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EFFECTS OF GLUCOSE OXIDASE IN MICE*

ROBERT N. FEINSTEIN, MARILYN COULON and JOHN E. SEAHOLM

Division of Biological and Medical Research, Argonne National Laboratory, Argonne, Ill., U.S.A.

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Abstract—The effects of glucose oxidase, which oxidizes glucose and produces H_2O_2, were studied in mice after intraperitoneal injection of supralethal doses of the enzyme. Such injections cause the appearance of hematin pigment in the plasma, up to 20 per cent of this pigment being methemoglobin. After high lethal doses of glucose oxidase, the hematocrit decreases (readings as low as 7 have been obtained), and the total hematin pigment of whole blood also decreases strikingly. After lower, but still lethal, doses of the enzyme, the hematocrit *increases* (readings as high as 80 have been obtained), and the percentage of hematin pigment in whole blood correspondingly increases. At least part of this loss of fluid from the blood is accounted for by increased water content of liver and kidney. After such doses of glucose oxidase, decreases were demonstrated in blood sugar and in liver glycogen.

THE ENZYME glucose oxidase catalyzes the reaction

$$\text{glucose} + \text{oxygen} \longrightarrow \text{gluconic acid} + \text{hydrogen peroxide}$$

In an attempt at the continuous production of H_2O_2 *in vivo*, glucose oxidase was injected into normal mice. The resulting effects on erythrocytes, hematin pigments, and carbohydrate stores proved sufficiently interesting to warrant a further investigation.

MATERIALS AND METHODS

All animals were CFl/Anl female mice, 3–5 months old. In any single experiment mice were of the same age (within a week). Four different commercial preparations of glucose oxidase were used: Sigma Chemical Co. (type III preparation), Dawe's Laboratories* (glucose oxidase, refined), Nutritional Biochemicals Corp., and Enzyme Development Corp. (Deoxin Special). Except where otherwise stated, the Dawe product was used to obtain the results shown.

For all blood studies blood was drawn from the orbital sinus, by the technique of Riley,[1] into heparinized capillary tubes which could then be used either: (*a*) as a source of whole blood, by transfer to micropipets or (*b*) as hematocrit tubes which, after centrifugation, gave not only the hematocrit value but also served as sources of plasma (measurable by volume) and packed cells (measurable by tube length, convertible to volume).

Total hematin pigment and per cent methemoglobin of whole blood, plasma, or packed blood cells were determined by the method of Hunter.[2] Blood glucose was

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* We wish to thank Dr. George E. Ward, of Dawe's Laboratories, for generous gifts of this product.

determined by the method of Nelson,[3] and liver glycogen was determined by the method of Good *et al.*,[4] followed by the glucose method of Nelson.[3]

Glucose oxidase assays were performed by a newly devised and unpublished technique which must at present be considered semiquantitative. Briefly, it consists of incubating the enzyme preparation with glucose, at 37°, in phosphate buffer, pH 6·0, in the presence of sodium azide ($2{\cdot}5 \times 10^{-4}$ M, final concentration) to inhibit catalase activity. (Almost all commercial glucose oxidase preparations are heavily contaminated with catalase as an impurity. The sole exception we have seen is the Deoxin Special, which shows only traces of catalatic activity.) Action was halted with H_2SO_4 (to a final concentration of 1 N), and the H_2O_2 formed was measured by the addition of $TiCl_4$ (to a final concentration of 0·3 N), the yellow color being measured at 420 mμ and compared with a standard calibration curve.

For dry weight determinations, tissues were weighed fresh and again after drying to constant weight at 100–105°. Dilution of Evans blue in plasma was measured in a group of mice of identical weight. Each mouse was given 0·25 ml of 2% Evans blue solution by intravenous injection via a tail vein: a single syringe and needle were used for the entire group. After 5 min, orbital sinus blood was withdrawn and centrifuged. After the hematocrit was read, 25 μl of plasma were withdrawn and added to 0·5 ml H_2O in microcuvets matched in the 6–110 semimicro adapter on the Coleman model 6B spectrophotometer; readings were taken at 600 mμ.

RESULTS

Toxicity and glucose oxidase activity

The LD_{50} after intraperitoneal injection was determined for each of the four enzyme preparations, and the glucose oxidase activities were determined as described above. The results are shown in Table 1. It will be observed that, although the four preparations vary widely (over 15-fold) in lethality and in glucose oxidase activity, the glucose

TABLE 1. LETHALITY AND GLUCOSE OXIDASE ACTIVITY OF SEVERAL COMMERCIAL GLUCOSE OXIDASE PREPARATIONS*

Source	LD_{50}, mg/kg	Glucose oxidase activity	Glucose oxidase per LD_{50}
Sigma	4	2·37	9·5
Dawe	13	0·68	8·8
Nutritional Biochemicals	21	0·46	9·7
Enzyme Development	70	0·145	10·2

* Glucose oxidase activity is expressed as millimoles H_2O_2 produced per milligram enzyme preparation, under standard conditions described in text.

oxidase activity of an LD_{50} is quite constant. This suggests that the toxicity of the preparations is indeed due to their glucose oxidase activity, rather than to some contaminant.

Survival time

It was soon realized that certain effects observed after injection of glucose oxidase were not only quantitatively, but also qualitatively, dependent on the dose administered.

Therefore most of the phenomena described below were investigated by means of three different doses. The enzyme employed was the Dawe product, and this was administered at 50, 250, and 1,250 mg/kg. It will be noted that even the lowest of these doses is approximately four times the LD_{50}. The median survival time for animals receiving an LD_{50} is about 2 days. Median survival times for mice receiving 50, 250, and 1,250 mg/kg are 4·5 hr, 1·5 hr, and 51 min respectively.

Hematocrit

The effects of the various doses of enzyme on the hematocrit are shown in Fig. 1. It will be observed that after 50 mg/kg, the hematocrit rises strikingly. Individual hematocrits as high as 80 have been observed; such blood flows extremely sluggishly, and its is difficult to obtain an adequate sample from the orbital sinus or even by decapitation. After enzyme doses of 250 or 1,250 mg/kg, the hematocrit decreases; individual values as low as seven have been seen.

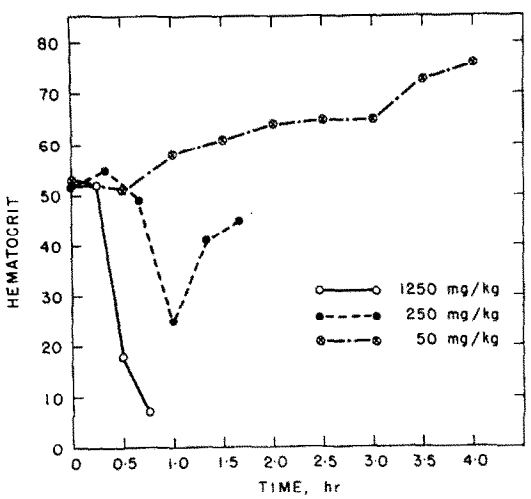

FIG. 1. Hematocrit values after intraperitoneal injection of glucose oxidase. Each point represents average value for three mice.

Hematin pigments

Optical absorption at 525 mμ has been used as a measure of total hematin pigments, since according to Hunter[2] this wavelength is isobestic for hemoglobin and methemoglobin. This value has been measured in whole blood, plasma, and packed red cells (after lysis) at various periods of time after the several dose levels of glucose oxidase.

Figure 2 presents the picture in whole blood. After 50 mg glucose oxidase/kg, the total pigment increases in a manner entirely comparable to the increase in hematocrit. After 250 or 1,250 mg/kg, pigment values decrease. The decrease, especially after 1,250 mg/kg, is less than would be expected from the decreased hemtocrit. This discrepancy is due to the fact that, as noted below, the plasma has become highly colored after these doses of enzyme, so that the plasma pigment now contributes heavily to the pigment of the whole blood.

Figure 3 shows the total hematin pigment found in the plasma. Although only traces of pigment appear in the plasma after 50 mg glucose oxidase/kg, the plasma contains considerable pigment after the higher doses. In fact, so highly colored are some of these plasmas that it was necessary sometimes to estimate the packed cell

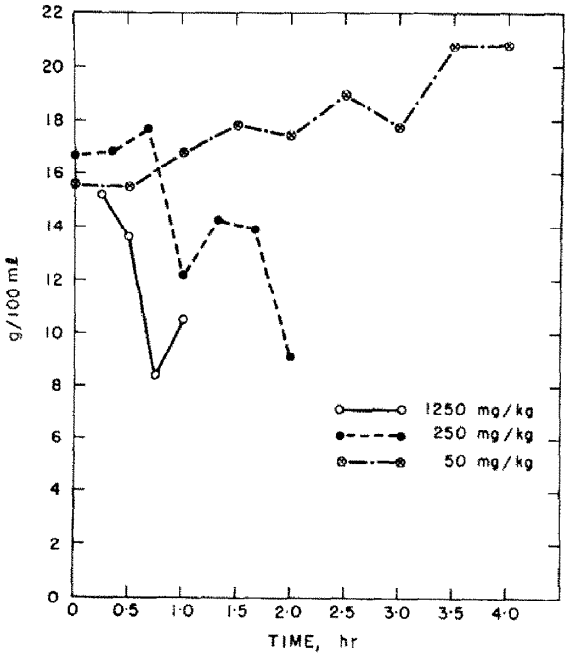

FIG. 2. Total hematin pigment of whole blood after intraperitoneal injection of glucose oxidase. Each point represents average value for three mice.

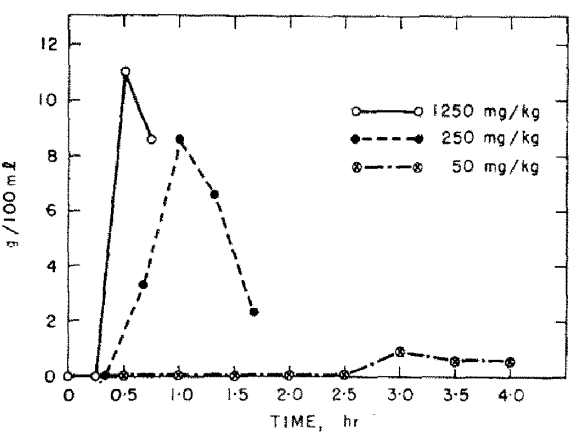

FIG. 3. Total hematin pigment in blood plasma after intraperitoneal injection of glucose oxidase. Each point represents average value for three mice.

level for hematocrit determination by gently lowering into the centrifuged capillary tube a light, footed glass rod and noting its point of initial settling. At the same time that the plasma is so heavily pigmented, pigment is also observed in the excreted urine. If the animal is sacrificed at this time, it is seen both macroscopically and

microscopically that its internal organs are heavily pigmented. This appeared especially true of the spleen.

The total hematin pigment was also measured in the packed blood cells after lysis. The only case in which a significant change was observed was that of the 1,250 mg/kg dose level. Here the total pigment per unit volume of cells decreased to approximately one-third of the original value in 45 min.

Methemoglobin formation

A cyanotic appearance is characteristic of animals injected with 250 or 1,250 mg glucose oxidase/kg. Analysis indicates that this is due to the formation of significant amounts of methemoglobin. Fig. 4 indicates that methemoglobin sometimes reaches

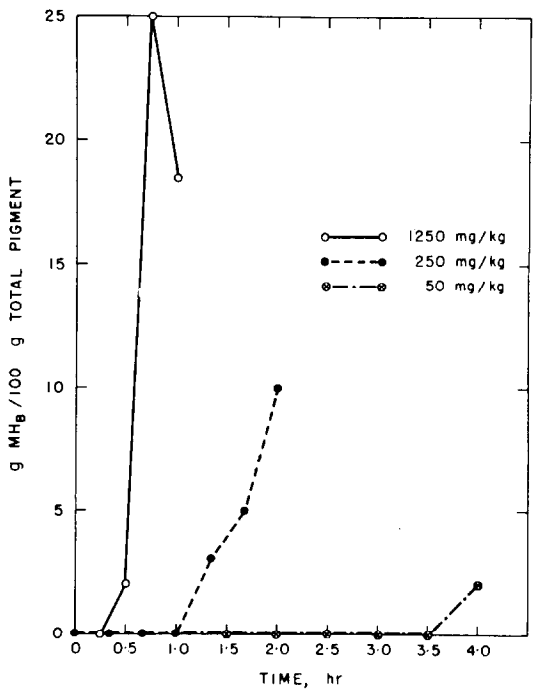

FIG. 4. Methemoglobin formation in whole blood after injection of glucose oxidase. Each point represents average value for three mice.

values as high as 25 per cent of the total pigment in whole blood, and Fig. 5 shows approximately the same result for plasma. The slight difference between the two is undoubtedly due to biological variation, since the data for whole blood and for plasma were obtained from different groups of animals. In no case has methemoglobin been detected in packed red cells after lysis.

Plasma volume and tissue water

The increase in hematocrit and in the hematin pigments of whole blood after 50 mg glucose oxidase/kg suggests that blood plasma volume has decreased, the lost fluids presumably entering the tissues. This possibility has been examined in two ways. In the first experiment, the results of which appear in Table 2, Evans blue was injected

intravenously into a series of normal and glucose oxidase-injected mice. All mice were of the same weight, and each animal received an identical injection of Evans blue, the same syringe and needle being used throughout. Table 2 shows clearly that the dye has become diluted into a lesser volume of plasma in the case of the enzyme-injected mice than in the normal animals.

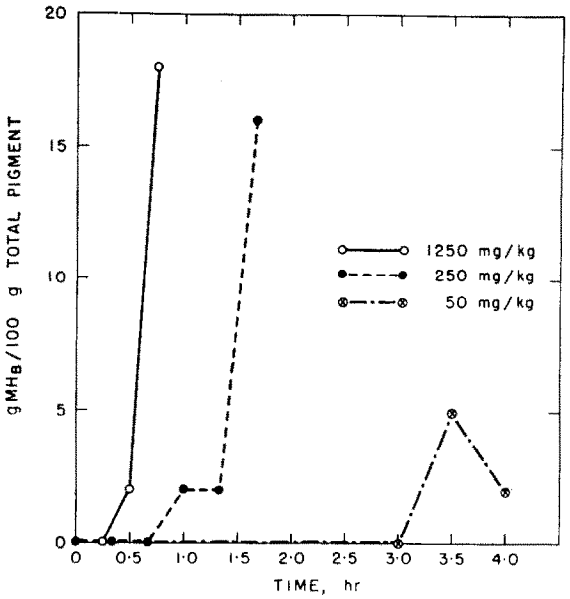

FIG. 5. Methemoglobin appearance in blood plasma after intraperitoneal injection of glucose oxidase. Each point represents average value for three mice.

TABLE 2. CONCENTRATION OF EVANS BLUE IN PLASMA

	Evans blue conc.
No enzyme	0·514
After enzyme,* 5 min	0·614
After enzyme, 3 hr.	0·93

* Enzyme was Dawe's glucose oxidase, 50 mg/kg intraperitoneally. Evans blue was a 2% solution, injected intravenously at 0·25 ml/mouse, from the same syringe and needle, into mice of identical weight. Three mice were used for each test. Evans blue concentrations shown are in plasma after centrifugation and are expressed as optical density at 600 mμ after 20-fold dilution with water.

The second set of experiments bearing on the question of a possible fluid shift is summarized in Table 3, which indicates that at least the kidney and liver have a significantly lower dry weight; i.e. a higher moisture content after injection of the enzyme.

Carbohydrate stores

If the injected glucose oxidase remained enzymatically active *in vivo*, one would expect decreased blood glucose and liver glycogen. The data of Table 4, typical of a larger mass of data, indicate that such effects are found after any of several doses of the enzyme.

TABLE 3. EFFECT OF GLUCOSE OXIDASE ON WATER CONTENT OF TISSUES*

Tissue	Per cent dry weight		Student's 't' test
	Control	Experimental	
Liver	39·1	33·0	4·22†
Intestine	21·8	20·5	1·53
Skin	26·4	28·8	0·91
Muscle	25·9	24·9	1·48
Spleen	23·3	22·0	2·36
Kidney	24·7	22·7	4·48†

* Each datum is mean of six animals sacrificed. Mice were fasted overnight. Tissues were weighed fresh and after achieving constant weight at 100–105°. 'Experimental' animals were sacrificed 3 hr after intraperitoneal dose of 50 mg Dawe's glucose oxidase/kg body weight.

† Statistical significance of $P \leq 0{\cdot}01$.

TABLE 4. EFFECT OF GLUCOSE OXIDASE INJECTIONS ON BLOOD SUGAR AND LIVER GLYCOGEN

Enzyme dose, mg/kg	Time	Blood sugar, mg/100 ml	Liver glycogen, per cent
50	Zero (no enzyme)	102	4·6
	1 hr	98	3·2
	2 hr	84	2·7
	3 hr	62	1·4
	3 hr (no enzyme)	104	3·7
1,250	Zero (no enzyme)	120	2·3
	30 min	30	1·7

DISCUSSION

The question of fundamental interest from our point of view is whether or not the group of symptoms described is actually due to hydrogen peroxide production. It seems to us that the syndrome described might be due to one or more of the following factors, based on the equation:

$$\text{glucose} + \text{oxygen} \longrightarrow \text{gluconic acid} + H_2O_2$$

(*a*) carbohydrate depletion, (*b*) hypoxia, (*c*) gluconate toxicity, (*d*) acidosis from gluconic acid formation, (*e*) H_2O_2 toxicity, (*f*) toxicity unrelated to the glucose oxidase itself (i.e. toxicity due to a contaminant).

No single experiment has yet been devised to decide definitively among these various possibilities. Evidence is at hand, however, to suggest that several of them may be eliminated from consideration. Thus, for example, the direct proportionality between

the enzyme activity and the toxicity of several commercial preparations of glucose oxidase (Table 1) suggests that the lethality is not due to a contaminant. Further, a heat-denatured glucose oxidase is nontoxic.

Carbohydrate depletion also seems an unlikely explanation, since we have found that the injection of glucose into mice injected with lethal amounts of glucose oxidase actually exacerbates, not relieves, the symptoms. Thus hematocrit and pigment changes appear sooner and to a greater degree in glucose-injected animals; survival time is shortened after lethal doses of the enzyme, and the lethality of approximately LD_{50} doses is increased.

We have done no experiments bearing directly on the possible role of a hypoxia. However, the syndrome we describe above seems totally unrelated to the picture of hypoxic death.

To estimate the possible role of gluconate toxicity or acidosis, we first estimated the amount of glucose that would be destroyed, under optimal conditions, by the dose of 50 mg glucose oxidase/kg acting over 3 hr; this comes to approximately 75 mg glucose. (It should be emphasized that this is an extreme maximal value; it assumes a slightly nonphysiological but enzymatically optimal pH, and no loss of enzyme activity by excretion, proteolysis, dearth of substrate, or maldistribution.) The injection of sodium gluconate equivalent on a molar basis to 100 mg of glucose was without effect on the animals.

The possible significance of an acidosis is less easily decided. The injection of gluconic acid (as 50% solution), in amounts equivalent on a molar basis to 100 mg of glucose, is rapidly lethal to all animals. If, however, the gluconic acid is merely diluted with an equal volume of water, the same total amount of gluconic acid is lethal to only a fraction of the mice, and any further reduction in concentration or amount renders it totally nonlethal. Lethal doses of gluconic acid will cause an increase in hematocrit, but we have not been able to find any time and dose that will produce the decreased hematocrit and massive cell lysis seen after higher doses of the enzyme. The question of the possible role of acidosis in the symptomatology described must therefore remain moot, although we are of the opinion that acidosis alone cannot account for all, or most, of the effects observed.

These considerations thus favor, by elimination, the concept that H_2O_2 is the causative agent. Experiments with catalase inhibitors also provide suggestive evidence bolstering this viewpoint. Sodium azide not only inhibits catalase but also a wide variety of other heme enzymes; 3-amino-1,2,4-triazole (AT) is a fairly specific inhibitor of catalase. Either of these agents, if injected in sublethal doses before the glucose oxidase, sharply decreases the LD_{50} of the injected glucose oxidase and shortens the survival time.

If, however, H_2O_2 is the causative agent, the question of how methemoglobin may be formed in the presence of abundant catalase must then be raised. (We have assayed the catalatic activity of whole blood from animals moribund from glucose oxidase injection; it has decreased, but not greatly.) A comparison of Fig. 4 and 5 indicates that methemoglobin never appears in whole blood samples before it appears in the plasma; this, combined with the fact that we have never detected methemoglobin in packed erythrocytes, suggests that H_2O_2 may oxidize hemoglobin to methemoglobin only after the hemoglobin has been released from the cells and thus possibly separated from the catalase.

It will be noted that the 'normal' values obtained for hematocrit and for total blood pigment are higher than those commonly quoted. This matter was tested, and it was clearly established that orbital sinus blood is significantly higher than decapitation blood in both respects:

	Orbital sinus	Decapitation	Student's 't' test
Hematocrit	52·8	49·0	8·09 (d.f. = 11)
g pigment/100 ml	15·73	14·99	4·29 (d.f. = 11)

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