TOCOPHEROL AND HEMOLYSIS *IN VIVO* AND *IN VITRO*

By Paul György and Catharine S. Rose

Nutritional Service of the Department of Pediatrics and the Gastro-Intestinal Section of the Medical Clinic, School of Medicine, University of Pennsylvania, Philadelphia, Pa.

Work of Houssay and Martinez,[1] published in 1947, suggested that the same mechanisms might be active in protecting the rat against the toxic effects of alloxan that had been found of value in protection of the liver against acute massive necrosis induced by dietary means. Among other effects of dietary factors, they observed longer survival after alloxan injection in rats which had received methionine (but not choline) as a supplement to a diet high in lard, or had been given a diet in which the lard was replaced by vegetable oils. It has recently been shown that tocopherol is a protective agent for the liver, and experiments were undertaken to determine whether the advantage of vegetable fats might depend on their content of tocopherol.

The rapid hemolysis of the blood of tocopherol-deficient rats, which was the major finding of this investigation, was observed first at autopsy.[2] The kidneys of rats which died a few hours after injection with 160 mg. per kg. of alloxan intraperitoneally were completely engorged with blood. Hemoglobinuria and hemoglobinemia were then followed, and the extent of hemolysis in some cases was astounding. Within 10 minutes after the injection, the centrifuged hematocrit tube showed the serum layer dark red in color, and, in about half an hour, the layer of cells, instead of being about 50 per cent of the total volume, was almost undetectable. In those animals which survived, the hemoglobin was excreted and there was no further hemolysis.

TABLE 1 shows the effect of variations in the tocopherol-deficient diet

TABLE 1

EFFECT OF DIETARY FACTORS ON EARLY MORTALITY AND HEMOGLOBINURIA FOLLOWING ADMINISTRATION OF ALLOXAN

Diet	*Rats dead in first two days* (%)	*Hemoglobinuria* (%)
High lard	55	100
High lard with tocopherol	25	0
High lard with yeast	15	60
High lard with yeast and tocopherol	0	0
High vegetable shortening ("Vream")	10	0
High "Vream" with yeast	15	0
Low fat	25	60
Low fat with tocopherol	0	0
Low fat with yeast	5	55
Low fat with yeast and tocopherol	5	0

and of supplementation with tocopherol on hemolysis. The diets used have been described previously.[2] The high fat diets contained 38 per cent of the lard or vegetable shortening. Animals in the low fat groups received 3 drops of corn oil daily and 3 drops of percomorph oil weekly as the only

fat in their ration. In the yeast groups, 5 per cent of yeast replaced an equal amount of carbohydrate. The tocopherol-supplemented animals received 3 mg. of mixed natural tocopherols daily. The rats, young females weighing 100 to 120 grams, were given the experimental ration for one month before being injected with alloxan.

With the low-tocopherol diets, the degree of hemolysis varied. There was an advantage of a low content of fat and, in some groups, benefit from yeast was observed. Tocopherol, whether it was given as a natural constituent of the dietary fat or as a separate supplement, gave complete protection in all cases, without a single exception. There was mortality in almost all groups during the first 2 days after alloxan injection. Tocopherol reduced the incidence of mortality, particularly in the high-fat groups, apparently by prevention of extensive red blood-cell destruction with the consequent blocking of the kidney.

To return to the original question: Would tocopherol protect the islets of the pancreas against destruction by alloxan? The data gave no support to this hypothesis. Diabetes, as measured by blood sugars taken 48 hours after injection of alloxan, was equally severe whether or not tocopherol was given (TABLE 2). Blood-non-protein nitrogen determinations indicated that

TABLE 2

EFFECT OF DIETARY FACTORS ON DIABETES, KIDNEY DAMAGE, AND SURVIVAL FOLLOWING ADMINISTRATION OF ALLOXAN

Diet	*Blood sugar 48 hours (mg. %)*	*Blood NPN 48 hours (mg. %)*	*Survival 7 days (%)*
High lard	500	200	5
High lard with tocopherol	490	90	15
High "Vream"	510	110	35
Low fat	660	170	45
Low fat with tocopherol	520	105	70

kidney damage might be somewhat more serious in the tocopherol-deficient animals, but histological examination showed the same type of injury in all cases. The increased nitrogen retention in the deficient groups was probably a result of the blocking of the kidney with blood. Equally, tocopherol showed no effect in prolonging the life of the rats beyond the two-day period already mentioned. On the basis of our experiments, the protective effect of tocopherol is limited to the red blood cell. It gave *no* protection against the development of alloxan diabetes.

Hemolysis has not been mentioned in connection with alloxan effects in rats but has been observed in rabbits, as reported a few years ago by Kennedy and Lukens[3] and within the past year by Gualandi and Campana.[4] These animals were presumably normal and there is no information about their vitamin E status.

Our further experiments were directed towards an explanation of the hemolysis and the way in which tocopherol protected against it. If one attributes any effect to alloxan itself, it must be one which occurs very

rapidly, since alloxan disappears from the blood stream within 2 or 3 minutes after intravenous injection. The main reactions of alloxan with which we must be concerned are shown in FIGURE 1. In neutral solution, alloxan

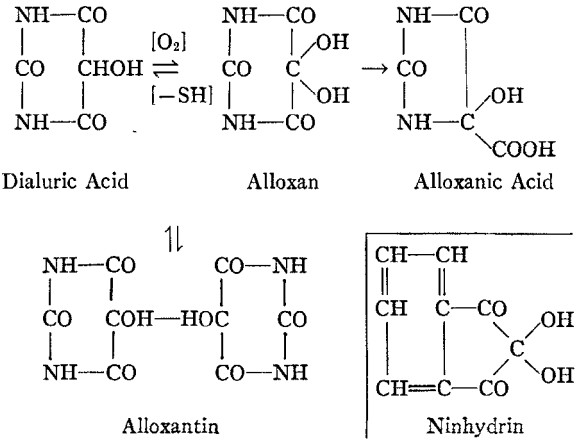

FIGURE 1.

is converted very rapidly to alloxanic acid by molecular rearrangement. Under physiological conditions this reaction is not reversible. In the presence of such mild reducing agents as the sulfhydryl compounds, alloxan is reduced to dialuric acid. With less than equivalent amounts of the reducing agent, an intermediate compound, alloxantin, which may be considered as a double molecule consisting of one molecule of alloxan and one molecule of dialuric acid, is formed. In dilute solution, alloxantin is almost completely dissociated into alloxan and dialuric acid. This system is readily reversible, dialuric acid being oxidized by molecular oxygen. In the presence of ammonia, alloxantin will form murexide, the ammonium salt of purpuric acid. Ninhydrin, the formula of which is given for comparison with that of alloxan, was also considered as a possible hemolyzing agent, since, as far as the triketone portion of the molecule is concerned, it is analagous to alloxan and forms a similar series of compounds.

A number of these compounds were tested *in vivo* on rats which had been kept for one month on a tocopherol-deficient ration. Alloxanic acid was without hemolyzing effect. Alloxantin showed about the same activity as alloxan, while dialuric acid proved to be about twice as effective. Ninhydrin was by far the most active material tested, giving extensive hemolysis at a level of 25 mg. per kg., about $\frac{1}{6}$ the required amount of alloxan. With 10 mg. per kg. of ninhydrin there was no hemolysis.

None of these substances caused any hemolysis of the blood of animals receiving supplements of tocopherol. Since ninhydrin was effective at such a low level, it seemed useful to see how high one would have to go to overcome the tocopherol effect. Ninhydrin is more toxic than alloxan and animals receiving over about 80 mg. per kg. do not survive. This did not

affect the experiment, however, since the hemolytic reaction is very fast. We continued to raise the dose in tocopherol-treated rats, giving 100, 200, and finally 500 mg. per kg. Two animals received this highest dose. One survived only 15 minutes, but the other lived for 1-½ hours. In neither was there any trace of hemolysis.

Simultaneously with these tests, we had been trying out the possibility of producing hemolysis *in vitro*. It was found that under appropriate conditions the red blood cells of animals deficient in tocopherol could be completely hemolyzed, while those of tocopherol-treated animals were never affected. Blood was collected from the tail of the rat into a tube containing normal saline and sodium citrate. The sample was centrifuged, washed, and made up in a 5 per cent suspension with saline. One volume of this suspension was mixed with one volume of phosphate buffer (.05 M monopotassium phosphate and .039 M sodium hydroxide; pH 7.4) containing the material to be tested, the tube was incubated at 37°C. for 15 minutes, and the progress of hemolysis was followed by observation of the decrease in opacity of the suspension.

A comparison of the results obtained *in vitro* with those previously found *in vivo* is given in TABLE 3. Alloxan did not cause hemolysis, nor did ninhydrin, which is comparable in structure. Under the conditions used, dialuric acid in a concentration of 0.7 millimols per liter of the mixture caused complete hemolysis in 15 to 30 minutes. There was hemolysis with alloxantin, but not so rapidly as with dialuric acid, which suggests that the results obtained with dialuric acid did not depend on the formation of alloxantin in the mixture and, rather, that the effect of alloxantin was due to the dialuric acid moiety. In both methods of testing, dialuric acid stands out as the active member of the system of compounds related to alloxan.

TABLE 3

HEMOLYSIS BY ALLOXAN AND RELATED COMPOUNDS IN TOCOPHEROL-DEFICIENT RATS

Compound	*Hemolysis*	
	in vivo	*in vitro*
Alloxan	+	—
Alloxantin	+	++
Dialuric acid	++	++++
Ninhydrin	++++	—

These experiments have answered the first question which arose in connection with tocopherol: whether its action was in the body fluids or in the red blood cell. Since washed red blood cells of animals which had received tocopherol were resistant to the hemolytic action of dialuric acid, the protection was a function of the cell itself. Another question was whether tocopherol was acting as such in the cell, or whether, under its influence, a more resistant cell was manufactured. This was tested by addition of synthetic tocopherol to the reaction mixture. Tween 80 was used to get the tocopherol into solution. Four grams of Tween were mixed with one

gram of tocopherol and the solution diluted 25,000 times with phosphate buffer. Control tests with Tween alone showed that it had no effect on hemolysis in the dilutions used. In the first experiments, the tocopherol was added to the suspension of the red cells of a tocopherol-deficient animal just before the dialuric acid. There was very definite protection. With a dialuric-acid concentration of 0.7 millimols per liter, tocopherol in a concentration of 0.009 millimols per liter or a ratio of about 1 mol to 80 mols of dialuric acid prevented hemolysis completely. To determine whether the added tocopherol was acting in the solution or in the cell, red blood cells were incubated with tocopherol for half an hour at 37°C., centrifuged, the supernatant fluid removed, and the cells resuspended in saline and treated with dialuric acid. When this procedure was followed the activity of the tocopherol was increased tenfold.

In these experiments, the requirement of tocopherol seemed to be more closely related to the concentration of cells than to the concentration of dialuric acid. The 0.009 mM. per liter of tocopherol protected equally against the hemolyzing effect of 0.7 and 1.4 mM. per liter of dialuric acid, but, with either concentration of dialuric acid, gave no protection when a 10 per cent suspension of red blood cells was used in place of the usual 5 per cent suspension.

Since the red blood cells were able to adsorb tocopherol from the surrounding medium, the cells of tocopherol-deficient rats were incubated with the plasma of tocopherol-treated animals, which, by calculation, contained enough tocopherol to afford complete protection. Not only was the plasma itself quite ineffective, it inhibited the effect of added tocopherol. It may be assumed that the added tocopherol was bound by the proteins of the plasma.

One cannot explain the effect of tocopherol as a reaction between it and dialuric acid. It is a mild reducing agent, but rather strenuous methods are necessary for reduction of dilauric acid. Tocopherol is most frequently considered as an antioxidant, and dialuric acid is readily autoxidizable. It is likely that it is some molecule or radical formed during the oxidation of dialuric acid that is the actual hemolyzing agent and which reacts with tocopherol.

It has been shown that alloxan is reduced by sulfhydryl compounds in the body, so alloxan and cysteine were tested together *in vitro*. The combination was found to behave like dialuric acid. Using 0.2 mg. of alloxan per ml. and varying amounts of cysteine, hemolysis was found to occur with as little as 0.01 mg. of cysteine per ml. This makes the system even more active than dialuric acid and supports the idea that a reactive intermediate is responsible for the hemolysis.

It was interesting to find that the three compounds used to reduce alloxan would themselves cause hemolysis. This reaction was slower than that with dialuric acid, never appearing in less than 2 hours, and occurred only with considerably larger amounts of reagent. The reaction resembled that of dialuric acid, however, in occurring only with cells deficient in tocopherol. These compounds are all autoxidizable. Ascorbic acid and dehydroascorbic acid are indeed analogous in structure to dialuric acid and alloxan. Cysteine

was the most active of the three compounds. On a molar basis, it was two to three times as effective as ascorbic acid and five times as effective as glutathione. Typical results obtained with cysteine are shown in TABLE 4.

TABLE 4
HEMOLYSIS *In Vitro* WITH CYSTEINE

Cysteine in mol./liter	*Degree of Hemolysis*		
	3	*7*	*18 hours*
5.2	—	—	++++
2.6	±	+	+++
1.3	+	++	Complete
0.65	—	—	++

There was most rapid hemolysis with 1.3 millimols per liter of cysteine, the rate decreasing with both higher and lower concentrations. The decrease in hemolysis at higher concentrations is typical of hemolyzing agents and is a result of a protective layer formed around the damaged cell. This was observed also with dialuric acid. The rate of hemolysis began to drop off above 0.2 mg. per cc., and with 0.75 mg. per cc. there was no hemolysis. That hemolysis was always delayed with cysteine, glutathione, and ascorbic acid may be due in part to overlapping of the hemolytic and protective levels.

The *in vitro* procedure has proved to be a simple and convenient method of studying some aspects of tocopherol metabolism.

The animals used in these studies were obtained from Sprague-Dawley and kept in our laboratory usually from 1 to 3 weeks before being put on experiment. The injection experiments, as well as the tests on washed red blood cells, had shown that when these animals had received a tocopherol-deficient diet for only one month all of their red blood cells could be hemolyzed. The blood of normal animals was not hemolyzed by dialuric acid *in vitro*. If the rats were fasted 48 hours, however, a procedure frequently followed when alloxan is used to produce diabetes, there was a slight degree of hemolysis, although probably not enough to have caused serious complication if alloxan was injected. When rats were tested at various intervals after being transferred to the tocopherol-deficient diet, in only 3 to 7 days the red cells showed sensitivity to dialuric acid which might be estimated as ++ on the usual scale of + to ++++, and after about 2 weeks showed the maximum rate of hemolysis. In contrast to these animals, a group may be mentioned which had received a supplement of 3 mg. of mixed tocopherols daily for a month and a half. After 3 months on the deficient ration without supplement, the blood of these animals was still almost completely resistant to hemolysis.

A confirmation of the fact that our "normal" animals bore a much closer resemblance to the deficient ones than to those receiving a generous allowance of tocopherol was found in the levels of plasma tocopherol. Treated animals had an average value of 1.0 mg. per cent, the stock rats about $\frac{1}{3}$

of this amount, 0.38 mg. per cent and the rats on the deficient diet, 0.27 mg. per cent.

Tests have been made to determine the amount of tocopherol necessary to protect the rats against hemolysis. Animals weighing 100 to 120 grams were placed on the high lard ration and given daily supplements of 0.1, 0.2, and 0.4 mg. of mixed natural tocopherols. After one week, only those receiving the highest dose were completely protected. Only a few animals have been tested at intermediate levels and no more precise definition of the protective dose can yet be given.

The rapid adsorption of tocopherol observed *in vitro* has been borne out by tests *in vivo*. A single dose of 1.5 mg. of tocopherol given orally to deficient rats gave complete protection in 24 hours. The protective effect did not disappear completely for about 10 days. Feeding of $\frac{1}{2}$ mg. of tocopherol for 3 consecutive days did not give as good protection as the single large dose. The tests with plasma *in vitro* have indicated that the process of tocopherol utilization in the living animal is not so simple as the tests in the synthetic medium might have suggested.

The low level of tocopherol in the newborn has been the subject of a number of recent investigations.[5] We have studied the blood of young rats and found that the cells of rats 1 or 2 hours old showed almost the maximum degree of hemolysis by dialuric acid. Litter-mates whose blood was tested the next day were already completely protected.

It is hoped that more extensive studies of this type may be made. The method gives an opportunity of studying a physiological activity of tocopherol *in vitro*. A comparison of different tocopherols would be of interest in this connection. Symptoms of deficiency, according to this classification, appear when the rat has been on a tocopherol-deficient diet for only a few days or weeks, and rigid exclusion of tocopherol from the diet for such a long period of time as is necessary to produce most of the physiological manifestations of deficiency in the rat is not required. The simplicity of the procedure would make it most useful as a method of bioassay for vitamin E. The measurements of protective and curative doses which have been discussed illustrate how such an assay might be carried out.

The fact that tocopherol is present in the erythrocyte to protect it against the effect of alloxan furnishes a teleological basis for an argument that it is there for that purpose. That hemolysis can be caused by cysteine, glutathione, and ascorbic acid, which are normal metabolites, makes this more probable. On the other hand, there is apparently no great need for the system under ordinary circumstances. Destruction of red blood cells has not been a characteristic feature of vitamin E deficiency. Our rats showed no signs of anemia before they were injected with alloxan. This defense may be of importance only under abnormal conditions of metabolism. The human adult is not likely to suffer from this deficiency except in such special cases as conditions involving failure of fat absorption. The experiment with the young rats suggests that the human fetus may be in a peculiarly defenseless position against such hemolyzing agents as have been discussed.

In summary, hemolysis caused by alloxan and related compounds seems

to be linked with the reversible oxidation-reduction relation between alloxan and dialuric acid, some intermediate of the reaction probably being the actual hemolyzing agent. Cysteine, glutathione, and ascorbic acid resemble dialuric acid in being autoxidizable and probably act in the same fashion in causing hemolysis. The protective action of tocopherol is within the red blood cell and may best be explained as an antioxidant effect.

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Discussion of the Paper

DR. P. GYÖRGY (*Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pa.*): The hemolysis demonstrable in rats with an average body weight of 100–150 Gm., only three to seven days after the animals were put on a tocopherol-free diet, appears to indicate an insufficient, or at least sub-optimal, intake of vitamin E by these animals while fed a regular stock diet. The hemolysis test may be applied for bioassay of vitamin E, requiring a much shorter preparatory period and a simpler technique than the usual resorption test.

Possible practical implications may be based on the following observations and its extrapolation to conditions in man: The newborn litter of rats kept on a normal stock diet (mixed grains and greens) shows positive hemolysis test, whereas, simultaneously, the red blood cells of the mother animal exhibit no hemolysis under the influence of dialuric acid. Thus, it may be assumed that the mother animal is sufficiently supplied with vitamin E and the newborn litter is deficient in vitamin E. Six to twelve hours after birth, the hemolysis test becomes negative in the newborn animals, probably under the influence of the colostrum, which is rich in vitamin E. These findings seem to support the view that either the transfer of vitamin E through the placenta to the fetus is limited or the metabolism of vitamin E in the fetus requires a higher supply than furnished by the regular stock diet of the mother animal.

Assuming that similar conclusions may be applied to man, the observation of extra-medullary hematopoiesis and erythroblastosis as—after Miller—the most specific finding in the newborn infants of diabetic and pre-diabetic mothers is of special interest. Such a finding should be the result of increased destruction of red blood cells, which in turn indicates exposure to some hematotoxic agent. The question arises whether such an agent could be similar in its action to alloxan, producing slow and continuous hemolysis in the vitamin E-deficient fetus and leaving intact the red blood cells of the mother, with her sufficient stock of vitamin E. The origin of this hemolyzing, perhaps alloxan-like, agent is probably the maternal metabolism. The agent may freely invade the circulation of the fetus, where its hemolyzing effect will become evident owing to the absence of

sufficient amount of protective vitamin E. The absence of anemia and the lack of its progression after birth may be explainable on the basis of a relatively low concentration of the toxic substance in the blood before birth, and its complete disappearance—by cutting off its source—after birth.

This theoretical consideration is open to exact critical study and is presented here only as a remote, but not entirely improbable possibility.

In Rh-incompatibility, several research workers, among them Sir Leonard Parsons (Birmingham, England), N. Philpott (Montreal), and the speaker have discussed the rôle of liver injury as a central pathogenetic factor in the outcome of the disease, including especially the hemorrhagic manifestations and also kernicterus. Hepatic injury follows some as yet unidentified phases of the antigen-antibody reaction, which in itself occurs obviously as the first chain in the events linked with the syndrome of Rh-incompatibility. The hepatic injury is characterized chiefly by acute zonal or massive necrosis. Protection of the liver may be attempted by methionine. Philpott has already presented preliminary evidence in favor of this view. We may now add the further possibility that during the sensitization-process, which is at the very bottom of Rh-incompatability, the normally low vitamin E stores of the fetus and the newborn are further depleted. Hepatic necrosis is one of the characteristic sequelae of vitamin E deficiency under particular conditions (see also the papers of Schwarz and Hove in this monograph and the relevant studies of the speaker). Edema and pulmonary hemorrhage are often encountered in very severe human erythroblastosis (*hydrops fetalis*) and seen also in experimental vitamin E deficiency in animals. The postulated but unproven vitamin E deficiency in human erythroblastosis is independent from the immunological antibody-antigen reaction of Rh-incompatibility. Studies are in progress jointly with Dr. Carl Bachman, Professor of Obstetrics at the School of Medicine, University of Pennsylvania, on the combined use of methionine and vitamin E in the prevention of the unspecific sequelae of Rh-incompatibility, such as hepatic necrosis, kernicterus, pulmonary hemorrhage, and edema. It is *not* expected that the anemia, based on the immunological antigen-antibody reaction, will be influenced by the administration of methionine and vitamin E.