A STUDY OF THE PROTEIN-NUCLEATES OF THE SPECIES OF THE GENUS BRUCELLA

I. CHEMICAL CONSTITUTION OF THE PROTEIN-NUCLEATES.

II. BIOLOGICAL PROPERTIES OF THE PROTEIN-NUCLEATES.

THESIS

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by

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A STUDY OF THE PROTEIN-NUCLEATES OF THE SPECIES OF GENUS *BRUCELLA*

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I. CHEMICAL CONSTITUTION OF THE PROTEIN-NUCLEATES*

There have been relatively few reports in the literature regarding the chemical composition and biological properties of bacterial "nucleoproteins", or protein-nucleates; or of the properties of the bacterial nucleic acids. Johnson and Brown (1) and Johnson and Coghill (2) have studied the "nucleoprotein", protein and nucleic acid of *Mycobacterium tuberculosis*. Similar studies have been carried out by Thompson and Dubos (3) on *Diplococcus pneumoniae*, by Ferramola (4) on *Bacillus anthracis* and by Mitra (5) on *Vibrio comma*. Studies on "nucleoprotein" and protein fractions of *Strepto coccus pyogenes* have been reported by Sevag, Lackman and Smolens (6) and by Heidelberger and Kendall (7) on *Streptococcus hemolyticus*. Preliminary studies on the "nucleoproteins" of the species of *Brucella* have been made by Topping (8) and Huddleson and associates (9,10,12).

In a previous study, Huston, Hershey and Huddleson (9) described the preparation of an antigenic and chemically defined substance which they called a "nucleoprotein", this now being termed a protein-nucleate. Further studies on this material resulted in its production as "Brucellergen", which is a suspensoid of the protein-nucleate and is used as a skin-testing agent in detecting undulant fever (brucellosis) in humans (12). Although clinical research has progressed on this material since 1934, very little study has been made of its chemical composition and biological properties.

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The present papers concern the chemical nature and biological properties of the protein-nucleate fraction of the smooth, intermediate rough and rough forms of the organisms. The smooth form will be designated by (S), the intermediate rough by (IR) and the rough by (R).

Isolation of the Protein-nucleate

The protein-nucleates were prepared from a number of smooth, intermediate rough and rough strains of *Brucella abortus*, *Brucella melitensis* and *Brucella suis* from the stock collection maintained in the Central Brucella Station. The one intermediate rough and truly rough strain studied were *Brucella abortus*. All gave typical reactions characteristic of their species as to dye bacteriostasis, hydrogen sulfide production, and agglutinogenesis.

The dried cells were prepared in a manner similar to that described in a previous publication (9). The dried organisms were ground in a ball mill for 7 days. The resulting fine powder was suspended in sufficient distilled water (pH 6.5) to make a suspension of approximately 2.5 per cent by weight, stirred by means of a mechanical stirrer at room temperature for 3 hours and allowed to stand in a cold room at 0° to 5°C. for 24 hours. After this operation, the insoluble material was separated by passing the mixture through a steam-driven Sharples laboratory centrifuge.

The cell residue was extracted a second time in the same manner, this time, however, adding sufficient normal sodium hydroxide to bring the pH to 8.0.

The first and second water extracts which made a clear to slightly opalescent pale yellow solution, were combined and precipitated with glacial acetic acid by bringing the pH to 3.5. The resulting precipitate was allowed to sediment over night in the cold. The following morning the precipitate was collected in the centrifuge and washed with distilled water. This precipitate was then stirred mechanically in cold distilled water, and N sodium hydroxide was added until the pH of the solution was 7.2, at which point all of the material dissolved completely, however, still remaining opalescent. This was reprecipitated and dissolved a second and third time. A neutral solution of the twice reprecipitated protein-nucleate was filtered through a Seitz filter. The water clear filtrates were acidified, the precipitated proteins washed with water and made up to a 1 per cent solution with water at pH 7.0. These were used for further study.

It is not our purpose here to argue pro or con as to the desirability of using this method for the isolation of the protein-nucleate in its least modified form. This is the method which has been satisfactory in producing an allergic skin testing agent (Brucellergen), and is taken for analysis at its own value. The method of Heidelberger and Kendall (7) was used for the preparation of one "nucleoprotein" fraction, and its analysis and properties have been compared with the others. This method consists briefly in: extraction of the centrifuged whole cells with acetone and ether; centrifugation and grinding of the cells; extraction with 0.2 N acetate buffer at pH 4.0; centrifugation and extraction of the residue with M/15 phosphate buffer at pH 6.5; precipitation of this extract with glacial acetic acid - this precipitate being the "nucleoprotein" fraction used. It was further purified as directed in the publication (7).

The Chemical Nature of the Protein-nucleate

As Osborne and Campbell (11) have so aptly phrased it - "In reality 'nucleoprotein' means rather a 'method of preparation' than a chemical substance." They have given evidence that protein-nucleates, prepared from the same tissue by slightly different methods had different ratios of protein to nucleic acid. Such variations could be expected when a polyvalent base (protein) unites in different proportions with a polyvalent acid (nucleic acid). Thus we have tried to use a constant procedure for the preparation of the protein-nucleates, and even though carrying out the procedures very carefully, small differences between different preparations of protein-nucleate from the same organism were noted.

These protein-nucleates, as already stated, were obtained by precipitation with acetic acid at their isoelectric point of pH 3.5 to 4.0. The white, flocculant precipitate is readily and completely soluble at pH 7.0. The solution does not coagulate on heating at neutral reaction and in the presence of electrolytes.

The total yield of protein-nucleate for the smooth strains of abortus, melitensis and suis was from 12.0 to 13.7 per cent of the dry weight of the cells; whereas the intermediate rough and rough strains gave 18.0 to 18.2 per cent of the dry weight of the cells. The nitrogen and phosphorus content are set forth in Table I.

Table I. Nitrogen and Phosphorus Content of Protein-nucleates

	N per cent	P per cent	N/P ratio
abortus 230 (S)	15.02	3.06	4.98
*abortus 161 (S)	14.82	2.72	5.44
melitensis 2194 (S)	14.91	2.89	5.16
melitensis 2425 (S)	15.18	2.74	5.54
melitensis 9G (S)	14.88	2.60	5.68
suis 1582 (S)	14.68	2.31	6.35
abortus 85 (IR)	14.76	3.31	4.46
abortus 805R (R)	14.83	3.35	4.42

* abortus 161 protein-nucleate prepared by method of Heidelberger and Kendall (11).

A 1 per cent solution gave the following biochemical reactions: Bials' and Molisch and Feulgens markedly positive, biuret, Rosenheims', Millons' and Sakaguchi positive, and labile sulfur negative.

It was found impossible to run optical rotation on most any concentration of protein-nucleates, due to their opalescence and slight yellow color.

Separation of the Protein-nucleate into its Components

Three methods were tried for the separation of biologically active protein fractions from the protein-nucleates. The first was that of Johnson and Brown (1) in which the dried material was extracted successively with 3 per cent and 5 per cent sodium hydroxide. By this method they obtained a yield of nucleic acid that was approximately 1 per cent of the original weight of the tubercle bacillus. However, the alkali metaproteinate formed from Brucella was found to be biologically inactive and not suited for further studies. However, the nitrogen partition figures correspond remarkably well with those of the biologically active proteins obtained by the second method.

The second method was that of Sevag, Lackman and Smolens (6), which consisted in splitting the protein-nucleate with sodium carbonate and separating the mixture by means of chloroform. A detailed example is as follows: 5 grams of the protein-nucleate was dissolved in 1.4 liters of distilled water containing 7.1 grams of anhydrous sodium carbonate and heated in a water bath at 50-55°C. for 2 hours. After the mixture was cooled and any insoluble residue removed by centrifugation, it was neutralized with acetic acid to pH 7.0.

As Sevag states, "The protein and nucleic acid were then present in a free state. To remove the protein from solution a water-insoluble chloroform-protein gel was formed. This was separated by centrifugation. Nucleic acid does not form such a combination; protein and chloroform seem to enter into a loose molecular combination, and because of the density of the chloroform this complex settles out. On the acid side of the pH at which the protein precipitates, a salt-like recombination with or a slight adsorption of nucleic acid may take place. This can largely be prevented by adjusting the acidity with acetic acid to a point between the isoelectric point of the protein component and neutrality."

Specifically, after hydrolysis and neutralization, 0.25 volume of chloroform and 0.1 volume of butyl alcohol were added. The mixture was shaken mechanically about 6 hours, and upon centrifugation separated into two layers. The lower layer consisted of a fairly stable chloroform-protein gel; the upper layer is an aqueous one. The upper layer can easily be decanted from the gel. If an excess of chloroform is used, a third layer is formed which consists of pure chloroform and is found in the bottom of the bottle. The upper aqueous layer is further shaken and centrifuged until a gel no longer forms at the interface.

Traces of non-protein component are removed by transferring the chloroform-protein gel to a wet filter in a funnel. The protein was liberated by treatment with one volume of alcohol. The product was then centrifuged and washed with water adjusted to pH 5 to 6 with acetic acid. The washed protein was dissolved by adjusting to pH to 7.5.

The supernatants from the protein-gel which contains the nucleic acid are combined. Although they appeared clear, they still contained protein. By concentrating _in vacuo_ to a smaller volume and reextracting with chloroform, the last trace may be removed. The nucleic acid was isolated from the protein-clear filtrate by treatment with 4 volumes of alcohol containing enough N hydrochloric acid to bring it to pH 5.0, chilling in the cold room and centrifuging. The above procedure was repeated to obtain a purer product.

A third method consisted of splitting the protein-nucleate as above, and then fractionally precipitating out the protein at its isoelectric point (pH 3.5 to 4.0) and the nucleic acid at pH 2.0 to 2.5. However, the composition of the purified nucleic acid was so variable on batches of the same organism that the method was abandoned.

Chemical Study of the Protein

The proteins studied were all prepared by the Sevag chloroform-gel method. The yield of protein from the protein nucleates of S strains varied from 70 to 77 per cent. From both the IR and R strain protein-nucleates, the protein yield was approximately 60 per cent.

The protein gives a positive biuret test for the peptide linkage; a positive Millons' test for tyrosine; a faint reaction in the Rosenheim test for tryptophane and quite a strong Sakaguchi test for arginine. The protein gives a positive Molisch test for carbohydrates; a positive reaction with Bials' test for pentoses.

Nitrogen partition (Hausmann numbers). The protein fractions from the S, IR and R strain preparations of protein-nucleate were analyzed for total nitrogen, amide nitrogen, humin nitrogen, diamino nitrogen, mono-amino nitrogen; the amino acids arginine, lysine, histidine and cystine and for phosphorus. The method for the nitrogen partition was based on Hewitts adaptation (13) of the Thimann method (14). The results are summarized in Table 2. As may be seen in this Table, the only significant differences in composition of the S, IR and R strain preparations were their diamino acid content.

These proteins do not in any way resemble histones or protamines, according to two main criteria. First, they do not possess the very high diamino nitrogen content of histones (19 to 30 per cent) and protamines (53 to 89 per cent); second, most histones and protamines are toxic in nature, but it will be shown later that the proteins under study were non-toxic. The proteins also fail to give many of the other tests characteristic of histones and protamines.

Carbohydrates. The Sorenson and Haugaard (15) procedure for carbohydrates was run on all protein fractions. While no actual quantitative

Table 2. Nitrogen Distribution of Proteins from *Brucella* Protein-nucleates.

	Melitensis (S)			Suis (S)		Abortus (S)			Abortus (TR)			Abortus (R)		
	Strain 2425	Strain 2194		Strain 1582		Strain 230	Strain 161		Strain 85	Strain 85		Strain 805R	Strain 805R	
	Per cent	Per cent	Mean	Per cent	Mean	Per cent	Per cent	Mean	Per cent	Per cent	Mean	Per cent	Per cent	Mean
Amide N	9.91	11.55	10.73	8.89	8.89	9.11	10.63	9.92	11.66	8.60	10.13	7.79	8.53	8.06
Humin N	2.49	4.29	3.36	4.39	4.39		3.19		5.28	2.97	4.12	3.40	4.43	3.91
Monoamino N	65.71	73.47	69.09	68.28	68.28	72.34	70.40		82.53	84.24	83.38	68.70	76.86	72.78
Diamino N	19.30	20.46	19.89	18.10	18.10	18.08	19.27	18.67	4.42	6.37	5.40	10.18	14.47	12.37
Arginine	5.39		5.39				3.57	3.57					4.59	4.59
Lysine	7.76		7.76				9.28	9.28					6.78	6.78
Histidine	6.15		6.15				6.42	6.42					3.10	3.10
Cystine	0.00		0.00		0.00		0.00	0.00			0.00		0.00	0.00
Phosphorus			0.90		0.98			0.80			0.53			0.52

determination was made, nor identification of the sugar made, it was quite evident that polysaccharides were present in very small quantities. Reducing sugars were present as such, and calculated as glucose (Schaffer-Hartman procedure) ran from 1.56 to 2.15 per cent on the various samples. After hydrolysis with dilute hydrochloric acid, reducing sugars are found to comprise from 2.00 to 2.87 per cent.

As the amino-sugar, glucosamine, has been found present in other Brucella fractions studied in this laboratory, its determination was attempted on this particular fraction. Using the technique of Palmer, Smythe and Meyer (16), no trace of glucosamine could be detected.

Chemical Study of the Nucleic Acid

The nucleic acids, when dried with alcohol and ether formed a white powder; when precipitated with hydrochloric acid the dry powder was readily soluble in distilled water made slightly alkaline. After precipitation with alcohol it was immediately soluble in water, giving a perfectly clear solution. The nitrogen and phosphorus analyses are given in Table 3. The nucleic acids all give negative biuret, Millons' and Rosenheims' reactions for the detection of protein components; the Molisch test for carbohydrate, Bials' test for pentose and Feulgens test for desoxypentose were positive.

The yield of nucleic acid from the protein-nucleate prepared from the S forms was approximately 25 to 30 per cent, whereas that from the IR and R form was about 40 per cent.

Determination of Purine Nitrogen. - Samples of nucleic acid were hydrolyzed in 5 per cent (by volume) sulphuric acid for 2-1/2 hours on a hot plate at 100°C. To each sample a hot solution of silver sulphate was added until the filtrate gave no further precipitation with this reagent.

Table 3. Analytical Data on Brucella Nucleic Acids

Nucleic Acid from	Total N %	Total P %	N:P Ratio	Purine N %	Purine N: Pyrimidine N Ratio
melitensis 2194 (S)	13.11	6.25	2.23	42.1	0.76
melitensis 2425 (S)	13.12	6.91	1.90	54.0	1.14
suis 1582 (S)	12.75	7.12	1.79	44.5	0.74
abortus 230 (S)	12.19	6.35	1.92	37.7	0.60
abortus 161 (S)	13.81	6.63	2.08	32.4	0.47
abortus 85 (IR)	14.50	8.84	1.64	57.3	1.32
abortus 805R (R)	13.50	7.96	1.69	63.0	1.69
thymonucleic acid*	16.78	9.89	1.69	49.9	0.97
yeast nucleic acid*	16.32	9.64	1.69	66.7	2.00
pancreas nucleic acid°	15.72	8.49	1.85	74.7	2.95

* Calculated according to Levene and Bass ((17) pp. 263, 274)

° Levene, P. A. and Jorpes, E. J. Biol. Chem. 86, 389, 1930.

After cooling, the precipitate was centrifuged and washed twice with silver sulphate solution. Nitrogen was determined on the purine precipitate and pyrimidine filtrate by the Micro-Kjeldahl method.

From Table 3 one may note the amounts of purine nitrogen closest to that of thymonucleic acid. In going from the S - IR - R forms, one notices that the amount of purine nitrogen tends to increase. Whether this difference in the chemical composition of the nucleic acid has anything to do with the physiological characteristics of the organism is a point which should be further examined.

Isolation and Identification of Purines. The purines were isolated from *Br. abortus* (S), *Br. melitensis* (S) and *Br. abortus* (R) according to Jones' directions for the isolation of guanine and adenine (18). The guanine obtained after hydrolysis of the nucleic acid was precipitated twice, including one treatment with norit. The crude guanine obtained from the various preparations was converted into guanine hydrochloride and recrystallized from 5 per cent hydrochloric acid. For analysis the guanine hydrochloride was dried in the air.

$C_5H_5N_5O \cdot HCl \cdot 2H_2O$. Calculated, N 31.3; found, N 31.9

The filtrate from the guanine obtained in the quantitative determinations was prepared for the isolation of adenine by precipitating the purines as cuprous salts and decomposing with hydrogen sulfide. The adenine was then precipitated as the picrate. After recrystallization from 25 per cent acetic acid it melted at 286-291°C.

$C_{11}H_8N_8O_7$. Calculated, N 30.65; found, N 31.3

Although guanine and adenine were isolated and derivatives prepared, accurate weighings of each were not made. However, from the amounts weighed for the nitrogen determinations and from what was left over, one could judge easily that they were present in an approximate ratio of guanine : adenine = 1:1.

Isolation and Identification of Pyrimidines. Approximately 2 gm. samples of nucleic acid from Br. abortus (S), Br. melitensis (S) and Br. abortus (R) were hydrolyzed in an oil bath at 150-160°C. for 5 hours with 20 cc. of 25 per cent sulphuric acid. After being cooled, the sulphuric acid was removed with barium hydroxide and the purines precipitated with silver in a dilute acid solution. The pyrimidines were then removed by the addition of more silver and barium hydroxide. The resulting precipitate was decomposed with hydrogen sulfide and the filtrate from the silver sulfide concentrated. No thymine came down.

From this solution the cytosine was precipitated with picric acid. After twice recrystallizing, the picrate sintered at 256°C., and rapidly melted at 266°C.

$C_4H_5ON_3$ $C_6H_3O_7N_3$. Calculated, N 24.71; found, N 25.14

After removal of the picric acid from the cytosine picrate mother liquors, it was concentrated and even dried, but uracil did not crystallize out. Portions of the above liquid before completely drying were tested by the Wheeler and Johnson color test (19), but in every case a negative test was obtained.

The color reaction described by Harkins and Johnson (20) was performed at various intervals during the above described procedure, but in no case did it give a positive reaction. In order to make certain the absence of this pyrimidine, the following experiment was performed: 4 gms. of mixed nucleic acids was hydrolyzed with 25 per cent sulphuric acid and the pyrimidine fraction isolated as previously described. The solution was then concentrated to a very small volume and tested for thymine by the method of Harkins and Johnson. Although the test is sensitive to 2 mg. of thymine in the presence of much larger quantities of cytosine, a completely negative test was obtained.

Nature of the Sugars

Pentose content - The determination of pentose was executed employing the method described by Hoffman (21). The sample was transferred into a 500 cc. distilling flask made according to the directions of the author. 50 cc. of 20 per cent hydrochloric acid were added and distilled with a current of steam for 3 hours, always keeping the temperature of the mixture constant at 103-105°C. The distillate, collected in a measuring flask, was titrated in this flask with 10 per cent sodium hydroxide from a burette to neutrality to phenolphthalein, and the liquid was then diluted to an equal volume, in the present case 500 cc. The standard solution containing furfural approximately equal to that expected in the distillate was placed in a flask of the same size and treated with hydrochloric acid and sodium hydroxide until the solution was neutral to phenolphthalein, care being taken that the sodium hydroxide added equals the amount added to the unknown. This solution was also diluted to the mark. 6 cc. of each test and standard solution were transferred to a test tube, each was treated with 0.5 cc. aniline and 4.0 cc. of glacial acetic acid, allowed to stand for 10 to 15 minutes in the dark, and then compared in a colorimeter. The amount of pentose was calculated on the basis of the experimental data of Hoffman, in which he can recover the furfuraldehyde from guanine nucleotide 98.0 per cent, adenine nucleotide

98.0 per cent, cytosine nucleotide 4.5 per cent and uridine nucleotide 13.7 per cent.

Table 4. Pentose Content of Nucleic Acids

From Brucella	Sample mgs.	Furfural recovered mgs.	% Pentose (calculated as a tetranucleotide)	% Pentose (Calculated as a hexanucleotide)
melitensis 2194 (S)	50	2.77	15.3	12.7
melitensis 2425 (S)	50	2.95	16.7	13.8
suis 1582 (S)	20	1.22	17.3	14.3
abortus 230 (S)	50	2.80	15.9	13.2
abortus 161 (S)	40	2.40	17.0	14.0
abortus 85 (IR)	50	2.52	14.3	11.8
abortus 805R (R)	40	1.97	13.9	11.5
tetranucleotide (yeast)			20.8	
hexanucleotide				25.6

Thymonucleic Acid Content - This determination was carried out by means of the method introduced by Widstrom (22). His method is based upon the fact that when thymonucleic acid is heated with any acid it undergoes a disruption of its desoxyribose component with the formation of a genuine aldehyde, which gives a color reaction with Schiffs' fuchsin sulfurous acid reagent, and the latter coloration is applicable to the estimation of the original nucleic acid under limited conditions. 1 cc. of the solution of the 3 different sized samples which comprised 0.5 to

10 mg. of thymonucleic acid was taken in a test tube and made predominately acid to Crego Red by the addition of 0.1 N hydrochloric acid and then 10cc. of citrate buffer solution of pH 2.0 were poured into it and thoroughly mixed. Upon heating in a boiling water bath just 2-3/4 minutes, the test tube was taken out and immediately cooled in running water. The slightly insoluble material was removed by centrifugation and 9 cc. of the clear solution treated with 3 cc. of the mixed reagent composed of two volumes of citrate buffer solution as above, and one volume of Feulgens reagent. On the other hand, simultaneously a similar procedure was undertaken with 1 cc. of the standard solution. The colors, after standing sealed for 24 hours, were matched in the colorimeter.

The results are given in Table 5.

Table 5. Thymonucleic Acid Content of the Nucleic Acids

Nucleic Acid from	per cent T.N.A.
melitensis 2194 (S)	40.9
suis 1582 (S)	34.1
abortus 161 (S)	46.3
abortus 230 (S)	33.5
abortus 85 (IR)	36.3
abortus 805R (R)	22.4

Glucosamine - Glucosamine, as determined by the method of Palmer, Symthe and Meyer (16) could not be detected in any of the nucleic acids.

Comparative Test of Dissolubility in Acetic Acid

The well defined nucleic acids, namely yeast and thymonucleic acids, differ in solubility in acetic acid so much that the latter dissolves readily in concentrated acetic acid, while the former does not. In this point the present nucleic acids behave in a similar manner as a mixture of the two, in that some pentose nucleic acid precipitates out on the addition of an excess of glacial acetic acid, whereas the desoxypentose nucleic acid may be readily precipitated from the clear supernatant by the addition of 4 volumes of alcohol.

DISCUSSION

The protein-nucleates prepared from the S, IR and R strains of the species of Brucella contain relatively the same nitrogen content; the phosphorus content, however, is relatively much higher in preparations from the IR and R forms than in the S forms. This difference was also noted in the per cent of nucleic acid isolated. The nucleic acid in the smooth forms is approximately 25 to 30 per cent, whereas that obtained from the variant and rough forms is approximately 40 per cent.

By using the chloroform-gel method of separation of protein and nucleic acid, a protein which possesses specific precipitating powers is obtained. The metaprotein obtained by hydrolysis with sodium hydroxide possesses very slight precipitating power.

The protein obtained from the protein-nucleate is one exception to the common "test-book" statement that protein-nucleates or "nucleoproteins" consist of a protein, usually a histone or protamine, combined with nucleic acids. The Brucella proteins in no way resemble either protamines or histones, but do resemble many common proteins obtained from plant tissues. In Table 6 are presented comparative nitrogen partition data for some plant and bacterial protein fractions. The protein for which Johnson and Brown give analysis is that for the total protein left in the bacterial cell after lipide extraction and separation of nucleic acid; the tuberculin protein of Seibert is the protein present in a culture filtrate of Mycobacterium tuberculosis; the others are all the protein fractions isolated from the protein-nucleate.

Brucella proteins contain traces of sugars, which support the now accepted statement that all proteins have very small amounts of sugars present.

The nitrogen to phosphorus ratios of the nucleic acids check well with the data given by Thompson and Dubos (3) on nucleic acid from Diplococcus pneumoniae, Sevag et. al. (6) on the nucleic acid from Streptococcus pyogenes and with the accepted structure of thymonucleic and yeast nucleic acid. The per cent of purine nitrogen more closely approximates that of thymonucleic acid than yeast or pancreas nucleic acid. The purines guanine and adenine are found in approximately the ratio of 1:1. In the case of the pyrimidines, cytosine has been found to be present, but neither uracil nor thymine could be detected. The pentose content for the various nucleic acids ranged between 14 and 17 per cent, which is equal to about three fourths of the theoretical value for a tetranucleotide, and about one half when calculated for a hexa-nucleotide. The desoxyribonucleic acid or thymonucleic acid content,

Table 6. Comparative Analyses of Plant and Bacterial Protein Fractions

Protein	Protein from Nucleoprotein of Pea Germ		Protein of Tubercle Bacillus	Protein from Nucleoprotein of B.anthracis	Proteins from Brucella protein-nucleates			Tuberculin Protein T.P.A.*	Water-sol. fraction from Staphylococcus aureus	
Observers	Keisel and Belozersky (26)		Johnson and Brown (2)	Ferramola (4)				Seibert and Mundy (27)	Hoffstadt and Clark (28)	
	water-sol	alkali-sol			S	IR	R		S	R
Amide N			11.83	9.34	9.84	7.97	8.06	12.30	13.08	15.24
Humin N			4.11	4.08	3.64	2.00	3.91	1.80	1.08	4.35
Monoamino N			47.39	72.76	69.58	82.57	72.81	75.80	46.94	35.22
Diamino N	17.00	12.04	26.06	13.82	18.88	5.40	14.47	12.90	38.90	44.77
Arginine	9.99	4.32	10.63		5.51		4.59	7.60	11.28	11.45
Lysine	4.90	5.26	3.69		7.22		6.98		9.04	19.38
Histidine	2.11	2.46	11.48		6.15		3.10		14.67	13.57
Cystine			1.26		0.00		0.00		≁	≁

* T.P.A. = tuberculin protein prepared by means of $(NH_4)_2SO_4$

≁ Includes both lysine and cystine

determined by the Feulgen reaction varied from 22.4 per cent for the acid prepared from the R strain to as high as 46.3 per cent in one of the nucleic acids prepared from a S strain. The dissolubility of the nucleic acid in glacial acetic acid tends to point out that the nucleic acid studied is most likely a mixture of two separate nucleic acids. Further evidence of this is shown by the data for the quantity of thymonucleic acid, the fact that the pentose values are about one half of what might be expected were it a mixture of two separate nucleic rather than a single type of acid containing both pentose and desoxypentose nucleotides. However, there have been several cases where there have been reported nucleic acids from bacteria that are a single acid composed of both pentose and desoxypentose nucleotides. Both Coghill (24) and Akasi (25) have found the presence of this "mixed type" of nucleic acid in *Mycobacterium tuberculosis* and *Eberthella typhi* respectively. Since in the nucleic acids described in this study neither thymine nor uracil were detected places it neither in the so-called "animal type" (desoxypentose) nor "plant type" (pentose) of nucleic acid. However, it is reasonable to conclude from the present data that the nucleic acids are of a special type of an unknown nature, and should be placed in a class independent of other well defined nucleic acids.

SUMMARY

A method is described for the preparation of the particular *Brucella* protein-nucleate; a method for separating it into its components, protein and nucleic acid are also given.

The protein-nucleate comprises approximately 14 per cent of the total dry weight of the cells in the case of the preparations from S strains, and

about 18 per cent in the case of the IR and R strains.

The protein component, which comprises about 70 to 75 per cent of the protein-nucleate prepared from the S strains and about 60 per cent of those prepared from the IR and R strain, is characterized by a nitrogen partition (Hausmann numbers). All proteins contain traces of sugars, but no amino-sugar.

Guanine, adenine and cytosine, but no thymine or uracil, were found present in the nucleic acids. Both pentose and desoxypentose sugars were present and studied quantitatively. There is a soluble and insoluble portion when mixed with an excess of glacial acetic acid, and further data tends to show that there are two nucleic acids present; the other alternative, a nucleic acid composed of both pentose and desoxypentose nucleotides is possible but improbable.

II. BIOLOGICAL ACTIVITY OF THE PROTEIN-NUCLEATES

The studies of the biological properties of the various protein-nucleates were undertaken with the following objectives in mind: precipitability, allergic activity; toxicity in the guinea pig and antigenicity. Protein-nucleates prepared from the three strains of the smooth Brucella organism, on intermediate rough and one truly rough strain were used in these studies.

Precipitation Studies

The precipitation studies were made with specific serums prepared from an S strain of each of the three species of Brucella, one IR strain and one R strain of abortus. Normal rabbits were injected intravenously with a 1 cc. suspension of living organisms, having a density comparable to 1 on the McFarland nephelometer. When the rabbits showed good sensitivity to a skin test dose of protein-nucleate, they were bled from the heart to obtain blood for serum. The collected serums were diluted 1:1 with physiological salt solution and sterilized by passing through a Seitz filter. All precipitation tests were made in small glass vials using 0.2 cc. serum layered with 0.2 cc. of the antigen dilution. The tubes of serum and antigen dilutions were incubated at 37°C. for 2 hours and then read. It was found that if they were placed in the cold room for 24 hours after being in the incubator, the precipitates disappeared and could not be read. In Table 7 are recorded the results of the cross precipitation studies. From this table one may readily see that all three of the S preparations of protein-nucleate are non-type specific.

Table 7. The Comparative Protein-nucleate Precipitin Reactions

Antiserum from Rabbit Sensitized to	Protein-nucleate from	Dilutions of Protein-nucleate					
		1:1T*	1:2T	1:4T	1:8T	1:16T	1:32T⁑
abortus 1247 (S)	abortus 230 (S)	+	+	+	+	+	+
	suis 1582 (S)	+	+	+	+	+	+
	melitensis 9G (S)	+	+	+	+	+	+
	abortus 85 (IR)	-	-	-	-	-	-
	abortus 805R (R)	-	-	-	-	-	-
suis 1630 (S)	abortus 230 (S)	+	+	+	+	+	+
	suis 1582 (S)	+	+	+	+	+	+
	melitensis 9G (S)	+	+	+	+	+	+
	abortus 85 (IR)	-	-	-	-	-	-
	abortus 805R (R)	-	-	-	-	-	-
melitensis 2414 (S)	abortus 230 (S)	+	+	+	+	+	+
	suis 1582 (S)	+	+	+	+	+	+
	melitensis 9G (S)	+	+	+	+	+	+
	abortus 85 (IR)	-	-	-	-	-	-
	abortus 805R (R)	-	-	-	-	-	-
abortus 85 (IR)	abortus 230 (S)	-	-	-	-	-	-
	suis 1582 (S)	-	-	-	-	-	-
	melitensis 9G (S)	-	-	-	-	-	-
	abortus 85 (IR)	-	-	-	-	-	-
	abortus 805R (R)	-	-	-	-	-	-
abortus 805R (R)	abortus 230 (S)	-	-	-	-	-	-
	suis 1582 (S)	-	-	-	-	-	-
	melitensis 9G (S)	-	-	-	-	-	-
	abortus 805R (R)	-	-	-	-	-	-
	abortus 85 (IR)	-	-	-	-	-	-

*T = Thousand
⁑ = All precipitin reactions were negative at 1:64T

This result and the results with the intermediate rough strain check with those of Topping (8) and former studies in this laboratory (9,10).

An experiment was performed to determine the effect of pH and aging on the stability of a protein nucleate prepared from a smooth strain, Br. abortus 230. The protein-nucleates were made up and stored in bottles in the cold room at pH's 3.5, 4.0, 5.0, 6.0, 7.0 and 8.0. Samples were withdrawn at the end of 1, 2, 3, 5, 7 and 11 months and tested for their precipitating ability against an abortus goat antiserum. There was only a slight, if any, deviation from the original precipitating power. Thus we find that pH and age do not appear to affect the stability of protein-nucleate solutions, at least up to the end of eleven months.

Allergic Activity of the Protein-nucleates

The comparative allergic activities and cross reactions of Brucella protein-nucleates were determined on sensitized rabbits. Normal rabbits were sensitized by an intravenous injection of a 1 cc. suspension of living Brucella cells suspended in sterile saline solution. The turbidity was made to compare with tube 3 of the McFarland nephelometer. This procedure will produce a high degree of skin reactivity in 30 days for measuring quantitatively the allergic activities of the various Brucella protein-nucleates. Each rabbit received an intradermal injection of 0.1 cc. of progressive dilutions of protein-nucleate ranging from 1:1,000 to 1:32,000. The intradermal reactions were observed at 24 and 48 hour intervals. The reactions were recorded on the forty-eighth hour, although they frequently persisted as long as five days.

In Table 8 are recorded the collective results of several repeated experiments designed to show whether there were possible quantitative differences in the allergic activity of the various protein-nucleates against rabbits sensitized to the five cultures of Brucella.

The allergic titers of the protein nucleates from the three S strains show approximately the same degree of response in the animals sensitized to the S strains. The protein-nucleates used on its homologous sensitized rabbit elicits a slightly higher titer with the exception of *Br. suis*. The *suis* preparation was found to have less biological activity in all studies made on it than the *Br. abortus* or *Br. melitensis* protein-nucleates. The IR strain provokes a reaction to a small degree in all the sensitized rabbits with the exception of the one sensitized to the very rough strain. The protein-nucleate prepared from the R strain induces no allergic response in any of the rabbits sensitized to the S or IR strains, but elicits a feeble reaction in the rabbit sensitized to the R strain.

Antigenicity of the Protein-nucleates

In this experiment guinea pigs were used to study the ability of the protein-nucleates to provoke antibodies. The rapid agglutination test was used to measure agglutinin production; the phagocytic system previously described (12) was used to measure opsonin production. The pigs were given an intraperitoneal dose and bled on the tenth day after injection and examined for antibodies.

It has been found that antibody production was moderately stimulated in 19 out of 20 guinea pigs with 10 mgs. of antigen injected intraperitoneally.

Table 8. The Comparative Allergic Activity of the Protein-nucleates.

Rabbit Sensitized to	Protein-nucleate	Skin Reactions of Dilutions of Protein-nucleate					
		1-1T	1-2T	1-4T	1-8T	1-16T	1-32T
abortus 230 (S)	abortus 230 (S)	12mm.	12mm.	10mm.	10mm.	7mm.	5mm.
	suis 1582 (S)	8mm.	8mm.	6mm.	5mm.	5mm.	3mm.
	melitensis 9G (S)	8mm.	5mm.	5mm.	3mm.	2mm.	2mm.
	abortus 85 (IR)	5mm.	5mm.	4mm.	2mm.	-	-
	abortus 805R (R)	-	-	-	-	-	-
suis 1582 (S)	abortus 230 (S)	20mm.	15mm.	10mm.	7mm.	3mm.	-
	suis 1582 (S)	12mm.	10mm.	5mm.	5mm.	3mm.	-
	melitensis 9G (S)	12mm.	10mm.	7mm.	5mm.	3mm.	-
	abortus 85 (IR)	5mm.	4mm.	3mm.	-	-	-
	abortus 805R (R)	10mm.	-	-	-	-	-
melitensis 2414 (S)	abortus 230 (S)	10mm.	9mm.	9mm.	7mm.	5mm.	2mm.
	suis 1582 (S)	8mm.	6mm.	5mm.	5mm.	3mm.	-
	melitensis 9G (S)	12mm.	12mm.	10mm.	10mm.	5mm.	4mm.
	abortus 85 (IR)	6mm.	5mm.	4mm.	3mm.	-	-
	abortus 805R (R)	10mm.	9mm.	-	-	-	-
abortus 85 (IR)	abortus 230 (S)	4mm.	-	-	-	-	-
	suis 1582 (S)	4mm.	-	-	-	-	-
	melitensis 9G (S)	3mm.	-	-	-	-	-
	abortus 85 (IR)	5mm.	4mm.	-	-	-	-
	abortus 805R (R)	4mm.	-	-	-	-	-
abortus 805R (R)	abortus 230 (S)	-	-	-	-	-	-
	suis 1582 (S)	-	-	-	-	-	-
	melitensis 9G (S)	-	-	-	-	-	-
	abortus 85 (IR)	-	-	-	-	-	-
	abortus 805R (R)	5mm.	-	-	-	-	-

It is interesting to note that the protein-nucleate prepared from the R strain very mildly stimulated opsonins and agglutinins and correspondingly, as we shall see, 3 out of 5 guinea pigs showed traces of antibody after injection with the protein obtained from the homologous protein-nucleate.

Toxicity of the Protein-nucleates

The toxic property of each preparation of protein-nucleate was arrived at by injecting guinea pigs, weighing from 300 to 450 grams, intraperitoneally with one to four cc. of the preparations, containing definite amounts measured on a dry weight basis. The criteria of toxicity are based on those observed by Pennell and Huddleson (23) when using preparations of endo-antigen:

"The temperature of the animal begins to drop after the second hour, and may continue to do so until death occurs, or the temperature may rise again to normal if the injection is not fatal. As the temperature drops, the animal becomes nervous and irritable. The hair on the back becomes roughened, and the abdomen may become very tense and distended."

However, of 15 pigs inoculated with varying amounts of protein-nucleate, only one showed a drop of more than 2°C. in temperature. The results would indicate that the protein-nucleate preparations are non-toxic.

Biological Activity of the Protein and Nucleic Acid

Each of the constituents obtained from the protein-nucleate, namely, the protein and nucleic acid has been examined for its ability to precipitate specific serum, allergic activity, for toxicity and for antigenicity.

The comparative precipitating power of the original protein-nucleate and its isolated components is shown in Table 9. These were all performed at the same time using a Br. abortus (S) goat antiserum. The results point to the fact that practically all the precipitating power lies in the protein portion of the protein-nucleates, and that the nucleic acid shows very low precipitating power and may be attributed to traces of

Table 9. Comparative Precipitin Titers of Protein-nucleate, Protein and Nucleic Acid*

	Precipitin Titer of Antigen		
	protein-nucleate	protein	nucleic acid
abortus 230 (S)	1:32,000	1:32,000	1:1,000
abortus 161# (S)	1:64,000	1:64,000	1:2,000
suis 1582 (S)	1:16,000	1:16,000	1:1,000
melitensis 2194 (S)	1:16,000	1:16,000	1:1,000
melitensis 2425 (S)	1:32,000	1:8,000	1:1,000
abortus 85 (IR)	-	-	-
abortus 805R (R)	-	-	-

*Prepared by method of Heidelberger and Kendall (11).

abortus goat antiserum was used in all titrations.

protein which have not been completely separated from the nucleic acid.

Allergic Activity of the Protein and Nucleic Acid

Allergic activity was determined in the same manner as previously described in this paper. The results are set forth in Table 10.

Table 10. Comparison of Allergic Activity of Protein-nucleate, Protein and Nucleic acid

Rabbit Sensitized to		Skin Reaction of Dilution of					
		1-1T*	1-2T	1-4T	1-8T	1-16T	1-32T
abortus 230 (S)	abortus 230 (S) protein-nucleate	12mm.	10mm.	10mm.	8mm.	5mm.	5mm.
	abortus 230 (S) protein	10mm.	9mm.	6mm.	5mm.	3mm.	3mm.
	abortus 230 (S) nucleic acid	-	-	-	-	-	-
	melitensis 2425 (S) protein	8mm.	8mm.	8mm.	5mm.	5mm.	-
	melitensis 2425 (S) nucleic acid	-	-	-	-	-	-
	melitensis 2194 (S) protein	10mm.	9mm.	8mm.	8mm.	5mm.	5mm.
	melitensis 2194 (S) nucleic acid	-	-	-	-	-	-
	suis 1582 (S) protein	12mm.	8mm.	6mm.	4mm.	2mm.	-
	suis 1582 (S) nucleic acid	-	-	-	-	-	-
	abortus 85 (IR) protein	4mm.	-	-	-	-	-
	abortus 85 (IR) nucleic acid	-	-	-	-	-	-
	abortus 805R (R) protein	-	-	-	-	-	-
	abortus 805R (R) nucleic acid	-	-	-	-	-	-

*T = thousand

It is quite evident from the results that practically all the allergic activity lies in the protein, and that the nucleic acid portion of the protein-nucleate plays no part in provoking an allergic reaction.

Antigenicity of the Protein and Nucleic Acid

In the case of the protein-nucleates, all but those prepared from the R strain showed highly antigenic powers, the R strain only very feebly eliciting antibodies.

Thirty guinea pigs were injected intraperitoneally with varying doses (5 to 25 mgs.) of the protein fraction. Of these, 14 showed marked antigenic powers, 11 showed a moderate stimulation. The protein from the R strain produced a very feeble response in the case of three pigs and none whatsoever in the other two. Thus the power of the protein to elicit antibodies closely parallels that of the protein-nucleates.

Seventeen guinea pigs were used to study the antigenicity of the n nucleic acids. Thirteen pigs showed no opsonins or agglutinins while four produced a very feeble opsonin response, but no agglutinins could be detected.

From the above study we have concluded that all the antibody stimulating power is present in the protein portion of the protein-nucleate, and that the nucleic acid plays no part.

Toxicity of the Protein and Nucleic Acid

Toxicity was again determined by the method already mentioned. In the case of the protein fraction, 22 guinea pigs were subjected to varying doses of all five of the different proteins. Of the 22, only 3 showed any symptoms of toxicity. The three showing toxic symptoms were each out of three different groups, each of which was injected with a different preparation. The isolated instances of toxicity could not be explained.

In the case of the nucleic acid fraction 15 guinea pigs were used. All injected pigs remained normal. Thus we may conclude that neither the protein nor nucleic acid possesses toxic properties.

DISCUSSION

Precipitation tests on the protein-nucleates indicate that the S strains are non-type specific in nature, and that those from IR and R strains do not have precipitating powers. The same type of results was obtained on the protein portion of the protein-nucleates. The nucleic acid component is inactive, thus the total biological activity resides in the protein portion of the protein-nucleate. These results are much in accord with those of Sevag et. al., in that a nucleic acid prepared from streptococcal "nucleoproteins" by hydrolysis with sodium carbonate were 'serologically inactive;' that the protein portion 'reacted with streptococcal antisera in precipitation and complement fixation tests'. However, they state that 'further work is in progress to clarify the serological specificity of these fractions.' Similar results were obtained by Ferramola (4) in his study of _B. anthracis_.

In comparing the allergic activities of the protein-nucleates there was found to be very little, if any type specificity. The IR and R strain sensitized rabbits reacted feebly to any of the protein-nucleate preparations. Again, the protein component shows all of the allergic activity while the nucleic acid shows none.

In comparing the allergic activities of the protein-nucleates there was found to be very little, if any type specificity. The IR and R strain sensitized rabbits reacted feebly to any of the protein-nucleate preparations. Again, the protein component shows all of the allergic activity while the nucleic acid shows none.

Both the protein-nucleate and its protein component moderately stimulate the production of antibodies, whereas the nucleic acid has no such power. The protein-nucleates, protein and nucleic acids were found to be non-toxic.

SUMMARY

The results of precipitation studies with the protein-nucleates and protein components from S strain preparations show that they are not type-specific, and that the nucleic acid possesses no precipitating power. The protein-nucleate and protein component from the IR and R strain reacted only slightly with homologous and heterologous antiserum. Their nucleic acids also possessed no precipitating power.

The protein-nucleate and protein component elicit non type-specific reactions in Brucella sensitized rabbits. The IR and R strain preparations elicit only a slight allergic reaction.

The protein-nucleate, protein and nucleic acid were found to be non-toxic.

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