4. Sellers, A. L., Griggs, N., Marmorston, J., Goodman, H. C., *J. Exp. Med.*, 1954, v100, 1.

5. Krakusin, J. S., Jennings, R. B., *Arch. Path.*, 1955, v59, 471.

6. White, H. L., Tosteson, D. C., Rolf, D., *Fed. Proc.*, 1960, v19, 365.

7. Longley, J. B., Banfield, W. G., Brindley, D., *J. Biophys. Biochem. Cytol.*, 1960, v7, 103.

8. Scholander, P. F., *Biol. Bull.*, 1954, v107, 260.

9. Pappenheimer, J. R., *Physiol. Rev.*, 1953, v33, 387.

10. Rennie, D. W., Reeves, R. B., Pappenheimer, J. R., *Am. J. Physiol.*, 1958, v195, 120.

11. Dickens, F., Weil-Malherbe, H., *Biochem. J.*, 1936, v30, 659.

12. Hong, S. K., Rahn, H., *Fed. Proc.*, 1957, v16, 61.

13. Longley, J. B., Burstone, M. S., *J. Histochem. Cytochem.*, 1958, v6, 89.

14. Lassen, N. A., Longley, J. B., Lilienfield, L. S., *Science*, 1958, v128, 720.

15. Lilienfield, L. S., Lassen, N. A., Rose, J. C., *Proc. South. Soc. Clin. Res.*, 1958, 32.

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Fractionation and Immunological Properties of a DNA-rich Preparation from *Brucella abortus*.*† (26463)

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Previously we described the isolation of DNA-rich preparations from *Brucella abortus* by extraction with 0.5% phenol(1), and the ability of such preparations to produce antisera containing antibodies that precipitated DNase-sensitive antigens(2,3). Subsequent studies(4) revealed that such antisera also contained antibodies to antigens insensitive to DNase treatment, and that only 5% of all immunized rabbits were capable of producing antibodies against DNase-sensitive antigens. This variability may be similar to that encountered in studies of *Lupus erythematosus*, in which antibody-like serum factors reacting with DNA were found irregularly, and to the irregularity with which antibodies to haptenes are produced. With this variability in mind, the present report is restricted to an analysis of a lot of DNA-rich material from *B. abortus*, hereafter referred to as preparation #12, which contained DNase-sensitive antigens. Preparation #12, as well as previously used preparations, consisted largely of DNA, but also contained some RNA, carbohydrate, and about 25% protein that could not be dissociated from the nucleic acid(1). An attempt was therefore made to establish more precisely the chemical basis for the immunogolical specificity by fractionating the preparation containing the antigens. Progress made in such purification and the properties of the several fractions obtained will be described.

Materials and methods. Immunizing antigens. Preparation #12 ("#12 prep") was obtained from mucoid cells of *Brucella abortus*, strain 19, according to Braun *et al.*(1). It was separated into several fractions by differential ultracentrifugation as described below. *Preparation of antisera.* New Zealand white rabbits (5-6 lb.) were immunized by intravenous injections of a saline solution of either the unfractionated preparation or its fractions. In the course of immunization, each rabbit received a total of 2.5 mg of nucleic acid, as determined by absorption at 260 mμ, or that quantity of a given fraction which was obtainable from 2.5 mg nucleic acid in the unfractionated preparation. Injections were spaced over a period of 4 weeks, with doses of unfractionated material ranging from 0.05 to 0.25 mg of nucleic acid and equivalent amounts of the fractions. The

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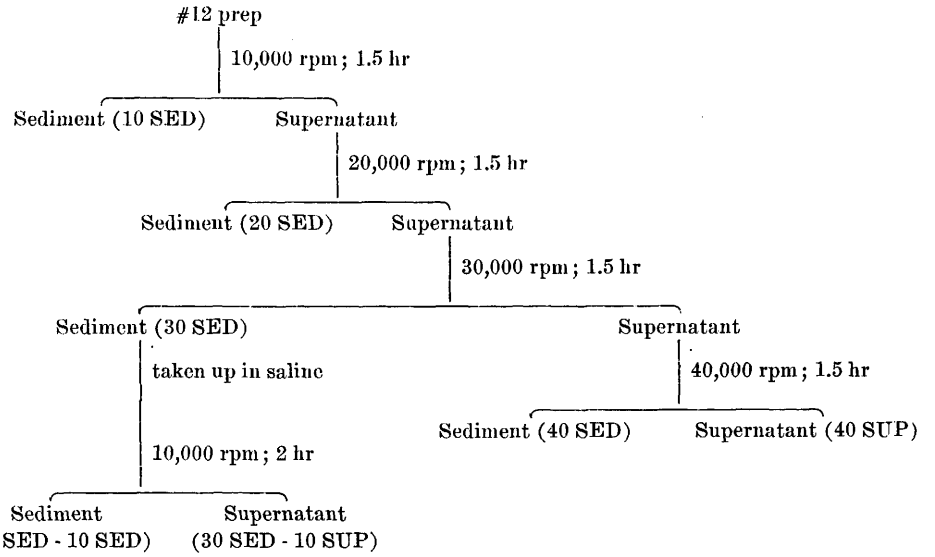

FIG. 1. Fractionation of "#12 prep" by differential centrifugation (Spinco ultracentrifuge, rotor 40, RCF = 6,590 × G at 10,000 rpm).

rabbits were bled by cardiac puncture one week after last injection and the sera were stored at −10°C. *Precipitin analyses.* The technique of Ouchterlony(5) was used for analysis of antigens. Although a series of concentrations of each antigen was tested, only results with optimal concentrations are given. Such solutions contained about 0.5 mg nucleic acid/ml; fractions were used at equivalent concentrations. Antisera were used undiluted or diluted with saline as much as 4-fold.

Results. Fractionation. In preliminary tests "#12 prep" proved heterogeneous electrophoretically, chromatographically, and ultracentrifugally. Differential ultracentrifugation was selected for purification because it gave the best resolution. Fig. 1 is a flow sheet for such fractionation, using a Spinco preparative ultracentrifuge with rotor #40. Each of the fractions, except the final supernatant, was taken up in 0.9% saline to the original volume, and all fractions were analyzed for DNA and protein. DNA was measured in terms of absorption at 260 mμ as compared to a standard solution of calf thymus DNA(6); however, since independent analyses had established that approximately 5-10% of the nucleic acid represented RNA, the values reported represent maximal amounts of DNA. Protein concentration was determined on the basis of the biuret reaction as modified by Zamenhof(7). The results (Table I) indicate that protein was not uniformly distributed among the fractions, its concentration being greatest in the fastest sedimenting fraction. The bulk of the protein was found in *10 Sed* and most of the nucleic acid was in the *40 Sup* fraction. The ultraviolet spectra of the slower-sedimenting fractions were typical for nucleic acids, whereas that of *10 Sed* showed no maximum at 260 mμ.

Reacting antigens in fractions. Results of the gel-diffusion analysis are shown in Fig. 2.

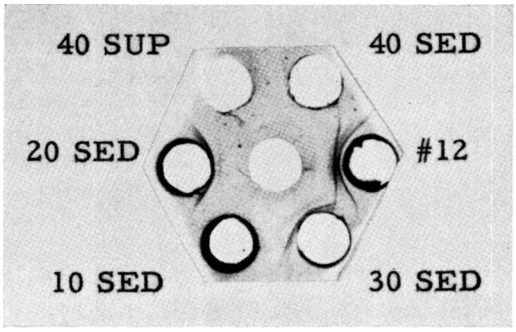

FIG. 2. Analysis of reacting antigens in a DNA-rich preparation from *Brucella abortus.* Center well contains antiserum pooled from 3 immunized rabbits, outer wells contain "#12 prep" and different fractions thereof.

TABLE I. Analysis of Fractions* of "#12 Prep."

Sample	DNA† mg/ml	DNA† % of original	Protein‡ mg/ml	Protein‡ % of original	DNA / Protein	O.D. (260) / O.D. (280)	O.D. (260)§ / O.D. (230)
#12 (original prep)	1.900	100	.930	100	2.2	1.89	1.56
10 SED	.180	9.5	.530	57.0	.33	1.68	.51
20 "	.122	6.4	.154	16.6	.79	1.83	.90
30 "	.222	11.7	.210	22.6	1.1	1.91	1.91
40 "	.356	18.7	.025	2.7	14.0	1.94	2.14
40 SUP	.922	48.5	.037	4.0	25.0	1.92	2.15
(30 SED - 10 SUP)	.209	11.0	.004	.4	52.0	1.94	2.24
(30 SED - 10 SED)	.060	.6	.200	21.5	.3	1.77	.58

* *Cf.* flow sheet, Fig. 1.

† Measured by optical density (Englander and Epstein, 1957), "#12 prep" contained 5-10% RNA; therefore these values represent maximal possible values for DNA.

‡ Measured by modified biuret method (Zamenhof, 1957).

§ For native calf thymus DNA, $\frac{\text{O.D. (260)}}{\text{O.D. (230)}} = 2.35$.

The antiserum was placed in the center well; one of the surrounding wells was filled with the immunizing "#12 prep," and the others with the fractions. The plates were kept at 23°C for 1 week for development. The *10 Sed* fraction gave no evidence of reaction with the antiserum diluted 1:3 with saline, but precipitated weakly when undiluted antiserum was used in another analysis, indicating a relatively low concentration of antibody directed against antigens in the *10 Sed* fraction. Both the *40 Sed* and *40 Sup* fractions reacted weakly at all concentrations. In the *20 Sed* fraction some antigens were detectable, whereas the reaction of the *30 Sed* fraction indicated that it had more of the reactive antigens and in higher concentration. The *30 Sed* fraction was therefore recentrifuged at 10,000 rpm in an effort to achieve further purification. A sub-fraction, *30 Sed - 10 Sup,* containing less than 2% protein was obtained. Fig. 3 shows the gel-diffusion analysis of antigens in these sub-fractions. Clearly, all of the reactive antigens were in the DNA-rich fraction *30 Sed - 10 Sup,* and no reactivity was detectable in the protein-rich fraction *30 Sed - 10 Sed.*

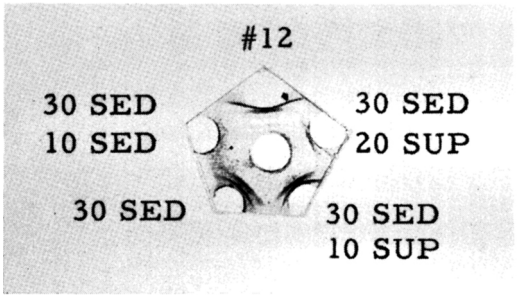

FIG. 3. Analysis of reacting antigens in sub-fractions of *30 Sed* derived from "#12 prep." Center well contains the antiserum pool; outer well contains the various fractions.

The *30 Sed* fraction subsequently was treated with DNase for 45 min at 26°C, then centrifuged at 10,000, 20,000 and 40,000 rpm for 2½ hr. After such enzymatic treatment, which resulted in a detectable reduction of reacting antigen, it was not possible to sediment remaining antigens even when speeds of 40,000 rpm for 2½ hr were employed.

Immunizing antigens in fractions. Because the reactive antigens were found predominantly in the *30 Sed* fraction, it was tested for its capacity to elicit the formation of precipitating antibodies in rabbits. A total quantity of *30 Sed* equivalent to 2.5 mg of 260 mμ-absorbing material in the crude preparation was injected intravenously into rabbits. An equal number of rabbits received the unfractionated preparation. All were bled by cardiac puncture, one week after the last injection, and the sera examined by gel-diffusion. The sera of rabbits injected with the *30 Sed* fraction, which had most of the reactive antigens, had no detectable precipitating antibody in contrast to the sera of rabbits which had received the crude DNA preparation. This suggests the necessity for

distinguishing between reactive and immunizing antigens (see discussion).

Rabbits that had received the *30 Sed* fraction and failed to give precipitins were subsequently injected with a mixture of fractions *10 Sed* and *30 Sed*. At the same time, rabbits which had previously yielded precipitating antibodies with the whole "12 prep," and had been allowed to rest until precipitins could no longer be detected in the serum, were given the *10 Sed* fraction alone. All of the sera now contained precipitins induced apparently by immunizing antigens in the *10 Sed* fraction but directed against reactive antigens in fractions other than *10 Sed*, such as *30 Sed - 10 Sup*. Subsequently, previously non-immunized rabbits were injected with the *10 Sed* fraction alone, and these rabbits also produced precipitating antibodies directed against antigens in fractions other than the immunizing fraction. It seems, therefore, that the protein-rich fraction *10 Sed* can contribute the immunizing antigens, but that the antigens reacting with immune sera are to be found among the DNA-rich fractions.

Discussion. These fractionation studies have demonstrated that in antigenically active DNA-rich preparations, obtained by 0.5% phenol extraction, protein is not uniformly distributed among fractions obtained by differential ultracentrifugation. This method of fractionation, therefore, has proved useful for an analysis of antigenic properties associated with materials differing markedly in their ratio of DNA to protein.

Reacting antigens were concentrated predominantly in fractions rich in DNA and were not proportional to the relative amounts of protein present. Thus the protein-rich subfraction of *30 Sed* contained no reacting antigens, whereas the DNA-rich supernatant *30 Sed - 10 Sup* showed undiminished reactivity. Furthermore, reactivity was not abolished by the action of proteolytic enzymes such as trypsin, chymotrypsin and panprotease. However, since the purest available preparations still contain traces of protein, the latter cannot be excluded definitely as a necessary part of the reactive antigens.

The likelihood that at least some of the several antigens present in the *30 Sed* fraction are in some manner associated with DNA is indicated by the finding that DNase treatment of the *30 Sed* fraction diminishes the intensity of the antigen-antibody reaction and decreases the molecular weight of the antigens so that they can no longer be sedimented at 40,000 rpm. The possibility that the antigens are complexes containing DNA has been suggested by our observation that treatment of the reacting antigen preparation with periodate, which acts primarily on carbohydrates but not on deoxyribose of DNA, results in a complete abolition of antigenic reactivity.

The protein-rich *10 Sed* fraction, which was capable of stimulating the formation of precipitating antibodies that reacted with the *30 Sed - 10 Sup* fraction (containing less than 2% protein), did not appear to have reactive antigens. Furthermore, the reactive fraction, *30 Sed - 10 Sup*, was unable to stimulate production of precipitating antibodies. It would appear, therefore, that a distinction must be made between the immunizing and reactive antigens. The failure to elicit antibodies with the relatively pure DNA fraction is consistent with the general experience of others who could not detect precipitating antibodies in the sera of animals injected with pure DNA(8). As yet no experimental data are available to explain the differences noted in the immunogenic capacities of purified DNA *vs.* DNA-protein.

While these studies have failed to yield pure antigens and thus have not enabled us to associate the DNase-sensitive antigens with a well-defined chemical entity, they have indicated at least that substantial concentrations of protein do not play a role in the antigen-antibody reactions here studied. Even though the specificity of antigens associated with DNA may not ultimately depend upon DNA itself, such antigens, nevertheless, would appear to be of considerable interest chemically and, above all, biologically.

Summary. A DNA-rich preparation, obtained from *Brucella abortus* by extraction with 0.5% phenol, was fractionated by differential ultracentrifugation, and its immuni-

logical properties were studied. The antigens that precipitated antibodies from the sera of rabbits injected with the crude DNA preparation were concentrated in a fraction consisting essentially of DNA and only about 2% protein. This fraction did not elicit the formation of precipitating antibodies, whereas a protein-rich fraction with no reactive antigens did. Treatment of the reactive antigens with DNase altered some of their properties. On the basis of present evidence, it is suggested that the DNase-sensitive antigens are complexes containing DNA.

1. Braun, W., Whallon, J., Phillips, J. H., *Nature*, 1957, v180, 1356.

2. Phillips, J. H., Braun, W., Plescia, O. J., *ibid.*, 1958, v181, 573.

3. ———, *J. Am. Chem. Soc.*, 1958, v80, 2710.

4. Braun, W., Plescia, O. J., Palczuk, N. C., *Proc. 5th Intern. Congr. Biol. Standardization*, 1959, 367.

5. Ouchterlony, O., *Ark. Kemi Min. Geol.*, 1948, v26B, 1.

6. Englander, S. W., Epstein, H. T., *Arch. Biochem. and Biophys.*, 1957, v68, 144.

7. Zamenhof, S., in Colowick, S. P., and Kaplan, N. O., eds. *Methods in Enzymology*, Academic Press, N. Y., 1957, vIII, 702.

8. Levine, L., Murakami, W. T., Van Vunakis, H., Grossman, L., *Proc. Nat. Acad. Sci.*, 1960, v46, 1038.

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Effect of ACTH on Sex Ratio of the Albino Rat.* (26464)

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Mammalian spermatogenesis results in an equal number of male and female producing spermatozoa. The actual sex ratio of most species, however, differs characteristically from the expected theoretical value of unity (1).

The factors which enter habitually into the reproductive process of those species and modify the sex ratio (number of males per 100 females) could be paternal, e.g. excess mortality of one type of spermatozoon after spermatogenesis or diminished aptitude of one type of spermatozoon for fertilization. Such and other differential properties of the 2 types of spermatozoa have been demonstrated and have become the subject of considerable research. On the other hand, the maternal milieu interne could be such as habitually to favor survival of, or fertilization by, one type of spermatozoon. These two possibilities may be largely synonymous.

In addition to these normal and species specific deviations from unity of the sex ratio further changes in sex ratio occur in several well authenticated situations. The old observation that the human sex ratio rises in war time was again verified during the second world war and statistical analysis suggests that this rise was not due to age of the parents or to the birth order(2). In many species including man, it has also been noted that the first born or the first litter tends to have a significantly higher sex ratio than the species as a whole. In the rat with a species specific sex ratio of 105 the ratio for first litters is 122(3). In some animals (cattle, sheep, pigs, wolf hounds) statistics indicate that the relative number of female births tends to rise during the cold months of the year. King has confirmed this for the rat(3).

These observations suggest that stress may be able to alter sex ratio. The possibility that the adrenal cortex may be involved in mediating these effects is strengthened by 2 observations of King and Donaldson made in 1929, i.e. before the relation between adrenal cortex and stress responses was understood. King recorded the sex ratio of the

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