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PROTEINS OF YEAST (*SACCHAROMYCES CEREVISIÆ*)*

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In the biological analysis of proteins yeast is commonly added to the diet in order to supply certain vitamins. Yeast protein is a good source of some of the nutritionally essential amino acids, and its addition to the experimental diet may in certain cases introduce errors by supplying amino acids in which the protein studied may be deficient. The magnitude of the error caused by the addition of yeast to the experimental diet could be estimated from the amino acid composition of the yeast proteins, but information on this point is very meager.

It is assumed that the protein in the yeast cell is present in conjugation with nucleic acid. At least, no one has obtained a preparation other than nucleoprotein or a derivative thereof. The proportion of nucleic acid to the protein varies widely in these preparations, judged from the amount of phosphorus found. In experimental diets whole yeast is generally added and not a yeast protein preparation. It has been found, however, that a direct acid hydrolysis of the whole yeast is not always suitable for amino acid determination. Cystine and histidine, for instance, were found for some reason not yet explained to be mostly decomposed.

As a rule, investigators in the past have used alkaline solvents in rather high concentration to extract the yeast protein, which undoubtedly changed the physical behavior and chemical composition of their preparations. Dreyer (1) used a 10 per cent ammonium carbonate solution and extracted 65.7 per cent of the

* The material in this paper was presented before the Twenty-seventh and Twenty-eighth meetings of the American Society of Biological Chemists (*J. Biol. Chem.*, **100**, xxxiii (1933); **105**, xix (1934)).

total yeast nitrogen. He claimed that there are two proteins in the yeast, an albumin and a globulin, but he did not consider them as nucleoproteins, which was undoubtedly the case. Thomas (2) separated two preparations by partial autolysis, a paranucleo-protein, slightly soluble in 10 per cent sodium chloride solution and non-coagulable by heat, and an albumin coagulable in water at 40°. He analyzed these preparations for their nitrogen distribution according to Hausman's method and determined the basic amino acids by the method of Kossel and Kutscher. Meissenheimer (3) determined the amino acid content of an autolyzed yeast digest, from which he calculated approximately the amino acid composition of the yeast protein.

Both water and 10 per cent sodium chloride solution extract surprisingly small quantities of nitrogenous material from the untreated yeast cell; even a 0.2 per cent aqueous sodium hydroxide solution removes only about half the total nitrogen. By subjecting yeast cells to the action of ether prior to extraction with protein solvents Buchner and Gruber (4) in 1899 were enabled to remove a considerable part of the yeast protein. Later (1902), Schröder (5) by employing the same method prepared the water-soluble and heat-coagulable yeast protein, and determined its basic amino acid content. He also showed the presence of cystine, leucine, tyrosine, and phenylalanine. Recently, Chibnall and his associates (6) in their extensive work on the proteins of green leaves and grasses have developed and very successfully applied the use of ether in the plasmolysis of leaves for the separation of proteins.

In the work described in this paper ether treatment of the yeast previous to extraction by solvents was applied. The time of ether and yeast contact was varied in order to show the effect of the ether treatment on the distribution of nitrogen as extracted by different solvents.

The action of ether is 2-fold: first, it kills the yeast cell; and secondly, by removing lipid substances it makes the cell wall more permeable to the solvents used. The experiments were carried out at low temperatures (6–8°); however, autolytic and enzymatic activities were not prevented. The increase of the coagulable protein to a maximum after which less protein was obtained as the time of ether contact with the yeast cell was increased serves as an

indication that such reactions were taking place within the dead yeast cell. The protein preparations obtained are not to be considered as representing components of the living yeast cell, but are undoubtedly secondary products. This conclusion is reached from the nature of the results demonstrated in the experimental part.

Van Slyke's nitrogen distribution method is not applicable to the yeast nucleoprotein preparations because of the presence of purine and probably pyrimidine bases. Although the purines are unstable during acid hydrolysis at the acid concentration and time duration stipulated by Van Slyke, they are not decomposed completely. The presence of the bases interferes with the nitrogen distribution in practically every phase of the Van Slyke method. The dibasic amino acids were determined quantitatively, therefore, in the yeast protein preparations as well as in the whole yeast hydrolysate by the Vickery and Block (7) method as described in the experimental part. The cystine, tryptophane, histidine, and lysine content of the yeast protein places it in a favorable position among those considered of good quality. The amino acid composition of brewers' and bakers' yeast proteins is very similar.

EXPERIMENTAL

Effect of Ether on Living Yeast Cell—The bakers' and brewers' yeasts used in this investigation were prepared by two commercial concerns without the addition of fillers and washed free from nutrients. The time factor in the ether treatment of the yeast cell was established on both yeast preparations as follows: 10 to 12 gm. of the yeast press-cake were weighed into a 100 cc. centrifuge flask, and 30 to 40 cc. of ether were added to cover the yeast. The liquefaction of the yeast, hastened by occasional stirring, takes place within a few minutes. The ether was poured off after the required time interval, and four extractions, each lasting from $\frac{3}{4}$ to 1 hour, were carried out on the residue with each solvent. The extracts were separated from the solids by centrifugation, syphoned off, and combined in a measuring cylinder, extracts of different solvents being kept separate. After the final extraction, the yeast residue was transferred to a Kjeldahl flask for the determination of unextracted nitrogen. All the samples and extracts

were kept at refrigeration temperatures of 6–8°. Nitrogen was determined in duplicate by the Kjeldahl method, aliquots from the extracts of each solvent being used (Table I).

As the time of the ether treatment increases, the unextracted nitrogen gradually diminishes, showing the source of extractable nitrogen. The water-soluble nitrogen increases with the time, while the salt-soluble nitrogen reaches a maximum and diminishes considerably toward the end of the experimental period. This behavior points to a continuous autolytic activity converting the original nucleoprotein of the yeast cell into the products here

TABLE I

Extraction of Nitrogenous Substances by Different Solvents in Percentages of Total Nitrogen of Ether-Treated Yeast

	Ether treatment of yeast	Water extract	10 per cent NaCl extract	0.2 per cent NaOH extract	Unextracted N	Heat-coagulable N in	
						Water extract	Salt extract
	<i>hrs.</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Bakers' yeast	Control	1.4	1.2	47.2	52.1		
	0.16	17.8	15.9	34.5	32.8	Trace	2.7
	1.0	19.5	21.0	27.5	34.0	"	3.5
	2.5	20.0	23.8	25.8	32.9		3.9
	18.0	30.1	25.8	33.4	13.4	9.9	8.3
	42.0	36.7	17.0	37.6	9.0	5.6	7.6
Brewers' yeast	Control	1.8	2.1	64.4	33.3		
	0.16	25.3	34.3	18.3	21.2		
	4.0	26.7	27.4	26.4	18.6		
	18.0	40.7	19.1	31.8	10.0		
	48.0	45.8	13.8	34.6	6.1		

separated. The higher the phosphorus content of the protein preparation, the more extensive was the proteolytic cleavage. The water-soluble protein preparation with the lowest phosphorus content should, therefore, represent in its amino acid composition the one nearest to that of the original yeast nucleoprotein.

Yeast Protein Preparations—The period of ether contact with the yeast which formed the largest amounts of coagulable protein was 18 to 20 hours. 500 gm. of yeast press-cake material were covered with ether, placed in a refrigerator for 18 hours, and occasionally stirred. After that the ether was syphoned off, and

1 liter of H_2O was added. The mixture was stirred vigorously and centrifuged. The water extraction was repeated twice. Each time approximately 1 liter of H_2O was added, and 1 hour was allowed for each extraction. The combined water extracts, amounting to approximately 2.5 liters, were acidified with 2 cc. of acetic acid and heated in a water bath. The water-soluble yeast protein coagulates at $47-48^\circ$, but to assure complete precipitation the temperature was brought to 60° . After cooling, the protein coagulum was separated by centrifugation. The precipitate was washed with distilled water and dehydrated in the usual way with alcohol followed by ether. The water-soluble yeast protein is also precipitable by ammonium sulfate at 65 per cent saturation.

The residue from the three water extractions was used for the preparation of the salt-soluble protein. The procedure was similar to that given above except that 10 per cent sodium chloride solution was used instead of water. The salt-soluble protein may be separated by heat coagulation ($48-50^\circ$), by the addition of ammonium sulfate up to 60 per cent saturation, or by acidification (pH 4.0) with acetic acid. Neither water dilution nor dialysis is applicable for the separation of the salt-soluble protein; the isoelectric point, pH 4.7, as determined by the method of Csonka, Murphy, and Jones (8), is a further indication that we are not dealing with a true globulin.

The yeast residue from the water and salt extractions was finally extracted with 0.2 per cent aqueous sodium hydroxide, the same volume and time of extraction being used as in the case of the previous extractions. The combined alkali extracts were acidified to pH 4 by the addition of acetic acid. It is difficult to separate this precipitate by centrifugation because of the high viscosity of the extracts. The precipitate settles overnight, however, and the supernatant liquid can then be syphoned off and the precipitate washed in acidified water. At this stage, especially if alcohol is added, the protein precipitate may be easily separated by centrifugation. The yield of these protein preparations is far from being quantitative and should be considered as minimal (Tables II and III).

Several batches of yeast were worked up for protein preparations which were subjected to elementary analyses. Phosphorus was

determined volumetrically by the ammonium phosphomolybdate method after the protein material was ashed in the presence of magnesium nitrate and the residue dissolved in dilute nitric acid. Nitrogen was determined by the Kjeldahl and sulfur by the peroxide methods, Parr's heat ignition bomb being used (9).

TABLE II

Yields of Protein Preparations Obtained from Ether-Treated (18 Hours) Brewers' Yeast and Calculated on 100 Gm. of Moisture-Free Yeast Containing 9.42 Gm. of Nitrogen

Solvents used in successive extractions in order stated	Moisture- and ash-free protein	Protein N in per cent of total yeast N
	<i>gm.</i>	<i>per cent</i>
Water.....	8.24	13.4
10% NaCl.....	2.08	3.6
0.2% NaOH.....	5.38	9.5
Total.....	15.7	26.5

TABLE III

Elementary Composition of Yeast Proteins Expressed in Percentages of Moisture- and Ash-Free Yeast Proteins

	Bakers' yeast			Brewers' yeast		
	Nitro- gen	Phos- phorus	Sulfur	Nitro- gen	Phos- phorus	Sulfur
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Coagulum from water extract by heat.....	15.65	0.968	0.906	15.29	0.55	0.684
Coagulum from salt extract by heat.....	16.26					
Protein from salt extract by acidification.....	16.06	0.29	0.790	16.19	0.33	0.733
Protein from alkali extract by acidification.....	14.80	2.83	0.500	16.37	3.39	0.510

The method of Folin and Ciocalteu (10) was used for the tyrosine determination, that of Sullivan for cystine, and May and Rose's method with slight modification for tryptophane, as given in a previous communication (11) (Tables IV and V).

Vickery and Block's (7) method was adopted with certain

changes for the determination of the basic amino acids. The changes simplified manipulations, required shorter time, permitted the use of smaller quantities of silver nitrate reagent and protein material, and included a colorimetric method for the determination

TABLE IV
Amino Acids in Percentages of Moisture- and Ash-Free Yeast Proteins

		Water-soluble protein by heat coagulation	Salt-soluble protein determined by		Alkali-soluble protein
			Heat	Acidification	
		<i>per cent</i>	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Bakers' yeast	Cystine	0.44	0.82	0.87	0.69
	Tryptophane	2.66	3.18	2.96	1.79
	Tyrosine	4.79	3.96	4.44	3.11
Brewers' yeast	Cystine	0.49		0.61	0.31
	Tryptophane	2.67		2.49	1.68
	Tyrosine	4.11		3.85	3.93
	Arginine	3.50			2.82
	Histidine	1.38			Trace
	Lysine	4.50			4.53

TABLE V
Diamino Acids in Yeast Proteins

	Schröder (water-soluble)	Thomas-Kolodziejska	
		Paranucleoprotein (salt-soluble)	Albumin (water-soluble)
	<i>per cent</i>	<i>per cent</i>	<i>per cent</i>
Arginine	3.22	3.58	3.95
	3.82		
Histidine	1.98	2.63	2.02
	2.36		
Lysine	8.68	4.09	7.14
	11.34		

of histidine based on the color test devised and published recently by Kapeller-Adler (12).

For the analysis approximately 5 gm. of protein material (more or less depending on the dibasic acid percentage of the material) are hydrolyzed for 36 to 40 hours at boiling temperature in 10

parts of 7 N sulfuric acid. The hydrolysate is then transferred quantitatively into a liter beaker, and most of the sulfuric acid is removed by the addition of slightly less than the calculated quantity of barium hydroxide dissolved in hot water, leaving the liquid slightly acid to Congo red indicator. The barium sulfate is removed by centrifugation and washed with 200 to 300 cc. of hot distilled water until the washings are practically colorless. The filtrate and washings are concentrated *in vacuo* and then transferred into a 500 cc. centrifuge bottle. The volume of liquid now should not be more than 100 cc. Concentrated silver nitrate solution is added until an excess of silver ion is present, as shown by the formation of a brown precipitate when saturated barium hydroxide solution is added to 1 drop of the clear supernatant liquid. Hot saturated barium hydroxide solution is added until pH 12 is reached, and the resulting mixture is cooled to room temperature and centrifuged. The precipitate contains the silver salts of histidine and arginine. The supernatant liquid, which should be slightly acidified with sulfuric acid, is set aside for lysine determine (Solution L). The silver precipitate in the centrifuge bottle containing both histidine and arginine is suspended in 80 to 100 cc. of distilled water, and concentrated sulfuric acid is added drop by drop until pH 3 to 4 is reached (blue to Congo red). It is stirred mechanically several times during a 1 hour period. A moistened strip of Congo red paper is placed in the upper part of the centrifuge flask to serve as a control in the next step of separating the histidine silver. Warm saturated barium hydroxide solution is added until the liquid changes the color of the test paper to purplish red. From here on cold saturated barium hydroxide is added until pH 7.4 is reached;¹ then the resultant mixture is centrifuged and the supernatant liquid set aside for arginine determination. In order completely to separate the arginine from the histidine the procedure described above is repeated twice in the same manner with the exception

¹ This is ascertained by placing a drop of the clear filtrate on a spot plate and adding brom-thymol blue indicator until a bluish green color is produced. Throwing down the silver precipitate by centrifugation greatly facilitates the settling of the subsequent precipitate formed by the addition of barium hydroxide solution; but the final test should be made after a thorough mixing.

that the precipitate is suspended each time in only about 50 cc. of water. The arginine-containing solutions are combined and designated Solution A.

Determination of Histidine—The histidine silver is decomposed with hydrogen sulfide after the precipitate has been suspended in distilled water and acidified with sulfuric acid. The silver sulfide is separated by centrifugation and the precipitate is washed twice, being suspended each time in 50 cc. portions of warm water saturated with hydrogen sulfide. The combined filtrate and washings containing the histidine are concentrated *in vacuo* to a small volume. This solution is now transferred into a 100 cc. Pyrex centrifuge tube, made up to 25 cc., and 1.25 gm. of concentrated sulfuric acid are added. The histidine is precipitated by adding an aqueous solution containing 10 per cent mercuric sulfate and 5 per cent by weight of sulfuric acid (Hopkins' reagent). After it has stood overnight in the refrigerator, it is centrifuged and the supernatant liquid discarded. The mercuric salt is decomposed with hydrogen sulfide in a manner similar to that described above for the silver histidine. The combined filtrates and washings are concentrated *in vacuo* to a volume of 25 to 30 cc., transferred into a 100 cc. Pyrex centrifuge tube, and brought to neutrality with saturated barium hydroxide solution. Copper carbonate is added in excess, and the tube is placed for $\frac{1}{2}$ hour in a boiling water bath, after which it is kept in the refrigerator overnight. It is then centrifuged, the supernatant liquid is poured off, and the precipitate is washed twice by suspending it in 5 cc. of cold water. The copper is removed with hydrogen sulfide from the combined liquids in the manner given for the removal of mercury or silver. The liquid is now concentrated *in vacuo* and made up to a volume of 75 or 100 cc. (Solution H).

The Kapeller-Adler (12) colorimetric test is employed for the histidine determination. 1 to 2 cc. of the purified histidine Solution H are measured into a Pyrex test-tube, and 1 cc. of a standard histidine solution¹ into another tube. The bromine reagent is added drop by drop to both test-tubes which are shaken vigorously until the liquids show a light yellowish tint that should remain permanent for 10 minutes. Then add 2 cc. of the ammoniacal ammonium carbonate mixture to each and place the tubes in a boiling water bath for 5 minutes. Cool, and transfer both stand-

ard and unknown to 10 cc. measuring cylinders. Bring the standard up to 4 cc. volume with the ammoniacal ammonium carbonate reagent and the unknown to a volume between 4 to 8 cc., which gives a reading within ± 2 mm. of that of the standard set at 20 mm. One preliminary determination is sufficient to make adjustment to fulfil these requirements.

Objection may be raised to the application of this colorimetric method to the analysis of protein preparations which may contain purines. However, when this method was applied in the analysis of the whole yeast cell, as referred to later, a negative result for histidine was obtained, showing that purines were not a color-contributing factor as the method was applied in this investigation.

Determination of Arginine—Solution A, which was immediately acidified with dilute sulfuric acid, is concentrated *in vacuo*, transferred, and, if necessary, filtered into a 500 cc. centrifuge bottle. The volume, including washings, required to transfer the concentrate should be not more than 50 cc. A test should also be made for the presence of excess silver ion and, if necessary, sufficient silver nitrate is added. The arginine silver is then precipitated by adding an equal volume of hot saturated barium hydroxide (and more, if necessary, to reach pH 12), centrifuged, and the supernatant liquid added to Solution L. The precipitate is suspended in 30 to 40 cc. of distilled water, the mixture acidified with sulfuric acid to pH 4, and the arginine silver is reprecipitated at pH 12 as given above. The arginine silver precipitate is now suspended in 50 cc. of cold saturated barium hydroxide solution and centrifuged. This one washing is generally enough to free it from nitric acid. The arginine silver precipitate is now decomposed with hydrogen sulfide according to the procedure described for the histidine. In an aliquot the nitrogen content is determined by the Kjeldahl method, and in the remaining portion most of the sulfuric acid is precipitated with barium hydroxide, leaving the solution faintly acid. The solution is then concentrated to a small volume and the required amount of flavianic acid (nitrogen $\times 5.61$) is added. When the solution is brought to boiling, the arginine monoflavianate is obtained as an orange precipitate. The precipitate, after standing overnight in a refrigerator, is filtered off by suction and washed first with a few cc. of cold water, then with alcohol, then with ether, and finally dried

at 105°. The weight of the arginine monoflavianate multiplied by 0.3566 gives the amount of arginine in the aliquot used.

Lysine Determination—Solution L combined with the washings is acidified with sulfuric acid and the silver removed by hydrogen sulfide. The clear liquid, including washings, is freed from mercuric sulfate and barium and concentrated *in vacuo* to a volume of approximately 50 cc. Concentrated sodium hydroxide solution is added to a slightly alkaline reaction and then an equal volume of alcohol. The ammonia thus liberated is removed by distillation *in vacuo*. The concentrate is now transferred to a 500 cc. Pyrex centrifuge tube and neutralized with sulfuric acid, and then enough concentrated sulfuric acid is added to make the final concentration 5 per cent by weight. The lysine is precipitated by the addition of an excess of phosphotungstic acid (100 per cent aqueous solution), and the precipitate is redissolved by heating it in a water bath. After standing in a refrigerator overnight, the lysine phosphotungstate precipitate is removed by centrifugation and washed twice with 25 to 30 cc. of chilled 2.5 per cent phosphotungstic acid in 5 per cent sulfuric acid. It is then dissolved in 50 per cent aqueous acetone solution and decomposed by an excess of warm saturated barium hydroxide solution at pH 10. The barium phosphotungstate precipitate is washed twice with cold saturated barium hydroxide solution, and the combined liquids are freed from barium by the addition of sulfuric acid in slight excess. The nitrogen content is determined in an aliquot by the Kjeldahl method, and the remainder of the solution is concentrated in the presence of barium carbonate (13) to a small volume. The precipitate is filtered off and washed. The combined liquids are concentrated to a sirupy consistency, preferably *in vacuo*, with a 100 cc. Claisen flask with a removable ground jointed bulb. Absolute alcohol is added to the sirupy residue until a slight turbidity is formed, which disappears after the addition of a few drops of water. At first only two-thirds of the required amount of picric acid (calculated by multiplying the nitrogen content of the aliquot used by 8.1) dissolved in hot absolute alcohol is added (13). The lysine picrate thus formed is allowed to stand in a refrigerator overnight and then centrifuged, the mother liquor being used to transfer the precipitate adhering to the Claisen flask quantitatively into the small centrifuge tube. The crude lysine

picrate is suspended in a mixture of 5 cc. of absolute alcohol and ether, and the washing is repeated twice. The remaining one-third of the calculated amount of picric acid is added to the mother liquor, and the precipitate, if any, treated as described above. Finally, both precipitates of the crude lysine picrates are combined and recrystallized from a small amount of water. After standing overnight at room temperature, the precipitate is filtered off, dried, and weighed. The solubility correction of 0.54 gm. per 100 cc. is added to the lysine picrate actually separated.

Amino Acid Content of Whole Yeast Cell—Cystine is a constituent of glutathione, which was shown by Hopkins (14) to be present in the yeast cell. For this reason it was of especial interest to ascertain the quantity of cystine in the whole yeast cell, since the amount present as part of the yeast protein molecule does not represent all the cystine. It was found, however, that the cystine is decomposed by acid hydrolysis of the yeast. Aliquots were taken, therefore, from the extracts obtained in the studies of ether effect on the brewers' yeast (Table I). The aliquots from the 18 hour experiment were slightly acidified with sulfuric acid and evaporated to a volume of 20 cc. in a flask in which later the hydrolysis was accomplished, after sufficient sulfuric acid had been added to bring the acid concentration to 6 N. A value of 0.3 per cent for cystine was found as determined by Sullivan's method as modified by the author (11). Bakers' yeast treated similarly was found to contain 0.27 per cent cystine, calculated on moisture-free yeast.² Arginine and lysine were determined by the method described above in a 50 gm. sample of brewers' yeast press-cake after hydrolysis for 42 hours at boiling temperature with 100 cc. of 7 N sulfuric acid. 7 gm. of concentrated sulfuric acid had been added previously to take care of the moisture in the press-cake. The analysis, calculated on a moisture-free basis, showed that bakers' and brewers' yeasts contained 1.32 and 1.37 per cent of arginine and 2.15 and 2.61 per cent of lysine, respectively. Histidine was not found in the hydrolysate. This negative result may explain Schenk's (16) erroneous statement that the yeast cell lacks histidine. An attempt to estimate the quantities of tryptophane and tyrosine in the whole yeast, according to the methods

² Prunty (15) found in two samples of dried brewers' yeast, which he analyzed, 0.92 and 0.52 per cent cystine.

of May and Rose, and of Folin and Ciocalteu, respectively, was unsuccessful.

SUMMARY

Cystine, tryptophane, tyrosine, arginine, histidine, and lysine were determined quantitatively in protein preparations obtained from bakers' yeast and from brewers' yeast. It was found that cystine and histidine undergo a change when yeast is hydrolyzed by acid. Because of the high percentages of nutritionally essential amino acids in yeast proteins the supplementing effect of yeast when added to experimental diets should be taken into consideration.

BIBLIOGRAPHY

1. Dreyer, G., *Z. ges. Brauw.*, **36**, 201 (1913).
2. Thomas, P., *Compt. rend. Acad.*, **156**, 2024 (1931). Thomas, P., and Kolodziejska, S., *Compt. rend. Acad.*, **157**, 243 (1913).
3. Meissenheimer, J., *Z. physiol. Chem.*, **104**, 229 (1919); **114**, 205 (1921).
4. Buchner, H., and Gruber, M., German patent 113,181, *Patentbl.*, **21**, 1127 (1900).
5. Schröder, R., *Beitr. chem. Physiol. u. Path.*, **2**, 389 (1902).
6. Chibnall, A. C., *J. Biol. Chem.*, **55**, 333 (1923); **61**, 303 (1924). Chibnall, A. C., and Nolan, L. S., *J. Biol. Chem.*, **62**, 179 (1924-25). Miller, E. J., and Chibnall, A. C., *Biochem. J.*, **26**, 392 (1932). Chibnall, A. C., Miller, E. J., Hall, D. H., and Westall, R. G., *Biochem. J.*, **27**, 1879 (1933). Pollard, A., and Chibnall, A. C., *Biochem. J.*, **28**, 326 (1934).
7. Vickery, H. B., and Block, R. J., *J. Biol. Chem.*, **93**, 105 (1931).
8. Csonka, F. A., Murphy, J. C., and Jones, D. B., *J. Am. Chem. Soc.*, **48**, 763 (1926).
9. Parr, S. W., *Ind. and Eng. Chem.*, **8**, 637 (1916).
10. Folin, O., and Ciocalteu, V., *J. Biol. Chem.*, **73**, 627 (1927).
11. Csonka, F. A., *J. Biol. Chem.*, **97**, 281 (1932).
12. Kapeller-Adler, R., *Biochem. Z.*, **264**, 131 (1933).
13. Calvery, H. O., *J. Biol. Chem.*, **83**, 631 (1929).
14. Hopkins, F. G., *J. Biol. Chem.*, **84**, 269 (1929).
15. Prunty, F. T. G., *Biochem. J.*, **27**, 387 (1933).
16. Schenk, M., *Woch. Brau.*, **22**, 221 (1905).