

Reduction of Plasma Urate Levels in the Cockerel with Polyethylene Glycol-Uricase¹

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ABSTRACT

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Uricase from *C. utilis* and uricase to which polyethylene glycol had been attached were injected into leghorn cockerels in an attempt to lower plasma urate levels. Twenty units of either enzyme reduced urate levels to near zero for 24 hr on initial

injection, whereas 10 U were effective for less than 6 hr. After injection with four-weekly doses of enzyme, unmodified uricase was ineffective in lowering plasma urate levels. Polyethylene glycol-uricase, however, was as effective as on the first injection. Both enzyme preparations appear in the lymph shortly after injection into the rat, although polyethylene glycol-uricase appears slightly sooner and maintains a more constant level than uricase.

Uricase (urate oxidase; EC 1.7.3.3) is absent in man and as a result uric acid is the end product of purine metabolism. This causes the human species to have a higher serum urate concentration than other mammals and increases the susceptibility of man to hyperuricemia and gout (Wyngaarden and Kelly, 1976). Treatment of these disorders generally involves drugs which decrease synthesis or increase elimination of uric acid. Unfortunately, the drugs presently in use, such as colchicine, probenecid and allopurinol, all cause side effects of varying severity (Gutman, 1968; Orgyzo *et al.*, 1966).

Gout and hyperuricemia also have been treated by the i.v. injection of uricase. In clinical tests, hog liver uricase could be detected in the plasma for several hours after injection (London and Hudson, 1957). Royer *et al.* (1967, 1968) injected fungal uricase into patients and showed lower uric acid levels *in situ*. Repeated injections produced a decrease in uricemia and urinary uric acid (Kissel *et al.*, 1968; Gross *et al.*, 1971). However, prolonged treatment caused antibody formation which resulted in reduced uricase activity (Kissel *et al.*, 1968; Brogard *et al.*, 1972; Fitzpatrick *et al.*, 1971).

Chen *et al.* (1981) modified uricase from hog liver and *Candida utilis* by covalent attachment of methoxypolyethylene glycol. Attachment of sufficient PEG eliminated the antigenicity of both uricases. If the antigenicity of uricase is removed, direct injection of the modified enzyme may be effective therapy

for hyperuricemia, especially acute hyperuricemia involving renal insufficiency.

This paper describes the *in vivo* efficacy of PEG-uricase in lowering blood urate levels in leghorn cockerels. The ability of PEG-uricase to enter the lymph system also is described.

Methods

C. Utilis uricase was a gift from Toyobo Company, Ltd. (Osaka, Japan). Uric acid was obtained from Fisher Scientific Company (Pittsburgh, PA). Leghorn cockerels (2-3 kg) and Swiss-Webster rats (250-300 g) were obtained from local suppliers. Two preparations of PEG-uricase were prepared as described previously (Chen *et al.*, 1981).

Plasma uricase. A uric acid standard solution is prepared by dissolving 60 mg of LiCO₃ in 20 ml of water followed by 40 mg of uric acid and diluting to 100 ml with water. A 2 mg/100 ml of uric acid assay solution in 0.1 M borax (pH 8.5) is prepared by adding 0.5 ml of standard solution to 9.5 ml of buffer. One milliliter of assay solution is placed in a cuvette at 37°C followed by 10 µl of appropriately diluted plasma (0.01-0.05 U of uricase). The reaction is followed at 290 nm for 5 min using a recording spectrophotometer. Activity is calculated from the slope by using the molar extinction coefficient for uric acid (1.22 × 10⁴). The reaction is linear during the initial 4 min and is proportional in the range of 0.01 to 0.1 U of uricase.

Reduction of serum urate levels with uricase and PEG-uricase. Cockerels were maintained in individual cages. Food (commercial laying mash) and water were provided *ad libitum*. Birds were injected *via* the brachial vein with 10 or 20 U of uricase or PEG-uricase (49%). After 1 hr, 2 ml of blood was drawn from the brachial vein with heparinized syringes for analysis of urate and uricase levels. Additional samples were taken at intervals up to 48 hr. Each sample was centrifuged immediately. An aliquot of the plasma (0.2 ml) was frozen for

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ABBREVIATIONS: PEG, polyethylene glycol; PEG-uricase (49%), uricase with PEG attached to 49% of the available amino groups; PEG-uricase (61%), uricase with PEG attached to 61% of the amino groups.

uricase assay. An aliquot of 0.5 ml was deproteinized and analyzed for urate by the procedure of MacRae (1977), except that reaction with uricase was done in cuvettes instead of in the Auto Analyzer. A reaction time of 5 min was sufficient for oxidation of urate.

Immunological protocol. Birds that had been given 10 U of uricase were then given three additional weekly i.v. injections of 10 U of uricase; those birds injected initially with 10 U of PEG-uricase (49%) were then given three additional weekly i.v. injections of 10 U of PEG-uricase (49%). Six days after the fourth injection, blood was drawn to check for circulating antibodies. The following day, the uricase-injected cockerels were given a final injection of uricase and the PEG-uricase (49%)-injected birds were injected with PEG-uricase (61%). The protocol is given in table 1.

PEG-uricase (49%) is slightly immunogenic in the mouse, whereas uricase with 57% or more of its amino groups modified by PEG is not (Chen *et al.*, 1981). PEG-uricase (61%) does not produce active anaphylaxis in the guinea pig against animals that have received a sensitizing dose of either uricase or PEG-uricase (61%). In addition, guinea pigs receiving a sensitizing dose of PEG-uricase (61%) do not respond to subsequent challenge with uricase (K. V. Savoca, unpublished observations).

Uricase levels in lymph after i.v. injection. The thoracic ducts of male Sprague-Dawley rats were cannulated by the technique described by Ford (1978). Animals were maintained under pentobarbital anesthesia during lymph collection. To maintain lymph flow, an i.v. infusion of Dulbecco's phosphate-buffered saline with 1 U/ml of heparin was given at a rate of 3 ml/hr; 20 U of heparin were administered i.v. after the operation to prevent clotting of lymph. Uricase or PEG-uricase (61%) were each injected i.v. (tail vein). Lymph was collected in tubes containing 0.1 ml of saline and 4 U of heparin. Hourly samples were collected for 25 hr. Samples were centrifuged to remove cells and were frozen for later uricase assay.

Results

Lowering of plasma urate. Normal urate levels in the blood of cockerels ranged from 5 to 10 mg/100 ml. Injection of 20 U of either uricase or PEG-uricase (49%) lowered blood urate to undetectable levels within 1 hr (table 2) on initial injection. The urate levels remained at or near zero for at least 24 hr and then began to rise until half the normal level was

TABLE 1
Injection schedule of uricase and PEG-uricase for production of antibodies

Day	Immunogen Injected (10 U)	
	Group 1	Group 2
1*	Uricase	PEG-uricase (49%)
8	Uricase	PEG-uricase (49%)
15	Uricase	PEG-uricase (49%)
22	Uricase	PEG-uricase (49%)
29*	Uricase	PEG-uricase (61%)

* Time studies of plasma uricase and urate levels were done after injections.

TABLE 2
Effect of i.v. injected urate oxidase and PEG-urate oxidase on serum urate levels in the cockerel, first injection

Treatment	Postinjection of Serum Urate				
	1 hr	6 hr	12 hr	24 hr	48 hr
	mg/100 ml				
Controls (4)*	5.8	7.8	7.1	5.7	
20 U of uricase (3)	0	0	0.9	0.8	3.5
20 U of PEG-uricase (3)	0	0	0.3	0	1.7
10 U of uricase (4)	0.5	2.4	4.0	4.5	
10 U of PEG-uricase (3)	0.1	2.7	3.0	4.6	

* Values in parentheses are the number of birds used.

reached at 48 hr. It appears that PEG-uricase (49%) is slightly superior at this stage, no doubt due to the higher level of enzyme present in the blood. We have observed earlier that PEG-uricase at about the 49% level of modification is immunogenic in the mouse. On initial injection, however, its plasma circulating life in mice greatly exceeds that of unmodified uricase (Chen *et al.*, 1981). Extended plasma half-lives on first injection is characteristic of PEG-enzymes, regardless of whether or not they have been modified to the extent of nonimmunogenicity.

Ten units of either enzyme lowers blood urate levels for a short time period. The levels rise within 6 hr and approach normal values after 24 hr. At either dose level, PEG-uricase (49%) maintains a higher level of activity in blood than the native enzyme.

After five weekly injections, native uricase is rapidly removed from the circulation (table 3). No enzyme is detectable in the blood after 1 hr and there is no effect on blood urate levels. Plasma from both sets of birds contained antibodies to uricase and PEG-uricase (49%), as determined by double diffusion in agar. PEG-uricase (61%) and PEG, however, gave no precipitation bands against any of the antisera. PEG-uricase (61%) still circulates in the plasma quite well and is apparently capable of lowering blood urate levels for a period of 24 hr.

Appearance of uricase and PEG-uricase (61%) in lymph. Both uricase and PEG-uricase (61%) appear in the lymph of the rat shortly after i.v. injection (fig. 1). As uricase

TABLE 3
Effect of i.v. injected urate oxidase and PEG-urate oxidase on serum urate levels in the cockerel, fifth injection

Birds were given weekly injections of 10 U of urate oxidase or PEG-urate oxidase. Urate oxidase levels in serum were monitored after the fifth injection.

Treatment	Post-Injection of Serum Urate			
	1 hr	6 hr	12 hr	24 hr
	mg/100 ml			
Controls (4)*	7.6	8.2	6.5	6.2
Uricase (4)	8.5	6.5		
PEG-uricase (4)	0.3	0.6	1.9	5.5

* Values in parentheses are the number of birds used.

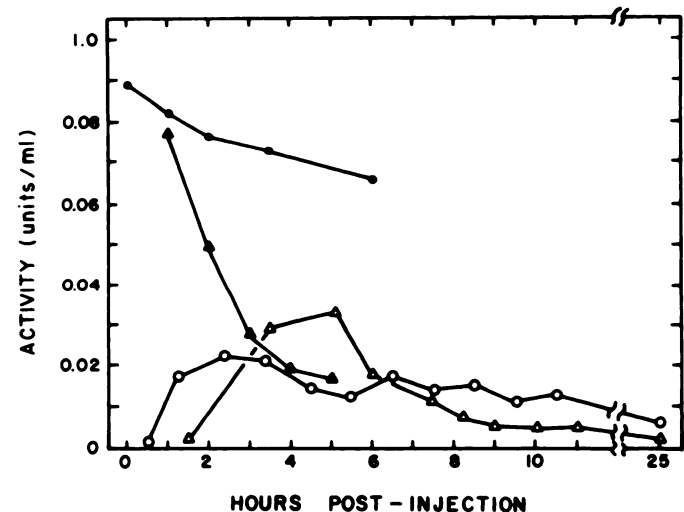


Fig. 1. Circulating life of urate oxidase and PEG-urate oxidase in blood and appearance in lymph after i.v. injection into rats. ●, PEG-urate oxidase level in blood; ▲, urate oxidase level in blood; ○, PEG-urate oxidase level in lymph; △, urate oxidase level in lymph.

disappears rapidly from the blood, it apparently concentrates somewhat in the lymph, with maximal activity occurring at 5 hr. PEG-uricase (61%) maintains a considerably higher level of activity in the blood, appears in the lymph slightly sooner and remains in the lymph at a more constant level than the native enzyme. The slight decrease of enzyme activity in the lymph between 4 and 6 hr may be a function of the collection procedure. The more rapid loss of uricase from lymph may be due to a generally greater rate of clearance from plasma. Earlier studies (Abuchowski *et al.*, 1977) indicate that the liver is the principal site of uptake.

Discussion

Effective treatment of hyperuricemia and gout by enzyme therapy will require an enzyme preparation capable of prolonged activity in blood even after repetitive injections. Chen *et al.*, (1981) showed that uricase from both hog liver and *C. utilis* circulated for extended time periods after modification with PEG. After a series of 39 injections in mice over a period of 90 days, the PEG-enzyme exhibited an unaltered circulating life and was easily detectable in blood 48 hr postinjection. Native uricase circulated for 24 hr on the first injection but was undetectable in 1 hr after the 13th injection. It appears the modified enzyme has potential therapeutic use.

The chicken presents an excellent model for enzyme therapy with uricase since it normally has elevated levels of blood urate. Both uricase and PEG-uricase (49%) were effective in decreasing urate levels. As might be expected, 20 U of enzyme decreased blood urate levels for a longer time period (24 hr) than 10 U (6 hr). However, after five weekly injections, the blood picture changed dramatically. Native uricase was rapidly removed within 1 hr after injection and failed to lower blood urate levels. PEG-uricase (61%) was as effective on the fifth injection as was PEG-uricase (49%) on the first injection. Apparently this preparation is nonantigenic since these same animals were immunized to PEG-uricase (49%). PEG-uricase (49%) after the fourth injection was removed as rapidly after injection as the native enzyme, due to the presence of antibody in these animals. This is an important consideration in therapy since it indicates that a properly modified PEG-enzyme can be used to treat a patient previously exposed to the native enzyme with no immunological consequences.

Normal individuals contain about 1100 mg of uric acid of which approximately $\frac{1}{3}$ is in the plasma and $\frac{2}{3}$ is in the extravascular fluid. The gouty patient has a uniformly elevated miscible pool of uric acid composed of urate deposits (tophi) and dissolved urate in the extravascular fluid. The extravascular fluid is collected by the lymphatic system and emptied back into the blood system. Treatment of gout and hyperuricemia

with PEG-uricase may be more effective if the modified enzyme enters the extravascular fluid. Detection of the modified enzyme in the lymph fluid assumes its presence in the extravascular fluid.

Both uricase and PEG-uricase enter the lymph. PEG-uricase was detectable slightly sooner than uricase and maintained a more constant level of activity. It may be assumed also that unaltered circulating life in the blood after repetitive injections translates into unaltered life in the extravascular fluid.

The properties of extended circulating life, nonantigenicity and ability to diffuse into extravascular spaces are of tremendous therapeutic importance for enzyme therapy. PEG-uricase successfully lowers blood urate levels in the cockerel. Its therapeutic effectiveness in humans is presently being studied.

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