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ACTION OF PURE β-D-GLUCOSE OXIDASE ON HIGHER ANIMALS

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Abstract—The action of pure β-D-glucose oxidase from *Penicillium amagasakiense* on higher animals by intravenous administration and *per os* was studied. In the case of intravenous administration, the values of LD_{50} were determined to be 3·50 mg/kg for rabbits, 0·80 mg/kg for rats, and 8·50 mg/kg for mice. When 1·72 mg/kg of β-D-glucose oxidase was administered to the rabbit, the blood glucose of rabbits rapidly decreased on account of the oxidation of β-D-glucose to D-glucono-δ-lactone, reached a minimum (54 per cent decrease) in about 15 min, and returned to normal 2–3 hr. The enzyme activity in blood reached a maximum in 3–7 min, diminished exponentially, and became zero in about 1 hr after the injection. The dissolved oxygen in blood also diminished slightly only for a while after the injection, and the ratio of β- to α-D-glucose distinctly lowered as supposed from the β-D-glucose oxidase reaction. The degree of decrease of blood glucose was almost proportional to the injected amount of β-D-glucose oxidase. On the contrary, in the case of administration *per os*, no action of the enzyme on mice, rats and rabbits was observed.

IT IS well known that β-D-glucose oxidase (EC 1.1.3.4), one of the flavin enzymes, oxidizes almost exclusively β-D-glucose to D-glucono-δ-lactone, which is spontaneously hydrolyzed to D-gluconic acid, in the presence of molecular oxygen with forming hydrogen peroxide. The enzyme has hitherto been used mainly for the preservation of canned and packed foods by elimination of oxygen or glucose[1, 2] and for the determination of glucose content in blood and urine.[3–6] On the application of the enzyme for medical purpose, Lettré[7] reported the curing effect of the enzyme for the ascites tumor of mouse. Very recently, Vincent and Perrier[8] described briefly the decrease of blood glucose of rabbits with crude enzyme preparation.

The authors studied independently the action of pure β-D-glucose oxidase on rabbits, rats and mice, and measured the changes of glucose amount, enzyme activity, oxygen content, and ratio of α- to β-D-glucose in blood after the injection of the enzyme. The values of LD_{50} of the enzyme on rabbits, rats and mice were determined when administered intravenously. The results in detail are described.

EXPERIMENTAL

Materials

Pure and crude β-D-glucose oxidase from *Penicillium amagasakiense* were kindly supplied from Dr. K. Kusai of Nagase & Co. Ltd., Osaka, Japan. The crude enzyme was purified by the method of Kusai.[9, 10] The ultracentrifugal analysis showed that the purified enzyme was homogeneous and its purity was checked by the measurement of the ratio of peaks of absorbance and the enzyme activity.[9] The molecular activity

was 34,000 moles of glucose per mole of enzyme per minute. D-Glucose, heparin sodium, sodium azide, acetic acid and sodium acetate were commercial samples. Male rabbits (2·3–3·3 kg), male Wistar strain rats (100–150 g) and male *dd* strain mice (18–22 g) were used after fasting for at least 12 hr in these experiments.

METHODS

Administration of β-D-glucose oxidase. β-D-Glucose oxidase dissolved in 0·1–0·3 ml of physiological saline was administered to rats (0·30–25·80 mg/kg) by injection into the tail vein or *per os*. The same enzyme solution (0·20–0·40 ml) was injected in the ear vein of rabbits (0·37–1·72 mg/kg), and 0·1 ml in the tail vein of mice (0·53–10·50 mg/kg).

Collection of blood. All of the blood used in these experiments was obtained from the heparinized animals. Each blood was collected from the ear vein of rabbits, provided that the ear was different from the one to which β-D-glucose oxidase was injected, and from the tail vein of rats with an injector at indicated times after the injection of β-D-glucose oxidase and from mice after decapitation.

Determination of blood glucose. After 0·1 ml of blood was added to 1·0 ml of distilled water, the mixture was immediately heated in boiling water for 30 sec. After cooling, 1·8 ml of 0·2 M acetate buffer (pH 5·6) and then 50 μl of β-D-glucose oxidase solution (10 mg/ml) were added to the mixture, and the oxygen consumption was measured by Beckman oxygen analyzer (Model 777). The glucose amount in blood was determined from the oxygen consumption by comparing with that of D-glucose solution after complete mutarotation as reported by the authors.[5, 6]

Determination of β-D-glucose oxidase activity in blood after injection of the enzyme. To a mixture of 0·1 ml of blood and 1·5 ml of 0·25% $K_3Fe(CN)_6$ as an oxidizing agent of hemoglobin to methemoglobin, 0·8 ml of 0·1 M acetate buffer (pH 5·6) was added. After standing for 5 min, 10 μl of 1·0% NaN_3 was added to 2·0 ml of the resultant mixture for the purpose of conversion of methemoglobin to azide-methemoglobin and inhibition of catalase in blood. When the dissolved oxygen in the solution became constant, enough amount (0·1 ml) of 10% D-glucose was added, and the initial velocity (ppm/min) of oxygen consumption was measured. The β-D-glucose oxidase activity was estimated from the calibration curve determined with the pure enzyme under the identical experimental condition, and expressed as μg of the pure enzyme per ml of blood.

Determination of partial pressure of oxygen in venous blood. About 1 ml of the venous blood was obtained with a syringe washed with physiological saline containing 0·02% heparin sodium. The partial pressure of oxygen in blood was directly measured by Beckman oxygen analyzer (Model 777) and expressed in terms of mm Hg.

Determination of the ratio of α- to β-D-glucose in blood after administration of β-D-glucose oxidase. The ratio of α- to β-D-glucose in blood was determined from the oxygen consumption of fresh and heated blood in the presence of β-D-glucose oxidase, because heating accomplishes mutarotation of blood glucose and denatures β-D-glucose oxidase in blood. The details of the method will be published in the near future.[11]

RESULTS AND DISCUSSION

While β-D-glucose oxidase was first administered to rats *per os*, they were not affected at all by administration of 25·8 mg/kg of the enzyme. On the other hand, when

β-D-glucose oxidase was injected intravenously, even 2·8 mg/kg of the enzyme induced remarkable cyanosis and ended in death within 2 hr. Since it was suggested that β-D-glucose oxidase could not be absorbed from the intestine or would be very rapidly digested in a gastrointestinal tract in the case of administration *per os*, only LD_{50} when administered intravenously was determined. The value of LD_{50} of β-D-glucose oxidase was estimated by the method of Behrens–Kärber[12] using four groups, each of which consisted of six animals. When the death within 48 hr after administration of the enzyme was regarded to be significant, the values of LD_{50} were determined to be 3·50 mg/kg for rabbits, 0·80 mg/kg for rats, and 8·50 mg/kg for mice. Thus, the value of LD_{50} of β-D-glucose oxidase for mice was about ten times as large as that for rats. The difference of LD_{50} between mice and rats will be discussed below.

The action of β-D-glucose oxidase on rabbits was tested considering the value of LD_{50} (3·50 mg/kg) for the rabbit. The effect of the enzyme on blood glucose was first examined and it was found that the significant decrease of blood glucose was observed even by injection of 0·37 mg/kg of the enzyme. The degree of decrease of

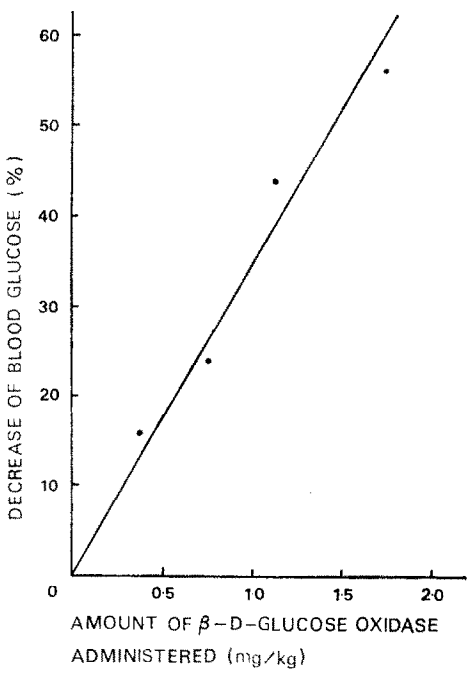

FIG. 1. Relation between the degree of decrease of blood glucose and the amount of β-D-glucose oxidase administered.

blood glucose increased in rough proportion to the amount of the enzyme administered at least so far as 1·72 mg/kg (Fig. 1). This may be a convincing evidence for the fact that the decrease of blood glucose depends really on the oxidation of blood glucose by β-D-glucose oxidase. A typical data of the action of β-D-glucose oxidase on blood glucose was shown in Fig. 2, Curve A. The blood glucose immediately started to decrease, reached a minimum in about 15 min, and returned to normal 2–3 hr almost completely after the injection. A report of Vincent and Perrier[8] on the action of crude

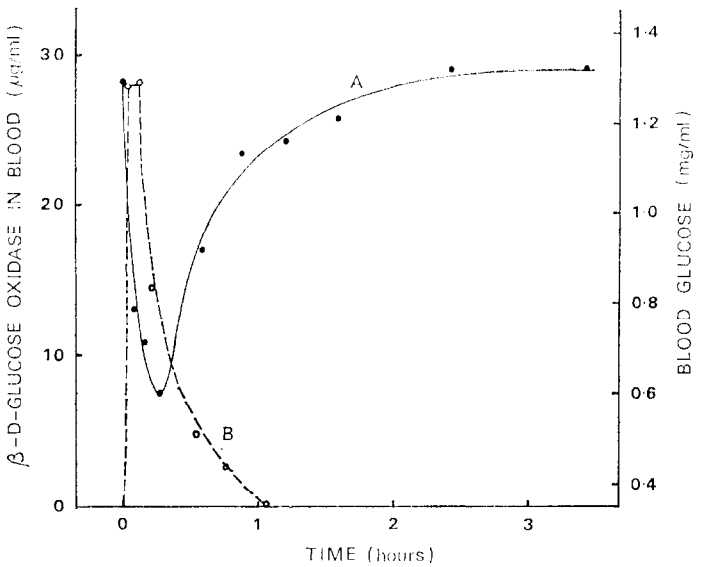

FIG. 2. Changes of amount of blood glucose and β-D-glucose oxidase in blood after administration of 1·72 mg/kg of β-D-glucose oxidase to male rabbit weighing 2·7 kg. Curve A, blood glucose, Curve B, β-D-glucose oxidase.

β-D-glucose oxidase on blood glucose recently appeared, and they described that intravenous injection of β-D-glucose oxidase into rabbit at a dose of 330 mg/kg induced a severe hypoglycemia (40 per cent decrease) followed by an intense hyperglycemia (80 per cent increase) and death. In contrast with their data, notwithstanding 54 per cent decrease of blood glucose was caused by administration of only 1·72 mg/kg of the pure enzyme, any subsequent hyperglycemia was not observed in our experiment as shown in Fig. 2, Curve A.

Since it was supposed from the fact described above that the β-D-glucose oxidase administered would be digested in the main organs or excreted from blood at a considerably high speed, it was intended to determine the change of the enzyme activity in blood. In fact, the activity quickly disappeared from blood, that is, increased to the maximum level in 3 min, started to decrease in 7 min and became zero in about 1 hr after the injection (Fig. 2, Curve B). Thus, the change of amount of blood glucose seemed to be consistent with the rise and fall of the enzyme activity in blood.

Since the difference between rats and mice for LD_{50} of the enzyme may depend on the divergence of the digesting speed in the body of both species for the enzyme, the activity of the enzyme in blood was determined. It was found that the enzyme activity in blood of both species changed like in the case of rabbit with the lapse of time and disappeared entirely in about 40 min after the injection. Therefore, the digesting activity in the body would not be concerned with the difference of LD_{50}. In our speculation, the damage of central nervous system, which is very sensitive organ to glucose and oxygen quantity in blood, would be the common cause of death, and hence the difference of LD_{50} between rats and mice will be due to the divergence of susceptibility of central nervous system to simultaneous decrease of glucose and oxygen in blood which is temporary but severe.

As supposed from the β-D-glucose oxidase reaction consuming molecular oxygen, the dissolved oxygen in blood should naturally decrease as far as the β-D-glucose oxidase activity is observed in blood. The dissolved oxygen was measured by estimation of the partial pressure of oxygen in blood, and the partial pressure (46·8 mm Hg) before the injection decreased to 41·8 mm Hg during 5 to 15 min after the injection and then it soon returned to normal (Fig. 3). From this result, it was found that even

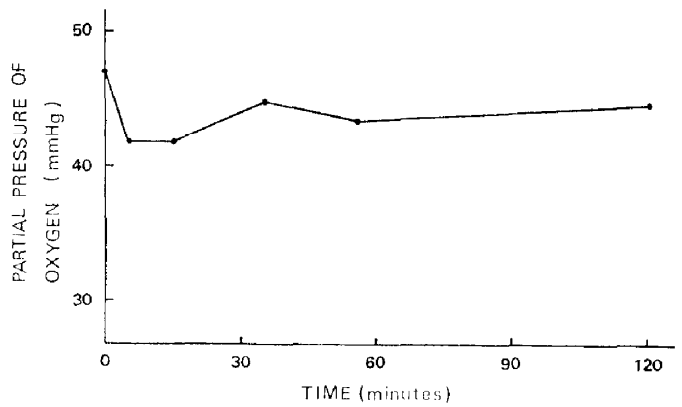

FIG. 3. Changes of the partial pressure of oxygen in venous blood of ear after administration of 1·50 mg/kg of β-D-glucose oxidase to male rabbit weighing 3·2 kg.

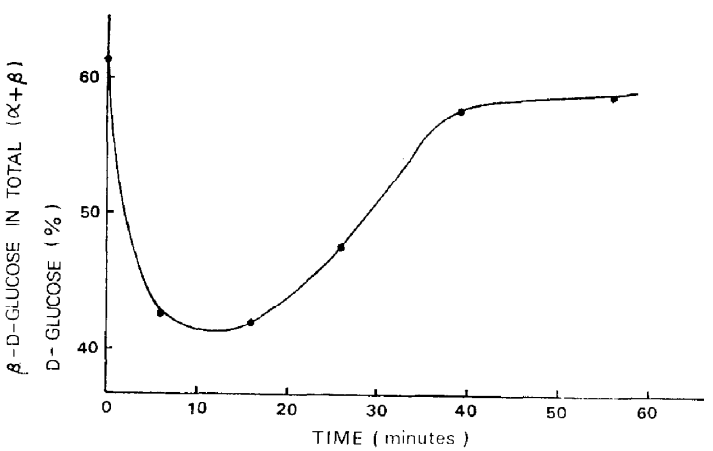

FIG. 4. Changes of the ratio of β- to α-D-glucose in blood after administration of 1·70 mg/kg of β-D-glucose oxidase to male rabbit weighing 3·3 kg.

enough amount (1·50 mg/kg) of β-D-glucose oxidase for decreasing blood glucose (ca. 50% decrease) caused a little decrease of the dissolved oxygen only while the enzyme activity in blood was so high, but further decrease of oxygen in blood was observed by the injection of large amount of the enzyme.

It was also expected from the fact that the β-D-glucose oxidase oxidizes only β-D-glucose that the ratio of β- to α-D-glucose in blood would diminish with the

progress of oxidation of β-D-glucose. As shown in Fig. 4, the ratio rapidly diminished with the decrease of blood glucose, reached a minimum in 10–15 min, and then returned to an almost normal level in 40 min after the injection. This result is also consistent with the change of the β-D-glucose oxidase activity in blood described precedently.

It became clear from the facts described in this paper that pure β-D-glucose oxidase may be administered to the patients when the quick decrease of blood glucose is required.

The authors are investigating the distribution and metabolism of injected β-D-glucose oxidase in higher animals as these problems are very interested from the points of the metabolism of injected exogeneous protein and the preventing action of animals for the protein. The results will be published elsewhere.

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