120 mm.) were added to the ethanol. In the course of 15 minutes the temperature rose to 40° and after 1 hr. two drops of concentrated hydrochloric acid were added to neutralize the caustic. The ethanol was distilled off at water pump vacuum on the steam-bath, and the residue was distilled giving 17 g. (94% yield) of material boiling at 113–118° (0.05 mm.). This distillate crystallized almost immediately and melted at 57–59°; recrystallization from petroleum ether (b.p. 60–70°) did not raise this melting point.

Anal. Calcd. for $C_9H_{12}N_2O_2$: C, 60.43; H, 6.59; N, 15.55. Found: C, 60.15; H, 6.72; N, 15.35.

A small sample of this material was converted to the semicarbazone in the usual manner. After crystallization from water the semicarbazone melted at 179–180°.

Anal. Calcd. for $C_{10}H_{15}N_5O_2$: N, 29.55. Found: N, 29.0.

Alkylation of 3-Methyl-6-pyridazone with 3-Chloro-2-butanone.—To a solution of sodium (4.2 g., 0.18 atom) in ethanol (200 ml.) there was added 3-methyl-6-pyridazone (18.0 g., 0.16 mole); this mixture was cooled to below 10°, and 3-chloro-2-butanone (21.3 g., 0.18 mole) was added dropwise with good stirring. The mixture was heated at reflux for 2 hr. and then evaporated at water pump vacuum on the steam-bath. The residue was taken up in benzene (100 ml.), and this solution was washed with two 50-ml. portions of 30% potassium carbonate solution. The benzene solution was dried over anhydrous potassium carbonate and then evaporated under vacuum. The residue was distilled, giving 8.7 g. (30% yield) of material boiling at 90–100° (0.1 mm.), $n^{25}D$ 1.5063. $n^{25}D$ of 3-(3-methyl-6-pyridazonyl-1)-butanone-2 is 1.5226. Seeding this material with 4-(3-methyl-6-pyridazonyl-1)-butanone-2 did not induce crystallization.

A sample of this material was converted to a semicarbazone in the usual manner. The crude semicarbazone melted at 179–183°; after three recrystallizations from water it melted at 194–197°, no depression when mixed with the semicarbazone of 3-(3-methyl-6-pyridazonyl-1)-butanone-2. A mixed melting point with the semicarbazone of 4-(3-methyl-6-pyridazonyl-1)-butanone-2 melted at 160–175°.

Preparation of Alcohols. 3-(3-Methyl-6-pyridazonyl-1)-butanol-2.—A mixture of dry isopropyl alcohol (100 ml.), aluminum isopropoxide (freshly distilled) (20.4 g., 0.1 mole) and 3-(3-methyl-6-pyridazonyl-1)-butanone-2 was distilled very slowly for 2 hr. through a 10-in. helices packed column; 10 ml. of distillate was collected. The mixture was cooled and acidified with 25.5 ml. of concentrated hydrochloric acid and then made strongly alkaline with sodium hydroxide (24 g., 0.6 mole) dissolved in water (50 ml.). This solution was extracted with two 100-ml. portions of benzene, and the combined benzene extracts were dried over anhydrous potassium carbonate. The benzene was evaporated at water pump vacuum on the steam-bath and the residue was distilled, giving 15 g. (82.5% yield) of material boiling at 95° (0.1 mm.) which crystallized and melted at 73–76°.

Anal. Calcd. for $C_8H_{14}N_2O_2$: C, 59.32; H, 7.74; N, 15.38. Found: C, 59.16; H, 7.93; N, 15.26.

3-(3-Methyl-6-pyridazonyl-1)-2-methylbutanol-2.—Methylmagnesium iodide was prepared in the usual manner from magnesium turnings (2.64 g., 0.11 atom) and methyl iodide (17.1 g., 0.12 mole) in absolute ether (125 ml.). This solution was cooled to 10° and with good stirring there was added slowly a solution of 3-(3-methyl-6-pyridazonyl-1)-butanone-2 (16 g., 0.089 mole) in absolute ether (50 ml.); a solid complex was formed which was decomposed by the addition of ammonium chloride (7 g.) in water (20 ml.). An excess of anhydrous potassium carbonate was added to the mixture and the ether was separated and the solid was washed with two 100-ml. portions of benzene. The benzene and ether solutions were combined and evaporated at water pump vacuum on the steam-bath. The residue was distilled giving 12 g. of material boiling at 80–90° (0.02 mm.). This distillate was heated under reflux for 2 hr. with a solution of semicarbazide hydrochloride (6.9 g.) and anhydrous sodium acetate (4.9 g.) in water (30 ml.) in order to separate unreacted ketone as the semicarbazone. The resulting solution was evaporated to dryness under water pump vacuum on the steam-bath and the residue was treated with benzene (100 ml.), and this suspension was filtered. Evaporation of the benzene solution followed by distillation gave 7 g. (41% yield) of material boiling at 95–100° (0.02 mm.) which crystallized and melted at 53–55°. A small sample after recrystallization from petroleum ether (b.p. 60–70°) melted at 57.5–58.5°.

Anal. Calcd. for $C_{16}H_{16}N_2O_2$: C, 61.20; H, 8.22; N, 14.28. Found: C, 61.31; H, 8.08; N, 14.20.

NEW YORK 11, NEW YORK

COMMUNICATIONS TO THE EDITOR

THE MECHANISM OF PEPSIN DENATURATION

Sir:

In 1930 Northrop[2] observed that the inactivation of pepsin was paralleled by the formation of acid insoluble pepsin. Subsequently Philpot[3] reported that the sedimentation constant (S) of pepsin showed a gradual decline between *p*H 5 and 11.

The gradual decline in sedimentation constant that Philpot observed has now been shown to represent the change from native to denatured pepsin and occurs over a very narrow range of *p*H, conforming to the enzyme kinetics of inactivation.[4] Single symmetrical boundaries, which spread at similar rates, were observed in the ultracentrifuge for both the native and denatured forms of pepsin. In 0.10 $\Gamma/2$ phosphate buffer the $S^{\circ}_{20,w}$ was 3.08 at *p*H 6.0 and 2.03 at *p*H 7.0 for the two forms, respectively.

To distinguish between frictional and mass changes in pepsin during inactivation, light scattering and viscosity methods were employed. At *p*H 6.42 the reduced intensity ($R = I_{90}r^2/I_0$) decreased uniformly to about 60% of its initial value. When the log of the fractional residual scatter $\{\log (R_t - R_\infty)/(R_0 - R_\infty)\}$ was plotted against time a linear relationship was observed. The kinetics agreed closely with that determined from the rate of formation of acid insoluble pepsin.

The viscosity of pepsin solutions was found to increase on inactivation. The rate of increase in viscosity paralleled the rate of enzyme inactivation; the data appear in Table I.

Since optical rotatory changes can serve as an indicator of molecular structural alterations in pro-

(1) Aided in part by grant No. C-1974 from the National Cancer Institute of the National Institutes of Health, Public Health Service, and by an Institutional Grant from the American Cancer Society.

(2) J. H. Northrop, *J. Gen. Physiol.*, **13**, 739 (1930).

(3) J. St. L. Philpot, *Biochem. J.*, **29**, 2458 (1935).

(4) J. Steinhardt, *Kgl. Danske Videnskab. Selskab Mat. fys. Medd.*, **14**, no. 11 (1937).

teins and polypeptides,[5] the rate of increase in levorotation was compared with the rate of enzyme inactivation and found to be indistinguishable.

TABLE I

THE KINETICS OF PEPSIN INACTIVATION BY FOUR DIFFERENT METHODS

All solutions were adjusted from unbuffered stock solutions, which were near *p*H 5.5, to their experimental *p*H values by the addition of relatively small volumes of concentrated buffers. The *p*H of the buffers were only slightly higher than the *p*H of the experiment. The pepsin concentration is for the total weight of sample. These crystalline preparations of pepsin (Worthington Biochemical Corporation) contain about 20% split products. The light scattering experiments were performed at room temperature, which was 28°; in the other procedures the temperature was controlled at 25.0°. An Ostwald viscometer was used with a flow time of 89.2 sec. The optical rotatory change was for a 2-dm. cell. Tris buffer is tris-(hydroxymethyl)-aminomethane.

	Method	Pepsin g./100 ml.	*p*H	Buffer	*M*	Net observed change	$k \times 10^2$ (min.$^{-1}$)
1	Light scatter	0.35	6.42	Cacodylic KNO_3	0.023 0.136	−60%	4.4
2	Viscosity	0.33	7.10	Imidazole NaCl	0.015 0.009	+3.1 sec.	4.8
3	Optical rotation	0.80	7.08	Imidazole KNO_3	0.045 0.008	−0.25°	5.7
4	Acid liberation	0.42	6.64	Tris NaCl	0.023 0.146	5.6 H/ mole pepsin)	3.4

Finally in the conversion of pepsin from a catalytically active protein to an inactive form (in the *p*H range 6.64–6.97), about 5.6 moles of hydrogen ions are liberated per mole of enzyme. In four experiments in this *p*H interval, the first order velocity constants agreed with the rate of inactivation[6] within 5%. The unmasking of these acidic groups is reflected by appreciable differences in the titration curves of native and denatured pepsin above *p*H 6. These groups are displaced to lower *p*H values in the denatured form.

The acid formed in the inactivation of pepsin can be most readily accounted for if it comes from hydrogen-bonded carboxyl groups. The intrinsic *pK* of carboxyl groups in a number of proteins is near 4.5.[7] The *pK* of these groups in pepsin are increased from their intrinsic values both by a sizable contribution from the electrostatic free energy[8] and by the free energy of hydrogen bonding.[9] It should be noted that part of the 5.6 moles of acid formed probably comes from non-hydrogen bonded ionizable groups since the change in the mass and shape of pepsin decreases the electrostatic free energy of ionization.[10]

It thus appears that the loss of pepsin enzyme activity is accompanied by the appearance of both hydrophilic and hydrophobic groups, gross changes in the mass and shape of its molecular kinetic unit and that the bio- and physico-chemical alterations are expressions of a single rate-determining process, which manifestly involves the rupture of carboxyl linked hydrogen bonds.

(5) P. Doty and J. T. Yang, THIS JOURNAL, **78**, 499 (1956); R. B. Simpson and W. Kauzmann, *ibid.*, **75**, 5139 (1953).

(6) M. L. Anson, *J. Gen. Physiol.*, **22**, 79 (1938). The test was modified to the extent that the concentrations of split products were determined by their absorption at 280 mμ.

(7) C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, **77**, 6414 (1955), *cf.* Table III.

(8) G. E. Perlmann, in "Advances in Protein Chemistry," M. L. Anson, K. Bailey and J. T. Edsall, Vol. X, Academic Press, Inc., New York, N. Y., has reported that the isoelectric point increases from a value near *p*H 1.0 to 1.7 when the single phosphate group of pepsin is removed by enzymatic means.

(9) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

(10) As the NaCl concentration is increased to 1*M* the acid liberated decreases to about 75% of its value in 0.15*M* NaCl. This would leave about 4 moles of acid originating from hydrogen-bonded groups.

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RECEIVED MARCH 30, 1956

BIOSYNTHESIS OF THE PYRIDINE RING OF NICOTINE[1]

Sir:

A biosynthetic connection between nicotinic acid, or its supposed antecedents, and the pyridine ring of nicotine has been proposed.[2,3,4] However, isotopic tracer experiments using carboxyl-C^{14} nicotinic[5] and anthranilic[6] acids and tryptophan-β-C^{14}[7] have failed to yield supporting evidence. Recently, Dewey, Byerrum and Ball[8] and Leete[9] reported that the pyrrolidine ring of nicotine, and in particular the carbon atom at position 2′ in the pyrrolidine ring, is derived from ornithine. If this is correct, obviously the labeled atoms of the three compounds listed could not be retained in the newly formed nicotine molecule. Therefore, the experiments cited above[5,6,7] are inapplicable.

Ring-labeled H^3-nicotinic acid was prepared by neutron irradiation of a mixture of the acid and lithium carbonate,[10] with carboxyl tritium being removed during the purification of the acid. Nicotinic acid containing C^{14} in both ring and carboxyl positions was obtained by neutron irradiation of nicotinamide.[11]

We have supplied these two preparations to *sterile* cultures of excised roots of Turkish tobacco[5] and have found substantial amounts of radioactivity in the nicotine produced by the roots during their growth (Table I). Lesser incorporation of C^{14} label was partly due to the use of limiting amounts of nicotinic acid, and also to the loss of nicotinic acid carboxyl carbon.[5] Even in this case, however,

(1) Work performed under the auspices of the U. S. Atomic Energy Commission at Brookhaven National Laboratory and at Columbia University under Contract No. AT(30-1)-1778.

(2) E. Winterstein and G. Trier, "Die Alkaloide," 2nd ed., Borntraeger, Berlin, 1931, p. 1031.

(3) G. Klein and H. Linser, *Planta*, **20**, 470 (1933).

(4) R. Dawson, *Plant Physiol.*, **14**, 479 (1939).

(5) R. Dawson, D. Christman and R. C. Anderson, THIS JOURNAL, **75**, 5114 (1953).

(6) R. Dawson and D. Christman, unpublished data.

(7) K. Bowden, *Nature*, **172**, 768 (1953).

(8) L. Dewey, R. Byerrum and C. Ball, *Biochem. et Biophys. Acta*, **18**, 141 (1955).

(9) E. Leete, *Chem. and Ind.*, No. **19**, 537 (1955).

(10) R. Wolfgang, F. Rowland and C. Turton, *Science*, **121**, 715 (1955).

(11) A. Wolf and R. C. Anderson, THIS JOURNAL, **77**, 1609 (1955); *cf.* R. C. Anderson, E. Penna-Franca and A. Wolf, Brookhaven National Laboratory Quarterly Progress Report, October 1-December 31, 1954.