COMMUNICATIONS TO THE EDITOR

DETECTION OF POLYMERIZATION RADICALS BY PARAMAGNETIC RESONANCE[1]

Sir:

We have directly verified the presence of free radicals formed during vinyl polymerization by means of paramagnetic resonance absorption spectroscopy. Cross-linked systems such as glycol dimethacrylate (GDM) in which relatively high concentrations of long-lived radicals may be expected have been employed.

GDM polymers were formed by heating monomer and an initiator at 60–100° in sealed glass tubes until gelation occurred. They were then cooled and the spectra of the partially polymerized samples observed at room temperature.[2] Identical spectra, except for intensity, were observed using one (and sometimes ten) weight per cent. of several initiators: benzoyl peroxide, azobisisobutyronitrile, *t*-butyl perbenzoate, and di-*t*-butyl peroxide. Irradiation of the gelled samples at room temperature with a Hanovia type 7420, 500-watt, mercury-vapor lamp increased the intensity of spectra, again without change in form. The radical concentration is estimated to be as high as 10^{-3} to 10^{-4} molar in some samples, and radicals are still observable in the sealed tubes after three months storage.

When GDM is diluted with methyl methacrylate (MM), we observe identical spectra except for a possible decrease in intensity, and with pure MM, spectra are only observable in a highly polymerized sample after intense irradiation.

The spectrum of GDM and MM polymers consists of five symmetrically located absorption peaks with four subsidiary peaks interspersed between the main lines. It spreads over a region of about 100 gauss centered about a value of the magnetic field close to the position expected for a free electron, and is similar in its essential details to the spectrum obtained from X-ray irradiated poly-(MM) by Schneider, Day, and Stein.[3] These authors attributed this complex spectrum to interactions between electrons trapped in holes at more or less regular intervals in the polymer matrix, but our work indicates that it represents the actual free radicals involved in the polymerization process (or, in their case, perhaps depolymerization) immobilized in the gelled or glassy system. The complex fine structure observed may be due to interactions between the unpaired electrons and the magnetic moment of neighboring protons, but we have been unable to devise a satisfactory splitting pattern from the presumed structure of the radical species believed to be present.

Spectra also have been observed in gels of vinyl methacrylate, divinylbenzene (a 55% technical material), and glycol diacrylate. Vinyl methacrylate gives a spectrum similar to GDM except that individual peaks are broadened and subsidiary peaks have disappeared. The spectrum from divinylbenzene at the level of microwave power normally used shows a single peak, but when the level is reduced, it splits into three partially resolved lines spread over 47 gauss between extreme points of maximum slope, indicating that saturation of the spin system is occurring. The spectrum from glycol diacrylate, which was only observed after moderate irradiation, is similar to that from divinyl benzene at low power levels, but the lines are not as well resolved.

This variation of spectrum with monomer structure is further evidence that we are actually observing the radicals involved in the polymerization process, and shows the utility of the method in both detecting and identifying the intermediates in free radical processes.

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(1) Supported in part by the Squier Signal Laboratory, U. S. Army Signal Corps.

(2) A preliminary account of our instrument, which employs a type 2K25 klystron at a wave length of 3.2 cm., has been published: J. M. Hirshon, R. L. White, and G. K. Fraenkel, *Rev. Sci. Instr.*, **23**, 772 (1952). A detailed account has been submitted for publication by J. M. Hirshon and G. K. Fraenkel to *Rev. Sci. Instr.*

(3) E. E. Schneider, M. J. Day and G. Stein, *Nature, Lond.*, **168**, 645 (1951).

A TECHNIQUE FOR STEPWISE DEGRADATION OF PROTEINS FROM THE AMINO-END[1]

Sir:

The techniques used for stepwise degradation of peptides by the phenyl isothiocyanate method[2] cannot be applied to proteins, since the phenylthiocarbamyl proteins are insoluble in the acid media needed for formation of phenylthiohydantoins (PTH). Saturation of the solution with guanidine hydrochloride has overcome this difficulty with insulin.[3]

A technique has now been developed which is considerably simpler and permits the stepwise release of PTH's from a number of proteins. The principal feature is the use of small strips of filter paper as carriers for the protein throughout the procedure. The distribution of the protein over a big surface renders it accessible to the reagents and prevents precipitation by acid.[4]

Aliquots of a protein solution (0.05 or 0.1 ml., 1–3 mg.) are applied evenly to about four-fifths of the area of 2–12 small strips (1 × 5 cm.) of Whatman No. 1, held suspended by chromatographic clamps and marked at one end. The papers are allowed to dry, then wetted with 0.05 ml. of 20%

(1) Aided by a grant from the National Foundation for Infantile Paralysis.

(2) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950); M. Ottesen and A. Wollenberger, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **28**, 463 (1953); B. Dahlerup-Petersen, K. Linderstrøm-Lang and M. Ottesen, *Acta Chem. Scand.*, **6**, 1135 (1952).

(3) H. Fraenkel-Conrat and J. Fraenkel-Conrat, *ibid.*, **5**, 1409 (1951).

(4) The use of paper strips was suggested by the work of J. Leonis (*Bull. Soc. Chim. Belges*, **61**, 524 (1952)), but it fulfills here a different and more crucial role.

phenyl isothiocyanate in peroxide-free dioxane and placed moist into an atmosphere saturated with pyridine, dioxane and water for 2–3 hours at 40°. They are then exhaustively washed with several changes of benzene and a mixture of equal parts of absolute alcohol and peroxide-free ether (until the supernatant after 2–3 hours of gentle shaking shows an o.d. of less than 0.025 at 270 mμ, usually after about 24 hr.). The strips are then dried, and placed in the bottom of a desiccator containing beakers with glacial acetic and 5.7 *N* hydrochloric acid. The desiccator is evacuated to about 100 mm. After 4–16 hours, depending on the protein, the papers are aerated to dissipate the acids and again extracted with the alcohol ether mixture. The PTH's in this solution show the characteristic maximum near 270 mμ, from which their amount and purity can be estimated.[3,5] They are identified either directly by chromatography[6] or after hydrolysis to the amino acids.[2,5] The paper strips are ready for the next cycle of addition of the reagent and release of the PTH.

This technique has been carried through as many as 14 steps with insulin. The yields of PTH are quite similar (about 1.7 equiv. per 6000 g.) through the first five steps and then decrease, sometimes gradually and sometimes more irregularly. Also with other proteins a characteristic amount of PTH is formed at each of several steps. This suggests a clean sequence of reactions. However, chromatographic analysis of the PTH's does not always bear out this conclusion. With insulin, oxytocin, and, according to Harris and Li, α-corticotropin[7] the N-terminal and adjacent peptide bonds are split through the first 5–7 steps, with little evidence for non-specific splitting. Then the chromatographic picture gets rather suddenly much more complex and no further identifications have as yet been possible.

The results (Table I) obtained have confirmed the known amino acid sequences of insulin[8] and oxytocin.[9] One of the remaining questions in the structure of insulin, the location of amide groups, has been answered for the four dicarboxylic residues near the N terminal end of the molecule. Tentative conclusions, in part confirmatory, have been reached for some other proteins. The sequence of eight amino acids of α-corticotropin, as determined by this method, is described by Harris and Li in THIS JOURNAL.[7]

TABLE I

AMINO ACIDS OBTAINED IN STEPWISE DEGRADATION OF PROTEINS[a]

Step no.	I	II	III	IV	V	VI
Insulin	gly, phe	ileu, val	val, asp NH_2	glu NH_2	glu NH_2, his[a]	
Insulin, A chain	gly	ileu	val	glu NH_2	glu[a] NH_2	glu[a]
Insulin, B chain	phe	val[a]	asp NH_2	glu NH_2	his	his[a]
Oxytocin	none[b]	tyr	ileu	glu NH_2	asp NH_2	none
Lysozyme	lys	val[a]	?	gly[a]	many	
Myoglobin	gly	leu[a]	ser[a]	glu[a]		
β-Lactoglobulin	leu	leu[a]	val[a]			
Protamine	pro	arg	arg[a]			

[a] Only the predominating amino acids are listed; cases in which others seemed in amount to approach the main product are indicated by *a*. Identification is then not unequivocal. [b] Cystine PTH is expected to be formed and to remain attached to the peptide.

(5) H. Fraenkel-Conrat and B. Singer, THIS JOURNAL, **76**, 180 (1954); H. Fraenkel-Conrat, in the Chemical Structure of Proteins, p. 102, J. and A. Churchill, Ltd., London, 1953.

(6) J. Sjöquist, *Acta Chem. Scand.*, **7**, 447 (1953).

(7) J. I. Harris and C. H. Li, THIS JOURNAL, **76**, 3607 (1954).

(8) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463, 481 (1951); F. Sanger and E. O. P. Thompson, *ibid.*, **53**, 535, 666 (1953).

(9) This sequence, established by du Vigneaud, *et al.* (V. du Vigneaud, C. Roessler and S. Trippett, *J. Biol. Chem.*, **205**, 949 (1953)), was actually confirmed by these authors by a modification of the Edman method.

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N- AND C-TERMINAL AMINO ACID SEQUENCES OF α-CORTICOTROPIN (ACTH)[1]

Sir:

The isolation from *sheep* pituitary glands of a polypeptide (α-corticotropin) possessing adrenal stimulating activity was recently reported from this laboratory.[2] The hormone was shown to contain 39 amino acid residues with a minimum molecular weight of 4,500. We wish to report the N- and C-terminal sequences obtained when stepwise degradation procedures were applied to α-corticotropin.

The phenyl*iso*thiocyanate (PTC) procedure of Edman[3] as modified by Fraenkel-Conrat[4] was employed for the investigation of the N-terminal sequence. In a typical experiment, 2.8 mg. of α-corticotropin trichloracetate (0.5 μM.) was dissolved in 50 μl. of 2% aqueous ammonia and the solution was applied to a strip (2 × 8 cm.) of Whatman No. 1 filter paper. After it was dried, the strip was treated with 50 μl. of 20% PTC in dioxane, and incubated in an atmosphere of aqueous pyridine for three hours at 40°. The strip was then thoroughly washed successively with benzene and 1:1 absolute alcohol/ether (peroxide-free). Cleavage of the phenylthiohydantoin (PTH) of the N-terminal amino acid was accomplished by placing the washed strip in an evacuated desiccator containing glacial acetic acid and 6 *N* hydrochloric acid for eight hours at room temperature, and the liberated PTH was extracted from the paper strip by shaking for one hour with 10 ml. of ethanol:ether (1:1). In this manner the PTH derivative of the N-terminal residue in α-corticotropin was obtained in a yield of 85%, and was identified as serine PTH by direct chromatography on paper in both the heptane/pyridine and the heptane/*n*-butanol/formic acid systems proposed by Sjöquist.[5]

A second application of the above procedure led to the isolation in 80% yield of a second phenyl-

(1) This investigation was supported in part by research grants from the National Institutes of Health, U.S. Public Health Service (G 20907), and Eli Lilly and Company.

(2) C. H. Li, I. I. Geschwind, A. L. Levy, J. I. Harris, J. S. Dixon, N. G. Pon and J. O. Porath, *Nature*, **173**, 251 (1954).

(3) P. Edman, *Acta Chem. Scand.*, **4**, 283 (1950).

(4) H. Fraenkel-Conrat, THIS JOURNAL, **76**, 3606 (1954); and in D. Glick, "Methods of Biochemical Analysis," Vol. II, Interscience Publishers, New York, in press. We are indebted to Dr. Fraenkel-Conrat for communicating to us the details of the paper strip procedure prior to its publication.

(5) J. Sjöquist, *Acta Chem. Scand.*, **7**, 447 (1953).