Radioprotective Properties of Some Heterocyclic Nitrogenous Compounds
Against X-radiation Injury to Serum Proteins in Mice

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The results obtained from this study suggest the following: The concentration of total serum proteins in mice is changed very little under the various treatments, while protein fractions showed significant alterations.

The concentrations of various serum proteins remain almost constant under normal conditions. Intraperitoneal administration of imidazole or benzimidazole at the mentioned doses induces rapid quantitative changes in the serum which are recovered in about 3 days after injection.

Whole-body X-irradiation at 750 r creates slow but progressive and persisting serious changes in concentration of serum protein fractions which end in death of animals 8—10 days after irradiation.

Whole-body X-irradiation of imidazole or benzimidazole protected animals results in quantitative rapid changes in concentration of serum protein fractions for about four days. There after a slow but steady restoration begins. The concentration approaches the normal levels towards the 10th day after irradiation.

Imidazole and benzimidazole proved to be good radioprotectants against the effects of X-radiation on serum protein fractions. Benzimidazole seems to surpass imidazole.

Because of the potential practical importance of certain chemical compounds that exhibit a protective character against lethal effects of ionizing radiation, excessive work has been done to identify the most effective non-toxic ones. Since Patt et al. 1 reported that the amino acid, cysteine, significantly the most effective non-toxic ones. Since Patt et al. 1 reported that the amino acid, cysteine, significantly increased the survival of irradiated rats, thousands of agents have been tested, THOMSON 2. Repeated investigations have been also carried out to study the mechanism through which such compounds manifest their protective effects. It seems likely, however, that all those compounds exert their effects by influencing the production of reactive radicals by radiation. The protection of the vital cellular functions is thus achieved either by production of hypoxia, i.e. reduction of oxygenation in tissues, or through reaction with the free radicals released by radiation to inactivate them. The biological difficulty with the application of these compounds in general is that they appear to be effective only at close to toxic levels. Moreover, almost none of these agents has been shown to be of benefit when given after radiation exposure. Most of the experiments were carried out on mice. The increase in 30 day survival has been usually taken as a parameter to assume that the protectant is effective in changing sensitivity. Less attention has been given to the studies on the actual pharmacological and biological rôle played by such compounds in the biological medium.

During the last few years, however, repeated investigations have been carried out at the “Laboratoire de Radiobiologie, Centre d'Etudes Nucléaires de Grenoble” and at the “Institut des Radioisotopes, Université de Grenoble” (France) on the radioprotective properties of some heterocyclic nitrogenous compounds. Among the most successful compounds tested are imidazole and benzimidazole. Imidazole has been found to exhibit a remarkable activity in this regard. Up to 85% of mice inoculated with 0.35 mg imidazole/g body weight 5—15 min before receiving a lethal radiation dose of 750 r survived (RINALDI and BERNARD 3). Benimidazole, on the other hand, is found to possess highly interesting properties. The percentage of survivals obtained with mice treated with benzimidazole (0.35 mg/g body weight) and exposed to lethal dose of radiation approaches 90% (RINALDI et BERNARD 4).

Being different from the usually studied thiols as radioprotectants, it seemed necessary to us to study the pharmacological responses of imidazole

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and benzimidazole, and to attempt to correlate such responses with their ability to protect against lethal effects of radiation.

In the present work, a radiosensitive parameter has been taken into consideration: the changes in protein metabolism. Since the serum proteins are the most readily available group of proteins having a wide variety of natural functions and biological properties, the changes in the concentration of various serum proteins due to different treatments form the subject of the present studies. Such changes have been the subject of repeated investigations by many previous authors (Muntz, Fischer et al., Kashkin et al., Alix et Pierotti, Kashkin and Aleksandrova, etc.).

It is generally known that the most important alterations include a decrease in the concentration of the albumin fraction, an early increase, then decrease, in γ-globulins, and an increase in the α- and β-globulins. However, the mechanisms underlying these changes are still poorly understood. Furthermore, very little information is available concerning the alterations in the specific protein fractions with time after irradiation.

**Materials and Methods**

This work has been conducted on male mice of the race C3H/Jax 11–12 weeks old, weighing 35–40 g and kept in rooms thermostatically controlled at 21 °C. The experimental animals were divided into four groups as follows: Group I untreated normal controls; Group II treated animals to which the drug, either imidazole or benzimidazole at a dose of 0.35 mg/g body weight, was administered intraperitoneally; Group III irradiated controls who received whole-body X-irradiation in a lethal dose of 750 r; Group IV irradiated pre-treated animals who received whole-body X-irradiation at a dose of 750 r five to ten minutes from time of drug injection. Imidazole was administered in a solution of 15 mg per ml in isotonic NaCl solution while benzimidazole in a solution of 7.5 mg/ml in 1.2 propandiol 10% solution (after Rinaldi and Bernard).

The source of radiation utilized was X-Ray Radiotherapy Unit: Massiot-Philips, manipulated under dosimetric control at 180 kV, 18 mA, and with a 0.5 mm Cu filter. The animals were placed 50 cm from the anode, the dose rate being 80 r/min under those conditions. During each X-ray exposure, the dose was measured with an integrating dosimeter, Massiot-Philips, fitted with an ionization chamber of 2 r.

During irradiation, the animals were kept in boxes of plexiglass, each divided into 11 compartments and having a lid 2 mm in thickness. The boxes were made to revolve slowly around their axes, once every one minute, to ensure isodose irradiation.

After irradiation, the animals were kept in numbers corresponding to the number of days during which it was proposed to examine the changes in serum protein composition and were fed normally. Being a lethal radiation dose (Storer, after Green p. 432), almost all the X-irradiated nonprotected animals died during the experimental period of 8–10 days following irradiation.

For the blood sampling, the animals were lightly anesthetized by ether and the blood was collected by a tuberculin syringe in amounts of 0.8–1.0 ml from every animal through cardiac puncture. The blood serum of each animal was stored in the frozen state for some few hours till experimentation. The sera of all the four groups of animals were subjected to exactly the same treatment and were investigated simultaneously.

The total serum proteins was determined photometrically by Biuret method (Weichselbaum).

Microelectrophoresis technique on cellulose acetate membranes (Grunbaum, Zec and Durrum and Grunbaum, Lyons and Zec) has been employed for quantitative analysis of serum proteins. Separation of serum protein fractions has been carried out with Beckman Model R-100 Microzone Electrophoresis system which permitted eight simultaneous electrophoretic analyses at the same time. The cell has been operated at room temperature for 20 minutes at a potential gradient of 250 volts in veronal buffer (Beckman-Buffer B-2), pH 8.6 and ionic strength of 0.075. Duplicate runs have been made for each sample. The membranes were then dried and developed in fixative dye ponceau S (Beckman) for 7 minutes, successively washed by acetic acid and ethyl alcohol and finally dried. They were then evaluated by photoelectric scanning with a Spinco-Model RA Analytrol. The final values obtained were the averages of two runs for each sample.
sample. The calculation of each protein component has been made by dividing the component areas of the electrophoretic pattern by dropping perpendicular lines from the maximum height between two peaks on the curve to the baseline.

As usually carried out at pH 8.6, electrophoresis separates the serum proteins into albumin, $\alpha_1$, $\alpha_2$, $\beta$ and $\gamma$-globulins. Since $\alpha_4$-globulin does not usually appear as a separate boundary in serum of mice, and is usually included with the albumin, under these conditions what is referred to here as albumin means the albumin plus $\alpha_4$-globulins and what is referred to as $\alpha$-globulins means $\alpha_2$-globulins.

## Results

### A. Total Serum Proteins

Intraperitoneal administration of imidazole and benzimidazole does not cause a detectable change in the level of total serum proteins. Irradiation of untreated and treated animals as well do not show an appreciable change throughout time post-irradiation.

### B. Serum Protein Fractions

#### Albumin

Administration of imidazole and benzimidazole is accompanied by a rapid and serious reduction in albumin levels (Figs. 1 and 2). Such pharmacological effect is more pronounced with benzimidazole. Restoration of normal albumin level takes place over a period of about three days after treatment.
X-irradiation does not create a sudden drop in albumin level, but the reduction gradually proceeds with varying rates over the whole period preceding death. The sharpest drop being between the fourth and fifth day after irradiation (Figs. 1 – 3).

In the irradiated animals treated with imidazole and benzimidazole, there is a sudden sharp drop in albumin level which is more pronounced with benzimidazole (Figs. 1 – 3). A more gradual drop continues for about one day after treatment after which a gradual increase in the albumin level begins. The rate of recovery proceeds steadily with benzimidazole and with slight fluctuations with imidazole. Almost full restoration is attained towards the tenth day after treatment (Figs. 1 – 3). Again restoration with benzimidazole is more complete towards the tenth day than with imidazole.

**α-Globulins**

Administration of imidazole and benzimidazole is accompanied by a sudden increase in α-globulins which is of more or less the same magnitude with both drugs (Figs. 4 – 6). The increased levels of α-globulins continue over a period of one day with benzimidazole but is of much shorter duration with imidazole (Fig. 6). After then, a rapid drop takes place followed by a more gradual drop turning the α-globulin levels towards normals in a period of about ten days after treatment.
a sharp drop takes place towards the second day after irradiation.

The moderately elevated level of $\alpha$-globulins is then retained for a long time extending up to the tenth day after irradiation. The recovery is not yet complete at the tenth day especially with imidazole.

$\beta$-Globulins

Administration of imidazole and benzimidazole is not apparently accompanied by a sudden change in $\beta$-globulin levels (Figs. 7–9). However, a slight increase is detected only with benzimidazole (Fig. 8). The level of $\beta$-globulins then gradually increases over a period of one day after treatment with imidazole and two days with benzimidazole. Afterwards, the level returns to normal values towards the third day after treatment.

Irradiation causes but a slight sudden increase after which the level of $\beta$-globulins gradually increases along the first day after irradiation. The level remains more or less unchanged from the first to the fourth day after irradiation, after then a remarkable gradual increase of varying rates takes place. This increase in the level of $\beta$-globulins continues until death of the animals (Fig. 9).

In the irradiated animals treated with imidazole and benzimidazole, there is a detectable sudden rise in the level of $\beta$-globulins which is more intensified over the first day after irradiation. The raised level of $\beta$-globulins continues with more or less the same magnitude for a period of about three days after which recovery is attained towards the fifth day after irradiation.

$\gamma$-Globulins

Administration of imidazole and benzimidazole creates a sudden and drastic jump in the level of $\gamma$-globulins of more or less the same magnitude
(Figs. 10—12). With benzimidacole, however, a further rise continues over the first day after treatment after which a sharp drop takes place rendering the level to normal towards the third day after treatment. From the fourth to the tenth day, repeated drastic fluctuations have been recorded, the maximal rise is on the fifth and ninth day after treatment while the minimal drop recorded is on the sixth and eighth day after treatment (Fig. 11). With imidazole the level of γ-globulins is progressively decreased returning to normal values on the third day after irradiation. No drastic fluctuations have been recorded and these are not much different than the fluctuations recorded in normal conditions (Fig. 10). Irradiation causes a progressive increase in the level of γ-globulins which continues along two days after irradiation, after which no remarkable change is recorded over a period extending from the second to the fifth day after irradiation. After then a sharp drop takes place returning the level to normal value towards the seventh day and then a further drop continues till the death of the animals (Fig. 10).

In the irradiated animals treated with imidazole and benzimidazole, there is a sudden sharp jump in the level of γ-globulins of more or less the same magnitude. The levels gradually decrease turning to normal around the fifth day after irradiation. With benzimidazole a curious fluctuation of the level is recorded towards the first day after irradiation (Fig. 11).

**Albumin/Globulin Ratio**

Intraperitoneal administration of imidazole and benzimidazole causes sudden increase in A/G ratio which is gradually recovered over three days after
treatment. The change is more pronounced with benzimidazole.

Irradiation causes no comparable sudden rise in A/G ratio but this gradually increases until the fourth day after irradiation. After then a sudden sharp rise intervenes between the fourth and sixth day followed by a more gradual rise from the seventh day until death.

In irradiated animals treated with imidazole and benzimidazole, a sudden sharp rise in A/G ratio takes place which is of more or less the same magnitude. The A/G ratio tends to decrease towards normal levels over a recovering period of about nine days after irradiation. Recovery is more steady and more pronounced with benzimidazole.

The results observed in this study suggest that the concentration of total serum proteins is very little changed due to the different treatments carried out. On the other hand, the various protein fractions show significant alteration. In healthy normal mice, however, the concentrations of the various serum proteins remain comparatively constant. Moreover, the samples of blood serum taken, under identical conditions of separation, give entirely coinciding electrophoretograms.

Intraperitoneal administration of imidazole and benzimidazole at a dose of 0.35 mg/g body weight,
induces rapid quantitative changes in the serum proteins of mice. These changes are detectable some few hours after treatment. They last, however, for about three days during which they are progressively recovered. These changes are characterized by a sudden decrease in the albumin fraction accompanied by a sudden rise in the concentration of $\alpha$-globulins and $\gamma$-globulins. The concentration of $\beta$-globulins undergoes a similar increase but is not detectable before one day after treatment. The albumin/globulin ratio is naturally elevated from normal values. Towards the fourth day after treatment, the quantitative concentration of serum proteins returns back to normal. The slight fluctuations which can be recorded after then are found to remain within the same magnitude of the variations of normal levels in healthy normal animals.

Whole-body X-irradiation at a dose of 750 r has been found to create changes in the serum protein fractions in mice characterized by a gradual decrease in the concentration of albumin fraction, an early increase then decrease in the concentration of $\gamma$-globulins and a gradual increase in the $\alpha$-globulins and $\beta$-globulins. The albumin/globulin ratio steadily rises throughout the post-irradiation time preceding the death of the animals. The increase is found to be at a faster rate between the fourth and fifth day after irradiation. Death of the animals, however, has not been found to be preceded by remarkable rapid changes in the concentrations of various serum protein fractions. Almost all the animals die during the experimental period of 8–10 days following irradiation.

Whole-body X-irradiation at a dose of 750 r of mice protected by imidazole and benzimidazole creates as well rapid changes in the concentration of serum protein fractions which are remarkable during the first two days following treatment. During such period of time, these changes are much more pronounced than those recorded in the similarly irradiated non-protected animals. During the third and fourth day after treatment, the changes in both groups of animals parallel each other in magnitude. From the fourth day onwards, a true but slow restoration intervenes in the irradiated protected animals. The restoration of the normal concentrations of various serum proteins is associated again with an increase in the albumin fraction while the amounts of $\alpha$-globulins, $\beta$-globulins and $\gamma$-globulins gradually decrease. This naturally necessitates the progressive decrease in the albumin/globulin ratio towards the normal values. The rate of restoration is found to be faster with benzimidazole than with imidazole. However, the normal concentrations of the various serum proteins are not fully restored before ten days after treatment.

### Discussion

It is already known that the concentrations of the various proteins in the circulation are the result of balance between filtration into the capillaries and return of the amount of protein in the circulation reflects the balance between filtration into the capillaries and return from the tissue spaces (Petermann, Putnam\textsuperscript{14}, p. 310). The determination of serum protein levels as an aid in diagnosis of disease or radiation sickness has much theoretical possibilities. The change in the albumin/globulin ratio seems to be the earliest pathological sign in the blood following a sub-lethal or lethal dose of radiation, the increase being perceptible as early as few hours after irradiation.

However, the serum protein pattern often reflects the total physiology and clinical state of the patient rather than a specific disease process (Luetscher\textsuperscript{15}). Being the result of balances between a number of opposing forces, the changes in the concentrations of serum proteins in disease and after sub-lethal and lethal doses of radiation cannot easily be attributed to a particular well-defined cause. The quantitative changes noted might have been related both to a change in the concentration of certain "normal" components of the serum proteins in the irradiated animals and to the penetration of new proteins into the blood which migrate in electrophoresis together with one of the serum protein fractions.

It is also suggested that serum proteins are lost more rapidly from the circulation owing to changes in the distribution between the extra and intra vascular space or to a more pronounced extra hepatic catabolism (Reuter et al.\textsuperscript{16a}).

Of the many quantitative changes that has been described in serum proteins, the most striking and


\textsuperscript{15} J. A. Luetscher, Physiol. Rev. 27, 621 [1947].
frequent is a decrease in albumin fraction. This may be either a primary change, or secondary to an increase in globulins and altered colloid osmotic pressure (Petermann-Putnam\textsuperscript{14}, p. 377). This change is of a great metabolic importance as albumin serves a transport function, carrying both undesirable and desirable ions to the site of elimination or need.

Due to the extreme importance of the liver in protein metabolism (Miller and Bale\textsuperscript{16}), liver disturbances are expected to change the concentrations of serum proteins markedly. The reduction in serum albumin has been recorded in most forms of liver diseases (Gutman\textsuperscript{17}, Ricketts and Sterling\textsuperscript{18}; and Sterling and Ricketts\textsuperscript{19}; after Petermann-Putnam\textsuperscript{14}, p. 319). This reduction can be attributed to decreased synthesis, since albumin is synthesised in the liver (Miller and Bale\textsuperscript{16}). In liver diseases moreover, elevated β-globulins are also seen while electrophoretic analysis shows an extreme elevation in γ-globulins as well with a possible relation to antibody formation (Petermann-Putnam\textsuperscript{14}, p. 331). The physicochemical and immunological similarities of γ-globulins, antibodies and the pathological serum globulins strongly suggest a common origin if not a common function (Putnam, p. 399). The pathological globulins are sometimes suggested to represent an immunological response to an unknown stimulus (Barr\textsuperscript{20}, after Putnam, p. 399).

It seems likely that the indirect radiation effects on the liver and on the protein metabolism play a remarkable rôle in the induction of certain changes in the concentrations of serum protein fractions after sub-lethal and lethal doses of radiation.

The magnitude of the pharmacological effects of imidazole and benzimidazole on the electrophoretic pattern of serum proteins seems to be of great interest as it is known that only some few diseases cause such gross abnormalities in protein metabolism that characteristic changes can be detected by electrophoretic methods (Petermann-Putnam\textsuperscript{14}, p. 337).

This points to the probability, of the interference of imidazole and benzimidazole with the biosynthetic activity in the liver. It has been found that the liver fixes a good deal of intraperitoneally administered benzimidazole\textsuperscript{14}C which is detectable 5 minutes after injection. The fixed substance has been almost entirely retained by the liver of rats for about one hour after which a rapid release of the substance takes place in less than six hours (Tyortyalian\textsuperscript{21}).

In the course of our studies, it has been also found that imidazole and benzimidazole exert remarkable pharmacological effects on the liver. They cause as well, a rapid serious depletion of the glycogen store in the liver. Such remarks are still fragmentary and not yet completely studied, but we do believe that the rôle played by imidazol and benzimidazole in the disturbance of protein metabolism should be the subject of extensive studies.

However, we should always take into consideration that the changes in the concentrations of serum proteins cannot be confined only to liver disturbances. Characteristic changes can be also detected in conditions of malnutrition, acute situations or infections and in presence of fever (Putnam, p. 310). In many disease states, the electrophoretic patterns show deviations in the total sum of protein fractions.

Moreover, we cannot overlook the radioprotective properties of some compounds on the serum proteins themselves. This has been proved by the work of Libby et al.\textsuperscript{22} who found that certain radioprotectants as cysteamine exert a remarkable protective activity on the bovine albumins against direct action of ionizing radiation through prevention of radical formation.

\textsuperscript{16} L. L. Miller and W. F. Bale, J. exp. Medicine 99, 125 [1954].
\textsuperscript{17} A.B. Gutman, Advances in Protein chemistry 4, 156 [1948].
\textsuperscript{18} W. E. Ricketts and K. Sterling, J. clin. Invest. 28, 1477 [1949].
\textsuperscript{19} K. Sterling and W. E. Ricketts, J. clin. Invest. 28, 1469 [1949].
\textsuperscript{20} D. P. Barr, Amer. J. Med. 9, 277 [1950].
\textsuperscript{21} C. Tyortyalian, Rapport C.E.A. R. 2899 [1965].