for approximately 30 percent of the dry weight of the cells.

By mechanical disruption of baker's yeast and its differential centrifugation in media of differing density, preparations of isolated cell walls have been obtained that are entirely free from intact cells and from other particulate cellular matter. From the clean cell-wall fragments, we have isolated a mannan-protein complex (*3*) that appears to be a major structural entity of the cell.

Cells from starch-free pound cakes of baker's yeast (*4*) suspended in water or in a 5 percent (by volume) aqueous solution of thiodiglycol (150 g of yeast, 50 ml of solution) were disrupted in the cold by agitation in a Waring Blendor with glass beads according to the method of Lamanna and Mallette (*5*). More than 90-percent breakage of cells was achieved after 90 minutes of agitation. Cell-wall fragments were isolated by differential centrifugation and repeated washing with distilled water, 8.5-percent sucrose solution, and phosphate buffer (*p*H 7.4, 0.1*M*). Washing with sucrose and with buffer served to eliminate completely the small particles which, in water, sediment along with cell-wall fragments.

After about 50 repetitions of washing and differential centrifugation, a fraction consisting exclusively of cell-wall material was obtained. This was lyophilized. All data are based on material prepared in this manner. By this method of preparation, it is possible to obtain a cell-wall fraction of constant composition. From two different batches of yeast, the nitrogen contents of the cell-wall preparations were 1.28 and 1.24 percent. These values are lower than that (2.1 percent) reported by Northcote and Horne, who stated that their preparation contained no unbroken cells but was contaminated by small particles which were difficult to eliminate.

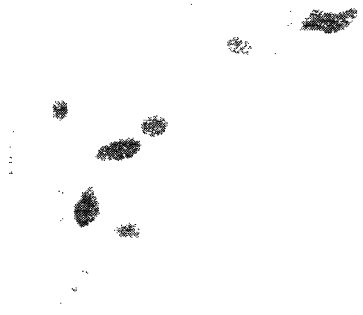

Fig. 1. Amino acids in hydrolyzates (6*N* HCl for 16 hours at 110°C) of isolated cell wall of baker's yeast as demonstrated by two-dimensional paper chromatography (solvents were a solution of butanol, acetic acid, and water and a solution of phenol and ammonia; ninhydrin development; cobalt chloride spray to intensify color and make record permanent): *1*, cysteic acid; *2*, aspartic acid; *3*, glutamic acid; *4*, serine; *5*, glycine; *6*, threonine; *7*, alanine; *8*, lysine; *9*, arginine; *10*, tyrosine; *11*, valine and methionine; *12*, leucine and isoleucine; *13*, phenylalanine; *14*, unidentified. Small amounts of hexosamine present were not detectable after vigorous hydrolysis.

Table 1. Composition of isolated cell wall of baker's yeast.

Component	Percentage by weight of dry cell wall
Total reducing sugar	84.4
Hexosamine	2.7
Total nitrogen	1.28
Hexosamine N	0.21
Protein N	1.07
Protein N (×6.25)	6.7
Phosphorus	0.34
Sulfur	0.14

The analytic data presented in Table 1 show that the clean cell-wall material contains 6.7 percent protein, 84.4 percent total reducing sugar (anthrone method, *2*), and 3.0 percent chitin (based on the hexosamine content). The high sulfur-to-protein ratio (2.1 percent) indicates that the protein may be of a pseudokeratin type (*6*). By means of paper chromatography of hydrolyzates of cell-wall material, the presence of 15 amino acids and one as yet unidentified substance was demonstrated (Fig. 1).

From preparations of clean cell-wall materials a major fraction (approximately 75 percent) was solubilized in 1*N* KOH on slight warming. The solubilized material was dialyzed against running tap water for 24 hours and then lyophilized, yielding a white powder, of which the major part was readily soluble in water. The residue formed a gel that was separated from the solution by centrifugation at 20,000*g*. The nature of this fraction is under study; it appears to be a glucan-protein. (Only glucose is found, in addition to amino acids, after acid hydrolysis). The water-soluble material that had resisted dialysis was found to contain mannan and protein, as shown by analysis for tyrosine (Folin) and polysaccharide (anthrone), ultraviolet absorption at 280 mμ and chromatographic analysis (Only mannose is found, in addition to amino acids, after acid hydrolysis.) Nucleic acid was absent as judged by absorption at 260 mμ. Three different preparations had nitrogen contents close to 1.10 percent (equivalent to 6.8 percent protein).

The mannan and protein appeared to be tightly bound, for the polysaccharide could not be precipitated as the copper complex by treatment with cold Fehling's reagent (*7*). In the ultracentrifuge, the mannan-protein appears to be monodisperse, giving at this stage of purification a sedimentation constant (uncorrected) of $S_{20} = 4.3 \times 10^{-13}$ at several concentrations in water and in buffer (*8*). The mannan-protein complex has been further purified by precipitation from aqueous solution on saturation with ammonium sulfate followed by dialysis and subsequent lyophilization. The amino acid composition of this material is being analyzed quantitatively, and biochemical, immunochemical, and physical studies on the mannan-protein are underway.

From fresh bottom yeast (brewer's yeast) Lindquist (*9*) obtained, by simple agitation of a thick suspension at room temperature, a material which he termed "cebrosan" that has some features in common with the mannan-protein we have isolated. Lindquist reported his purified material contained 1.2 percent nitrogen, but it had the property of a yeast mannan—that is, it was precipitable by Fehling's solution in the cold. The material we have isolated appears to be an almost homogeneous structural entity of the cell wall, consisting of protein and mannan in the ratio (by weight) of 1/12. Evidence has been obtained that this cell-wall protein plays a role in cellular division (*10*).

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References and Notes

1. D. H. Northcote and R. W. Horne, *Biochem. J. (London)* **51**, 232 (1952).
2. C. W. Chung and W. J. Nickerson, *J. Biol. Chem.* **208**, 395 (1954).
3. Investigations supported in part by a grant (E-251) from the National Institutes of Health, U.S. Public Health Service.
4. We are indebted to E. T. Palumbo, manager of Yeast Plant No. 2, Anheuser-Busch Inc., Old Bridge, N.J., for making available generous supplies of fresh yeast.
5. C. Lamanna and M. F. Mallette, *J. Bacteriol.* 67, 503 (1954).
6. W. H. Ward and H. P. Lungren, *Advances in Protein Chem.* **9**, 243 (1954).
7. A. R. Ling, O. N. Nanji, F. J. Paton, *J. Inst. Brewing* **31**, 326 (1926).
8. The collaboration of H. O. Singher (division of biochemistry, Ortho Research Foundation) in physical studies on this material is gratefully acknowledged. A report of the details of this work is in preparation.
9. W. Lindquist, *Biochim. et Biophys. Acta* 10, 580 (1953).
10. W. J. Nickerson and G. Falcone, in preparation.

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Inhibitory Mechanism of Chlortetracycline on D-Amino Acid Oxidase

Yagi *et al.* (*1*) found that *p*-aminosalicylic acid quenched the fluorescence of riboflavin in aqueous solution by the formation of a complex and that the inhibitory action of *p*-aminosalicylic acid on D-amino acid oxidase is partly due to the complex formation between

p-aminosalicylic acid and flavin adenine dinucleotide.

After these results were obtained, many organic substances were examined in our laboratory to determine whether they had a quenching action on flavins. Chlortetracycline was found to be a strong quencher. First the *p*H-fluorescence curve of chlortetracycline was examined. No fluorescence was observed below *p*H 7.0, and the blue fluorescence was strengthened with increasing *p*H values. Therefore in the experiments reported here, the *p*H was fixed at 6.0, a value at which chlortetracycline has no fluorescence but flavins have yellow fluorescence. Chlortetracycline (*2*) and flavin adenine dinucleotide, prepared by the method of Yagi *et al.* (*3*), were dissolved in water and mixed with phosphate buffer. The fluorescence was measured at 20°C, using a fluorometer designed by us (*4*).

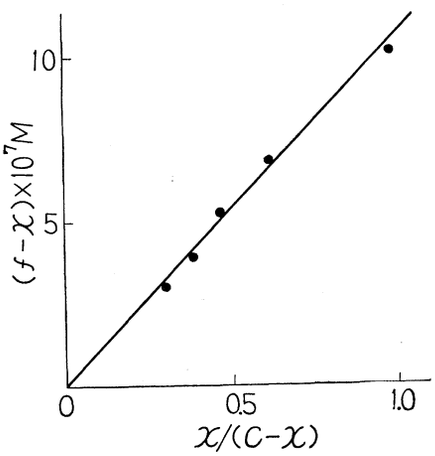

Fig. 1. Quenching of fluorescence of flavin adenine dinucleotide by chlortetracycline. The quantities, f and $(f-x)$, correspond to the intensity of fluorescence of flavin adenine dinucleotide in the absence and presence of chlortetracycline, and x was calculated from these values.

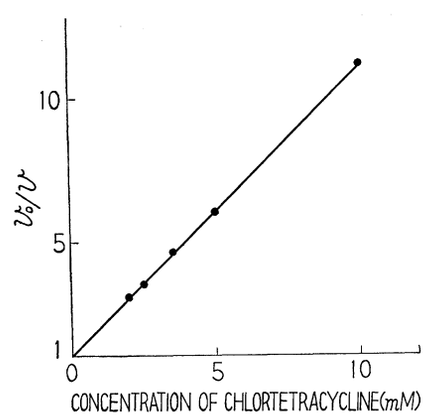

Fig. 2. Inhibition of chlortetracycline on D-amino acid oxidase. Each cup contained 2 mg of oxidase protein and $6.25\times10^{-2}M$ (final concentration) of DL-alanine. After the equilibrium temperature was reached, $1.6\times10^{-7}M$ (final concentration) of flavin adenine dinucleotide and graduated concentrations of from $2\times10^{-3}M$ to $1\times10^{-2}M$ of chlortetracycline were added from the side vessel simultaneously. V was 143 μlit/30 min and v_0 was 87.5 μlit/30 min.

Assuming that the complex is nonfluorescent and that the reaction is bimolecular, the dissociation constant K of the complex will be presented by the following formula.

$$K=\frac{(f-x)\ (c-x)}{x}$$

where f is the molar concentration of flavin adenine dinucleotide, c is that of chlortetracycline, and x is that of the complex in the tested solution. In the measurements, f and $(f-x)$ correspond to the intensity of fluorescence in the absence and presence of chlortetracycline, respectively. Each tested sample contained $1.5\times10^{-6}M$ of flavin adenine dinucleotide and graduated concentrations from $1\times10^{-6}M$ to $5\times10^{-6}M$ of chlortetracycline. When $(f-x)$ was plotted against $x/(c-x)$, a straight line was obtained, as is shown in Fig. 1, and the value of K as calculated from the slope of this line was $1.1\times10^{-6}M$.

The influence of the complex formation by chlortetracycline of a complex with flavin adenine dinucleotide on the activity of D-amino acid oxidase was then analyzed with purified oxidase protein (protein E), which was prepared from hog kidney by the method of Negelein and Brömel (*5*), using a Warburg manometer. The relationship between the activity of D-amino acid oxidase and the flavin adenine dinucleotide concentration is

$$v=\frac{Vf}{K_F+f}$$

where v is the reaction velocity in the presence of a concentration f of flavin adenine dinucleotide, V is the maximum velocity obtained by increasing the concentration of flavin adenine dinucleotide, and K_F is independent of f and v. Using this formula, the dissociation constant of flavin adenine dinucleotide with oxidase protein was calculated as $1.1\times10^{-7}M$.

If chlortetracycline forms a complex with flavin adenine dinucleotide or competes with the latter to combine with oxidase protein and $K\gg K_F$, the activity of the enzyme can be shown as

$$v=\frac{Vf}{K_F\left(1+\frac{i}{K}\right)+f}$$

where i is the concentration of chlortetracycline, K is the constant of its dissociation from a complex with oxidase protein or with flavin adenine dinucleotide. Therefore, K can be evaluated from the following formula as described by Burton (*6*):

$$\frac{v_0}{v}=1+\left\{1-\frac{v_0}{V}\right\}\frac{i}{K}$$

where v_0 and v are the reaction velocity in the absence and presence of chlortetracycline, respectively.

In the experiments, chlortetracycline and flavin adenine dinucleotide were placed in the side vessel of the manometer flask. When, after a temperature equilibrium had been reached, the contents of the side vessel were emptied into the main chamber containing oxidase protein and DL-alanine, the inhibiting effect of chlortetracycline on the reaction velocity was demonstrated. Measured values of v_0/v, plotted against the concentration of chlortetracycline, gave the straight line shown in Fig. 2, which indicated that only one inhibitory factor was present. From the line's slope, K was calculated to be $3.9\times10^{-4}M$. This inhibition may be considered to be due to the formation of a complex by chlortetracycline with flavin adenine dinucleotide.

The difference in the value for K obtained by fluorescence measurement and the value for K obtained by enzymatic research may be attributed to variation in *p*H and temperature. Therefore, $3.9\times10^{-4}M$, the value obtained for K, is considered to be the constant of the dissociation of chlortetracycline from its complex with flavin adenine dinucleotide at *p*H 8.3 and 38°C in the presence of the enzyme. The values obtained for v_0/v when, after incubation of oxidase protein, DL-alanine, and chlortetracycline in the main chamber for 10 minutes, flavin adenine dinucleotide was added from the side vessel nearly agreed with those shown in Fig. 2. However, each value obtained closely approached 1 when, after oxidase protein, DL-alanine, and flavin adenine dinucleotide had been incubated in the main chamber for 10 minutes, chlortetracycline was added from the side vessel.

From these results, it may be supposed that chlortetracycline can combine with flavin adenine dinucleotide when it is free from oxidase protein and cannot react with flavin dinucleotide when it is combined with protein. The formation of a complex by chlortetracycline with flavin adenine dinucleotide may be one of the factors in the etiology of the ariboflavinosis that is caused by this antibiotic.

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References and Notes

1. K. Yagi and Y. Matsuoka, *Biochem. Z.*, in press; K. Yagi, *et al., ibid.*, in press.
2. Furnished by the Lederle Co.
3. K. Yagi, *et al., J. Biochem.* (*Japan*) 43, 93 (1956).
4. K. Yagi and T. Arakawa, *Vitamins* (*Japan*) 6, 523 (1953).
5. E. Negelein and D. Brömel, *Biochem. Z.* 300, 225 (1939).
6. K. Burton, *Biochem. J.* (*London*) 48, 458 (1951).

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