Table IV. Inhibitory Effect of Ammonium Sulfate[a]

$(NH_4)_2SO_4$, M	Units	% Activity Remaining	Units	% Activity Remaining	Units	% Activity Remaining
0	17.5	100	11.8	100	5.88	100
0.0095	13.5	77.1	9.90	83.9	4.38	74.6
0.062	6.90	39.4	3.89	33.0	2.53	43.0
0.17	5.40	30.8	3.65	30.9	1.85	31.5
0.33	5.50	31.4	3.70	31.4		

[a] Regular test system.

to an alteration of the clottable proteins, probably by binding of chloride anions, and that the enzyme does not compete with the inhibiting agent for the substrate.

Table IV presents data for the inhibitory effect of ammonium sulfate. It can be seen that ammonium sulfate exerts its inhibitory effect in a manner similar to that of sodium chloride and that this inhibition is approximately twice that due to the sodium chloride.

Figure 4 presents a plot of the data of Tables III and IV, where the per cent remaining activity (average) is plotted against concentrations of the inhibiting salts. In carrying out routine analyses in the presence of known concentrations of the above inhibitory salts, units obtained were corrected for inhibition by multiplying by the factor, 100/% activity, obtained from the plots.

No attempt was made to investigate either the influence of the inhibitory salts on the value of the constant, c, or the possibility of preferential inhibition of one of the enzymes by a given salt.

Literature Cited

(1) Balls, A. K., Hoover, S. R., *J. Biol. Chem.* **121,** 737 (1937).
(2) Bodansky, O., *Ibid.*, **120,** 555 (1937).
(3) Gawron, O., Cheslock, K. E., *Arch. Biochem. Biophys.* **34,** 38 (1951).
(4) Gawron, O., Draus, F., Division of Biological Chemistry, 122nd Meeting, ACS, Atlantic City, 1952.
(5) Goldstein, A., *J. Gen. Physiol.* **27,** 529 (1944).
(6) Jansen, E. F., Balls, A. K., *J. Biol. Chem.* **137,** 459 (1941).
(7) Lineweaver, H., Burk, D., *J. Am. Chem. Soc.* **56,** 658 (1934).
(8) Straus, O. H., Goldstein, A., *J. Gen. Physiol.* **26,** 559 (1943).

Received for review October 7, 1955. Accepted January 3, 1956. Division of Biological Chemistry, 122nd Meeting ACS, Atlantic City, N. J., September 1952. Abstracted in part from the master's thesis of Frank Draus.

MEAT PIGMENTS

Factors Affecting the Oxidation of Nitric Oxide Myoglobin

K. A. WALSH and DYSON ROSE
Division of Applied Biology, National Research Laboratories, Ottawa, Canada

The stability of nitric oxide myoglobin, the pigment found in cured, unheated meat, was investigated by measuring its rate of oxidation in solution. The rate of oxidation was first-order and was affected by light, pH changes, temperature variations, nitrite concentration, and certain inhibitors. Three distinct mechanisms were observed: a rapid oxidation by nitrous acid, a slow autoxidation in air, and a photo-oxidation.

When meat is cured in brine or dry salt mixtures, myoglobin, the major pigment of the fresh meat (*11*), is converted to nitric oxide myoglobin (MbNO). During storage, this characteristic red pigment tends to change to undesirable brown and gray hues on exposed surfaces. The rate of discoloration is increased by light (*5, 8, 14*), but can be retarded by the exclusion of air (*14*), by wrapping the meat in red cellophane to filter out undesirable light (*14*), and by dipping in sodium nitrite after the sulfhydryl groups have been released by heating (*16*).

Kampschmidt (*5*) recently showed that light accelerated the oxidation of nitric oxide myoglobin and suggested that the reaction proceeded by a dissociation followed by an oxidation to metmyoglobin (MMb). Other information concerning the stability of nitric oxide myoglobin has been inferred from studies with nitric oxide hemoglobin, a protein with similar but not identical properties.

To provide more information on the properties of the pigment of cured, unheated meat, studies were undertaken on the influence of various factors on the stability of pure nitric oxide myoglobin in solution.

Reagents

Buffers above pH 5.8 were mixtures of sodium phosphates; below 5.8, mixtures of sodium acetate and acetic acid. Hydrosulfite reagent was made immediately before use as 0.2% sodium hydrosulfite in 0.04M buffer at pH 6.0. Nitrite reagent was 1% sodium nitrite in water.

Crystalline myoglobin was isolated from horse hearts by the method of Theorell (*12*). Stock solutions (about 0.4mM) were prepared by dissolving the crystals in water and dialyzing free of ammonium sulfate. They were stored at 0° C. The total pigment concentrations of these solutions and nitric oxide myoglobin solutions were determined by iron analyses by the modified bipyridine method (*2*), assuming one atom of iron per molecule of myoglobin derivative.

Concentrated nitric oxide myoglobin was prepared immediately before use by combining one part of stock myoglobin, one part of nitrite reagent, and two parts of hydrosulfite reagent.

Standard nitric oxide myoglobin was prepared by diluting concentrated nitric oxide myoglobin to about 0.04mM in a final buffer concentration of 0.048M.

Dilute nitric oxide myoglobin solutions were prepared for oxidation rate measurements by dialyzing concentrated nitric oxide myoglobin against 0.04M buffer at 4° C. to reduce the sodium nitrite

level below 1 p.p.m. After dialysis the pH and ionic strength were varied as desired, nitrite or inhibitors were added as required, and the solution was diluted to about 0.04mM nitric oxide myoglobin and a final buffer concentration of 0.016M.

Standard metmyoglobin was prepared by adding to stock myoglobin a tenfold molar excess of potassium ferricyanide. Buffer and water were then added to give a final myoglobin concentration of 0.04mM and a buffer concentration of 0.016M.

Methods

Solutions of nitric oxide myoglobin were incubated in the light and dark under controlled chemical and physical environments. Five-milliliter aliquots of dilute nitric oxide myoglobin were placed in containers with flat circular bottoms (24 sq. cm. in area) and incubated for suitable intervals in either a lighted or completely dark bath maintained at 0.5° ± 0.05° C. unless otherwise stated. The lighted bath was illuminated through a pebble-glass bottom by a bank of six 20-watt G.E. White 80 fluorescent lights supplied with a constant line voltage. The light intensity at the surface of the containers in the lighted bath was approximately 800 foot-candles, as measured with a Weston illumination meter (Model 603). Where necessary, the light intensity was varied by placing wire screens of different mesh sizes between the light source and the bath.

Immediately after incubation the percentage of total pigment remaining as nitric oxide myoglobin was estimated by determining the absorbances of the solutions in a Cary recording spectrophotometer and calculating the extinction coefficients, E, at 547, 565, and 577 mμ. The percentage of the total pigments as nitric oxide myoglobin was then calculated by comparison with standard nitric oxide myoglobin and metmyoglobin solutions, assuming a two-component system (*1*). This method of calculation was justified, because except where oxymyoglobin was formed, only one end product could be distinguished and its spectrum was identical with that of metmyoglobin.

As nitric oxide myoglobin is not stable in solution, the absorbance values of the standard solutions were always measured without delay. The extinction coefficients found were 12.2 and 10.9 per millimole per cm. at 547 and 577 mμ, respectively, the peaks of maximum absorbance, and 10.4 at the minimum at 565 mμ. The absorbance obeyed Beer's law at these wave lengths and was not affected by excess nitrite or pH variations between pH 5.0 and 7.5. The metmyoglobin solutions were stable and obeyed Beer's law at the three wave lengths, but the E values depended upon the nitrite level (*15*) and the pH (*1*). E values of metmyoglobin standards were therefore determined for each experimental pH and nitrite level.

Nitrite concentration was measured wherever necessary by the sulfanilic acid method (*10*).

Results

The rate of loss of nitric oxide myoglobin was studied at various pH values, ionic strengths, nitrite levels, temperatures, and light intensities. Under all these conditions the reaction rate was first-order in the light and in the dark. Assuming that the dark reaction proceeded in the light according to the same kinetics that it followed in the dark, the photo-oxidation rate constant (k_{photo}) was calculated as the difference between the reaction rate constants in the light (k_{light}) and in the dark (k_{dark}). A typical experiment is illustrated in Figure 1.

The effect of pH upon the rate of nitric oxide myoglobin oxidation in the light and in the dark was studied between pH 5.0 and 7.5 in the presence or absence of 200 p.p.m. of sodium nitrite (Figure 2). In the absence of nitrite, increasing pH had no effect on the dark reaction but increased the rate of photo-oxidation. In the presence of nitrite, decreasing pH below 6.3 markedly accelerated the oxidation in both light and dark. Above pH 6.3 k_{photo} was slightly reduced by nitrite, while k_{dark} was not affected.

The accelerating effect of nitrite at low pH was examined further by varying nitrite and maintaining pH 5.6 (Figure 3). Nitrite stimulation was very significant above 100 p.p.m. of nitrite, and the rate of reaction in the dark was a linear function of the square of the nitrous acid concentration (Figure 4) as calculated from a dissociation constant for nitrous acid of 6×10^{-4} (*9*).

The ionic strength was varied from 0.03 to 1.0 at pH 6.8 with suitable additions of chloride, phosphate, acetate, citrate, and sodium ions. No relation could be found between the k_{photo} or k_{dark} and the ionic strength.

The influence of temperature upon the k_{photo} and the k_{dark} was studied between 0.5° and 26.3° C. at pH 6.3 (Table I). The dark reaction had a Q_{10} of 5.0 and an energy of activation of 22 kcal., while the photo-oxidation had a Q_{10} of 1.6 and an energy of activation of 7.0 kcal.

The influence of light intensity upon the rate of photo-oxidation was measured at pH 6.8. The rate of reaction

Figure 1. Typical first-order plots for destruction of nitric oxide myoglobin in light and dark

800 foot-candles, 75 p.p.m. $NaNO_2$, 0.5° C., pH 6.7

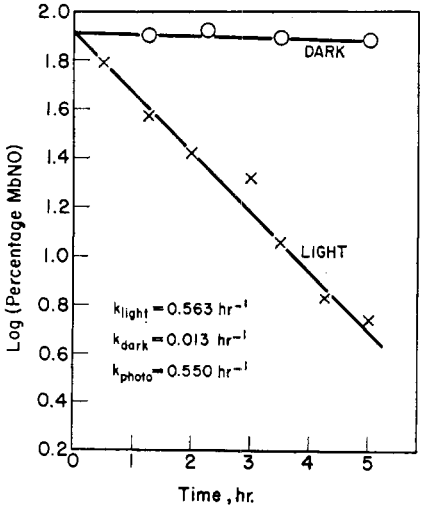


Figure 2. Effect of pH on nitric oxide myoglobin oxidation rates in the presence and absence of sodium nitrite

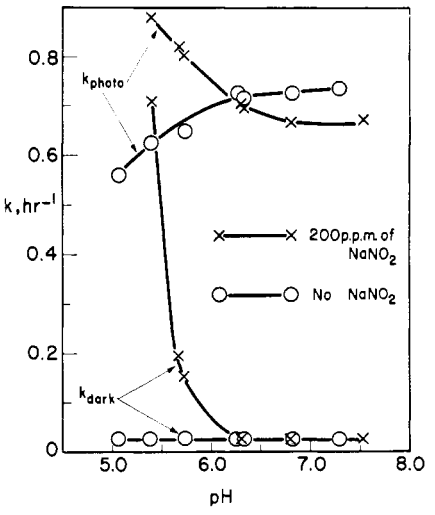
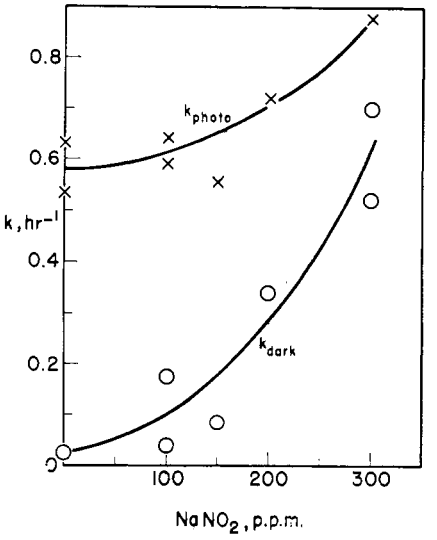


Figure 3. Effect of sodium nitrite at pH 5.6 on the rate of oxidation of nitric oxide myoglobin



appeared to be a curvalinear function of light intensity, increasing more than proportionately with increasing light intensity (Table II).

Various substances were tested for their ability to retard the rate of reaction in the light or dark at pH 6.8 (Table III). Oxidation in the dark was studied at 20° C. to give an easily measured reaction rate. The photo-oxidation was not affected by sorbic acid (2,4-hexadien-1-ol) but was retarded by dextrose, potassium iodide, and potassium thiocyanate. The dark reaction was not affected by dextrose, sorbic acid, and potassium thiocyanate, but potassium iodide appeared to be mildly inhibitory. In the dark and in the light ascorbic acid, hydroquinone, and reduced glutathione stabilized the red color, as judged visually, but the absorption spectrum indicated that some oxymyoglobin was being formed (cf. *5*), making calculation of the rate of nitric oxide myoglobin loss impossible.

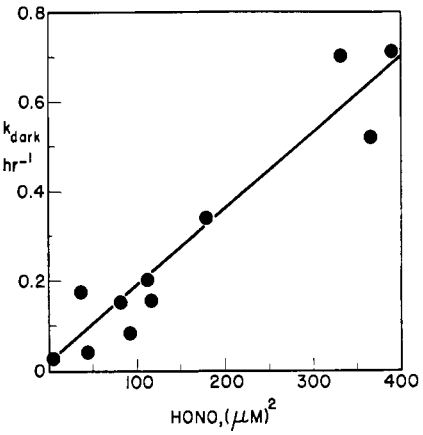


Figure 4. Relationship between square of nitrous acid concentration and rate of oxidation of nitric oxide myoglobin in dark

The measurements of inhibitory effects were repeated in the presence of 200 p.p.m. of sodium nitrite at pH 6.8. Under these conditions reduced glutathione did not cause oxymyoglobin formation and did not affect the photo-oxidation rate, but did increase the rate of oxidation in the dark (k_{dark} increased from 0.39 to 0.71). Nitrite had no significant effect on the action of the other substances.

Discussion

Nitric oxide myoglobin is rather unstable in purified solutions exposed to air. Oxidative mechanisms destroy the pigment slowly in the dark and more rapidly in the light.

Nitric oxide myoglobin is probably destroyed in the dark by an autoxidation of the type: MbNO + $^1/_2O_2$ → MMb + NO_2^-. The reaction proceeds independently of pH, is very sensitive to temperature, and is not readily inhibited.

Table I. Influence of Temperature on Rate of Oxidation of Nitric Oxide Myoglobin

Temp., °C.	k_{dark}, Hr.$^{-1}$	k_{photo}, Hr.$^{-1}$
0.5	0.025	0.700
11.05	0.113	1.04
20.15	0.437	1.65
26.32	1.13	...

Table II. Effect of Light Intensity on Rate of Photo-Oxidation of Nitric Oxide Myoglobin

Light Intensity, Foot-Candles	k_{photo}, Hr.$^{-1}$	Ratio, k_{photo}/Foot Candles
0	0	...
232	0.143	0.00062
429	0.289	0.00067
561	0.421	0.00075
799	0.705	0.00088

Table III. Effect of Various Substances on Rate of Oxidation of Nitric Oxide Myoglobin

Substance Added	Concentration	k_{dark} at 20° C., Hr.$^{-1}$	k_{photo} at 0.5° C., Hr.$^{-1}$
Nil	...	0.39	0.73
Dextrose	0.5%	0.42	0.54
Sorbic acid	2.0m*M*	0.37	0.70
KI	2.0m*M*	0.31	0.60
KSCN	2.0m*M*	0.35	0.54

The addition of sodium nitrite to nitric oxide myoglobin promotes a second type of oxidation, particularly at low pH levels. The rapid increase in the rate of nitric oxide myoglobin oxidation in the dark as the pH is brought below 6.3 can be explained as an oxidation of nitric oxide myoglobin by nitrous acid formed from nitrite. The linear relationship between the square of the nitrous acid concentration and k_{dark} suggests that two molecules of nitrous acid oxidize one of nitric oxide myoglobin—i.e., 2 HONO → N_2O_3 + H_2O and N_2O_3 + MbNO → MMb + NO_2^- + 2 NO. The reaction is first-order with respect to nitric oxide myoglobin in the system studied, because the large sodium nitrite–nitrous acid ratio keeps the nitrous acid level essentially constant at any level of nitrite.

The production of oxymyoglobin in the dark in the presence of ascorbic acid, hydroquinone, or reduced glutathione can be explained as a reduction of metmyoglobin to reduced myoglobin, which readily oxygenates to oxymyoglobin. This is supported in part by the observation (*4*) that ascorbic acid does reduce metmyoglobin. The acceleration of the dark reaction by the combined effects of glutathione and nitrite at pH 6.8, where there is not sufficient nitrous acid to oxidize nitric oxide myoglobin directly, may have been due to the formation of oxidized glutathione (*6*) by nitrous acid. The oxidized glutathione could increase the rate of oxidation by acting as an oxidation-reduction mediator between nitrous acid and nitric oxide myoglobin. Since the liberation of protein sulfhydryl groups in meat does not accelerate pigment oxidation by nitrite (*16*), either these groups are not oxidized by nitrous acid or their oxidation products are not suitable electron acceptors or mediators.

The destruction of nitric oxide myoglobin in the light is a photo-oxidation and the reaction follows first-order kinetics. The activation energy of 7 kcal. suggests that there is at least one nonphotochemical reaction involved. Since only nitric oxide myoglobin and metmyoglobin could be detected by examination of the absorption spectrum, any intermediate would have to be present at very low concentration. A peroxide intermediate is not likely, because sorbic acid had no effect on the rate. A dissociation mechanism (*5*, *13*) would lead to the formation of an intermediate mixture of reduced myoglobin and oxymyoglobin. However, the measured rates of photo-oxidation are much more rapid than the known (*3*) rates of oxidation of this mixture, and the absence of measurable amounts of this intermediate mixture makes the dissociation mechanism highly improbable. Formation of oxymyoglobin in the presence of ascorbic acid cannot be accepted as evidence for a dissociation mechanism (*5*), because ascorbic acid can reduce metmyoglobin (*4*) and lead to a formation of oxymyoglobin even in the dark.

A more plausible mechanism for the photo-oxidation would involve the formation of an activated MbNO* molecule by absorbed light, which would either deactivate to nitric oxide myoglobin or give up an electron to oxygen to form metmyoglobin and a free nitrite ion.

$$\mathrm{MbNO} \underset{}{\overset{\text{light}}{\rightleftharpoons}} \mathrm{MbNO^*} \xrightarrow{^1/_2\,O_2} \mathrm{MMb} + \mathrm{NO_2^-}$$

Such a mechanism is supported by the observation that known, nonselective deactivating agents (*7*), potassium thiocyanate and potassium iodide, had inhibitory effects. Other nonselective deactivating agents such as phenols could not be evaluated in this system because their oxidation products interfered with the spectrophotometric measurements.

It appears that nitrous acid can supplement oxygen as an electron acceptor for the photo-oxidation, since nitrite increases the photo-oxidation rate below pH 6.3. Above pH 6.3 the inhibitory action of the nitrite must be due either to the reducing action of the nitrite ion or to a deactivating effect on the proposed activated MbNO* molecule.

Summary

Factors affecting the rate of oxidation of nitric oxide myoglobin to metmyoglobin have been studied. Three distinct pseudo-first-order mechanisms were observed: an autoxidation, an oxidation by nitrous acid, and a photo-oxidation.

The autoxidation was not affected by pH within the range 5.0 to 7.5 or by nitrite in the absence of nitrous acid—i.e., above pH 6.3. The Q_{10} of autoxidation was 5.0.

The rate of oxidation by nitrous acid was directly proportional to the square of the nitrous acid concentration, and was therefore markedly influenced by nitrite concentration and by pH.

The rate of photo-oxidation was dependent on light intensity and temperature (Q_{10} = 1.6), and decreased slightly with increasing acidity. Nitrite reduced the rate slightly at pH levels above pH 6.3, but increased it progressively with increasing acidity from pH 6.3 to 5.4. Evidence is presented that the photo-oxidation proceeds by an active molecule mechanism and not by a light-catalyzed dissociation.

Literature Cited

(1) Austin, J. H., Drabkin, D. L., *J. Biol. Chem.* **112,** 67 (1935).
(2) Delory, G. E., *Analyst* **68,** 5 (1943).
(3) George, P., Stratmann, C. J., *Biochem. J.* **51,** 418 (1952).
(4) Gibson, Q. H., *Ibid.*, **37,** 615 (1943).
(5) Kampschmidt, R. F., J. AGR. FOOD CHEM. **3,** 510 (1955).
(6) Lemoigne, M., Monguillon, P., Desveaux, R., *Compt. rend.* **206,** 947 (1938).
(7) Privault, M., *Ibid.*, **184,** 1120 (1927).
(8) Ramsbottom, J. M., Goeser, P. A., Shultz, H. W., *Food Inds.* **23** (2), 120 (1951).
(9) Ray, P. C., Dey, M. L., Ghosh, J. C., *J. Chem. Soc.* **111,** 413 (1917).
(10) Rider, B. F., Mellon, M. G., *Ind. Eng. Chem., Anal. Ed.* **18,** 96 (1946).
(11) Shenk, J. H., Hall, J. L., King, H. H., *J. Biol. Chem.* **105,** 741 (1934).
(12) Theorell, A. H. T., *Biochem. Z.* **252,** 1 (1932).
(13) Urbain, W. M., Jensen, L. B., *Food Research* **5,** 593 (1940).
(14) Urbain, W. M., Ramsbottom, J. M., *Ibid.*, **13,** 432 (1948).
(15) Watts, B. M., *Advances in Food Research* **5,** 1 (1954).
(16) Watts, B. M., Erdman, A. M., Wentworth, J., J. AGR. FOOD CHEM. **3,** 147 (1955).

Received for review November 17, 1955. Accepted January 26, 1956. Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada. Issued as N.R.C. No. 3891.

WINE CONSTITUENTS

Comparative Analysis of Fusel Oils from Thompson Seedless, Emperor, and Muscat of Alexandria Wines

ROBERT M. IKEDA
Department of Food Technology

A. DINSMOOR WEBB
Department of Viticulture and Enology

RICHARD E. KEPNER
Department of Chemistry, University of California, Davis, Calif.

The compositions of fusel oils from three varieties of *Vitis vinifera*—Thompson seedless, Emperor, and Muscat of Alexandria—are reported. The differences in composition are evaluated with respect to the possible effects of variations in fermentation conditions, distillation conditions, treatment of the fusel oil after removal from the column, and differences due primarily to the variety of grape fermented. Consideration of the low boiling alcohols found and the amino acids that would be required to form them by the Ehrlich mechanism indicates that the over-all formation of fusel oils must be extremely complex. Further studies of the varietal effect upon fusel oil composition would be of value in selecting the best grape variety for any particular type of brandy.

FUSEL OILS GENERALLY have been considered to be mixtures of alcohols with boiling points in the range extending from a temperature slightly above that of the ethyl alcohol boiling point to a temperature slightly higher than that of the boiling point of *n*-hexyl alcohol. Investigations of fusel oils from many different source materials have demonstrated that the principal components always consist of these simple alcohols (*6, 8, 9, 11, 14, 19*). The substances other than the alcohols through *n*-hexyl alcohol present in fusel oils to the extent of 5 to 15% have been investigated less thoroughly and reported to consist mainly of very high boiling esters (*12, 15, 16, 19*), alcohols with boiling points higher than that of *n*-hexyl alcohol (*12, 15, 16*), pyrazine derivatives (*2, 15, 16*), and, in certain cases, terpenes (*10, 12, 17*).

The composition of a fusel oil is determined by the nature of the substance fermented, the fermentation conditions (yeast type, temperature, aeration, agitation, sulfur dioxide content), the distillation procedure, and the washing treatment of the fusel oil after removal from the distilling column. The investigation of Schicktanz, Etienne, and Steele (*14*) has demonstrated that the fermentation, distillation, and washing procedures employed by two different distilleries definitely can influence the composition of the fusel oil from a common source material. More recently Jensen and Rinne (*6*) have reported differences not only in composition but also in constituents in fusel oils obtained from fermentation of paper mill sulfite waste liquors from 15 mills in Finland.

This paper reports the compositions of three fusel oils obtained from distillations of wines from three different varieties of grapes (*Vitis vinifera*). Two of the samples, the Thompson Seedless and the Emperor, were obtained from a large winery-distillery located in the San Joaquin Valley in California, and presumably were fermented, distilled, and washed under identical plant operating conditions. The fusel oil sample from Muscat of Alexandria wine was obtained by use of pilot plant scale equipment in these laboratories. An attempt was made to duplicate in the pilot plant as nearly as possible the fermentation and subsequent processing conditions used in the large distillery.