Europ. J. Cancer Vol. 17, pp. 21–28.
© Pergamon Press Ltd. 1981. Printed in Great Britain

0014-2964/81/0101-0021 $02.00/0

Effects of Misonidazole, Irradiation and Hyperthermia on Lysosomal Enzyme Activity in Mouse Tumours*

G. M. BARRATT and E. D. WILLS

Department of Biochemistry, The Medical Colllege of St. Bartholomew's Hospital, Charterhouse Square, London, EC1M 6BQ, United Kingdom

Abstract—*Male C3H mice bearing transplanted tumours were treated with hyperthermia, γ-irradiation and the radiosensitising drug misonidazole. The activity of tumour lysosomal acid phosphatase and β-glucuronidase was determined using quantitative cytochemical techniques which measure both lysosomal membrane permeability and enzyme activity. Misonidazole* (0.5 mg/g) *had no effect on the membrane permeability or enzyme activity of tumour lysosomes* 1 hr *after injection; but* 25 hr *after the drug treatment the permeability of the lysosomal membrane to the substrate was increased to* 1.7 *times control. Increases in the lysosomal enzyme activity and membrane permeability were, however, observed* 1 hr *after combined treatment with misonidazole and irradiation, although neither the drug nor irradiation given alone affected the lysosomes* 1 hr *after treatment. Twenty-five hours after treatment of tumours with* 0.5 mg/g *misonidazole given* 25 min *before irradiation of tumours with* 1 krad, *permeability of the lysosomal membrane had increased to* 2.3 *times the control. The effects of the irradiation and the radiosensitisers were thus synergistic. Hyperthermic treatment of tumours at* 43°C *for* 1 hr *caused increases in the lysosomal membrane permeability and enzyme activity measured immediately after exposure, but misonidazole reduced both membrane permeability and enzyme activity. These experiments have demonstrated that misonidazole and irradiation act synergistically to cause increased lysosomal activity, but that misonidazole depresses the effect of hyperthermia on lysosomes.*

INTRODUCTION

MISONIDAZOLE (Ro-07-0582) is one of a group of compounds which sensitise the normally radioresistant hypoxic tumour cells to radiation and are therefore potentially very useful in radiotherapy [1–3]. The drug also has a cytotoxic effect alone on hypoxic cells [1, 4, 5]. The selective toxicity of misonidazole towards cultured cells is increased by hyperthermia [6, 7], misonidazole enhances the hyperthermic damage to EMT6 tumour cells grown *in vivo* [8, 9] and exposure of cells to misonidazole at temperatures above normal increased the cytotoxicity of the drug [10]. The radiosensitising effect of the drug in murine tumours is also increased by elevated temperatures [11].

Activation of lysosomal enzymes or enzyme release has been demonstrated after the irradiation of mouse mammary tumours [12, 13] and increases in the permeability of the lysosomal membrane and the activity of the lysosomal enzymes occur following hyperthermic treatment of tumours [14]. Misonidazole synergistically acts with both irradiation and hyperthermia to reduce the rate of growth of tumours, and this investigation has been designed to study the effects of these radiosensitisers on tumour lysosomes.

Effects of misonidazole administration, both alone and in combination with irradiation and hyperthermia, have been studied on the lysosomes of C3H mammary tumours. Lysosomal enzyme activities have been measured using quantitative cytochemical techniques developed by Wills and co-workers [12, 14–16].

Accepted 8 July 1980.
*This investigation was supported by the National Cancer Institute, Grant No. CA 5R01-18286.

MATERIALS AND METHODS

Animals

Male C3H mice were obtained from the

Department of Radiobiology of St. Bartholomew's Hospital Medical College. Small pieces of spontaneously-arising tumour from a female mouse were implanted in one thigh. The tumours were used in experiments when they had grown to a diameter of about 1 cm.

Experimental plan

Three tumour-bearing mice were used in most experiments to investigate the effect of each treatment described.

Hyperthermia

The tumours were treated with hyperthermia *in vivo* by warming the legs of the animals in a water bath. The mice were anaesthetised with 40 mg/kg of pentobarbitone sodium (Sagatal) when misonidazole was given to the animal, or 60 mg/kg if misonidazole was not used, injected intraperitoneally, and were placed in a vertical perspex tube which fitted into a metal rack with 2 holes to accommodate the feet. The rack was placed in a small perspex waterbath around which water from a larger bath was circulated. The temperature of the latter was controlled by a toluene/mercury regulator, and was adjusted so that the smaller bath reached the desired temperature, which in these experiments was 43°C. The temperature was monitored throughout the experiment using a thermometer accurate to 0.1°C, and did not vary more than a maximum of 0.3°C from the mean of 43°C. The temperature within the tumour was between 41.8°C and 42.2°C. This was measured with a probe attached to a Light Instruments electric thermometer, reading to 0.05°C accuracy.

Radiation

γ-rays from a ^{60}Co source in a 3000 Ci Gammabeam unit were used. Animals were first anaesthetised as described, and placed in a perspex box. The box was placed behind 10 cm of lead blocks, with a small conical hole 1–2.5 cm in diameter, to permit irradiation of the tumour and shield the remainder of the body. The dose given to the tumour was 1 krad, at a dose rate of 400 rad/min; while the rest of the body received only 0.1% of this dose.

Preparation of sections

Animals were killed by breaking the neck. The tumour was dissected out, washed and cut into pieces about 3 × 3 × 3 mm, discarding the extremities and any necrotic areas of tumour. The blocks were frozen and mounted according to the method of Chayen *et al.* [17]. Sections were cut at 8 μm on a Cambridge rocking microtome in a Bright's cryostat. The cabinet temperature was −20°C and the knife temperature −40°C. Sections were stored in the cryostat for 1–2 hr before use.

Injections

Misonidazole was given in single intraperitoneal injections, as a suspension in about 0.5 ml of 0.9% (w/v) NaCl solution.

Cytochemical procedures

Lysosomal acid phosphatase activity was measured in sections by the Gomori lead sulphide method described by Aikman and Wills [15] and Clarke and Wills [12]. Incubation times of up to 1 hr were used. The absorbance was read on a Vickers M85 scanning and integrating microdensitometer, at 550 nm, ×40 objective, and No. 2 spot size. Ten readings were taken from each of three sections for each incubation time, and for the control. The mean density of the control was subtracted from the mean density obtained for each incubation time, and average values were calculated from readings obtained from tumours that had been given the same treatment. The progress curve obtained by plotting absorbance against time was used to determine rates of enzyme action [15].

β-Glucuronidase was determined by the method of Fishman and Goldman [18], described by Chayen *et al.* [17]. Readings were taken at 635 nm, ×40 objective, and spot size 2, and plotted as for acid phosphatase.

RESULTS

The effect of misonidazole on the lysosomal enzyme activity in tumours

Single doses of 0.5 mg/g of misonidazole were given to groups of three mice which were killed either 1 or 25 hr after treatment.

One hour after injection, misonidazole did not cause a significant increase in the rate of acid phosphatase activity or of β-glucuronidase activity as compared to controls. In contrast, the initial rates of both acid phosphatase and β-glucuronidase were increased to about 1.6 times control 25 hr after the injection of misonidazole, although the overall rates were not affected (Figs. 1 and 2). The increase in the rate over the first 20 min of

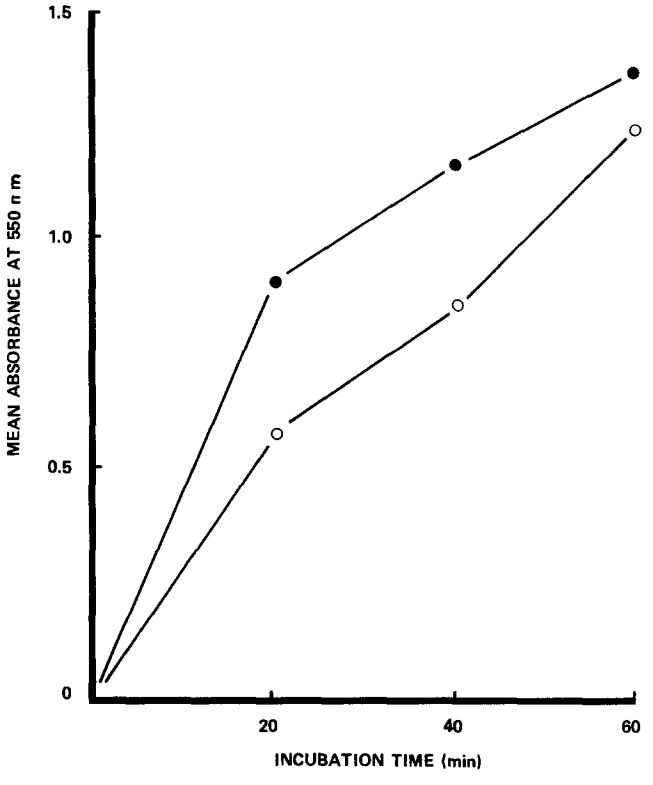

Fig. 1. *Lysosomal acid phosphatase activity in mouse tumours after treatment with misonidazole.* ○, *Sections cut from untreated tumours.* ●, *Section cut from tumours from mice treated with* 0.5 mg/g *misonidazole* 25 hr *before death.*

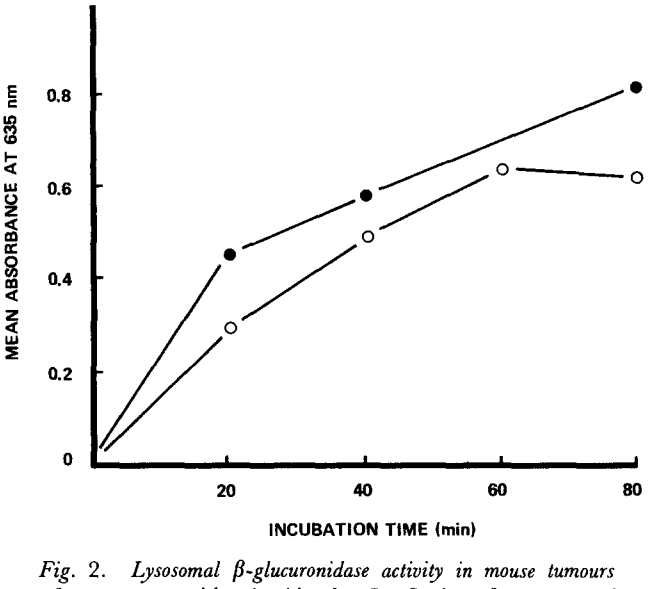

Fig. 2. *Lysosomal β-glucuronidase activity in mouse tumours after treatment with misonidazole.* ○, *Sections from untreated tumours.* ●, *Sections from tumours from mice treated with* 0.5 mg/g *misonidazole* 25 hr *before death.*

incubation in medium indicates that the lysosomal membrane had been rendered more permeable to the substrates of acid phosphatase and β-glucuronidase by the administration of misonidazole.

The effect of local irradiation on the lysosomal enzymes activity tumours

One hour after local irradiation of tumours with 1 krad, the rates of both acid phos-

phatase and β glucuronidase were not significantly different from the control value but 25 hr after irradiation the initial rate of acid phosphatase activity was increased to 1.7 times control. The initial rate of β-glucuronidase was increased to 1.27 times the control but this was not significantly different from the control

The effect of misonidazole combined with local irradiation on the lysosomal enzyme activity of tumours

Tumours were irradiated with 1 krad 25 min after the injection of 0.5 mg/g of misonidazole. One hour after this treatment the activities of both acid phosphatase and β-glucuronidase enzyme activity were increased significantly (Table 1). These increases represent an interaction between misonidazole and irradiation, because neither treatment alone affected the lysosomes within 1 hr.

The effect of hyperthermia on the lysosomal enzyme activity in tumours

The initial rates of both acid phosphatase and β-glucuronidase were increased in tumours examined immediately after warming in a water bath at 43°C for 1 hr (tumour temperature 42°C), as were the overall rates (Table 2). Thus hyperthermia alone increased both the lysosomal membrane permeability and lysosomal enzyme activity.

The effect of misonidazole in combination with hyperthermia on the lysosomal enzyme activity in tumours

Misonidazole (0.5 mg/g) was injected into mice 30 min before treatment in a water bath at 43°C for 1 hr. The initial rate of acid phosphatase activity measured immediately after this treatment was not different from the control, and represented a significant decrease

Table 1. *Lysosomal enzyme activity in C3H mouse tumours after treatment with* 0.5 mg/g *misonidazole followed by* 1000 rad *local irradiation*

Treatment	Acid phosphatase activity as % control (±S.E.M.) Rate over: 0–20 min	0–60 min	β-glucuronidase activity as % control (±S.E.M.). Rate over: 0–20 min	0–80 min
1 hr after MIS-irradiation	121 ±12 (<0.002)*	143 ±12 (<0.001)	146 ±12 (<0.05)	133 ±25 (<0.01)
25 hr after MIS-irradiation	226 ±58 (<0.05)	169 ±37 (<0.01)	150 ±21 (N.S.)	177 ±20 (<0.001)

*Values in parentheses are *P* values for a two-sample *t*-test comparing the results with the effect of irradiation alone. N.S. denotes $P > 0.05$, not significant.

Twenty-five hours after the combination treatment the activities of the two lysosomal enzymes were substantially increased (Table 1) and the initial rate of acid phosphatase was raised to 2.3 times the control. This value was consistent with an additive effect of the two treatments on the lysosomal membrane. The overall rate of acid phosphatase activity was also increased (Table 1, Fig. 3), whereas neither misonidazole nor irradiation alone had increased this rate. This showed a synergistic interaction between the treatments on the lysosomal enzyme activity in tumours.

when compared to the effect of heat alone (Table 2). The overall rate was greater than the control, but was also significantly less than the value obtained after heat treatment alone.

The rates of β-glucuronidase activity were also significantly reduced by misonidazole and hyperthermia as compared to both control activity and the activity after hyperthermic treatment (Table 2).

Misonidazole had thus stabilised the lysosomes against damage and prevented the increase in membrane permeability and enzyme activity caused by hyperthermia.

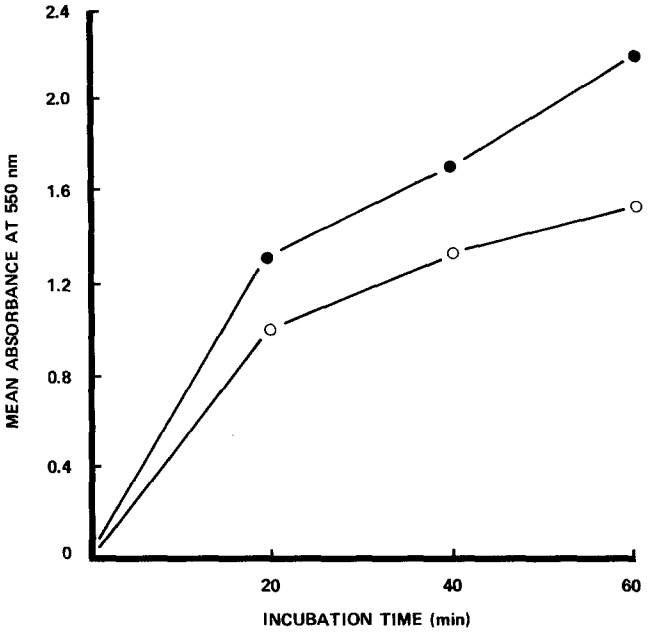

Fig. 3. Lysosomal acid phosphatase activity in mouse tumours after treatment with misonidazole and local irradiation. ○, *Sections from tumours of mice treated with* 1000 rad 25 hr *before death.* ●, Sections from tumours of mice treated with 0.5 mg/g *misonidazole followed* 25 min *later by* 1000 rad 25 hr *before death.*

Table 2. Lysosomal enzyme activity in C3H mouse tumours measured immediately after treatment with misonidazole and hyperthermia

Treatment	Acid phosphatase activity as % control (±S.E.M.). Rate over:		β-glucuronidase activity as % control (±S.E.M.). Rate over:	
	0–20 min	0–60 min	0–20 min	0–80 min
Hyperthermia— 1 hr at 43°C	149 ±17	168 ±16	115 ±14	140 ±12
P	<0.001	<0.001	<0.02	<0.01
0.5 mg/g MIS and 1 hr at 43°C	104 ±5	136 ±5	60 ±31	70 ±29
P	<0.001	<0.001	<0.001	<0.001

N.S. denotes $P > 0.05$, not significant.

The effect of misonidazole in combination with hyperthermia and irradiation on the lysosomal enzyme activity in tumours

Misonidazole (0.5 mg/g) was given 25 min before irradiation, which was followed by hyperthermia. Immediately after this treatment the activity of lysosomal acid phosphatase was significantly more than the activity after heat alone (Table 2) and also greater than the activity 1 hr after misonidazole treatment or after irradiation (Table 1). The rate over the first 20 min incubation was 195 ± 15% of the control value and over 0–60 min incubation it was 246 ± 15% of the control. The combined effects of hyperthermia, irradiation and misonidazole were therefore much greater than the effect of either hyperthermia or misonidazole given alone.

DISCUSSION

The rate of lysosomal enzyme activity measured over the first 20 min of incubation in the quantitative cytochemical method is indicative of the permeability of the lysosomal mem-

brane [15]. Misonidazole increases the initial rate (Figs. 1 and 2) and thus causes labilisation of the lysosomal membranes. The effect may be directly on the lysosomal membrane, or indirect, and caused by effects on other subcellular organelles or even on remote tissues. The toxicity of misonidazole is believed to be a result of the production of nitro or nitroso radicals, since cell killing was reduced by the simultaneous administration of cysteamine [6]. These radicals may react with sulphydryl groups on membrane proteins to cause permeability increases in the lysosomes. Modification of proteins may be the primary cause of hypoxic cell killing by misonidazole, because no chromosome damage could be detected in Chinese hamster cells even after a dose that killed 99% of the cells [6]. However, the results in Fig. 1 show that one consequence, primary or secondary, of this free radical activity is the labilisation of lysosomes. This may serve to amplify the cell damage caused by misonidazole by allowing substrates access to the lysosomal enzymes, leading to further modifications of cellular components.

Local irradiation to the tumour also causes increases in the lysosomal membrane permeability 1 day after treatment. In an earlier investigation Clarke and Wills [12] showed that the lysosomal enzyme activity in mouse C3H tumours was increased 24 hr after whole body irradiation. The experiments described in this paper also demonstrate activation 24 hr after tumour irradiation and indicate clearly that the tumour lysosomes respond directly to irradiation. The effects observed after whole body irradiation are not therefore likely to be due to abscopal influences. The effect of irradiation may involve free radical-induced lipid peroxidation of the lysosomal membrane and increased peroxidative activity has been demonstrated in lysosomes after irradiation *in vivo* and *in vitro* [19, 20]. From the results shown in Table 1, it is clear that the misonidazole and irradiation can affect the lysosomal membrane independently. However, the two treatments did interact to cause an increase in the activity of acid phosphatase and β-glucuronidase (Table 1, Fig. 3). The activation may be a result of the large increase in membrane permeability caused by the combination treatment, with the large influx of substrate activating the enzymes; or structural changes in the membrane may cause activation of membrane-associated enzymes. Alternatively, the enzymes might be directly affected by free radicals, or by factors released from the damaged nucleus as a result of the combination treatment.

We have shown previously that hyperthermia causes lysosomal enzyme activation [14] which can be demonstrated very shortly after the hyperthermic treatment.

Although it has been demonstrated that misonidazole and hyperthermia act synergistically to reduce the rate of tumour cell growth [8, 9], our experiments on lysosomes did not demonstrate an increase in lysosomal enzyme activity, and, in contrast, misonidazole treatment protected the lysosomes, especially as demonstrated by measurements of β-glucuronidase (Table 2). When, however, treatments with misonidazole and hyperthermia were combined with irradiation, a marked increase in lysosmal acid phosphatase activity was demonstrated (Results, para. vi).

The results described in this paper and those of earlier investigations from this laboratory [12, 14] have shown that irradiation, hyperthermia and misonidazole used separately cause increased activation of lysosomal enzymes and all these treatments reduce the rate of tumour cell growth. Misonidazole, which enhances the effect of irradiation on tumour regression, also enhances the effect of irradiation on lysosomal enzyme activation. This group of experimental findings therefore demonstrates a good positive correlation between the effects of these treatments on lysosomal enzyme activation and on tumour regression or cell killing. However, misonidazole did not enhance the effect of hyperthermia on lysosomes although it does enhance hyperthermic damage to cells [8, 9]. This group of experiments, therefore, in contrast to the previous group, throws doubt on an important role for lysosomes in hyperthermic damage and this view has been supported by the experiments of Hofer *et al.* [21] who showed that modification of lysosomes with drugs did not affect the response of murine carcinoma cells to hyperthermia or to combination of hyperthermia and irradiation.

An important aspect of lysosomal involvement in cellular damage is the time scale of events. Lysosomal activation after irradiation cannot be demonstrated in tumour cells until 15–18 hr after the irradiation (Table 1) [12] and a similar time interval must elapse after irradiation of HeLa cells before lysosomal enzyme activation can be demonstrated [22]. In contrast to this effect, hyperthermia causes activation of lysosomal enzymes within 1 hr after treatment [14]. After 24 hr recovery takes place so that the lysosomal enzyme activity

recovers normal control values. The radiosensitising drug, misonidazole, also causes activation of lysosomal enzymes shortly after treatment (Figs. 1 and 2).

The transitory activation of lysosomal enzymes may, however, be important in the future development of the cell by digesting vital cell components or releasing hydrolytic enzymes which cause damage which is shown subsequently in the life cycle of the cell.

At present, therefore, although there are unequivocal demonstrations of lysosomal enzyme activation after irradiation and hyperthermia, the importance of lysosomes in causing cell death and tumour regression must await the development of a better understanding of the role of lysosomes in treated cells.

Acknowledgements—The authors would like to thank the Department of Radiobiology, St. Bartholomew's Hospital Medical College, for assistance with supply and breeding of C3H tumour-bearing mice.

REFERENCES

1. S. A. Hill and J. F. Fowler, Radiosensitising and cytocidal effects on hypoxic cells of Ro-07-0582 and repair of X-ray injury, in an experimental mouse tumour. *Brit. J. Cancer* **35,** 461 (1977).
2. N. J. McNally, J. Denekamp, P. W. Sheldon and I. R. Flockhart, Hypoxic cell sensitisation by misonidazole (Ro-07-0582) *in vivo* and *in vitro*. *Brit. J. Radiol.* **51,** 317 (1978).
3. P. W. Sheldon and S. A. Hill, Further investigations of the effects of the hypoxic cell radiosensitiser Ro-07-0582 on local control of a mouse tumour. *Brit. J. Cancer* **36,** 198 (1977).
4. J. Denekamp, Cytotoxicity and radiosensitisation in mouse and man, *Brit. J. Radiol.* **57,** 636 (1978).
5. I. J. Stratford, Split dose experiments with misonidazole. *Brit. J. Cancer* **38,** 130 (1978).
6. E. J. Hall, M. Astor, C. Geard and J. Biaglow, Cytotoxicity of Ro-07-0582—Enhancement by hyperthermia and protection of cysteamine, *Brit. J. Cancer* **35,** 809 (1977).
7. I. J. Stratford and G. E. Adams, The effect of hyperthermia on the differential cytotoxicity of a hypoxic cell radiosensitiser Ro-07-0582 on mammalian cells *in vitro*, *Brit. J. Cancer* **35,** 307 (1977).
8. M. Bleehen, D. J. Honess and J. E. Morgan, Interaction of hyperthermia and the hypoxic cell sensitiser Ro-07-0582 on the EMT6 mouse tumour. *Brit. J. Cancer* **35,** 299 (1977).
9. D. J. Honess, J. E. Morgan and N. M. Bleehen, The hyperthermic potentiation of the cytotoxic effect of misonidazole on the EMT6 mouse tumour; relevance of *in vitro* measurement of the *in vivo* effect. *Brit. J. Cancer* **37,** Suppl. 3, 173 (1978).
10. K. C. George, D. G. Hirst and N. J. McNally, The effect of hyperthermia on the cytotoxicity of the radiosensitiser Ro-07-0582 in a solid mouse tumour. *Brit. J. Cancer* **35,** 372 (1977).
11. K. G. Hofer, M. G. Hofer, J. Ieracitano and W. H. McLaughlin, Radiosensitisation of hypoxic tumour cells by simultaneous administration of hyperthermia and nitroimidazoles. *Radiat. Res.* **70,** 362 (1977).
12. C. Clarke and E. D Wills, Lysosomal enzyme activation in irradiated mammary tumours. *Radiat. Res.* **67,** 435 (1976).
13. J. E. Paris and D. Brandes, The effect of X-rays on the functional status of lysosomal enzymes of mouse mammary glands. *Cancer Res.* **31,** 392 (1971).
14. G. M. Barratt and E. D. Wills, The effect of hyperthermia and radiation on lysosomal enzyme activity of mouse mammary tumours. *Europ. J. Cancer* **15,** 243 (1979).
15. A. A. Aikman and E. D. Wills, Studies on lysosomes after irradiation. I. A quantitative histochemical method for the study of lysosomal permeability and acid phosphatase activity. *Radiat. Res.* **57,** 403 (1974).
16. A. A. Aikman and E. D. Wills, Studies of lysosomes after irradiation. II. Lysosomal membrane permeability and acid phosphatase activity of lymphoid and other tissues after whole body irradiation. *Radiat. Res.* **57,** 416 (1974).

17. J. Chayen, L. Bitensky, R. G. Butcher and L. W. Poulter, *A Guide to Practical Histochemistry*. Oliver & Boyd, Edinburgh (1969).
18. W. H. Fishman and S. S. Goldman, A coupling technique for β-glucuronidase employing the substrate naphthol-AS-B1-β-D-glucosiduronic acid. *J. Histochem. Cytochem.* **13,** 441 (1965).
19. E. D. Wills and A. E. Wilkinson, Release of enzymes from lysosomes by irradiation and the relationship of lipid peroxide formation to enzyme release. *Biochem. J.* **99,** 657 (1965).
20. E. D. Wills and A. E. Wilkinson, The effect of irradiation on lipid peroxide formation in subcellular fractions. *Radiat. Res.* **31,** 732 (1967).
21. K. Hofer, B. Bruzzard and M. G. Hofer, Effect of lysosomal modification on the heat potentiation of radiation damage and direct heat death of BR8 sarcoma cells. *Europ. J. Cancer* **15,** 1449 (1979).
22. C. Reynolds and E. D. Wills, The effect of irradiation on lysosomal activation in HeLa cells. *Int. J. Radiat. Biol.* **25,** 113 (1974).