immune precipitate, when mixed with the homologous antiserum, gave no precipitation under the same experimental conditions.

The precipitates were centrifuged at 0°C and washed twice with 1 cc saline also at 0°C. The N contents of the precipitates were determined by micro-Kjeldahl method. A control precipitin experiment was done by adding known amounts of the same lot of Type I Pneumococcus polysaccharide to the same lot of antiserum as that used in preparing the original immune precipitate under the same experimental conditions. The amount of precipitate N was plotted against the amount of polysaccharide. The amount of polysaccharide recovered at different pH's in the recovery experiment was then read from the control precipitin curve. The results are shown in Table I. It will be noted that antigen was recovered only in comparatively more acid solutions, where the recovery of antibody was previously shown[2] to be over 50%. When the pH was higher than 3.38, the amount of antigen recovered was too small to be estimated accurately.

Summary. Antigen was recovered from Type I Pneumococcus immune precipitate from which some antibody had been removed by treatment with acid. This experiment substantiates our previous finding that the recovery of antibody from immune precipitate by the action of acid or alkali is due to a shift in the antigen-antibody equilibrium. The present finding also suggests a possible method for the isolation of pure antigen which may be useful when it cannot be obtained otherwise.

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Further Studies on Type-Specific Protein of *Corynebacterium diphtheriae.*

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In a previous study[1] it was found that a serologically active, type-specific protein could be prepared by mild alkaline extraction at low temperature from the well-known Park 8 strain. In view of this finding it seems of interest to extend this observation to other types of *C. diphtheriæ* in order to determine (1) whether the method of

[1] Wong, Sam C., and T'ung, T., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 824.

extraction has a general application and (2) whether the protein is responsible for type-specificity of all types.

Method. The organisms employed for the present investigation were selected at random from the representative types of Sia and Huang's scheme of classification of *C. diphtheriæ*.[2] Seven strains belonging to 5 serological types and including the cultural types of gravis, intermediate and mitis were used. All but one (strain No. 7) were virulent. The growing of the organisms, the method of preparing the protein antigens, and the production of immune sera with the 7 strains were the same as those previously outlined.[1] In addition, immune sera of the following strains were also prepared: 8, 9, 10, 11. In all instances 2 rabbits for each strain, making a total of 22, were used. The precipitin ring test was performed at room temperature by carefully laying various dilutions of the antigen over a constant amount of undiluted immune serum and the results, read 2 hours later. The protein content of each preparation was estimated by determining the total nitrogen with the Micro-Kjeldahl method. The nitrogen value was multiplied by the protein factor 6.25 to give the concentration in milligrams. In the table one mg of protein is expressed as 1:1000 dilution.

In the course of this investigation several difficulties arose. In the first place the diphtheria bacilli are essentially poor antigens. While a type-specific agglutinating serum could be obtained generally in about 3-4 weeks of immunization in rabbits, a precipitin serum either for the proteins or for the polysaccharides may require several months. In 2 instances (strains 2 and 4) antibodies could not be detected by various dilutions of the polysaccharide although precipitins were present when tested with the protein antigens at high concentrations. Satisfactory sera for strains 2, 4 and 5 were obtained only after 6 months of immunization. New animals were used for a few of the strains without any apparent success. In the second place, sera of rabbits immunized with *C. diphtheriæ* invariably acquired anticomplementary properties. This fact renders the complement-fixation test inapplicable for the detection of type-specific antibodies. In the third place, serological activities of the proteins varied with different preparations. At times it was almost impossible to decide whether the protein was inactive or whether the immune serum was unsuitable. In general, however, the potency of an immune sera for the specific protein antigen may be estimated by its precipitin titer for the polysaccharide. The higher the titer for the

2 Sia, R. H. P., and Huang, C. H., *Ibid.*, 1939, **41**, 348.

latter the more suitable is the serum for the demonstration of the type-specific antigen.

Chemical Studies. All the protein preparations obtained gave the usual color reactions for proteins (biuret, xanthoproteic, Hopkins-Cole, and Millon). They could be precipitated by neutral salts such as ammonium sulfate and sodium sulfate, by heavy metals such as copper sulfate or lead acetate, and by trichloracetic and tannic acids. The Molisch test for polysaccharide in 1:500 dilution of the protein was negative. Extraction of proteins with ether did not destroy type-specificity but their serological activities were reduced. This indicates that the specific component is not a lipoid-complex.

In our previous report[1] we found that the type-specific protein prepared from Park 8 was heat-labile, being converted into a group specific protein upon heating at 56°C for 30 minutes. Subsequent study on this and other type-specific proteins, however, showed that this observation has no general application and appears to vary with different preparations which in turn depend upon a number of undetermined factors. It seems that the more active the preparation the conversion is more likely to occur although complete destruction of serological activity by heat has yet to be observed. Indeed, all the protein preparations employed in the present study in concentrations of 5-10 mg could withstand heating at 122°C for 20 minutes without alteration of type-specificity nor diminution of serological activity. However, the phenomenon of type to group-specific conversion could be demonstrated with certain preparations particularly from some strains (Park 8 and No. 3). Furthermore all proteins prepared by 1% potassium hydroxide extraction of organisms in boiling water bath are group-specific although the serological activity in general

TABLE I.
Type-specificity of Proteins as Shown by Precipitin Reactions.

		Protein						
Serum	Type	1	2	3	4	5	6	7
1	D41	1:100,000	1:5,000	—	—	—	—	—
2*	D41	1: 5,000	1:1,000	—	—	—	—	—
3	D43	—†	—	1:100,000	1:5,000	—	—	—
4	D43	—	—	1: 10,000	1:1,000	—	—	—
5	D14	—	—	—	—	1:5,000	—	—
6*	6287	—	—	—	—	—	1:10,000	—
7*	X	—	—	—	—	—	—	1:5,000
8	D25							
9	D30	—	—	—	—	—	—	—
10	D40							
11	1219							

*Sera which have been previously absorbed.
†Indicates negative to 1:500 dilution of the protein.

was low. The mechanism underlying these reactions is being studied.

Serological Studies. The results are presented in Table I. It is to be noted that all sera except strains 2 and 4 used in these studies were very potent when tested with the polysaccharide, precipitin titers ranging from 1:200,000 to 1:500,000 being observed. An analysis of the data reveals several points of interest. In the first place proteins from certain strains appear to be more active in serological test than others. For example antigens prepared from strains 1, 3, 6, 7 gave a specific reaction with the homologous type sera in dilutions of 1:50,000 to 1:100,000 while those from strains 2, 4 and 5 reacted in only 1:5000. It is conceivable that the marked difference in serological activities of the various antigens may be due either to denaturation of the antigen during preparation or to unsuitable serum. In the second place none of the proteins reacted with heterologous type-sera produced with strains 8, 9, 10 and 11. In the third place all the protein antigens are strictly type-specific with the exception of those from strains 6 and 7. Even here there was a quantitative difference, the protein reacting with the homologous serum to a much higher titer. In order to show the type-specificity, reciprocal absorption tests were carried out between the undiluted immune sera of strains 2, 6 and 7 and the organisms. The results showed that the common precipitins were promptly removed. The titers of the resulting type-specific sera, however, were significantly lowered when retested with the homologous protein. In case of sera of strains 6 and 7 there was a ten-fold drop in the precipitin titer. This may explain the marked cross reactions observed in the slide agglutination test with these strains.

Comment. The result of the present study shows clearly (1) that the method, consisting of alkaline extraction of bacilli previously defatted, is applicable to all the types studied and (2) that the protein is the cellular component responsible for type-specificity. For the demonstration of the latter point, serum from superimmunized animals in general is necessary. Furthermore, if marked cross reaction occurs in the agglutination test it is also likely to occur with the protein antigens. The difference, however, is quantitative and the common antigenic factor could be easily removed by reciprocal absorptions between immune serum and the heterologous organism. It may be mentioned that the third cellular component of *C. diphtheriæ,* the lipoids, obtained from all the 7 strains are, like the polysaccharides,[3] group-specific. This is in agreement with the observation previously reported on Park 8.[1]

[3] Wong, Sam C., and T'ung, T., *Chinese Med. J.*, Suppl. III, 1940, in press.

Conclusion. From the observations made on the chemical and immunological studies of the polysaccharide, lipoid, and protein fractions obtained from representative serological and cultural types it is justifiable to conclude that the alkali-soluble protein is the cellular constituent responsible for type-specificity in *C. diphtheriæ*.

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Thyroid Enlargement Following Liver Feeding in Rats.

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That the feeding of liver to animals produces an enlargement of the thyroid gland was reported by Hunt,[1] who found that the thyroid of mice fed on a diet of oatmeal and liver was larger and more vascular than of those on a diet of eggs, crackers and milk. Marine[2] produced a hyperplasia of the thyroid in brook trout by feeding them with liver and heart. Burget[3] found that rats kept under hygienic conditions and fed a high protein diet, consisting of fresh liver and lean beef mixed with a little oatmeal or bread crumbs, developed a hyperplasia of the thyroid. All the above reports of enlargement of the thyroid were interpreted as due to the high protein diet.

Recently, Remington[4] reported that in a low iodine goiter-producing diet, the replacement of a part of the wheat gluten by dried pig liver aggravated the degree of goiter, while purified casein prevented goiter formation, and yeast had no effect. This led Remington to believe that the liver in his goiter-producing diet was responsible for the enlargement of the thyroid. The writer (Hou[5]) in the same year reported that rats fed on a soybean-millet diet with dried liver powder as the only source of animal protein developed a marked hypertrophy of the thyroid gland. The enlargement was over 10 times when the animal was fed on the millet and liver diet for over a year. It becomes of great interest to know which constituent or constituents

1 Hunt, Reid, *J. Am. Med. Assn.*, 1911, **57**, 1032.
2 Marine, D., *J. Exp. Med.*, 1914, **19**, 70.
3 Burget, G. E., *Am. J. Physiol.*, 1917, **44**, 492.
4 Remington, R. E., *J. Nutrition*, 1937, **13**, 223.
5 Hou, H. C., *Chinese J. Physiol.*, 1936, **10**, 659; 1937, **12**, 488.