estrogens are approximately the same. 4) When administered alone, these estrogens produce no significant change in either carbonic anhydrase titers or G/M ratios of the endometrium. 5) The linear relationship with negative slope which is obtained between the logarithmic dose of the estrogens and the endometrial carbonic anhydrase content of the progesterone-treated Clauberg rabbit suggests the usefulness of the carbonic anhydrase method as an assay procedure for anti-progestational activity. 6) Comparison of the log-dose-response curves of progesterone with and without estradiol indicates that estrogen may depress reactivity of the endometrium to progesterone rather than neutralize or inactivate progesterone in the body.

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Acid Hydrolysis of Erythropoietin.* (24391)

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We have recently isolated a heat stable erythropoietic factor, erythropoietin, and have shown it to be an acidic glycoprotein(1). Because several workers have suggested that erythropoietin is not a protein (2,3,4) it was of interest to investigate the possibility that this glycoprotein subserves a carrier function. Since dialysis of erythropoietin does not reduce its activity(5), any 'carried factor' would be firmly bound. Therefore, erythropoietin was submitted to acid hydrolysis and the degradation products studied biologically and qualitatively by chromatography.

Materials and methods. Purified erythropoietin was isolated from acidified, boiled plasma filtrates of anemic rabbits by DEAE-cellulose ion-exchange chromatography as previously described(1). It was dissolved in distilled water, 3 mg/ml. Five 1 ml aliquots were hydrolyzed in a water bath at 80° C for 1 hour with 5 ml H_2SO_4 in final concentrations of 0.005 N, 0.01 N, 0.04 N, 0.08 N and 0.10 N respectively. A 1 ml aliquot brought to 5 ml with distilled water was carried through the procedure as a control. The hydrolysates were separately dialyzed in the cold against repeated changes of distilled water for 5 days. The dialysates, concentrated in vacuo, and protein dialysands were lyophilized and stored. Separations of 1 mg of the hydrolyzed protein were carried out on Whatman 3 MM paper at 5 ma using veronal buffer pH 8.6, ionic strength 0.075. The strips were stained with bromphenol blue. Seven hour descending chromatograms of the dialysates in an ethyl acetate:pyridine:H_2O (10:4:3) solvent system(6) were prepared. A mixture of neuraminic (sialic) acid, d-glucosamine, d-mannose, d-glucose, d-galactose and l-fucose served as a reference material. After development and drying, the chromatograms were sprayed with aniline hydrogen phthalate reagent(7) and then heated at 110° C for 15 min. One half mg, in 0.9% NaCl, respectively of dialyzed 0.01 N hydrolysate of erythropoietin and in tact erythropoietin were injected subcutane-

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ously daily for 4 days into rats. Reticulocyte levels and 24 hour uptake of Fe^{59} by the total red cell mass were then determined by previously described methods(1).

Results. Hydrolysis of erythropoietin in 0.005 N acid reduced its paper anodic mobility to 40% of the control. Hydrolysis in 0.01 N acid reduced mobility to a minimum, 25% of normal. Heating erythropoietin in water for 1 hour at 80° C did not affect mobility.

No carbohydrates could be detected on the paper chromatograms of the dialysate from erythropoietin heated in aqueous solution. A weak spot for neuraminic acid appeared on the chromatogram of the 0.005 N dialysate. A strong spot of neuraminic acid appeared on the chromatogram of the 0.01 N dialysate, along with the first faint spots for fucose and hexoses. Chromatograms of the dialysates from 0.04 N and stronger acid hydrolysis revealed spots for neuraminic acid, glucosamine, fucose, galactose and mannose. No neuraminic acid could be detected in the dialysands of 0.01 N or stronger hydrolysis. These results indicate that neuraminic acid is the most labile carbohydrate moiety of erythropoietin, and that the decrease in anodic mobility of the hydrolyzed protein may be due to loss of neuraminic acid. For the first time galactose, mannose and fucose are established as component parts of the erythropoietin molecule.

Erythropoietin from which all neuraminic acid has been removed, has no erythropoietic activity (Table I). Previous data indicate that neuraminic acid alone does not stimulate erythropoiesis (unpublished). Thus, it would appear that the intact mucoprotein molecule is necessary for activity. These findings do not support a concept that erythropoietin behaves as a 'carrier' protein.

TABLE I. Assay of Erythropoietic Activity.

Material inj.	Reticulocytes (%)	Fe^{59} incorporation in RBC (%)
.01 N hydrolysate of erythropoietin	1.8 ± .6*	24.8 ± 2.9*(8)
Erythropoietin	5.7 ± 1.8	43.2 ± 3.8 (4)
Saline	1.3 ± .6	24.0 ± .4 (4)

* Mean ± S.D.

Figures in parentheses indicate No. of animals in group. Underlined values statistically significant, P = 0.01 or less.

Since the neuraminic acid of erythropoietin is labile to very mild acid hydrolysis, and its presence is probably necessary for biological activity, care should be taken not to let the pH of solutions containing erythropoietin decrease below 3. We have previously demonstrated reduction in erythropoietic activity of 'anemic' plasma treated with perchloric acid (5).

Summary. Purified erythropoietin has been subjected to mild acid hydrolysis. This mucoprotein thus has been further characterized and shown to lose erythropoietic activity upon hydrolytic removal of neuraminic acid.

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