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Time-dependent RNA synthesis in different skin layers after wounding. Experimental investigations in vital and postmortem biopsies*

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Summary. Incision wounds were made on the outer ear of rats and two biopsies were taken for examination after different survival times. In each case a biopsy was made of vital tissue and a second of postmortem tissue after refrigeration for 24 h. The biopsies were exposed to a solution containing the RNA precursor ^{3}H-cytidine for 1 h, washed and fixed in formalin. Sections 5 µm thick were then autoradiographically prepared and automatically evaluated using Quantimet 920. The intravital specimens showed a significant increase in ^{3}H-cytidine incorporation in the basal cell layer after survival times of 10–24 h. No increase was seen in the stratum corneum, corium or cartilage tissue. The investigated distance from the wound margin did not have any significant bearing on the results. The ^{3}H-cytidine incorporation rate in postmortem tissue was practically identical with that of vital tissue, but no increase was observed in the rate of RNA synthesis in the basal cells as a function of the age of the wound. It may therefore be assumed that this method provides no additional information as to the age of wounds in postmortem examination.

Key words: RNA synthesis – Skin – Wound – Survival time – Vital – Portmortal

Zusammenfassung. Nach einer Schnittverletzung an der Rattenohrmuschel wurden Biopsien mit unterschiedlichen Überlebenszeiten entnommen, wobei eine Biopsie jeweils vital entnommen wurde, eine zweite postmortal – nach einer 24 h Lagerung unter Kühlschrankbedingungen. Die Biopsien wurden je 1 h in einer Lösung exponiert, die einen RNA-Vorläufer, ^{3}H-Cytidin, enthielt, anschließend gewaschen und in Formalin fixiert. 5 µm dicke Schnitte wurden autoradiografiert. Die Auswertung erfolgte automatisch über Quantimet 920. Durch Zugabe von alpha-Amanitin konnte eine Hemmung des Einbaues von ^{3}H-Cytidin erreicht werden, als Hinweis darauf, daß der postmortale Cytidin-Einbau den Vorgang der RNS-Synthese repräsentiert. Bei intravitaler Biopsie nach einer Traumatisierung wurde eine signifikante Zunahme der Einbaurate von ^{3}H-Cytidin in der Basalzellschicht bei Überlebenszeiten zwischen 10 und 24 h beobachtet. Keine Zunahme der Einbaurate fand sich in der Hornzellschicht, dem Korium oder der Knorpelzellschicht. Dieser Befund war unabhängig von der Entfernung vom Wundrand. In postmortal entnommenen Biopsien war die Einbaurate von ^{3}H-Cytidin praktisch identisch wie in den vital entnommenen Biopsien; eine Zunahme der Syntheseleistung der Basalzellen in Abhängigkeit vom Wundalter konnte demgegenüber nicht festgestellt werden. Unter dem Aspekt der Wundaltersbestimmung am Leichenmaterial ist somit zunächst davon auszugehen, daß die beschriebene Methode keine zusätzlichen Informationen über das Überlebensintervall einer Verletzung geben kann.

Schlüsselwörter: RNS-Synthese – Haut – Wunden – Überlebenszeit – vital – postmortal

Introduction

Studies have shown (Lindner 1967) that practically all types of metabolic cell activity are induced at the wound margin following injury (trauma), including stimulation of DNA and RNA synthesis in local cells (for literature see Oehmichen 1990). Isolated examinations of RNA synthesis at the wound margins as a function of the age of wounds have been carried out since 1974. Buris (1974) noted an increase in RNA incorporation in muscles only 15 min after traumatization; Lorup (1977) describes the incorporation of the RNA precursor uridine in fibroblasts after 6 h, with a peak at 36 h and normalization after 72 h. However, no systematic examination of RNA synthesis in various skin layers as a function of the age of wounds has been made to date.

* Dedicated to Professor O. Pribilla on the occasion of his 70th birthday

Offprint requests to: M. Oehmichen

An examination of this nature could give an insight into the detailed course of RNA synthesis and hence provide information as to the time of activation of local cell systems. From a forensic point of view there is also the question of the detection of RNA synthesis in postmortem tissue. This possibility has already been postulated in a pilot study (Oehmichen and Zilles 1984). However, for forensic purposes additional theoretical criteria for estimation of the age of a wound would be expected under such circumstances.

The experimental model used in the present study permitted the examination of vital and postmortem RNA kinetics on the same wound in identical animals, thereby providing an excellent means of obtaining information on vital and postmortem synthesis at the wound margin.

Materials and methods

Animals. Female HAN-SPRD rats (Sprague Dawley), approx. 6 months old, were fed ad libidum with water and altromine.

Experimental method. A straight, smooth-edged scissors cut about 6 mm in length was made on both outer ears of rats under ether anaesthetic. The animals were allowed to survive for different periods of time and specimens were taken from each of 7 rats after 0, 10, 20, 24, 28, 32, 36, 40, 48, 60, 72, 96, 120, 192 h.

The *vital* specimens were removed under ether anaesthetic using a punch (biopsy punch, 3 mm diameter, Stiefel, Offenbach, FRG) at the end of the specified periods. The animals were then killed under general anesthetic by ether intoxication with simultaneous deoxidization and stored in a refrigerator at 4°C. After 24 h further tissue was removed from the second side of the same ear wound for the *postmortem* biopsy.

In vitro cytidine incorporation. Immediately after removal the tissue was exposed to a solution including radioactive cytidine: 1 ml contained 10 μCi ^{3}H-cytidine (specific activity 28.4 mm Ci/mmol; Amersham-Buchler, Braunschweig, FRG) dissolved in Basal Medium Eagle solution (BME Earle, code 47350; Serva-Feinbiochemica, Heidelberg, FRG) with the addition of 0.1 ml of a mixture of penicillin (10,000 IU/ml) and streptomycin (10 mg/ml; code 47970; Serva-Feinbiochemica), nystatin (100,000 IU/ml; code 5453-15; Lederle, Wolfratshausen, FRG) and L-glutamine solution (200 mmol/l; code 47425; Serva-Feinbiochemica).

The specimens were incubated in this solution for 1 h at 37°C and 2.2 atm oxygen pressure, after which non-radioactive ("cold") cytidine (2-deoxycytidine HCl, code 18370; Serva-Feinbiochemica) was added to prevent further incorporation of radioative cytidine.

Histological and autoradiographical preparation. The tissue blocks were immediately washed 3 times in BME Earle, then fixed in formalin and embedded in paraffin. Serial sections (4 μm) were coated by dipping in film emulsion (Kodak NTB2, emulsion thickness: 1 μm ± 0.5 μm) and exposed under dehydration (silica gel) for 4 weeks in the dark. The specimens were then developed (developer: Kodak D 19; developed for 2 min at 20°C; fixing solution: Kodak Ekaflo Fixer) and the nuclei stained with eosin. The sections were covered in Eukid.

Morphometry. A Quantimet 920 (= QTM; Image Analysis Application Laboratory, Cambridge Instruments, Cambridge, England; cf. Prensky 1971; Jenkinson 1983, 1985) was used to measure the number of reduced silver grains in the film layer in relation to the total area. The radioactivity was expressed as the quotient of "area of reduced silver grains" divided by "total area". The number of reduced silver grains reflects the amount of radioactivity, which in turn provides information on the in vitro incorporation rate of cytidine.

The width of the viewing field was set at 200 μm and activity measured in four different skin layers:

1. Stratum corneum
2. Basal cells
3. Corium
4. Cartilage

In each layer three adjoining fields (areas) for two nonconsecutive levels of the specimen were measured and the mean grain density (activity) was determined. In addition, three segments (200 μm each) of each layer of skin were measured at varying distances from the wound margin and the mean grain density again determined. Readings for the following segments were obtained in this way:

Segment 1: near wound margin (0–200 μm)
Segment 2: medium distance (201–400 μm)
Segment 3: long distance (401–600 μm)

As the measurements were controlled on the monitor by the examiner, obvious artefacts (e.g. silver grain accumulations, vessel lumens, hair follicles) could be detected and electronically excluded.

Statistical evaluation. Mean values and standard deviations were calculated for each group of 7 animals and for all survival intervals. The significance was determined by Levene's test and the T-test (Sachs 1984), with a level of < 0.05 being regarded as significant. The following values were statistically recorded.

a) Vital/postmortem specimens
Change in activity as a function of survival time (Levene's test)
b) Vital/postmortem specimens
Change in activity as a function of the distance from the wound margin (Levene's test)
c) Difference in activity between vital and postmortem specimens (T-test)

Inhibition tests. Inhibition tests were carried out to establish whether the postmortem incorporation of ^{3}H-cytidine was indicative of RNA synthesis or a non-specific process. A 40 mg/ml concentration of alpha amanitine was used (MW 915.0; code: 77028; Serva-Feinbiochemica) which inhibits RNA polymerase and prevents incorporation in RNA to 90% (cf. Wieland 1972).

The rats were divided into two groups of 4 and sections of tissue were taken after 24 h (post mortem) from the undamaged outer ear. The specimens from 1 group were then exposed to ^{3}H-cytidine and from the second group to ^{3}H-cytidine + alpha amanitine for 60 min. The subsequent preparation, autoradiography and evaluation were carried out as described.

Results

RNA synthesis inhibition

Postmortem biopsy tissue taken from the outer ear was exposed to a solution containing ^{3}H-cytidine for 1 h. Specimens from identical animals were exposed under identical conditions except that 40 mg/ml alpha amanitine was added to the exposure solution. The activity with and without the addition of alpha amanitine was plotted (Fig. 1). It can be seen that the incorporation rate was significantly reduced by the RNA inhibitor.

RNA synthesis in vital and postmortem specimens

It was established that after a survival time of more than 60 h no change in RNA synthesis was observable at the

wound margin. The following comments (vital and postmortal biopsies) therefore refer exclusively to intervals of up to a maximum of 60 h.

The RNA kinetics at the wound margin were plotted graphically as a function of the survival time (Figs. 2–9).

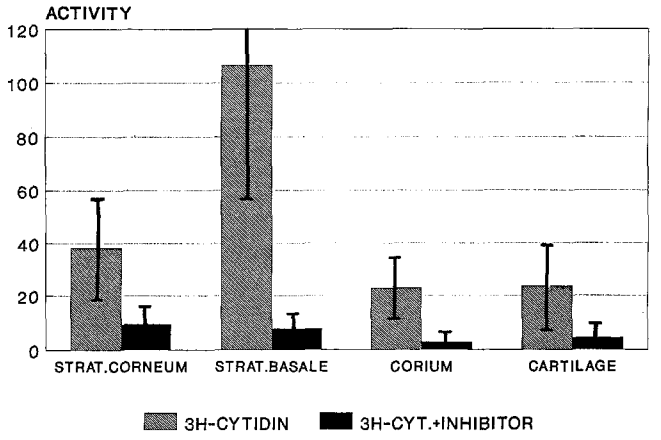


Fig. 1. Inhibitory effect of alpha amanitine on the RNA incorporation rate after postmortem (24-h interval) exposure of intact skin in a solution containing either ^{3}H-cytidine only or ^{3}H-cytidine + alpha amanitine (inhibitor). The distinct inhibitory effect in different skin layers is demonstrated as an indication of real postmortem RNA synthesis

The following observations may be made with regard to vital tissue:

1. RNA synthesis activity in three skin layers (stratum corneum, corium, cartilage) was on average 15 units (Figs. 2, 4, 5), while the mean rate for the basal cells was 50 units (Fig. 3).
2. A significant change in the rate of synthesis was only observable in basal cells (Fig. 3). The change occurred after 10 h in the form of an increase in activity to 180 ± 22.5 units. The mean activity was still 110 ± 68.4 units after 20 h survival time and 77.1 ± 51.2 units after 24 h, after which the rate of synthesis normalized.

The readings for postmortem biopsy tissue were also plotted in the same graphs (Figs. 2–5). It can be seen that with or without injury the basic activity in the basal cells was 3–5 times greater than in other skin layers. In none of the skin layers, however, was a significantly higher activity observed for any of the survival intervals.

RNA synthesis as a function of the distance from the wound margin

In theory it is possible that the rate of RNA synthesis might vary as a function of the distance from the wound

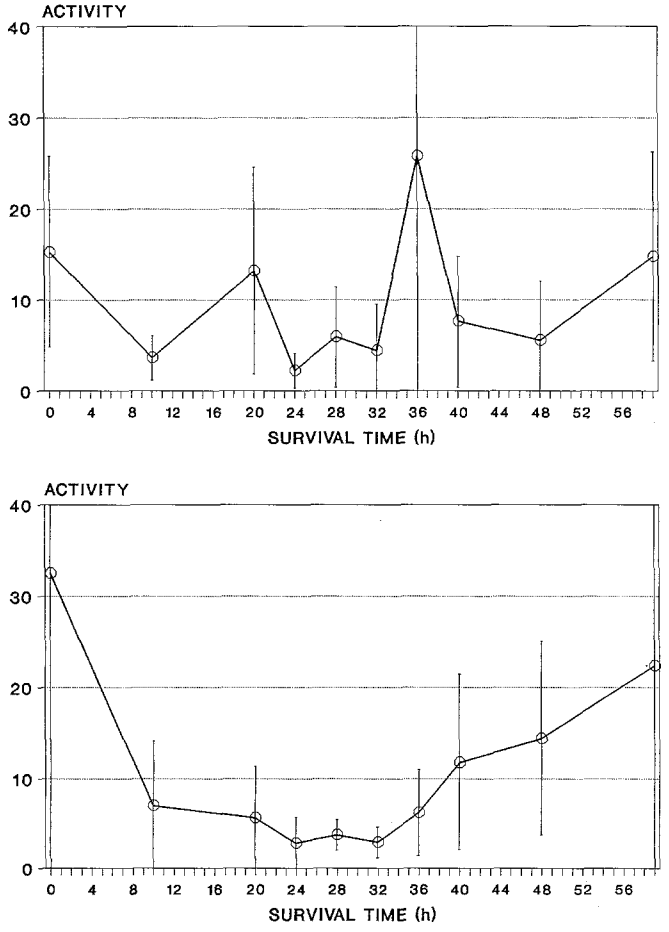


Fig. 2. Vital *(above)* and postmortem *(below)* RNA incorporation rate as a function of survival time: stratum corneum

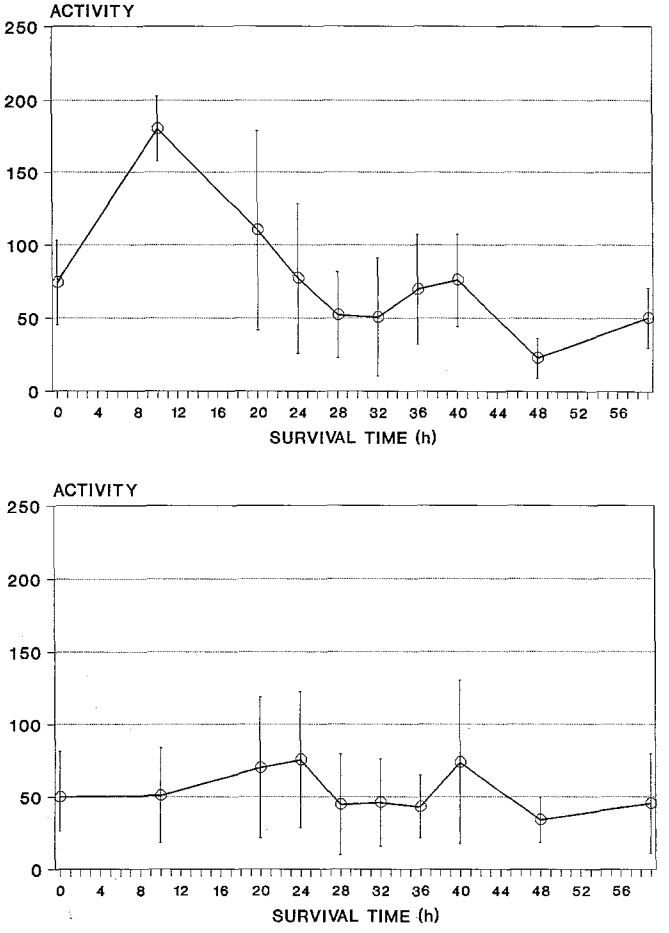
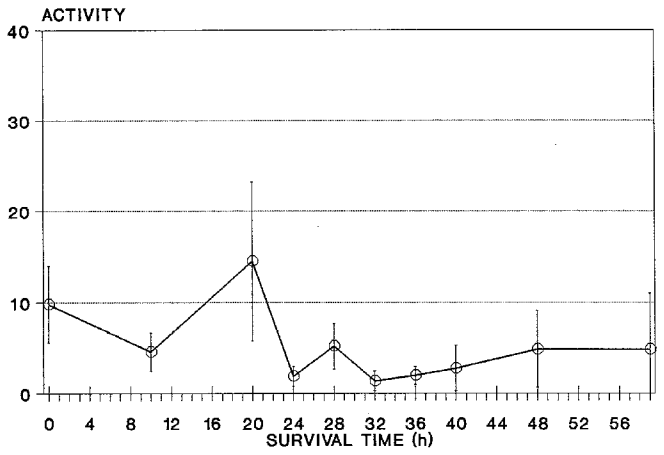


Fig. 3. Vital *(above)* and postmortem *(below)* RNA incorporation rate as a function of survival time: stratum basale epidermis



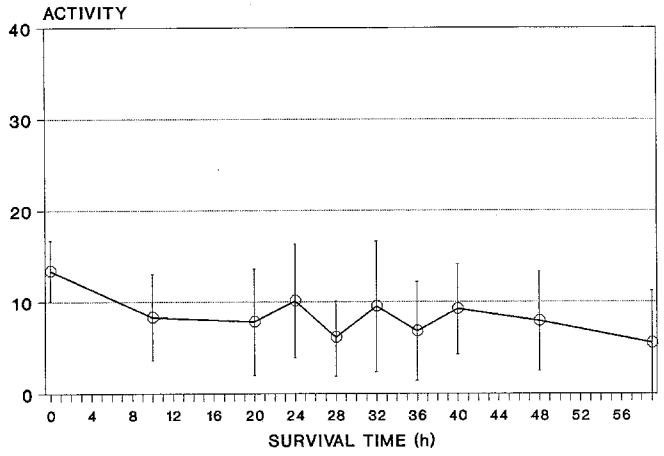
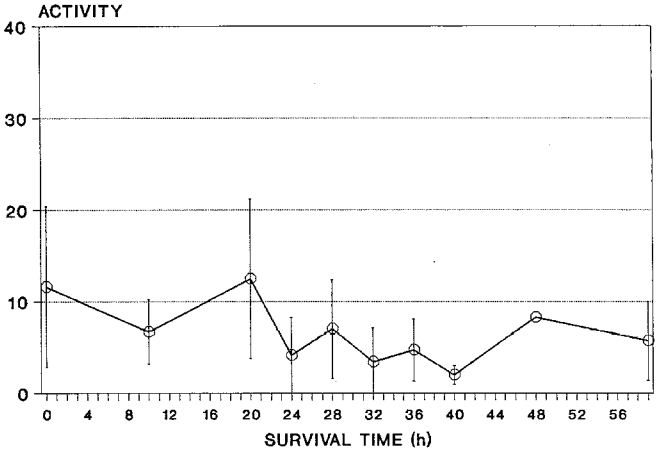


Fig. 4. Vital *(above)* and postmortem *(below)* RNA incorporation rate as a function of survival time: corium



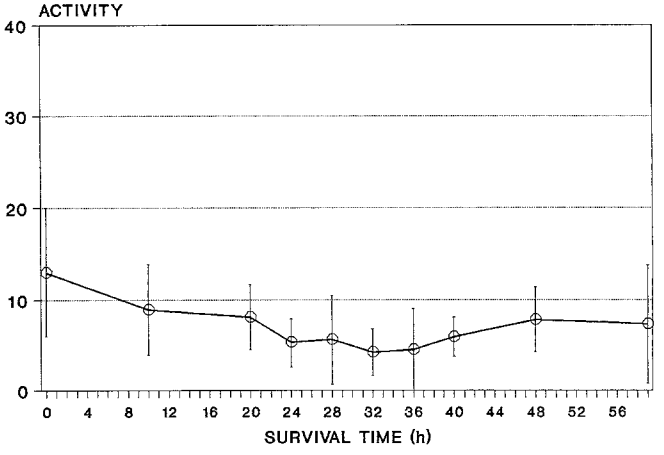


Fig. 5. Vital *(above)* and postmortem *(below)* RNA incorporation rate as a function of survival time: cartilage

margin. For this reason readings were taken at three different distances. The mean values are shown in the graphs (Figs. 6–9). A relationship between RNA activity and the distance from the wound margin was not observed in either the vital or the postmortem tissue under the measuring conditions described here.

Discussion

Inhibition of ^{3}H-cytidine incorporation

The inhibition test verified that in our experimental model RNA incorporation can be inhibited by alpha amanitine, i.e. ^{3}H-cytidine incorporation is an indicator of RNA synthesis even under postmortem conditions. However, residual activity can still be observed after inhibition. This enrichment could be due to background staining or to diffusion and thus represents a non-specific process. On the other hand, the considerations below should also be taken into account. Cytidine is not incorporated solely into RNA, but also into DNA. Feinendegen and Bond (1964) demonstrated that with very rapidly proliferating HeLa S_3 cells approx. 5% of the label was found in DNA after brief incubation with ^{3}H-uridine. Parallel studies in mice with ^{3}H-cytidine and ^{3}H-uridine, however, show that both nucleosides have the same relative incorporation scheme (Schultze 1968). The residual activity might be attributable to the incorporation of the RNA precursor in DNA.

Given the fact that RNA synthesis takes place, it is important to establish what information is provided by the number of reduced silver grains. Extensive studies, particularly that of Schultze (1968), have shown that some of the incorporated RNA can be released through the fixing process. The number of reduced silver grains therefore gives no information as to the absolute quantity of newly formed RNA per unit time, but merely provides an indication of the relative quantity.

^{3}H-cytidine incorporation as a function of wound age

Without injury the rate of RNA synthesis in the basal cell layer was more than 3 times that in the other cell layers examined. A significant increase in the incorporation of ^{3}H-cytidine was only observed in the survival range 10–24 h. All other skin layers showed unchanged rates of incorporation.

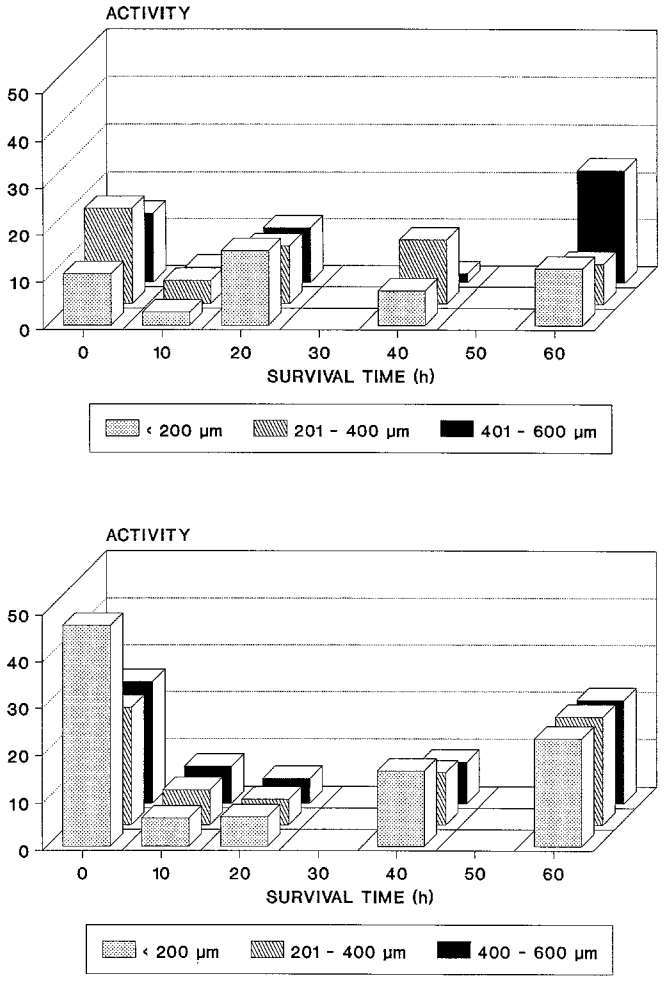


Fig. 6. Vital *(above)* and postmortem *(below)* RNA incorporation rate as a function of survival time and distance from the wound margin: stratum corneum

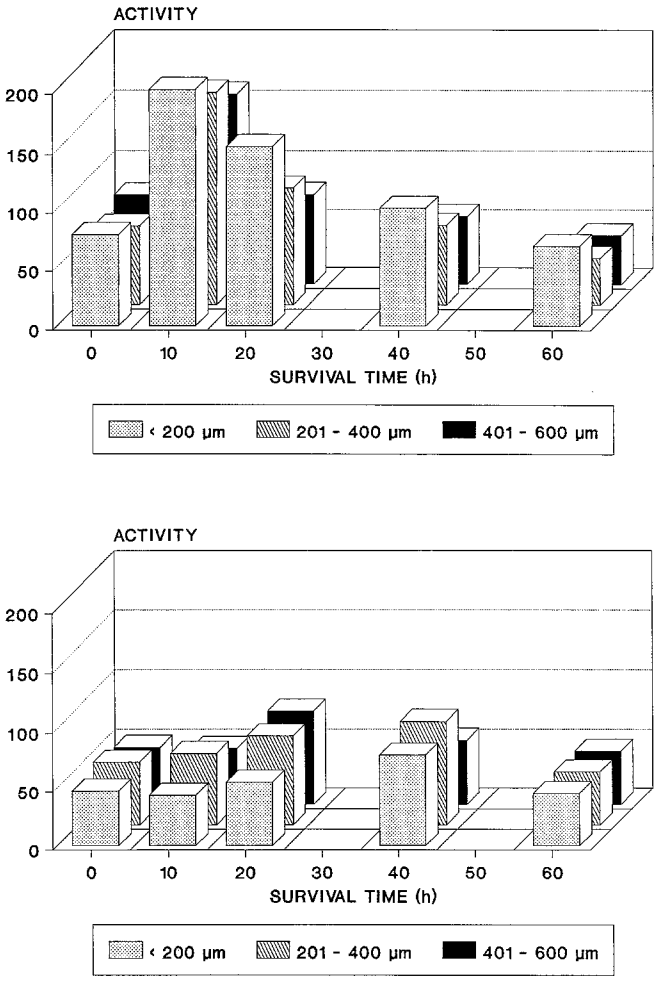


Fig. 7. Vital *(above)* and postmortem *(below)* RNA incorporation rate as a function of survival time and distance from the wound margin: stratum basale epidermis

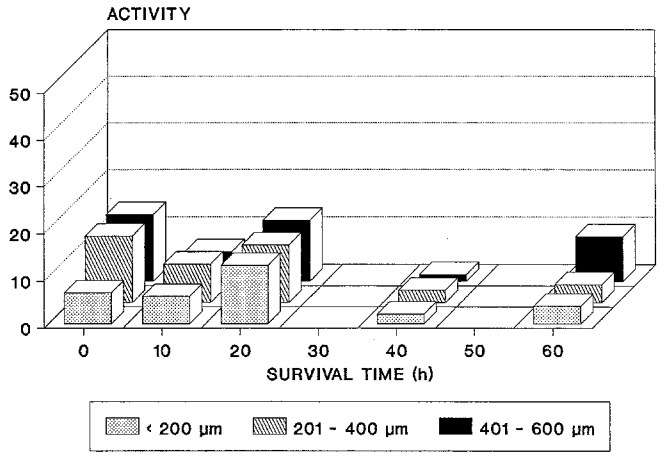
An increased rate of RNA synthesis in the basal cell layer has been known for a long time and has been qualitatively demonstrated by means of histochemistry (Brachet 1942). These observations were confirmed by Nolte (1947) and Hardy (1952) and finally by the biochemical investigations of Davidson and Waymouth (1944). As for the kinetics of RNA synthesis at the wound margin, only the above cited studies of muscles and fibroblasts are available. The fibroblast study in particular shows that the increase in RNA synthesis peaks after 36 h in the basal cell layer, as against 10 h in our study.

It is known that at the wound margin DNA synthesis, in particular, and synthesis of the basal cells is stimulated by injury. It is therefore useful to establish whether the increase in the rate of cytidine incorporation is connected with DNA synthesis. Clement-Noel (1944) and Washburn (1954, 1960) indicated such a connection. It is assumed that under normal circumstances RNA synthesis of the basal cells is dependent on the proliferative activity, whereas that of the higher epidermal layer is dependent on the degree of keratinization.

It has been noted that RNA synthesis takes place throughout the entire interphase and stagnates in the mitosis phase. It is at its lowest during the G_1 phase, increases steadily during the S phase and remains constant in the G_2 phase (Schultze 1968). The following time data are available for the various phases: the S phase in the epidermis of rats is calculated to be 4.8–8 h (Pilgrim and Maurer 1965) and the G_2 phase in the ear of mice, 4.8 h (Sherman et al. 1961). If it is further assumed that the start of the increase in the number of DNA synthesizing basal cells is only observable to any substantial extent approx. 20 h after traumatization (Oehmichen and Schmidt 1988), our observations would posit a direct causal link. It is striking, however, that DNA synthesis remains at a plateau for a period of 48 h for survival times up to 72 h, whereas the incorporation of ^{3}H-cytidine returns to a normal level within 24 h following a brief increase.

^{3}H-cytidine incorporation in postmortem specimens

Cytidine incorporation also indubitably takes place in postmortem tissue, and one result of this is, that the basal cell layer again shows a silver grain density at least 3 times that in the other skin layers. With survival times



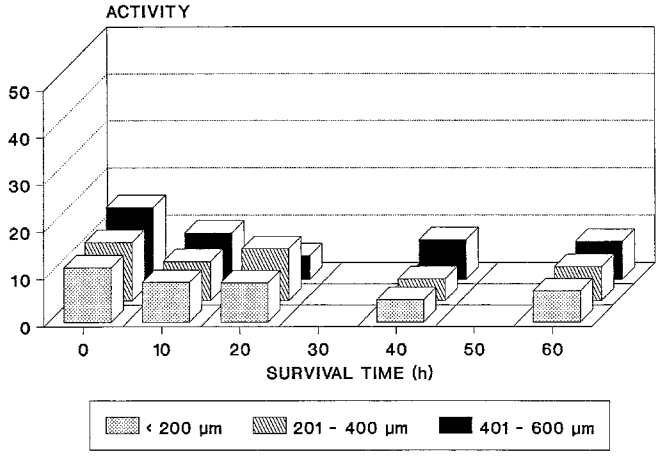
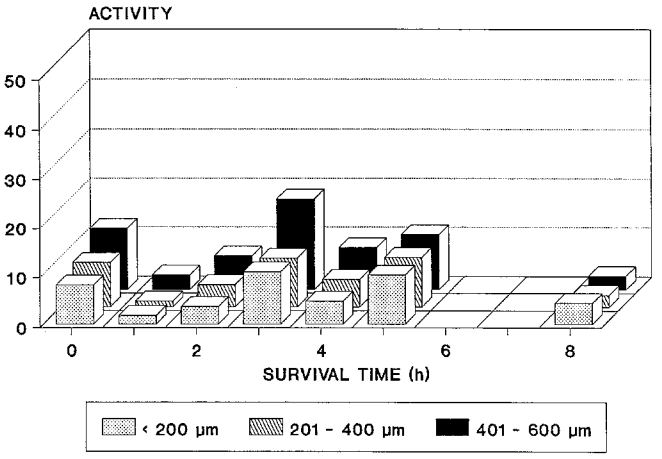


Fig. 8. Vital *(above)* and postmortem *(below)* RNA incorporation rate as a function of survival time and distance from the wound margin: corium



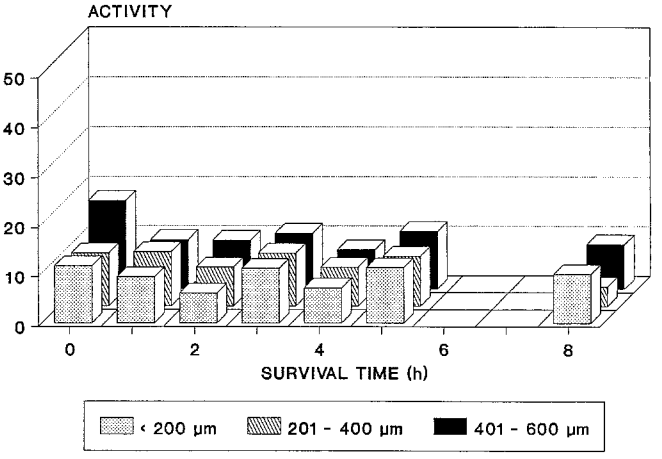


Fig. 9. Vital *(above)* and postmortem *(below)* RNA incorporation rate as a function of survival time and distance from the wound margin: cartilage

of 10 h and above, however, no increase in silver grain density can be seen at the wound margin.

This phenomenon is surprising in that DNA synthesis is observable in postmortem tissue to virtually the same extent as in intravital tissue. It must therefore be assumed that basic cell activity occurs in postmortem tissue without additional stimulation by the wound leading to an increase in RNA synthesis. In vital tissue this stimulation led to significantly higher RNA synthesis in the basal cell layer 10 h after trauma. Either this stimulation decreased during the postmortem interval of 24 h and the RNA synthesis peak had already been surpassed, or the induced stimulation itself is absent.

With regard to the validity of this method of study for estimating the age of wounds on corpses, the following conclusion may be drawn: when a body is examined 24 h after the cessation of cardiovascular activity, no reliable indications as to the age of the wound can be obtained with the method described here.

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