

A STUDY OF THE CHEMICAL CONSTITUTION AND BIOLOGICAL PROPERTIES
OF THE ENDO-ANTIGEN OF THE BRUCELLA GROUP OF MICRO-ORGANISMS

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A STUDY OF THE CHEMICAL CONSTITUTION AND BIOLOGICAL PROPERTIES
OF THE ENDO-ANTIGEN OF THE BRUCELLA GROUP OF MICRO-ORGANISMS

Thesis

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Chemical Constitution and Biological
Properties of the Endo-antigen
of the Brucella Group of Micro-
organisms.*

Chemical fractionation of the bacterial cell in order to obtain pure and suitable antigens has received considerable attention during the last few years. It has already been demonstrated that chemically defined and reproducible antigens separated from the bacterial cell serve as better immunizing agents, and furnish more suitable materials for the study of antigen-antibody systems and bacterial allergy than do the intact bacterial cells.

Considerable progress has already been made in the chemical fractionation of Brucella cells. In previous studies (1), (2), two significant highly antigenic fractions were separated which from preliminary investigations appeared to be of sufficient importance to warrant a further and more detailed chemical and biological study. The isolation of one of those, the B-substance, has recently been confirmed by Schapira (3). Although it is neither carbohydrate nor protein, its antigenic activity appears

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similar to that which is delegated to the specific polysaccharides in many other organisms. The albuminoid fraction from Brucella cells, a combination of the S-substance with a protein-like group, exhibits in addition to the precipitation of immune sera, the property of being extremely toxic for normal guinea pigs upon intraperitoneal injection. This same toxic property upon injection into normal guinea pigs is also exhibited by a dense suspension of the intact bacterial organisms.

The symptoms and signs shown by the guinea pig following the intraperitoneal injection of a toxic dose of the albuminoid, of the more highly purified endo-antigens or of a dense suspension of intact cells begin to appear about two hours after injection. The animal remains quiet, its coat becomes roughened, and it hangs its head in a state of stupor. At the fourth hour the animals show signs of peritoneal irritation characterized by rigidity and spasms of the abdominal muscles. The head is momentarily retracted on the back. Later the spasms involve other muscles of the body, causing the animal to jump upward when disturbed. If the reaction continues for 8 to 10 hours an opaque exudate containing neutrophiles collects in the left eye. The leukocyte count falls to 2,000, chiefly due

to the disappearance of neutrophiles from the peripheral blood.

The administration of a dose which produces no lowering of the temperature raises the leukocyte count, often to 20,000, the increase in count being due chiefly to an increase in the neutrophiles.

The temperature begins to fall from 1 to 2 degrees the second hour after injection, and continues downward until the animal's death, preceding which a temperature of 34° to 32°C. is noted.

Death occurs from 6 to 18 hours after injection depending upon the toxicity of the material. The temperature never rises more than 2 degrees above normal following the administration of a non-lethal dose. At autopsy, the only gross changes noted are a small amount of clear viscous exudate containing lymphocytes and neutrophiles in the peritoneal cavity, petechial hemorrhages in the parietal peritoneum and extensive hemorrhages in the mucosa of the stomach and in the intestines. The reactions in the guinea pig are similar to if not identical with those described by Vaughan and Vaughan (4) in their studies of bacterial poisons.

The S-substance was reported to stimulate no anti-body production, to elicit non-specific skin reactions and to precipitate specific serums. The albuminoid, on the other hand, was found to

produce specific antibodies in normal animals and specific skin reactions upon intradermal injection into sensitized subjects.

Gwatkin (5) has described the preparation of an alcohol precipitate prepared from an aqueous extract of moist Brucella organisms, as well as an extract involving autolysis of dried and ground Brucella cells. These preparations were found extremely toxic for normal guinea pigs. The alcohol precipitate, characterized by qualitative tests only, was found to be polysaccharide in nature. In work reported previously from this laboratory (1) it has been shown that precipitates and extracts prepared in a manner similar to those used by Gwatkin contain a variety of components which may be separated by further chemical treatment. It was thus believed that some further purification of the toxic principle should be possible.

The early work on the toxic fraction indicated that it was liberated in a soluble form in the course of autolysis of the bacterial cells. Autolysates prepared at varying pH and under a variety of conditions were subjected to fractionation by dialysis, alcohol precipitation and by tri-chloro acetic acid precipitation. Many of the fractions obtained exhibited toxicity for normal guinea pigs.

The fractions were, however, chemically ill-defined and constituted no improvement of the previously obtained albuminoid. This work was further complicated by the fact that occasionally autolysates were obtained which were non-toxic. Extracts of ground bacterial cells in N/2 NaOH, fractionated by various means, gave results similar to those obtained by autolysis.

Digestion of ground Brucella cells at pH 8 with trypsin and erepsin and thorough centrifugation of the undigested residue furnished extracts that were more constant in their toxicity than those obtained by the first methods used. Fractionation of the digests by alcohol precipitation provided little of interest. Precipitation with phosphotungstic acid, basic lead acetate and with silver nitrate, and decomposition of the precipitates yielded a biologically significant oil-like constituent which in large doses produced a typical toxic reaction and ⁱⁿ addition produced an immediate but temporary respiratory paralysis. This fraction was not antigenic.

In view of the close similarity between the toxic property of Brucella extracts and that of Vaughan and Vaughan's⁽⁴⁾ "soluble protein poison" which was prepared from a variety of proteins,

their chemical separation procedure was carried out on Brucella cells. A fraction was obtained similar in nature to their preparations which produced, upon intraperitoneal injection into normal guinea pigs, a toxic reaction entirely analogous to that given by injection of the albuminoid or of whole organisms. The fraction however, had no antigenic properties whatsoever.

The isolation of a toxic antigenic fraction from *Salmonella aertrycke* by Raistrick and Topley (6) and by Boivin, Mesrobianu and Mesrobianu (7) as well as from a number of other organisms by the latter authors, suggested the application of their procedures to the Brucella group. A modification of the method of Boivin and collaborators yielded a product which was found to be extremely toxic and at the same time a specific and highly active antigen. This product, moreover, was found similar to if not identical with the S-substance, which implies that there is a close connection between the new fraction and the albuminoid previously discussed.

Isolation of the Endo-antigen

The endo-antigen was prepared from a number of strains of Brucella abortus, Brucella melitensis and Brucella suis in the stock collection maintained in this laboratory. The dried cells were

prepared in a manner similar to that described in a previous publication (1). The dried organisms were ground in a ball mill for 7 days. The resulting fine powder was suspended in sufficient distilled water to make a suspension of 5 per cent by weight. Toluene was added at the same time with the water. The suspension was digested under toluene by 0.5 per cent trypsin and 0.5 per cent Na_2CO_3 at 37°C . for two and one-half days. At the end of this time 0.5 per cent trypsin was again added and digestion was allowed to proceed again for two and one-half days. To the tryptic digest suspension was added tri-chloroacetic acid to a dilution of N/4. At the end of three hours the mixture was neutralized and submitted to dialysis in running tap water, followed by similar treatment in distilled water until dialysis was complete. The dialyzed suspension was concentrated to as small a volume as was practicable by distillation at 40°C . in vacuo. The resulting concentrate was precipitated with 10 volumes of alcohol containing a few drops of glacial acetic acid. The precipitate was centrifuged and resuspended in a small volume of distilled water and again precipitated with alcohol, which procedure was repeated 5 times, or until the supernatants contained no further extractable material.

The final alcohol precipitate represents the endo-antigen. The yield in a large number of such preparations has varied from 15 to 30 per cent of the dry weight of the bacterial cell; the average for all preparations being roughly 25 per cent. The endo-antigen forms unstable acidic suspensions in distilled water. These suspensions may be converted into deeply opalescent solutions by adding N/1 NaOH until no increase in clarity is observed. The liquid may then be adjusted to pH 7 with no increase in turbidity. The preparations from Br. abortus, Br. melitensis and Br. suis are grossly similar.

The similarity of this product to the earlier mentioned albuminoid is shown in that suspensions of the finely ground albuminoid in distilled water, digested with trypsin and treated as above, yield preparations exactly analogous to those obtained from the pulverized whole cell.

Chemical Nature of the Endo-antigen.

The endo-antigen gives a positive, though very slow reaction in the Molisch test for carbohydrates; a positive reaction with Bial's test for pentoses; a negative reaction to the di-phenylamine test for ketoses; positive reactions to the biuret test for the peptide-

linkage and Millon's test for tyrosine; a very faint reaction in the Rosenheim test for tryptophane.

The nitrogen content of the endo-antigen (micro-Kjeldahl method) is from 5 to 10 per cent of the total weight. Amino nitrogen (method of Van Slyke) is absent. There are no reducing sugars present as such. After hydrolysis with dilute hydrochloric acid, however, reducing sugars calculated as glucose (Schaffer-Hartmann procedure) have been found to comprise from 4 to 12 per cent of the fraction. Phosphorus and sulphur are not present. Determinations of acetyl groups by the method of Kuhn and Roth (8) give an average value of 6 per cent. Acetic acid has not been isolated in this procedure and, because of data to be considered later, it has seemed possible that this value may represent some other volatile acid produced by the hydrolysis employed in the procedure.

A typical analysis of endo-antigen is given. This particular preparation was obtained from cells of Br. melitensis strain 606.

Total N: 9.403 per cent

Amino N: Absent

Reducing sugars before hydrolysis: Absent

Reducing sugars after hydrolysis: 5.93 per cent

Sulphur: Absent

Phosphorus: Absent

Acetyl groups: 3.48 per cent.

At this point it is perhaps well to consider a similar examination of a sample of S-substance prepared from the same strain of organisms as was the above endo-antigen. The S-substance gives a weekly positive Molisch reaction upon standing; the biuret reaction is a light pink; the Rosenheim test is negative; with Bial's reagent the reaction is positive but much weaker than that given by the endo-antigen. Millon's reaction ^{is} ~~was~~ weakly positive. The analysis of the S-substance is as follows:

Total N: 5.86 per cent

Amino N: Absent

Reducing sugars before hydrolysis: Absent

Reducing sugars after hydrolysis: 8.13 per cent.

Sulphur: Absent

Phosphorus: Absent

Acetyl groups: 48.43 per cent.

It will be seen that the data for the endo-antigen and those for the S-substance correlate closely with the exception of the acetyl content. The probably meaning of the high figure for the acetyl content of the S-substance will be considered later. The analytical data for the S-substance differ from those previously presented in that reducing sugars are found present after hydrolysis and the biuret test is found weakly positive. This latter reaction, checked several times to assure its authenticity, is very weak and might be easily overlooked. The reducing sugars upon hydrolysis are detectable only by the use of a quantitative reagent and do not afford reduction sufficient for visible detection.

Acetylation of the Endo-antigen

Since the endo-antigen presumably contained determinable acetyl groups, the importance attributed to the acetyl groups of the specific polysaccharide of *Pneumococcus* Type 1 (9) suggested a study of the effect of acetylation upon this substance. A

portion of endo-antigen prepared from cells of Br. abortus strain 1151, was precipitated with acid alcohol and the precipitate packed tightly by centrifugation. The precipitate, still moist with alcohol, was suspended in acetic anhydride containing a small amount of freshly fused sodium acetate. The mixture was warmed to start the reaction and was then heated in a boiling water bath for one-half hour. At the end of this time the mixture was neutralized, then dialyzed in running tap water and in distilled water until dialysis was complete. The acetyl content of the endo-antigen before acetylation was 2.23 per cent, after acetylation, 7.74 per cent.

Because of the interesting effect of the above treatment on the biological properties of the endo-antigen, to be discussed later, a second preparation which showed little biological activity from the same strain of organisms, was acetylated. Acetyl content was 3.17 and 3.57 per cent before and after acetylation respectively.

The method used in determining acetyl groups involved saponification in methyl alcoholic NaOH. A number of the solutions left after distillation of the acid from the saponification mixtures were

collected, reduced in volume and precipitated with alcohol containing glacial acetic acid. This precipitate was tested biologically and was then acetylated and retested. The acetyl content, which was assumed to be zero after saponification, increased to 29.03 per cent after acetylation.

For comparison with the endo-antigen a sample of S-substance was also acetylated. Its apparent acetyl content increased from 48.43 per cent before, to 68.19 per cent after acetylation.

Felton and Prescott (10) have shown it possible to restore completely antigenicity to the S-substance of *Pneumococcus* Type 1 after removal of the acetyl groups, by allowing it to stand at 4°C. in 25 per cent NH_4OH . In view of their remarkable findings, a portion of endo-antigen from *Br. melitensis* strain 748 was heated for 1/2 hour in N/2 NaOH at 86° C. Upon cooling, the suspension was precipitated with alcohol without neutralization. One portion of this precipitate was acetylated and one portion was suspended in 25 per cent NH_4OH and allowed to stand overnight in the icebox.

It was then precipitated with alcohol and ether, resuspended in distilled water and reprecipitated. In Table 1 is presented the chemical data obtained for the three preparations. It may be noticed that treatment with NH_4OH had no influence upon the N content of the material. It is also apparent that heating with $\text{N}/2 \text{ NaOH}$ for one-half hour reduced both the nitrogen content and the reducing sugars determinable upon hydrolysis. The acetyl content on the other hand shows an unexpected increase by this treatment. The effects of those procedures on the biological properties of the endo-antigen are considered in a later portion of the paper.

Table 1

Chemical Properties of an Endo-antigen
Treated with N/2 NaOH, Acetylation
and NH₄OH

	Per cent of total N	Per cent of Reducing Sugars After Hydrolysis	Per cent of Distillable Acid Formed in Acetyl Determination
Endo-antigen from Br. melitensis strain 748	9.35	4.53	3.34
Endo-antigen heated 1/2 hr. at 80°C. in N/2 NaOH	7.00	3.37	6.05
Endo-antigen acetylated after heating in N/2 NaOH	5.34	3.22	14.85
Endo-antigen treated with NH ₄ OH after heating in N/2 NaOH	6.88	2.52	6.84

Table 2

Acetyl Content of Several Preparations
Before and After Acetylation

	Per cent of Distillable Acid Formed in Acetyl Determination	
	Before Acetylation	After Acetylation
Endo-antigen from Br. abortus str. 1151	2.28	7.74
Inactive endo-antigen from Br. abortus str. 1151	3.17	3.54
Material recovered after saponification in the acetyl determination	0.00	29.03
3-substance from Br. melitensis str. 306	48.43	68.19

Data showing the effect of acetylation on the acetyl content of several preparations are presented in Table 2. That the acetylation procedure may have some effect on the endo-antigen other than addition of acetyl groups is suggested by the one experiment in which the acetyl content of the endo-antigen was unchanged by the process although its biological properties were found to have increased. The acetyl content of the re-acetylated product, which can hardly entirely represent acetyl groups, suggests that acetylation has brought about an hydrolysis, forming some distillable acid other than that representing acetyl groups. The product is known to have been partially broken down already by saponification. This explanation is borne out by the acetyl contents of the products obtained when the endo-antigen was heated in $N/2$ NaOH and treated with NH_4OH , both of which should have been acetyl-free. The extremely high figures obtained for the S-substance would, by analogy, suggest the S-substance to be a partially hydrolyzed endo-antigen. Final analysis of the effect of acetylation must await identification of the products obtained in the acetyl distillation.

Acetone Extraction

When the endo-antigen was precipitated with alcohol containing

a few drops of glacial acetic acid, and the resultant centrifuged precipitate was thoroughly extracted with acetone containing a few drops of 5N HCl, an intensely yellow extract was obtained. No pigment was extracted by acetone except in the presence of HCl which seemed to act as a catalyst. After distillation of the acetone from the extract there remained an orange-brown, semi-solid mass with a peculiar sweetish-pungent odor. Nitrogen was absent. When submitted to saponification by refluxing for four hours in 4 per cent alcoholic KOH, the material formed a deep reddish-brown solution. Upon cooling and diluting the saponification mixture with three volumes of distilled water, ether extraction removed much of the pigment. After distillation of the ether from this extract there remained a yellow oily material having a spicy fragrance. The small quantities of this material which have been obtained in a pure state have precluded its adequate characterization. The compound gives the characteristic red color given by ketones and aldehydes with diazo-benzene sulphonic acid. This, together with its physical properties and its spicy odor, suggest it to be a di-ketone. It was shown to be responsible for the brilliant red ring sometimes encountered in testing the endo-antigen with Molisch reagent.

shown to be responsible for the brilliant red ring sometimes encountered in testing the endo-antigen with Molisch reagent.

Acidification of the aqueous soap solutions and extraction with ether yielded, upon distillation of the ether, a slightly pigmented fatty acid. The fatty acid was added to that obtained by subsequent ether extraction of the endo-antigen after preliminary examination had shown their similarity.

In a single experiment the aqueous solutions remaining after the removal of the unsaponifiable and the fatty acid fractions were examined. From these were obtained a suggestion of the presence of polysaccharides consisting of a slowly developing Molisch reaction and 2.53 per cent of reducing substance calculated as glucose. No polysaccharides were isolated and no osazones could be obtained. There was no trace of glycerol or of any other alcohol.

Hydrolysis of the acetone extract with a 1 per cent solution of H_2SO_4 in alcohol was attempted in a single experiment. The orange-brown semi-solid was recovered unchanged.

That this acetone extract was not entirely extraneous matter merely accompanying the endo-antigen is indicated by the fact that intraperitoneal injection into normal guinea pigs proved it to be

midly antigenic. Doses of from one to five milligrams were found to elicit agglutinins and opsonins in low titers for Brucella cells.

Ether Extraction

Further material was removed from the acetone extracted endo-antigen by thorough extraction with ether. The ether extract was nitrogen-free. To these ether extracts, in many instances, were added the ether soluble fatty acid obtained upon saponification of the acetone extract. Upon removal of the ether by distillation there remained a straw-colored waxy residue. This residue was saponified by refluxing for 4 hours in 4 per cent alcoholic KOH. The unsaponifiable fraction consisted of a minute amount of the fragrant yellow oil obtained in the acetone extract. The aqueous soap solutions were acidified and thoroughly extracted with ether. The ether extract was washed with distilled water until free from acid. It was then dried over Dehydrite and reduced to a small volume by distillation. Quantities of white stocky needles were obtained having an odor resembling tar soap. After crystallization they were found to have become less soluble in ether but were soluble in acetone. The fatty acid was saturated and quickly became liquid at 25° C. and atmospheric pressure. No further study of this compound

has been made. Its resemblance to the acetone soluble, saturated, liquid fatty acids first noted by Anderson (11) in his studies of the lipides of the tubercle bacillus is obvious.

Examination of the aqueous solutions remaining from saponification was undertaken in a single experiment. There was a suggestion of polysaccharide in a weakly positive Molisch reaction. There was no trace of glycerol. There was obtained, however, a small amount of crystalline material which was benzoylated by the Schotten-Baumann reaction. Saponification of the benzoylated product and back titration suggested an alcoholic nature for the crystals.

Intraperitoneal injection of the ethereal fraction had no antigenic or toxic effects. This fraction may represent extraneous material accompanying the endo-antigen. It is present, however, even when the endo-antigen is prepared from previously defatted cells.

The acetone and ether fractions together, represent from 10 to 15 per cent of the total weight of the endo-antigen. In the particular separation just discussed, these two extractions removed 13.4 per cent of the total weight of the endo-antigen.

Acetone-Ether Extracted Endo-antigen

The endo-antigen, which was usually an amber color in solution or a gray powder when dried, became white after the two extractions. Upon suspension in distilled water it became a salmon-pink in color. The resuspended material was found to be acidic and quite insoluble. After neutralization, it became partially soluble and suffered no loss in its biological properties. Further addition of N/1 NaOH brought about further solution of the material, but was found, in contrast to the findings before extraction, to reduce the biological properties of the fraction.

The extracted endo-antigen still gave similar reactions to all the qualitative tests to which it had previously been subjected. The Millon's test for tyrosine was much enhanced, while the Rosenheim reaction for tryptophane had become extremely faint and even absent in some cases. In comparison to the unextracted endo-antigen, the nitrogen content was slightly higher, running from 8 to 12 per cent, while sugars determinable as glucose upon hydrolysis were lower, 3 to 8 per cent. values for acetyl group content varied from 5 to 10 per cent

The chemical constitution of the acetone-ether extracted endo-antigen from Br. melitensis strain 606 is as follows:

Total N: 12.0 per cent

Amino N: Absent

Reducing sugars before hydrolysis: Absent

Reducing sugars after hydrolysis: 4.0 per cent

Acetyl groups: 5.42 per cent

After lipid extraction, a series of tests was employed to identify the nitrogenous compounds. The analysis indicated the presence of amino acids or amino-sugars and of pyrrol rings only.

Boivin and co-workers (12) reported the splitting of the antigen obtained from Salmonella aertrycke by hydrolysis in dilute acetic acid into toxic non-specific and non-toxic specific fractions. Portions of extracted endo-antigen were hydrolyzed by refluxing for one hour in N/5 acetic acid, one hour in N/2 acetic acid, one hour in N/2 HCl, one hour in N/1 HCl, eight hours in N/2 sulfuric acid, one hour in N/4 NaOH and one hour in N/2 NaOH. The results were very unsatisfactory. A number of fractions were obtained, each of which was an amorphous powder containing polysaccharide and nitrogen, and many of which retained some part or all of their biological activity. From these experiments it was learned that only hydrolysis for one hour in N/5 acetic acid had no material effect on the biological properties of the endo-antigen. There was noted a gradual destruction

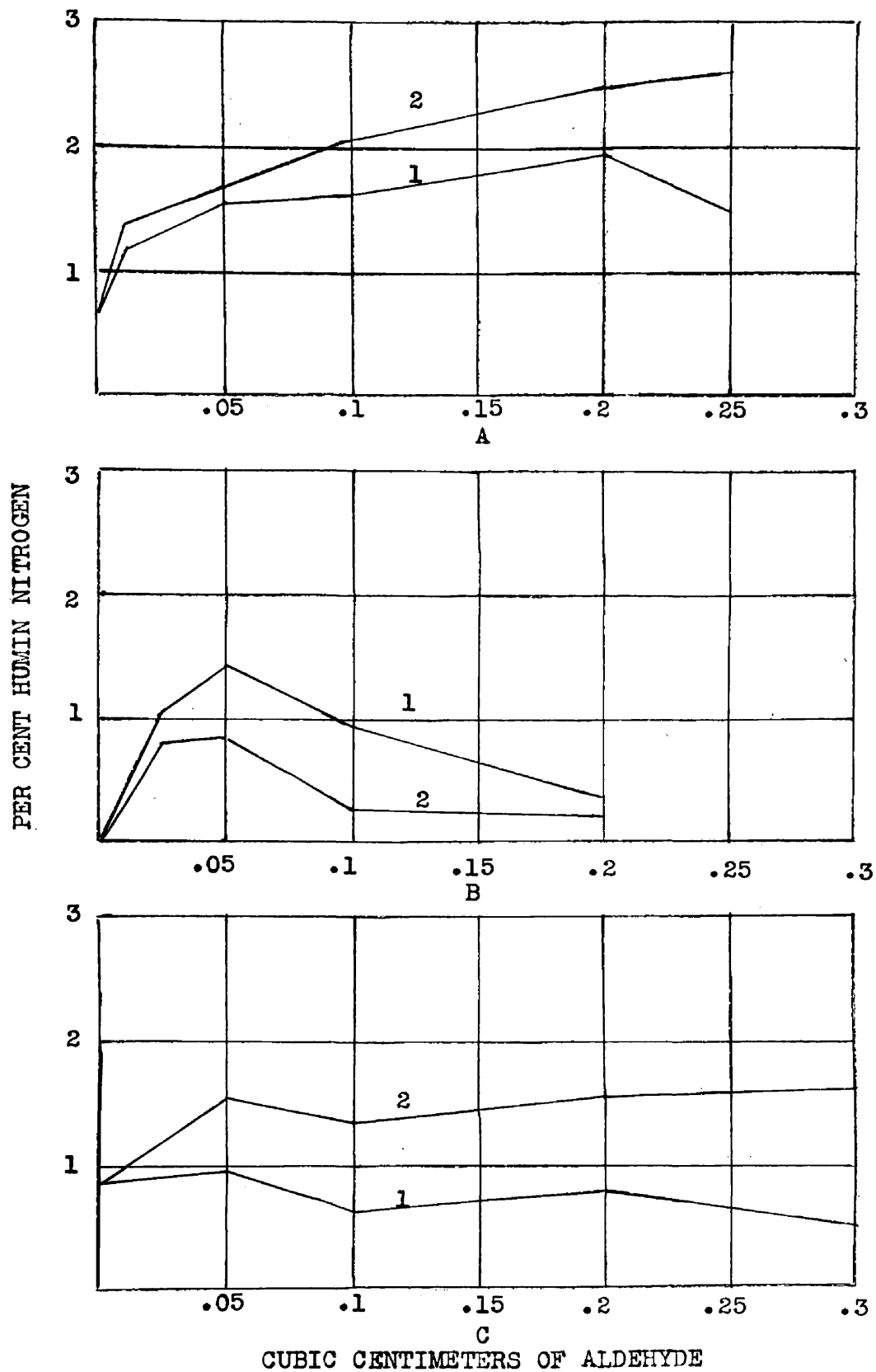
of these properties as the strength of the acid and the length of hydrolysis was increased. After eight hours in N/1 sulphuric acid the hydrolytic products showed only slight precipitation with specific serum. On the other hand, hydrolysis with N/4 or with N/2 NaOH for one hour completely destroyed the biological activity of the fraction.

An attempt was made to isolate the polysaccharide constituent of the endo-antigen by the method used by Fränkel and Jellinek (13) in the isolation of the polysaccharide from egg albumin. Hydrolysis for three hours in 10 per cent barium hydroxide in flowing steam failed to liberate any free polysaccharide. Attempts to produce osazones failed. The modification method of Levene and Mori (14) entailing longer hydrolysis produced similar results.

The repeated production of a pink to light lavender color upon the addition of acids, particularly of sulphuric acid, to the extracted endo-antigen suggested that tryptophane might be present in the substance even though the Rosenheim reaction was but faintly positive. The method evolved by Gortner and Holm (15) for the quantitative determination of tryptophane and the minimum determination of tyrosine in the course of their studies on humin formation was decided upon, in that it would

allow a complete nitrogen partition to be run on each sample. These workers found that hydrolysis of a protein with 20 per cent HCl in the presence of increasing amounts of formaldehyde produced a gradual increase and finally a decrease in the humin nitrogen formed. At the peak of this curve the humin nitrogen represented a quantitative determination of tryptophane in the protein. Repeating this procedure with benzaldehyde in the place of the formaldehyde, the amount of humin nitrogen formed rose to a still higher value. The difference between the peak value of the formaldehyde curve and that of the benzaldehyde curve represented a minimum determination of the tyrosine of the protein. The method of Van Slyke (16) was used in the further partition of the nitrogen of the fraction after removal of the humin.

From the curves shown in Graph 1, it is apparent that the percentages of tryptophane and tyrosine in the three species of *Brucella* vary one from the other. While the percentages obtained from Br. melitensis and Br. suis form curves approximately similar to those expected from the data of Gortner and Holm, the values for the benzaldehyde curve of Br. abortus differ widely from the expected.



Graph 1. Curve 1. - Humin formation in the presence of formaldehyde. Curve 2 - Humin formation in the presence of benzaldehyde.

A. - Br. melitensis; B - Br. abortus; C - Br. suis

According to the method, this could only mean that tyrosine is not present. The percentages of the two amino acids for the three species of Brucella are given in Table 3.

Table 3

Distribution of Tryptophane and Tyrosine in the Endo-antigens of the Three Species of <u>Brucella</u>				
	Per cent of Tryptophane Nitrogen	Per cent of Tyrosine Nitrogen	Per cent of Tryptophane	Per cent of Tyrosine
<u>Br. melitensis</u>	2.46	0.60	18.92	8.45
<u>Br. abortus</u>	1.49	none	11.46	none
<u>Br. suis</u>	0.91	0.66	7.00	9.29

The results tabulated in Table 3, show that the endo-antigens from the three species of *Brucella* vary widely in their tryptophane and tyrosine content. That the differences in percentages bear no relation to the total nitrogen content of the endo-antigen is shown by the fact that the total nitrogen of the three endo-antigens was: Br. melitensis 8.11 per cent, Br. abortus 9.08 per cent, Br. suis 10.07 per cent. The surprising feature of these data is that while the Rosenheim test for tryptophane was extremely faint for all three endo-antigens, the Millon's test for tyrosine was brilliant in all three cases. The possible significance of this fact will be discussed later.

It is apparent that the tyrosine and tryptophane nitrogen account for but a small portion of the total nitrogen. In every instance the remainder of the nitrogen fell into two classes, namely, acid-amide nitrogen and mono-amino acid nitrogen. The acid-amide nitrogen values remained almost constant throughout, averaging 1.57 per cent. This nitrogen is generally regarded as being derived from acid-amide linkages $-\text{CONH}_2$. Gortner and Holm have shown, however, that in a 24 hour hydrolysis there may be an appreciable amount of deamination of the amino acids, which nitrogen occurs in this fraction. The remainder

of the nitrogen of the endo-antigen fell into the class containing the mono-amino mono-carboxylic, and the mono-amino decarboxylic acids. There was no indication of the presence of any of the basic amino acids.

The Sugar Acid

The solutions remaining from the nitrogen determinations of each endo-antigen were united. These solutions were concentrated to a small volume in vacuo at 40° C. Addition of 5 volumes of alcohol to each of those produced a precipitate, which upon standing formed a gummy residue. This gummy precipitate was thoroughly examined from only the endo-antigen of Br. melitensis. It was re-dissolved in distilled water, decolorized by boiling with Norite. Re-precipitation with alcohol produced an almost colloidal white precipitate, which upon collecting became a colorless sirupy material. This material gives a slowly positive Molisch reaction; nitrogen is absent; Bial's test is negative; there is no reduction of Benedict's reagent; with FeCl_3 a deep yellow color is given which is produced by many alpha-hydroxy acids. The material is acidic, titrating slowly. For complete titration it is necessary to heat in a sealed tube in N/1 alcoholic KOH. The fraction

appears to be an anhydride or a lactone of a sugar acid. Because of its sirupy nature, equivalent weights obtained are meaningless. The compound is optically inactive. Attempts to prepare saccharic and mucic acids from it have been futile.

That this compound is a lactone of a sugar acid seems highly probable. Since it is optically inactive, this suggests that it is produced in the course of the hydrolysis. It is difficult to conceive that its precursor could have been the usual type of reducing sugar as those are completely destroyed by prolonged heating in acid solution. There is a possibility that it is formed from a sugar acid present as such in the original endo-²antigen, or that it is formed by oxidation and deamination of an amino sugar.

Lead Acetate Precipitation

The alcoholic supernatants from the above precipitation of the sugar acid of each endo-antigen were combined and freed from alcohol by distillation in vacuo at 40° C. The aqueous concentrates were thoroughly precipitated with neutral lead acetate, the bulky precipitate consisting largely of lead chloride. The lead precipitates were decomposed with H₂S. The supernatants from the sulphide precipitates, upon evaporation on the steam bath, deposited crystals

of lead chloride. Occluded to the lead chloride in a manner which resisted separation during four recrystallizations from hot water were small quantities of the fatty acid, the yellow fragrant oil and the sugar acid previously mentioned. There were no nitrogenous products. These materials were detected because of the persistent antigenicity of the crystals. They were eventually liberated by saponification. This surprising ability of an inorganic salt to carry with it through repeated crystallization traces of an antigenic material, is worthy of further investigation.

The behavior of the three endo-antigens differed somewhat, however. With the endo-antigen from Br. suis needle-like crystals separated before and during concentration for the precipitation of the sugar acid and upon concentration for precipitation with lead acetate. This crystalline deposit was obtained only from Br. suis and has not yet been characterized chemically. The Br. melitensis endo-antigen differed from the other two, in that while the lead sulphide precipitates from the Br. suis and Br. abortus preparations could be decomposed with HCl with the formation of $PbCl_2$, the lead sulphide precipitate from the Br. melitensis endo-antigen could not be decomposed. It was found to contain organic matter.

It is significant to note that injection into normal rabbits of the combined products obtained from lead acetate precipitation (although they were nitrogen-free and had been hydrolyzed in strong acid, produced agglutinins and opsonins for the intact Brucella cells. This is possibly due to the presence of small quantities of the diketone fraction which has been previously noted as antigenic. In an earlier preliminary report (17) mention has been made of a hydrocarbon, a probable isomer of b-anthraquinone carboxylic acid, obtained from the lead acetate precipitates of Br. melitensis and Br. abortus but not from Br. suis. Subsequent work has shown this product to be impure. The anthraquinone derivative is present only in traces and not in significant quantities as was previously supposed. It is possibly an artifact. The antigenicity previously attributed to it seems due to traces of the diketone fraction.

The Remaining Nitrogenous Constituent

The supernatants from the lead precipitation yielded upon concentration, an orange sirupy material which became partially crystalline in the ice box, but a sirup again at room temperature. This sirup was thoroughly examined only in the case of the Br. melitensis endo-antigen. Upon saponification in 4 per cent alcoholic KOH an

insoluble precipitate of inorganic matter is formed. There is no trace of lipide present. The aqueous solutions from this saponification contain nitrogen and give a positive Molisch reaction. They do not reduce Benedict's reagent; no osazone is produced; and Bial's test is negative. The nin-hydrin reaction is positive. Although the color developed in the Elson-Morgan test for glucose-amine and chondrose-amine (18) deepens in proportion to the nitrogen content of the samples being tested, neither of these compounds was isolated.

In Table 4 are presented, as complete as possible, summarized data of the chemical constitution of the endo-antigens. In consideration of the chemical examinations of the endo-antigens, sufficient data have been presented to demonstrate their dissimilarity to any previously described bacterial products. The differences in the distribution of the tyrosine and tryptophane in the endo-antigens from the three species of *Brucella*, as well as the differing behavior of the hydrolytic products, demonstrate that the three endo-antigens are not chemically identical. Although quantitative methods of determining the sugar acid and the remaining unidentified nitrogenous fraction have not yet been developed, the amounts

of each obtained preclude the presence of any other constituents in the endo-antigen. In fact, assuming the sugar acid to arise from the reducing sugars demonstrable in the endo-antigen, and assuming the nitrogenous constituent to represent even a very small mono-carboxylic amino acid, the percentages already add up to more than 100 per cent. This can only indicate that some of the fractions are identical and are being counted twice as would be the case if the residual nitrogen, the reducing sugars and the sugar acid all had their origin in an amino-sugar.

An attractive but at present purely speculative correlation of the chemical data is possible if one considers the proposition of the endo-antigen being a combination of an amino-sugar with a diketone. That amino-sugars may form a variety of hetero-cyclic compounds, including those containing a pyrrol ring has been demonstrated (19). Their combination with diketones to form such compounds is the basis of their colorimetric determination (18). Precedence for such a combination is thus established. The fact that the tryptophane and tyrosine determination do not correlate with the qualitative tests for these compounds might indicate their presence not as actual entities, but as formed by the com-

Table 4

Summary of the Chemical Constitution of
the Endo-antigens of Brucella

Per cent	<u>Br.melitensis</u> endo-antigen strain 606	Lipide-extracted <u>Br.melitensis</u> endo-antigen strain 606	<u>Br.abortus</u> endo-antigen strain 1151	<u>Br.suis</u> endo-an- tigen strain 1635	S-sub- stance strain 606
al N.	9.40	12.00	6.08	8.86	5.86
ino N	-	-	-	-	-
lucing sugars efore hydrolysis	-	-	-	-	-
lucing sugars ter hydrolysis	5.93	4.00	6.49	4.4	8.13
fur	-	-	-	-	-
sphorous	-	-	-	-	-
tyl groups	3.48	5.42	5.82	10.03	48.43
tone soluble llow oil	8.53		6.00	9.00	
ty acid	4.87		9.00	5.57	
ptophane N	2.13	2.46	1.26	0.77	
osine N	0.51	0.60	0.00	0.52	
ptophane	16.38	18.92	9.74	5.98	
osine	7.31	8.45	0.00	7.93	
ar acid	Percent not determined	Percent not determined	Percent not determined	Percent not determined	
identified itrogenous omponent	Percent not determined	Percent not determined	Percent not determined	Percent not determined	
isch test	+ Slow	+ Very slow	+ Slow	+Slow	+Slow
l's test	+	+	+	+	Weak
ret test	+	+	+	+	Very faint
lon's test	+	2+	+	+	+
enheim test	Faint	Faint or negative	Faint	Faint	Nega- tive

bination of other compounds. The data disclose that a diketone is present in, or closely associated with, the endo-antigen. There is also evidence that the final nitrogenous residue gives a color reaction for an amino-sugar. All of these data could be correlated with such an hypothesis.

It is hoped that further work may complete the identification of each of the constituents of the endo-antigen, and reveal the manner in which they are combined.

Biological Activity of the Endo-antigen

The studies of the biological properties of various preparations of the endo-antigen were undertaken with the following objectives in mind: precipitability; antigenicity; toxicity and nature of the toxic reaction in the guinea pig; sensitizing activity; immunizing value and therapeutic property. Three types of material, viz. endo-antigen, lipide-extracted endo-antigen and acetylated endo-antigen, were used in these studies.

Precipitation studies: The precipitation studies were made with serums prepared for each of the three species of Brucella. Normal goats were injected intravenously with suspended living bacterial cells from one agar slant of growth. When the serums of the goats

reached an agglutination titer of 1-10,000, the goats were bled to obtain a large amount of serum. The collected serums were diluted one-half and sterilized by passing through a Seitz filter. All precipitation tests were made in small glass vials using 0.2 cc. of serum layered with 0.2 cc of the antigen solution. The tubes of serum and antigen dilutions were incubated at 37° C. for 2 hours and then allowed to stand in the cold room at 10°C. over night. The antigen dilutions were made on a dry weight basis. The results are recorded by giving the highest dilution of antigen showing precipitation.

In Table 5 are recorded the results of an experiment to determine the effect of pH, heating and aging on an endo-antigen prepared from Br. abortus strain 1151. The antigen was divided into three parts, one of which was adjusted to pH 6, one to pH 7 and the other to pH 8. These were then divided into equal parts, one of which was left unheated and the other heated at 80°C. for 3 hours in a water bath. The results indicate that the precipitability of the endo-antigen is only slightly affected by heat, age and alkalinity.

Table 5

Effect of Heat, pH and Age on <u>Br. Abortus</u>			
Endo-antigen Strain 1151			
Age of Antigen	pH	Precipitation Titer of Antigen	
		Unheated	Heated
One week	6	409,600	409,600
	7	409,600	409,600
	8	409,600	409,600
Three months	6	409,600	204,800
	7	409,600	204,800
	8	409,600	102,400

Since much emphasis has been placed on the importance of acetyl groups in antigens, several experiments were made to determine the effect of acetylation on the precipitability of several endo-antigens. The results of one experiment comparing the precipitability of 4 antigens before and after acetylation are recorded in Table 6. It is quite evident from the results that acetylation enhances the precipitating property of the endo-antigens. The S-substance is unaffected.

Table 6

Effect of Acetylation on the Ability of Endo-antigens to precipitate Specific Serums		
	Precipitation Titer of Antigen	
	Before Acetylation	After Acetylation
Endo-antigens from <u>Br. abortus</u> strain 1151	345,000	1,500,000
Inactive endo-antigen from <u>Br. abortus</u> strain 1151	57,600	921,000
Material recovered after saponification in the acetyl deter- mination	1,800	921,000
S-substance	409,600	409,600

The effect of acetone and ether extraction on the precipitating ability of the endo-antigen was studied. Representative results are presented in Table 7. The data indicate that extraction of the endo-antigen with acetone containing HCl and with ether causes some loss of ability to precipitate specific serum. The precipitating ability is by no means destroyed, however.

Table 7

Effect of Lipide Extraction on the Ability of endo-antigens to Precipitate Specific Serum		
	Precipitation Titer of Antigen	
	Before Extraction	After Extraction
	573,440	192,560
Endo-antigen from <u>Br. abortus</u> strain 1151		
Endo-antigen from <u>Br. melitensis</u> strain 748	437,400	128,000
Endo-antigen from <u>Br. suis</u> strain 1635	349,820	316,200

Extremely significant results were obtained in the study of precipitating ability of the endo-antigen after treatment with N/2 NaOH at 80°C. for one-half hour and with subsequent acetylation or treatment with NH_4OH of the product thus obtained. The results of one experiment are presented in Table 8. While heating the endo-antigen in N/2 NaOH destroys the ability of the material to precipitate specific serum, this ability is restored either by acetylation or by allowing the material to stand in NH_4OH , thus demonstrating that the beneficial effect which acetylation has been shown to have on the precipitating ability of the endo-antigen is probably not due to the addition of acetyl groups, but to some other influence. These results correlate with those presented by Felton and Prescott (10) in their work on the specific substance of *Pneumococcus* Type I. The amount of precipitate produced by the acetylated and by the NH_4OH treated material was not so heavy as that produced by the original endo-antigen, indicating possibly a partial depolymerization.

Table 8

Precipitation of Specific Serum by an Endo-antigen After Chemical Treatment	
Endo-antigen from <u>Br. melitensis</u> strain 748	Precipitation Titer of Antigen 512,000
Endo-antigen heated 1/2 hour at 80°C. in N/2 NaOH	None
Endo-antigen acetylated after heating in N/2 NaOH	182,000
Endo-antigen treated with NH ₄ OH after heating in N/2 NaOH	512,000

Toxicity: In the preliminary studies of the toxicity of the endo-antigen it was found that various preparations differed considerably in their effect on the guinea pig. From those early experiments it was learned that the differences were due chiefly to the size of dose used and the pH of the preparation. The administration of too large a dose as well as of too small a dose of a particular antigen failed to elicit a reaction in the guinea pig. The toxic activity of each preparation of endo-antigen was arrived at by injecting guinea pigs, weighing from 350 to 450 grams, intraperitoneally with one to two cubic centimeters of the preparations, containing definite amounts measured on a dry weight basis. The criteria of their toxicity for the guinea pig are as follows:

Upon intraperitoneal injection into normal guinea pigs, the first symptoms are rarely noticeable before the end of the first hour and much more frequently at the end of the second or third hours. The temperature of the animal begins to fall and may continue to do so until death occurs, or the temperature may rise again to normalcy if the injection is not fatal. As the temperature drops the animal becomes irritable and nervous. The hair on the back becomes roughened. The abdomen may become very tense

and distended and a white purulent exudate may appear in the left eye. Toward the end of a fatal injection the animal is frequently seized with periodic spasms. Upon post-mortem examination the most usual findings are an intense congestion of the blood vessels of the mesentery, hemorrhagic areas in the mucosa of the stomach wall, the intestines, the greater omentum and the genital organs. Of these, the mucosa of the stomach is most often and most severely affected. The spleen is often congested with blood.

In Table 9 are set forth the results of one of several experiments which show the effect of pH, heat and aging on the toxicity of an endo-antigen prepared from cells of Br. abortus strain 1151. It was found that the antigens, shortly after preparation show their greatest toxicity in 20 mg. doses. Slight increasing or decreasing of the dose from the optimums, results in little or no reaction in the guinea pig. In determining the toxicity of a particular antigen at intervals after preparation, the usual procedure followed was to inject guinea pigs with doses of 1,5,10,15,20, and 25 mg. The results would reveal any decrease or increase in toxicity. The results in Table 9 are tabulated only for the amount of antigen

which produced the maximum reaction or death of the guinea pig.

Preparations that were allowed to age at 10°C., especially those made at or below pH 7 and heated, increased in toxicity. The previous size doses then failed to elicit reactions in the guinea pig while smaller ones were effective. Preparations made at a pH above 7 tend to decrease slightly in toxicity with age.

Table 9

The Effect of pH, Heat and Aging on the
Toxicity of the Endo-antigen

Age of Antigen	pH	Toxic Dose	
		Unheated	Heated
One week	6	20 mg. Died 24 hrs.	10 mg. Died 4 days
	7	20 mg. Died 24 hrs.	20 mg. Died 7 days
	8	20 mg. Temp. fell to 35.5°C. Recovered	10 mg. Temp. fell to 37°C. Recovered
Two months	6	20 mg. Died 10 hrs.	5 mg. Temp. fell to 37.5°C. Recovered.
	7	1 mg. Temp. fell to 37.5°C. Recovered	20 mg. Died 10 hrs.
	8	25 mg. Died 7 hrs.	20 mg. Died 9 hrs.
Four months	6	20 mg. Died 10 hrs.	5 mg. Temp. fell to 33.2°C. Recovered
	7	1 mg. Temp. fell to 36°C. Recovered	20 mg. Died 6 hrs.
	8	25 mg. Died 7 hrs.	20 mg. Died 4 hrs.

The effects of acetone and ether extraction on the toxic property of the endo-antigen were quite significant. Representative results are recorded in Table 10. In each case it is seen that the toxic activity of the endo-antigen is markedly increased by the extractions.

Table 10

Effects of Lipide Extraction on the Toxicity of the Endo-antigen		
	Toxicity	
	Before Extraction	After Extraction
Endo-antigen from <u>Br. abortus</u> strain 1151.	20 mg. Temp. fell to 36.6°C. Re- covered	1 mg. Temp. fell to 37.1°C. Re- covered.
Endo-antigen from <u>Br. melitensis</u> strain 748	20 mg. Died 4 hrs.	5 mg. Temp. fell to 37.2°C. re- covered
Endo-antigen from <u>Br. suis</u> strain 1635	10 mg. Temp. fell to 37.4°C. Recov- ered.	5 mg. Temp. fell to 34.4°C. Re- covered.

The effect of acetylation on the toxicity of the endo-antigen is less clear. While the toxicity was never destroyed it appeared to be considerably reduced. In endo-antigens where a lethal dosage was found before acetylation, none could be discovered afterwards although a variety of dosages showed mild toxicity. The same findings occurred when the endo-antigen was heated with N/2 NaOH at 80°C. for one-half hour. The original toxicity was not restored in the latter by treating with NH_4OH or by acetylation. One may summarize that the toxic property of the endo-antigen is not easily destroyed, but once impaired, its original toxicity cannot be restored.

Antigenicity: As a rule guinea pigs were used to study the ability of the endo-antigen to provoke antibodies. The rapid and test-tube agglutination tests were used to measure agglutinin production; the phagocytic system previously described (20) was used to measure opsonin production. Those pigs in which a given intraperitoneal dose of antigen was non-toxic or which failed to succumb following a systemic reaction were bled on the tenth day

after injection and examined for antibodies. Preliminary examinations showed that the antibodies reached their peak by the tenth day.

In Table 11, are presented representative data from one of several experiments which illustrate the antibody response to varying doses of heated and unheated antigen. It is interesting to note that antigenicity and toxicity correlate very closely. When large doses of the antigen fail to provoke a toxic reaction in the guinea pig, they also stimulate little if any antibodies. A one milligram dose of the antigen, if toxic, appears to be just as capable of stimulating antibodies as larger doses. The phagocytic system was found to be much more delicate than an agglutinin or precipitin system for measuring the antigenicity of the endo-antigens. Many times opsonins were produced when the presence of agglutinins could hardly be detected.

There was found to be little difference between the antigenicity of heated and unheated antigens. The storing of antigens in a cold room at 10°C. for four months does not impair their antigenicity.

Table 11

The Antigenic Activity of the Endo-antigen							
Antigen	Dose	Toxicity	Agglutinin Titer	Opsonic Activity Cells (a)			
				Ma	Mo	S	N
Unheated	20 mg.	Negative	-	0	1	8	16
	5 mg.	Slight	+1/25	18	5	2	0
	1 mg.	Slight	+1/500	18	7	0	0
Heated (b)	20 mg.	Negative	-	6	9	10	0
	10 mg.	Slight	+1/100	20	5	0	0
	5 mg.	Slight	+1/100	21	4	0	0
	1 mg.	Slight	P1/25	16	9	0	0

(a) Ma-Cells showing marked phagocytosis

Mo-Cells showing moderate phagocytosis

S--Cells showing slight phagocytosis

N--Cells showing no phagocytosis

(b) At 80°C. for 2 hrs.

When the endo-antigen is freed of its lipide content, the antigenicity is unimpaired. It still correlates with the toxicity of the material, however.

In Table 12 are recorded the effects of acetylation on the antigenicity of the endo-antigen. It is seen that antigenicity is unquestionably enhanced by the process. This is demonstrated most clearly in the case of the product recovered from acetyl determination and in the case of the S-substance. Although the toxicity of the endo-antigen is changed by acetylation so that there is no lethal dose determinable and so that there is little gradation with dosage, the antigenicity is increased regardless of the size of the dose. The correlation between toxicity and antigenicity is, therefore, no longer present.

Table 12

Effects of Acetylation on the Antigenicity
of the Endo-antigen and of the S-substance

	Dose		Agglutinin Titer		Opsonic Activity (a)							
	Before Acetylation	After Acetylation	Before Acetylation	After Acetylation	Before Acetylation				After Acetylation			
					Ma	Mo	S	N	Ma	Mo	S	N
Endo-antigen from <u>Br. abortus</u> strain 1151	20 mg.	20 mg.	P 1/50	P 1/100	22	3	0	0	24	1	0	0
Inactive endo-antigen from <u>Br. abortus</u> strain 1151	20 mg.	20 mg.	P 1/50	+ 1/500	7	6	6	6	25	0	0	0
Material recovered after saponification in the acetyl determination.	20 mg.	15 mg.	Negative	+ 1/50	16	5	4	0	25	0	0	0
S-substance from <u>Br. melitensis</u> strain 606	15 mg.	15 mg.	Negative	+ 1/50	8	13	4	0	25	0	0	0

(a) Ma - Cells showing marked phagocytosis

Mo - Cells showing moderate phagocytosis

S -- Cells showing slight phagocytosis

N -- Cells showing no phagocytosis

In the experiment in which the endo-antigen was heated in N/2 NaOH for one-half hour at 80°C. antigenicity was completely destroyed. Treatment with NH_4OH failed to restore the antigenicity while acetylation brought about but a very slight restoration. As it was noticed in the study of this treatment on precipitating ability of the endo-antigen that partial hydrolysis had probably occurred, it may be supposed that this explains the failure of acetylation to restore complete antigenicity in this case.

Allergic Activity of Endo-antigens: The comparative allergic activities of endo-antigens prepared from the three species of Brucella were determined on sensitized rabbits. The rabbits were sensitized by one intravenous injection of 1 cc. of living cells suspended in sterile saline solution. The turbidity of the suspension was made to compare with tube 2 of the McFarland Nephelometer. This procedure will produce a high degree of skin sensitiveness to Brucella in 30 days for measuring quantitatively the allergic activities of various preparations. When antigens prepared from each of the three species of Brucella were compared as to their allergic activities, successive dilutions ranging from 1-5000 to 1-1,280,000 were made in sterile

saline solution. An injection of 0.1cc of each dilution of the three antigens was then made intradermally into a rabbit sensitized to Br. abortus, one to Br. suis and one to Br. melitensis. The intradermal tests were observed at 24 hour intervals for 5 days. The reactions were recorded at the 48th hour, although they often persisted for as long as 5 days.

In Tables 13 and 14 are recorded the results of two of several experiments designed to determine quantitative differences in the allergic activity of acetylated and lipid extracted endo-antigen in rabbits sensitized to Br. abortus, to Br. suis and to Br. melitensis. Rabbits sensitized with Br. abortus were found to show the same degree of allergic response to both Br. abortus and Br. suis, acetylated and lipid extracted endo-antigens. The allergic titers of the two melitensis antigens were considerably lower than those from Br. abortus or Br. suis. The allergic titer of Br. suis acetylated antigen was considerable higher than the Br. abortus or Br. melitensis antigens in Br. suis sensitized rabbits. Lipid extracted antigens induce little or no allergic response in dilutions of 1-5000 and above in Br. suis sensitized rabbits. Rabbits sensitized to Br. melitensis showed about the same allergic response

to dilutions of the acetylated antigens and to dilutions of the lipide extracted antigens. The allergic titer of the melitensis antigens was either low or negative in the Br. melitensis sensitized rabbits.

It is apparent from the results that rabbits, when sensitized to the different species of Brucella show a considerable difference in their allergic response to varying dilutions of endo-antigens prepared from each of the three species. If one employs the proper dilutions of acetylated and lipide extracted endo-antigens it should be possible to detect the species of Brucella responsible for an existing state of allergy. It is believed that in these antigens may be found the means for detecting the infecting species of Brucella in human beings.

Endo-Antigens

[illegible]

Comparison of the Allergic Activity of Lipid Extracted Endo-antigens

[illegible]

Immunizing value of the endo-antigen: All attempts to immunize guinea pigs with various preparations of the endo-antigen in varying size and number of injections against experimental Brucella infection have thus far failed. Guinea pigs in groups of five have been injected intradermally, subcutaneously and intraperitoneally with from one to nine successive doses of the antigens in amounts varying from 2 mg. to 20 mg. After an interval of 30 days the pigs were exposed to infection by placing one drop of a dense suspension of a newly isolated anaerobic strain of Br. abortus in the conjunctiva. At the end of five weeks after exposure, the treated pigs as well as an equal number of untreated controls were killed and the organs examined for gross changes and cultured. All pigs treated with varying size doses of the endo-antigens as well as the untreated controls, showed evidences of infection, grossly and culturally. Since guinea pigs are not easily immunized against Brucella infection and since those used in these experiments were exposed to a massive dose of the virulent culture, the results do not necessarily mean that the antigens are without immunizing value.

Studies are being conducted to determine the immunizing value of the antigens against *Brucella* infection in cattle and human beings. While the studies are far from being complete, the results thus far show that if the proper size dose of the lipide extracted endo-antigen is injected intradermally, cattle and humans develop a high *Brucella* opsonin titer which persists for more than 6 months.

Therapeutic value of the endo-antigen: Several experiments were carried out on small groups of guinea pigs 30 days after exposure to a virulent culture of Br. abortus, to determine whether the course of infection could be altered by the administration of endo-antigen. The endo-antigen was used intradermally and subcutaneously at intervals of 3 days and 7 days in 5 and 10 mg. doses. One group of pigs received one injection, another 3 injections and another 9 injections. Infected guinea pigs are very allergic to *Brucella* antigens, and consequently many pigs died within 24 hours following the injection of the larger amount of endo-antigen. When the surviving pigs were killed 5 weeks after the last injection of antigen in each series of experiments, all showed gross changes in the organs characteristic of *Brucella* infections. The organism was cultured from all pigs exposed to infection.

Biological Activity of the Constituents of the Endo-antigen

Each of the constituents obtained from the endo-antigens, has been examined for ability to precipitate specific serum, for toxicity and for antigenicity. None have been found to be toxic and none have been found to precipitate immune serum, although the lipid fractions could not be examined for precipitating ability because of their insolubility in water or in saline. The portion of the endo-antigen removed by acetone extraction has, however, been found to produce agglutinins and opsonins for Brucella upon injection into normal animals. This fraction is nitrogen free. Likewise, the material precipitated by lead acetate, after hydrolysis in 20 per cent HCl, has been found antigenic. This activity may also be due to the diketone fraction, as a small amount of this material was removed from the lead acetate precipitate.

Physiological Studies

Blood sugar: It was noticed by Delafield (21) that the toxic antigenic fraction isolated by Raistrick and Topley (6) from Salmonella aertrycke had a hyperglycemic effect when injected into animals. The endo-antigen from Brucella was examined for this activity.

In a single experiment a guinea pig was bled from the heart immediately before, 3 hours after and 24 hours after intraperitoneal injection of 5 mgs. of endo-antigen. Blood sugar was run by the Somogyi-Shaffer-Hartmann method (22). The following results were obtained:

	Blood sugar in mgs. per 100 cc.
Before injection:	137 mgs.
3 hours after injection:	174 mgs.
24 hours after injection	97 mgs.

The endo-antigen is thus seen to bring about in injected animals a hyperglycemia which is followed by a hypoglycemic condition.

Basal metabolism: A study was made of the effect of injections of the endo-antigen on the basal metabolism of the guinea pig. A glass metabolism chamber was arranged so that a regulated amount of

dried, CO₂ free air could be drawn through it. The moisture and CO₂ of the air leaving the chamber were removed by passage through Dehydrite and Ascarite respectively. A normal metabolism was run on each animal before its use in the experiment. The animals used varied greatly in size and age. The variation in basal metabolism of the normal animals is explainable largely by this factor. Young guinea pigs invariably had a higher metabolic rate than did the older animals. The amounts of endo-antigen injected varied with the body weight. The first tests were run for a period of 8 hours. A second set of tests were run for 4 hours. The results of these tests are recorded in Table 15.

Table 15

The Effect of Endo-antigens on Basal Metabolism				
Antigen	Calories per kilogram per hour			
	4 hour period		8 hour period	
	Before Injection	After Injection	Before Injection	After Injection
<u>Br. abortus</u>				
10 mgs. per kg.	3.35	4.03	8.6	8.8
20 mgs. per kg.	2.56	3.80	11.8	4.7
30 mgs. per kg.	2.56	4.15	3.7	0.65
<u>Br. suis</u>				
10 mgs. per kg.			8.3	4.1
20 mgs. per kg.			2.8	2.56
30 mgs. per kg.			4.5	3.2
<u>Br. melitensis</u>				
10 mgs. per kg.			3.1	3.0
20 mgs. per kg.			10.5	7.7
30 mgs. per kg.			2.5	0.97

In most cases, if the animal is observed over a period of 8 hours, a definite decrease in the basal metabolism is noted. If, however, the time of observation is limited to 4 hours the data show a definite increase in the calories consumed per kilogram per hour. In other words, upon intraperitoneal injection of the endo-antigen, the basal metabolism of the animal is at first stimulated, and is then depressed, the depression usually being more than sufficient to counteract the increase.

DISCUSSION

The endo-antigens prepared from the three species of the Brucella differ from any previously described bacterial fractions. Although their structures have not yet been fully elucidated it appears from the data presented that they may be described as neither protein nor carbohydrate in nature, but a combination of certain units of both of these classifications with a lipide fraction. The endo-antigens are thus somewhat similar to the preparations described by Boivin and coworkers (7, 12), but differ from these in their stability, their incapability of being separated into specific non-toxic and toxic non-specific fractions and in their containing no phosphorus or sulfur.

In previous work on the chemistry of the Brucella group a soluble specific substance, the S-substance, was found to exist in Br. melitensis but could not be prepared from the other two species (1, 2, 3). A very similar substance was obtained, however, by chemical treatment of a protein-like fraction, the albuminoid, occurring in all three species. The present work has shown the identity or close similarity of the endo-antigen with the S-substance, and has shown that the endo-antigen may be prepared from the albuminoid

of all three species. The implication is, therefore, that the endo-antigen is united with a protein in the bacterial organism, occurring in a free state only in Br. melitensis.

The quantitative determination of tyrosine and tryptophane in the endo-antigens of Br. melitensis, Br. suis and Br. abortus demonstrates clearly that the three endo-antigens are not chemically identical. There has been, as yet, no clear demonstration of species variation in the other constituents of the endo-antigen, but this must obviously appear upon further investigation.

The relationship of acetyl groups to the biological properties of the endo-antigens does not emerge clearly from the work accomplished. Although distillable acid, which is believed to be acetic acid, is obtained in the determination of acetyl groups, the biological properties of the endo-antigen do not correlate closely with the acetyl content, as would be expected if the groups possessed the importance delegated to the acetyl content of the specific polysaccharide of the Type 1 Pneumococcus (9). On the other hand, acetylation of the endo-antigen brings about a remarkable increase in its biological properties, although, here too the increase in antigenicity does not always correlate with the increase in acetyl

groups. It has been shown further that when the ability to precipitate specific serum has been lost by the endo-antigen, this property may be restored either by acetylation or by allowing the endo-antigen to stand in 25 per cent NH_4OH at 10°C ., in a manner similar to that demonstrated for the restoration of the specific polysaccharide of *Pneumococcus* Type 1 (10). One must conclude, therefore, that the presence of acetyl groups in the endo-antigens is not requisite to their biological activity, but that their presence induces a state which is necessary for biological activity and which can be reproduced in their absence by other means.

The role of the lipides in the endo-antigen is not clear from the present work. Whereas the lipide-extracted endo-antigen is increased in toxicity and antigenicity it shows a decline in the ability to precipitate specific serum. It has also been noticed that the lipide-extracted endo-antigen is much more sensitive to the presence of alkali than is the unextracted antigen. It seems, thus, that the lipides play a part in the antigenic pattern of the endo-antigen but do not contribute to toxicity or antigenicity.

The surprising dependence of the toxicity and antigenicity of the endo-antigen upon the optimum dosage, and the disappearance of

this dependence after acetylation, do not admit ready explanation.

Similar evidence of antigenicity in small doses but not in large doses has been noticed with the specific polysaccharide of Type 1 Pneumococcus (9) and with the Forssman antigen (23). The significance of this observation on the application of the endo-antigen to other animals and in attempted immunization is being investigated.

Although the experimental data are not yet complete, the endo-antigens show some promise of value as immunizing, therapeutic, and diagnostic agents. Attempts to experimentally immunize guinea pigs have, it is true, been unsuccessful although the immunizing injections stimulated excellent antibody production in the animals.

Because of work done in this laboratory on guinea pigs with agents known to immunize other animals, the use of guinea pigs in immunizing experiments seems questionable. Similar results have been obtained by Gwatkin (24) in his work with Brucella fractions. Incomplete work on the immunizing value of the endo-antigens for cattle and humans appears very promising.

The therapeutic value of the endo-antigen for infected guinea pigs

has been found to be negligible. Here, as with experimental immunization, the use of guinea pigs as experimental animals seems to be of little value. There is excellent evidence that at least a part of the activity of Brucellin, a therapeutic agent developed in this laboratory and used very successfully in humans (25, 26), is due to the presence of endo-antigen in that product. The endo-antigen has been isolated from this agent. Experimental therapy in humans with the endo-antigen is being investigated.

As diagnostic agents the endo-antigens have been investigated only in regard to their ability to elicit skin reactions in sensitized animals. As shown in the experimental part, the acetylated and lipide-extracted endo-antigens show some species differentiation, which gives hope for the development of an agent capable of differentiating infections due to Br. abortus, Br. suis and Br. melitensis.

SUMMARY

A method is described for the preparation from cells of Br. abortus, Br. melitensis and Br. suis of a highly antigenic fraction, the endo-antigen, which is toxic for normal guinea pigs, and which precipitates immune serum in dilutions of from 1-500,000 to 1-5,000,000.

Endo-antigens obtained from the three species of Brucella are grossly similar. The endo-antigen comprises roughly 25 per cent of the bacterial cell. While containing the same or similar constituents, however, the endo-antigens from the three organisms have been shown to differ markedly in the distribution of some of these constituents.

Positive reactions are given to the Molisch test, the biuret test, Millon's test, Bial's test and a very slight reaction to the Rosenheim test. The nitrogen content of the fraction varies from 6 to 8 per cent. Reducing sugars are absent before hydrolysis. Calculated as glucose after hydrolysis reducing sugars represent from 4 to 12 per cent of the endo-antigen. Amino-nitrogen, phosphorus and sulfur are absent. In the determination of acetyl groups, distillable acid representing an average of 6 per cent of the endo-antigen is obtained. This acid is presumably acetic acid although

that product has not been isolated.

From the endo-antigen there may be extracted by acetone and ether a compound having the properties of a di-ketone, and an acetone soluble, saturated liquid fatty acid. These two compounds represent from 10 to 15 per cent of the fraction.

The acetone-ether extracted product still reacts positively to the above mentioned qualitative tests. Tryptophane and tyrosine have been found to represent 18.92 per cent and 8.45 per cent, 11.46 per cent and 0 per cent, 7 per cent and 9.29 per cent of the extracted endo-antigens of Br. melitensis, Br. abortus and Br. suis respectively.

From the remaining 65 to 70 per cent of the original fraction there has been obtained an unidentified nitrogenous fraction and an optically inactive sugar acid. These are obtained in quantities such as to preclude the occurrence of any further compounds in a significant amount.

The endo-antigen is shown to be relatively stable in the presence of dilute acid, and dilute alkali, upon heating, and upon long standing. Its activity is not completely destroyed by hydrolysis with dilute acids, but is destroyed by similar treatment with dilute alkali.

The ability to precipitate specific serum is lessened by extraction with acetone and ether, but is enhanced by acetylation or by treatment with 25 per cent NH_4OH . The toxicity and antigenicity of the endo-antigen are shown to be dependent upon proper dosage, an over-dosage as well as an under-dosage giving poor results. The toxicity and antigenicity are increased by lipid extraction of the endo-antigen. Acetylation causes a distinct decline in toxicity but a marked increase in antigenicity.

The endo-antigen elicits specific skin reactions in sensitized animals, the lipid-extracted and the acetylated endo-antigens showing some species specificity in this reaction. Injections of endo-antigen have failed to protect normal guinea pigs from subsequent exposure to virulent Brucella organisms and have failed to alter the course of the disease in infected pigs. Experiments with cattle and humans, however, seem to promise immunizing and therapeutic value to the endo-antigen.

Injection of the endo-antigen causes a hyperglycemia followed by a hypoglycemia in experimental animals. The basal metabolism of injected animals is at first stimulated and then depressed. A leukopenia, chiefly due to the disappearance of neutrophils from the

peripheral blood, follows injection of the endo-antigen into normal guinea pigs.

The endo-antigen may be produced from the previously described albuminoid fraction of the Brucella, thus accounting for the toxicity of that fraction and suggesting that this albuminoid is a combination of the endo-antigen with a protein-like group. The endo-antigen is shown to be similar to or possibly identical with the previously described S-substance, the latter being probably a partially hydrolyzed endo-antigen.

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