SUMMARY

The effect of pyridine derivatives on yeast alcohol dehydrogenase has been described. These compounds have been found to compete with DPN or DPNH for the active site on the enzyme. Three classes of compounds have been studied: pyridine bases, N^1-methylpyridinium salts, and analogues of DPN.

A number of pyridine compounds have been found to show inhibitory powers different for the DPN reduction than for the DPNH oxidation. The inhibition of pyridine bases appears to be due to their ionized forms.

Although pyridine bases inhibit the yeast alcohol dehydrogenase reaction, some of these compounds could actually, at low concentrations, stimulate the rate of reaction.

A partial reaction sequence and an enzyme-coenzyme model are proposed to explain the experimental data.

REFERENCES

1 J. VAN EYS, *Federation Proc.*, 15 (1956) 374.
2 H. THEORELL AND R. K. BONNICHSEN, *Acta Chem. Scand.*, 5 (1951) 1105.
3 J. E. HAYES, JR., AND S. F. VELICK, *J. Biol. Chem.*, 207 (1954) 225.
4 A. P. NYGAARD AND H. THEORELL, *Acta Chem. Scand.*, 9 (1955) 1300.
5 A. P. NYGAARD AND H. THEORELL, *Acta Chem. Scand.*, 9 (1955) 1551.
6 H. VON EULER, *Ber. deut. Chem. Ges.*, 75 (1942) 1876.
7 N. G. BRINK, *Acta Chem. Scand.*, 7 (1953) 1090.
8 P. FEIGELSON, J. N. WILLIAMS, JR. AND C. A. ELVEHJEM, *J. Biol. Chem.*, 198 (1951) 361.
9 L. J. ZATMAN, N. O. KAPLAN, S. P. COLOWICK AND M. M. CIOTTI, *J. Biol. Chem.*, 209 (1954) 453.
10 N. O. KAPLAN AND M. M. CIOTTI, *J. Biol. Chem.*, 221 (1956) 823.
11 N. O. KAPLAN, M. M. CIOTTI, F. E. STOLZENBACH AND N. R. BACHUR, *J. Am. Chem. Soc.*, 77 (1955) 815.
12 M. E. PULLMAN, S. P. COLOWICK AND N. O. KAPLAN, *J. Biol. Chem.*, 194 (1952) 593.
13 H. H. JAFFÉ AND G. O. DOAK, *J. Am. Chem. Soc.*, 77 (1955) 4441.
14 L. P. HAMMETT, *Physical Organic Chemistry*, 1940, McGraw-Hill Book Co., New York, Chapter VII.
15 H. H. JAFFÉ, *Chem. Revs.*, 53 (1953) 191.
16 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 56 (1934) 658.
17 N. O. KAPLAN, M. M. CIOTTI AND F. E. STOLZENBACH, *J. Biol. Chem.*, 221 (1956) 833.
18 N. O. KAPLAN, S. P. COLOWICK, L. J. ZATMAN AND M. M. CIOTTI, *J. Biol. Chem.*, 205 (1953) 31.
19 J. VAN EYS, M. M. CIOTTI AND N. O. KAPLAN, *Biochim. Biophys. Acta*, 23 (1957) 581.
20 E. S. GUZMAN BARRON AND S. LEVINE, *Arch. Biochem. Biophys.*, 41 (1952) 175.
21 B. L. VALLEE AND F. L. HOCH, *J. Am. Chem. Soc.*, 77 (1955) 821.

Received July 28th, 1956.

YEAST ALCOHOL DEHYDROGENASE*

II. PROPERTIES OF THE CATALYTICALLY ACTIVE SITE*

JAN VAN EYS, MARGARET M. CIOTTI AND NATHAN O. KAPLAN

The McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Md. (U.S.A.)

In the previous paper a reaction scheme and a model of the enzyme-coenzyme complex of yeast alcohol dehydrogenase** were proposed[1]. In the development of the theoretical rate in the presence and absence of a pyridinium ion, it was assumed that

* Contribution No. 161 of the McCollum-Pratt Institute. Supported in part by grants from the Rockefeller Foundation and the National Cancer Institute of the National Institutes of Health (Grant No. C-2374C).

** Abbreviations used in this paper are: ADH: alcohol dehydrogenase; DPN and DPNH: oxidized and reduced diphosphopyridine nucleotide, respectively; Py-3AlDPN and Py-3AlDPNH: oxidized and reduced analogs of DPN of pyridine-3-aldehyde, respectively; pCMB: *para*-chloromercuribenzoate.

References p. 587.

the enzyme has multiple sites, all of which are active and independent in the binding of the coenzymes and in the reaction catalysis. Under these circumstances the relative rate in the presence and absence of a pyridinium ion should be inversely proportional to the concentration of the pyridinium ion. Experimentally, however, the relative rate is proportional to the logarithm of the concentration of the pyridinium ion. This can be the result of one of three possibilities: (1) The sites are not equal and independent, (2) there is more than one pyridinium ion required per site, and (3) there is more than one site for binding, but only a few of these sites are active at one time in catalyzing the enzymic reaction. In the studies of HAYES AND VELICK[2] it has been shown that there are four equal and independent sites for binding DPN and DPNH. This is in agreement with VALLEE's observation that there are four zinc atoms per molecule of enzyme[3]. However, there is no evidence that all four sites are independently active.

In this paper evidence will be presented which suggests that only one site is active in catalyzing the reaction of alcohol oxidation. Furthermore, some indications as to the chemical nature of the reactive center will be reported.

MATERIAL AND METHODS

The source and preparation of the enzyme and coenzymes has been described[1]. The ultracentrifugal separation analysis of coenzyme binding was performed according to VELICK *et al.*[4]. Enzyme concentration was measured spectrophotometrically using a molar extinction coefficient at 280 mμ of $1.89 \cdot 10^5$ [2]. The extinction of DPNH was taken to be $6.22 \cdot 10^3$ at 340 mμ[5]; that for Py-3AI DPNH at 355 mμ $7.0 \cdot 10^3$ [6].

Sulfhydryl groups were determined with pCMB as described by BOYER[7]. When the reduction of 2,6-dichlorophenolindophenol was measured, a molar extinction of $1.9 \cdot 10^5$ at 610 mμ (pH 7.0) was used[8]. At the isosbestic point (520 mμ) an extinction of $8.1 \cdot 10^4$ was employed.

RESULTS

Coenzyme binding

Yeast ADH, as commercially obtained, contains an appreciable amount of ethanol which cannot be dialyzed out without excessive inactivation of the enzyme. Consequently, when the ultracentrifugal analysis of HAYES AND VELICK[2] was tried, no reliable results could be obtained with DPN, since a significant amount was converted to DPNH. However, the binding of DPNH could be easily verified. By the same technique it could also be shown that Py-3AlDPNH was bound to the enzyme. Analysis of the data as described[2] gave again four coenzyme molecules per molecule of enzyme for either DPNH or Py-3AlDPNH. The dissociation constant for both complexes was approximately $1.5 \cdot 10^{-5}$ moles/liter.

Spectrophotometric evidence for enzyme-coenzyme complex

As noted in the previous paper[1] the yeast enzyme differs from the horse liver alcohol dehydrogenase, in that DPNH does not show a spectral shift with the yeast protein. This spectral shift does occur when the reduced coenzyme is bound to the liver enzyme. This difference holds true for APDPNH and Py-3AlDPNH.* Similarly, the DPN-hydroxylamine complex observable with the mammalian enzyme[9] does not form with the yeast enzyme. When one uses, however, Py-3AlDPN and hydroxylamine, a complex is observed with yeast ADH. This complex has a maximum at 315 mμ (Fig. 1). These results indicate that the liver and the yeast enzymes may have some similarity in the formation of a ternary complex.

* N. O. KAPLAN and M. M. CIOTTI unpublished observations.

References p. 587.

The molar extinction of the Py-3AlDPN/hydroxylamine complex at 315 mμ is $6.0 \cdot 10^3$. When the data were treated in the manner of HAYES AND VELICK, *i.e.*:

$$r = n - K(r/C_f) \quad (1)$$

where r stands for the number of complexes bound to the enzyme, n the number of binding sites per enzyme, and C_f the concentration of free coenzyme, a plot is obtained as illustrated in Fig. 2.

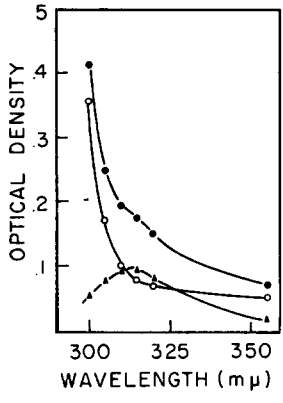

Fig. 1. The spectrum of enzyme and enzyme-Py-3AlDPN-hydroxylamine complex, O = free enzyme, ● = enzyme plus Py-3AlDPN. The coenzyme was also added to the blank cuvette. ▲ = difference spectrum. The spectra were taken in 0.1 *M* phosphate buffer, pH 7.5. Enzyme concentration 130 mμ *M*, hydroxylamine 0.02 *M*, final volume 1.0 ml.

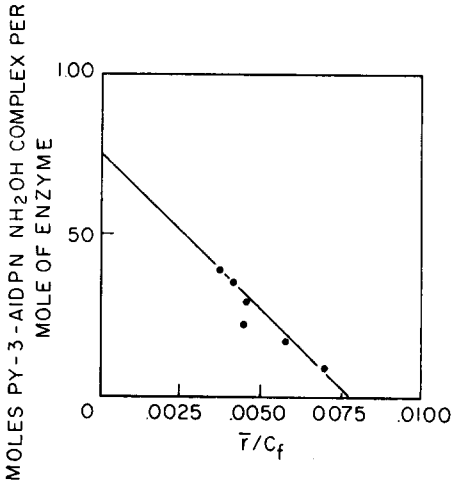

Fig. 2. A plot of Py-3AlDPN/hydroxylamine complex bound per mole of enzyme *vs.* this ratio over concentration of free Py-3AlDPN. Phosphate buffer, 0.04 *M*, pH 7.0, hydroxylamine 0.02 *M*, enzyme: 140 mμ *M*. Final volume 1.0 ml. The intercept corresponds to the maximal number of complexes bound per mole of enzyme, at infinite coenzyme concentration.

The intercept n equals 0.80, or one mole of complex is formed per mole of enzyme. This value did not change over the whole pH range of 5.2–8.2. The dissociation constant (K) was the same at all pH values (10^{-2} moles/liter).

It must be concluded that not all sites are capable of accepting or forming this complex, even though four moles of coenzyme are bound. If one assumes that this complex is similar to the DPN-ethanol-enzyme complex[9], it appears that only one site is involved in forming the activated ternary complex.

Enzyme denaturation

Apart from complex-formation, the active site participates in other reactions, as is indicated by the acid denaturation of the enzyme. When yeast ADH is placed in a buffer of pH 5.0 or lower, a slow precipitation is observed. The rate of precipitation can be followed by observing the increased turbidity in the Beckman spectrophotometer at an arbitrary wavelength. Usually 550 mμ was chosen. This denaturation is illustrated in Fig. 3. The denaturation was relatively independent of the buffer used. The reaction sequence responsible can be pictured as follows:

$$E + n.\,H^+ \underset{}{\overset{K}{\rightleftharpoons}} E\text{-}n\mathrm{H} \xrightarrow{k_1} E_{d_1} \xrightarrow{k_2} E_{d_2} \longrightarrow \cdots \xrightarrow{k_x} E_{dx} \quad (2)$$

References p. 587.

where E_{dx} is the xth member of a sequence of denatured forms of the enzyme. The rate of denaturation can be described as:

$$(dE_{dx}/dt) = (k_1 \cdot k_2 \cdot \cdots \cdot k_x)\ K\ (E)\ (H^+)^n \quad (3)$$

A plot of

$$\log (dE_{dx}/dt) = -n\text{pH} + \log (k_1 \cdot k_2 \cdot \cdots \cdot k_x)\ K\ (E) \quad (4)$$

will yield as slope the number of hydrogen ions involved. When the rate of denaturation is plotted *vs.* pH, an indication of the dissociation of the proton acceptor involved can be obtained. These graphs are presented in Fig. 4. From the data the number of hydrogen ions involved was found to be 0.9, which indicates that one proton reacts with one enzyme molecule. The proton acceptor on the protein appeared to have a pK between 3.0–3.5.

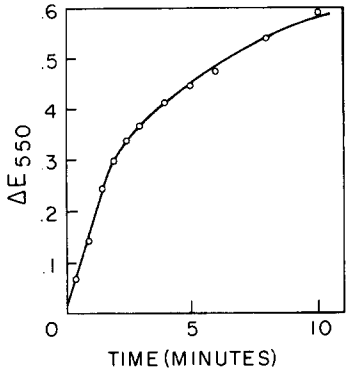

Fig. 3. The rate of denaturation of yeast ADH. Phosphate buffer 0.04 M, pH 4.0. Enzyme concentration: 13 mμM. Final volume 3.0 ml. The increase in optical density is a measure of increased turbidity.

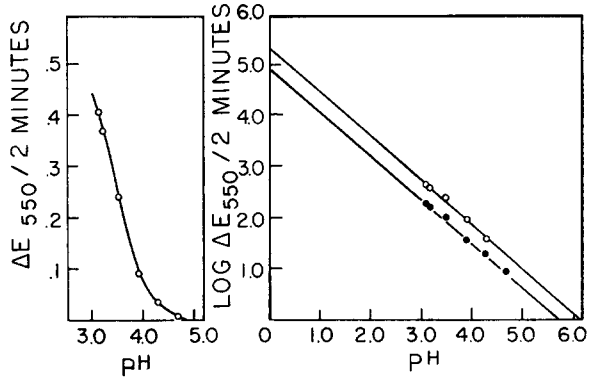

Fig. 4. A. A plot of the rate of denaturation versus pH. Enzyme concentration: 18 mμM. Acetate buffer 0.05M. Final volume 3.0 ml. B. A plot of the logarithm of the rate of denaturation *vs.* pH. ○ = enzyme concentration: 18 mμM, ● = enzyme concentration: 9 mμM. Acetate buffer 0.05 M.

Reduction of 2,6-dichlorophenolindophenol and denaturation

2,6-Dichlorophenolindophenol has been found to react with sulfhydryl groups of proteins[10]. Although it is a two electron dye, 2,6 dichlorophenolindophenol will react in a one to one ratio with each sulfhydryl grouping[8,10,11]. Yeast ADH, however, was an exception to this rule. The dye did not react with the protein unless a low pH was employed, even though the dye is more easily reduced at higher pH values. The rate of reduction at pH 4 was twice that at pH 5, while at pH 7.0 and higher very little reduction occurred. The extent of reduction was but 8.5 moles of dye reduced per mole of protein. The correlation between the rate of dye reduction at different pH values and the denaturation rate at these pH values suggested that the potential proton acceptor on the protein was an unreactive sulfhydryl group. When the total number of sulfhydryl groupings was measured with pCMB a value of 18.5 moles S–H was found per mole protein. This value is in fair agreement with the data of BARRON[12].

Site of denaturing groups

Acid denaturation of the yeast ADH could be prevented, or at least slowed by the

References p. 587.

presence of DPN*, DPNH, Py-3AlDPN or beta-picoline. If the sites on the enzyme are equal and independent, and if each site yields, after adding a proton, an enzyme equally susceptible to denaturation, a protective agent (P) must cover all n sites to be effective in protecting the enzyme. Thus the equilibrium between protected enzyme (E-P) and susceptible enzyme (E_s) can be written as:

$$(E_s) + n.\,(P) \rightleftharpoons^{K} (E\text{-}P) \tag{5}$$

Solving for E_s and substituting $E_t - E_s$ for E-P we get:

$$E_s = E_t/(K\,(P)^n + 1) \tag{6}$$

where E_t stands for total enzyme concentration. Substituting equation (6) in the rate equation for denaturation, *i.e.*, equation (3), we get:

$$(dE_{dx}/dt) = K(H^+)(E_t)/(K(P)^n + 1) \tag{7}$$

For the relative rate v_r of denaturation in the presence and absence of a protecting agent (equation (7) divided by equation (3)) we thus get:

$$v_r = 1/(K(P)^n + 1) \tag{8}$$

which can be rewritten as:

$$\log[(1/v_r) - 1] = n\log\,(P) + \log K \tag{9}$$

When the data for beta-picoline protection are plotted in this fashion, we get the plot of Fig. 5. The data agree well with the predicted linear relationship. Different experiments yielded a value for n ranging from 2.4 to 3.3.

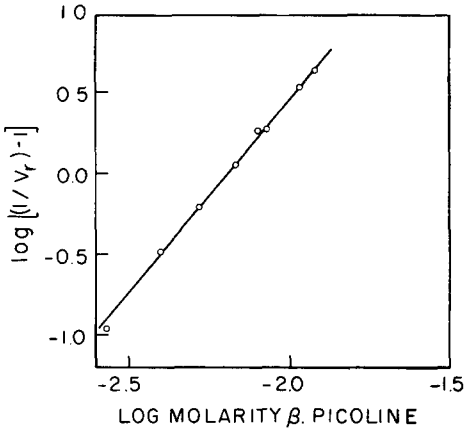

Fig. 5. The effect of beta-picoline on the acid denaturation of yeast ADH. Enzyme concentration: 9 mμM. The plot is the logarithm of the reciprocal of the relative rate minus one *vs.* the logarithm of the concentration of beta-picoline. See text for details. The slope is an indication of the number of beta-picoline ions per mole of enzyme.

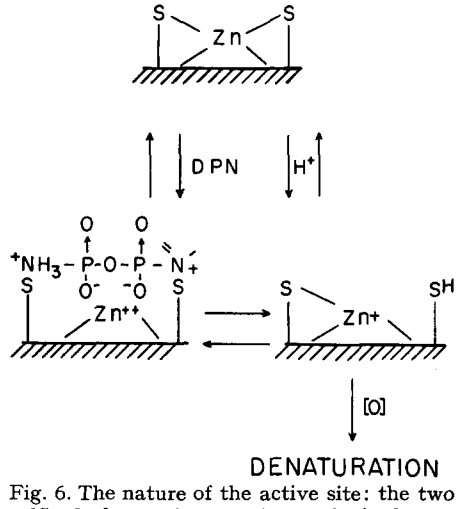

Fig. 6. The nature of the active site: the two sulfhydryl groupings are in non-ionized combination with the zinc.

DISCUSSION

The picture which suggests itself for the active site of yeast ADH is one that contains unreactive sulfhydryl groups. That sulfhydryl groups are present on the active site

* At low concentrations DPN actually somewhat accelerates the denaturation reaction. This is in line with the low affinity of the enzyme for DPN. Once the Zn-S complex is broken, the enzyme is susceptible to denaturation (see Fig. 6).

References p. 587.

is inferred from the observations of BARRON[12] and from the fact that the hydroxylamine – Py-3AlDPN complex can be broken up with pCMB. These sulfhydryl groups must be bound in such a way that acid will release the grouping. A possible reason for this lack of reactivity is illustrated in Fig. 6 where the sulfhydryl groups are bound to the zinc. The denaturation can be pictured as a sequence of reactions: the proton adds to one of the sulfhydryl groups, yielding a free –SH grouping which is susceptible to oxidation. Thus the denatured enzyme probably is the result of the formation of randomly crosslinked disulfide groupings. Breaking up of one sulfhydryl zinc linkage would tend to strengthen the second zinc sulfide bond*. The native enzyme appears to be completely reduced. This picture is compatible with the previously postulated enzyme-coenzyme complex[1].

It appears probable that only one site on the enzyme is at one time engaged in alcohol oxidation. The reasons for this possibility are as follows. First, all sites are equal and independent for coenzyme binding, yet only one forms the Py-3AlDPN-hydroxylamine complex. Secondly, in the event of acid denaturation all four sites are equally effective in accepting a proton, and thus all four sites must be occupied for the enzyme to be protected. Therefore, four moles of beta-picoline will be required per mole of enzyme to completely protect it, if the sites are equal for beta-picoline. Experimentally, the measure is maximally 3.3 ions per four sites. Thus, only one pyridinium ion is required per site. However, the effect of the pyridinium ions on the rate of alcohol oxidation is logarithmically related to the concentration of the pyridinium ion[1]. It is clear from the above that this relationship is not the result of the fact that the sites are not equal and independent for the pyridinium ion. Furthermore, the experimental data show but one beta-picoline ion per site. The logarithmic proportionality, then, is most logically explained by the assumption that only one site is active at a given time. It must be mentioned that this does *not* imply that only one DPN is required per enzyme molecule, but rather only one DPN out of four reacts with an ethanol molecule at a time.

These postulations require an obligatory reaction sequence:
enzyme ⟶ enzyme–coenzyme ⟶ enzyme–coenzyme–substrate. This sequence does not involve a substrate enzyme complex, and is in accord with our previous proposal[1]. The same reaction sequence appears to be operating for heart muscle lactic dehydrogenase[13] and hence may not be unique for the yeast ADH.

SUMMARY

Yeast alcohol dehydrogenase binds four moles of the reduced pyridine-3-aldehyde analog of DPN (Py-3AlDPN) or DPNH per mole of enzyme. No DPN-hydroxylamine complex is formed with yeast ADH. However, one mole of Py-3AlDPN-hydroxylamine complex is formed per mole of enzyme.

When acid denaturation of the enzyme is measured, pyridine derivatives inhibit the rate. For inhibition close to four moles of pyridinium ions are required per mole of enzyme, but only one hydrogen ion is required to give an enzyme species susceptible to denaturation. Evidence is presented which indicates that yeast ADH contains unreactive sulfhydryl groupings on the active sites; the nature of this unreactive sulfhydryl group is discussed.

Discussion of the possibility that one of the four DPN binding sites of the enzyme at a time is engaged in the conversion of DPN to DPNH, is presented.

* That at least two sulfhydryl groups are present per active site can be calculated from the data of BARRON[12]. It is also indicated by the observation that deamino DPN does not protect against the acid denaturation (J. VAN EYS, M. M. CIOTTI and N. O. KAPLAN, to be published). Free pyridine derivatives do inhibit the denaturation. Thus at least two different sulfhydryl groupings are implied.

References p. 587.

REFERENCES

1 J. VAN EYS AND N. O. KAPLAN, *Biochim. Biophys. Acta*, 23 (1957) 574.
2 J. E. HAYES JR., AND S. F. VELICK, *J. Biol. Chem.*, 207 (1954) 225.
3 B. L. VALLEE AND F. L. HOCH, *J. Am. Chem. Soc.*, 77 (1955) 821.
4 S. F. VELICK, J. E. HAYES JR. AND J. HARTING, *J. Biol. Chem.* 203 (1953) 527.
5 A. KORNBERG AND B. L. HORECKER, *Biochem. Preparations*, 3 (1953) 23.
6 N. O. KAPLAN, M. M. CIOTTI AND F. E. STOLZENBACH, *J. Biol. Chem.*, 221 (1956) 833.
7 P. D. BOYER AND H. L. SEGAL, in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, The Johns Hopkins Press, Baltimore, 1954, p. 528.
8 R. E. BASFORD AND F. M. HUENNEKENS, *J. Am. Chem. Soc.*, 77 (1955) 3873.
9 N. O. KAPLAN AND M. M. CIOTTI, *J. Biol. Chem.*, 211 (1954) 431.
10 G. RAFTER AND S. P. COLOWICK, *Federation Proc.*, 14 (1955) 267.
11 G. RAFTER, *Federation Proc.*, 15 (1956) 334.
12 E. S. GUZMAN BARRON AND S. LEVINE, *Arch. Biochem. Biophys.*, 41 (1952) 175.
13 G. W. SCHWERT AND Y. TAKENAKA, *Federation Proc.*, 15 (1956) 351.

Received July 28th, 1956

THE INFLUENCE OF DIETARY PROTEIN ON THE INCORPORATION OF ^{14}C-GLYCINE AND ^{32}P INTO THE RIBONUCLEIC ACID OF RAT LIVER

C. M. CLARK, D. J. NAISMITH AND H. N. MUNRO

Department of Biochemistry, The University, Glasgow (Scotland)

Two dietary factors influence the course of ribonucleic acid (RNA) metabolism in the liver. Protein intake plays a dominant role in determining the amount of RNA in the liver[1,2], whereas energy intake affects its rate of synthesis as measured by ^{32}P incorporation[2]. The influence which protein intake exerts over the amount of RNA in the liver does not appear to involve changes in the rate of RNA formation; thus the feeding of a protein-free diet causes a large reduction in the amount of RNA without affecting the absolute rate of ^{32}P uptake by RNA[2]. An alternative way in which the dietary supply of protein could influence the amount of RNA in the liver is by regulating its rate of breakdown. In the course of studying the influence of energy intake on ^{14}C-glycine incorporation into RNA, we obtained evidence strongly suggesting that the rate of RNA breakdown diminishes after a meal containing protein. This evidence emerged when we used rats which were trained to eat protein-containing or protein-free diets at fixed times each day; in consequence, it was possible to know with certainty whether the animals were in the post-absorptive state or were actively absorbing nutrients at the time of injection with isotopes. When the animals were fasting after meals containing protein, there was evidence of a considerable breakdown of RNA. After feeding protein, the picture rapidly changed to one which we have interpreted as indicating cessation of breakdown. These findings have led us to conclude that the intensity of protein synthesis determines the stability of liver RNA. This may have some bearing on the role of RNA in protein synthesis. A preliminary account of these experiments has already appeared[3].

References p. 599.