Enzymic actions on the human urinary erythropoietic-stimulating factor

Although there is widespread acceptance of the view that erythropoiesis is regulated by a circulating erythropoietic-stimulating factor, disagreement still exists concerning its general chemical constitution[1]. To obtain some knowledge of the structure of ESF we have subjected the factor to the action of several types of enzymes.

The ESF present in the urines of children with thalassemia major[2] was precipitated with 4 vol. absolute ethanol at -10° and exhaustively dialyzed against 0.16 *M* NaCl and 0.025 *M* Na_2HPO_4 at pH 7.4 in Visking 20/32 casings. Enzyme concentrations were generally 0.75 mg/ml, except for carboxypeptidase which was 1.5 mg/ml and neuraminidase which contained 1.3 μg, or 6000 units/ml*. Trypsin was activated with 0.015 *M* $MgSO_4$, and papain was activated with 0.005 *M* cysteine. Neuraminidase was used without activator.

The factor was digested for 5 h at 37° with each enzyme at near optimal pH with the exception of the peptic digestion. Similar aliquots of the ESF preparation were exposed to the same conditions used for the enzyme digestions, but without added enzymes. The ^{59}Fe uptake in the starved-rat-erythrocyte assay of FRIED *et al.*[3] was employed to determine the ESF concentrations in the incubates. The relative % ^{59}Fe incorporation into the erythrocytes of groups of 3–5 rats receiving 2 daily 2-ml injections of the incubation mixtures is shown in Table I.

It is apparent that the urinary ESF is more stable in alkaline than in acid solution. For this reason, a suboptimal pH was selected for the peptic digestion. In general,

TABLE I

EFFECT OF DIGESTION UPON ACTIVITY OF HUMAN URINARY ESF

pH	*Enzyme added*	*Relative % ^{59}Fe uptake*
8.2	None	100.0
7.4	None	87.6
6.0	None	68.2
4.7	None	40.9
4.7	Pepsin	12.7*
7.4	Trypsin	6.6*
6.0	Papain	0.0*
7.4	Carboxypeptidase	108.0
8.2	Aminopeptidase	33.1*
7.4	Ribonuclease	97.0
6.0	Lysozyme	103.1
4.7	Hyaluronidase	27.7
6.0	Neuraminidase	22.1*

* Significantly lower in activity ($P < 0.05$) compared to the pH control.

Abbreviation: ESF, erythropoietic-stimulating factor.

* Crystalline pepsin, trypsin, ribonuclease and carboxypeptidase-DFP were obtained from the Worthington Biochemical Corporation, Freehold, New Jersey. Lysozyme and hyaluronidase were secured from the Mann Research Laboratories, New York and the leucine aminopeptidase was supplied by the Bios Laboratories, Inc., New York. Neuraminidase was kindly prepared and supplied by Dr. G. L. ADA, University of Melbourne, Australia.

the proteolytic enzymes, pepsin, trypsin, papain and aminopeptidase, but not carboxypeptidase, produced significant inactivation. Among the non-proteolytic enzymes studied, hyaluronidase and neuraminidase diminished activity, whereas ribonuclease and lysozyme were ineffective.

From these data, it is inferred that the active portions of ESF consist of both carbohydrate and polypeptide moieties. Susceptibility to aminopeptidase suggests the presence of an unblocked polypeptide approach to the active region from an N-terminal position. Resistance of the C-terminal position to carboxypeptidase could have occurred if this end were blocked by carbohydrate or by a slowly lysed peptide bond. Inactivation with neuraminidase indicates that the terminal carbohydrate derivative of human ESF is neuraminic acid in agreement with LOWY *et al.*[4] who noted that rabbit plasma ESF is also susceptible to neuraminidase.

Evidence that the effects of these enzymes were exerted directly upon ESF *in vitro* and were not due to depression of erythropoiesis within the recipient rats has also been obtained and will be included with complete details in a forthcoming paper.

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Negative-staining electron microscopy of proteins at pH values below their isoelectric points. Its application to hemocyanin

The negative-staining technique of BRENNER AND HORNE[1] is a very important method for studying the structure of protein molecules with the electron microscope. In this method contrast is obtained by the use of potassium phosphotungstate at pH 7.2. Because of the negative charge of the phosphotungstate ions, this method is applicable only at pH values above the isoelectric point, where the protein molecules also carry negative charges and thus do not take up the contrasting ion.

We are studying the structure of the molecules of *Helix pomatia* hemocyanins at different pH values and wished to extend this method below the isoelectric point. Uranyl acetate appeared to give the best result as a contrasting agent. It is clearly seen from Fig. 1 that uranyl ions do not interact with the protein. Uranyl acetate