nor in the appearance of the colonies which they formed on heart-infusion agar and on eosin-methylene-blue agar. All of the strains failed to utilize citrate, and showed negative Voges-Proskauer and positive methyl-red tests. Tests for penicillinase production were done by a method previously employed with *Staph. aureus*(4). *E. coli* strains 2 and 3 produced penicillinase in these tests while the other 4 did not; this was true of both the parent strains and those which had been subcultured in the antibiotics singly or in pairs. In order to visualize more clearly the changes that have been described, the results shown in Tables II and III are summarized in Table IV.

Summary and conclusions. The data presented confirm and extend the observations previously made with staphylococci and some streptococci using other antibiotic combinations. For *E. coli,* and within the limitations of the methods employed, it was shown that following repeated subcultures in pairs of antibiotics, resistance generally developed more slowly and to a smaller degree than when the same organisms were exposed to the same antibiotics individually. There were wide variations, however, depending mainly on the antibiotics used, but perhaps also with the strain of organism. Cross-resistance to neomycin frequently accompanied increases in resistance to streptomycin resulting from exposure to the latter, alone or in combinations. Likewise, cross-resistance to oxytetracycline and chlortetracycline from exposures to chloramphenicol and to chloramphenicol and chlortetracycline following subcultures on oxytetracycline accompanied the development of resistance to the homologous antibiotic, whether present alone or paired with another agent.

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Amyloidosis and Renal Lesion Induced in Mice by Injection with Freund-Type of Adjuvant.* (20808)

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During attempts to produce injury to collagen in the mouse by subcutaneous injections of homologous collagen incorporated in the Freund-type of adjuvant, it was noted that deposits of amyloid occurred in certain tissues of the animals. Subsequent studies revealed that collagen played no role in the induction of amyloidosis and that the injury was due to the adjuvant. The Freund-type of adjuvant has been shown to enhance in the animal host circulating antibody formation against a variety of antigens(1) and to potentiate the production of allergic encephalitis (2). Another reaction to this adjuvant has been the development of amyloidosis. Although numerous reports have appeared in which this type of adjuvant has been used in many animal species, amyloidosis has been reported only in the duck following injection of killed malarial parasites combined with paraffin oil and killed tubercle bacilli(3). Experimental amyloidosis has been induced in several species of animals with diverse materials but only after long periods of treatment and with highly variable incidence. The observation that repeated injections of adjuvant induce amyloid in the mouse may offer a more efficient method of inducing amyloid-

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osis for the study of the pathogenesis of this disease in the experimental animal.

Materials and methods. W-Swiss, H-line mice† of both sexes 6 to 9 weeks old, weighing 15 to 20 g were employed. Their diet was made up of "Big Dog" pellets, water, bread and milk. The adjuvant was prepared as previously described(4), and consisted of a water-in-oil emulsion in which killed microorganisms were suspended. The oil phase consisted of an autoclaved mixture of 1.5 cc of Arlacel A‡ (Mannide monoleate), an emulsifying agent, and 8.5 cc of Bayol F,§ a paraffin oil of low viscosity; in this mixture were suspended 8 mg of killed and dried *M. butyricum,* or *Nocardia asteroides,*‖ or Group A streptococci,¶ type 6, strain S43/101/7. The aqueous phase consisted of 30 cc of 1-10000 dilution of acetic acid** in distilled water. As the latter was added by drops to the oil phase it was emulsified by repeatedly filling and emptying a syringe in the mixture, no needle was attached to the syringe. The emulsion was stored for several weeks at 4°C without evident separation. The mice were injected subcutaneously or intramuscularly at 7-day intervals. Untreated paired litter mates sacrificed simultaneously with the experimental animals served as controls. The tissues were fixed in 10% neutral formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin, congo red, and, in some instances, with crystal violet for metachromasia. Specimens of liver and spleen were treated with Lugol's solution and sulfuric acid for amyloid.

Pathologic findings. No deaths occurred during the experimental period. All animals were sacrificed 7 days after the last injection. The spleen and liver were usually found to be greatly enlarged, and in many instances the kidneys appeared large, pale and waxy. Gross specimens of spleen, liver, and kidney failed to give the characteristic stain for amyloid with Lugol's solution and sulfuric acid. The *spleen* was 3 to 4 times normal in size and firm with a tense capsule. The Malpighian corpuscles were grossly visible and the cut surface had a glassy appearance. Microscopic examination showed the loss of normal architecture and a homogeneous acidophilic deposit surrounding the corpuscles. This material was also found in the walls of the central arterioles, beneath the capillary endothelium of the follicles and walls of the vascular sinuses (Fig. 1). These deposits showed the characteristic congo red stain for amyloid. The red color was less intense than in human material employed as a control. The crystal violet stain for metachromasia was characteristic: the amyloid stained red-violet and tissue blue. The *livers* of the treated mice were enlarged, firm, pale and the cut surface appeared glassy. The amount of amyloid was less than in the spleen. It was most extensive in the sinusoids of the lobules between the endothelial and liver cells, resulting in pressure atrophy of the latter. Periportal round cell infiltration was also prominent in many of the sections. Amyloid was also found in the hepatic arteries and branches of the portal veins (Fig. 2). The involved *kidneys* were enlarged, pale, waxy, and almost bloodless. On cut section, the corticomedullary junction was lost. The capsules were not adherent and the surface was smooth. Microscopically, the striking features were the marked dilation of the tubules, often appearing as multiple cystic areas and the dilation of Bowman's space of the glomeruli (Fig. 3). The tubular epithelium was atrophied and the tubules contained colloid casts but no inflammatory cells. The glomerular capillary loops were ischemic and larger arteries were free of blood. Amyloid deposits were found in the medulla near the papilla where it occurred in the capillary walls beneath the endothelium. The tip of the

† Obtained from the Rockefeller Institute for Medical Research.

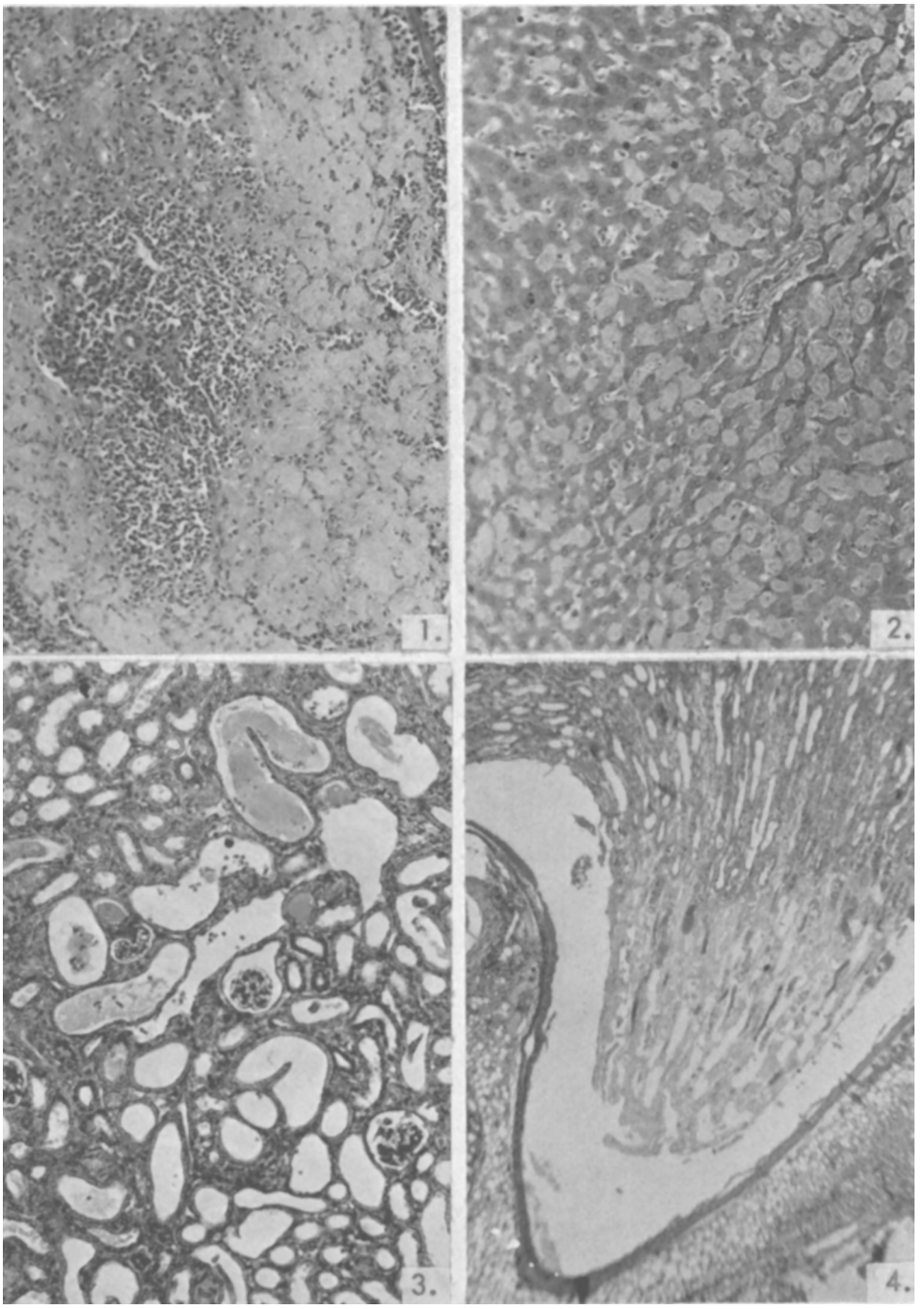
‡ Distributed by the Atlas Powder Co., Wilmington, Del.

§ Distributed by the Stanco Distributors, New York, N. Y.

‖ *M. butyricum* and *Nocardia asteroides* were kindly supplied by Dr. Jules Freund of Public Health Research Institute, City of New York.

¶ Group A streptococci kindly supplied by Dr. R. C. Lancefield of the Hospital of the Rockefeller Institute.

** Acetic acid was used as a solvent for the collagen, but it is not essential.



Microscopic appearance of mouse tissues after 10 weekly injections of Freund-type adjuvant.

FIG. 1. Section of spleen showing perifollicular infiltration of amyloid. × 90.

FIG. 2. Section of liver with moderate infiltration of amyloid and compression atrophy of hepatic cells. × 90.

FIG. 3. Section of kidney showing marked dilation of tubules, atrophy of tubular epithelium, colloid-like casts, and some interstitial fibrosis; glomeruli show little blood, small deposits of amyloid, and dilation of Bowman's space. × 100.

FIG. 4. Section of kidney showing necrosis of tip of papilla with deposits of amyloid lining capillary walls. × 75.

papilla was necrotic (Fig. 4). The glomeruli were only slightly involved by amyloid but patches of it were demonstrated by crystal violet staining. The interstitial tissue showed no evidence of acute inflammatory reaction but small areas of fibrosis were found in some instances. No obstruction was found in the ureters.

The injection sites did not ulcerate and appeared as encapsulated areas containing material similar to that injected which on culture revealed no growth. In those animals showing amyloid, the spleen was invariably involved; the liver less frequently; and the kidney lesion was never observed unless there was involvement of both liver and spleen. Amyloid was not found in other organs. Although studies were not made for plasma cells in all tissues, no foci of these cells were found in bone marrow sections. In 40 control litter mates neither amyloid nor renal lesions were found, nor have these lesions occurred spontaneously in older mice of the W-Swiss H-line as observed in Strain A mice by Gorer (5) and by Dunn(6).

Production of amyloid by various components of the Freund adjuvant. Since the Freund-type of adjuvant consists of 3 different components, an oil, emulsifying agent, and killed mycobacteria, it seemed of interest to determine which of these components were essential for the production of the amyloid. A group of 30 mice was injected with 0.2 cc of whole adjuvant every 7 days for 10 injections and killed 7 days after the final injection. Of this group 29 mice (Table I) showed amyloidosis; the spleen was involved in all and the liver in 16; the renal lesion occurred in 16. When the emulsifying agent, Arlacel A, and the paraffin oil, Bayol F, were employed in 10 mice under similar conditions no amyloid deposits or renal lesions were seen. This finding suggested that the killed *M. butyricum* were essential. However, *M. butyricum* alone also failed to induce amyloidosis. When *M. butyricum* was suspended in either Arlacel A or Bayol F, amyloid and the renal lesion did appear but the incidence was less than when the complete adjuvant was employed (Table I). It seemed desirable to determine whether or not some other microbial agent which had acid fast properties similar to the mycobacteria might be effective in inducing amyloidosis. *Nocardia asteroides* was selected because this organism has been shown to potentiate sensitization(7). Of 20 mice treated 13 showed amyloid of the spleen and liver; and 4 the renal lesion (Table I).

TABLE I. Incidence of Amyloidosis and Renal Lesion in Mice Injected with Components of Freund-Type Adjuvant.

Material inj.	No. of mice used	No. of mice showing		
		Amyloid—Spleen	Amyloid—Liver	Renal lesion
Arlacel, Bayol, & *M. butyricum*	30	29	16	16
Arlacel & Bayol	10	0	0	0
M. butyricum	10	0	0	0
Arlacel & *M. butyricum*	10	6	6	1
Bayol & *M. butyricum*	10	5	5	2
Arlacel, Bayol & *N. asteroides*	20	13	13	4
Arlacel, Bayol & Group A streptococci, Type 6, Strain S43/101/7	20	3	0	0

It was also of interest to determine whether bacteria without acid fast properties, such as Group A streptococci, had the capacity to induce amyloid when combined with the paraffin oil and emulsifying agent. Group A streptococci, type 6, strain S43/101/7 were employed in combination with Arlacel A and Bayol F. Of 20 mice injected 3 developed amyloid in the spleen; the liver and kidney were not involved.

Time of appearance of amyloid. To determine approximately when the amyloid first could be detected 20 mice were injected weekly for from one to 10 weeks with the complete Freund adjuvant employing *M. butyricum,* and 2 mice were killed each week, 7 days after the last injection. Amyloid first appeared in the spleen following the 4th injection. All mice receiving more than 5 injections had amyloidosis. The amount of amyloid in the tissues increased with the number of injections. The renal lesion was not observed in this series until after the 10th injection. These results are in contrast with those of the caseinate method employed in

other studies(8-12) for which at least 30 injections over a 6-week period are required for the earliest deposition of amyloid. Whether the number of injections or the time interval is most important for amyloid deposition is not answered by this experiment; but earlier workers(9,11,12) employing the caseinate method, observed that both the number of injections and duration of administration rather than the quantity of material introduced are the significant factors.

Summary. A method has been described for the induction of amyloidosis in mice by injection of the Freund-type adjuvant. The *M. butyricum*, or oil and emulsifying agent alone were not effective. *M. butyricum* with either the oil or emulsifying agent was capable of inducing amyloid. *N. asteroides* or Group A streptococci could be substituted for *M. butyricum* but the incidence of amyloidosis was less. The present method, as compared with previous ones, requires fewer injections, and it produces a higher incidence of amyloidosis in a shorter time.

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Heat Studies on a Thermophilic Bacteriophage.* (20809)

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Inactivation of bacteriophage by heat has been observed by several investigators, and seems to be a general phenomenon(1-3). Nanavutty(2), who exposed coli-dysentery phage to various temperatures and Smith and Krueger(3), employing vibrio-phage, found that their respective phages were not inactivated at a constant rate; heat inactivation curves (50° to 65°C) obtained by them were concave and became asymptotic with the time-axis. In contrast, the inactivation of a staphylococcus phage at various temperatures followed a monomolecular reaction and the temperature coefficient was characteristic of protein denaturation(4).

Koser(5,6) isolated a thermophilic phage from sewage-polluted river water and observed the optimum temperature for the lytic activity of the virus to be about 50°C, its ability to lyse the host at 57° to 58°C, its survival at 70°C for 30 minutes, and its destruction at 75°C. Heat resistance tests demonstrated that despite activity at higher temperatures, the phage was inactivated at about the same temperature (70° to 75°C) as the coli-dysentery phages. A thermophilic phage studied by Adant(7) was found to be active at 52°C, but was destroyed at 75°C when exposed for 30 minutes.

Recently we isolated from green house soil a bacteriophage capable of lysing a thermophilic bacterium at 65°C. Because the virus was active at high temperatures, heat inactivation studies were made since it was thought that it might be more resistant to heat than phages thus far reported. This investigation

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