FROM THE INSTITUTE OF CANCER RESEARCH, RADIUMSTATIONEN, DK-8000 AARHUS, DENMARK.

# HISTOLOGIC AND HISTOCHEMICAL REACTIONS IN A MOUSE MAMMARY CARCINOMA FOLLOWING EXPOSURE TO COMBINED HEAT-ROENTGEN IRRADIATION

K. OVERGAARD and J. OVERGAARD

Heating of experimental tumours in situ might in many cases effect an elective and total destruction of all the tumour cells. The necessary values of temperature and time of application (curative heat dose = cHD) in the temperature range of 41 to 42.5°C were determined revealing a regular mutual relation (OVERGAARD & OVERGAARD 1972 a). The compilation of all the mutually combined temperature and time values formed a contingent regular line in a logarithmic system. It was designed "standard heat dose" (StHD) and could be used as working unit for the heat effect independent of the actual temperature range.

Similar experimental tumours were exposed successively to a local heat dose in the size of 1–1/8 StHD in the temperature range of 40.5 to 42.5°C and to local roentgen irradiation, 400 to 1 600 R (OVERGAARD & OVERGAARD 1974). Though most of the used partial doses separately were without any curative effect, cures were obtained in a reproducible number of cases depending on the size of doses of both of the components. The sequence of the components and the interposition of an interval of 24 hours between the applications did not influence the results.

The existence of a synergistic curative effect between heat and roentgen irradiation on tumour tissue was supposed. As this synergism has not been seriously considered,

Submitted for publication 17 June 1974.

no information on the microscopic appearances of tumours treated that way is available and no suggestions as to the nature of this reaction have been advanced. Therefore a series of histologic and histochemical examinations was performed concurrently with the afore-mentioned curative experiments.

## Method

More than 600 $C_3H$ mice inoculated with a highly malignant anaplastic mammary carcinoma (HB) were exposed to successive heat and roentgen irradiation (cHR) covering all combinations of doses and variations of sequence and intervals put in use in the curative experiments. Moreover, many intermediate dose combinations and a number of higher and lower doses were used. Animals were killed at short intervals for microscopy following a part of the treatment or at its end to obtain a conception of the development of the tissue reaction from the end of treatment to the final scar formation. Microscopy of the site of tumour and adjacent tissues was performed in all cured cases.

The animals were killed by suboccipital luxation; the tumour and its surroundings were excised and fixed in Helly's fluid, embedded in paraffin, routinely stained in Heidenhain's iron-haematoxylin, Mayers' haematoxylin-eosin and Harris' haematoxylin-sirius dye for connective tissue. Histochemical examinations of the acid phosphatase activity were performed on quickly frozen, unfixed tumour tissue. Seven $\mu$ cryostate sections were incubated at 37°C for 30 min in Gomori's medium for acid phosphatase and treated by the method of BARKA & ANDERSON (1963).

The technique employed permits of a reliable estimation only of the oecologic reactions in the tissues; only the main features of the internal reaction in the individual cell can be assessed, however.

## Basic Investigations

Mostly, the untreated tumour forms a central bulk of densely packed, uniformly resting tumour cells (B cells) displaying a small number of mitoses. The cells are large and bright with indistinct cell membranes. The nuclei are large and vacuolated with a sparse granular and partly reticular chromatin structure and mostly include one large nucleolus (Fig. 1). The stroma and the vascularity are sparse. Commonly, spots of spontaneous necrosis are present, even in small tumours.

Surrounding this bulk a narrow rim of active A cells appears, placed singly or in small clusters inside or outside some streaks of loose connective tissue encircling the tumour. Moderate irregular outgrowths of tumour cells may invade adjacent tissue. The mitotic activity in this area is higher.

Microscopically, tumours treated with a curative heat dose revealed intense shrinkage of the cytoplasm and nuclei in all tumour cells within a few hours (OVERGAARD & OVERGAARD 1972 a). These alterations progressed by pyknosis and karyolysis, and all the cells disintegrated completely within a few days. No mitotic activity occurred.

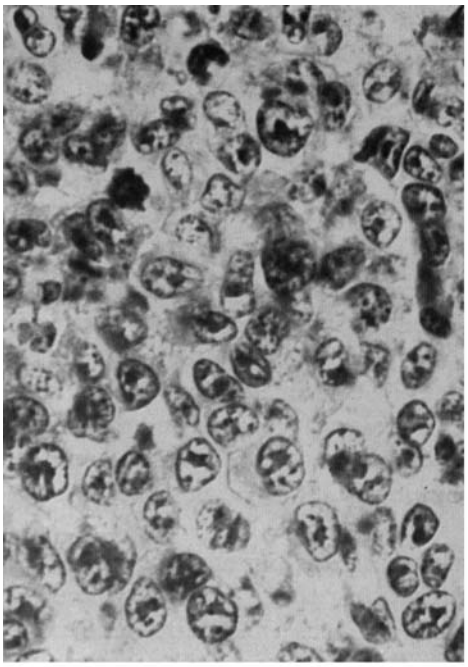


Fig. 1

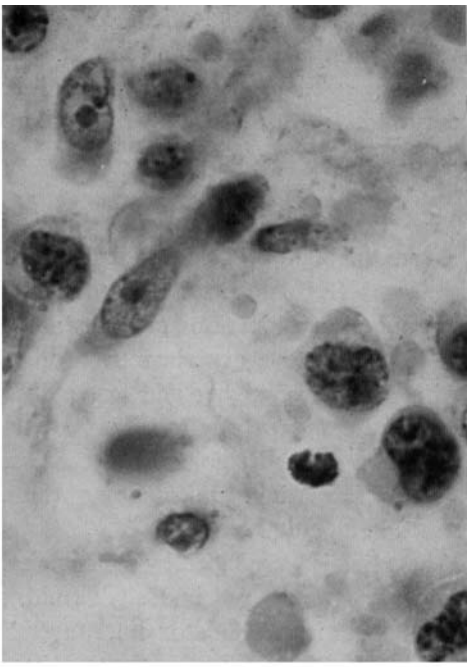


Fig. 2

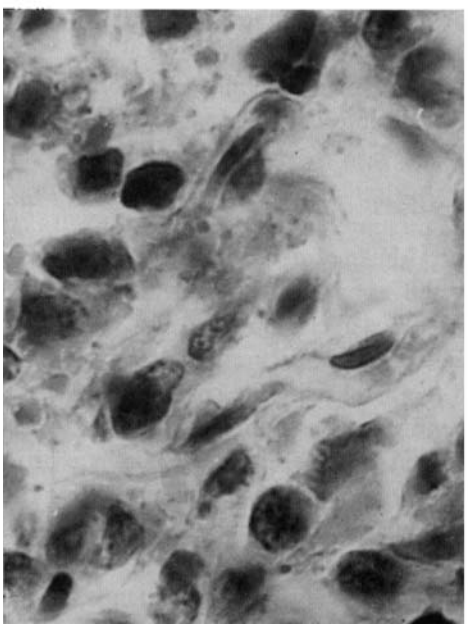


Fig. 3

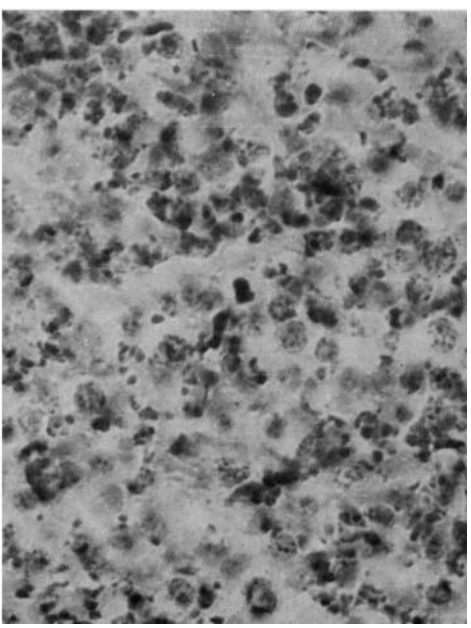
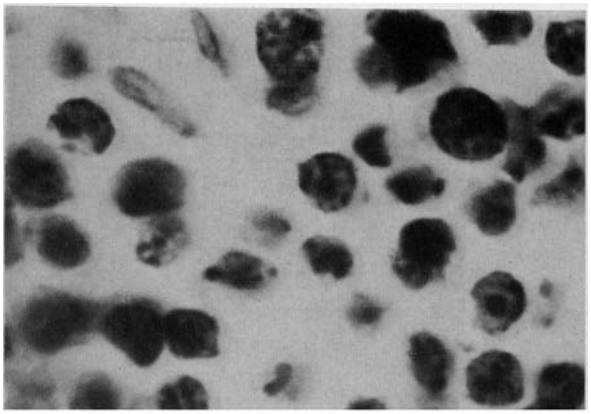
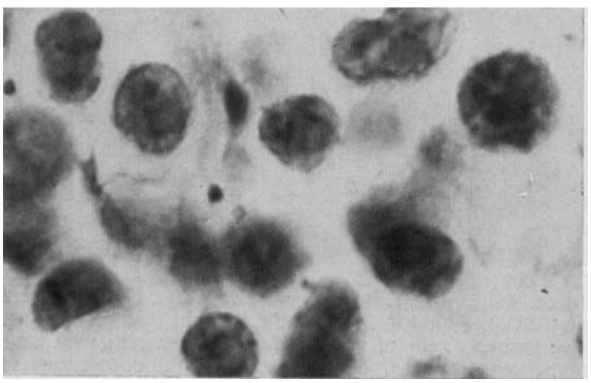


Fig. 4

(For legends see opposite page.)



a



b

Fig. 5. Rapid shift of cellular appearance by roentgen irradiation to preheat-treated tumour tissue. a) Central tissue 24 h after heat treatment 42.5°C/30 min (1 600 ×). Tumour cells isolated. Nucleus moderately shrunken; chromatin condensed in large clumps. b) 1 h after cHR treatment 42.5°C/30 min; 24 h 800 R (1 600 ×). Nuclei somewhat larger. Nuclear membrane clearly marked. Chromatin splitting up in minor granules.

The non-malignant stromal cells remained uninjured and grew rapidly, isolating the disintegrating tumour cells and forming a scar tissue.

After exposure to smaller heat doses, some tumour cells may be destroyed completely, but many displayed disturbances of various types. The number of injured

---

Fig. 1. Untreated tumour. Densely packed large cells, poorly demarcated. Nucleus large, bright with scattered chromatin spots and more intensely stained central nucleolus; abundant mitoses in the more peripheral regions, few mitoses in the more central parts. 525 ×.

Fig. 2. Central tumour tissue 2 h after cHR treatment, 200 R 42.5°C/15 min (1 350 ×). Tumour cells isolated. Cytoplasm sparse. Nucleus unaltered size. Chromatin condensed in irregular, unsharp granules; nuclear membrane demarcated. Stroma cells without definite alterations.

Fig. 3. Central tumour tissue 4 h after the same treatment as in Fig. 2 (1 350 ×). Tumour cells isolated, sparse cytoplasm. Nucleus with irregular granular concretions, some more diffusely condensed. Stroma cells shrunken with condensed nuclei, some demarcation of fibrils.

Fig. 4. Central tissue 6 days after cHR treatment 42.5°C/30 min, 1 600 R (405 ×). Highly decaying tumour tissue. Cell structures blurred. Most nuclei broken up in a large number of fine granules placed in the central region of the decaying cell. The appearance very similar to common spontaneous necrosis; scattered remnants of mitoses indicate the treatment induced nature of the process.

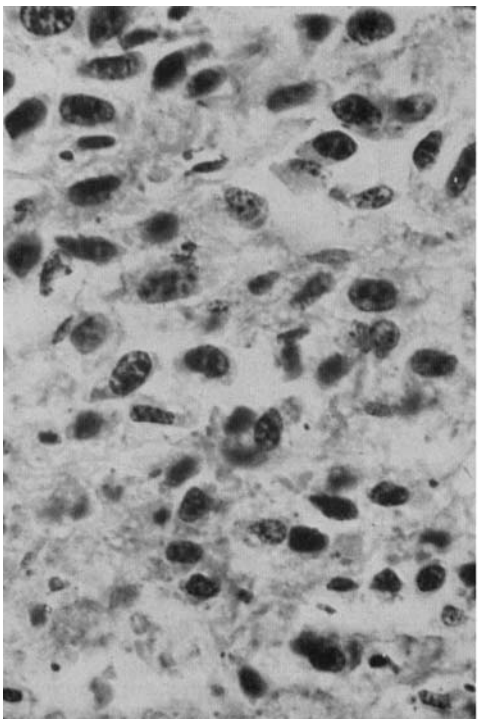


Fig. 6. Tumour tissue in the border zone. 4 h after cHR treatment 800 R 41.5°C/30 min (620 ×). Tumour cells loosely placed in connective tissue. Different forms of chromatin alterations, cells with 'large dark nuclei'; some cells with one single chromatin clot; other cells with a few medium-sized clumps and clear karrhyoplasm.

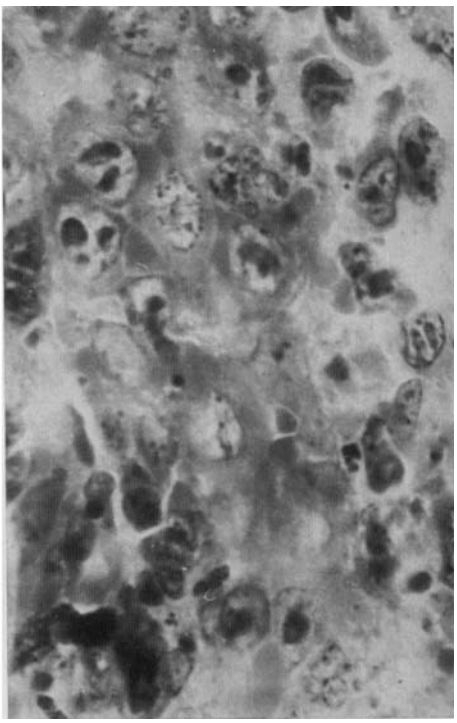
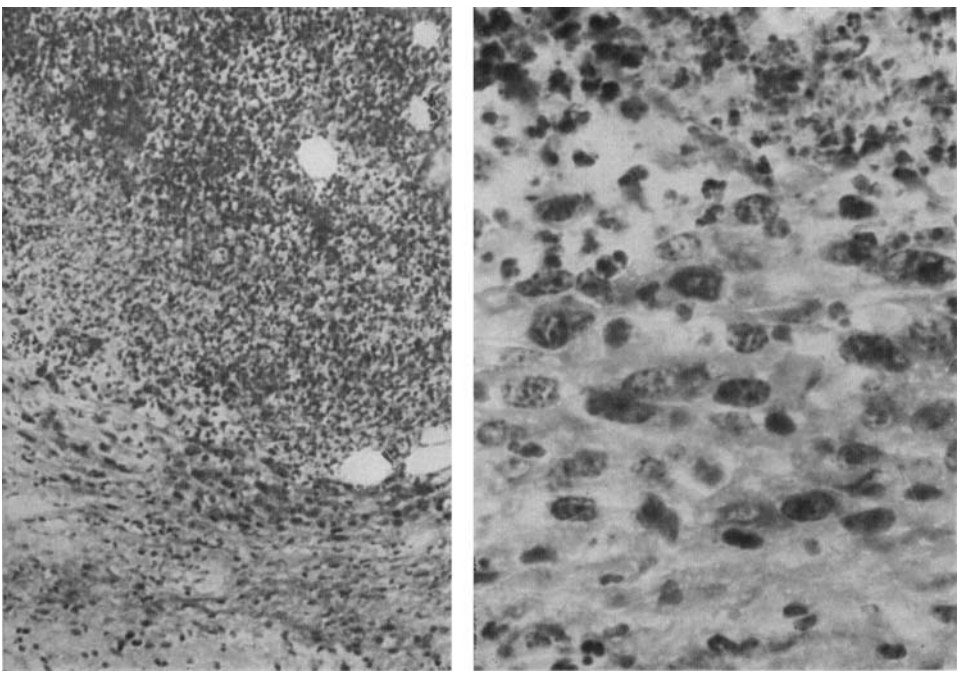


Fig. 7. Border zone 2 days after cHR treatment 800 R 24 h; 41.5°C/30 min (620 ×). Tumour cells of variated forms. Most cells with large nuclei, displaying chromatin concretions of different size; some cells with chromatolytic decay. Connective tissue unhurt (adjoining central tissue downward left).

cells and the prominent types of injuries depended on the size of the heat dose. Following relatively low heat doses, a number of mitotic irregularities—especially arrested metaphases—were visible. Some tumour cells apparently remained uninjured, and pluricentric newgrowth of tumour always occurred within a few days (OVERGAARD & OVERGAARD 1972 a).

Microscopically, following roentgen irradiation with doses within the experimental range, tumour cells mostly displayed cellular enlargement with a large, somewhat 'empty' nucleus, the chromatin being usually condensed in one or a few lumps. The nucleolus was large. Within one or a few days a number of irregular mitoses were present. Later, many cells disintegrated—some of them with great cytoplasmic growth, but invariably some cells remained uninjured, and new tumour growth could occur.

Usually, rapid growth of the connective tissue was encountered.



a b

Fig. 8. a) 4 days after cHR treatment 42.5°C/30 min, 800 R (87 ×). Marginal part of tumour. Mass of highly decaying central tumour tissue, sharply delineated by a narrow border zone containing necrobiotic tumour cells in different stages of decay, placed in the loose ring-like streaks of connective tissue. b) Detail of (a) (570 ×).

## Special Findings

Just after the end of combined heat-roentgen (cHR) treatment, moderate hyperaemia might occasionally but not constantly be present. Almost at the same time, initial signs of a very special reaction could also be revealed. The alterations were widely different in the central (hypoxic) part of the tumour and in the peripheral (euoxic) rim of tumour cells.

A fine granulation of the nuclear chromatin occurred a few hours after cHR treatment in the central tumour cells. The nucleolus was not visible; the nuclear border was distinct, often somewhat angulated. The cell borders were marked, and the cells isolated; cytoplasmic staining more intense. No clear shrinkage of cells or nuclei was visible (Figs 2, 3). At intervals of a few hours the reaction was uniform, quite independent of sequence of the components. At intervals of 24 hours the tissue could immediately after the completed cHR treatment display some similarity to the alterations produced by the factor first applied (Fig. 5 a), but in a very short time ($\frac{1}{2}$ to 2 hours) the type of reaction changed to the type just described (Fig. 5 b).

During the next few days the nuclear granules became finer and more indistinct;

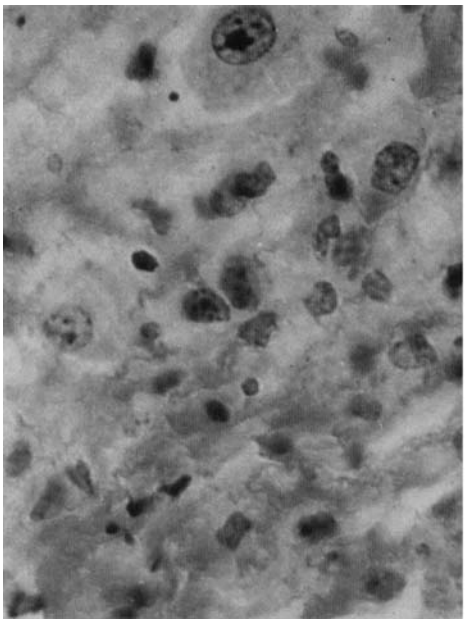


Fig. 9. 13 days after cHR treatment 800 R 42.5°C/30 min (545 ×). Progressing necrobiotic decay of tumour cells. Growth of connective tissue.

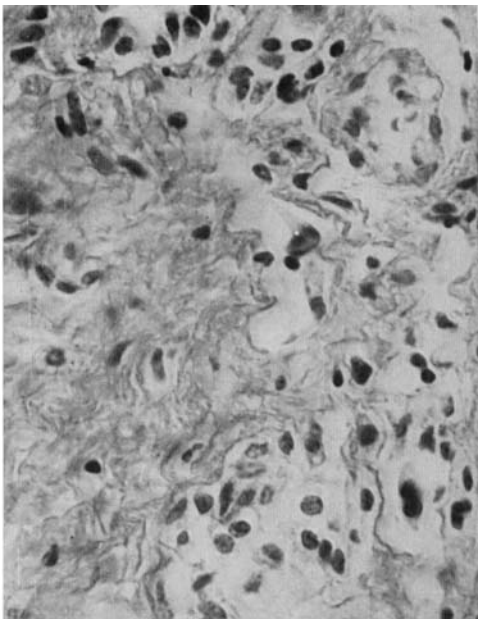


Fig. 10. 21 days after cHR treatment 42.5°C/60 min, 800 R (545 ×). Unclear remnants of tumour cells in more mature fibrotic connective tissue.

the cellular and nuclear borders disappeared. Existing mitoses and the stromal tissue were somewhat resistant, but in the following days the decay progressed, and the central part of tumour was totally transformed to an amorphic necrosis (Figs 4, 8 a). If no newgrowth of tumour occurred in the peripheral part, this necrosis could remain unchanged for 2 to 3 weeks before it was invaded by connective tissue.

In the peripheral zone of the tumour the reaction was quite different: Within hours heterogeneous chromatin alterations appeared in many cells. Cells with 'diffuse dark nuclei', cells with lumps of chromatin in an 'empty' nucleus or fine chromatin granules were intermingled with cells without any definite alterations (Fig. 6). In a couple of days, mostly cells with a clear 'empty' nucleus containing a small number of chromatin fragments were present (Figs 7, 8 b). No tendency to shrinkage of the nucleus or cytoplasm appeared; on the contrary, a number of cells had a large amount of cytoplasm combined with a sparse irregular chromatin. No fresh mitoses were seen; existent mitoses were arrested.

In the following weeks, the tumour cells gradually disappeared with progressive karyolysis, reduction of the cytoplasm and blurring of the cellular borders (Fig. 9). The loose connective tissue augment isolated the tumour cells in the network (Fig. 10), and finally—in about a month—all tumour cells disappeared and a scar was formed.

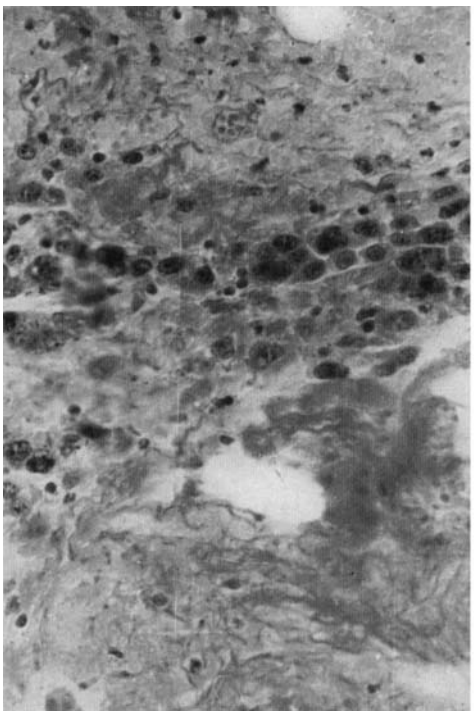


Fig. 11. 6 days after cHR treatment 42.5°C/30 min, 800 R (570 ×). New growth of tumour along the border of central necrosis. A few new necrobiotic tumour cells in the peripheral parts of tumour.

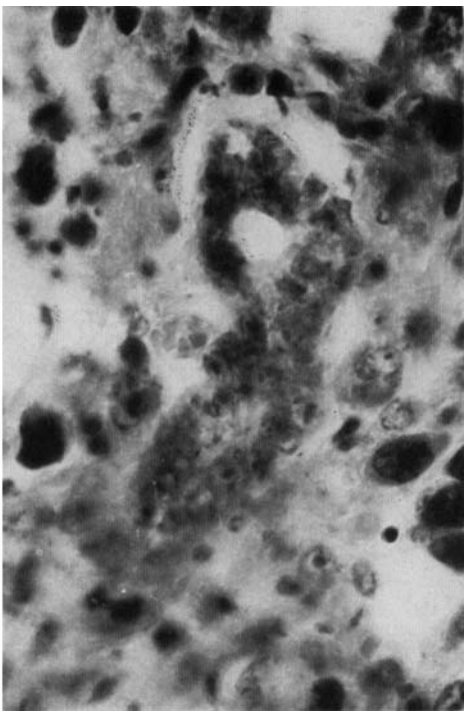


Fig. 12. 11 days after cHR treatment 42.5°C/60 min, 1 600 R (570 ×). Undamaged glandular tissue (hostess) from the normal mamma included in decaying tumour tissue.

During the first week it was impossible to point out any viable tumour cell. About this time it could be possible, in cases of unsuccessful treatment, to recognize a fresh 'normal' mitosis, and soon some newgrowth tumour cells could be demonstrated, commonly as a halo around the central necrosis (Fig. 11). Apart from a moderate number of macrophages, no cellular reaction occurred in the tissue. The surrounding normal tissue was quite unaltered. The final scar consisted of irregular streaks of connective tissue in highly varying amounts. In some cases, normal mammary gland tissue remained (from the hostess) enclosed in the scar (Fig. 12). In deep-seated tumours, normal visceral organs (liver, spleen, pancreas, adrenals, kidney or lung) could be intimately continuous with or partly enclosed in the scar.

Microscopically, it was impossible to distinguish between cases treated with high and with low subcurative doses. In all cases total destruction of the central part of tumour occurred, and the real difference could be only small variations in the number of viable cells in the border zone. Heat doses of curative size or higher displayed a similar appearance. Possibly the peripheral reaction was somewhat 'speeded up'.

Histochemical examinations of untreated tumour tissue revealed evidence of a

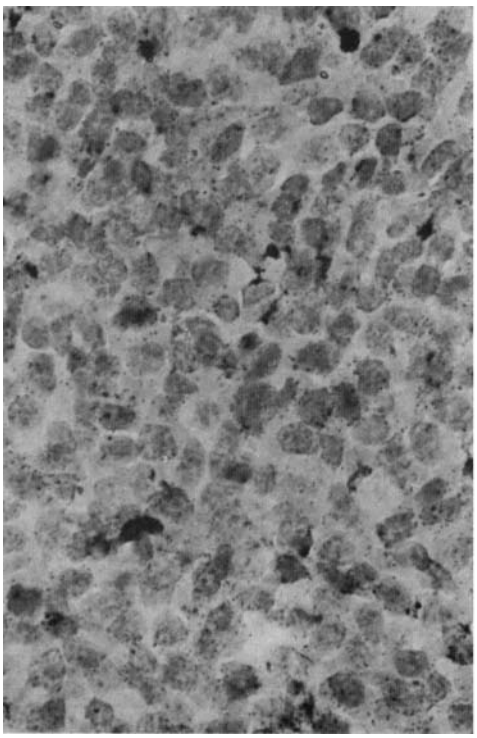


Fig. 13. Acid phosphatase reaction in untreated tumour tissue. Few small and uniform positive granules indicate a very slight lysosomal activity (365 ×).

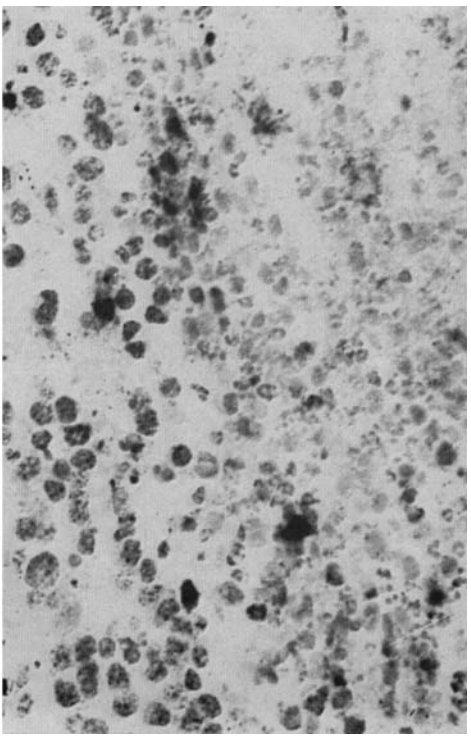


Fig. 14. 48 h after roentgen irradiation (1 000 R); an appearance almost similar to the untreated tumour, the peripheral cells with a slightly more intense positive reaction, however (215 ×).

very slight activity of lysosomal enzymes in all parts of tumour except in scattered areas of spontaneous necrosis (Fig. 13). After roentgen irradiation with doses within the range used no definite alterations in activity were observed during the first two days (Fig. 14). Just after isolated heat doses in the range used an increase in active granules was observed all over the tumour, in particular, in the more central parts. The activity reached a maximum at about 24 hours (Fig. 15). After a completed cHR treatment in all the used ranges intensification of the activity occurred. Even in the isolated and invasive cells at the tumour periphery some activity was seen (Fig. 16). In about 48 hours, the reaction had decreased in most of the tumour, signs of total necrosis appeared in the tissue; only in a narrow peripheral rim (the border zone) some signs of activity persisted.

## Discussion

In previous literature it has been supposed that heat might enhance the curative radiation effect by setting up a state of hyperaemia (SCHMIDT 1909, THEILHABER 1914)

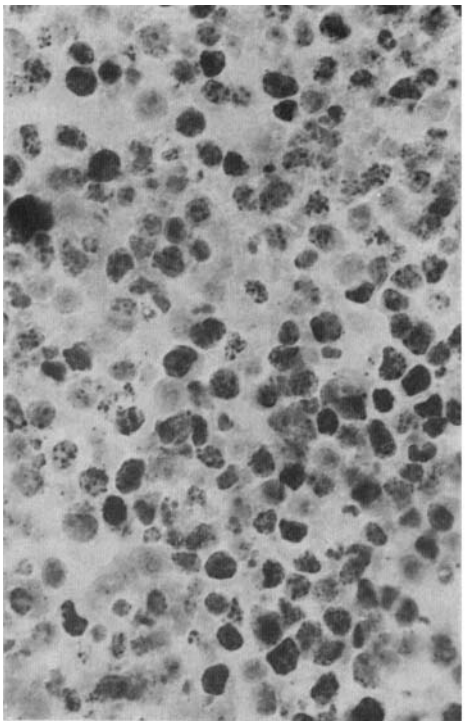


Fig. 15. Heat therapy 42.5 °C/30 min, 24 h after treatment. Closely packed small tumour cells with increased number and size of granules demonstrate an active lysosomal reaction (365 ×).

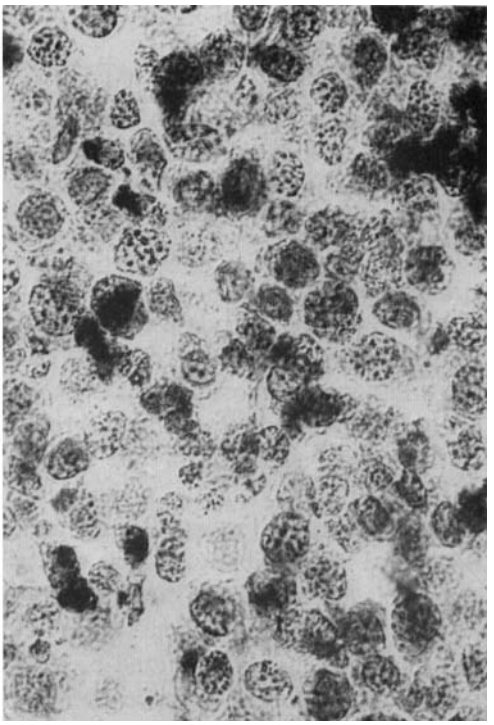


Fig. 16. Large peripheral cells 24 h after combined therapy 42.5 °C/30 min, 800 R. Increased acid phosphatase and distribution with large dense granules in the tumour cells (520 ×).

or some synchronization of the mitotic activity (BERKMAN & DESSAUER 1937, MARTIN & SCHLOERB 1964, RAO & ENGLEBURG 1965, SAPOZINK et coll. 1973, SISKEN et coll. 1965, WESTRA & DEWEY 1971), but no supportive evidence has been published.

The present results may give some hints on the nature of the reaction: In the central tissue reaction a very intense lysosomal activity is common for heat and cHR reaction. However, the appearance of the cellular decay is quite different. After exposure to heat, the heavy shrinkage of the cytoplasm, nuclei and chromatin in the tumour cells and the undamaged stroma with rapid formation of connective tissue are in sharp contrast to the cHR reaction, in which there is no shrinkage of cells, but fine granulation (Fig. 5) of the disintegrating chromatin and secondary destruction of the stroma are characteristic features. Here the terminal phase is very similar to the spontaneous necrosis, the inclusion of some decaying mitoses being the principal difference (Fig. 4).

As no significant difference in the content of acid phosphatase and catepsin D seems to exist (OVERGAARD & OVERGAARD 1972 b), some alterations in the cellular

pH might perhaps explain this finding. The existence of such a mechanism has not been demonstrated, however.

Some features in the later part of the cellular decay in the border zone may bear resemblance to the roentgen reaction. Presumably, some metabolic potential may be left in cells whose reproductive functions have been severely injured as well by cHR as by roentgen irradiation. However, after separate exposure to roentgen irradiation with doses in the ranges used a large number of irregular mitoses occur within a few days, while the total absence of mitotic activity in cHR-treated tumour cells is a striking feature.

As also mitotic irregularities may be demonstrated (MARTIN & SCHLOERB, RAO & ENGLEBURG, SAPOZINK et coll., SISKEN et coll.) after separate exposure to heat in low doses, it seems possible that two different injuries in the mitotic apparatus (separately, reparable or below a vital threshold) may work together to effect a blockade of the mitotic activity. Only the experimental evidence exists, but previous results (KEMP & JUUL 1931, JUUL & KEMP 1933) suggest the existence of some differences in the way heat and roentgen radiation hamper mitogenesis, and these authors also mentioned the possibility of some synergism between the factors.

Possibly a confirmation of the suggestions concerning the missing mitogenesis may be provided by recent findings: Some frequent and mostly reparable radiation lesions in malignant cells (single-strand breaks) were distinctly aggravated and might even lead to death after heat application (ORMEROD & STEVENS 1971, BEN-HUR et coll. 1972).

In addition, the high lysosomal activity may presumably play a role in the rapid and severe cellular destruction. As this activity is confined to the tumour cells—just as in the heat reaction—the special metabolic relations in tumour cells are presumably of importance.

**Acknowledgements**

This work was supported by grants from the Krista and Viggo Petersen Foundation and the Danish Cancer Society.

## SUMMARY

In mouse experiments a high curative effect on implanted tumours was obtained by successive application of low local heat and roentgen doses. Histologic and histochemical examinations of tumours so treated revealed a special reaction in the tissues. A total and rapid destruction of all hypoxic tumour tissue was observed and (in successful cases) a total blockade of all mitotic activity in euoxic tumour tissue was followed by a long-drawn necrobiotic decay of the existent tumour cells. The disturbances are electively bound to the tumour cells, only some normal tissues may secondarily be injured. A rapid and intensive lysosomal activity in all the tumour cells may probably be of importance in the reaction.

## ZUSAMMENFASSUNG

In Mäuseexperimenten wurde durch aufeinanderfolgende Anwendung lokaler Erwärmung und Röntgenbestrahlung ein hoher kurativer Effekt bei implantierten Tumoren erhalten. Histologische und histochemische Untersuchungen derartig behandelter Tumoren wiesen spezielle Reaktionen der Gewebe auf. Es wurde eine totale und rasche Destruktion aller hypoxischen Tumorgewebe beobachtet und (bei erfolgreichen Fällen) eine vollständige Blockierung jeglicher mitotischen Aktivität im gut-Sauerstoff versogten Tumorgeweben, der ein langanhaltender nekrobiotischer Zerfall der vorhandenen Tumorzellen folgte. Diese Störungen betrafen ausschliesslich die Tumorzellen, lediglich etwas Normalgewebe mag sekundär geschädigt werden. Eine rasche und intensive lysosomale Aktivität in allen Tumorzellen ist wahrscheinlich für diese Reaktionen von Bedeutung.

## RÉSUMÉ

L'application locale successive de petites quantités de chaleur et de petites doses de rayon roentgen sur des tumeurs greffées à des souris a donné un effet curatif très important. Les examens histologiques et histochimiques des tumeurs ainsi traitées ont montré une réaction spéciale des tissus. On a observé une destruction totale et rapide de tout le tissu tumoral hypoxique et (dans les bons cas) un blocage total de toute l'activité mitotique dans le tissu tumoral euoxique a été suivi par une altération nécrobiotique évoluant lentement des cellules tumorales existantes. Les troubles sont électivement liés aux cellules tumorales, seule une partie des tissus normaux peut être lésée secondairement. Il est probable qu'une activité lysosomique rapide et intense dans toutes les cellules tumorales a une importance dans cette réaction.

## REFERENCES

BARKA T. and ANDERSON P. J.: Histochemistry, theory, practice and bibliography. Hoeber, New York 1963.

BEN-HUR E., BRONK B. V. and ELKIND M. M.: Thermally enhanced radiosensitivity of cultured Chinese hamster cells. Nature (Lond.) 238 (1972), 209.

BERKMAN T. und DESSAUER F.: Versuch, die fraktionierte Bestrahlung durch Provokation sensibler Zellphasen zu unterstützen. (Provokationsbestrahlung.) Strahlentherapie 58 (1937), 551.

JUUL J. und KEMP T.: Über den Einfluss von Radium- und Röntgenstrahlen, ultraviolettem Licht und Hitze auf die Zellteilung bei warmblütigen Tieren; Studien an Gewebekulturen. Strahlentherapie 48 (1933), 457.

KEMP T. und JUUL J.: Der Einfluss der Wärme auf die Zellteilung. Arch. exp. Zellforsch. 9 (1931), 602.

MARTIN R. J. and SCHLOERB P. R.: Induction of mitotic synchrony by intermittent hyperthermia in the Walker 256 rat carcinoma. Cancer Res. 24 (1964), 1997.

ORMEROD M. G. and STEVENS U.: The rejoining of x-ray-induced strand breaks in the DNA of a murine lymphoma cell (L5178Y). Biochim. biophys. Acta (Amst.) 232 (1971), 72.

OVERGAARD K. and OVERGAARD J. (a): Investigations on the possibility of a thermic tumour therapy. I. Short-wave treatment of a transplanted isologous mouse mammary carcinoma. Europ. J. Cancer 8 (1972), 65.

— — (b) Investigations on the possibility of a thermic tumour therapy. II. Action of combined heat-roentgen treatment on a transplanted mouse mammary carcinoma. Europ. J. Cancer 8 (1972), 573.

— — Radiation sensitizing effect of heat. Acta radiol. Ther. Phys. Biol. 13 (1974), 501.

RAO P. N. and ENGLEBURG J.: HeLa cells. Effects of temperature on the life cycle. Science 148 (1965), 1092.

SAPOZINK M. D., DESCHNER E. E. and HAHN E. W.: Induction of mitotic synchrony by intermittent hyperthermia in Ehrlich carcinoma in vivo. Nature (Lond.) 244 (1973), 299.

SCHMIDT H. E.: Zur Röntgenbehandlung tieferliegender Tumoren. Fortschr. Röntgenstr. 14 (1909), 134.

SISKEN J. E., MORASCA L. and KIBBY S.: Effects of temperature on the kinetics of the mitotic cycle of mammalian cells in culture. Exp. Cell Res. 39 (1965), 103.

THEILHABER A.: Die Entstehung und Behandlung der Karzinome, p. 146. Karger, Berlin 1914.

WESTRA A. and DEWEY W. C.: Variation in the sensitivity to heat shock during the cell-cycle of Chinese hamster cells in vitro. Int. J. Radiat. Biol. 19 (1971), 467.