INFRARED SPECTROPHOTOMETRY OF HANSENULA AND SACCHAROMYCES WHOLE YEAST CELLS AND YEAST CELLULOSE

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Received for publication March 1, 1954

A recent report by Stevenson and Bolduan (1952) on the application of infrared spectrophotometry for identification of bacteria has stimulated further interest in the use of such methods for studying certain physiological processes of microorganisms. Levine *et al.* (1953*a, b*) used infrared spectrophotometry for the identification of glycogen in whole bacterial cells, whereas Bailey and co-workers (1953) observed the effect of antibacterial agents on the infrared spectrum of *Micrococcus pyogenes* var. *aureus* and are continuing their work towards the use of this tool for relating chemical structure to mechanism of activity.

During the course of studying the nitrogen metabolism of *Hansenula anomala*, it was found expedient to determine the infrared spectrum of this yeast in an attempt to detect certain metabolic intermediates. This investigation subsequently led to a characterization of the whole cell spectrum of *H. anomala* from 6.0 to 12.0 μ and an association of this spectrum with that of the cellulose fraction of this yeast. These spectra were then compared with the corresponding spectra of *Saccharomyces cerevisiae*.

MATERIALS AND METHODS

A stock culture of *H. anomala*, N.R.R.L. Y365, was maintained at refrigeration temperature on an agar slant containing glucose, 5 per cent; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 per cent; KH_2PO_4 , 0.1 per cent; and $(\text{NH}_4)_2\text{SO}_4$, 0.1 per cent.

A small loopful of this yeast was transferred to three main types of liquid media for culture. Their respective compositions were as follows: medium 1—glucose, 1.0 per cent; KNO_3 , 0.1 per cent; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 per cent; and KH_2PO_4 , 0.1 per cent; medium 2—glucose, 5.0 per cent; KNO_3 , 0.1 per cent; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 per cent; and KH_2PO_4 , 0.1 per cent; media 1*a*, 2*a*—media 1*a* and 2*a* differed from their counterparts by having $(\text{NH}_4)_2\text{HPO}_4$ as the nitrogen source in

place of KNO_3 ; medium 3—glucose, 5.0 per cent; peptone (Difco), 0.5 per cent; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25 per cent; and KH_2PO_4 , 0.2 per cent. Five hundred ml of each medium were placed in 2 L flasks and inoculated with *H. anomala*. Then they were incubated for 14 and 24 hours in stationary cultures or 4 days on a shaker. The cells grown for 4 days on a shaker were harvested, washed, and resuspended in one L of nitrogen-free medium and aerated for 24 hours in order to attain nitrogen starvation, according to the technique of Virtanen and co-workers (1949). Cells were also grown in stationary cultures for one week in media 2, 2*a*, and 3. All cultures were incubated at 30 C.

The cells grown under the above conditions were centrifuged for 10 minutes at 2,400 rpm and washed twice with distilled water. The cells of each type of growth were smeared with a loop on a silver chloride plate, and the films that were thus formed were allowed to dry at room temperature. These were then used for spectrophotometric examination.

The cellulose was separated from the remainder of the whole cells by suspending the washed cells in 150 ml of 5 per cent NaOH, similar to the method used by Forziati and Rowen (1951) for bacteria with the exception that a mineral oil layer was spread over the alkaline yeast suspension instead of a nitrogen atmosphere in order to prevent oxidation of the cellulose during the separation process. After 3 days' standing at room temperature, the oil was removed from the surface of the suspension with a separatory funnel. The alkali-cellulose was centrifuged down and washed with 5 per cent NaOH. The supernate was discarded. The washed alkali-cellulose was then spread on a glass plate with a 15 mil film applicator and the cellulose regenerated by steeping in 0.5 per cent acetic acid for 30 minutes. The film was then washed with water until all traces of acid were removed as indicated with

litmus paper. The glass plate with the cellulose film was allowed to dry at room temperature. The cellulose film was removed from the glass plate with a razor edge and mounted on a cardboard disc with a 3 by 15 mm slit. The thickness of the dried film averaged $7.6\ \mu$.

The infrared spectrophotometric characteristics of the alkali soluble but the alcohol insoluble fraction of *H. anomala* were also determined. Four day old cells, grown in medium 3, were harvested, washed twice with water, and then digested in one per cent NaOH for 3 days at room temperature, while covered with a mineral oil layer. This was done in order to separate the yeast cellulose from the remainder of the cell material (Shirk and Greathouse, 1952). This is as effective as the 4 per cent NaOH (Forziati and Rowen, 1951) and reduces the degree of hydrolysis of the cell proteins by the alkali (Vickery, 1922). The yeast cellulose was removed by centrifugation, and the supernate was acidified to a pH of 7.0 with 10 per cent HCl. Four volumes of 95 per cent ethanol were added. The alcohol precipitated a white substance which was removed by centrifugation, redissolved in water, and reprecipitated. Hereafter, we shall refer to the white substance as the alcohol precipitate. A portion of the alcohol precipitate was transferred to a silver chloride plate, dissolved in a drop of water, then spread evenly across the plate in the path of the infrared beam and allowed to dry. The infrared spectrum of the alcohol precipitate was recorded from 6 to $12\ \mu$.

About 10 g of *S. cerevisiae* (Fleischmann's Yeast, Standard Brands, Inc.) were suspended in 150 ml of distilled water. The cells were centrifuged and the supernate discarded four times. The washed whole cells were smeared on a silver chloride plate, as above. A yeast cellulose film was prepared from *S. cerevisiae* as was done with *H. anomala*. *S. cerevisiae* cellulose was also prepared by digesting the yeast cells in 3 per cent NaOH at 100 C and then in 0.5 N acetic acid at 75 C to remove the trapped glycogen, according to the method of Northcote and Horne (1952).

For our cellulose I material, we used a wood pulp cellulose (Solka-Floc BW200, Brown and Company, 99 per cent α -cellulose) from which the lignin and tannins had been removed by treatment with 5 to 6 per cent alkali and sulfite, respectively. The cellulose was mulled in mineral oil (Stanolind), and the slurry was used to obtain an infrared spectrum. In order to obtain the de-

sired spectrum in the 6.5 to $7.5\ \mu$ region, mineral oil was placed in a cell in the path of the reference beam to compensate for the absorption by the mineral oil in that region. A regenerated wood pulp cellulose film was used for a cellulose II sample. All the spectrophotometric measurements were obtained with a Perkin-Elmer, model 21, double beam recording spectrophotometer.

EXPERIMENTAL RESULTS AND DISCUSSION

A characteristic whole cell spectrum of *H. anomala* grown for 4 days or one week, under each of the experimental growth conditions indicated above, is shown in figure 1 (A). Certain associations may be made with the absorption bands of the whole cell spectrum and specific chemical groupings. Among these are the absorption bands at $6.05\ \mu$ and $6.45\ \mu$ which are generally associated with the strong absorption of the C=O vibrations and the deformation of the N-H group of the peptide linkage, respectively (Darmon and Sutherland, 1947; Elliott and Ambrose, 1950; Klotz and Griswold, 1949; Klotz *et al.*, 1949). The weaker band at $6.9\ \mu$ may be ascribed to the deformation of the CH₂ group in polypeptides (Darmon and Sutherland, 1947) and that at $7.0\ \mu$ to the CH bending in proteins (Klotz and Gris-

Figure 1. Infrared spectra of whole yeast cells, yeast cellulose, purified wood cellulose, and regenerated cellulose.

wold, 1949; Klotz *et al.*, 1949). The yeast cellulose spectrum (figure 1 (B)) also has minor absorption bands in these regions.

The $8.0\ \mu$ band of the whole cell spectrum may be assigned to the $P=O$ vibrations of nucleic acids (Gore, 1950; Fraser and Fraser, 1951). Other more intense bands for nucleic acid absorption appear at about 9.3 and $9.5\ \mu$ (Fraser and Fraser, 1951). These are due to C_1-O-C_4 vibrations in the sugar residues and to the $C-O-P$ linkages of nucleic acids (Bellamy and Beecher, 1952a, b). Carbohydrates also absorb strongly in the 9 to $10\ \mu$ region (Kuhn, 1950; Forziati and Rowen, 1951; Shirk and Great-house, 1952). Levine *et al.* (1953a) working with the whole cell spectra of enteric bacteria have assigned the band at about $8.0\ \mu$ largely to nucleic acid absorption and the 8.6 to $10\ \mu$ band partly to nucleic acid absorption and partly to polysaccharide absorption. However, the intensity of our yeast cellulose absorption in the 8.6 to $10\ \mu$ band (see figure 1 (B)) indicates that this substance probably contributes in large measure to the characteristic absorption of the whole cell spectrum in that region, particularly by the presence of the doublet at about the 9.3 to $9.6\ \mu$ region in both the whole cell spectrum and the yeast cellulose spectrum. The lack of other substances in high concentration which may absorb in this region was verified by the nature of the infrared spectrum of the alcohol precipitate (see figure 2) prepared from the cell material soluble in one per cent NaOH according to the procedure listed above. The infrared spectrum of the alcohol precipitate has strong absorption in the $6\ \mu$ region and weaker bands at higher wavelengths. This spectrum is similar to the infrared spectrum

Figure 2. Infrared spectrum of soluble *Hanse-nula anomala* cell material precipitated by alcohol after digestion with NaOH.

of proteins (Darmon and Sutherland, 1947; Elliott and Ambrose, 1950; Klotz and Griswold, 1949; Klotz *et al.*, 1949) for the reasons stated earlier in this paper.

Differences in carbohydrate concentration of nitrogen source and of length of incubation time were made in an attempt to alter the cell composition or yeast cellulose orientation; however, all the different samples gave similar patterns for the whole cells, whether incubated for 4 days or one week. The infrared spectra for the cellulose fractions of the various yeast cultures likewise were similar to each other. The fact that the whole cell spectra remain constant would indicate that regardless of the variations in growth conditions that were made, the cell material remained qualitatively the same as detected by infrared spectrophotometry.

Levine *et al.* (1953a) found that the infrared spectra of 18 hour cultures of enteric bacteria differed from those of older cultures. We tested the effect of incubation time on the infrared spectra of *H. anomala* by culturing the yeast in medium 1a for 14 and 24 hours at 25 C. The washed cells were spread on a silver chloride plate, and the infrared spectrum was recorded. Differences in the spectra of these cells were noted only in the 9 to $10\ \mu$ region. These are indicated in figure 3. The 24 hour cells had an infrared spectrum similar to that of older cells (figure 1 (A)). It was characterized by a doublet in the 9.3 to $9.6\ \mu$ region, whereas the 14 hour cells had one broad absorption band in the same region. Preliminary experiments on the spectra of the cell material insoluble in 4 per cent NaOH indicate that the insoluble substance from the 14 hour cells has little or no absorption in the 9 to $10\ \mu$ region, whereas the insoluble material from the 24 hour cells has strong absorption bands in this region, which is somewhat different from that of the yeast cellulose spectrum of the older cells (figure 1 (B)) in that there is an attenuation of the absorption at the $9.3\ \mu$ region of the doublet.

The whole cell spectrum of *S. cerevisiae* was like that of *H. anomala* (see figure 1 (A)). The *S. cerevisiae* cellulose spectrum regardless of the method of preparation, whether by the method of Forziati and Rowen (1951) or Northcote and Horne (1952), was identical with cellulose spectrum of *H. anomala*.

It is noteworthy that the yeast cellulose spectra which we studied were similar to that of type II

cellulose and not like bacterial cellulose which has a spectrum like that of type I cellulose, as reported by Forziati and Rowen (1951). The typical pattern of type I cellulose found in bacteria, wood, and cotton is characterized by distinct absorptions of nearly equal intensities at wavelengths of about 7.0, 7.3, 7.4, and 7.5 μ in the 7 μ to 8 μ region. A similar pattern was obtained by us with wood pulp spectrum of cellulose I, which is shown in figure 1 (C). In the region of 8.5 to 10 μ , type I cellulose has distinct and sharp maxima at approximately 8.6, 9.0, 9.4, and 9.6 μ with the greatest absorption at 9.4 μ (figure 1 (C)). With type II cellulose, or regenerated cellulose, or the yeast cellulose obtained by us from *H. anomala* or *S. cerevisiae* (see figure 1 (B) and (D)), the absorption at 7.4 is absent, but there is a definite absorption at 7.3 μ . In the 8.5 μ to 10.0 μ band, the absorption at 9.0 μ , 9.4 μ , and 9.6 μ is absent as distinct and definite entities as in cellulose I, but these absorption areas merge into each other to produce a deep and broad pattern of absorption in the 9.0 to 9.8 μ area, with two slight absorption maxima at essentially the 9.4 and 9.6 μ regions. The absorption in the 8.6 μ area is present in type II cellulose, but less intense than in type I cellulose. Also, the absorption at about 11.2 μ is more intense in type II cellulose than in type I cellulose.

The cellulose of *S. cerevisiae* was identified by Hassid and co-workers (1941) and by Bell and Northcote (1950) as a 1:3 β -glucan. This indicates that cellulose II, a 1:4 β -glucan, and *S. cerevisiae* yeast cellulose, a 1:3 β -glucan, have similar infrared spectra. Whether *H. anomala* yeast cellulose is a 1:4 β -glucan of type II cellulose or a 1:3 β -glucan will be resolved by further investigation.

The infrared spectrum of cellulose bears certain similarities to the glycogen spectrum, as reported by Levine *et al.* (1953b, c), and has a strong absorption band at about 9.6 μ which is for all practical purposes in the same region as the characteristic glycogen wavelength at 9.75 μ . We are in agreement with Levine and co-workers (1953b, c) that the infrared method for the determination of glycogen and similar substances in whole microbial cells should be applied only where interfering polysaccharides are absent and where absolute values are not of utmost importance; otherwise, the interfering polysaccharide should be removed, or the glycogen separated from the

Figure 3. Infrared spectra of *Hansenula anomala* whole yeast cells after growth for (A) 14 hours, and (B) 24 hours at 25 C.

cellular materials by chemical means prior to analysis.

ACKNOWLEDGMENT

The authors are pleased to thank Dr. P. H. Lindenmeyer of the Visking Corporation, Chicago, Illinois, for his advice and assistance in producing the infrared spectra.

SUMMARY

The infrared spectra of whole cells of *Hansenula anomala* were obtained under various conditions of growth and compared with the spectra of the cellulose fractions of these cells. The whole cell spectrum of *H. anomala* after 14 hours' growth differed from that of cells 24 hours and older in the 9 to 10 μ region. After 24 hours, the whole cell spectrum did not change. Similarities were noted in the infrared spectra of the whole cells and the yeast cellulose which indicated that the yeast cellulose contributed substantially to the whole cell spectrum, especially in the 9.0 μ to the 10.0 μ region. Further, regardless of the variations in growth conditions, the whole cell spectrum and the yeast cellulose spectrum of the old cells remained the same. This would indicate that

the cell material remains qualitatively constant under varied growth conditions and also that the yeast cellulose orientation, as determined by infrared spectrophotometry, does not change. The infrared spectra of *Saccharomyces cerevisiae* whole cells and *S. cerevisiae* cellulose were like the whole cell spectra of *H. anomala* and *H. anomala* cellulose, respectively. Both yeast cellulose spectra were found to be similar to the infrared spectrum of type II cellulose, rather than type I cellulose which is present in bacteria.

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