

## THE CHEMICAL NATURE OF RESIDUE ANTIGEN PREPARED FROM YEAST.

By J. HOWARD MUELLER, Ph.D., AND JOSEPH TOMCSIK, M.D.

(From the Department of Bacteriology of the College of Physicians and Surgeons,  
Columbia University, New York, and the Department of Bacteriology  
and Immunology of Harvard Medical School, Boston.)

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It has been shown, in previous communications (1, 2), that extracts of a great many different species of bacteria (tubercle bacilli, pneumococci, *Bacillus influenzae*, *Staphylococcus pyogenes aureus*, streptococci, and meningococci), prepared in such a way as to be as free as possible from coagulable proteins and acid-insoluble proteins, contain substances which specifically precipitate with homologous immune sera, produce delayed skin reactions of the tuberculin type in infected animals, and do not induce antibody formation on repeated and energetic administration to rabbits. Since these properties seem to represent the antigenic haptophore group of the bacteria, and promise to possess considerable importance in connection with the pathology of infection, because of their violent reactions with the sensitized animal (2) it has appeared essential to study their chemical properties and constitution in greater detail. As a result of a series of chemical analyses of preparations from various bacterial sources, reported by one of the writers with Wayman and Zinsser (3), two points were established: (1) that the material as prepared was very low in nitrogen, and, therefore, probably contained a considerable quantity of a non-nitrogenous substance; and (2) that the small yield, coupled with the colloidal nature of the substance and the presence of several impurities, made the task of purifying and establishing the nature of the active compound almost impossible from bacterial sources at our command. Because it had been possible to obtain preparations having similar biological and serological properties from every bacterial species with which the attempt had been made, it seemed possible

that other varieties of microorganisms, such as the yeasts, might serve equally well, and that perhaps by using a commercial bread yeast or brewer's yeast enough residue could be obtained for further study. It did not seem impossible that facts gleaned from the study of such material could be applied to further investigation of the smaller quantities obtained from bacteria.

Accordingly, rabbits were injected intraperitoneally first with dead, later with live yeast, using a strain isolated in the spring of 1923 from Fleischmann's compressed yeast.

After several injections at intervals of 5 to 6 days, rabbits were bled and the serum tested for agglutinating properties against suspensions of the yeast. Agglutination was definite but slow up to a serum dilution of about 1:100, or a little better, but did not increase materially after several further injections. The comparatively low titer may be due in part to the large size of the yeast cells, causing them to settle rapidly out of suspension, and in part, perhaps, to an inherent antigenic weakness on the part of yeast. We have found that some rabbits will yield a much higher titer serum than others, and have had one in our series which failed to produce even a trace of antibody when injected in parallel with others which had reached a fairly high level. Normal rabbit sera, used as controls, gave, of course, no agglutination with the yeast suspension.

The next step consisted in the preparation of an extract of yeast cells by a method similar to that used in the preparation of bacterial residue antigens.

For this purpose the "starch-free" grade of Fleischmann's yeast was used. A small quantity was extracted with saline, after bringing to pH 9 to 10 with NaOH, by shaking for several hours. The material was centrifugalized, acidified slightly with acetic acid, filtered, neutralized, and precipitated with alcohol. The precipitate, taken up in saline, gave a good precipitin test (ring) with the yeast serum, but not with the serum of normal controls.

Material prepared in this way was low in nitrogen, having between 1.0 and 2.0 per cent, and gave a strong Molisch test. The yield, however, was small, most of the yeast cells remained viable after extraction, and probably much of the specific substance was lost.

About this time Heidelberger and Avery (4) reported their work on the specific precipitable substance of pneumococcus, a substance almost certainly identical with Zinsser's residue antigens. Their results were similar to those already obtained by us in so far that the

nitrogen was low (1.2 per cent) and that the bulk of their preparation was carbohydrate in nature. Because of the fact that the specific properties of their preparation increased as the nitrogen content diminished down to their minimum figure, they believed that the active material was non-nitrogenous and that the carbohydrate was the specific substance. While their work was convincing, we did not feel that the presence of two nitrogenous bodies, one inactive, the other, represented by the remaining 1.2 per cent of nitrogen, active, had been completely excluded. If such a supposition were correct, their material would contain about 90 per cent carbohydrate, and 10 per cent of some material with roughly the same nitrogen content as protein, the two being extremely difficult to separate by means of the physical methods of fractional precipitation with alcohol, salting out with  $(\text{NH}_4)_2\text{SO}_4$  and dialysis, which had been used. Such a nitrogenous compound, when pure, should give a ring test up to a dilution of 1:30,000,000. However, in view of their work, and of the invariably strong Molisch test given by residue antigen preparations from other bacterial sources, and now from yeast, it seemed that one must attach some significance to the complex carbohydrate material in these extracts.

Several such compounds have been isolated from yeasts, and reported in the literature. One, particularly, the so called yeast gum, described by Salkowski (5), and more fully by Oshima (6), and by Meigen and Spreng (7), seemed to merit closer investigation. It was extracted by them from yeast with hot potassium hydroxide, precipitated by Fehling's solution as the copper compound, and freed from copper by solution in dilute HCl and reprecipitation with alcohol. It is described as being free from nitrogen and phosphorus, and hydrolyzing with acid to dextrose and mannose. It had attracted attention mainly because of its frequent presence in invertase preparations.

We found at once that our yeast residue antigen preparations gave a precipitate with Fehling's solution, and that this precipitate carried with it the specific precipitable substance of the yeast. The preparation of yeast gum by a method practically identical with Salkowski's was therefore undertaken.

*Preparation of Yeast Gum.*

1 pound of Fleischmann's starch-free yeast was suspended in 2 liters of 1 per cent NaOH, heated in a large evaporating dish for  $\frac{1}{2}$  hour on the water bath, neutralized with glacial acetic acid to faint acidity to litmus, and filtered through paper. To the filtrate, Fehling's solution was added until a precipitate began to separate, requiring between 200 and 300 cc. of the reagent. The bulky precipitate, which clumps and settles out as a friable gum, was washed two or three times by pressing out in water, ground in a mortar with water to which strong HCl was added drop by drop, until the blue precipitate had completely dissolved, and the solution was somewhat green. Three volumes of alcohol were then added, and the mixture allowed to stand for some hours, until the precipitate had settled. The latter was again dissolved in water, a few drops more of HCl added, and reprecipitated by alcohol. The precipitate, after settling out, was filtered on a suction filter, washed with alcohol and ether, and dried. It still contained a trace of copper, gave a non-specific ring, probably because of the copper, in a dilution of 1:100, and specific ring tests with anti-yeast sera, at 1:1,000 and 1:10,000. The yield was about 10 gm.

By minor modifications of this method, using NaOH up to 2 per cent, and heating up to 1 hour, similar yields were obtained, until in all some 60 gm. of the yeast gum had been collected, giving a good specific ring test up to 1:10,000 in 15 to 20 minutes, and 1:100,000 in 2 to 3 hours.

At this stage the work was interrupted in the spring of 1923, to be resumed in the fall. It was necessary at this time to reisolate a strain of yeast for immunization purposes, and to inject new rabbits with the strain thus obtained. The serum obtained precipitated the yeast gum prepared in the spring in the same dilution previously obtained (1:100,000 in 3 hours), but it proved impossible to prepare a yeast gum by Salkowski's method from yeast then obtainable which gave any precipitate whatever with the immune sera. The gum obtained although apparently similar to the earlier material was completely inactive serologically. After many fruitless attempts to obtain an active material by the method used so satisfactorily a few months earlier, we were forced to abandon that method of preparation. Because of the ease with which active gum was at first made, with several different concentrations of NaOH, and periods of heating, the most obvious explanation for the difficulty, which, however, we have not been able to confirm, is that either the commercial

strain of yeast, or the method of manufacture, changed during the summer, and that the specific material of the product now marketed is more susceptible to destruction by hot alkali than that which had been obtainable a year previously. Whatever the explanation, the 60 gm. of material prepared by the hot alkali method have retained their activity, and have served for a considerable amount of work on purification which will be described below.

The yeast obtainable through the present winter has agglutinated with the immune sera just as well as that used earlier. Residue antigens prepared by Zinsser's original method have shown good specific rings with the sera, but for quantity production it has been necessary to modify the original method of preparation to the extent of avoiding, as far as possible, treatment with hot alkali. The method finally adopted, after a number of experiments in various directions, is as follows:

Fleischmann's compressed yeast is autoclaved without the addition of water, at 15 pounds for from 2 to 3 hours. This is best carried out in tall beakers, filled not over one-third full. At the end of this stage, the yeast has become semifluid, like thick cream. A little solid NaCl, 10 to 15 gm. to a pound of yeast, is stirred into the mass, and it is poured into boiling water, 2 liters to a pound, covered, and allowed to boil slowly for about 2 hours. After partial cooling, the suspension is poured into tall glass jars, and allowed to settle overnight. The supernatant is siphoned off, the sediment filtered by suction, and the combined supernatant and filtrate run through a Sharpless centrifuge.

Fehling's solution, in the proportion of 150 to 200 cc. to a liter of extract is now added, and the mixture warmed in a double boiler for a few minutes until the precipitate begins to separate and contract. The supernatant is carefully decanted, the gummy precipitate pressed together with a spatula, and finally washed two or three times by pressing out in water.

From this point the method is exactly as described originally, dissolving in dilute HCl, precipitating with alcohol, and again dissolving and precipitating if desired.

The yield is only about one-half that previously obtained; namely, 5 gm. from a pound of material.

These two preparations were about equally active as shown by the precipitin test against an anti-yeast rabbit serum. Analyses showed the following.

	Hot NaOH preparation.	Autoclave preparation.
	<i>per cent</i>	<i>per cent</i>
Total N.....	0.51	1.21
“ P.....	0.42	0.61
Reducing substances after acid hydrolysis, calculated as glucose.....	84	
Specific rotation.....	+83°	

*Further Purification of Hot NaOH Preparation.*

25 gm. of this preparation were dissolved in 500 cc. of water, centrifuged from a small amount of fine white precipitate, during which process some material was lost by a broken tube, and about 400 cc. of alcohol added slowly, with stirring until a definite permanent precipitate had formed. This was allowed to settle overnight, the supernatant poured off, and the precipitate washed with alcohol and ether, and dried. Marked Precipitate I.

To the supernatant fluid 200 cc. more of alcohol was added. A fine, colloidal precipitate was formed, which, on standing, settled out as a sticky syrup on the bottom and sides of the beaker. It was washed with alcohol and ether, and dried, and marked Precipitate II.

Further addition of alcohol to the supernatant from Precipitate II produced only a minimal amount of precipitate, and was not investigated further.

	Weight.	Total N.	Titer.
	<i>gm.</i>	<i>per cent</i>	
Precipitate I.....	3.5	0.73	1:100,000 in 1½ hrs.
“ II.....	16.2	0.19	1:100,000 “ 1½ “

Precipitate I was fractionated further by dissolving in water and adding alcohol up to the first visible permanent precipitate, and allowing to stand overnight. This precipitate (No. IA) was dried, and the filtrate precipitated further with alcohol, leading to a syrupy material like Precipitate II. Precipitate IA was a dark-colored, granular material. It contained 1.65 per cent N and gave a ring test at a dilution of 1:100,000 in 3 hours.

An aqueous solution of Precipitate II gave no precipitate with the following reagents: tannic acid, picric acid, phosphotungstic acid, MgSO<sub>4</sub> (saturated), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (saturated), lead acetate, mercuric chloride, and mercuric cyanide.

A further attempt at purification by dialysis through a fish bladder against running water for 4 days resulted in a practically quantitative recovery of the amount used, with no change in precipitin titer or nitrogen content.

*Further Purification of Autoclave Preparation.*

60 gm. of material, resulting from 12 pounds of compressed yeast, were dissolved in about 700 cc. of water, a fine white undissolved residue was centrifuged off, dried, and saved as "insoluble material." The clear solution was fractionated by successive additions of alcohol, as in the preceding preparation, four fractions being separated, the first with about one-half volume, the last with about three volumes of alcohol. After removing the fourth precipitate, the supernatant was evaporated to dryness and the residue reserved. A summary of the separation follows.

Fraction.	Volumes alcohol to precipitate.	Weight.			Titer.
		gm.	N per cent	P per cent	
Insoluble.....		5.2	1.59	0.72	
Precipitate I....	One-half.	8.8	2.52	1.23	Trace in 1:200,000 in 2 hrs.
"    II....	One.	30.2	0.69	0.47	" " 1:400,000 " 2 " *
"    III....	" and one-half.	10.8	0.67	0.49	" " 1:400,000 " 2 " *
"    IV....	Three.	5.4	0.96	0.48	+ " 1:200,000 " 2 " *
Residue.		3.3	0.88	0.42	

\* Trace in 1:800,000 in 6 hours.

Reserving a small amount of Precipitates II and III, the remainders were mixed together, dissolved in 2,000 cc. of water, and reprecipitated by Fehling's solution. About 30 gm. of the gum were used for this purpose, and after washing the precipitate, dissolving, and reprecipitating twice with three volumes of alcohol, it was dissolved in water containing somewhat more HCl than that necessary to give the solution a clear, yellowish green color, and poured into seven volumes of alcohol. After standing overnight, it was filtered off and dried. Yield—20 gm., which still contains a faint trace of copper. The precipitin titer is unchanged, 1:400,000 in about 2 hours.

A small trial lot of this preparation showed that the specific reaction was unchanged upon treatment for as long as 15 hours with N HCl and N NaOH at room temperature, although a few minutes of heating with either reagent destroys it. Accordingly, the remainder of the 20 gm. was dissolved in 400 cc. of N HCl, 2,800 cc. of alcohol added, and the precipitate allowed to settle overnight. The precipitate was filtered off, dissolved in 400 cc. of water, and again thrown down by seven volumes of alcohol, and finally dissolved in 400 cc. of N NaOH, alcohol added as before, and the precipitate, after settling, taken up in 150 cc. of water. To this solution was added six volumes of glacial acetic acid, the precipitate separated as rapidly as possible by centrifuging and washing with alcohol, and dried. Yield—12 gm.

This material gives a definite ring at 1:400,000 in  $\frac{1}{2}$  hour, but the 1:800,000 tube remains negative after several hours. It contains 0.56 per cent N, 0.028 per cent amino nitrogen by Folin's colorimetric method (8) before hydrolysis, and 0.089 per cent amino nitrogen after heating with 50 per cent HCl for 5 hours in a sealed tube at about 105°. The latter figure is little more than an approximation, because of the necessity for evaporating the HCl and decolorizing the residue with charcoal. The phosphorus content was still 0.48 per cent. Its specific rotation was +90°.

The description of the products obtained in the attempted purification of the starch gum, makes it apparent that we must face the same difficulty of interpretation of results as outlined above in the discussion of Heidelberger and Avery's pneumococcus work. Is the yeast gum the substance which carries the specific properties of the yeast residue antigen, and does its molecule contain a low percentage of nitrogen and phosphorus; or are the latter two elements combined in some other substance which it has been impossible to separate from the gum merely through failure to discover a suitable method? While we admit that the point is not firmly established experimentally, we are inclined to adhere to the former view, because of the following considerations.

Referring to the fractional precipitation of the hot NaOH preparation by alcohol, Precipitate IA contains 1.65 per cent N, while Precipitate II contains only 0.19 per cent. Hence, if the specific property were following the nitrogen, No. IA should be nearly ten times as active as No. II, which is not the case. As a matter of fact, there is very little difference between the two, as would necessarily be the case if the yeast gum were the active agent. Precipitate IA probably contains about 85 per cent gum, against 97 to 98 per cent in Precipitate II, a difference insufficient to recognize by the precipitin test. Moreover, we have carried out numerous experiments with extractions of various sorts, cold acid and alkali, autolysis, etc., and have never obtained a preparation which would give a precipitin test without also giving a gummy precipitate with Fehling's solution, the precipitin test being, as a rule, roughly parallel with the purity of the gum.

We must also leave open for the present the question of whether or not the yeast gum contains nitrogen or phosphorus in its molecule. The earlier workers state that it is nitrogen-free, but give no account



of how this was determined, and from the description of their method of preparation, certainly nothing approaching the efforts made in purifying our autoclave preparation were carried out by them.

The fact that the autoclave preparation fractions show a somewhat higher titer than the hot sodium hydroxide fractions is without significance, since a better serum was available for the latter part of the work. The difference in nitrogen content of these two purified preparations, 0.19 per cent in one and 0.56 per cent in the other, would seem to indicate that an impurity is still present in the autoclave preparation at least.

#### *Attempts to Immunize Rabbits with Yeast Gum.*

Previous attempts by Zinsser to immunize with bacterial residue antigen, and by Heidelberger and Avery with their pneumococcus specific substance, have been uniformly negative. We attempted in several different ways to bring about antibody production by injection of yeast gum, all with completely negative results.

In one experiment a rabbit was given intravenous doses averaging 50 mg. of dry gum, dissolved in saline at 5 day intervals for over 2 months. On several bleedings after the period of injections it showed no precipitins whatever.

In a second experiment, it was determined that the gum disappeared rather quickly from the circulation when given intravenously, but when administered intraperitoneally in doses of 0.25 gm., it appeared promptly in the blood, and was present for several hours after the injection. The rabbit was therefore given 0.35 gm. of the gum intraperitoneally every 12 hours for a week. The serum of the rabbit at all times during the week, and for about a week afterward, gave a ring test when diluted slightly with saline and layered upon yeast immune serum. But at no time, up to 2 weeks after the disappearance of the substance from the serum, could any evidence of precipitin production be found. The animal was then given a single intraperitoneal injection of 0.5 gm. compressed yeast killed at 60° for  $\frac{1}{2}$  hour, and a week later its serum showed a titer of about 1:10,000 with the gum. This merely served as a control on the rabbit to show that it could produce antibodies to yeast.

The third experiment was based upon Landsteiner and Simms' (9) experiments in connection with heterogenetic antibody production, and the so called "haptenes." They found that by mixing an alcohol extract of horse kidneys with dilute human serum or pig serum and injecting rabbits, antibodies to guinea pig cells were formed as was the case when suspensions of horse kidney were injected. They suggested that possibly the bacterial residue antigens were com-

parable to these haptenes, and served to carry specificity, but did not become antigenic until combined with protein.

Using Landsteiner and Simms' method we prepared, therefore, two solutions, one containing 12 cc. of 5 per cent yeast gum and 230 cc. of saline, the other, 12 cc. of 5 per cent gum and 230 cc. of a 1:8 dilution of horse serum. 0.25 per cent of phenol was added to each. Two series of three rabbits each were injected intravenously with 5 cc. of the solutions at 7 day intervals, until seven injections had been given. Bleedings on the 7th and 14th days after the last injection showed no trace of precipitin against yeast gum, although the three rabbits receiving the serum-gum mixture all showed high titer precipitin with horse serum.

These experiments on immunization, like earlier ones with similar material from other sources, are uniformly negative, but because of the considerable quantity injected into some of the animals, they make it highly improbable that the nitrogen in the gum is in the form of protein.

#### CONCLUSIONS.

Residue antigen recognizable by the precipitin test can be prepared from yeast as from bacteria.

The active material appears to be identical with a complex carbohydrate, the "yeast gum" of Salkowski.

In the purest form of it obtained small amounts of both nitrogen and phosphorus are still present, either as impurities or as part of the molecule.

The yeast gum is not antigenic in the sense of producing antibodies.

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