hag and Mandel (13) with poliovirus. They reported the IgM (19S) type of antibody to be present only in the early immune response sera and not in late sera.

Since antiserum to phage DS6A did not neutralize significantly bacteriophages D29, R1, or Leo, this serum appears to be specific for phage DS6A (Table I). The conclusion may also be made that mycobacteriophages DS6A, D29, Leo, and R1 are serologically unrelated in neutralization tests.

Summary. The antigenicity of mycobacteriophage DS6A was determined in rabbits. The concentration of antibody produced was low (*K* values 10 or less). The amount of antibody produced appears to be dependent on the method of immunization. Bacteriophage DS6A appears to be serologically unrelated to mycobacteriophages D29, R1, and Leo by neutralization tests.

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Collagen Biosynthesis in Normal and Cirrhotic Rat Liver Slices* (33839)

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The fibrosis that accompanies hepatic cirrhosis, either that produced in animals experimentally (for example, by CCl_4, ethionine, or choline-free diet) or that occurring in man (viral, alcoholic-nutritional or biliary) is one of the main features of the disease.

Two mechanisms have been proposed for the development of fibrosis: (a) condensation or collapse of preexisting hepatic stroma (2), and (b) induction of fibroblastic proliferation and *de novo* collagen deposition (5). The latter mechanism is supported by evidence showing a net increase of total collagen content or an increase of hydroxyproline content per unit weight of liver (3, 7). The present experiments were designed to explore the capacities of normal and cirrhotic rat liver slices to synthesize protein containing radioactive hydroxyproline in the course of incubation with labeled proline.

Materials and Methods. Male Wistar albino rats weighing from 60 to 70 g were fed *ad libitum* with Purina chow. Cirrhosis was produced by intraperitoneal injection, 3 times/week, of 0.15 ml of a 1:7 solution of CCl_4 in mineral oil; each rat received a total of 20 injections.

Two days after receiving the last injection, a rat was deprived of food but not of water during 16 hr. It was stunned by a sharp blow to the head, decapitated, and the liver was removed to ice-cold Ringer–$NaHCO_3$ buffer.

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[1] Fellow of the Helen Hay Whitney Foundation (1962–1967).

Thin slices were cut by hand, and a pool was made of liver slices from 5 rats. The slices were washed twice with cold buffer and then incubated for 4 hr in Erlenmeyer flasks under an atmosphere of O_2:CO_2 (95:5%), at 37° in a Dubnoff shaking incubator at 100 cycles/min.

The incubation medium contained per flask: 1 g of liver slices, 2.5 ml of buffer and 5 μCi (0.5 ml) of uniformly labeled proline-^{14}C [The Radiochemical Centre, Amersham, Bucks; sp act, 120 mCi/mmole]. Zero-time controls contained, in addition to the former, 20 μmoles of KCN. Protein biosynthesis was stopped by rapidly chilling the flasks and homogenizing its contents in a tightly fitting Potter–Elvehjem device, in the presence of 0.5% deoxycholate, pH 8.0.

The proteins were precipitated by addition of an equal volume of ice-cold 10% trichloroacetic acid (TCA), and then washed five times with one volume of cold 5% TC. Collagen in its denatured form was extracted from the protein precipitate by treatment with one volume of 5% TCA at 75 ° for 90 min.

The 5% TCA extract containing denatured collagen was dialyzed repeatedly against large volumes of distilled water for 24 hr and against 0.5 *M* acetic acid for 72 hr, in order to remove all traces of free proline-^{14}C.

Aliquots of the dialyzed extracts were hydrolyzed with 6 *N* HCl for 12 hr at 100–110° *in vacuo*. The HCl was removed under reduced pressure and the sample was resuspended in 1 ml of 0.2 *M* pyridine buffer, pH 3.2, and applied to a 20 × 1.2-cm column of Bio-Rad AG 50W-X8, 200-400 mesh H^+ form. The amino acids were eluted with the same buffer, and 2-ml fractions were collected at a rate of 30 ml/hr. Aliquots of 0.2 ml were used for determination of proline (8). Hydroxyproline was determined in a 0.2-ml aliquot by the method of Woessner (10). Total ninhydrin-reactive material was determined by the method of Rosen (6). Before each set of experiments, a standard mixture containing 4 μmoles of proline and 2 μmoles of hydroxyproline was chromatographed on the same column using identical conditions. Aliquots of 0.5 ml were dried on stainless steel planchettes and counted in a Nuclear Chicago thin window, gas-flow counter.

Thin-layer chromatographic separation of proline and hydroxyproline was performed by the technique of Myhill and Jackson (4). Autoradiography of thin-layer plates was performed with Dupont's Cronex III radiographic plates using an exposure of 1 week.

Results and Discussion. In all of these experiments, the incorporation of proline into protein precipitable with TCA was 2 to 3 times higher in liver slices from cirrhotic rats than in those from normal rats. In the case of the cirrhotic livers as compared with normal, the amount of collagenous protein extracted into hot TCA was 5–30 times greater. (The wide range obtained with the cirrhotic liver preparations reflects the different responses of individual animals to the toxic action of CCl_4.) The controls (KCN blanks) did not shown more than 50 counts above background.

In every case, good separation of hydroxyproline from proline was achieved by column chromatography. Figure 1 depicts a typical chromatogram of a hydrolyzate obtained from the protein of a cirrhotic rat liver. As determined by specific colorimetric assays, no more than one tube between the two peaks of elution contained both proline

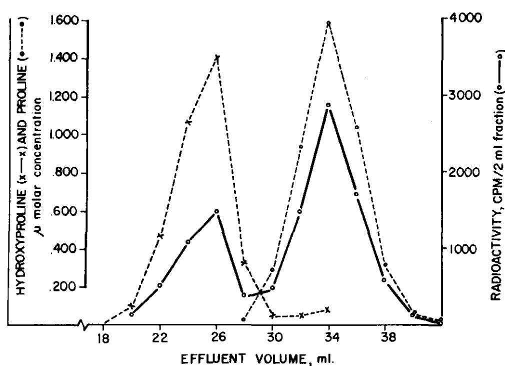

FIG. 1. Chromatography of a collagen hydrolyzate obtained from cirrhotic rat liver on a 20 × 1.2-cm column of AG 50W-X8, 200-400 mesh: 2-ml aliquots were collected at a flow rate of 25 ml/hr; aliquots of each tube were withdrawn for proline and hydroxyproline analysis and ^{14}C radioactivity determination.

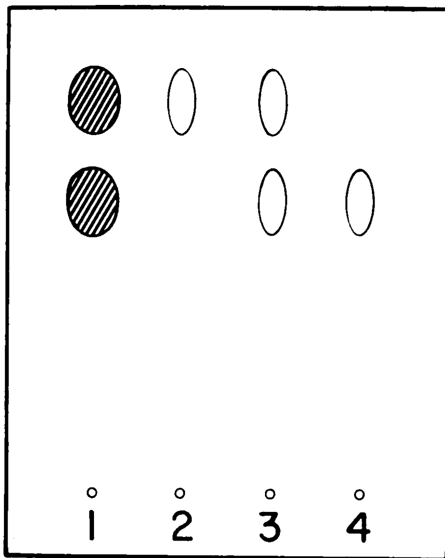

FIG. 2. Thin-layer chromatography of proline and hydroxyproline obtained from cirrhotic rat liver collagen hydrolyzates: the imino acids were developed with 0.4% isatin in *n*-butanol containing 4% acetic acid; the hatched zones represent the areas developed by autoradiography; (1) Sample: (2) proline std.; (3) proline hydroxyproline std.; (4) hydroxyproline std.

and hydroxyproline. Ninhydrin determination of 570 mμ showed that no other amino acids were present in the hydroxyproline and proline peaks. As shown in Fig. 1, radioactivity closely followed the elution pattern of the two imino acids.

The presence of radioactive hydroxyproline was also checked by thin layer chromatography and autoradiography. As shown in Fig. 2, the two radioactive spots have the same mobilities as hydroxyproline and proline standards, respectively, run on the same plate.

Total radioactivity of hydroxyproline plus proline recovered from the chromatographic column accounted for about 90% of the radioactivity incorporated into collagen. This indicates that there was a low degree of labeling of aspartic and glutamic acids.

Total values of hydroxyproline and proline recovered from the chromatographic column are shown in Table I. As shown, the total hydroxyproline content of the cirrhotic liver (μmoles/g of wet liver) was 4–8 times the content of hydroxyproline in normal liver, and that the proline:hydroxyproline ratio of cirrhotic liver is closer to that found in pure collagen. As Gottlieb *et al.* (1) have suggested, ratios greater than 1.5 may indicate the presence of proline-rich contaminants. Preliminary evidence suggests that the hydroxyproline-containing protein is, indeed, collagen, and that the site of synthesis is the liver stroma and not the Glisson's capsule (Huberman and Rojkind, unpublished results). The hydroxyproline-containing protein was extracted into cold 0.5 *N* acetic acid and digested with highly purified bacterial collagenase. The ^{14}C-radioactivity then became dialyzable. As was shown by Urivetzky *et al.* (9), digestibility of a protein by a purified specific bacterial collagenase in a proline-incorporating system can be used as a proof that the protein is collagen.

As shown in Table I, there is a correlation between the initial content of hydroxyproline, and the degree of incorporation of radi-

TABLE I. Collagen Bound Hydroxyproline-^{14}C and Proline-^{14}C from Normal and Cirrhotic Rat Liver Slices.[a]

Expt. no.		Hydroxyproline			Proline		
		(μmole/g)	(cpm/g)[b]	(sp act.)[c]	(μmole/g)	(cpm/g)[b]	(sp act.)[c]
Normal	I	0.429	38	79	0.712	565	795
	II	0.467	43	92.5	1.205	226	229
Cirrhotic	I	1.640	600	366	1.885	2239	1185
	II	2.967	3810	1285	4.979	11,058	2220
	III	3.569	3570	1000	4.242	7381	1740

[a] "*In vitro*" incubation with 5 μCi of proline-U-^{14}C.

[b] (cpm) are total counts less background with correction for self absorption.

[c] Specific activity is expressed as cpm/μmole of hydroxyproline or proline.

oactivity into protein extractable with hot TCA. The lower the initial hydroxyproline content, the less the incorporation. These experiments suggest that the capacity of the liver to synthesize collagen depends on the extent of liver damage produced by the toxic agent employed. This impies that the increase in the total collagen content in the liver of rats treated chronically with CCl_4 is due mainly to biosynthesis of collagen.

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Reaction of a Cobra Venom Factor with Guinea Pig Complement and Generation of an Activity Chemotactic for Polymorphonuclear Leukocytes* (33840)

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Since cobra venom was shown to destroy serum complement in 1903, much work has been done on the biological properties of this interesting substance (1, 2). Recently it has been reported that a purified nontoxic factor from the venom of cobra, *Naja naja*, selectively destroyed C′3 in human serum (3).[1] In guinea pig serum, on the other hand, a purified nontoxic factor from cobra, *Naja haje*, destroyed both C′3 and C′5, and destruction of C′3, C′5, C′6, C′8, and C′9 on administration of venom factor to guinea pigs has been reported (4–6). The generation of anaphylatoxic activity in the guinea pig serum treated with cobra venom has been described (7). In animals treated with cobra venom factor (CVF), the death caused by injection of Forssman antibody was prevented (4), the Arthus reaction was inhibited (6, 8), survival time for xenografts was prolonged (9), and the ability to generate slow-reacting substance of anaphylaxis was impaired (10). Thus, cobra venom factor is a useful experimental tool for studying inflammatory processes initiated by antigen–antibody reactions.

The present paper reports the results of *in*

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[1] Abbreviations: C′1, C′4, C′2, C′3, C′5, C′6, C′7, C′8, C′9: first, fourth, second, third, fifth, sixth, seventh, eighth, and ninth components of the complement system.