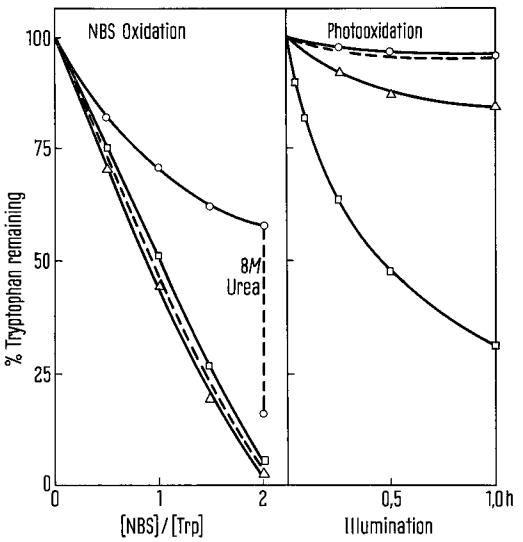
in 0.1 *M* sodium acetate buffer, pH 4, at 25 °C for 30 min essentially according to SPANDE and WITKOP[9]. Loss of FMN from NBS-treated flavodoxin was noted by spectral measurement of the protein after dialysis. Photochemical oxidations with 450 nm light from a xenon lamp monochromatized by an Aminco-Bowman spectrophotofluorometer were carried out in aerobic solutions of 0.1 *M* sodium phosphate buffer, pH 7.5, at 25 °C. These conditions have been found satisfactory for photooxidation of tryptophan in the presence of FMN[10]. Tryptophan was assessed by the colorimetric method of SPIES and CHAMBERS[11], which is insensitive to the oxidation products of this amino acid.

Results and discussion. Results obtained upon treatments of tryptophan alone, flavodoxin, flavinyl tryptophan with 5 methylene groups between chromophores, and tryptophan in the presence of FMN in the ratio known for flavodoxin is shown in the Figure. Under the conditions used for the oxidation with NBS, tryptophan alone, with FMN, and in the flavinyl peptide is extensively and almost equally oxidized. The same behavior was also found for the flavinyl tryptophan with only 1 methylene group between chromophores. Hence, such tight complexing as occurs between flavin and tryptophan portions of the synthetic peptides does not prevent the ready and complete chemical oxidation of the amino acid. On the other hand, only about half of the tryptophan residues of flavodoxin are oxidized by similar treatment, whereupon FMN is released and activity lost. Most of the remaining tryptophan in this flavoprotein becomes susceptible to oxidation by NBS in 8 *M* urea, as indicated. Apparently 2 of the 4 tryptophans are much less exposed. As shown by the data for photooxidation in the figure, the flavin coenzyme in flavodoxin cannot act as photosensitizer for oxidation of tryptophan residues in flavodoxin, as is also true for the flavinyl tryptophan with 1 methylene group between chromophores, under conditions where ready photooxidation of tryptophan in the presence, but not absence of FMN occurs. Even the flavinyl tryptophan with 5 methylene groups between chromophores loses but little of the tryptophan portion. Hence, tight complexing between flavin and tryptophan does prevent ready and complete photochemical oxidation of the amino acid.



Oxidation by N-bromosuccinimide (left) and aerobic illumination at 450 nm (right) of tryptophan alone (dashed lines), within flavodoxin (○), in a flavinyl-(5-methylenes)-tryptophan peptide (△), and together with FMN in solution (□). All solutions were initially equimolar with respect to tryptophan.

Overall, the present results indicate that 2 of the 4 tryptophan residues of flavodoxin are relatively buried and that at least one of these could be tightly complexed with FMN. It should also be recalled that a cysteine sulfhydryl has been implicated in the binding of the coenzyme[3]. NBS treatment might also oxidize this function and lead to a loss of activity, and photochemical treatment would not necessarily involve tryptophan[12].

Zusammenfassung. Zwei der vier Tryptophan-Reste in Flavodoxin sind relativ gut abgeschirmt, wie das Ausmass der Oxidation durch N-Bromsuccinimid zeigt. Ausserdem kann wahrscheinlich mindestens einer dieser Tryptophan-Reste mit FMN einen stabilen Komplex bilden, wie die Unempfindlichkeit gegenüber Photooxidation nahelegt.

D. B. MCCORMICK

Graduate School of Nutrition and Biochemistry Section, Cornell University, Ithaca (N.Y. 14850, USA), 15 September 1969.

[9] T. F. SPANDE and B. WITKOP, in *Methods in Enzymology* (Ed. C. H. HIRS; Academic Press, New York 1967), vol. 11, p. 498.
[10] M. B. TAYLOR and G. K. RADDA, in *Methods in Enzymology* (Ed. D. B. MCCORMICK and L. D. WRIGHT; Academic Press, New York 1970), in press.
[11] J. R. SPIES and D. C. CHAMBERS, Analyt. Chem. *21*, 1249 (1949).
[12] This work was supported in part by Grant No. AM-04585 from the National Institutes of Health, and in part by funds made available from the State University of New York. I wish to thank Drs. R. W. F. HARDY and E. KNIGHT JR., of the Central Research Department, E.I. duPont de Nemours and Company, for their generous gift of flavodoxin.

Turnover of Hepatic Collagen in Reversible and Irreversible Fibrosis

Diffuse hepatic fibrosis in the rat produced by ethionine intoxication is characterized by a parallel increase in the amount of collagen and in the number of cells. Therefore, there is a constant hydroxyproline (OHPr)/DNA ratio[1]. The fibrosis is reversible upon replacement of ethionine by methionine when 5 times the normal collagen content of the liver is catabolized within 14 days. During the period of rapid fiber resorption, the half-life of hepatic collagen is only 10 days[2].

In early carbontetrachloride (CCl_4) induced hepatic fibrosis, the OHPr/DNA ratio similarly remains constant. Later the ratio increases[3]. These observations pose the following questions: (a) Does hepatic fibrosis remain reversible when the OHPr/DNA ratio is increased? (b) Is

[1] F. HUTTERER, R. RUBIN, E. F. SINGER and H. POPPER, Cancer Res. *21*, 206 (1961).
[2] F. HUTTERER, E. RUBIN and H. POPPER, Expl. Molec. Path. *3*, 215 (1964).
[3] E. RUBIN, F. HUTTERER and H. POPPER, Am. J. Path. *42*, 715 (1963).

excess collagen synthesis or decreased collagen catabolism the main factor in the disproportionate increase of collagen in the later stages?

Materials and methods. Sprague-Dawley female rats with an initial body weight of 150 g, were fed Rockland Farms' rat diet and were given 0.1 ml CCl_4 in mineral oil s.c. twice weekly. They were kept in a constant environment and their food intake was regulated. Groups of 10 rats were killed at intervals. CCl_4 treatment of 2 other groups of 20 rats each was discontinued after 60 and 140 days, respectively. 10 rats from each group were sacrificed 40 and 90 days after discontinuation of CCl_4 treatment.

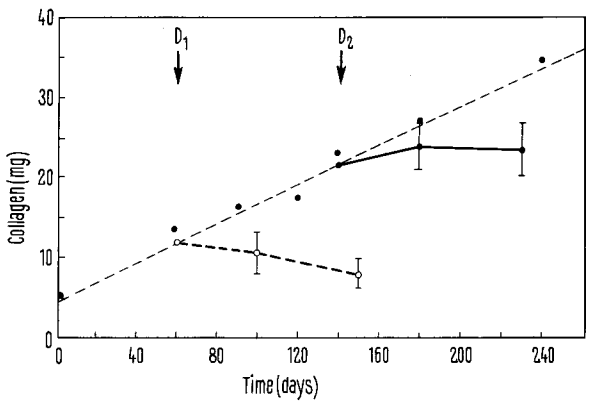


Fig. 1. Thin dotted line represents the least squares plot of hepatic collagen increase during CCl_4 treatment ($y = 4.27 + 0.12x$). Heavy dotted line and solid line represent the hepatic collagen content after discontinuation of CCl_4 treatment after 60 days (D_1) and 140 days (D_2), respectively.

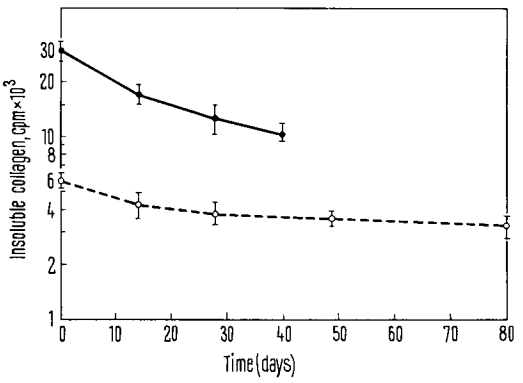


Fig. 2. Semilogarithmic plot of radioactivity of insoluble collagen of the liver after glycine-C^{14} injection. Solid line, glycine-C^{14}, was given after 60 days of CCl_4 treatment. Dotted line, glycine-C^{14}, was given after 140 days of CCl_4 treatment. Time: 0 represents the maximum labeling of insoluble collagen obtained 72 h after glycine-C^{14} administration.

Insoluble collagen		Reversible phase		Irreversible phase	
Rate constant	k_1		0.0189		0.0023
	k_2		0.2334		0.0991
Initial activity	A_1	21,740		3,984	
(cpm)	A_2	8,208		1,727	
Half life (days)	$t_1{}^1/_2$		36.7		301.3
	$t_2{}^1/_2$		2.9		6.9

Another 2 groups of rats were given glycine-C^{14} (u.l.), 10 μc/100 g body weight i.p. on the 60th and 140th day of CCl_4 treatment, respectively, and were killed in groups of 10 at various intervals up to 100 days after the isotope injection. Hepatic OHPr was determined as described previously[1]. The alkali-soluble collagen and insoluble collagen of liver were extracted, purified, and the radioactivity measured as described previously[2]. The morphological characteristics and the changes of OHPr/DNA ratio were described in detail elsewhere[3].

Results and discussion. The progressive increase of the collagen content during CCl_4 treatment is shown in Figure 1. Discontinuation of the CCl_4 treatment after 60 days resulted in a significant decrease in the hepatic collagen content ($p < 0.01$). Discontinuation of CCl_4 treatment after 140 days, on the other hand, did not result in a significant decrease of the collagen content. Therefore, under the conditions of the experiment, hepatic fibrosis was reversible after 60 days of CCl_4 treatment and irreversible after 140 days.

The uptake of glycine-C^{14} into the insoluble collagen was almost 10 times higher in the reversible phase than in the irreversible phase (Figure 2).

The semilogarithmic plot indicates that the system is multicompartmented in both phases. Instead of the usual free hand fitting[4] of multiple exponentials to the data, we applied the iterative method of SHEPPARD[5] and solved a set of equations $y = \Sigma_n . A_n e^{-k_n t}$, where y is the radioactivity at any time, t; A is the initial activity of the component and k is the rate constant, with a computer. The program was written in MAD language. In the reversible phase there were 2 components of insoluble collagen with half life of 3 days and 37 days, respectively. In the irreversible phase there were also 2 components with half lives of 7 and 301 days. The A_n values indicate that the longer lived components accounted for 70% of the radioactive uptake.

The half life of the major component of the insoluble collagen is approximately 10 times longer in the irreversible phase than in the reversible phase. Thus a decreased rate of collagen catabolism, rather than increased rate of synthesis distinguishes the irreversible phase from the reversible phase. The cause of this decreased rate of collagen catabolism is not known. Decrease of hepatic lysosomal hyaluronidase[6], or the increase of hyaluronidase resistant Chondroitin Sulphate-B[7] in the irreversible phase may contribute to the decreased rate of degradation[8].

Zusammenfassung. Werden Ratten bis zu 60 Tage lang mit Carbontetrachlorid behandelt, dann ist die Vermehrung des hepatischen Kollagens reversibel; bei einer Behandlung von mehr als 140 Tagen bleibt sie jedoch bestehen.

F. HUTTERER, M. EISENSTADT and E. RUBIN

Department of Pathology, Mount Sinai School of Medicine of The City University of New York, New York (New York 10029, USA), 18 August 1969.

[4] D. S. RIGGS, *The Mathematical Approach to Physiological Problems* (Williams and Wilkins, Baltimore 1963).
[5] C. W. SHEPPARD, *Basic Principles of the Tracer Method* (Wiley and Sons, New York 1962).
[6] F. HUTTERER, Biochem. biophys. Acta *115*, 312 (1966).
[7] F. HUTTERER and P. BACCHIN, Fedn. Proc. *27*, 412 (1968).
[8] Supported by U.S.P.H.S. Grant No. AM03846, NIH.