cines grown in different tissue cultures systems developed delayed dermal hypersensitivity when skin tested with vaccine materials. Animals immunized with alum containing vaccines developed more severe reactions than did animals immunized with aqueous vaccines. The addition of DPT vaccine to RS virus preparations also appeared to intensify the observed reactions. In addition, animals immunized with bovine kidney parainfluenza vaccine developed delayed dermal hypersensitivity when skin tested with bovine kidney RS vaccine. It appeared that both viral and host cell components of these vaccines were responsible for the observed reactions. Partially purified RS antigen A and B did not elicit delayed dermal hypersensitivity in animals immunized with these vaccines.

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Effect of Gamma Irradiation and Radioprotectors on Alkaline Phosphatase and ATPase* (33424)

M. NOAMAN, M. K. HAMDY, AND W. O. CASTER

Departments of Food Science and Home Economics Nutrition, University of Georgia, Athens, Georgia 30601

Doses of ionizing radiation produce biochemically important changes in tissues resulting in metabolic derangement which, in time, may lead to cellular damage or even death of the cells. A dose of ionizing radiation lethal to mammals involves an infinitesimal energy absorption and a corresponding low number of biochemical changes per cell. Hollaender (1) estimated that a radiation dose of 0.1 krad deposits within the nucleus (assumed to be 1 μ^3) 6×10^3 eV of energy. Since most radiochemical reactions involving organic materials require 10–20 eV for bond rupture, it is reasonable to estimate that chemical changes will occur in only some 600 molecules out of the hundreds of millions of molecules present in the nucleus. The extent to which radiation-induced chemical transformations in cells and tissues are associated with specific chemical groupings is largely unsolved. Comparatively few radiochemical changes can result in a severe biological damage suggesting that ionizing radiation may exert a considerable degree of specificity on metabolism and on the complex high polymer substances of living organisms. One possibility is that the "target" molecules are part of the catalytic system of the cells (2).

Dale (3, 4) made one of the earliest attempts to determine if the effects of X-rays

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on living systems could be due to inactivation of enzymes. Kuzin (5) reported changes in activity of alkaline phosphatase (AP) in several rat organs following whole-body irradiation with 0.3, 1, and 3 krads. He stated that no changes were detected in the levels of AP in the brain tissue, the pancreas, kidneys, or testes and only showed an increase in activity in the spleen, the thymus gland, and the duodenum. He also reported that inactivation of AP was evident in liver and brain of white rats 24 hr after whole-body irradiation with 5 krads. On the other hand, Ludewig and Chanutin (6) found no change in AP activities in the liver of rats receiving a total body X-ray of 0.5 krad.

The activity of adenosinetriphosphatase (ATPase) of kidneys, spleen, and liver of irradiated rats receiving a dose of 0.8 krad was not altered during the first day after irradiation (7). Dilute solutions of ATPase showed partial reduction in activity when irradiated with 0.01 krad, and more complete reduction using 1.0 krad of X-ray, and that the addition of glutathione completely reactivated the partially inactivated enzyme (8). Previous results (9) showed that whole-body γ-irradiation (750 R) enhanced the activities of the lysosomal enzymes in the first 3 days after irradiation. However, the extent of increase, as a function of time, differed from one enzyme to the other and also from one organ tissue to another. Both cysteine and 4-amino-1-napthol (4A1N) protected some, but not all, of the lysosomal enzymes, and 4A1N exerted by far the best protection (10). In view of these findings, the authors decided to explore the effects of γ-irradiation on the activities of two nonlysosomal enzymes, AP and ATPase, both *in vivo* and *in vitro*. The *in vitro* effects of cysteine and 4A1N as radioprotectors to these enzymes were also examined.

Materials and Methods. Irradiation and procurement of tissues. Two ^{60}cobalt sources were used: the first (14.5 R/min) was used for the irradiation of rats (*in vivo* experiments); the second (120.5 R/sec) Gammacell 200, Atomic Energy of Canada Ltd., for the irradiation of enzymes (*in vitro* experiments). The dose rates of both sources were calibrated by the ferrous sulfate dosimetry method of Spinks and Woods (11). The former source was used to expose 60 white Sprague Dawley rats (3 months old, approximately 300 g, Holtsman Co., Madison, Wisconsin) to whole-body γ-irradiation of 750 R ($LD_{50/14\ days}$). Nine nonirradiated rats served as controls. One nonirradiated and 6 irradiated rats were sacrificed by decapitation at definite time intervals postirradiation, and the brain, heart, liver, and small intestine were immediately removed. Each organ was wrapped in Cryovac packaging film, immersed in ice and used for analysis within 1–3 hr. The organ samples (irradiated and nonirradiated) were weighed, minced, and then thoroughly homogenized in cold distilled water (2°) in 50-ml precooled Omni-mixer cups (Servall) at a speed of 16,000 rpm for 3 min (heart and small intestine) and for 2 min (brain and liver) and the homogenate was stored at 2° until used. The correlation coefficients presented throughout the text were computed on an IBM 7094 computer. In each case, correlations larger than 0.63 and 0.76 are significant at the 5% and 1% level of probability, respectively.

Enzyme assay in the in vivo *system.* Alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) and adenosinetriphosphatase (ATPase, EC 3.6.1.8) activities of the homogenates were determined according to the method of Valentine *et al.* (12) with disodium-*p*-nitrophenylphosphate as substrate for AP, and according to Lowry and Lopez's procedure (13) with disodium adenosinetriphosphate as substrate for ATPase. Appropriate controls and reagent blanks were included for all enzyme assays. Protein determinations were conducted with alkaline copper reagent of Gornall *et al.* (14) and used to express specific activities of the enzyme in the homogenates. These specific activities were based on amount of end-product formed per unit time and per milligram of wet tissue weight. The results reported herein represent the average data obtained from 6 irradiated rats (sacrificed at the same specific interval) and then computed as percentage of control. The latter represents average data secured from 9 nonirradiated rats.

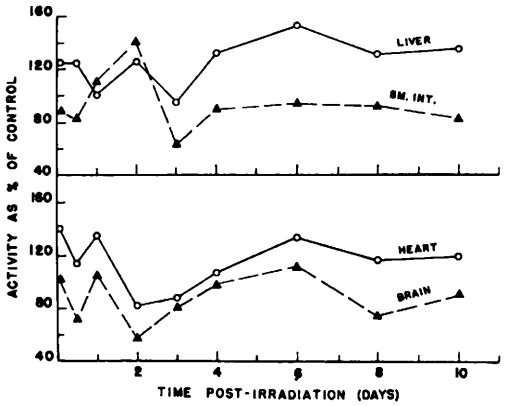

FIG. 1. Effect of whole-body γ-irradiation (750 R) on free activity of AP in rat tissues.

Irradiation and enzyme assay of system in vitro. Aqueous solutions of the enzymes (0.12 μM) alone or with either 0.1 mM of DL-cysteine or 0.1 mM 4-amino-1-napthol (4A1N) were irradiated in air at 20° and immediately after irradiation the activities of the enzyme solutions were assayed as previously outlined. Specific activities were based on amount of end-product produced per unit time and per unit molecular weight of dried enzyme: alkaline phosphatase, mμM nitrophenol/0.12 μM dried enzyme; ATPase, μM phosphate/0.12 μM enzyme. The molarity of the enzyme solutions were calculated on the basis of a molecular weight of 8×10^4 for AP and $\sim 8 \times 10^5$ for ATPase. Enzymatic activities were also computed as percent of nonirradiated control (enzyme alone) and as percentage of nonirradiated enzyme–chemical mixture in the radioprotection experiments.

The D_{37}, the dose in krads for 63% inactivation of the enzyme (15), was used to demonstrate the effects of irradiation and protection of cysteine and 4A1N on the enzymes. From dose residual activity curves the D_{37} was determined graphically for each treatment.

Chemicals. Adenosinetriphosphate and p-nitrophenyl phosphate were obtained from Nutritional Biochemical Corp. Sigma Chemical Co. supplied alkaline phosphatase (mucosa type VI) and ATPase (potato, adenosine-5′-triphosphatase and adenosine-5′-diphosphatase).

Results. Effect of irradiation on enzyme activities in vivo. Figures 1 and 2 illustrate the variation of the free activity of AP and ATPase in tissue of 4 rat organs as a function of time postirradiation. All tissues studied showed similar changes in the period from 3–8 days, but these early changes were different from one organ to another. The data on AP activities in brain and heart showed a somewhat similar pattern but differed in magnitude. Both had triphasic decreases occurring at 12 hr, 2 and 8 days postirradiation. It was also noted that each organ had three major activity peaks: the first at 3 hr, the second at 1 day, and the third at 6 days; the values being 103, 105, and 112%, respectively, for the brain, and 141, 135, and 134% (significant at the 1% level), respectively, for the heart. From 3–12 hr the enzyme activities in the liver and the small intestine had contrasting trends; an increase in liver and a decrease in small intestine that were significant at the 5% level. On the second day both organs had a significant increase of 126% in the liver (compared to control) and 140% in the small intestine. Thereafter, the liver maintained a higher level of AP activity, whereas the small intestine was constantly below control to the tenth day.

Three hr following irradiation, ATPase activities in all 4 organs decreased significantly (at the 1% level) and was 51–60% of control followed by a marked increase after 12 hr (Fig. 2). From these intervals on, ATPase activities of the heart and small intestine

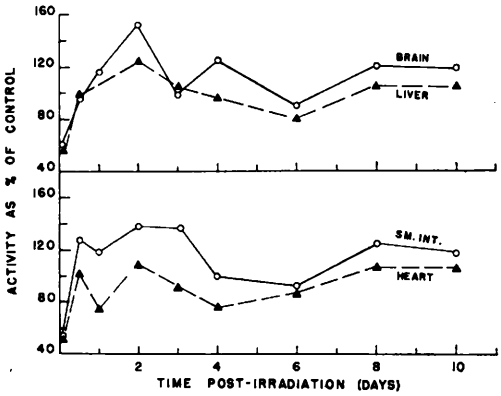

FIG. 2. Effect of whole-body γ-irradiation (750 R) on free activity of ATPase in rat tissues.

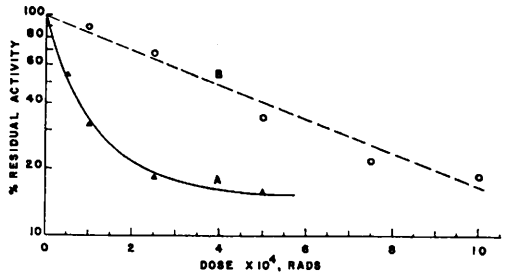

FIG. 3. Effect of γ-irradiation on AP and ATPase *in vitro*. Curve A, AP (0.12 μ*M*); B, ATPase (0.12 μ*M*).

were similar, having biphasic decreases: in the former at 1 and 4 days, and in the latter at 1 and 6 days. The ATPase pattern of activity in the brain and liver were different at 4 days, at which time the brain increased sharply to 124%, and the liver decreased to 96% (of control). However, similar peak increases in these organs occurred at 2 and 8 days where the values were 152% (significant at 1% level) and 120%, respectively, for the brain, and 124% and 105%, respectively, for the liver. It is of interest to report that the correlations between the ATPase activities in all organs ranged from +0.61 to +0.88 and the changes in liver ATPase showed +0.88 correlations with that of heart and small intestine. The correlation between ATPase in brain and liver was +0.68.

Effect of irradiation on enzyme activities in vitro. Figure 3 shows the effect of γ-irradiation (*in vitro*) on the activities of AP (curve A) and ATPase (curve B). These results were computed as percentage of the nonirradiated enzyme. It can be observed that both enzymes were markedly inactivated as a function of dose absorbed. With initial irradiation of 5 krads, AP activity decreased to 55% and continued to decrease to 32, 18, and 16% of the nonirradiated enzyme at 10, 25, and 50 krads, respectively. The calculated D_{37} value was 9 krads. The ATPase was less radiosensitive and had a D_{37} value of 55 krads. Again, there was progressive enzyme inactivation from 90% of the control at 10 krads, to 69% at 25 krads, 35% at 50 krads, 22% at 75 krads, and 19% at 100 krads. Inactivation of ATPase followed first order kinetics, while inactivation of AP followed first order kinetics at dosages up to 20 krads only.

Effect of cysteine and 4A1N on enzymatic activities. Studies were conducted to determine the effect of these chemicals on the enzymatic activities of AP and ATPase prior to irradiation. The concentrations used were 0.1 m*M* cysteine and 0.1 m*M* 4A1N in the reaction mixture. The activities of both the AP–cysteine mixture and AP–4A1N mixture were 95% of control (nonirradiated enzyme alone) whereas when cysteine was added to ATPase, the activity decreased to 90% and with 4A1N, the activity decreased to 94% of control. These data indicated that ATPase was slightly more sensitive to the addition of these chemicals.

Effect of radioprotectors. Cysteine (0.1 m*M*) and 4A1N (0.1 m*M*) exhibited notable radioprotective effects on both enzymes. In the presence of cysteine, the D_{37} of AP changed from 9 krads to 21 krads, and the D_{37} (in krads) changed from 55 to 87 in the case of ATPase. This indicated that cysteine had a proportionately greater protective effect on AP than noted for ATPase. The cysteine dose factors (CDF) were 2.3 and 1.6, respectively. The AP also showed greater protection when irradiated in the presence of 0.1 m*M* 4A1N. The 4A1N dose factor (4A1NDF) was 20.0 whereas ATPase had a 4A1NDF of 2.7. The CDF and 4A1NDF are defined herein as the relative increase in D_{37} due to the presence of the radioprotector and computed as follows:

$$CDF = D_{37}\ (\text{cysteine})\ /D_{37}\ (\text{no cysteine}),$$

$$4A1NDF = D_{37}\ (4A1N)\ /D_{37}\ (\text{no } 4A1N).$$

When the enzyme was mixed with either cysteine or 4A1N, better radioprotection at lower doses of irradiation was noted (Fig. 4). For example, when the AP–cysteine mixture was irradiated (curve A), the percentage of residual activity changed from 62 at 5 krads to 23 at 50 krads, whereas the ATPase–cysteine mixture (curve D) changed from 93% at 10 krads to 33% at 100 krads. A somewhat similar pattern was noted for irradiated enzyme–4A1N mixtures: the percentage of residual AP activity (curve B) changed from 83 (5 krads) to 68 (50 krads); ATPase activity (curve C), 98 (10 krads) to 52 (100 krads).

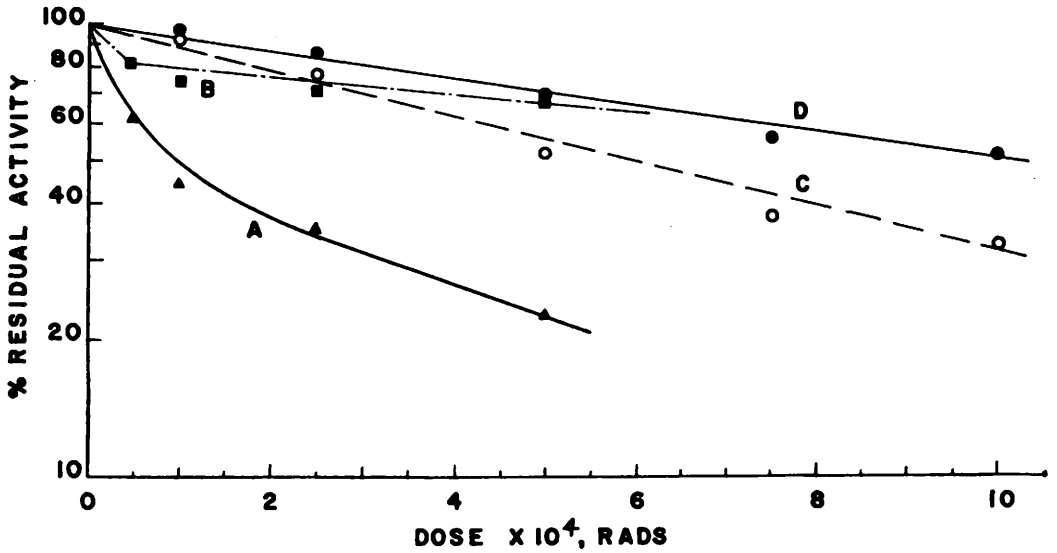

FIG. 4. Radioprotective effect of 0.1 m*M* cysteine and 0.1 m*M* 4A1N on AP and ATPase: (A), AP and cysteine; (B), AP and 4A1N; (C), ATPase plus cysteine; (D), ATPase plus 4A1N.

Discussion. Kuzin (5) reported that the radiosensitivity of enzymes, *in vitro*, is highly variable and upon irradiation of dilute solution, pure enzyme inactivation may occur with a wide range of doses. He also stated that the protection of enzymes from ionizing radiation is a characteristic feature when protein, glucose, and other substances are present and that many enzymes which are readily inactivated when irradiated in pure solution are quite resistant in their natural environment.

The AP activities in the 4 organs of the irradiated rats showed two dissimilar patterns: (a) a significant increase in both the heart and liver within 3 hr of whole-body γ-irradiation and an average activity, compared to control, during the entire experimental period (10 days) of 115.2% for the heart and 114.3% for the liver; (b) a significant decrease within 12 hr in both the brain and small intestine and an average of 88.1 and 93.2% (of control), respectively, during the entire experiment. The changes in AP activity showed no consistent relationship to those of the ATPase in any tissue. The correlations between these two enzymes in heart, brain, small intestine, and liver were —0.20, —0.67, +0.13, and —0.33, respectively. The intertissue correlations for these enzymes ranged from +0.59 to —0.70 with more than half of the values between +0.17 and —0.35. The statistical data related to these enzymes showed little, if any, correlation with the lysosomal enzyme data reported previously (9). One exception was the case of AP of the heart which was significantly, but negatively, correlated with acid phosphatase in the same organ ($r = -0.83$) and with brain arylsulfatase ($r = -0.87$). Mollura *et al.* (16) reported that mice receiving a dose of 10 krads (whole-body irradiation) failed to produce any apparent change in AP activity in various tissues when measured at intervals from 2 hr to 14 days after exposure. On the other hand, enzyme activity of AP was reported by Hajdukovic and Raskovic (17) to increase in both the spleen and the kidney of mice subjected to 1 krad of whole-body X-irradiation.

With respect to ATPase activity, a significant decrease within 3 hr postirradiation was noted in all 4 organs followed by an increase above normal level after 2 days. The average activities of this enzyme for the entire experimental period were: 108.3, 89.2, 98.1, and 112.3% (of control) in brain, heart, liver, and small intestine, respectively. It appears that the effect of whole-body γ-irradiation on this enzyme was slight and was not selective.

Maxwell and Ashwell (18) reported an increase of ATPase activity in various isolated cell fractions of spleen from mice 1–2 days after irradiation with 640 R. Peterson *et al.* (19) found that irradiation of isolated mitochondria did not enhance the activity of ATPase with doses of 0.8–1.0 krad. In addition, Prokudina (20a) demonstrated an increase in ATPase activity of the spleen upon irradiation of rat with a dose of 4.5 krads, whereas a dose of 1.5 krads did not alter ATPase activity of the spleen (20b). Kuzin (5) stated that with minimum lethal doses of radiation, one must consider both a direct effect of radiation on the metabolizing organ and the effect of humoral factors. Bacq and Alexander (21) stated that the primary lesion causing death of cells was an alteration in the permeability of certain intracellular structures. Their proposal was substantiated by the fact that the earliest biochemical changes were detected by increases in enzymatic activity.

The free AP activity *in vivo* was slightly affected by 750 R of whole-body γ-irradiation, but drastically inactivated at higher doses of irradiation *in vitro*. This *in vitro* inactivation was very rapid in the dose range of 5–10 krads and slow between 20 to 55 krads. Barron *et al.* (8) found that purified sulfhydryl (SH) enzymes such as ATPase and phosphoglyceraldehyde dehydrogenase were more sensitive to irradiation *in vitro* than some non-SH enzymes and that inactivation could be prevented and, even more significantly, reversed by the addition of glutathione. Our results revealed that ATPase (*in vitro*) was moderately radioresistant compared to AP. This observation is not in line with those reported by Barron *et al.* (8) who used lower concentration of enzyme than employed in the present investigation. It was also noted that the inactivation curve of Fig. 3(B)corresponded to the single exponential predicted from the one-hit "target theory" (15). The 4-amino-1-napthol was a better radioprotector than cysteine for both AP and ATPase. Maximum protection (20-fold) afforded by 4A1N was noted for AP. The protective effect of 4A1N may be attributed to its reaction with free radicals to form less active forms or stable radicals. Bacq and Alexander (21) pointed out that cysteine protects enzymes by absorption of the radiation energy. It seems unwarranted to explain the protective phenomenon on the basis of a single mechanism because enzyme inactivation can be influenced by many factors such as irradiation dose, nature of irradiated enzyme, change of temperature, presence of impurities, oxygen and/or water.

Summary. Whole-body γ-irradiation (750 R) affected both AP and ATPase activities in 4 rat organs with similar changes in the period from 3 to 8 days postirradiation. However, the magnitude of these changes was different from one organ to another. Solutions of ATPase and AP (*in vitro*) were inactivated by irradiation. The former enzyme was radioresistant as evidenced by its D_{37} of 55 krads compared to D_{37} of 9 krads for AP. Cysteine (0.1 m*M*) altered the D_{37} of both AP and ATPase to 21 and 87 krads, respectively, whereas 4A1N (0.1 m*M*) changed the D_{37} of AP to 180 and ATPase to 148 krads. Maximum protection (20-fold) afforded by 4A1N was noted for AP.

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Changes in Human Serum High-Density Lipoproteins Induced By Disulfide-Exchange Reagents.* (33425)

LOUIS COHEN AND JULIANA DJORDJEVICH

Department of Medicine, University of Chicago School of Medicine, Chicago, Illinois 60637

In 1962, Shore and Shore (1) found evidence suggesting that lipoproteins of density (D) 1.125–1.20 g/ml contain three identical peptide chains per molecule. In the same year Scanu *et al.* (2) also presented evidence consistent with a similar interpretation for the protein part of the entire alpha$_1$ lipoprotein density range (D 1.063–1.21) (HDL), and later, additional evidence of protein homogeneity was reported by Sandbar and Alaupovic (3). Although an antigenic heterogeneity of HDL present in aged plasma was recently demonstrated by Levy and Fredrickson (4), this was not ascribed to differences in protein composition, but rather to differences either in protein lipidation or polymerization of some basic monomeric unit. Thus, the bulk of the evidence indicates that the HDL protein is homogeneous.

There is less agreement about the cystine content of this protein. Although the presence of cystine had not been found by some (2, 3), more recently using two different methods it has been demonstrated to be present by Shore and Shore (1) and Levy and Fredrickson (4).

In the present report evidence is presented that suggests that the protein component of HDL is heterogeneous in its polypeptide composition and consists of at least two different polypeptides. One variety, called polypeptide C, reacts with a variety of reagent thiols and thiol-disulfide reagent combinations, suggesting it contains cystine-disulfide bonds. The other polypeptide is not reactive with these reagents or their combinations.

Methods. Isolation of HDL. Lipoproteins of density 1.063–1.21 (HDL) were isolated from 80 different sera by ultracentrifugal flotation using a modification of methods described elsewhere (5). Five milliliters of fresh serum were made to D 1.063 by adding 4 ml of a salt (NaCl + KBr) solution of D 1.134; the mixture was spun at 30,000 rpm for 24 hr in a Model L Spinco ultracentrifuge using a 30.2 rotor. The top 3 ml were then removed. The infranatant fluid was then layered over with a salt solution of D 1.063, and the sample was respun for 24 hr. The top 3 ml were removed and the density of the infranatant fluid was increased to D 1.21 by mixing with solid KBr; this mixture then was made to appropriate volume by adding a NaCl-KBr solution of D 1.21. It was spun as before, but only the top 1 ml containing the HDL was removed. It was placed at the bottom of a 9-ml Lusteroid centrifuge tube, carefully layered over with 8 ml of a salt solution of D 1.21, and respun as before. The top 1 ml was removed and dialyzed over-

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