Oxygen Consumption in Gamma Irradiated Mouse Testes

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Radiation/Testes/O_2 /Consumption
    An elevation in respiration accompanies the irradiation of mouse testes at 600 rad following a delay of two weeks. The high metabolic activity is maintained for several weeks after the initial exposure and is most probably associated with the recovery phase of the testes when spermatogonia A are repopulating the germinal epithelium. The correlation between these two events is indicative of the proliferation of cells which, while escaping death from irradiation, have sustained considerable and perhaps permanent injury which is subsequently expressed as the aberrant metabolism observed. Using the metabolic inhibitor DNP reveals little that is directly ascribable to the effects of gamma irradiation since the pattern of inhibition for each is essentially similar in control and irradiated samples. The use of ATP, on the other hand, suggests that metabolic control in regenerating testes may be highly modified. Alternative hypotheses are also discussed.

INTRODUCTION

The irradiation of mouse testes is accompanied by predictable responses, the most consistent of which is weight loss[1,2]. Experimental evidence clearly shows that the atrophy of the testicular tissue is primarily due to the killing of radiosensitive spermatogonia[3,4]. Estimations of comparative sensitivities to radiation damage among germinal cells make it clear that these cells are indeed the most sensitive elements and are therefore, the most rapidly depleted[5]. However, the depletion of the germinal epithelium is also exacerbated by the additional radiation-induced destruction of spermatogonia B whose sensitivity nearly equals that of spermatogonia A[6]. For reasons not yet understood, the regeneration of these latter cells is greatly delayed[6] and may, in fact, not ensue for many weeks following a 600 rad exposure[7] unlike stem cells which initiate repopulation about seven days after a similar exposure[6]. The available data seem to imply that an optimal number of stem cells may be necessary for the regeneration of spermatogonia B to commence. Between the loss of sensitive spermatogonia A and the recovery of B cells, upon which the regeneration of

the germinal epithelium depends, there exists an extensive period of several weeks (at 600 rads) during which the epithelium consists largely of remaining Sertoli cells and regenerating spermatogonia A.

In addition to weight loss, the irradiation of testicular tissue elicits distinct increases in oxygen consumption. The cause for the elevation in the rate of oxidative processes has been variously ascribed to the activity of radioresistant cells whose inherently high oxidative activity was previously masked by the intact epithelium[8, 9] to changes in oxidative phosphorylation by reason of the similarity between the effects of 2,4-dinitrophenol (DNP) and the elevation in oxygen uptake following the irradiation of kidney slices[10]. Radiation-induced disturbances in the pituitary-gonadal axis, resulting in increased stereodogenesis, has also been implicated[11].

Attempts to further characterize the underlying causes of the increased oxygen consumption in testes that are deteriorating, have revealed perturbations in the activity of individual enzymes[12-17]. Unfortunately, a comprehensive view of metabolism is not easily provided by the study of individual enzymes since a mere reconstruction of isolated events does not necessarily reflect the whole. I have thus measured oxygen consumption in irradiated testes in the presence of metabolic inhibitors as a means of probing the injurious effects of radiation on the functional integrity of metabolism. Given the detailed knowledge about the cellular events associated with the damaged epithelium following exposure, an attempt was also made to establish a correlation between either the depletion or the repopulation phases of the testes and the attendant changes in metabolism.

MATERIALS AND METHODS

The data presented in this study are the result of three separate irradiation experiments carried out over a period of two years. All animals were derived from an SW stock and maintained locally with free access to food and water. Irradiation was performed at the Flagstaff Community Hospital with a Picker C4W/60 cobalt therapy unit. Mice were anesthetized in groups of eight by the intraperitoneal injection of sodium pentobarbitol (80 mg/kg). They were arranged along the periphery of a 15 × 10 cm field area with only the scrotum and tail exposed. A Lucite platform was suspended over the area upon which lead blocks were placed so as to provide additional shielding of the unexposed parts. Exposures of 600 rads were administered from a distance of 70 cm at a rate of 51 rads per minute.

Measurements of oxygen were made using a Gilson Differential Respirometer equipped with 20 channels and according to the methods of Umbreit et al.[18]. Two experimental and one control mice were used each week after exposure beginning on the day of irradiation. Each mouse was lightly anesthetized and killed by cervical dislocation. The dissected testes were rapidly weighed to the

nearest 0.1 mg, decapsulated in 5 ml of cold TKMS buffer (tris-HCl, 0.05M; KCl, 0.025M; MgCl$_2$, 0.005M; sucrose 0.25M; pH 7.5) and macerated by finely cutting with scalpels. A homogeneous suspension was obtained by forceful aspiration through a 5 ml pipette. Two ml of the macerate were placed in each of two Warburg flasks. Fresh 20% KOH (200 μl) was placed in the center wells with a folded strip of Whatman No. 1 paper for enhanced CO_2 capture. Following an equilibration period of 20 minutes at 37°C, readings were taken at 15 minutes intervals for a total of 90 minutes.

To measure the effects of the inhibitors (DNP and ATP), 8 to 10 testes were pooled, weighed, decapsulated and macerated in cold TKMS buffer. Four ml of the macerate were dispensed into individual Petri plates and 1 ml of various ATP concentrations were added. A constant weight ratio of ATP to tissue of 1/16.16 was maintained throughout. In experiments in which the concentration of ATP was held constant, as in the case with amino acids, the inhibitor was added directly to the macerate and 1 ml of dissolved casein hydrolysate was subsequently added in the appropriate dilutions. The concentration of amino acids in casein was estimated on the basis of an average molecular weight of 120 daltons. A similar procedure was followed when DNP was used with an inhibitor to tissue weight ratio of 1/425. Control Petri plates consisted of 4 ml of macerate to which 1 ml of TKMS was added.

Uneven delivery of the samples into the Warburg flasks required that the amount of tissue in each flask be determined by differential weighing (to the nearest mg) before and after filling. The values thus obtained were converted to actual weight of tissue by taking into account its volume (1.195 ml per gm) and its percent partitioning within the macerate as follows: if 1.5 gm of testes were added to 30 ml of TKMS, the final volume became, 30 ml + (1.5 gm $\times$ 1.195) = 31.79 ml. The % tissue partitioning was calculated to be: (31.79 ml − 30 ml):31.79 = 5.6% tissue. If the differential weight of the macerate was 2.001 gm, then the tissue weight within the flask was: 2.001 x 0.056 = 0.112 gm.

Weekly samples of blood were obtained from the retro-orbital sinus of lightly anesthetized mice. Total hemoglobin was determined by the Drabkin method[19]. Electrophoresis of hemoglobin samples was conducted on cellulose acetate[19]. Hematocrit values were derived from centrifuged heparinized blood samples in capillaries.

RESULTS

General Observations

Regarding such parameters as hemoglobin contents, electrophoresis, and hematocrit, no specific pattern could be detected and no statistically significant differences could be observed between irradiated and control animals. It was surmised that the changes observed were probably reflecting normal internal

fluctuations whose nature was not known. The weight of the animals did not change during the duration of testing. In contrast, a marked decrease in testicular weight became apparent beginning three weeks after exposure. The rapid decline in weight reached its maximum during the fourth week at a level which represented 40% of the original weight. In terms of actual weight loss, irradiated testes atrophied from an average of 230 mg to a low of 90 mg. Slow recovery of testicular weight was initiated 7 weeks after irradiation.

Oxygen Consumption

For the purposes of these experiments, it seemed reasonable to measure respiration without an exogenous supply of energy so as to provide a better assessment of the intrinsic metabolic properties of the tissues. Under those conditions, in the presence of 0.25 M sucrose, respiration could easily be maintained for 90 minutes. The contribution of the substrate to the total oxidation
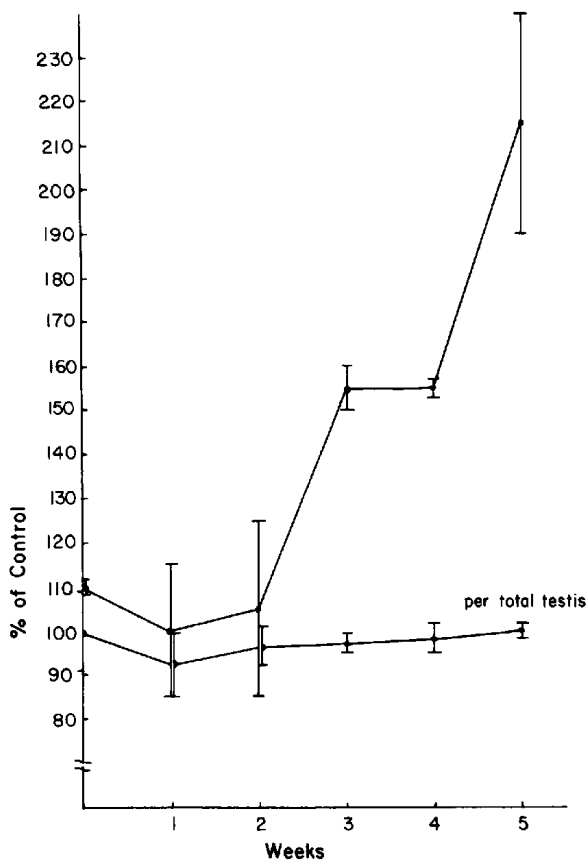

Fig. 1.   Respiration in irradiated testes. Vertical bars represent ± 1 standard deviation of data collected during a 2 year period.

of the tissues was found to be minimal as was that of sperm isolated by filtration through bolting cloth (80 mesh) and tested under identical conditions (not more than 6% of the total consumption after 90 minutes). The eventual loss of sperm, following irradiation, was, therefore, not considered significant in the evaluation of respiration in testes.

The irradiation of testicular tissue induced a gradual increase in oxygen uptake by the third week after exposure (Fig. 1). By the fifth week, the increase reached a peak value of more than 200%. That the elevation in uptake was an inherent property of the injured tissue was shown in two ways. First, plotting the results in terms of total testicular weight revealed that the high rate of oxidation was independent of weight loss. Secondly, a separate experiment was performed to ascertain that the enhanced oxidation was not artifactually induced by a more efficient oxygen diffusion due to the reduced amount of tissue present in the same volume of buffer. A series of known dilutions of normal testes were analyzed under the same conditions and shown to consume identical quantities of oxygen after proper correction for the differences in weight. The results are, therefore, correctly representative of specific activity and the enhancement seen is indeed an intrinsic property of the irradiated tissues.

Effects of DNP

Under the conditions of this study the effects of DNP are inhibitory in both control and irradiated samples. Inhibition is initially pronounced at low concentrations of the inhibitor but reaches a maximum of 42% in irradiated testes and 34% in control samples; a difference not considered significant. It was also observed that the enhanced respiration of irradiated tissues was in no way influenced by the addition of DNP as indicated by the fact that the pattern of inhibition was identical in both tissues. No specific effects of gamma irradiation could, therefore, be correlated with the inhibitory patterns of DNP.

Effects of ATP

High concentrations of ATP result in strong inhibition culminating in the total suppression of oxidation at 20 mM (Fig. 2). As in the case with DNP, the overall elevation in metabolic activity of irradiated testes is evident and remains unaffected by the presence of ATP. But a significant departure from parallelism is seen when high concentrations of ATP are tested in irradiated tissues. In contrast to the inhibition observable from 14 to 20 mM in control tissues, no suppression in activity can be detected in experimental testes. That the results in control tissues are due to the specific effects of ATP can be shown by measuring oxygen uptake in the presence of ATP and equimolar concentrations of $MgCl_2$. As expected, total chelation of Mg^{++} by ATP vitiates the inhibitory effects (Fig. 2).
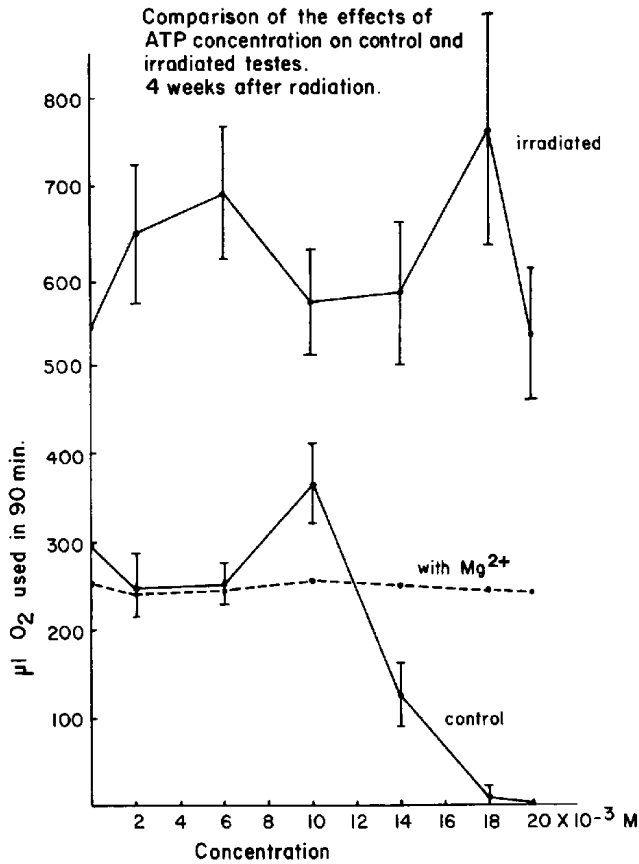
Fig. 2.   Effects of ATP. Pools of 8 to 10 testes were macerated in
          cold TKMS buffer, pH 7.5. To 4 ml of the macerate 1 ml of
          APT was added to the final concentrations indicated. Du-
          plicate flasks were incubated each containing 2 ml of the
          macerate. The vertical bars represent ± 1 S.D. from 3 sepa-
          rate experiments. The specific effects of ATP in control
          tissues are shown by its chelation of equimolar concentra-
          tions of Mg^{2+}.

Effects of ATP and Exogenous Amino Acids

In view of the refractoriness of irradiated tissues to the effects of ATP, it
was of interest to attempt to delineate which metabolic pathway was being
affected at various concentrations. It was surmised that the inhibition of glyco-
lysis could be distinguished from that of the aerobic pathway by the mitigating
effects of an exogenously supplied pool of free amino acids some of which are
known to enter an unperturbed Krebs cycle. In contrast, severe or complete
inhibition was expected at concentrations of ATP where aerobic enzymes are

suppressed in addition to those of anaerobic glycolysis. In this latter case, escape from inhibition would not be expected to occur even by the provision of an exogenous supply of amino acids.

The results of such an experiment are shown in Fig. 3. When increasing concentrations of free amino acids are given to normal tissues in conjunction with various amounts of ATP, three principal patterns emerge. On the one hand, 20 mM ATP is completely inhibitory irrespective of the amount of free amino acids available; a condition strongly suggesting that both anaerobic and aerobic pathways are suppressed. At concentrations of 3 mM and less, the influence of ATP is greatly diminished and the metabolic activity resembles that of control samples which are essentially independent of amino acid metabolism. At a con-
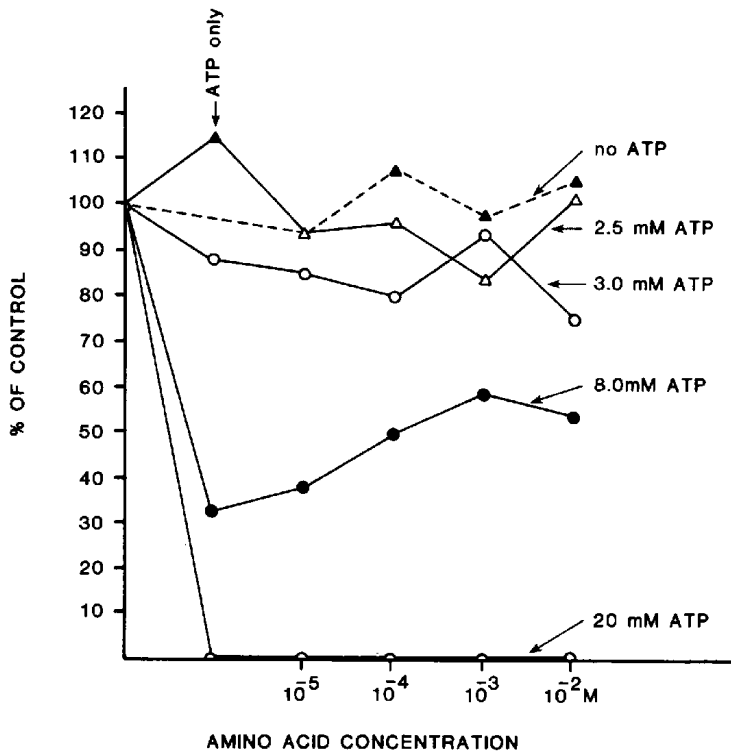

Fig. 3.   Effects of ATP on the amino acid utilization of irradiated testes. Pools of 8 to 10 testes were macerated in TKMS buffer, pH 7.5. ATP, at the concentrations indicated, was directly added to the macerate. 1 ml of casein hydrolyzate was added to each of several Petri dishes containing 4 ml of the macerate. Amino acid concentration was based on an average molecular weight of 120 daltons. Duplicate flasks were run with 2 ml of the macerate in each. Each point represents the average of three separate determinations.

centration of 8 mM, a definite escape from the inhibitory effects of ATP can be seen in proportion to the concentration of amino acids provided. It would thus appear that at concentrations higher than 8 mM, ATP affects the Krebs cycle enzymes as well as glycolysis and no escape is, therefore, possible. The enhancement of activity at 8 mM ATP implies that amino acids can be successfully utilized as an alternative substrate when glycolysis is uniquely blocked at that concentration of inhibitor.

The evidence seems to indicate that the range of ATP concentration at which it is inhibitory in normal testes coincides with the activity of the Krebs cycle enzymes. It follows, therefore, that irradiated tissues may possess aerobic enzymes which have been modified by the gamma exposure so as to lessen the regulatory effects of ATP, a condition highly favorable to the maintenance of an elevated metabolic activity.

DISCUSSION

The increase in the metabolic activity of irradiated testes is concurrent with testicular weight losses but is only evident after a quiescent period of two weeks following exposure, and coincides with extensive cell death[20, 6]. Thus, the repopulation of the germinal epithelium and the increased metabolic activity must be linked to cells that have originally escaped the radiation damage. The data, therefore, contradict the previous assertions that inherently more active cells are being unmasked by the death of others[8, 9]. The postulated "unmasking principle" would have revealed distinct increases in oxidation during the first two weeks after exposure when cell death is maximal.

The data are consistent with the proposition that radioresistant and therefore surviving cells may be most vulnerable to incipient but permanent genetic-metabolic damage. It can then be assumed that the ultimate repopulation of testes is derived from a small stock of cells which, while escaping death, must have sustained irreversible injury.

With DNP, uncoupling of oxidative phosphorylation could not be demonstrated under the conditions of this experiment even though the range of concentrations was adequate[21]. Its specific effects on oxidative phosphorylation in irradiated testes, therefore, could not be evaluated. The observed inhibitory effects could be accounted for by several previously reported phenomena; osmotic collapse of mitochondria[22, 23], impairment of substrate transport[22], and inhibition of specific dehydrogenases[24, 25].

The inhibitory pattern of ATP within the concentration range used conforms to the results of Lardy and Parks[26] in which the addition of excess amounts of ATP induced a reduction in the incorporation of $^{14}CO_2$ into succinate. The inhibition could be mitigated by equivalent concentrations of Mg^{++} indicating

that the function of ATP was to chelate Mg^{++} which is essential for metabolism. In this study, aerobic respiration has been shown to be preferentially affected by ATP at concentrations above 8 mM leading to its complete inhibition at 20 mM. Accordingly, the irradiation of testes leaves glycolysis unaffected since the enhancement of respiration is unperturbed by the exposure. This is contrary to a previous report which indicated that several anaerobic enzymes were depressed following irradiation and that a concommittant accumulation of glycogen resulted from this apparent decline in glycolytic activity[16]. The evidence presented also indicates that Krebs cycle enzymes are radioresistant as they have previously been shown to be even at lethal doses[14]. The underlying causes for such resiliency are not known but modifications of molecules may be involved with the result that radiation-induced escape from regulation by ATP could, in fact, provide uniquely favorable conditions for the maintenance of such an elevated metabolism. Possible perturbances in Mg^{++} metabolism should also be considered.

Irrespective of the inhibitor, the elevation in oxygen uptake remains high. This could be viewed as an acceleration of reactions involving oxygen. As an example, metabolic enzymes irradiated *in vivo* are activated in contrast to purified enzymes which are depressed[27]; a difference attributed to the protective effects of bound substrates and other cytoplasmic constituents.

An alternative model for the radiation-induced elevation of oxygen consumption is provided by Kurnick[28,29] who suggested the presence of natural inhibitors in normal tissues. The enhanced activity is thought to result either from the radiation-induced destruction of these inhibitors or from an impairment of their synthesis.

Due to the apparent lack of response to either DNP or ATP enhancement of oxygen consumption after radiation could also be conceived in non-metabolic terms. The delay in the enhancement of metabolic activity could reflect the gradual cumulation of oxidized products observable only two weeks after exposure. Thus still higher doses should considerably accelerate these reactions with the ultimate result of shortening the interval between exposure and the initiation of the elevated oxygen uptake. Mendelsohn's investigations are relevant to this hypothesis since enhancement of oxidation was observed within 30 minutes after an extremely high exposure of more than 100,000 rads[10]. It is not likely that such a large dose could support the kind of metabolic integrity necessary for the maintenance of an elevated activity unless oxygen utilization is conceived in non-metabolic terms. To this end, Roberts *et al.*[30] have proposed that oxygen may serve as a scavenger of free radicals formed during exposure.

The information provided in this study is insufficient to delineate the precise mechanisms involved in the induction of an increase in metabolic activity by gamma rays. Additional work should ellucidate between the above mentioned alternatives and thus provide tangible working hypotheses. Given the ubiquity

of the response a search for its causes may be valuable for an appropriate under-
standing of the effects of radiation in testes and in other tissues as well.

ACKNOWLEDGEMENT

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