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COMBINED EFFECTS OF TOTAL BODY X IRRADIATION AND RADIANT ENERGY THERMAL BURNS. III. OSMOTIC AND MECHANICAL FRAGILITY OF THE ERYTHROCYTE

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Encl: (1) U. S. Naval Radiological Defense Laboratory UNCLASSIFIED Report USNRDL 419 entitled "Combined Effects of Total Body X Irradiation and Radiant Energy Thermal Burns. III. Osmotic and Mechanical Fragility of the Erythrocyte"

1. This report provides additional data on osmotic fragility and data on mechanical fragility of red blood cells of rats during a 30-day interval following exposure to thermal and ionizing radiation injury.

2. It appears that either thermal or radiation injury, by the removal of fragile cells and by promoting a more rapid red blood cell turnover, is associated with an increased resistance to osmotic hemolysis. It is important to note that the increased resistance to osmotic hemolysis associated with a dose level of 500 r, does not develop at a level of 100 r. In the combined traumata the results are considered to be additive.

3. Mechanical fragility is increased by lethal but not recovery doses of ionizing radiation, and by lethal combinations of radiation and burns when neither trauma is lethal alone.

4. The significance of these findings is that a quantitative appraisal of degree of ionizing radiation injury is possible. Increased mechanical fragility may be a prognostic sign of lethality in irradiated animals either in the presence or absence of other complicating traumata such as burns.

5. This report is forwarded for information.

A. R. BEHNKE
By direction

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23 November 1953

ABSTRACT

The osmotic and mechanical fragility of rat erythrocytes have been measured during the 30 day interval following severe and moderately severe burns, 100 and 500 r of whole body ionizing radiation, and combinations of the two traumata.

It has been found that, although it is difficult to demonstrate an increased fragility as early as 4 hr after severe burns, there is most probably an increased resistance to osmotic hemolysis 24 hr later as the result of removal of the most fragile cells. This is a transient finding and normal fragilities are found on the third day. During the recovery period an increased resistance to osmotic hemolysis develops which is discussed as a possible result of increased red cell turnover.

Irradiation produces an increased osmotic resistance at the 500 r dose level but not at 100 r. This change is correlated with removal of fragile cells and increased red cell turnover.

Combination of the traumata brings about essentially additive results insofar as osmotic fragility is concerned.

The mechanical fragility was found to be increased by lethal doses of ionizing radiation and by lethal combinations of ionizing radiation and burns when neither trauma was lethal alone. It is thought that the increased mechanical fragility is a sign of impending death in radiated animals either in the presence or absence of other complicating traumata.

INTRODUCTION

A recent report from this Laboratory[1] has described the effect of various doses of ionizing radiation on both the immediate and long term mortality resulting from thermal burns. For the purpose of elucidating the mechanisms causing increased mortality from the combined traumata, the changes in erythrocyte fragility following thermal burns, ionizing radiation, or a combination of both were investigated.

Although increased osmotic fragility of the red cell with an associated hemoglobinuria is a frequently discussed early complication of thermal burns, little laboratory work and only limited clinical studies have been devoted to a careful investigation of these changes. One of the most important contributions in this field is the work of Shen and his colleagues at Harvard Medical School[2]. They have also investigated the resistance of the erythrocyte to a standardized mechanical trauma and have attempted to correlate changes in this parameter with osmotic fragility changes.

It has been shown by Goldschmidt, et al.[3], that even though a defect in the erythrocyte cannot be demonstrated by usual means, the resistance of the erythrocyte to heat is markedly lowered by 500 or 600 r of whole body ionizing radiation. This occult defect could be of great significance in the initial and continuing hemolysis associated with thermal trauma when ionizing radiation is superimposed. The aims of the work described herein have been twofold — to define the changes in osmotic and mechanical fragility associated with thermal burns, and to evaluate the effect of ionizing radiation given concomitantly.

EXPERIMENTAL DESIGN AND METHODS

Animals

Female rats of the Sprague-Dawley strain, weighing initially between 140 and 180 g and approximately six weeks old, from the Laboratory

colony were used in all experiments. They were housed in individual cages after treatment and fed Purina "Checkers" and water *ad lib*.

Treatment

All animals were clipped and depilated using a commercial strontium sulfide depilatory according to the method of Kuhl, et al.[4] Immediately after depilation the animals were assigned to experimental groups by means of a table of random numbers. Approximately 72 hr following depilation the rats were anesthetized with pentobarbital sodium, 4.5 mg/100 g body weight subcutaneously, and burned, irradiated, or a combination of both according to the following protocol.

The experimental groups were assigned the following designations: Group I — control (C); Group II — 500 r total body X irradiation (X_5); Group III — 100 r total body X irradiation (X_1); Group IV — 4 radiant energy strip burns (25 per cent body area) plus 100 r (B_4X_1); Group V — 2 strip burns (15 per cent body area) plus 500 r (B_2X_5); Group VI — 2 strip burns (15 per cent body area) (B_2); Group VII — 4 strip burns (25 per cent body area) (B_4).

A second experiment described in the text designed to test the effect of a uniformly fatal treatment was assigned the following group designations: Group I — control (C'); Group II — 475 r total body irradiation (X_{475}); Group III — 4 radiant energy strip burns (25 per cent body area (B_4'); Group IV — 4 strip burns (25 per cent body area) plus 475 r total body irradiation (B_4X_{475}).

Irradiation was accomplished with a 250 KVP Westinghouse therapy unit operated at 15 ma. The irradiation factors were as follows: target to skin distance, 40 in.; 0.5 mm Cu, 1 mm Al filter, half value layer 1.5 mm Cu. The dose rate was approximately 23 r/min. Irradiation preceded burning by 20 to 60 min.

Those animals which were burned were treated by positioning the anesthetized rats on a rotary turntable behind an asbestos insulated shield with rectangular openings 2.5 x 12 cm. The rats were held tightly in the proper position by means of an elastic cloth sling. Radiant energy was provided by a Mitchell-Nelson carbon arc projector operated at 225 amps and 70 V D.C. The source provided a concentrated source of radiant energy 3.5 cm in diameter at the focus. The turntable was rotated past the focused beam at a speed of one revolution every 42 seconds. Such treatment yields an exposure time of approximately 0.4 sec with a total energy delivered of 9.0 cal/sq cm. The radiation was measured at each rotation of the table by a black body radiometer. No corrections for non-absorbed radiation have been made. These conditions correspond to the thermal radiation received at approximately 6000 ft from the

nominal atomic bomb as described by Brooks[5]. The visible strip burn from a single exposure was slightly overlapped for each succeeding exposure to produce a contiguous burn. The two-strip burns gave mean burn areas which were approximately 15 per cent of the total body area, while the four-strip burns gave mean burn areas of approximately 25 per cent of the total body area as measured by the method described by Davis, et al.[6] This latter burn produces about 15 per cent mortality in unirradiated rats while the former is not lethal under the same conditions.

Histological examination of the lesions revealed that these burns are essentially equivalent to the characteristic "white burn" described by Sheline, et al.[7] The burns were applied to the back and sides of the animals and did not interfere with ambulation or excretory functions.

Blood sampling was accomplished by withdrawal of approximately 5 ml of blood from the exposed right ventricle into a clean, siliconized 5 ml syringe. The blood was carefully expelled into a siliconized 10 ml Erlenmeyer flask containing a small quantity of heparin. The method used for determining the osmotic fragility of the erythrocyte was that of Shen, Ham, and Fleming[2] using the NaCl-phosphate buffer recommended by Ponder[8].

Hematocrits were done by the micro method in use in this Laboratory and described elsewhere[9].

Mechanical fragilities were done by the method of Shen, Castle, and Fleming[10] which utilizes rolling contact of the blood with small glass beads as a measure of resistance to mechanical destruction.

Reticulocyte counts were done in a wet preparation after staining the cells with a solution of brilliant cresyl blue in 0.9 per cent saline. One thousand cells were counted and the ratio of reticulocytes to mature cells was converted into a value for number of circulating reticulocytes per cu mm of blood.

RESULTS

Osmotic Fragility

For purposes of calculation the osmotic fragility data were plotted in the usual fashion — the per cent hemolysis against the osmotic

tonicity. From the resulting sigmoid curve the tonicity values for 20 per cent hemolysis (H_{20}), 50 per cent hemolysis (H_{50}) and 80 per cent hemolysis (H_{80}) were read. In Figs. 1 through 7 are plotted the changes in osmotic and mechanical fragility as a function of time after exposure. The associated hematocrit and reticulocyte concentrations are provided for purposes of correlation. The changes in hematocrit and reticulocyte levels as a result of the treatments discussed have been reported elsewhere[6]. The asterisked points are those which have been established as significantly different from the control values at the 0.05 probability level by the method of Tukey[11] for comparison of individual means in the analysis of variance. The osmotic fragility data are plotted as the mean tonicity, expressed as gram per cent NaCl, required to produce 20 per cent hemolysis (H_{20}); 50 per cent hemolysis (H_{50}); and 80 per cent hemolysis (H_{80}). Of these three values only the H_{50} was subjected to statistical analysis by the method described. It can be seen that in the untreated animals there is a definite trend toward the establishment of a more osmotically fragile population of red cells as the animals age. Therefore, it is especially important to compare the treated groups only with the control groups sacrificed on the particular days in question.

The burned animals, either 25 per cent (B_4) or 15 per cent (B_2) body area, demonstrate a small fall in the H_{50} tonicity, i.e., increased resistance, on Day 1 followed by a return to normal values on Day 3. This early fall has a p value of only 0.20 and therefore is of a low order of significance. After the third day a steady decline in the H_{50} tonicity is seen. This increased resistance of the erythrocyte in the burned animal is significantly different from the control group at Day 8 for the B_4 group and at Day 11 for the B_2 group. In the B_4 group this trend continues downward throughout the period of study, while the B_2 animals stabilize at the new level after the second week. This increased resistance to osmotic destruction of the erythrocyte is not simply a shift of the normal curve of hemolysis versus tonicity with a preservation of its shape, but is a shift of the median hemolytic saline concentration (H_{50}) accompanied by an increased slope of the hemolytic response curve. This increased slope can be seen in the increased spread between the H_{20} and H_{80} values.

It is necessary to sample the burned animals earlier than Day 1 in order to detect any increased fragility. The B_4' animals were sampled at 4 hr and were found to have, in general, increased fragilities at this time. However, even at this early time it was not a uniform finding. All animals did have a marked hemoglobinuria and visible hemoglobinemia during the first 24 hr.

Irradiation at the 500 r level (X_5) produces an over-all pattern of increased resistance much like that found in the burned groups. However, the change is smaller and the spread of the osmotic hemolysis curve as measured by the range of tonicity from H_{20} to H_{80} has returned to

normal limits at Day 30 after showing a marked increase from Day 11 through 21. This return of the H_{80} to H_{20} tonicity range to normal on Day 30 is accompanied by a hematocrit and reticulocyte count within control limits. These latter findings are associated with the recovery of the animal from the hemorrhagic defect due to irradiation. One hundred roentgens (X_1) has no significant effect on osmotic fragility.

Combination of the two traumata, B_2X_5 and B_4X_1 groups, results in essentially the same picture as found with the burn alone. However, the increased resistance found in the B_2X_5 group develops a good deal more rapidly and the H_{50} tonicity is significantly below either the B_2 or B_4 group on Days 5 and 8. This trend is reversed by Day 21 and on Day 30 the H_{50} value for the B_2X_5 group is the same as for the B_2 group and X_5 group.

Mechanical Fragility

No significant changes in the mechanical fragility are found in the burn groups at any time. If any increased resistance to mechanical disruption of the erythrocyte is occurring it is difficult to demonstrate its significance statistically. It can be seen in Figs. 2 and 5, however, that the plotted values do show a very small downward trend with time.

Irradiation with 100 r whole body ionizing radiation produces no detectable change in the mechanical fragility, nor does 475 r, but at 500 r a small increase is evident, being significant at Days 8 and 11 as shown in Fig. 8. Burning, although of itself having no demonstrable effect, sharply enhances the increased mechanical fragility associated with radiation injury (Fig. 8).

To rule out the possibility that the mechanical fragility changes in the X_5, B_2X_5 and B_4X_{475} groups are due simply to the sharply reduced hematocrit, normal cells were diluted with plasma from the same animal to adjust the final hematocrit to the range of interest. The results of these experiments indicate a reduction in fragility from 10 per cent, with an hematocrit of 40, to 7 per cent, with an hematocrit of 15. This small drop in mechanical fragility with cell dilution is of no significance with respect to the magnitude of the changes and, indeed, if correction for this effect were made, there would be a slightly greater net increase in the fragility of the B_4X_{475}, X_5, and B_2X_5 groups. The results of Shen, et al.[10], on the effect of hematocrit changes in human blood are in accord with these observations.

DISCUSSION

Osmotic Fragility

An almost universal early finding in severely burned rats and a frequent observation in severely burned patients is a marked hemoglobinuria which usually disappears by the second or third post-burn day. To what extent this rapid early destruction of erythrocytes contributes to the early and long term erythropoietic load on the organism and to what extent later fragility changes can account for secondary thermal anemia is a question which has attracted a good deal of attention from clinicians and investigators in the field of thermal burns. Shen and his coworkers[2], after analyzing clinical data from a series of severely burned patients, observed that an increased osmotic fragility of the erythrocyte was associated with the period of hemoglobinuria, but that no significant anemia could be demonstrated in the same interval, i.e., 24 to 48 hr post burn. In the series of rats reported here it has not been possible to demonstrate any consistent and significant increase in the osmotic fragility of the erythrocyte at 1 day after burning in either burned or burned and irradiated animals; in fact, findings to the contrary are more common. However, it has been shown in this Laboratory[6] that a significant decrease in red cell mass is found within a short time after burning. The failure to find a consistent early increase in osmotic fragility is probably merely due to a rapid clearing from the circulation of heat damaged cells and, indeed, Shen has found most marked changes in osmotic fragility occurred in those patients whom they have been able to observe within a very few hours of the burn episode. The increased resistance to osmotic hemolysis which seems to be present in all burn groups, whether irradiated or not, on the first post-burn day is almost certainly due to the removal of a proportion of the more fragile or heat damaged cells following the burn episode apparently leaving a residue of more resistant cells.

Of greater significance is the later increased resistance found in the B_2, B_4, and X_5 groups and in the two combined injury groups, B_4X_1 and B_2X_5.[4] It has been reported by Davis, et al.[6], that a "false anemia" exists in burned rats, an anemia that is characterized by hyperplasia in erythropoietic tissues, reticulocytosis in the peripheral blood, normal red cell volume, and expanded plasma volume. The conclusion of these authors is that a hemolytic process exists which is accompanied by a compensating erythropoiesis, with no net change in circulating red cell volume even though the peripheral indices of red cell count and hemoglobin are lowered. The net result of these two competing activities is a normal steady state red cell volume, the cells of which have a mean age much lower than untreated animals. This young population manifests itself in increased resistance to osmotic hemolysis. Davis, et al.[12], have clearly

demonstrated a shift towards a more resistant red cell population in rats in which a young cell population has been produced by acute bleeding. The osmotic resistance of this young cell population was characterized by a shift of the 50 per cent hemolytic tonicity (H_{50}) to lower concentration and a spreading out of the curve. In the present data these conditions are found. There is a shift of the H_{50} in the direction of increasing hypotonicity and a spreading of the curve as represented by the increased interval between the H_{20} and H_{80} hemolysis concentrations. The hematocrit of the burned animals remains essentially unchanged throughout this period, but it can be seen in Figs. 2 and 5 that the increased resistance is closely correlated with the post-burn reticulocytosis. Careful consideration of the data of Shen[2] shows that this increased resistance is also seen in his patients during recovery from severe burns.

The possibility that the increased resistance can be attributed to the continuing preferential destruction of certain erythrocyte groups which are more fragile to osmotic hemolysis, leaving a residue of resistant cells, cannot be excluded, but it is felt that in burned animals this phenomenon must play a minor role. Other studies in this Laboratory with radioactive iron indicate that over and above the primary red cell destruction occurring during the hyperthermic episode erythrocytes are destroyed in an accelerated random fashion without regard for cell age.

The explanation for the increased resistance found for the X_5 group is not as clear-cut as in the case of the burns. The change is not as pronounced as for the B_4 group and is occurring in the face of a rapidly falling hematocrit and a low level of circulating reticulocytes. It is possible that the key to this problem really lies in a combination of two effects, namely, preferential removal of fragile cells accompanied by a falling hematocrit at the early stages and later, when the reticulocytosis manifests itself, the production of young cells which are osmotically more resistant.

In the combined injury there is essentially a simple additivity of the irradiation and burn effects. The 25 per cent burn, 100 r group (B_4X_1) shows simply the effect of the B_4 treatment while the 15 per cent burn, 500 r group (B_2X_5) shows essentially a simple additive effect of the B_2 and X_5 treatments. It is noteworthy that again in the B_2X_5 animals the resistance develops before the reticulocytosis is evident.

Mechanical Fragility

The slightly increased mechanical fragility found for the 500 r irradiated group (X_5) and for the B_2X_5 and B_4X_{475} groups is seen to immediately precede the period of maximum blood loss as characterized by the falling hematocrit. This increased fragility rapidly disappears and by the eleventh and fifteenth day when the hematocrit is at its lowest value, it

is again within the control range. The mechanism whereby burning succeeds in increasing the mechanical fragility of the erythrocyte in the irradiated animal is not easily explained. However, the temporal relationship of the increased fragility to the time of beginning deaths in each of the groups in which it is seen and in which most of the deaths will occur leads one to a feeling that the change is one that precedes death. In general, the later deaths from the combined lesion is essentially associated with radiation pathology. The late effects of burning seem to be merely potentiation of the radiation effectiveness and in this sense the potentiation of radiation mortality.

A difficulty inherent in studies of this kind is that it is impossible to predict which animal would die if not sacrificed experimentally, and the study, by its nature, is essentially one restricted to animals surviving to the day of sacrifice. Those animals given 475 r, just enough below the 500 r level to reduce the mortality to zero, show no increase in mechanical fragility. This finding again would seem to strengthen the position that the increase in mechanical fragility is a finding only in the dying animal.

This increase in fragility is associated with the beginnings of the hemorrhagic diathesis of radiation at which time large quantities of erythrocytes have been seen to appear in the thoracic duct lymph[12]. By the seventh day red blood cell levels in the thoracic duct lymph reach values around one million per cu mm and then slowly decline on succeeding days. The trauma to the red cell associated with this extravasation and the lymphatic collection of red cells with return to circulation may well be one of the primary causes of the defect observed. As the hemorrhagic tendency is probably a principal contributor to final death it may well be that the finding reported is an early measurable indication of an early occult extravasation of erythrocytes.

SUMMARY

The osmotic and mechanical fragility of rat erythrocytes have been measured during the 30 day interval following severe and moderately severe burns, 100 and 500 r of whole body ionizing radiation, and combinations of the two traumata.

It has been found that, although it is difficult to demonstrate an increased fragility as early as 4 hr after severe burns, there is most probably an increased resistance to osmotic hemolysis 24 hr later as the

result of removal of the most fragile cells. This is a transient finding and normal fragilities are found on the third day. During the recovery period an increased resistance to osmotic hemolysis develops which is discussed as a possible result of increased red cell turnover.

Irradiation produces an increased osmotic resistance at the 500 r dose level but not at 100 r. This change is correlated with removal of fragile cells and increased red cell turnover.

Combination of the traumata brings about essentially additive results insofar as osmotic fragility is concerned.

The mechanical fragility was found to be increased by lethal doses of ionizing radiation and by lethal combinations of ionizing radiation and burns when neither trauma was lethal alone. It is thought that the increased mechanical fragility may be a sign of impending death in radiated animals either in the presence or absence of other complicating traumata.

Approved by:

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Sciences Division

For the Scientific Director

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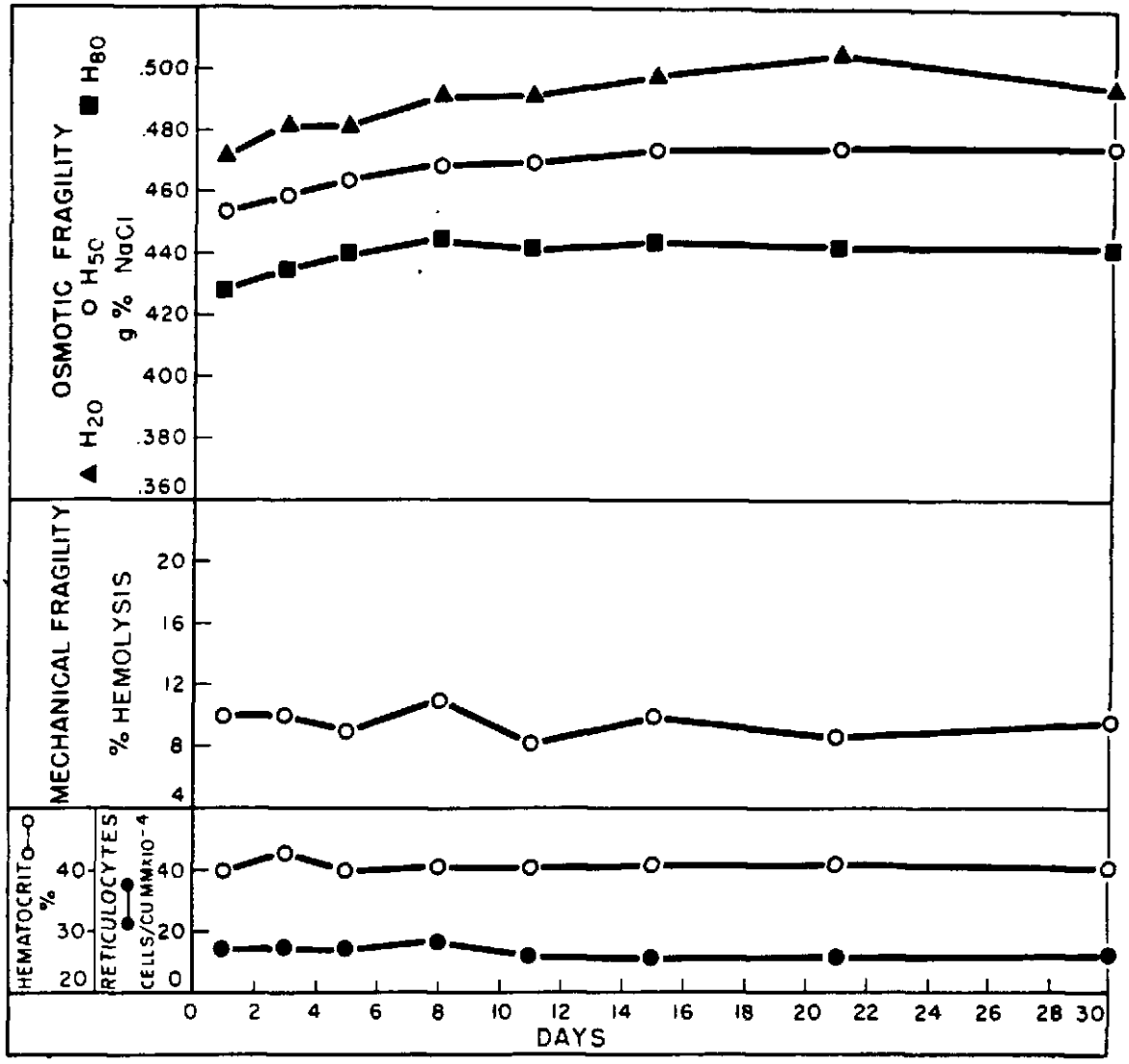

Fig. 1 Osmotic and Mechanical Fragilities, Hematocrit, and Reticulocyte Levels after Burning and/or Irradiation — Control (C).

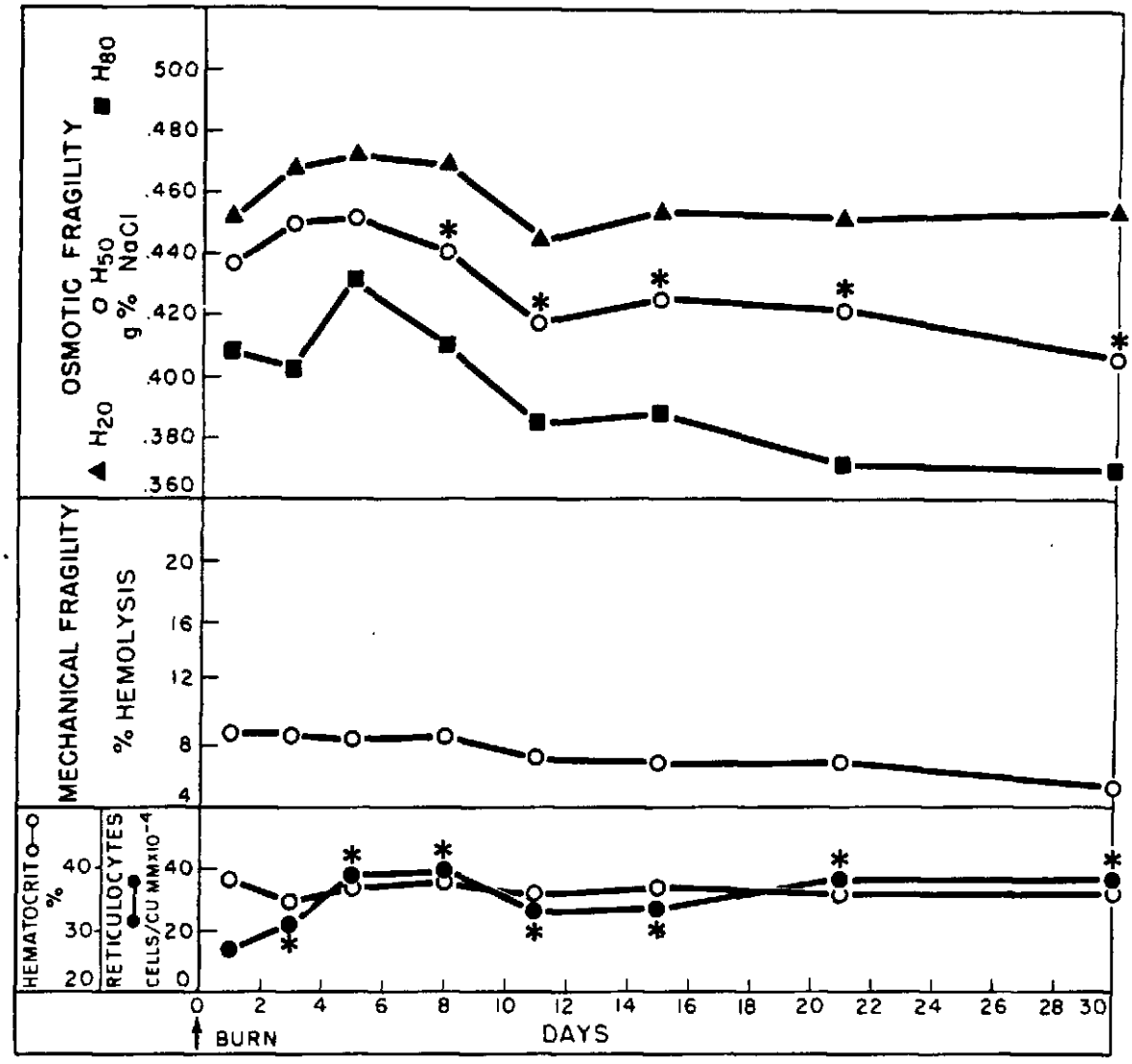

Fig. 2 Osmotic and Mechanical Fragilities, Hematocrit, and Reticulocyte Levels after Burning and/or Irradiation — 25 Per Cent Burn (B_4). Those values which are significantly different from the control on the day of sacrifice are indicated by the asterisks. The H_{50} values, only, of the osmotic fragilities have been statistically analyzed.

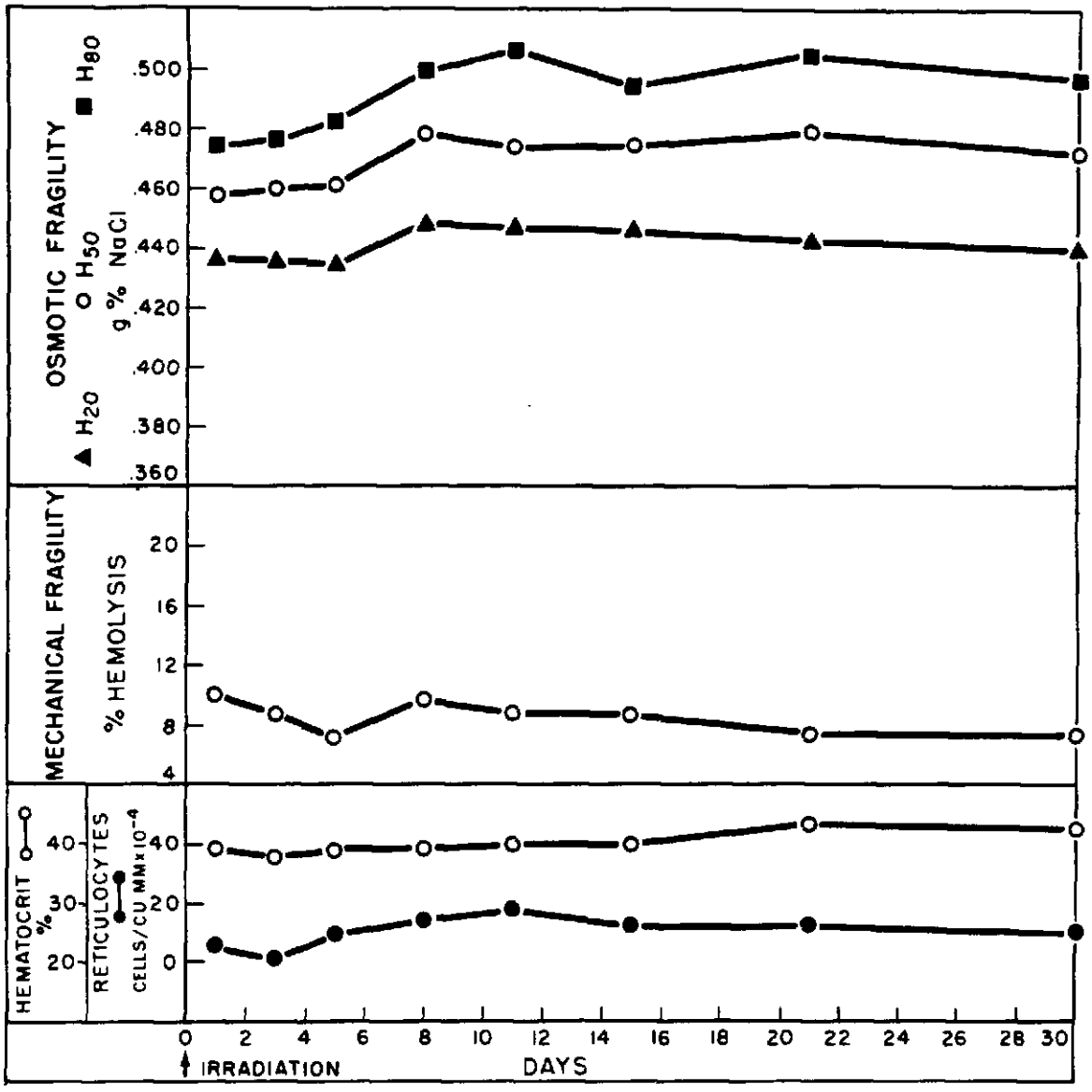

Fig. 3 Osmotic and Mechanical Fragilities, Hematocrit, and Reticulocyte Levels after Burning and/or Irradiation — 100 r Whole Body Irradiation (X_1). Those values which are significantly different from the control on the day of sacrifice are indicated by the asterisks. The H_{50} values, only, of the osmotic fragilities have been statistically analyzed.

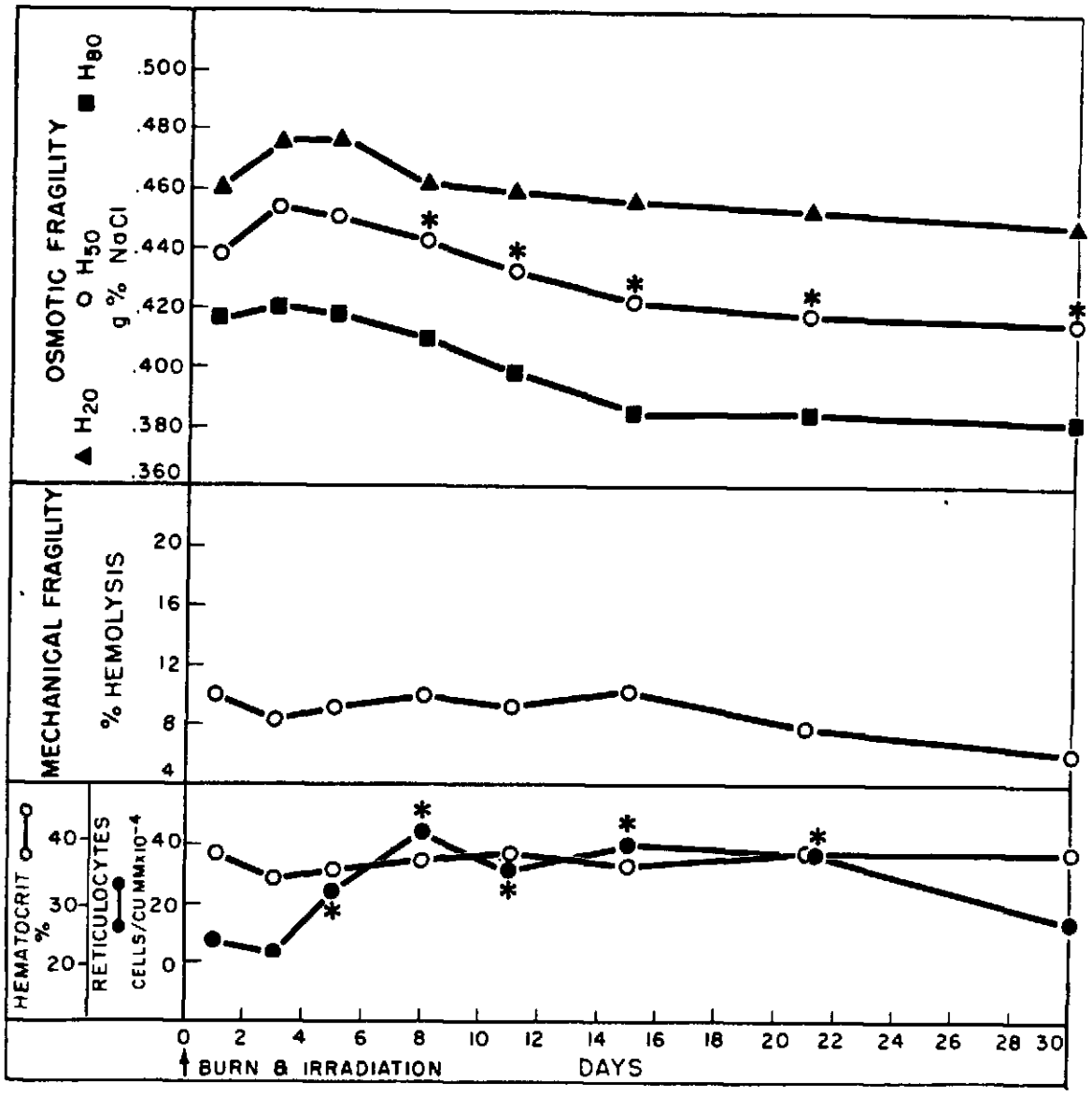

Fig. 4 Osmotic and Mechanical Fragilities, Hematocrit, and Reticulocyte Levels after Burning and/or Irradiation — 25 Per Cent Burn and 100 r Whole Body Irradiation (B_1X_1). Those values which are significantly different from the control on the day of sacrifice are indicated by the asterisks. The H_{50} values, only, of the osmotic fragilities have been statistically analyzed.

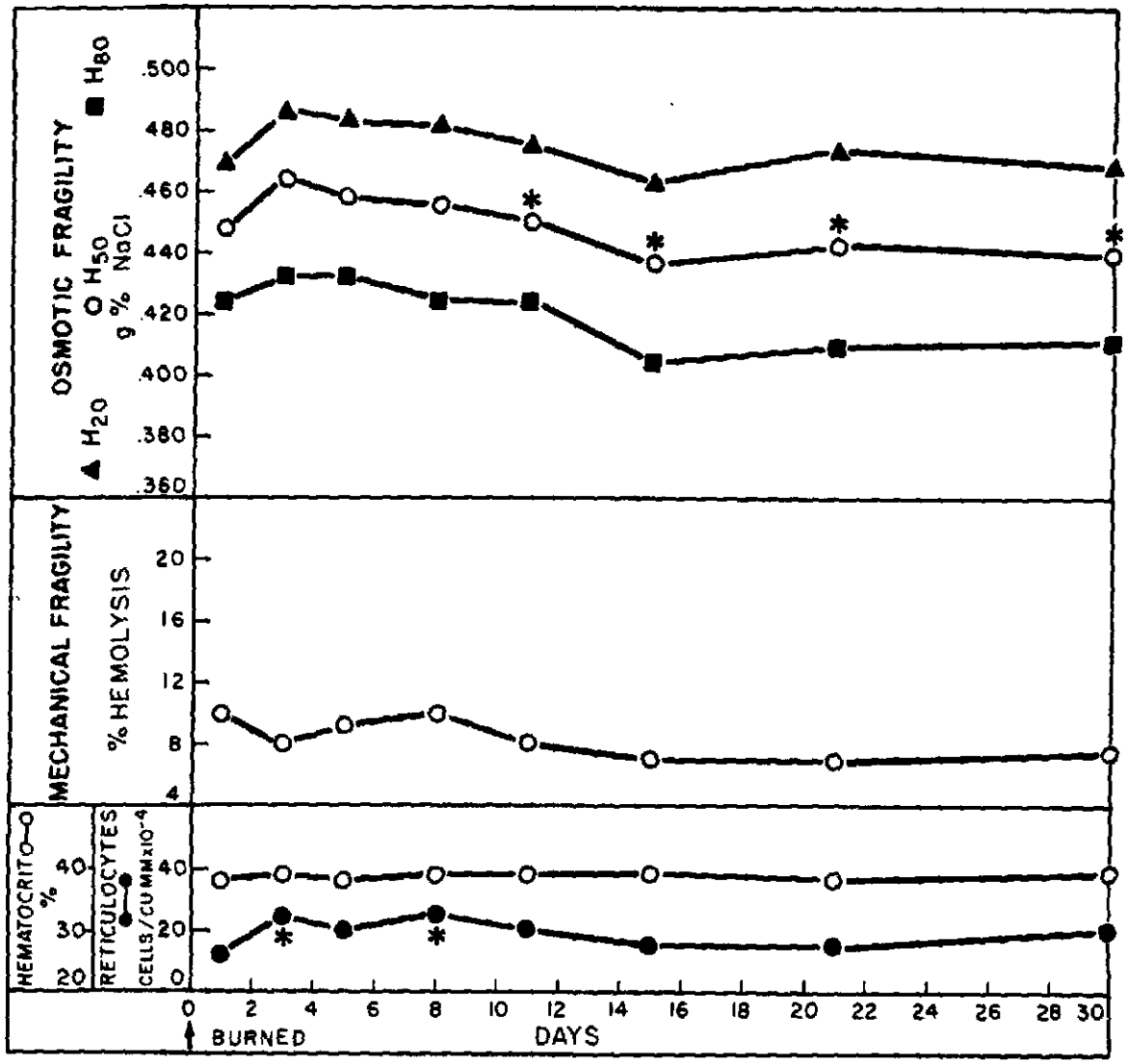

Fig. 5 Osmotic and Mechanical Fragilities, Hematocrit, and Reticulocyte Levels after Burning and/or Irradiation — 15 Per Cent Burn (B_2). Those values which are significantly different from the control on the day of sacrifice are indicated by the asterisks. The H_{50} values, only, of the osmotic fragilities have been statistically analyzed.

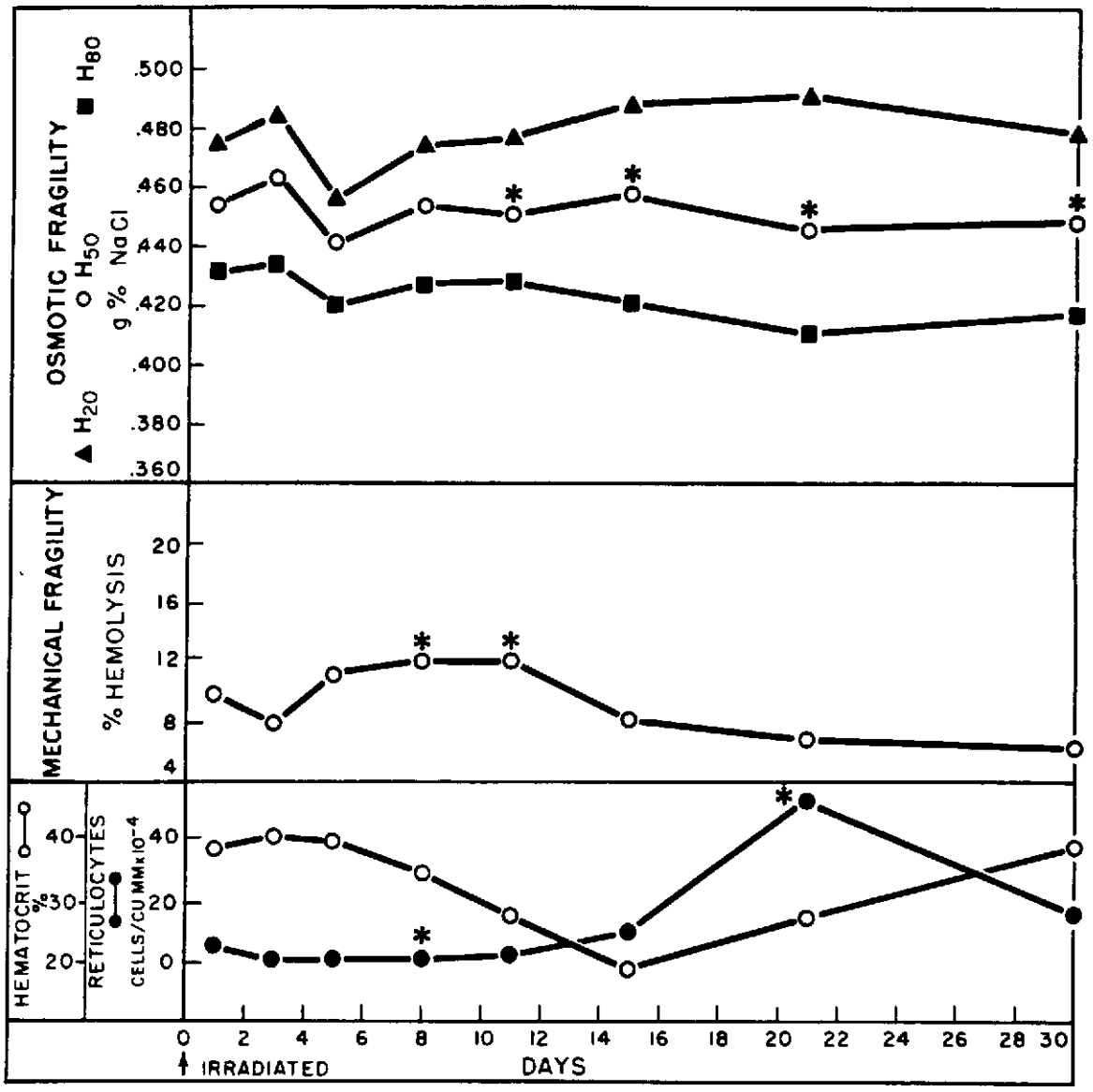

Fig. 6 Osmotic and Mechanical Fragilities, Hematocrit, and Reticulocyte Levels after Burning and/or Irradiation — 500 r Whole Body Irradiation (X_5). Those values which are significantly different from the control on the day of sacrifice are indicated by the asterisks. The H_{50} values, only, of the osmotic fragilities have been statistically analyzed.

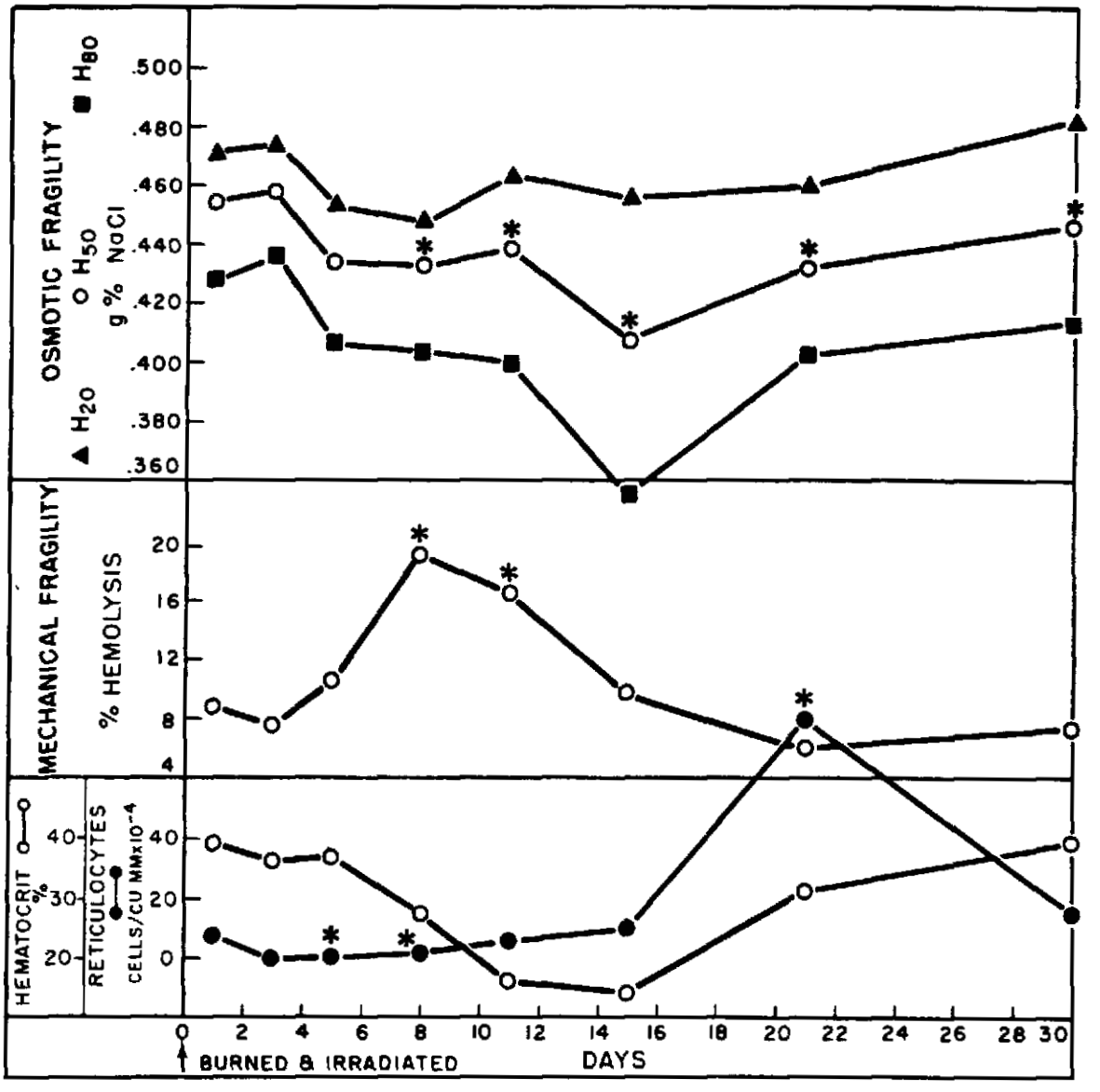

Fig. 7 Osmotic and Mechanical Fragilities, Hematocrit, and Reticulocyte Levels after Burning and/or Irradiation — 15 Per Cent Burn and 500 r Whole Body Irradiation (B_2X_5). Those values which are significantly different from the control on the day of sacrifice are indicated by the asterisks. The H_{50} values, only, of the osmotic fragilities have been statistically analyzed.

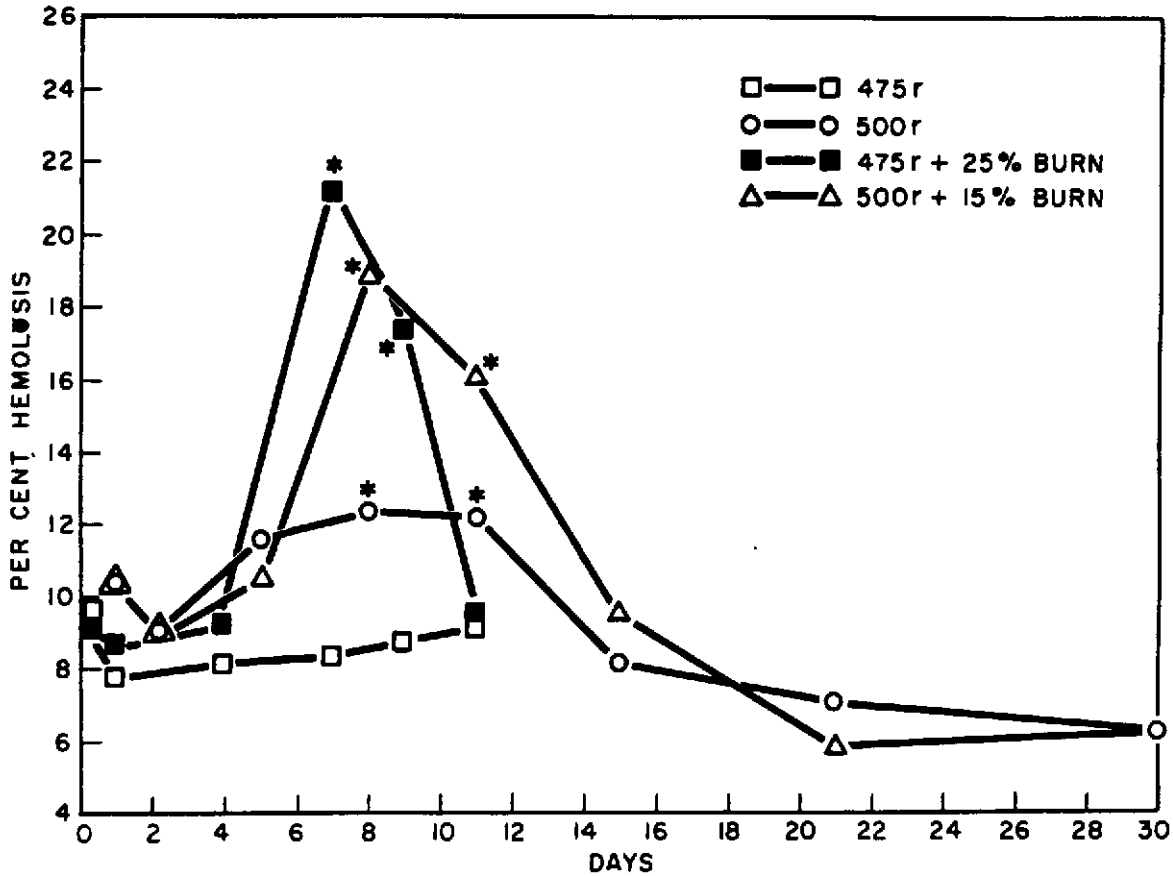

Fig. 8 Comparison of Mechanical Fragility for Irradiated and Combined Burned and Irradiated Animals. Those values which are significantly greater than the control values are indicated by the asterisks.

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2. X radiation - Physiological effects
3. Thermal radiation - Pathological effects
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