Discussion of Results

It is evident from a consideration of results obtained that the method here presented of determining sulfur in insoluble sulfates may be extended to the determination of sulfur in oils and rubbers when one uses a gas-tight bomb instead of the open crucible. All of the sulfur in the organic material is converted to calcium sulfide when heated to 700° in contact with calcium hydride. The water-soluble sulfide thus formed may be determined by iodimetry. The method lends itself most readily to determinations in rubbers and oils of high sulfur content, and presents itself as a rapid method for the determination of sulfur in this type of material.

Summary

1. Sulfur may be determined in sulfurated oils by fusion of the oils with calcium hydride, followed by an iodine titration on an acidified solution of the fusion residue.

2. A method of determining the percentage of sulfur in some rubbers is presented.

3. By this method it is possible to determine the percentage of sulfur in free sulfur, sodium thiosulfate and similar sulfur-containing compounds.

MADISON, WISCONSIN

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COMPOSITION OF THE GUM PRODUCED BY ROOT NODULE BACTERIA

BY E. W. HOPKINS, W. H. PETERSON AND E. B. FRED

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One of the most striking characteristics of the root nodule bacteria is the gummy or viscous nature of the colonies or streak cultures. The mucinous appearance of the cultures has been reported by many investigators[1] and has been attributed to the formation of dense capsules by the organisms. What function the gum performs in the metabolism of the bacteria or in their relation to the host plant is not known. Grieg-Smith[2] suggests that the gum is used by the plant and built into nitrogenous compounds, while Mazé[3] is of the opinion that the gum itself is the nitrogenous compound supplied the plant by the bacteria. That the latter assumption is

[1] (a) M. W. Beijerinck, *Bot. Ztg.*, **46**, 754 (1888); (b) G. F. Atkinson, *Bot. Gaz.*, **18**, 157 (1893); (c) L. Hiltner, *Centr. Bakt.*, II Abt., **6**, 273 (1900); (d) M. Dawson, *Phil. Trans. Roy. Soc.*, **B193**, 51 (1900).

[2] R. Grieg-Smith, *Centr. Bakt.*, II Abt., **30**, 552 (1911).

[3] M. Mazé, *Ann. de l' Inst. Pasteur*, **12**, 1 (1898).

incorrect was shown by Buchanan[4] and Fred,[5] who found that the purified gum contained no nitrogen.

The properties and composition of the gum have not been thoroughly investigated. Much of the work has been of a qualitative nature, and in no case have the hydrolysis products been conclusively identified.

The nitrogen-free gums of clover[6] and of bean[4] root nodule bacteria were found to give on hydrolysis reducing sugar which was assumed to be glucose. According to Beijerinck,[7] the slime producing strains of *B. radicicola* form a "cellulan" a type of gum which, contrasted with the dextrans and levulans, is not fermentable by *Granulobacter saccharobutyricum*. Grieg-Smith[8] prepared gum from cultures of lupine and pea nodule bacteria which had specific rotations of +29.7 and 31.7°, respectively. These gums contained small amounts of nitrogen and on hydrolysis gave a sugar which from its reducing power and optical rotation appeared to be almost entirely glucose. A phenylosazone, separated into two fractions with melting points 205 and 193°, thus similar to those of glucose and galactose, was reported, but the author gives no information as to how these were obtained or analyses of their composition. Owing to the lack of particulars, it is impossible to decide whether the author had an osazone of galactose or merely a slightly impure glucosazone. It is well known that a slight impurity markedly lowers the melting point of an osazone. In a later paper by the same author,[2] the gum of bean root nodule bacteria was reported as consisting of glucose and galactose, and the specific rotation of the gums of bean, pea and lupine root nodule bacteria is given as +29°. Kramár[9] hydroyzed *B. radicicola* gum, and prepared a phenylosazone which was apparently glucosazone. Its melting point was 205°. As far as present knowledge goes, the gum of the root nodule bacteria presumably contains glucose, but there has as yet been no conclusive proof of the presence of this sugar. As to the question of the existence of galactose as a constituent of the gum, the evidence is much less convincing than that for glucose. In our own work the gum has been found on hydrolysis to give glucose and a uronic acid, but no fructose, mannose, galactose or pentoses have been found among the hydrolysis products.

Experimental

Preparation of Samples.—The gums studied in this work were produced from cultures of root nodule bacteria of three different cross-inoculation groups. The strains used were *Rhizobium meliloti* 100 (alfalfa), three

[4] R. E. Buchanan, *Centr. Bakt.*, II Abt., **22**, 371 (1908).

[5] E. B. Fred, *Virginia Agr. Expt. Sta. Report*, 145 (1911–1912).

[6] G. E. Gage, *Centr. Bakt.*, II Abt., **27**, 7 (1910).

[7] M. W. Beijerinck, *Fol. Microbiol.*, 1, 377 (1912).

[8] R. Grieg-Smith, *Proc. Linn. Soc. of New South Wales*, **31**, 264 (1906).

[9] E. Kramár, *Centr. Bakt.*, I Abt., **87**, 401 (1921).

batches, preparations 1, 2 and 3; *Rhizobium trifolii* 205 (clover), three batches, preparations 4, 5 and 6; *Rhizobium trifolii* 201 (clover), one batch, preparation 7; *Rhizobium leguminosarum* 311 (pea), two batches, preparations 8 and 9. The media used in the preparation of these samples and the yields of gum in grams per liter are given in Table I.

Table I

The Composition of the Culture Media Used in the Different Batches

	Alfalfa Preparation, g./liter			Red clover Preparation, g./liter				Pea Preparation, g./liter	
Compounds	1	2	3	4	5	6	7	8	9
Sucrose	...	10.0	10.0	...	...	...	...	...	...
Mannitol	10.0	...	...	10.0	10.0	10.0	6.0	10.0	6.0
Peptone	...	0.5	0.5	...	...	...	...	...	...
KNO_3	...	...	...	0.2	0.2	0.2	...	0.2	...
$NaNO_3$	...	...	...	...	...	...	0.05	...	0.05
NaCl	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.1
$MgSO_4$	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.1
K_2HPO_4	0.4	2.0	2.0	0.5	0.5	0.5	0.4	0.5	0.4
$CaCO_3$	0.4	...	...	1.0	3.0	...	...	...	...
$CaSO_4$	...	...	...	0.1	0.1	0.1	0.1	0.1	0.1
Yeast water, 10%	5 cc.	...	...	...	...	...	...	...	...
Agar	16.0	...	...	...	20.00	20.0	15.0	...	13.0
Yields of gum (dry weight), g. per liter	0.686	0.481	0.706	0.530	0.665	1.052	0.295	1.019	0.290

The general procedure for preparing the samples was first to test the purity of the cultures at the end of fermentation by inoculating sterile litmus milk and potato slants. All contaminated cultures were discarded. In the case of the agar cultures, the surface growth was washed from the agar and filtered through a thick layer of absorbent cotton to remove any pieces of agar. The liquid cultures were so viscous that dilution was usually desirable. The gum solution and bacterial suspension was then run through a Sharples supercentrifuge rotating at a speed of about 42,000 r. p. m. This treatment removed most of the bacteria from suspension, while the gum remained dissolved in the liquid. Concentration of the gum solution was effected in a vacuum pan at 60° and the gum precipitated by adding two volumes of acetone. This precipitate was redissolved in warm water and again precipitated, and a second resolution and precipitation conducted in the same manner for further purification of the gum. The stringy coagulum, when dried, gave a grayish-yellow cake, which was ground to an impalpable powder and dried to constant weight.

It was observed that when calcium carbonate was used in the medium, the gum precipitated as a stringy mass, while in the other media, it came down as flocculent particles. Better precipitation of the gum was found to take place in all cases when calcium chloride was added to the gum solution. This procedure was then followed to insure a maximum yield of the gum. This improved precipitation may be due to a chemical union of calcium with the gum—later work showed that the gum molecule contains a sugar acid.

Analytical Methods.—The ash was determined by ignition of 50-mg. samples. *Uronic acid anhydride* determinations were made by the pro-

cedure of Dickson, Otterson and Link.[10] The term "uronic" acid is here applied to glucuronic and galacturonic acids. Decarboxylation of the uronic acids is effected by means of hydrochloric acid and the carbon dioxide driven off estimated by absorption in alkali. For *pentosan* determinations, the Youngburg[11] micro-method of distillation with phosphoric acid was used. *Sugars* were determined by the micro-method of Stiles, Peterson and Fred.[12]

Composition of Gum.—In Table II are given the analytical results. The ash content of the samples is not consistent. Gum from agar cultures contained less ash than that from liquid cultures inasmuch as the separation from the salts of the medium was more easily accomplished in the former cases.

TABLE II

ANALYTICAL DATA ON THE DRY GUM PRODUCED BY THE ROOT NODULE BACTERIA

Prepn.[a]	Sample	Ash, %	Uronic acid anhydride, ash-free basis, %	Pentosans (apparent) furfural × 1.71 (ash-free), %	Pentosans (actual)[b] (ash-free), %	Reducing sugar as glucose (ash-free basis), %
1	Rhizobium meliloti 100 a	6.1	4.1	7.5	6.2	72.8
2	Rhizobium meliloti 100 b	52.8	6.8	4.3	2.2	
3	Rhizobium meliloti 100 c	26.2	4.5	5.9	4.5	
4	Rhizobium trifolii 205 a	15.5	22.1	11.3	4.5	
5	Rhizobium trifolii 205 b	9.6	24.8	16.4	8.6	
6	Rhizobium trifolii 205 c	7.9	22.3	16.1	9.2	75.2 67.3
7	Rhizobium trifolii 201	9.2	25.3	10.5	2.7	82.2 75.6
8	Rhizobium leguminosarum 311 a	21.6	19.3	10.3	3.9	61.2 52.8
9	Rhizobium leguminosarum 311 b	10.8	22.0	12.6	5.8	

[a] In Sample 1, calcium carbonate in medium but no calcium chloride was added to the gum solution. In Samples 2 and 3, no calcium carbonate in medium and no calcium chloride was added to the gum solution. In Samples 4 and 5, calcium carbonate was present in the medium and calcium chloride was added to the gum solution. In Samples 6, 7, 8 and 9, no calcium carbonate in medium, but calcium chloride was added to the gum solution. [b] (Total furfural − furfural from uronic acid) × 1.71.

The uronic acid content of the gums shows great variation. Pea and red clover nodule bacteria gums contain between 19.3 and 25% of the acid anhydride, while the gum of alfalfa nodule bacteria contains only about one-fourth as much anhydride.

In the calculations of the pentosan figures given in Table II, the furfural obtained by the Youngburg method of analysis was multiplied by 1.71, the average figure given in Kröber's tables[13] for a like calculation. The

[10] A. D. Dickson, H. Otterson, and K. P. Link, THIS JOURNAL, **52,** 775 (1930).

[11] G. E. Youngburg, *J. Biol. Chem.*, **73,** 599 (1927).

[12] H. R. Stiles, W. H. Peterson and E. B. Fred, *J. Bact.*, **12,** 427 (1926).

[13] C. A. Browne, "A Handbook of Sugar Analysis," John Wiley and Sons, Inc., New York, 1912.

general trend of the figures is toward high values when the uronic acid content is high, and low figures for the samples which contain less uronic acid.

The sixth column in Table II is headed "Pentosans (actual)." Both uronic acids and pentoses will yield furfural when heated with phosphoric acid. If the amount theoretically obtainable from the uronic acid is subtracted from the total furfural a small difference is left. This remainder was calculated to pentosans, and the values are given under the column headed "Pentosans (actual)." The figures vary from 2.2 to 9.2%.

The last column of Table II gives the percentage (ash-free basis) of reducing sugar calculated as glucose which was yielded on hydrolysis of the gums. The amount of reducing substance obtained ranged from 52.8 to 82.2% of the ash-free gum. Although the reducing effect was calculated as glucose, the reduction is not necessarily due to this sugar alone, since other substances such as furfural or uronic acid would likewise reduce Fehling's solution. Our results are not in agreement with those of Grieg-Smith,[8] who reported that the gums of pea and lupine nodule bacteria were hydrolyzed quantitatively to reducing sugar.

Hydrolysis of Gums, and Identification of Sugars by Chemical Means

Tests were made on Preparation 1 (alfalfa nodule bacteria) to ascertain what conditions of hydrolysis gave the maximum yield of sugars. Five per cent. (by weight) sulfuric acid was used as the hydrolytic agent. Although refluxing the acid solution of the gum for ten hours produced the maximum yield of reducing sugar (72.9), autoclaving for two hours at 15 pounds' steam pressure was a more convenient method of hydrolysis and gave only a slightly lower yield of sugar (68.4%). The latter method was therefore adopted as the general procedure. The acid was neutralized with barium carbonate, the barium sulfate filtered off, washed free of reducing sugars and the filtrate evaporated to about 50 cc. At this point decolorization was effected with bone charcoal and the filtrate again evaporated to a small volume. On pouring this sugar solution into four volumes of 95% alcohol, a flocculent precipitate formed at once. This precipitate was probably uronic acid. Ehrlich and von Sommerfeld[14] obtained barium galacturonate from the hydrolysis of pectins at this point. The precipitate gave a strong naphthoresorcinol test for uronic acid. A mucic acid test was negative, so that the acid seems to be *glucuronic*. All of the preparations of which the hydrolysis products were studied (Numbers 1, 6, 7, 8) gave strong naphthoresorcinol tests. Work is now in progress to identify this acid by the preparation of its characteristic derivatives.

Hydrolysis of Preparation 1.[15] (Alfalfa Nodule Bacteria Gum.)—The carbon content of this preparation was found to be 40.6% when analyzed by the micro-method of Lochte.[16] Analysis for total nitrogen by the Kjeldahl method gave no nitrogen beyond the experimental error.

The gum was hydrolyzed by the procedure already given and glacial acetic acid was added to the concentrated sugar solution. Crystallization took place after several days. The crystals were filtered off, washed with glacial acetic acid, redissolved in a minimum

[14] F. Ehrlich and R. v. Sommerfeld, *Biochem. Z.*, **168**, 263 (1926).

[15] The work on the preliminary hydrolyses, the crystallization and identification of glucose was performed by Mr. W. B. Sarles.

[16] H. L. Lochte, This Journal, **48**, 1301 (1926).

quantity of water and again recrystallized after the addition of glacial acetic acid. After filtering and washing, the crystals were dried to constant weight in a vacuum desiccator. The weight of sugar obtained was 0.3158 g. This sugar was dissolved in 25 cc. of water, a drop of ammonia was added to bring about constant rotation and readings were made in a 200-mm. tube at 20°: reading, +7.75° Ventzke scale, $[\alpha]_D^{20}$ +53.2°; $[\alpha]_D^{20}$ for *d*-glucose, +52.8°.

Hydrolysis of Preparation 7. (**Red Clover Nodule Bacteria Gum.**)—The sugar was used for qualitative tests, and osazone preparations. Qualitative tests: aniline hydrochloride and phloroglucinol tests for furfural were positive. A uronic acid, as well as a pentose, would yield furfural under the conditions of these tests. Grieg-Smith[8,2] reports that the gums which he examined gave furfural on hydrolysis.

To the sugar in solution, phenylhydrazine hydrochloride and sodium acetate were added in amounts such that the ratio of the three compounds was 1, 2, 3. Yellow crystals formed after seventeen minutes heating in a boiling water-bath. The crystals were filtered off, recrystallized twice from a mixture of pyridine and 50% alcohol and twice from pyridine alone. The crystals occurred in fan-shaped aggregates of yellow needles. The melting point was 204°.

The osazone of glucose was prepared in the same way and recrystallized four times from pyridine. The preparation melted at 204°. Browne[13] gives the melting point of phenylglucosazone as 204–205°.

Hydrolysis of Preparation 6.—The phenylosazone of the reducing sugar was prepared as previously indicated and purified by six recrystallizations from pyridine. Its melting point was 204°.

Qualitative tests for mannose (hydrazone test) and fructose (Seliwanoff test) were made on the sugar solution after hydrolysis of the gum with negative results in both tests. When mannose and fructose equivalent to 5% of the total reducing sugar present were added to the hydrolysis product, positive tests were obtained. It is probable, therefore, that if these sugars are contained in the gum molecule, they constitute only a small percentage of the total sugar.

Hydrolysis of Preparation 8. (**Pea Nodule Bacteria Gum.**)—The phenylosazone of the sugar was prepared, and recrystallized seven times from pyridine. The melting point was 204°. Qualitative tests were made for mannose and fructose as under the hydrolysis of Preparation 6, and both tests were negative.

Identification of Sugar by Fermentation Tests

The above evidence shows that glucose is a constituent of the gum, but that fructose and mannose are absent. The work of Grieg-Smith[8,2] indicates the possibility of the presence of galactose. Pentoses may also be present, as was suggested by the furfural figures (Table II). The chemical determination of such a mixture of sugars would be difficult, if not impossible. It should be possible, however, by means of fermentation tests to prove the presence or absence of these sugars. This method of sugar determination has been suggested and used in various ways. Kluyver[17] used yeast alone for the sugar determination, while Sherrard and Blanco[18] used yeast fermentation followed by fermentation with bacteria. By means of galactose and non-galactose fermenting yeast, the presence or absence of this sugar could be demonstrated. Likewise, the use of two

[17] A. J. Kluyver, "Biochemische Suikerbepalingen," Thesis, Delft, 1914, 223 p.

[18] E. C. Sherrard and G. W. Blanco, *Ind. Eng. Chem.*, **15**, 611 (1923).

strains of bacteria, one fermenting xylose but not arabinose, the other using arabinose and not xylose, might be used in the determination and identification of any pentoses present. The organisms selected for this purpose were the following: *Schizosaccharomyces pombe, Saccharomyces cerevisiae,* yeast, Honey B and lactic acid-producing organisms Nos. 19 and 36. The *Saccharomyces cerevisiae* yeast was obtained from a single cell isolation. The yeast called "Honey B" was isolated from fermenting honey. *Schizosaccharomyces pombe* and Honey B ferment glucose but not galactose, while the *Saccharomyces cerevisiae* ferments both glucose and galactose. Culture No. 36 ferments xylose, but leaves arabinose untouched, while Culture 19 ferments arabinose, but not xylose. Analysis of a mixture of known sugars by means of these organisms was first made to determine whether the method would give satisfactory results. The sugar solutions were added to a 10% extract of fresh starch-free yeast and the P_H adjusted to 5.2; 10-cc. samples of this solution were pipetted into test-tubes, the tubes plugged with cotton and sterilized. Inoculations of the tubes were made as indicated in Table III, and after eight days' incubation at 28° the remaining sugar was determined in 1 cc. drawn from each culture tube. The remaining liquid was resterilized, sterile calcium carbonate added and inoculated with the pentose-fermenting bacteria. Sugar analyses were made on the cultures after ten days' incubation at 37°. Table III gives the results of the experiment, and indicates that satisfactory checks can be obtained by this method. The slight decrease in sugar in the uninoculated controls (Tubes 7 and 8) is probably due to loss on sterilization.

TABLE III

ANALYSIS OF KNOWN MIXTURES OF SUGAR BY MEANS OF YEASTS AND BACTERIA

	Tubes 1 and 2		Tubes 3 and 4		Tubes 5 and 6		Tubes 7 and 8	
	Yeast fermentation							
Yeast	*Saccharomyces cerevisiae*		*Schizosaccharomyces pombe*		Honey B		Uninoculated	
Type of sugar fermented	Glucose and galactose		Glucose		Glucose		....	
	1	2	3	4	5	6	7	8
Before, mg.	101.5	101.5	101.5	101.5	101.5	101.5	101.5	101.5
After, mg.	27.3	26.6	49.9	49.2	48.7	47.8	99.7	99.7
Should be present, mg.	24.2	24.2	49.0	49.0	49.0	49.0	101.5	101.5
	Bacterial fermentation							
Bacteria	No. 36	No. 19	No. 36	No. 19	No. 36	No. 19	Uninoculated	
Type of sugar fermented	Xylose	Arabinose	Xylose	Arabinose	Xylose	Arabinose		
Before, mg.	27.3	26.6	49.9	49.2	48.7	47.8	99.7	99.7
After, mg.	11.8	13.6	12.4	12.4	13.4	12.5	94.3	95.4
Should be present, mg.	11.4	12.8	11.4	12.8	11.4	12.8	101.5	101.5

The sugar solution in each tube contained the following amounts of sugar: glucose, 52.5 mg.; galactose, 24.8 mg.; xylose, 12.8 mg.; arabinose, 11.4 mg.; total, 101.5 mg.

The fermentation test just described was applied to the hydrolyzed solution of Preparations 6 and 8.

Fermentation Test on Preparation 6. (Red Clover Nodule Bacteria Gum.)—0.532 g. of sugar in solution was taken for the fermentation test. This solution was concentrated, yeast water added and the P_H adjusted to 5.2; 10-cc. portions of this solution were then pipetted into each of four test-tubes and the tubes sterilized. Three of the tubes were inoculated with yeast which would ferment only glucose, Honey B and *Schizosaccharomyces pombe*, and the other one with the yeast which would ferment both glucose and galactose, *Saccharomyces cerevisiae*. The tubes were incubated at 28°. Eight days after inoculation the cultures were made up to 10 cc., and 1 cc. was removed. This was then made up to 5 cc., and 1 cc. of this last dilution taken for micro sugar analysis.

As has been indicated, the presence of pentoses was suggested by the furfural data (Table II). The pentoses present would be most likely to be arabinose and xylose, it being more probable that there would be only one of these in the molecule. In order to decide this question, the solutions fermented by the yeast were submitted to bacteria fermentations. Yeasts do not normally attack pentoses, especially in the presence of a hexose, so that the pentose, if present, should still be in solution. The control fermentation of the mixture of known sugars showed that the pentoses were not attacked by the yeast. The remaining sugar solution after the yeast fermentation was again sterilized in tubes and sterile calcium carbonate added. Pure cultures of the lactic acid bacteria were inoculated into the tubes of sugar solution, and incubated at 38° for twelve days. At the end of that time the contents of each tube were made up to 10 cc., and 1 cc. of this solution was taken for micro sugar analysis.

The results of the two fermentations are given in Table IV. The yeast fermentation gave results which showed that the hexose was glucose, or that if galactose was also present, it was so in insignificantly small amounts. The difference between the amount of sugar fermented by the two bacterial cultures was so slight as not to permit the conclusion that pentoses were present. Typical fermentation data are given in detail in Table IV. The extent of recovery of galactose when added to the gum sugar solution was tested in later experiments: 98–100% was destroyed by the galactose-fermenting yeast and 3.8% fermented by the non-galactose fermenting yeast.

TABLE IV

FERMENTATION OF SUGARS FROM PREPARATION 6 (RED CLOVER ROOT NODULE BACTERIA GUM)

	Tube 1	Tube 2	Tube 3	Tube 4
	Yeast fermentation			
Yeast	Honey B	Honey B	*Saccharomyces cerevisiae*	*Schizosaccharomyces pombe*
Type of sugar fermented	Glucose	Glucose	Glucose and galactose	Glucose
Before, mg.	267	267	267	267
After, mg.	26.6	24.7	22.3	26.6
Sugar fermented, mg.	240.4	242.3	244.7	240.4
	Bacterial Fermentation			
Bacteria	No. 36	No. 19	No. 36	No. 19
Type of sugar fermented	Xylose	Arabinose	Xylose	Arabinose
Before, mg.	26.6	24.7	22.3	26.6
After, mg.	16.0	16.6	15.6	16.8
Sugar fermented, mg.	10.6	8.1	6.7	9.8

A second fermentation experiment (data not given) was conducted in the same manner as the one above. *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were the yeasts used in this experiment, since Honey B grew more slowly and gave no additional information. The percentage of sugar fermented by the yeasts was higher in this experiment than in the first. The reducing substance remaining after the yeast fermentation is probably uronic acid, which is generally believed to be unfermentable by yeasts. The controls of known sugars show that the yeast fermentation is complete to the extent of nearly one hundred per cent. In the gum sugar solutions the bacteria do not, then, ferment only the portion of the hexose left by the yeast, but also a part of some other reducing substance still in solution. It may be possible that they are able to ferment the uronic acid. At the same time this fermentation does not indicate the presence of a pentose. The difference in percentage of fermentation of the sugar by the yeast between the first and second fermentations may be explained by the fact that in the first fermentation calcium carbonate had been used to neutralize the acid after hydrolysis of the gum, while in the second fermentation, barium carbonate was used. Since barium salts are generally less soluble than calcium salts, there was probably more complete removal of the uronic acid when barium carbonate was used. The reducing material left after the yeast fermentation appears to be the uronic acid, or its salts.

In the second bacterial fermentation of the sugars produced by hydrolysis of Preparation 6, the sugar contents of only two of the six tubes used in the fermentation could be determined. The other four tubes contained some material which prevented the appearance of the usual iodine end-point. In these cases, after titration to the end-point, the blue color reappeared.

Fermentation Test on Preparation 8. (Pea Root Nodule Bacteria Gum.)—The fermentation test was conducted in the same manner as was given under the fermentation of Preparation 6, two complete sets of fermentations being made in this case as in the one above. In Table V it will be seen that the extent of fermentation of the sugar by yeast is not as great as it was on the sugars from Preparation 6. The presence of galactose is not indicated by the results obtained.

The percentage of hexose fermented by the yeast in the second fermentation (Prepn. 8) was larger than it was in the first. This is probably due, as seemed to be the case with the fermentation of sugars from Preparation 6, to the use of barium carbonate instead of calcium carbonate as the neutralizing agent of the hydrolysate in the second fermentation.

The bacterial fermentation in the second fermentation gave results which did not indicate the presence of a pentose.

Table V gives a summary of the results of the fermentations on the hydrolyzed gum of Preparations 6 and 8. The figures given under each preparation are the average of two complete fermentation tests like that represented in Table IV. A comparison of the percentages of sugar fermented by the galactose and non-galactose fermenting yeast indicates that if galactose is present at all, it is so in amounts of 1% or less. Likewise, the bacterial fermentations demonstrated that there is less than 1% of a pentose present, and it is likely that no pentose is present at all. The small differences between these fermentations cannot safely be attributed to any other cause than the biological variation inherent in such a method. The results of the yeast fermentation tests show that the hexose obtained on

hydrolysis of the gum of both red clover and pea root nodule bacteria gum was glucose. The bacterial fermentations do not give evidence of the presence of a pentose sugar.

TABLE V

THE COMPOSITION, AS SHOWN BY FERMENTATION TESTS, OF THE GUM SUGAR OBTAINED FROM HYDROLYSIS OF PREPARATIONS 6 AND 8

		Preparation 6 Percentage of total sugar		Preparation 8 Percentage of total sugar	
Yeast	Glucose fermenting	92.6	93.1	82.9	83.2
	Galactose and glucose fermenting	93.6		83.6	
Bacteria	Xylose fermenting	3.2	3.0	5.6	5.2
	Arabinose fermenting	2.8		4.7	
Unfermented reducing substance			4.3		11.6
			100.4		100.0

Conclusions

Root nodule bacteria of three cross-inoculation groups were grown in pure culture on synthetic media, and the gum produced was precipitated with acetone. The gum of the root nodule bacteria of alfalfa, *Rhizobium meliloti* 100, was nitrogen-free. The carbon content of this gum was variable, being 40.6% in one sample, and 36.4% in another. Glucose was crystallized from the gum solution after hydrolysis, and identified by its specific rotation (+53.2°).

Glucosazone was prepared from hydrolyzed gum produced by pure cultures of red clover root nodule bacteria, *Rhizobium trifolii* 205 and 201, and pea root nodule bacteria, *Rhizobium leguminosarum* 311. Fermentation tests of the sugar from these two gums by pure cultures of known yeasts also indicated that the sugar was glucose. Fermentation tests by pure cultures of pentose-fermenting lactic acid bacteria showed the absence of a pentose sugar.

The gums of *Rhizobium meliloti* 100, *Rhizobium trifolii* 205 and 201, and *Rhizobium leguminosarum* 311 contain uronic acid in amounts varying between 4.1 and 25.3% ash-free basis.

All of the results up to the present time indicate that these gums of the root nodule bacteria are complexes of glucose and a uronic acid, probably glucuronic acid.

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