCl. The two eluates were neutralized, exhaustively dialyzed against water, and reduced in volume. The first eluate, which absorbed strongly at 280 mμ but did not have any material containing hexuronic acid, was lyophylized and 10 mg were recovered. The second eluate did not absorb at 280 mμ but had a large amount of material containing hexuronic acid. This was precipitated by addition of 0.5 g of $BaCl_2$ and ethyl alcohol to a final concentration of 40 percent, and then dried with ether. The yield was 378 mg, a portion of which was analyzed.

Table 1. Analyses of preparations of protein-polysaccharide (PP) from cartilage of bovine nasal septa. Results are precentages.

Hexuronic acid*	Hexosamine†	Nitrogen‡	Sulfate sulfur§	Hydroxyproline§
Unfractionated PP, K salt				
23	18.1	5.49	3.50	0
Light fraction of PP, K salt				
28.4	24.2	4.27		0
Papain-treated light fraction, K salt				
30.6	26.8	2.48		
Hyaluronidase-treated light fraction, K salt				
9.2	7.2	6.0		
Barium chondroitin sulfate from alkaline degradation of light fraction				
30.7	28.0	1.95		

* Dische's method (*9*) for hexuronic acid, glucuronolactone as a standard. † Blix's method, as described by Gardell (*10*) for total aminosugars. ‡ Micro-Kjeldahl for nitrogen, the samples being digested with 70 percent perchloric acid for 35 minutes. § Conductometric titration with a standard solution of $BaCl_2$ for sulfate sulfur. ¶ Stegemann's method (*11*) for hydroxy-proline.

The antiserum obtained was found to produce two precipitation bands when diffused against the potassium salt of the unfractionated protein polysaccharide and it produced one band when diffused against the potassium salt of the light fraction.

Serums taken from the rabbits before immunization and samples of normal rabbit serum selected at random failed to show any bands of precipitation when tested with comparable amounts of the same antigens. No precipitation bands were detected when the antiserum was diffused against the "papain-treated" and "hyaluronidase-treated" light fraction, nor with the protein and carbohydrate moieties of the light fraction recovered after alkaline degradation.

Duplicate 1-ml portions of absorbed antiserum were added to increasing amounts of bovine light fraction (25 to 700 μg) and saline solution was added to a total volume of 1.20 ml. These mixtures, including controls containing 700 μg of light fraction with 1 ml of normal rabbit serum and 1.0 ml of antiserum without light fraction, were incubated at 37°C for 2 hours; they were stored at 4°C for 15 days and centrifuged at 20,000g for 30 minutes. The sediments obtained were washed twice with 1.5 ml of ice-cold saline, dissolved in 1 ml of 0.05*N* NaOH, and analyzed for hexuronic acid. No material containing hexuronic acid was found in the controls. Figure 1 shows the amount of the hexuronic acid precipitated by the antiserum, expressed as percentage of the amount initially present.

No evidence of precipitation reaction was observed when absorbed rabbit antiserum to the bovine protein polysaccharide was tested with protein polysaccharides obtained from costal cartilage of man and pig, and from skeletal cartilage of rat, chicken embryo, and salamander.

The absence of precipitation between antiserum to bovine protein polysaccharide and the protein polysaccharides from costal or skeletal cartilage of other species indicates that the antigens present in the immunizing preparation do

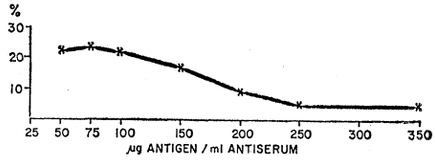

Fig. 1. Recovery of hexuronic acid in antigen-antibody precipitates, expressed as percentage of the amount initially present.

have a tissue or a species specificity. It seems that the specificity of the precipitins cannot be entirely attributed to the structure of either the protein or polysaccharide moiety of the antigen in view of the observed absence of precipitation when each moiety was separately tested against the specific antiserum.

It is possible, however, that this specificity is a function of the secondary and tertiary structure of the protein polysaccharide as a whole, the antigenic determinants consisting of carbohydrate and amino acid residues linked in three-dimensional arrangements specific for different tissues or different species. This hypothesis is consistent with the substantial difference in structure and physical properties found in protein polysaccharides extracted from cartilage of two different species (*8*). Moreover, it may explain the low recovery of material containing hexuronic acid in the antigen-antibody precipitates of the experiment illustrated in Fig. 1. In fact, if the antigenicity of the protein polysaccharide depends, even in part, on its tertiary structure, disruption or rearrangement of it may cause a loss of antigenic determinants without producing detectable chemical differences. It is possible that treatment with organic solvents and dehydration during extraction may produce a heterogeneous population of particles, only part of which retain the structural requirements necessary for the reaction with the sepcific precipitins.

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References and Notes

1. R. W. Quinn and K. P. Singh, *Proc. Soc. Exptl. Biol. Med.* **95**, 290 (1957); R. W. Quinn and R. Cerroni, *ibid.* **96**, 268 (1957).
2. J. D. Gregory and L. Roden, *Biochem. Biophys. Res. Commun.* **5**, 430 (1961).
3. J. Sandson and D. Hamerman, *J. Clin. Invest.* **41**, 1817 (1962).
4. I. Malawista and M. Schubert, *J. Biol. Chem.* **230**, 535 (1958); B. R. Gerber, E. C. Franklin, M. Schubert, *ibid.* **235**, 2870 (1960).
5. E. A. Kabat and M. M. Mayer, *Experimental*

Immunochemistry (Thomas, Springfield, Ill. 1961), pp. 85-88.
6. J. G. Feinberg, *Nature* **194**, 307 (1962).
7. ECTEOLA, Brown Company, Berlin, N.H.
8. B. Johnson and M. Schubert, *J. Clin. Invest.* **39**, 1752 (1960).
9. Z. Dische, *J. Biol. Chem.* **167**, 189 (1947).
10. S. Gardell, *Methods Biochem. Anal.* **6**, 297 (1958).
11. H. Stegemann, *Physiol. Chem.* **311**, 41 (1958).
12. Supported by grant AM 06029-02 of the U.S. Public Health Service and by a fellowship from the Helen Hay Whitney Foundation.
* Established investigator of the American Heart Association.

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Hemoglobin Solubility and α-Chain Structure in Crosses between Two Inbred Mouse Strains

Abstract. *In genetic crosses between mouse strains with low and high solubilities of hemoglobin, solubility segregated as a Mendelian unit and appeared to be determined by the alleles controlling α-chain structure. New data eliminate close linkage between the α-chain locus and genetic markers in linkage groups III, IV, XI, XIII, and XVI. Simplified methods are presented for screening differences in α-chain peptides and solubility.*

Hemoglobins of mice from various inbred strains differ in the primary structure of their α- and β-chains (*1*) and in their solubility in buffered salt solutions (*2*). Alleles at the *Hb*-locus (linked to albinism) affect β-chain structure and hemoglobin solubility (*3*). Each mouse classified as Hb^sHb^s contains one major hemoglobin of either intermediate or low solubility (*3*, *4*). Mice classified as Hb^dHb^d carry unequal amounts of two major hemoglobins whose non-α-chains differ from each other and from the β-chain of all Hb^s hemoglobins which have been studied (*1*). These Hb^d hemoglobin complexes tend to be highly soluble over a wide range of experimental conditions (*3*, *4*).

Popp has suggested that all heritable solubility differences among hemoglobins from Hb^sHb^s mice are controlled by alleles at a single *Sol* locus (*5*) and has named four alleles in this series (*6*). He has also postulated that genes at the *Sol*-locus affect the primary structure of the α-chain of hemoglobin. In Hb^sHb^s segregants from a cross NB × BALB/c (defined by Popp as $Sol^3 \times Sol^2$), he demonstrated changes in the two-dimensional electrophoretic and chromatographic patterns of tryptic peptides associated with differences in hemoglobin solubility (*7*). However, differences in α-chain-peptide pattern, hereafter called "fingerprint" pattern, are not always demonstrable in Hb^sHb^s mice differing in hemoglobin solubility. For example, no α-chain-peptide difference has been found between Popp's prototype for Sol^1 (C57BL) and his prototype for Sol^2 (SEC), between C57BL (Sol^1) and BALB/c (Sol^2), or between C57BL (Sol^1) and HBS (Sol^2) (*1*, *8*). These findings are subject to two different interpretations: there might be differences in α-chain structure which do not affect "fingerprint" patterns; or the difference in solubility between C57BL, SEC, and HBS could be controlled by alleles at a locus distinct from that determining α-chain structure in NB and BALB/c hemoglobins. We have tested these two possibilities by studying segregations for hemoglobin solubility and for α-chain structure in mice produced in crosses between the chinchilla-colored ($c^{ch}c^{ch}$) SEC/1Re-*Se* inbred strain (hereafter called SEC), and the albino (*cc*) SWR/J inbred strain (hereafter called SWR), both of which are homozygous for Hb^s (that is, they contain only a single β-chain).

We have limited our crosses to strains which have only one hemoglobin, since the presence, even in heterozygotes, of extra hemoglobins with differing non-α-chains introduces large solubility differences which modify and obscure effects of α-chain substitutions. The hemoglobin of our strain SEC mice is very insoluble (Sol^2, optical density of standard preparations, 0.1 to 0.2 in our test system), while that of SWR has a higher solubility, similar to but not identical with that of C57BL/6J (*4*, *9*). The α-chain of SEC contains peptide α4, also found in C57BL/6J, while that of SWR contains α4H, staining for histidine (*10*), also found in strain FL/Re (*1*). Previously reported techniques for studying α-chain inheritance by classification of the peptide composition of mouse hemoglobins (*11*) required two-dimensional "fingerprinting" to identify α4H (from FL/Re); however, we find that one-dimensional chromatography of crude hemoglobin digests is quite adequate for distinguishing the presence or absence of a single dose of α4H (*12*).

Table 1. Segregation of differences in hemoglobin solubility, α-chain structure, and coat color in 24 mice produced by backcrossing (SEC × SWR) F_1 × SEC, demonstrating complete correspondence between high hemoglobin solubility [measured as optical density (O.D.) of hemoglobin remaining in solution, values above 0.30] and positive histidine stain in peptide α4 on chromatograms, versus low hemoglobin solubility (optical density below 0.20) and absence of histidine stain in peptide α4. Coat-color difference assorts independently of both hemoglobin characteristics. Twelve mice in each experiment.

O.D. Hb. solub. (mean ± S.E.)	Histidine peptide α4	Color genotype	
		$c^{ch}c^{ch}$	$c^{ch}c$
0.13 ± .01 (0.09 − 0.17)	All negative	4	8
0.38 ± .01 (0.30 − 0.45)	All positive	4	8

Twenty-four progeny from backcrosses of (SEC × SWR)F_1 mice to the SEC inbred strain were classified (at the Jackson Laboratory) according to albino alleles carried ($c^{ch}c^{ch}$ or $c^{ch}c$ by progeny test) and according to solubility of hemoglobin (*13*, *14*). The mice were individually identified by ear punch and shipped to the University of Kentucky where their hemoglobins were classified according to whether they contained α4 or α4H. Prior knowledge of the solubility was withheld from the investigators at the University of Kentucky. Results of this experiment are given in Table 1. Exact agreement was obtained between hemoglobin solubility and the nature of the α-chain. However, it is likely that this difference is not due to the presence or absence of histidine in peptide α4, since strain SEC hemoglobin has α4 (no histidine) but is much less soluble than either C57BL (α4) or SWR (α4H), both of which have similar solubilities. The low solubility characteristics of SEC hemoglobin and the Sol^2 allele previously reported to segregate independently of the β-chain is shown here to be due to an undefined feature of the α-chain of SEC mice, which has not been visualized in "fingerprint" studies reported to date. Our data also confirm independent assortment (*5*) between genes at the α-chain and albino loci (Table 1).

Popp has reported breeding tests eliminating for several *Sol*-alleles both sex-linkage and close linkage with a variety of genes on several different autosomes (*6*, *9*). Because of the great advantage which would accrue from finding a close genetic linkage between the α-chain structural locus and a marker gene with clear-cut expression, we have employed the solubility method to extend the series of *Sol*-linkage tests, using efficient methods of backcrossing as follows: for dominant markers (for