separated out gradually, which weighed 43 mg. and melted at 195~196°. Recrystallization from acetone-ether gave prisms, m.p. 196~197.5°. IR λ_{max}^{Nujol} μ: 5.36, 5.60 (anhydride), 5.79 (carbonyl). *Anal.* Calcd. for $C_{14}H_{16}O_5$: C, 63.32; H, 6.10. Found: C, 63.12; H, 6.20.

Summary

The reaction of 2,5-dialkylhydroquinones, 2-methyl-5-ethylhydroquinone (II), 2,5-diethylhydroquinone (III), 2-methyl-5-propylhydroquinone (IV), and 2,5-dipropylhydroquinone (V), with maleic anhydride was investigated. Of these hydroquinone homologs, (II) and (III) gave the maleic anhydride adducts, (VIIa) or (VIIb) and (VIII), respectively.

The effect of alkyl groups on the reactivity of the hydroquinone homologs is discussed.

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25. Komei Miyaki, Makoto Hayashi, and Tsutomu Unemoto: Degradation Pathway of Ethanolamine in *Proteus morganii.*

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Reports on the metabolism of ethanolamine in the past have been concentrated on oxidative deamination forming hydroxyacetaldehyde and ammonia in animals[1] or decomposition into ethylene glycol and ammonia by *Clostridium* genus in microörganisms.[2]

Recently, it has been found in this laboratory that the presence of ethanolamine ($10^{-3}M$) in a medium containing glucose and glutamate stimulated the growth of *Proteus morganii.*[3] It was also revealed that the cells and ethanolamine-adapted cells of this bacteria effected decomposition of ethanolamine into ammonia and acetaldehyde, irrespective of aerobic or anaerobic conditions, instead of oxidative deamination like ordinary monoamines and diamines. The acetaldehyde here formed then produces acetic acid and ethanol. Consequently, the presence of an enzyme that decomposes ethanolamine into ammonia and acetaldehyde was assumed and it was designated as ethanolamine dehydrase, in accordance with the example of serine.[4]

It is interesting that such a new pathway for decomposition of ethanolamine has been found only in *Pr. morganii.*

Experimental Method

Materials—Ethanolamine, N-methylethanolamine, N,N-dimethylethanolamine, choline chloride, phosphorylethanolamine (2-aminoethyl dihydrogen phosphate), and 2-hydroxypropylamine used in this work were all prepared in this laboratory. Other reagents used were commercial products.

Enzyme Preparation—Throughout all the experiments, the washed cells of *Pr. morganii* AS-21 (in the collection of the Institute of Food Microbiology, University of Chiba) cultured in a medium (pH 7.0) containing 0.5% yeast extract and 1% glucose at 30° for 16~18 hrs. was used as the enzyme preparation. Its suspension in a final concentration of 0.1~0.2 mg. Kjeldahl N/cc. was used. The enzymatic reaction seemed to proceed somewhat more actively under anaerobic than aerobic conditions. As will be described

* Okubo, Narashino, Chiba-ken (宮木高明, 林 誠, 畝本 力).

1) A. Weissbach, D. B. Sprinson: J. Biol. Chem., **203**, 1013(1953).
2) G. Cohen, B, Nisman, M. Raynaud: Compt. rend., **225**, 647(1947).
3) H. Momiyama: Nippon Eiseigaku Zasshi, **11**, 296(1957).
4) E. Chargaff, D. B. Sprinson: J.Biol. Chem., **151**, 273(1943).

later, all experiments were carried out under anaerobic conditions in N_2 atmosphere in order to avoid oxidation of metabolic products. In ordinary experiment, 0.1*M* phosphate buffer of pH 7.0 was used and the final concentration of a substrate was 0.01 *M*.

Determination of Ammonia—NH_3 was separated by Conway's microdiffusion method[5] and determined by Akamatsu's colorimetry,[6] using the phenol-hypochlorite reagent.

Separatory Determination of Ethanol and Acetaldehyde—EtOH and AcH were separated by Conway's microdiffusion method, allowed to stand over night, and absorbed in acid dichromate to be oxidized to AcOH. The dichromate reduced during this process was determined by iodometry and the value obtained here was taken as the amount (A) which corresponds to EtOH and AcH. Next, the amount of dichromate reduced was determined with the product obtained by diffusion after addition of 50 micromoles of semicarbazide and the value so obtained was taken as the amount (B) which corresponds to EtOH. The amount of EtOH was calculated by dividing B by 4, and AcH by dividing (A−B) by 2. Blank tests were carried out in all the experiments.

Determination of AcOH—AcOH was determined by the method of Ochoa, *et al.*[7] that uses the acetokinase of *Escherichia coli*. The cell-free acetokinase of *E. coli* was applied to the test solution in the presence of adenosine triphosphate, magnesium ion, and hydroxylamine to derive AcOH to hydroxamic acid and the latter was determined by colorimetry. The presence of EtOH and AcH did not affect the determination of AcOH.

Identification of AcH—A mixture of 1 millimole of ethanolamine and 50 cc. of 0.1*M* phosphate buffer (pH 7.0) containing ca. 5 mg.-N of the cells of *Pr. morganii* was incubated at 30° with aeration by N_2 gas. The apparatus was connected to a vessel containing 2,4-dinitrophenylhydrazine saturated in 2*N* HCl and the precipitate formed in the solution after 5 hrs. was recrystallized from 50% EtOH solution to 55 mg. of crystals melting at 147~147.5° (uncorr.), showing no depression on admixture with 2,4-dinitrophenylhydrazone of AcH. *Anal.* Calcd. for $C_8H_8O_4N_4$: C, 42.86; H, 3.60; N, 24.99. Found: C, 43.47; H, 3.51; N, 25.24.

Identification of EtOH—A mixture (50 cc.) of the same composition as above was incubated for 4 hrs., the cells were removed by centrifugation, and its supernatant solution was submitted to distillation with fractionating column, collecting 3 cc. of the initial distillate. This fraction contained alcohol corresponding to 384 micromoles and an aldehyde corresponding to 12 micromoles. Of this fraction, 0.4 cc. was submitted to column chromatography over Celite by the procedure reported by Neish.[8] The EtOH fraction revealed the presence of 48.5 micromoles of alcohol and there was no substance in other fractions that consumed acidic dichromate, indicating that the alcohol detected in 3 cc. of the initial fraction was EtOH alone.

Identification of AcOH—The residue left after distillation of EtOH in the above procedure was acidified and extracted with five 50-cc. portions of Et_2O. The combined Et_2O extract was dried over anhyd. Na_2SO_4 and Et_2O was evaporated. Titration of the residue with 0.1*N* NaOH revealed the presence of an acid corresponding to 130 micromoles of AcOH.

This procedure was repeated until an amount of the acid corresponding to ca. 24 mg. of AcOH had been collected. The acid fraction was neutralized, dried, and derived to *p*-nitrobenzyl derivative in accordance with the method of Reid.[9] Minute amount of crystals, m.p. 76.5~77.5° (uncorr.), was thereby obtained and they showed no depression in the melting point on admixture with *p*-nitrobenzyl acetate.

Results and Discussion

Enzymatic Formation of NH_4OH from Ethanolamine—As indicated in Fig. 1, NH_4OH was formed quantitatively.

Effect of Hydrogen Ion Concentration and Substrate Concentration on the Enzyme—Examination of the optimal pH and optimal substrate concentration of the ethanolamine dehydrase gave a pH- and pS-activity curves respectively shown in Figs. 2 and 3. The optimal pH was 7.0~8.0 and optimal concentration of the substrate, 4×10^{-3} to 3×10^{-2}*M*.

Distribution of Ethanolamine Dehydrase—Distribution of ethanolamine dehydrase in various microörganisms was examined by measuring formation of NH_4OH. It was thereby learned, as shown in Table I, that within the limit of present experiment, this enzyme was specific to *Pr. morganii*.

5) E. J. Conway: "Microdiffusion Analysis and Volumetric Error," 2nd ed., Crosby Lockwood and Son, London (1947).
6) S. Akamatsu: J. Biochem. (Tokyo), **39**, 203(1952).
7) I. A. Rose, M. Grunberg Manago, S. R. Korey, S. Ochoa: J. Biol. Chem., **211**, 737(1954).
8) A. C. Neish: "Analytical Method for Bacterial Fermentations," Natl. Research Council Can., Rept. 46-8-3, 2nd revision, NRC 2952(1952).
9) E. E. Reid: J. Am. Chem. Soc., **39**, 124(1917).

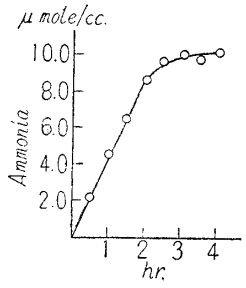

Fig. 1. Production of Ammonia from Ethanolamine by *Proteus morganii*

Concentration of ethanolamine: 0.01*M*; concentration of cell suspension: 0.12 mg.-Kjeldahl N/cc.; pH: 7.0 (0.1*M* phosphate buffer); incubation: 30°

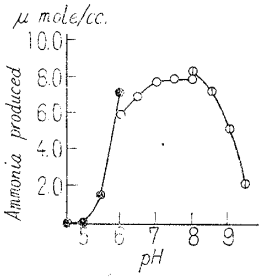

Fig. 2. Apparent pH-Activity Curve of Ethanolamine Dehydrase

Concentration of ethanolamine: 0.01*M*; concentration of cell suspension: 0.18 mg. N/cc.; —○—: 0.1*M* phosphate buffer; —①—: 0.1*M* borate buffer; —●—: 0.1*M* phthalate buffer; incubation: 30 mins. at 30°

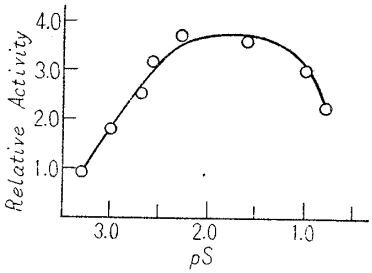

Fig. 3. Apparent pS-Activity Curve of Ethanolamine Dehydrase

Concentration of cell suspension: 0.2 mg. N/cc.; pH: 7.0 (0.1*M* phosphate buffer); incubation: 10 mins. at 30°

TABLE I. Enzyme Activity of Various Bacteria

Bacterium		Activity
Proteus morganii	AY	+
" "	AM-15	+
" "	AS-21	+
" "	AS-2	+
" "	AO-3	+
Pr. vulgaris	YO-1	−
" "	YO-2	−
Pr. mirabilis	OM-10	−
" "	OM-11	−
Pr. rettgeri	OR-5	−
" "	OR-6	−
Serratia marcescens	F-51	−
Aerobacter aerogenes	S-2	−
Escherichia coli	S-8	−
" "	S-15	−
Alkaligenes metalcaligenes	N-33	−
Pseudomonas jaegeri	1	−
" "	2	−
Ps. aeruginosa	7	−
" "	AHH-23	−
Micrococcus pyogenes var. *aureus*		
" "	209p	−
Bacillus subtilis	W-6	−
Bac. cereus	K-22	−
Achromobacter liquidum	AN-20	−

Specificity to Substrate—Specificity of ethanolamine dehydrase to various amine substrate was examined and it was found that the enzyme was entirely inert to N-methylethanolamine, N, N-dimethylethanolamine, choline, and 2-hydroxypropylamine, the enzyme reacting specifically with ethanolamine alone.

A slight activity, about one-tenth of that of ethanolamine, was found against phosphorylethanolamine. In this experiment, the intact cells were employed so that it is still not clear whether this 2-aminoethyl dihydrogen phosphate reacts after being converted to ethanolamine by phosphatase or this amine itself can be a substrate. Probably, it seems that the metabolism of this amine is due to the former, since *Pr. morganii* can hydrolyze 2-aminoethyl dihydrogen phosphate into ethanolamine and inorganic phosphate.

Stability of the Enzyme—This enzyme loses its activity completely on being heated at 60° for 5 mins., but the activity decreases only slightly even after 48 hrs. on lyophilization. The activity is also lost completely by drying with acetone, trituration with kieselguhr or alumina, and extraction by sonic vibrator. Attempted extraction of cell-free enzyme by addition of glutathione and/or adenylic acid, in accordance with the case of serine dehydrase,[10] and addition of pyridoxal phosphate[11] were all to no avail.

TABLE II. Effect of Various Substances on the Activity of Ethanolamine Dehydrase

Substance		Inhibition (%)
Ethylenediaminetetraacetic acid		1
8-Hydroxyquinoline		6
Monoiodoacetic acid		42
NH_2OH	$10^{-2}M$	96
„	10^{-3}	82
„	10^{-4}	51
NH_2NH_2		15
$NH_2CONHNH_2$		10
NaN_3		6.5
KCN		7.0
As_2O_3		12
$*Mn^{2+}$		5.0 (stimulation)
$*Fe^{3+}$		8.0
$*Co^{2+}$		2.5
$*Cu^{2+}$	$10^{-2}M$	93
	10^{-3}	58
	10^{-3}	27.5

Concentration: ethanolamine, $0.01M$ and inhibitor (except hydroxylamine and Cu^{2+}), $0.001M$; pH 7.0 in phosphate buffer; * $0.2M$ tris(hydroxymethylamino)methane buffer; incubation, 20 mins. at 30°.

Effect of Inhibitors—As shown in Table II, a strong inhibition was effected by hydroxylamine, monoiodoacetic acid, and bivalent copper ion, although the effect of hydroxylamine diminished by addition of pyridoxal phosphate. The coenzyme of serine dehydrase is said to be pyridoxal phosphate but it is still difficult to conclude whether pyridoxal phosphate has any connection with the coenzyme of ethanolamine dehydrase since the data obtained in the present series of experiments were with intact cells.

Periodical Variation in the Amount of NH_4OH, AcH, EtOH, and AcOH—As has already been described in the Experimental Method, metabolic decomposition of ethanolamine by *Pr. morganii* results in the formation of AcH, EtOH, and AcOH besides NH_4OH. Periodical variation in the amount of these substances was examined and, as indicated in Fig. 4, NH_4OH was found to be formed linearly and quantitatively with passage of time. Acetaldehyde also forms with passage of time, but, as long as ethanolamine is present, the amount of these three substances formed increases at about the same rate. When ethanolamine has quantitatively been decomposed, formation of AcH ceases and it is rapidly consumed to form

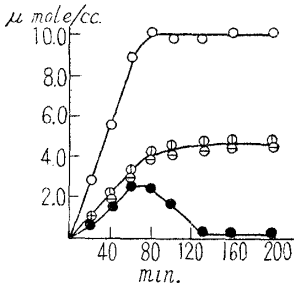

Fig. 4. Production of Ammonia, Acetaldehyde, Ethanol, and Acetic Acid from Ethanolamine by *Proteus morganii*

Concentration of ethanolamine: $0.01M$; concentration of cell suspension: 0.14 mg. N/cc.; pH: 7.0 ($0.1M$ phosphate buffer); —○—: ammonia; —①—: ethanol; —⊖—: acetic acid; —●—: acetaldehyde; incubation: 30°

10) W. A. Wood, I. C. Gunsalus: J. Biol. Chem., **181**, 171 (1949).
11) D. E. Metzler, E. E. Snell: *Ibid.*, **198**, 363 (1952).

EtOH and AcOH. At any time during this period, the total amount of AcH, EtOH, and AcOH present was equimolar with the amount of NH_4OH.

Examination of the metabolism of AcH by *Pr. morganii* showed that the formation of EtOH and AcOH was essentially the same as in the case of using ethanolamine as the substrate, as indicated in Fig. 5. In this case, equimolar amounts of EtOH and AcOH as that of AcH consumed are formed and

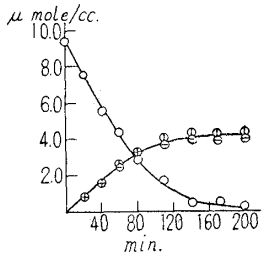

Fig. 5. Production of Ethanol and Acetic Acid from Acetaldehyde by *Proteus morganii*

Concentration of acetaldehyde: ca. 0.01*M*; concentration of cell suspension: 0.2 mg. N/cc.: pH: 7.0 (0.1*M* phosphate buffer); incubation: 30°; —○—: acetaldehyde: —①—: ethanol; —⊖—: acetic acid

the so-called enzymatic dismutation has taken place. This enzyme system has not been obtained in cell-free state as yet but it is assumed that its mechanism is the same as that of glycerol fermentation by yeast during alkali addition.

The foregoing facts suggest that the metabolic pathway of ethanolamine by *Pr. morganii* should be represented as follows:

$$NH_2\text{-}CH_2\text{-}CH_2OH \longrightarrow NH_3 + CH_3CHO \longrightarrow {}^1/_2\ C_2H_5OH + {}^1/_2\ CH_3COOH$$

Summary

The enzyme system that decomposes ethanolamine into ammonia and acetaldehyde was found in *Proteus morganii* and it was named ethanolamine dehydrase. The acetaldehyde formed during the course of this decomposition was also found to undergo concurrent dismutation to be changed into ethanol and acetic acid.

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