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THE RELATIONSHIP OF BIOCHEMICAL AND ENZYMIC ABNORMALITIES OF CELLS TO RADIATION SUSCEPTIBILITY AND TO THEIR INJURY BY OTHER AGENTS.

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ABSTRACT

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3. Principal Investigator: Alf S. Alving, M. D., The University of Chicago.

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Investigations have been initiated to explore the relative susceptibility of mammalian cells to X-radiation and to injury by other agents. The cells chosen for initial studies have been primaquine-sensitive human erythrocytes and sheep erythrocytes. The latter have been found to be biochemically similar to primaquine-sensitive human erythrocytes. They have an unstable glutathione, deficient activity of the enzymes of the hexosemonophosphate shunt and low catalase activity. Sheep erythrocyte glutathione inactivates not only the glucose-6-phosphate dehydrogenase but also the 6-phosphogluconic dehydrogenase. Nevertheless, intravenous and parenteral administration of primaquine to sheep did not induce acute intravascular hemolysis.

Under certain conditions, radiation of both primaquine-sensitive human erythrocytes and sheep erythrocytes caused a significant fall in the activities of glutathione reductase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. Normal human erythrocytes showed no significant loss in the activity of these enzymes. Radiation of blood from a sheep receiving 240 mg. of primaquine intramuscularly each day for 11 previous days resulted in a marked increase in activity of the three above named enzymes, the greatest increase being in the glucose-6-phosphate dehydrogenase. There was a small but consistent decrease in the glutathione content of radiated erythrocytes averaging 2.7 mg. per hundred ml. of red cells. Investigations of the mechanism leading to these changes is being continued.

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INTRODUCTION:

The present investigations were initiated and conducted by Dr. Paul Carson to explore the relative susceptibility of abnormal cells to X-radiation and to injury by other agents. The abnormal cells chosed for the initial studies have been human primaquine-sensitive erythrocytes. These have been shown to have a low reduced glutathione content[1], deficient glucose-6-phosphate dehydrogenase[2], increased glutathione reductase[3], increased aldolase[4], and decreased catalase activities [5], and increased oxidized pyridine nucleotide coenzymes[6]. This constellation of abnormalities, especially the low reduced glutathione and decreased catalase, suggests that these cells might be more susceptible to X-radiation than normal cells. Furthermore, the glucose-6-phosphate dehydrogenase has been shown to be differentially susceptible to inactivation by erythrocyte ghosts[7].

These investigations have included a search for cells with

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similar abnormalities in animals, since an experimental animal would be invaluable in increasing both the scope and speed of the investigations. To date, sensitivity to primaquine type hemolysis has been found only in human beings. However, we have found that sheep erythrocytes possess a biochemical pattern of great similarity to primaquine-sensitive erythrocytes.

METHODS AND MATERIALS:

These investigations have been performed using methods and materials described in the list of references. Briefly, hemoglobins have been determined in the Beckman DU spectrophotometer using Drabkin's solution as described by Crosby[8]. Hematocrits have been determined using an International Microhematocrit centrifuge. Glutathione has been determined by the method of Grunert and Phillips[9], using the technique described by Flanagan, et al[10]. Blood has been prepared for enzymatic assay after anti-coagulation with either heparin or ACD solution. Blood specimens were

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refrigerated immediately after being drawn and hemolysates prepared by washing the erythrocytes three times in cold 0.145 molar NaCl, hemolyzed by freezing and thawing three times in a dry ice acetone bath, adjusted with cold water to hemoglobin levels usually of 4 Gms. percent, and centrifuged at approximately 29,000g for 60 minutes in a Lourdes refrigerated centrifuge to remove the "ghosts". The glutathione stability test was performed as described by Beutler[11].

EXPERIMENTAL:

1. Metabolism of sheep erythrocytes and effect of primaquine *in vivo*.

A. Table I shows representative hematocrit, hemoglobin, reduced glutathione and glutathione stability values obtained from four healthy ewes. The data show that the glutathione content of the red blood cells is similar to that found in normal human erythrocytes in three of the sheep and higher in one of the sheep.

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However, the glutathione stability tests are markedly positive.

B. Figure 1 shows the activities of glutathione reductase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase when assayed under the same conditions as used for human erythrocytes. Comparative values for the same enzymic activities are shown in the figure by dashed lines for hemolysate prepared from primaquine-sensitive blood. The assays were performed by a modification of the coupled reactions described by Carson, et al[2]. The figure shows that not only the glucose-6-phosphate dehydrogenase but also the 6-phosphogluconic dehydrogenase, the TPN dependent enzymes of the hexosemonophosphate shunt, are even less active than the deficient glucose-6-phosphate dehydrogenase from the primaquine-sensitive erythrocytes. The activity of glutathione reductase is also considerably less than that found in the human erythrocyte, but is more than adequate for coupling reactions with TPN dependent enzymes. Also shown on the figure are average oxidized coenzyme values for normal

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human erythrocytes, primaquine-sensitive erythrocytes, and the erythrocytes of the sheep whose enzyme activities are shown on this figure. The TPN level is more like that of the normal human cell; the DPN level is more than twice the value of the human erythrocytes.

Figure 2 shows representative values for the same enzymes using a constant amount of hemolysate, but determining the activities as a function of time. To obtain these data, it was necessary to use 2.5 ml. of hemolysate in each reaction, which is five times the amount needed (0.5 ml.) to show the same degree of activity when human hemolysates are used. Once again, the relative deficiency of both enzymes of the hexosemonophosphate shunt is shown. There is good activity of glutathione reductase. Hemolysates of sheep erythrocytes from which the ghosts have not been removed exhibit a marked inactivation of both the glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase. In human hemolysates, this effect is principally on the glucose-6-phosphate dehydrogenase and not on the 6-phosphogluconic dehydrogenase. There was little

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effect of stromain on the glutathione reductase activity.

The activity of catalase of sheep cells as determined by the method of Beutler[12] is approximately 1/30 that of human red cell catalase.

The sheep chosen to receive primaquine weighed 140 lbs. Therefore, dosages comparable to those used in human beings were given. Figure 3 shows the effect of the oral ingestion of 60 mgm. of primaquine per day, twice the standard test dose used in human volunteers. A 14-day course of primaquine resulted in no significant fall in the hematocrit, hemoglobin or Chromium 51 survival curve. The half life of this "apparent survival curve" is approximately 12.6 days as compared to an average half-life for the human survival curve of 27 to 33 days. The glutathione level of the erythrocytes did not fall, as it does in primaquine-sensitive human beings. There was, in fact, a rise in the glutathione during the course of primaquine.

[illegible] absorption of the primaquine, another course was

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given of 240 mg. intramuscularly every day for 14 days. It was possible that the ruminant sheep had either failed to absorb the oral primaquine or the primaquine might have been metabolized by the sheep intestinal flora which include protozoa normally. Again, there was no hemolysis, as shown by lack of significant fall of the hematocrit, hemoglobin and Cr^{51} survival curve values. As yet to be explained is the marked difference in half life of the Cr^{51} survival curve which, in this experiment was 17 days. There was an initial sharp rise in the glutathione, which was not maintained. The occasional sudden fall of hematocrit shown in the figure was paralled by the hemoglobin level; it was felt to be real and not due to any variation in technique.

II. The effect of X-radiation on human and sheep erythrocytes.

In each case reported, the specimens to be radiated were sealed in a Hamilton tube, immersed in an ice bath in a round lucite holder which was placed on a rotating turn table and exposed to a total dosage of 20,000r over an average time of 40 to 45 minutes. Radiation

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was delivered from a GE Maxitron 250 with a 250 kilovolt port at 30 milliamperes with a 0.5 millimeter aluminum filter at a target distance of 43.7 centimeters. In each case control specimens sealed in Hamilton tubes were kept refrigerated without receiving radiation.

Reduced glutathione was measured before and after radiation. A small but consistent decrease was found ranging from 0.5 mg. to 4.8 mg., averaging 2.7 mg.

After radiation, both control and radiated specimens were processed for the analyses shown. Figure 5 shows the results obtained on one sample of primaquine-sensitive blood. In this instance there was a decrease in the 6-phosphogluconic dehydrogenase but no significant decrease in the glucose-6-phosphate dehydrogenase activity except possibly at the 2.5 ml. of hemolysate level. Values for radiated hemolysate not shown in the figure are less than the values for the control specimens but greater than the values obtained on the radiated whole blood.

Figure 6 shows the results obtained on normal human ery-

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threocytes made up to a 50% hematocrit in 0.145M NaCl. There was no significant difference in the glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase activities before and after radiation. There was an apparent loss of glutathione reductase activity in those reactions containing one or more ml. of hemolysate.

Figure 7 shows the results obtained on primaquine-sensitive human erythrocytes also made up to 50% hematocrit in saline. Here there was a definite loss of activity of all three of the enzymic activities shown, the loss being greatest for glucose-6-phosphate dehydrogenase.

Figure 8 shows the results obtained when sheep blood was exposed to 20,000r. There was a marked fall in glucose-6-phosphate dehydrogenase activity in both whole blood and hemolysate compared to the control blood. The fall was greatest in the whole blood, with the irradiated hemolysate giving intermediate values. Similar results were obtained for the 6-phosphogluconic dehydrogenase activities.

Figure 9 shows the results obtained on whole sheep blood

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before and after radiation, when the assays were performed as a function of time. Here, in contrast to the assay using increasing amounts of hemolysate, there was no loss of activity in any of the three enzymic activities.

Figure 10 shows the results of radiation on specimens exposed and assayed in exactly the same way as in Figure 9, except that the blood was obtained from a sheep which had been receiving 240 mg. of primaquine intramuscularly per day for 11 days. There was marked increase in activity of the three enzymes, the increase being greatest for the glucose-6-phosphate dehydrogenase activity.

DISCUSSION:

The data presented in this progress report are to be considered preliminary. Nevertheless, the results obtained thus far are significant.

Sheep erythrocytes differ from human erythrocytes in being

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much smaller and spherocytic, with an average red blood cell count between 7,000,000 and 8,000,000 per cubic millimeter. In terms of the abnormalities known to exist in human primaquine-sensitive erythrocytes, however, the sheep erythrocytes are remarkably similar. We have found that the sheep red cell glutathione is unstable, that the hexosemonophosphate pathway of carbohydrate metabolism is even less than that of primaquine-sensitive erythrocytes. The sheep "ghosts" inactivate not only the glucose-6-phosphate dehydrogenase but also the 6-phosphogluconic dehydrogenase. They affect the glutathione reductase activity only slightly. The catalase activity of the sheep is far less than that of the human primaquine-sensitive erythrocyte which, in turn, is less than that of the normal human erythrocyte. The oxidized coenzymes have been determined in only one instance, so that we do not know the range of values for sheep by this method. It is apparent that probably the DPN content of sheep erythrocytes is significantly higher than that found in human erythrocytes, either normal or primaquine-sensitive. The glutathione reductase activity is considerably

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less than that of human erythrocytes, either normal or primaquine-sensitive, but nevertheless is more than adequate for coupling with TPN dependent enzymes. Sheep glutathione reductase can utilize DPNH to a very much smaller extent than can human erythrocytes.

In spite of these findings, the sheep given large doses of primaquine, both orally and parenterally, fail to undergo acute intravascular hemolysis. This failure of primaquine to induce hemolysis in the sheep whose erythrocytes are positive by all known tests for primaquine sensitivity is of the utmost significance, since we do not yet know the exact cause of hemolysis of the primaquine-sensitive erythrocyte..

One possibility is that the metabolic product of primaquine, which causes hemolysis in the primaquine-sensitive individual, may not be formed by the sheep. Therefore, current investigations include a comparative study of the products found in human and sheep plasma during the administration of primaquine.

If the metabolic product of primaquine which causes hemo-

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lysis in human beings is formed in the sheep, and presented to the sheep erythrocytes in the circulation, investigation of the mechanism of protection of the sheep from primaquine-induced hemolysis will be necessary. This also would be presumptive proof that the cause of hemolysis in the primaquine-sensitive individuals lies in still another abnormality yet to be discovered.

If, on the other hand, the primaquine is metabolized differently by the sheep, presumably in the liver, the comparative mechanism of primaquine metabolism in sheep and human beings would require investigation. Such investigation might lead to means of modifying the metabolism of primquine by use of an additional agent to prevent the hemolytic activity.

Whether the metabolic product of primaquine which causes hemolysis is the same product which exerts curative effect in malaria is not known. Many other agents have been found which cause intravascular hemolysis in individuals whose erythrocytes show the abnormalities originally discovered at the Army Medical Research Project at Stateville. Some of these agents are not antimalarial. Therefore, the possibility exists that one of the

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metabolic products of primaquine may be antimalarial whereas another may produce the hemolytic reaction.

The effect of radiation on normal, primaquine-sensitive and sheep erythrocytes has revealed that, under certain conditions, there is an apparent loss of activity of the enzymes thus far studied. This is associated with a small but consistent decrease in the glutathione level. Normal human erythrocytes have shown no loss in enzymic activity on exposure to 20,000r. Primaquine-sensitive erythrocytes have shown losses of activity, as have the sheep erythrocytes.

The conditions resulting in loss of activity on exposure to radiation require further investigation. The conditions fall into three classes:

1. the amount and method of delivery of radiation;
2. the preparation of sample for radiation and,
3. the conditions of assay of the enzymes following radiation.

With regard to preparation of sample, it is worthy of note that radiated

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whole blood showed greater losses of activity than did hemolysates. As might be expected, erythrocytes in a saline medium were also more susceptible to radiation than those protected by plasma substances such as glucose. Since whole red blood cells have been shown to exert an effect exactly like that of the ghosts on inactivating the glucose-6-phosphate dehydrogenase of human hemolysates, and since the hemolysates thus far irradiated contained no ghosts, investigation of the effect of ghosts on susceptibility of the enzymes to radiation damage will be necessary.

With regard to conditions of enzyme assay following radiation, losses of activity were shown principally in those systems where the assays were performed by use of increasing amounts of hemolysate. Little if any change could be shown using a constant amount of hemolysate and assaying the activity of the enzyme as a function of time. Thus, the radiation effect may have altered the protein structure so that inhibition of the enzyme activities occurs at higher levels of enzyme concentration. Or, more glutathione could be lost in the protein precipitate made before

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measurement of the reduced glutathione.

The most striking radiation effect was the marked increase in activity, particularly of glucose-6-phosphate dehydrogenase, which occurred in the blood obtained from a sheep which had been receiving large doses of primaquine. This increase is probably a function of the conditions of enzyme assay, or may be associated with peculiarity of the particular sheep used, since it has a higher glutathione level than the three other sheep examined. Thus, we cannot as yet interpret this striking finding without further investigation of the effect of radiation on blood obtained from both human individuals and sheep receiving primaquine.

Current investigations are being expanded to include additional parameters of cellular metabolism.

SUMMARY:

1. Sheep erythrocytes are biochemically similar to primaquine-sensitive human erythrocytes.

2. Administration of large doses of primaquine to sheep.

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both orally and parenterally, failed to induce hemolysis.

3. Under certain conditions, radiation of both primaquine-sensitive and sheep erythrocytes at a dosage level of 20,000r caused loss of enzymic activities. Normal human erythrocytes showed no significant loss of enzymic activities.

4. Blood from a sheep receiving parenteral primaquine showed a significant increase in enzymic activities after radiation.

5. The significance of these findings is discussed.

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TABLE 1.

Sheep	Hematocrit	Hemoglobin gm./ 100 ml.RBC	GSH mg.100/ml. RBC	GSH stability mg./100 ml.RBC
1.	48.50	----	68.9	1.2
2.	49.50	14.5	66.0	26.1
3.	32.50	10.2	60.7	----
4.	39.50	12.2	82.1	22.0
4.	36.00	11.3	86.9	29.5
4.	34.00	10.9	85.6	32.4
4.	28.00	9.6	91.4	10.7
4.	38.5	12.9	84.9	23.6
4.	35.5	11.9	91.0	3.4

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