back-to-back in roller tubes, and maintained another 8 days in vitro. With this procedure there was no direct physical contact between the two tissues other than the common culture medium. Muscle cultures exposed to spinal cord explants in this parabiotic condition and control muscle cultures were assayed for AChE activity. In muscle cultures, which were exposed to the media bathing the physically separated spinal cord explants for 8 days, AChE activity was almost twice that of the control cultures (Table 1), an indication that direct contact between nerve and muscle is not essential for regulation of AChE activity.

The results also indicate that the trophic effect can be mediated by a diffusible substance produced by spinal cord tissue. We therefore sought to determine whether a brain–spinal cord extract would promote AChE synthesis. For that purpose, dissociated muscle cultures (see above) were maintained in nutrient fluids containing 5 percent brain–spinal cord extract of 10-day chick embryos in place of ordinary embryo extract (brain and spinal cord excluded). After 10 days in vitro, experimental groups of muscle cultures grown in medium with brain–spinal cord extract and control muscle cultures were assayed for AChE activity. The AChE activity was higher in the muscle cultures grown in the presence of brain extract as compared to the control muscle cultures grown in the absence of brain extract (Table 1). These results show that brain–spinal cord extract is effective in inducing AChE activity in muscle cultures.

Our results agree with both in vitro and in vivo experimental findings of trophic effects of neurons as they related to the control of cholinesterase levels in skeletal muscle (*1*), and in addition confirm the finding of Lentz (*4*) that this effect can be produced in the absence of functional synapses.

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12. The reason for the differences in AChE activity in the control groups shown in Table 1 is not known. These values were obtained from three separate experiments. In each experiment the control and experimental muscle cultures were obtained from embryonic muscle subjected to the same dissociation procedure and subsequently subjected to the same culture medium and environment. It is possible that slight variations in the trypsinization procedure, in the culture medium, or in the environment are responsible for the variations in AChE activity. Similar variations in AChE activity of cells grown in culture have been reported [I. Werner, G. R. Peterson, L. Shuster, *J. Neurochem.* **18**, 141 (1971)].

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Australia Antigen (Hepatitis B Antigen): A Conformational Antigen Dependent on Disulfide Bonds

Abstract. *Reduction and alkylation of purified hepatitis-associated Australia antigen (hepatitis B antigen) resulted in a total loss of serologic activity. The reduced and alkylated protein formed a single band with a sedimentation coefficient of 31S on analytical ultracentrifugation, and no subunits were detected by Sephadex gel filtration. Although this preparation induced a delayed hypersensitivity response when injected into guinea pigs, it failed to stimulate humoral antibody formation. The data suggest that hepatitis B antigen is a conformational antigen critically dependent upon the disulfide bonds of the protein moiety.*

The discovery of Australia antigen and its association with post-transfusion "serum" hepatitis caused by hepatitis type B virus (*1*) stimulated studies of the natural history, epidemiology, and prevention of hepatitis type B (*1*, *2*). Purified hepatitis B antigen, prepared devoid of plasma protein by zonal ultracentrifugation, has been used for studies of structure and composition (*3*). Determining the immunochemical basis of the serologic specificity of the hepatitis B antigen is essential for understanding its molecular biology. The fact that the antigen is unusually resistant to enzymatic proteolysis (*4*) necessitated an alternate approach to its structural analysis. The antigen was found to contain 6.5 moles of cysteine per 100 moles of protein (6.5 mole percent) (*5*). Reduction and alkylation of the disulfide bonds of the cystine residues resulted in complete loss of antigenic activity.

Hepatitis B antigen was purified from the plasma of a healthy carrier by a combination of isopycnic banding and rate sedimentation on cesium chloride gradients (*5*). The purified antigen, composed exclusively of particles 20 nm in diameter, was devoid of detectable human plasma proteins as determined by gel diffusion analysis with antiserum to normal human serum. Inert indicator red cells, coated by exposure to antigen solution (0.5 mg/ml) and then to 1.25 m*M* chromic chloride, were not agglutinated by serial dilutions of antiserum to human serum, but were agglutinated by antiserum to hepatitis B antigen at a dilution of 1 : 6000 (*6*).

A solution of purified protein (0.5 mg/ml) in 0.55*M* tris(hydroxymethylaminomethane (tris) buffer, *p*H 8.0, was treated with 0.2*M* β-mercaptoethanol for 3 hours at 37°C, chilled to 0°C, and alkylated with 0.2*M* iced

Table 1. Effect of various treatments on serologic specificity of hepatitis B antigen. The antigen solution (0.5 mg/ml, in tris-HCl buffer, *p*H 8.0) was treated with 0.2*M* β-mercaptoethanol or 0.05*M* dithiothreitol with and without alkylation with 0.2*M* iodoacetamide. All materials were dialyzed against saline, then against 1.0*N* acetic acid. The reduced alkylated and reduced nonalkylated proteins were tested with antiserum to hepatitis B antigen by hemagglutination inhibition (HI) and countercurrent electrophoresis (CE). A + for hemagglutination inhibition indicates a titer comparable to control, untreated antigen.

Treatment	HI	CE
β-Mercaptoethanol	+	+
β-Mercaptoethanol, iodoacetamide	−	−
β-Mercaptoethanol, urea	+	+
β-Mercaptoethanol, urea, iodoacetamide	−	−
Iodoacetamide	+	+
Dithiothreitol	+	+
Dithiothreitol, iodoacetamide	−	−
Acetic acid	+	+
Urea	+	+
Control (untreated)	+	+

solution of twice crystallized iodoacetamide. After 1 hour at 4°C the reduced and alkylated material was dialyzed against saline at 4°C, followed by extensive dialysis against 1.0*M* acetic acid. Reduction was also performed in 6.0*M* urea, with and without alkylating agent, and in 0.05*M* dithiothreitol by incubation for 1 hour at 25°C. A solution of reduced and alkylated hepatitis B antigen (1 mg/ml), tested by analytical ultracentrifugation (Beckman model E), showed a single band with a 31*S* sedimentation coefficient (*7*). The treated antigen was also tested for possible subunit structure by chromatographic separation of a 1-ml sample (3.0 mg/ml) placed on a 100-ml column of Sephadex G-200 packed in 1.0*M* acetic acid. The protein came out as a single peak in the void volume of the column.

Hepatitis B antigen was treated with various chemical agents (Table 1) and dialyzed against 0.15*M* NaCl before being tested with antiserum to hepatitis B antigen by countercurrent electrophoresis (*8*) and hemagglutination inhibition (*6*). Reduction of the disulfide bonds by either β-mercaptoethanol or dithiothreitol, followed by alkylation with iodoacetamide, resulted in complete loss of antigenic activity.

Reduction alone, or treatment with 6.0*M* urea or acetic acid in combination with reducing agents, did not affect the serologic activity of hepatitis B antigen, nor did reduction without alkylation. Similarly, treatment with 0.2*M* iodoacetamide alone did not affect antigenic activity. These results suggested that conformation of the antigenic determinant is critically dependent upon the disulfide bonds of the protein moiety. Presumably the reduced and alkylated protein undergoes partial reoxidation during prolonged storage because serologic activity was partly restored after the preparation had been refrigerated for 3 months. Similar results were reported by Sukeno *et al.* (*9*).

The immunogenicity of reduced and alkylated hepatitis B antigen was tested in four guinea pigs injected through the foot pad with 0.2 ml of a solution of treated antigen (3.0 mg/ml) mixed with complete Freund's adjuvant. Four other animals were similarly injected with purified native hepatitis B antigen. After 2 weeks, serum samples were obtained from each guinea pig for detection of primary humoral antibody, and each animal was given two intradermal injections, one of native antigen and the other of reduced and alkylated antigen (100 μg each). A delayed hypersensitivity reaction characterized by marked erythema and induration was observed at both injection sites in all animals. Control unimmunized animals and animals immunized with complete Freund's adjuvant alone failed to show any delayed hypersensitivity reaction to an identical dose of each antigen given intradermally. Serum samples obtained a week after the intradermal injections showed a remarkable secondary antibody response in the animals immunized with native hepatitis B antigen, but the animals immunized with reduced and alkylated antigen showed no detectable serological activity against the native molecule (Table 2). Thus, while delayed hypersensitivity is induced by both treated and native antigen, the specific humoral antibody detectable by hemagglutination assay and countercurrent electrophoresis is induced only by native antigen (*10*). The failure of reduced and alkylated antigen to induce antibody formation against native antigen supports the contention that the molecule is a conformational antigen critically dependent upon the integrity of the disulfide bonds of the protein moiety. Further, the molecular properties inducing humoral response were disrupted, whereas those responsible for delayed hypersensitivity were not. Such functional dissection of a chemically defined antigen, glucagon, was reported by Senyk *et al* (*11*). Our results may have practical implications in the understanding of bioimmunology of carrier state for hepatitis B antigen in man.

Table 2. Primary and secondary humoral responses and delayed hypersensitivity responses of guinea pigs immunized with native hepatitis B antigen or reduced and alkylated hepatitis B antigen. Antibody was tested by hemagglutination assay (*6*). The titer of specific antibody is expressed as reciprocal of the highest dilution of the guinea pig serum reacting with red cells coated with hepatitis B antigen. The antibodies with titer greater than 2000 were also detectable by countercurrent electrophoresis. The animals immunized with either native or treated antigen showed delayed hypersensitivity to both types of antigen; this indicates cross-reactivity for the delayed response. This response was characterized by erythema of approximately 25-mm diameter and induration of 4 to 5 mm. Abbreviations: *N*, number of animals; P, primary; S, secondary; DH, delayed hypersensitivity; HBAg, native hepatitis B antigen; RA-HBAg, reduced and alkylated hepatitis B antigen.

N	Antigen	Antibody		DH response to	
		P	S	HBAg	RA-HBAg
4	HBAg	64	10,000	+	+
4	RA-HBAg	0	0	+	+
2	Freund's	0	0	−	−

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References and Notes

1. Short-incubation viral hepatitis, commonly called infectious hepatitis, is produced by type A virus; and long-incubation viral hepatitis, commonly called serum hepatitis, is produced by type B virus. The Australia antigen is now designated hepatitis B antigen in conformity with the nomenclature suggested by the Viral Hepatitis Committee of the National Research Council.
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5. G. N. Vyas, E. E. Williams, G. G. B. Klaus, H. E. Bond, *J. Immunol.* **108**, 1114 (1972). Hepatitis B antigen was purified by zonal centrifugation with two successive isopycnic bandings on cesium chloride gradients in a B-15 rotor followed by rate sedimentation on cesium chloride gradients in a B-14 rotor. Three distinct peaks are separated by rate sedimentation. The first peak consists exclusively of particles of 20-nm diameter, the second peak consists of filamentous and spherical forms of the 20-nm particles, and the third peak consists predominantly of 40-nm particles. An extinction coefficient was determined for purified antigen from the first peak ($E^{0.1\%}_{1\,cm} = 3.726$). Analysis of amino acid composition revealed cysteine, 6.5 mole percent; methionine, 5.5 mole percent; and tyrosine, 2.3 mole percent. Because tryptophan is destroyed by acid hydrolysis and the extinction coefficient was high relative to the tyrosine content, we determined the tryptophan content by the method of M. K. Gaitonde and T. Dorey [*Biochem. J.* **117**, 907 (1970)]. The ratio of tyrosine to tryptophan was 0.8.
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