TABLE I. Comparison of Effects of Hydroxylamine Hydrochloride and Alanosine on Certain Bacterial Amino Acid Carboxy-lyases.[a]

Carboxy-lyase	Percentage of control velocity in the presence of	
	Hydroxylamine	Alanosine
Arginine	10	112
Lysine	7	126
Histidine	14	220
Tyrosine	24	105
Aspartic acid	47	161
Glutamic acid	27	29

[a] Each enzyme preparation was present at 0.5 mg/ml, substrate was 5×10^{-3} *M*, and hydroxylamine and alanosine were 6×10^{-4} *M*. Velocity was measured at 30° in phosphate-acetate buffer, pH 5.5.

M were totally devoid of any inhibitory action on the glutamic acid C-L from either rat brain or squash.

Discussion. From the foregoing data, two points emerge as worthy of consideration. First, two examples of a high degree of selectivity have been demonstrated: the selectivity of the inhibitor for only one protein species of three that perform the identical catalytic function, and the selectivity for only glutamic C-L of the several C-L's tested from microbial sources. Secondly, while hydroxylamine has been shown to be a competive inhibitor of glutamic C-L (7), a fundamental difference exists between its action and that of alanosine in that other bacterial C-L's are sensitive to hydroxylamine but are not inhibited by alanosine. Further, the inhibitory action of hydroxylamine on glutamic C-L is readily and completely antagonized by pyridoxal phosphate (7), while that of alanosine is not.

The insensitivity of the brain glutamic C-L, if it extends to other mammalian species, may be fortuitous in the sense that an undesirable side effect due to depletion of cerebral γ-aminobutyric acid may be obviated. Aside from other toxic effects that will likely be encountered in subsequent studies, the clinical manifestations of glutamic C-L suppression would certainly be expected to impede therapeutic application of the antibiotic.

Summary. Alanosine is an inhibitor of glutamic acid carboxy-lyase (C-L) of bacterial origin, but not of those from squash and rat brain. Its action is competitive in respect to glutamic acid, but noncompetitive in respect to pyridoxal phosphate. Other bacterial amino acid C-L's tested, while sensitive to hydroxylamine, are not inhibited by alanosine.

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Received July 1, 1968. P.S.E.B.M., 1968, Vol. 129.

Inactivation of Erythropoietin by Tissue Homogenates* (33439)

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In recent reports Fisher and co-workers (1, 2), Kuratowska (3), and Zanjani *et al.* (4) described a factor in renal extracts capable of inactivation of erythropoietin. *In vitro* studies in our laboratory have confirmed the existence of such a renal factor, but have shown it also to be present in homogenates from livers and spleens. Kinetic measurements

* Supported in part by Grant H-4612 and H-6374 from National Institutes of Health.

TABLE I. Inactivation of Urinary Erythropoietin (66-hr ^{59}Fe utilization ± 1 SE).

Human urinary erythropoietin (1 unit/ml/mouse)	Incubation with renal homogenate for 1 hr			
	Saline		Plasma (0.5 ml)	
	4°	37°	4°	37°
W.B. 12.0%	1.5 ± 0.36	0.4 ± 0.07	6.0 ± 1.3	2.3 ± 0.38
C.G. 12.4%	1.3 ± 0.16	0.2 ± 0.01	6.7 ± 0.07	1.2 ± 0.14

of the inactivation process suggest it to be a nonenzymatic reaction.

Materials and Methods. Homogenates were prepared from the kidneys of normal white New Zealand rabbits. The kidneys were minced, suspended in cold 0.02 *M* hypotonic phosphate buffer at pH 7.2 (1 ml/g tissue), homogenized first with a tissue grinder, either manually or with a motor-driven nylon pestle, and then sonicated with Heats Systems Sonifier using several 3–5-sec bursts of 90-W output. The entire extraction procedure was carried out at 4°C with intervening chilling in an ice bath. The homogenate was then screened through a fine stainless-steel mesh, frozen in a dry-ice bath, and stored at —20°C. Homogenates of spleen, liver, and bone marrow were prepared in the same manner. Normal human ACD plasma and human urinary or plasma erythropoietin were used. Urinary erythropoietin was obtained from patients with aplastic anemia (C.G., W. B., and L.S.) or severe iron deficiency anemia (N.G.). The urine was precipitated twice with 80% absolute ethanol and the precipitate was washed with ether before lyophylization (5). Plasma erythropoietin was obtained from patients with aplastic anemia (C.G. and R.P.) or severe iron-deficiency anemia (N.G.). In most studies equal volumes of erythropoietin diluted in saline or in normal human plasma, and of tissue extracts were incubated together in a water bath immediately prior to the assay. Assays for erythropoietic activity were carried out in Swiss Webster female mice rendered polycythemic by transfusion. The technique used was modified slightly from that described by Rosse and Waldman (6). The mice received 0.9 ml packed red cells i.p. on days 0 and 1. On days 6 and 7 they received the test material (0.5 ml s.c.q. day), on day 8 they received 1 μCi ^{59}Fe i.p. and on day 11, the 66-hr utilization of ^{59}Fe was determined using an estimated blood volume of 7% of body weight. Each assay consisted of a minimum of 4 mice and all animals with a hematocrit of less than 55% were discarded. The concentration of erythropoietin is reported either as 66-hr utilization of ^{59}Fe or in units, as determined from a dose-response curve (Standard B Erythropoietin).

Results. Study 1. Table I shows that 1 unit of human urinary erythropoietin dissolved in 1 ml of saline was completely inactivated when incubated for 1 hr at 37°C with 1 ml of renal homogenate from 1.0 g of renal tissue. The inactivation was less complete when incubated at 4°C or when erythropoietin was dissolved in normal plasma rather than in saline.

Study 2. Table II shows that plasma erythropoietin diluted with saline was also completely inactivated after 1 hr of incubation at 37°C with renal homogenate. The amount of plasma in this study was less than the amount of plasma used in study 1. The difference between complete inactivation of plasma erythropoietin diluted with saline and the partial inactivation of urinary erythropoietin suspended in plasma suggests that plasma erythropoietin is not specifically protected by a physiologic carrier function of plasma pro-

TABLE II. Inactivation of Plasma Erythropoietin (66-hr ^{59}Fe utilization % ± 1 SE).

Human plasma erythropoietin		Incubation with renal homogenate 37° for 1 hr
C.G. (0.13 ml)	17.1 ± 1.7	0.12 ± 0.01
R.P. (0.09 ml)	2.0 ± 0.20	0.13 ± 0.01
N.G. (0.30 ml)	14.5 ± 0.82	0.09 ± 0.01

TABLE III. Protective Effect of Plasma (66-hr ^{59}Fe utilization ± 1 SE %).

Plasma erythropoietin (C.G.)	Kidney homogenate suspended in:	66-Hr ^{59}Fe utilization (%)
1 unit	13% plasma + 87% saline	0.16 ± 0.04
1 unit	50% plasma + 50% saline	0.66 ± 0.13
1 unit	100% plasma + 0% saline	7.30 ± 1.05
Controls		
1 unit in saline		13.4%
1 unit in plasma		14.0%
Kidney homogenate in saline		0.33 ± 0.09
Kidney homogenate in plasma		0.12 ± 0.02

teins but rather that normal plasma interferes with the inactivating process. This conclusion is supported by the fact that plasma erythropoietin diluted with normal plasma and incubated with a plasma extract of the kidney showed considerably less inactivation than when saline was used as the diluting and extracting medium (Table III).

Study 3. The renal homogenate did not show significant TAME-esterase activity, and the addition of 50 μg or 250 μg of soy bean trypsin inhibitor (Worthington, 3 × cryst.) per ml of incubating mixture did not alter the inactivating potency of renal homogenate. Dialysis of the renal homogenate against distilled water or against 0.005 *M* Na_2 EDTA did not change its inactivating properties. Heating of the homogenate to 100°C for 30 min with subsequent rehomogenization by means of sonification did only partially abolish its inactivating property (Table IV).

Study 4. The kinetics of inactivation were examined by incubating 1 unit of urinary erythropoietin (C.G.) for 15, 30, 60, 120, and 180 min with two concentrations of renal homogenate. As can be seen from Fig. 1, the inactivation at the lower concentrations of renal homogenate was only partial despite prolonged incubation, suggesting that inactivation was caused by a nonenzymatic reaction.

Study 5. Inactivation of urinary erythropoietin by various tissue extracts is shown in Table V. Spleen and liver extracts inactivate almost completely the erythropoietin while bone marrow extract was found to have only slight inactivating potency. For all tissues,

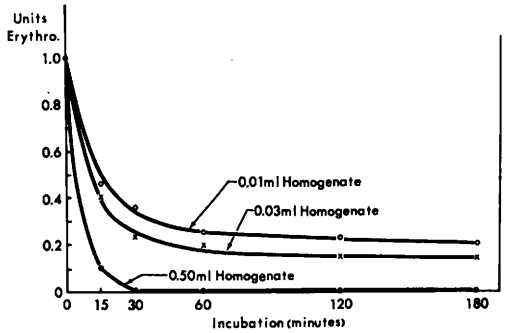

FIG. 1. Assays of 1 unit of urinary erythropoietin (C.G.) after incubation at 37°C for 15, 30, 60, 120, and 180 min against three concentrations of kidney homogenate in a saline medium: 0:50 ml/ml, 0.03 ml/ml, and 0.01 ml/ml.

TABLE IV. Inactivation of Urinary Erythropoietin with Unheated and Heated Renal Homogenates (66-hr ^{59}Fe utilization ± 1 SE %).

	Incubation for 1 hr with:		
	Saline	Unheated renal homogenates	Heated renal homogenates
W.B.	15.2 ± 1.3 (21)	0.4 ± 0.1 (23)	12.0 ± 1.0 (10)
C.G.	14.2 ± 1.2 (25)	0.3 ± 0.1 (20)	12.8 ± 1.9 (14)
L.S.	19.7 ± 1.9 (8)	0.2 ± 0.1 (10)	10.0 ± 1.6 (19)

TABLE V. Human Urinary Erythropoietin (units/ml after 1 hr incubation at 37° with tissue homogenate).*

	Control		Kidney		Boiled kidney		Liver		Spleen		Bone marrow	
	Saline	Plasma	Saline	Plasma	Saline	Plasma	Saline	Plasma	Saline	Plasma	Saline	Plasma
W.B.	1.00 (21)	1.30 (4)	0.00 (23)	0.15 (6)	0.76 (10)	1.00 (5)	0.11 (8)	0.18 (9)	0.12 (7)	0.20 (7)	0.60 (5)	0.40 (3)
C.G.	1.00 (25)	0.75 (5)	0.00 (20)	0.11 (8)	0.85 (14)	1.30 (4)	0.20 (8)	0.35 (8)	0.00 (4)	0.30 (4)	0.60 (7)	1.40 (4)
L.S.	1.00 (8)	1.20 (4)	0.00 (10)	0.00 (3)	0.35 (19)	0.40 (9)	0.00 (4)	0.40 (4)	0.00 (4)	0.12 (4)	0.50 (13)	0.60 (8)
Mean	1.00	1.08	0.00	0.09	0.65	0.90	0.07	0.31	0.04	0.21	0.57	0.80

* Figures in parentheses indicate the number of assay animals.

the capacity to inactivate was less when plasma was used as the suspending medium.

Discussion. The studies presented here show that homogenates of normal rabbit kidneys, livers, and spleens are capable of inactivating urinary and plasma erythropoietin. The inactivating process was modified but not abolished by heating to 100°C for 30 min and kinetic studies suggested it to be a nonenzymatic reaction. However, the data do not rule out the possibility that there was product inhibition of a degradative-type enzyme. Normal plasma was found to provide partial protection against inactivation, but since this protection was rendered to both urinary erythropoietin and to plasma erythropoietin, it does not appear to be dependent on a specific carrier function of plasma proteins (3).

The possible physiologic significance of this reaction is challenging but still completely conjectural. The finding that homogenates of normal livers and spleens have inactivating properties as well as the fact that plasma ameliorates the inactivation process appear to weaken the hypothesis proposed by Fisher *et al.* (2) that a renal inhibitor has a specific pathogenetic relationship to the anemia of renal failure. The near absence of inactivating potency of bone marrow homogenates is noteworthy but might be related to the lower density of nucleated cells in this organ.

Since partial inactivation of erythropoietin appears to occur even at 4°C, these studies may explain why it has proved so difficult to pinpoint the site of erythropoietin production in the kidneys or to extract significant amounts of erythropoietin from kidneys obtained from anemic animals. The protective effect of normal plasma may also explain difficulties we have encountered in repeating and evaluating studies indicating that erythropoietin is generated by the action of a renal erythropoietic factor on normal serum (7). Although this hypothesis is extremely attractive, we still need more precise identification and separation of the various factors involved (REF, renal inhibitor, serum substrate, serum inhibitor) before it should be accepted.

Summary. Homogenates prepared from

normal rabbit kidneys were found to contain a strong inhibitor of human erythropoietin obtained from human urine or from human plasma. The kinetics of the inactivation suggests it to be a nonenzymatic process. Dialysis against water or EDTA saline did not change the activity of the inhibitor and heating to 100°C did not completely abolish its activity. The addition of normal plasma reduced the inhibitory activity of the renal homogenate, a reduction that was directly proportional to the amount of normal plasma added and was observed both with urinary erythropoietin and with plasma erythropoietin. Homogenates prepared from normal rabbit spleens and livers were also found to contain an inhibitory principle that was modified by the addition of normal plasma. Homogenates from normal rabbit bone marrow were almost devoid of inhibitory activity.

We express our appreciation for the valuable association of Mr. Orin Miller, Miss Karen Dorian, and Miss Donna McDowall.

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Received July 1, 1968. P.S.E.B.M., 1968, Vol. 129.

Hemagglutinins in Sera of Africans of Gambia* (33440)

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Thirty-eight sera collected from Africans in the Medical Research Council Laboratory at Fajara, Gambia, British West Africa were studied for antierythrocyte activity. This report brings results obtained by testing these sera against (i) mammalian erythrocytes of foreign species origin, (ii) human blood group A and B erythrocytes, and (iii) human blood group O erythrocytes exposed to trypsin.

Methods. For titrations, twofold increasing saline dilutions of the sera were prepared in 0.1-ml volumes. To each test tube was added 0.1 ml of 1% suspension of three-times washed erythrocytes. Following 2 hr of incubation at room temperature, the test tubes were centrifuged at 500 rpm for 2 min and agglutination was recorded after shaking the test tubes gently. The titer was expressed as the reciprocal of the highest serum dilution at which definite agglutination was noted.

Trypsinized erythrocytes were prepared by mixing 10 vol of 2% erythrocyte suspension with 1 vol of 1% trypsin solution. The mixture was incubated at 37° for 10 min. Thereafter the erythrocytes were washed with cold saline.

For the indirect Coombs' test, the test serum, at various dilutions, was mixed with an equal volume of a 1% erythrocyte suspension. The mixture was incubated for 1 hr at room temperature. Thereafter the erythrocytes were washed 3 times with saline and resuspended to 1% concentration. One-tenth of this suspension was mixed with 0.1 ml of a Coombs' serum at a proper dilution, and

* Supported by a research grant from Ortho Pharmaceutical Corporation.