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INVESTIGATIONS CONCERNING THE PATHOGENESIS AND THERAPY OF COMBINED INJURIES *

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Summary

In preliminary tests, the question was investigated in how far the radiation lethality of test animals (NMRI mice) was modified not solely by open wounds (this had been investigated already in previous tests) but also by closed wounds (aseptic muscle necrosis), by the effects of heat and low temperatures as well as by a physical state of severe exhaustion (having forced them to swim).

Although the influence of the individual types of stress being different, a trend was ascertained with combined injuries, demonstrating that the radiation lethality increased either not at all or just a little or even decreased significantly in the presence of simultaneously or prior to irradiation inflicted traumata, whereas traumata inflicted after irradiation mostly resulted in a high degree increase in radiation lethality.

In subsequent tests the question concerning the causes for these changes in lethality were investigated. By applying the methods of electrophoresis for investigating the serum proteins, it was ascertained, especially when wounds were inflicted after irradiation, that high degree displacements were present in the serum protein picture with a reduction of the albumins and a relative increase in the α_2- and β-globulins. Prealbumins and gamma globulins showed considerable modifications as well. It is certain that these changes resulted in disturbances of the flow characteristics of the blood plasma and, therefore, were contributory to the increase in the lethality rate.

Further tests were made on the content of corticosteroids and cholesterol in the adrenal gland of mice. An irradiation as well as a skin lesion resulted in an increase of the corticoid content in two phases, i.e., two hours and two days after irradiation respectively surgery. If, however, a wound is inflicted two days prior to irradiation, the radiation effect will take affect at a time of increased adrenal activity. By this a limited protective effect seems possible. A wound inflicted two days after irradiation causes a long-lasting steroid increase of high degree as reaction to this very severe course of combined injuries.

In hematologic investigations the CFU (Colony-forming-units) in the spleen of mice, having skin lesions inflicted two days prior to irradiation, were examined. In comparison to radiation exposed control animals (without wounds) a three times higher occurrence of endogenous splenic nodules (CFU) was demonstrated as a result of an increased proliferation of the hematopoietic tissue. An open wound, evidently, affects a stimulating effect on the stem-cells of the bone marrow.

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I. PRELIMINARY STATEMENT OF PROBLEM

/1*

The effects of combined injuries on mammals within the scope of this research project have been investigated since 1965. Reports I through VII included literature searches on the statement of the problem as well as special investigative findings made using rats and mice.

Report I was chiefly devoted to combined injuries appearing in Hiroshima and Nagasaki. The findings of the "Joint Commission", a research group formed by American and Japanese physicians in October 1946, were referenced [1]. This commission selected from the abnormally affected population 5185 wounded individuals from H. (Hiroshima) and 4107 wounded from N. (Nagasaki) and used these to determine the percentage proportions of persons who had been affected by only one, two or three types of wounds. Accordingly, it was found that 60.5% in H. and 57.7% in N. were found to have been inflicted with one type of wound. Further, 34.5% in H. and 37.1% in N. had two types of wounds and, finally, 5.0% in H. and 5.2% in N. were found to have three types of wounds. In this case, the wounded persons in the third group had been injured by the effects of the shockwave, thermal radiation as well as nuclear radiation. The percentage figures are remarkably similar in both cities although, of course, they are only valid for wounded persons who survived the effects of both atomic explosions by at least 20 days. The patients dying within this first period were not taken into consideration in this statistic. The prudent estimate made including the deceased permits the assumption that approximately half of all the wounded were inflicted with combined injuries.

Modern estimates concede a still higher degree of probability to combined injuries. Accordingly, Geiger, a doctor in the Armed Forces of the GDR [2], reports that in the case of nuclear warfare carried out using rocket weapons, calculations must call for 65-70% of the wounded to suffer from combined injuries (Table 1).

/2

Single injuries 30-40%	Radiation injuries (including fallout)	15-20%
	Burns	15-20%
	Mechanical wounds	up to 5%
Combined injuries 65-70%	Burn + wound + radiation damage	20%
	Wound + radiation damage	40%
	Mechanical wound + radiation damage	5%
	Mechanical wound + burn	5%

Table 1. Probable distribution of types of injuries in a nuclear war (according to Geiger: "Fundamentals of Military Medicine" [Grundlagen der Militärmedizin]).

* Numbers in the right margin indicate pagination in the original text.

As a consequence of calculations of this kind which allowed the number of combined injuries to appear to be a great deal higher than the number of those affected by "pure" radiation damage, a great number of investigations were carried out in the fifties in the Soviet Union, which were published as well.

Accordingly, Professor Krupko, Director of the Clinic for Traumatology and Orthopedics at the S. M. Korov Academy for Military Medicine, reports that almost the entire collaborating working group of his chair had been occupied for a whole year researching various questions having to do with the behavior and treatment of combined injuries [3]. An "All Union conference" took place in Moscow from 9-12 June 1958 at which exclusively questions of combined radiation damage were discussed and more than 60 reports were given [4].

Most of the investigations carried out by surgeons, traumatologists and pathologists and a few radiation biologists were heavily oriented toward practical applications. In addition to the pathogenesis of combined injuries, they chiefly applied to the possibilities of a suitable therapy for such wounds. The following subject areas were essentially investigated by the Russian authors:

Shock pressure injuries and total-body irradiation /3
Burns and total-body irradiation
Open mechanical wounds and total-body irradiation
Fractures and total-body irradiation
Abdominal injuries and total-body irradiation

The special investigations carried out since 1965 on mice and rats apply essentially to the combination of open skin wounds with total-body irradiations. Further combinations of radiation exposure with burns, crush syndrome, abdominal operations and various drugs were only examined in a few experimental series. In addition, stress forms such as heat and cold reactions combined with specific wounds as well as severe states of exhaustion were likewise not combined with radiation effect. In this case, preliminary testing is involved whose results are provided in this report.

The mice and rats inflicted by us with injury combinations of total-body irradiation and open skin wounds were investigated from various aspects and using different techniques. These included histological, hematological, bacteriological, biochemical and lethality-statistical investigations. Their findings are presented in detail in Reports III-VII of this research project. On the basis of these special investigative findings and the results published in the bibliography, it will be sought in the following to represent and describe the injury profile resulting as a consequence of a combined effect of total-body irradiation and open mechanical wounds.

The injury model used by us is essentially as follows. Mice or rats are placed in ether narcosis and receive one or several coin-sized skin wounds on the back which involve less than 5% of the body's surface. The

skin wounds remain open and completely untreated, and lead to no or only a very slight lethality on the part of the test animals. The animals having these wounds are stressed with a total-body irradiation at approximately the same time or at various times before or after inflicting the wound. If these skin wounds are produced after the irradiation, it follows that this leads to a very severe disease profile affected with a high lethality /4
which is to be described in the following. The mice cower for a few days after the wound operation with arched backs unmoving in their cages. Their hair becomes dishevelled and the parts of the skin not covered with fur are now cyanotic. The animals lose up to 30% of their body weight. After an initial inappetence, they increasingly drink water since they have clearly lost much liquid through the open skin wounds. The quantities of urine passed decrease drastically. The consumption of solid nourishment ceases or is at least interrupted for several days. Taurine and urea are increasingly found in the urine [5] which suggests an increased proteolysis as a consequence of an acidotic metabolic condition. The histological investigations [5,6] show figures which likewise point to a histohypoxia. Changes within the meaning of a "shock kidney" are most striking. There are expansions of the lumina of the tubules in the area of the proximal tubule components and the medulla-cortex boundary as well as flattened endothelium. Severe hypoxic changes are found in the cardiac muscle cells with vacuolization, fiber homogenizations and interstitial edema. The right heart ventricle is often severely expanded and the vessels in many organs are dilated, accordingly choked with tight fillings of erythrocytes in some, the histological profile shows the result of protracted shock with clear microcirculatory disturbances. There is no basis for a septic action since a bacterial infection is, as revealed by further bacteriological investigations [7], substantially to be excluded as the cause of death of these mice.

Some data from Russian publications will be quoted in the following; thus, it is confirmed by Polyakov [8] that the shock syndrome plays a decisive role with combined injuries when he writes that "shock already causes drastic modifications of metabolic processes during the latent periods of radiation sickness. The reduction and oxidation processes in the tissues are severely inhibited ... Even a completely insignificant mechanical trauma can cause a shock at the critical point of radiation sickness ... The arterial blood pressure drops off sharply and can only /5
be raised up with difficulty".

The detailed investigations of Mitrofanov [9] on oxidation and reduction processes with tissue respiration also suggest a corresponding synergism of trauma and radiation effect. The dogs examined by him showed as a consequence of combined injuries an inhibition of the capability for oxidation on the part of the cells in the cerebrum, subcortex, brain stem, liver, kidneys, cardiac muscle and in the lungs. This explained the demonstrable disturbances of the formation of energy-rich phosphorus compounds and the tissue hypoxia characteristic of shock. The changes of cell respiration in the brain and in other tissues was confirmed by the drop in body temperature, lowering of the blood pressure, slowed down respiration and inhibition of reflexes with the dogs under examination. The animals studied especially showed at the critical point of radiation sickness an

increased sensitivity with respect to additional traumata which was expressed by a shorter excitation stage or even by its absence and through a quicker development of a severe torpid stage of shock.

In addition, the changes in the red blood cell formula after radiation and the wound inflicted two days later [10] found by us with rats suggest disturbances of the microcirculation; i.e., in the long run shock symptoms. In this case, anemia occurred. Examinations of the erythrocyte fragility [11] showed no increase in the mechanical or osmotic readiness for hemolysis but rather an increase in the resistance of erythrocytes with combined injuries. Further, we found, in the case of rats receiving combined injuries, a significant increase in the numbers of reticulocytes as an expression of the reinforced regeneration of the erythropoiesis. The anemia which commences earlier can be accepted as a consequence of a peripheral hemodynamic disturbance as can be observed with combined injuries and to a somewhat lesser extent in the case of "pure" radiation damage. The microcirculation disturbances in the peripheral stream bed could cause the creation of aggregations of erythrocytes in the capillaries thereby reducing the number of erythrocytes in the blood vessels.

In addition, the clearly demonstrable delay in healing time of the /6
wound with a persisting radiation sickness is certainly not to be explained by local radiation damage of the granulation processes in the wound but by the general damage of the organism. Smirnov [12] has described the histology of such wounds. Whereas a profileration of the adventitial cells, fibroblasts and blood capillaries was to be observed three days after inflicting the wound in the case of the non-irradiated control animals and a wide layer of granulation tissue already existed after five days, the wounds of irradiated rats, if the course of their sickness was especially critical, soon showed necroses on the wound bottom with no proliferations of the capillaries at all. Proliferations were demonstrable in the case of fibroblasts and adventitial cells on a delayed and scattered basis. The necroses were spread further reaching into the subcutaneous connective tissue as well as in the uppermost layers of the back muscles and a wound development of this type led to the death of the test animal. The proliferation processes were likewise severely disturbed on a temporary basis in the case of the surviving animals. However, after this phase, the healing of the wound took place with a delay similar to that of non-irradiated animals.

Undoubtedly, open, still-unclosed wounds represent a great danger for the life of the test animals. We were able to prove in special investigations [15] that mice which had been irradiated with a LD_{26} (here 510 R) and two days later inflicted with an open skin wound, perish in 90% of the cases. If the wound is immediately closed again, it was possible to reduce the mortality to 18%.

In addition, Russian authors have carried out investigations on the problems of therapy and their findings have been published. In addition to treatment of shock, the local wound therapy is of the greatest importance for survival from the consequences of combined injuries. Accordingly,

Razgovorov [13] requires application of the primary wound suture on the basis of his findings derived from experiments on dogs, rabbits and guinea pigs. In his investigations, he inflicted back wounds on the animals which penetrated deep into the muscle system. He then sutured them after they had remained open and untreated for up to 24 hours. The edges and bottom of the wound were excised with application of a local anesthetic. After /7
a careful hemostasis, the wound was sutured with silk threads. Guinea pigs and rabbits received no antibiotics whereas the dogs received 200,000 internat. units penicillin on a daily basis. The wounds healed in most cases in spite of a severe radiation sickness existing at the same time. When the wounds were not sutured, it followed that the course of the radiation sickness became considerably worse. The average survival times were shortened to 9-13 days in contrast to the dogs which had been surgically treated and died only after 17-21 days or even, on the other hand, survived the combined injuries. Sectioning revealed extensive hemorrhages in the vicinity of the open wounds which were not detectable in the case of the sutured wounds. Razgovorov comes to the conclusion that an open wound represents a very dangerous focus of infection which, nevertheless, can be extensively neutralized by radical techniques of excision and compact suturing. In addition to the early application of large doses of antibiotics, this is the method of choice in the case of such combined injuries.

Blinov as well turned in a special study [14] to the question of the use of antibiotics. Since, in the case of guinea pigs which had been irradiated and inflicted with skin wounds, local infections with separations appeared in 30% of the cases in spite of primary treatment of wounds, antibiotics were used in a number of comparative experiments. Streptomycin and penicillin were placed in the wound after excision. After the wound was sutured, up to 40,000 internat. units streptomycin were injected over the period of a day into the vicinity of the wound. The experimental result showed a clear reduction in bacterial flora in the area of the wound. If the wounds should open again, no necrotic masses on the wound bottom would be found as was the case in the treatment without antibiotics.

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II. ON THE LETHALITY OF MICE WHICH HAVE BEEN STRESSED BY TOTAL-BODY IRRADIATIONS IN COMBINATION WITH OPEN SKIN WOUNDS, ASEPTIC MUSCLE NECROSES, EFFECTS OF HEAT AND LOW TEMPERATURES OR SEVERE PHYSICAL EXHAUSTION /9

II.1 Introduction

In the 1972 Interim Report (Report VII) of this research project [1], reference has already been made concerning the beginning of a series of investigations which have now been concluded and are described in detail in the present report. At the same time, there will be further examinations of the question as to what extent the time factor affects the prognosis and progress of illness from combined injuries. Stresses such as the effects of cold, heat shock, severe exhaustion and aseptic muscle necroses which had not yet been specifically investigated by us were combined with radiation affect whereby the above-named morbific agents acted upon the test animals in varying time intervals before or after a total-body irradiation.

Chapters II-V describe the results of the investigations on the content of cortocoids in the adrenal glands, on the protein fractions in the serum and the stem cells in the bone marrow of mice which have been placed under stress with open wounds and irradiation. In addition, the question of lethality of the animals as a function of the time of wounding and irradiation was discussed in these investigations.

II.2 Material and Methodology

II.2.1 Test Animals and Their Maintenance

Twenty-eight hundred and ninety-two approximately eight week-old male mice (30-34 g) and female mice (22-25 g) of the NMRI stock of our own breeding were used. The radiation sensitivity of the animals has been discussed in detail in Reports IV-VII of this research project. In all investigative series, the lethality of the animals was checked for 30 days following application of the last intervention (wound or irradiation).

The mice were kept on chaff (shavings) in groups of five in macrolon cages. The environmental temperature in the test animals' stalls was kept constant at 23-25° C with a relative humidity of about 50%. The animals received standardized feed (Altromin R10) and water as needed. /10

II.2.2 Conditions of Irradiation

X-ray apparatus type MG 300 of the firm C.H.F. Mueller, Hamburg, focus-animal-interval 40 cm, tube voltage 250 kV, with 12 mA, dose rate 70 R/min., inherent filtering of the tubes 6 mm Al, half value thickness 2.5 mm Cu. The groups of five animals were kept during irradiation in a shallow plastic container. The dose rate is continuously measured during the irradiation (with the duplex-dosimeter of PTW).

II.2.3 Open Skin Wounds

After a superficial shearing of the back hairs by machine, a template is used to draw a dime-sized color mark 1.7 cm in diameter in the middle of the back skin of the animals. These color marks were subsequently excised during ether narcosis. The wound produced in this manner, going down to the fascia, remains open and completely untreated. As a consequence of the modified tension of the skin under narcosis, the wound usually is widened in diameter by 1 to 3 mm. The wound surface produced in this way amounts to 230 to 320 mm^2 and accounts, according to the computations of Lee [2], to less than 5% of the body surface.

II.2.4 Aseptic Muscle Necrosis (with Formalin)

During ether narcosis, 0.1 ml of a 3% formalin solution is injected into the gluteal muscle system of the rear left extremity. The 3% formalin solution was always prepared from a 37% formaldehyde solution (Merck). The cannulas whose size was 16 mm x 0.5 mm are changed after 10 injections in order to reduce the danger of tearing vessels.

II.2.5 Effective Heat (Heat Shock) /11

In an incubator (firm Haraeus) heated up to 50° C, each group of five mice was exposed continuously to heat for 12 minutes. The animals are located in a 25 x 14 x 15 cm size wire cage whose floor has been insulated by cardboard and shavings against direct heat contact between the animals and the wire cage. A sufficient supply of air for breathing is provided by opening a ventilation flap on the upper side of the 40 x 30 x 35 cm size incubator and by keeping a gap separation opening of the door. The temperature is monitored during the test by the constant checking of a thermometer.

II.2.6 Effect of Cold

The animals are exposed to a constant temperature of -20° C in a spacious freezer chest (522 l). At the same time, they are housed in individual plastic cages in order to avoid mutual heating owing to their body heat. The temperature is checked a number of times during the test. The presence of sufficient air for breathing is taken care of owing to the size of the freezer.

II.2.7 Exhaustion from Forced Swimming

The animals must swim to the point of exhaustion in a plastic tub with smooth vertical walls with a continuously-checked water temperature of 28-30° C. The depth of the water of 25 cm prevents the animals from supporting themselves on the floor of the tub. During each test, 25 mice swim at a time on a surface of approximately 11,000 cm^2. When the animals submerge and risk drowning, they are then taken from the water in time and dried off. After 120 minutes, the test is over for all animals. Even the mice with the shortened swim times are used for the test.

II.3 Findings /12

II.3.1 Total-Body Irradiation and Open Skin Wounds

225 female mice of the NMRI stock Hannover were subjected to an open skin wound (cf. II.2.3) 8, 4, 2 days or 1 hour before irradiation with 600 R or irradiated with 600 R (cf. II.2.2) and 6 hours, 2, 4, 8 or 14 days after infliction with an open skin wound. The lethality of the 40 control animals irradiated with 600 R amounted to 15% and that of the 25 control animals inflicted with wounds without additional irradiation amounted to 0%. The findings of this investigation are depicted graphically on Figure 1.

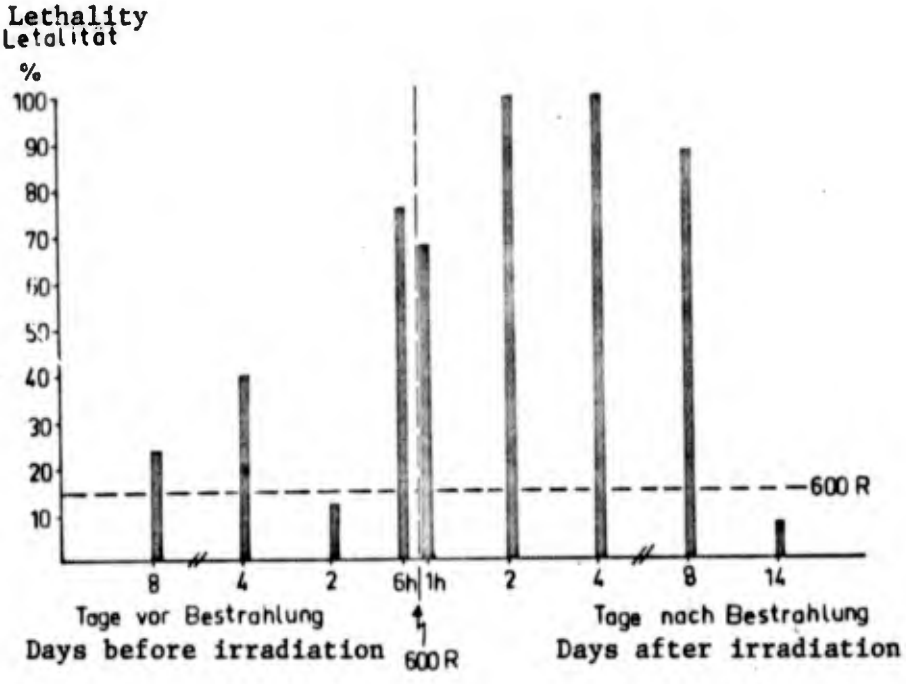

Figure 1. Lethality of female mice which had been inflicted with open skin wounds at various times before or after irradiation (600 R).

The figure reveals that the lethality of the mice deviates relatively little from that of the pure radiation effect (15%) unless the wound is inflicted a number of days before the irradiation. The rise in mortality to 40% in the case of wound infliction 4 days before irradiation, nevertheless, already statistically significant when computing the significance according to the χ^2-method using the value $p = 0.05$. The lethality increases are highly significant using $P = 0.001$ if the interventions are /13 applied approximately at the same time (6 hours before or one hour after) or 2, 4 or 8 days after irradiation.

In further investigations, 270 female mice of the same animal stock were irradiated with 400, 500, 550, 600, 650, 700 and 750 R and inflicted

with open skin wounds (SW) two days beforehand (SW + ϟ 2 d p.op.) or two days afterward (ϟ + SW 2 d p.r.). The results are shown in Table 2.

Dose Dosis (R)	SW HW + ϟ 2 d p.Op. Letalität % Lethality	"Pure" irradiation "reine" Bestrahlung Letalität % Lethality	ϟ + SW HW 2 d p.r. Letalität % Lethality
400	-	0	4
500	-	7	44
550	-	10	56
600	12	15	100
650	56	71	100
700	84	92	-
750	100	100	-

Table 2. Lethality of mice which were inflicted with open skin wounds two days before or after irradiation.

The application of the wound preceding irradiation led indeed to a drop in radiation lethality. However, the differences are not statistically significant ($p = 0.1$-0.05 and $p = 0.3$). Nevertheless, when the skin wound is made two days after irradiation, it follows that the mortality of the mice rises to highly significant values ($p < 0.001$).

II.3.2 Total-Body Irradiation and Aseptic Muscle Necrosis

Preliminary Experiment:

Before carrying out the main experiment, the concentrations and quantities of formalin were determined which produce an aseptic muscle necrosis at the same time allowing survival of the mice.

For this purpose, 35 female mice were treated by injecting each one with 0.2 ml in the muscles of the upper part of the thigh in decreasing concentrations of 10, 8, 6, 4, 3, 2 and 1%.

In the range of 10 to 6%, all animals died immediately following injection. 12% of the mice died within 36 hours when 4% was used. When a formalin solution of 3 to 1% was used, not one of the animals died. These findings are shown in Table 3 as well as in Figure 2. /14

Further, it was to be determined what changes were caused by the corrosive and albumin-precipitating effect of the formalin. A superficial wound which the body began to close by a scab developed at the point of application following a locally-confined fall of hair. After sacrificing

Formalin concentration Formalin-konzentration %	Mortality rate Sterblich-keitsrate	%
10	5/5	100
8	5/5	100
6	2/5	40
4	1/5	20
3	0/5	0
2	0/5	0
1	0/5	0

Table 3. Lethality of mice with various formalin concentrations.

the animals, dead tissue demarcated from the lower areas could be determined whereby, in a few cases, parts of the femur were affected. The creation of a relatively large superficial wound is not desirable for the problem under investigation here since the creation of an aseptic trauma can only be achieved with a closed coat of hair. An open wound was prevented only through the application of 0.1 ml i.m. (intramuscular) of a 3% solution of formalin.

Main Experiment:

772 female mice were injected with 0.1 ml of a 3% formalin solution (II.2.4). The exposure to radiation (600 R) took place at time intervals 1 hour, 2, 4, 6 and 8 days after application of formalin as well as in the same time intervals before injection.

It can be seen from Figure 3 that modifications of radiation lethality come about owing to the additional stress by a muscle necrosis. /16

A quite significant increase in lethality occurs when the formalin application is undertaken after irradiation whereas, in the case of formalin application before irradiation, the lethality on the average almost equals the control value or the values are located preponderantly in the dispersion area of the controls.

The lethality forms an exception when the injection takes place one hour before the application of radiation. This lethality is differentiated from that of the control value with a significance of $p = 0.02$. The significance of the differences of lethality of the animals treated with the formalin solution to those control animals irradiated with 600 R may be seen from Table 4.

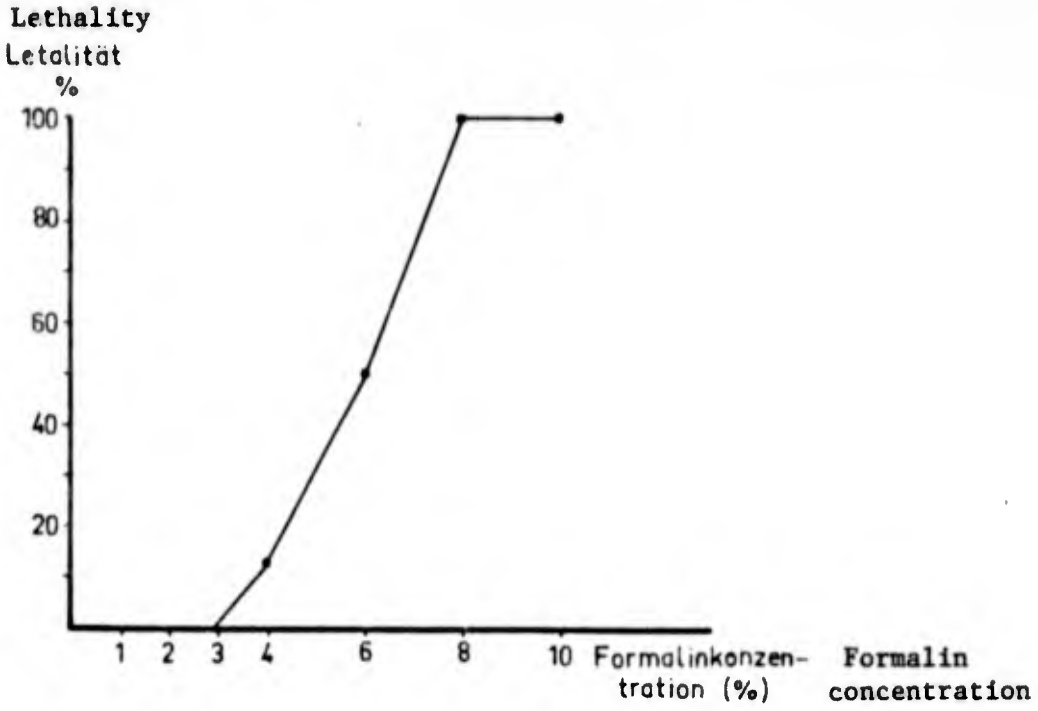

Figure 2. Lethality of mice following injection of 0.1 ml of various concentrations of formalin.

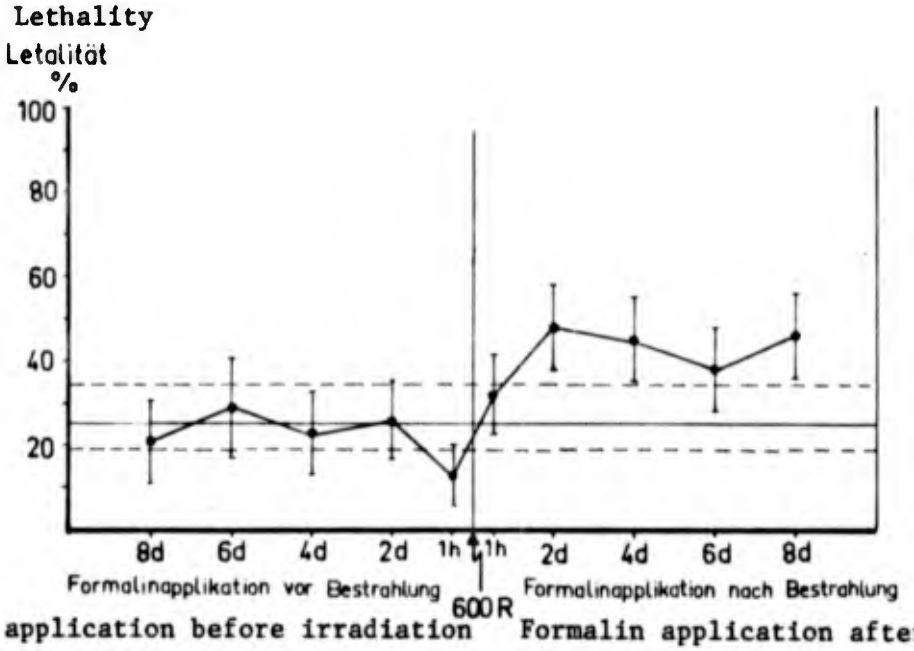

Figure 3. Lethality of mice as a function of the time interval between irradiation (600 R) and creation of an aseptic muscle necrosis.

	8d	6d	4d	2d	1h	1h	2d	4d	6d	8d
	Before irradiation					After irradiation				
p =	0.50	0.80	0.70	1.00	0.02	0.50	0.01	0.01	0.20	0.01

Table 4. Significance of differences in lethality of combined injuries with respect to the control animals (irradiation with 600 R).

From this, it can be determined that the treatment before irradiation demonstrates no significant differences referring the lethality to the control value until the 1h value is reached.

In the case of formalin application after the irradiation, the probability of exceeding lethality of the first hour and the sixth day is not significant (p = 0.5 and p = 0.2). The differences of the remaining values 2d, 4d and 8d are significant with p = 0.01.

II.3.3 Total-Body Irradiation and Effect of Heat (Heat Shock)

890 male mice of the NMRI stock of a special breed were given an exposure to radiation of 600 R (lethality 36%) or radiated with the same dose after they had been stressed 8, 6, 4, 2, 1 days or 2 hours before and/or 2 hours, 1, 2, 4, 6 or 8 days after the radiation with a heat effect of 50° C of a 12 minutes duration (cf. II.2.5).

Owing to the exposure to heat, a well-defined tachypnea and tachycardia made their appearance 2-3 minutes after beginning of the experiment. /17
Still another three minutes later, the animals became exceedingly restless and showed a severe sweat development. Further a slight exophthalmus as well as cyanosis at the extremities was to be observed. After ten minutes, the mice showed severe apathy and exhaustion and some were affected by spasmotic twitches. The animals gave the appearance of heat shock which led to a lethality of 8%. The combination of this exposure to heat with application of radiation (600 R) at various times before and after irradiation led in most cases to increases in radiation lethality. Figure 4 shows the result of these investigations in graphic form.

It can be seen that the heat shock causes no decrease but rather only increases in the radiation lethality whereby the exposures to heat made after irradiation cause much more well-defined increases in lethality. The effects of the heat shock two days before irradiation as well as 1, 2, 4, 6 and 8 days after irradiation lead to significant increases in lethality ranging from p = 0.5-0.02 to p = 0.01-0.001.

II.3.4 Total-Body Irradiation and Effect of Cold /18

350 male mice of the NMRI stock of our own breed were exposed to a radiation load of 600 R (lethality 64%)* or they were radiated with the same dose and two days or 5 minutes before or 2 or 8 days after irradiation exposed to the effects of cold at -20° C for a period of 30 min. (cf. II.2.6).

* The $LD_{50/30}$ of these NMRI mice was higher than with those used in the previous investigations (II.2.3). In this case, we are dealing with a group (of the same stock) from an earlier investigative series.

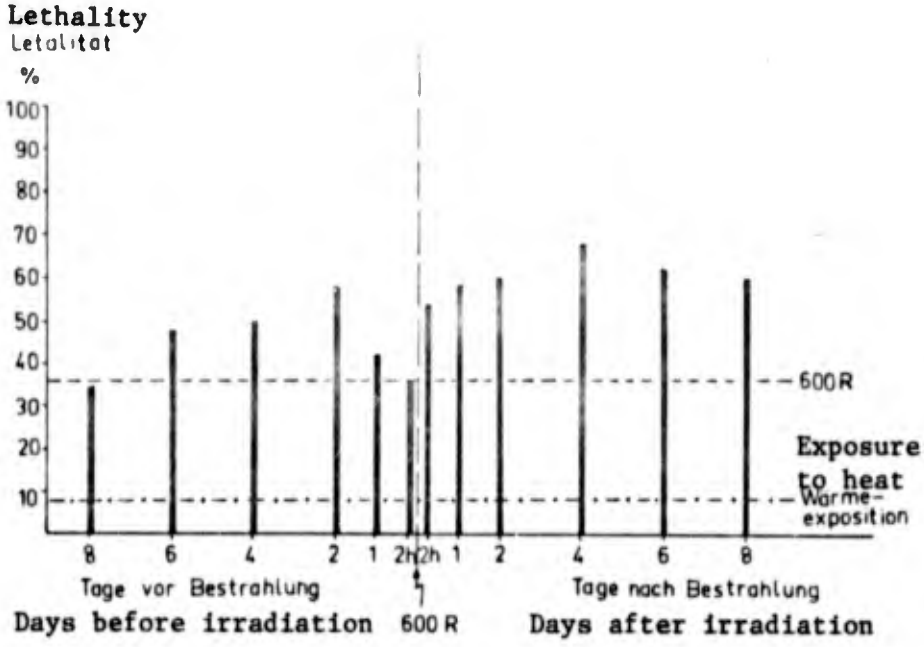

Figure 4. Lethality of male mice which were stressed heat effects (+50° C, 12 min.) at various times before or after irradiation (600 R).

Preliminary testing revealed that a dwell of 60 min. in the cooling chest leads to a lethality of 30% whereas all test animals survive a dwell of 30 min. For this reason, a 30 min. exposure to cold was used in the main experiment. Figure 5 provides a graphic portrayal of the result of this experiment.

When the cold test is carried out two days or 5 min. before irradiation, it follows that the lethality is lowered ($p = 0.05$-0.02 or $p = 0.05$). No noteworthy changes in radiation lethality occurred with exposures to cold after the irradiation.

II.3.5 Total-Body Irradiation and Exhaustion (Forced Swimming) /19

350 male mice of the NMRI stock of our own breed were exposed to irradiation of 600 R (lethality 64%), and two days or 5 min. before or 2 days or 8 days after irradiation subject to severe bodily exhaustion through swimming (cf. II.2.7). Half of the animals had to be taken from the water before conclusion of the testing time in order to avoid their drowning. This was the case with the first animals 98 min. after beginning

the test. Owing to this measure, the lethality of the exhaustion tests amounted to 0%. Figure 6 graphically portrays the results of these investigations. The increases of lethality with respect to the radiation effect alone in the case of exhaustion of the animals 2 days before irradiation ($p = 0.05$-0.01) as well as in the case of exhaustion of the animals 2 or 8 days after irradiation ($p < 0.001$ or $p = 0.01$-0.005) are significant. The lethality of the mice is changed considerably when the exhaustion takes place directly before irradiation ($p < 0.001$).

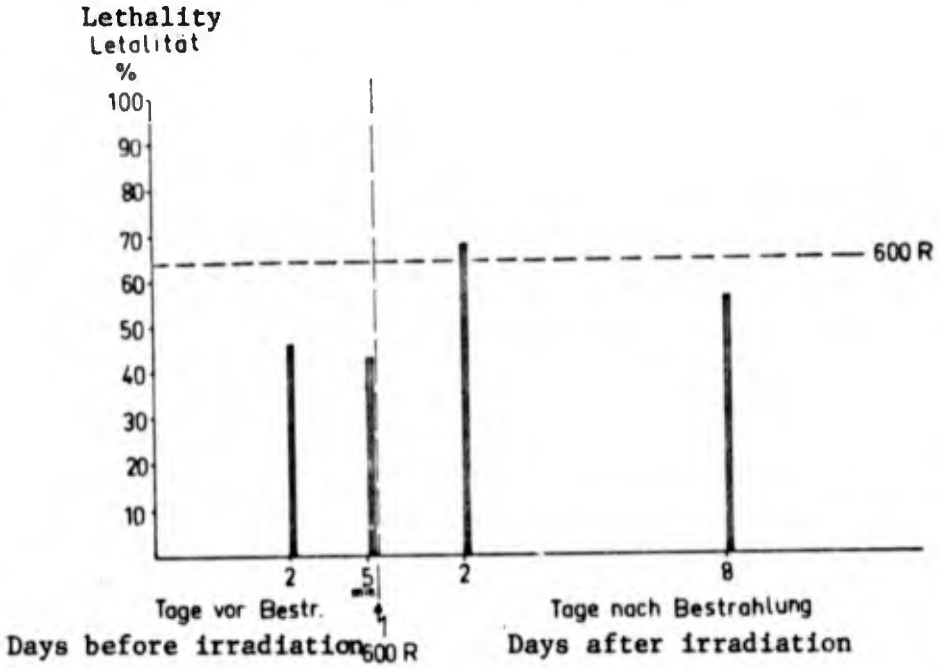

Figure 5. Lethality of male mice stressed with the effects of cold (-20° C, period 30 min.) at various times before or after irradiation (600 R)

II.4 Discussion of Results

/20

The combinations of open skin wounds with total-body irradiations caused, as Figure 1 reveals, only slight increases or even decreases in radiation lethality when the application of the wound precedes irradiation and to intense increases in mortality when the skin wound is made after irradiation. These findings correspond approximately to those already reported in earlier investigations and are described in Reports V-VII [1, 3,4].

In our effort to interpret these changes in lethality, the question of possible bacterial infection through the paths of the open skin wounds was also discussed. The bacteriological investigations carried out [3, 5,6] allow us to regard this possibility as highly improbable although a completely satisfactory answer could not yet be made through these results.

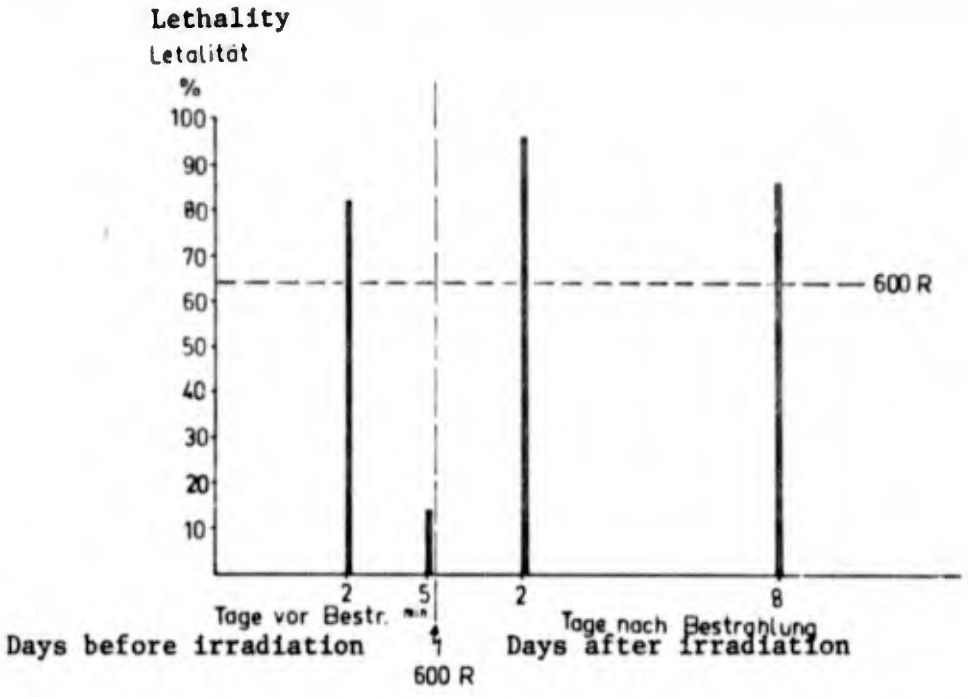

Figure 6. Lethality of male mice who were forced to swim for periods up to 120 min. at various times before or after irradiation (600 R).

The histological investigations of the organs of mice who had been inflicted with an open skin wound 2 days after total-body irradiation and thereby perishing in a very high percentage, revealed in the tissue sections likewise no bacterial accumulations at all or other indications suggesting bacterial infection [3,7].

The exposure of the organism with a closed wound should enable examination whether (without an open wound serving as an entry path for germs) subsequent to an irradiation can likewise lead to such increases in lethality. The creation of an aseptic muscle necrosis by injection of formalin appeared to be a suitable model for this. Figure 3 reveals that no modification in radiation lethality occurred when the muscle necrosis was inflicted before the irradiation with the exception of the time 1 hour before irradiation. In this case, there was a significant drop in radiation lethality.

If the above-mentioned muscle necroses are produced after the irradiation, it follows that the lethality of the test animals increased over those affected by purely radiation effect.

In the case of the combination of total-body irradiation and severe body exhaustion as well, as Figure 6 reveals, there occurs a considerable drop in radiation lethality when the swim test takes place directly before the irradiation. The lethality of the mice was significantly increased when they carried out their swim test leading to exhaustion 2 or 8 days after irradiation. Indeed, an increase in lethality also occurred here when the animals were forced to swim 2 days before the irradiation. /21

The combination of heat shock and total-body irradiation showed another pattern in that the applications of heat before as well as after irradiation led to an increase in radiation lethality. At the same time, it must nevertheless be taken into consideration that with these combined injuries, the heat shock itself involves a lethality of 8% whereas the other forms of stress described in this connection are burdened with no characteristic lethality. If this 8% is added to the radiation lethality of 36%, it follows that a total lethality of 44% is obtained. When comparing this value with those of the combined injuries, no significant increases or decreases in mortality can be determined with the exception of a lethality increase when heat shock is applied 4 days after irradiation ($p = 0.02-0.01$).

The investigations involving undercooling of the animals lead, as Figure 5 reveals, to decreases in radiation lethality in the case of exposure to cold beforehand and to no noticeable changes in mortality in the case of a reversed sequence of both traumata.

The effects of the five above-mentioned stress forms on radiation lethality can only be interpreted in connection with the results found by us in earlier investigations. First of all, it can be assumed that the exposures to heat and cold undertaken by us before or after irradiation as well as the swimming test lead to no additional bacterial infections. In addition, this conclusion can be made, although with considerably less certainty, with regard to muscle necrosis following formalin injection. With the exception of the effect of cold, these stress forms lead to increases in radiation lethality when they act on the mouse organism after /22
irradiation. This is, nevertheless, not the case on every day of actual experimentation. Significant decreases in mortality occurred in no case in this series of exposure to radiation and traumata. Indeed, the increases in lethality were considerably less than with the production of open skin wounds. With the exception of muscle necroses, heat shock, cold reaction and severe exhaustion are only transitory injuries. Nevertheless, they could lead to irreparable states of shock with an organism previously injured as a consequence of irradiation. This could have the consequence of an increase in mortality. This lethality increasing far higher owing to the effect of open wounds could be explained by the longer-lasting wound process. Even the aseptic muscle necrosis is a process which heals slowly although its effect on the radiation injuries increases its lethality to a far lesser extent. In this case, there is a difference with respect to the open wound in that the animals with the wounds could lose liquid and especially protein albumin materials through the skin defect. It appears that this circumstance conditions the high mortality of the test animals to a particular degree. The following chapter shall, for this reason, be devoted to the changes in serum proteins with combinations of total-body irradiations in skin wounds, and it shall, at the same time, be sought to explain the very high mortality of animals with this kind of combined injury.

The phenomenon of a decrease in lethality owing to stress effects preceding irradiation was possible to demonstrate in the experiments

through the effect of cold, muscle necroses, and especially through the exhaustion experiment. This finding was especially clear when the trauma was applied directly before the irradiation. We were able to describe such an effect in previous investigations especially in the case of abdominal operations such as splenectomies, test laparotomies and with skin wounds which are immediately resutured [3,8]. It had been possible to achieve a significant drop in mortality even with open skin wounds when the operations preceded irradiation in the case of earlier investigations although not in the case of the present ones.

The possible causes for this effect have been discussed by us in detail [3,9]. In this regard, consideration was given especially to the possibility of a pituitary-adrenal gland-reaction as a consequence of the stress preceding the irradiation and its protective function with respect to the subsequent radiation exposure. Chapter 4 of this report following will examine the behavior of the corticoids in the case of combinations of skin wounds with total-body irradiations. Chapter 5 as well will take up the same type of problems. Reports will be made concerning investigations applying to the question as to what extent a wound can have effects on the bone marrow and, indeed, in the direction of stimulating formation of blood which then has reactions again on the severity of radiation damage and consequently on the lethality of the test animals. /23

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III. SERUM PROTEIN CHANGES IN MICE EXPOSED TO TOTAL-BODY IRRADIATION IN COMBINATION WITH OPEN SKIN WOUNDS /25

III.1 Introduction

Reports IV and VI of this research project discuss the results [1,2] of "Electrophoretic albumin determinations in rats which have been exposed to total-body irradiations in combination with open skin wounds". The investigations show as a consequence of interaction of radiation effect and open wounds a reduction in the total protein the serum down to 76% of the normal value, considerable losses of the albumin component, a lesser reduction of gamma globulins and increases in the α_1-, α_2- and β-globulins in the serum.

Such investigations had not yet been carried out with mice although in available literature there can be found reports on investigations of blood protein not combined with the effects of radiation. Thus, Sassen et al. [3] reported that in mice prealbumins, albumins, α_1-globulins and γ-globulins decrease in their relative composition following lethal and even sublethal irradiation whereas α_2- and β-globulins increase. Mathe [4] et al. likewise reported a rise in α_2-globulin and a drop in albumin and γ-globulin. Grabar [5] and Kerschkin were able to make somewhat different statements. Using immunoelectrophoresis, they observed a shortening of the precipitation line in the prealbumin and γ-globulin, a reduction of the precipitation lines in the β-globulin area and, finally, new precipitation lines in the α_2-globulin region. Granson [6] et al. demonstrated an abnormal component having its mobility in the electrical field in the α_2-globulin region. It appears 3-4 days following irradiation with 950 R and is demonstrable up until death. Shekarchi [7] and Makinodan investigated the composition of serum proteins following irradiation with simultaneous administration of AET (aminoethylisothiouroniumbromide) and isologous bone marrow. It was only possible to determine a reduction in the albumin content and an increase in the globulin content after 950 R.

In our own investigations which will be described in the following, /26
we shall examine to what extent the serum-protein profile is modified with mice not only after radiation damages but also after combined injuries and to what extent conclusions can be drawn as to the lethality of the test animals.

The model for a combined injury will be, as in previous investigations, the interaction of total-body irradiation and open skin wounds whereby the skin wounds will be inflicted at various times before or after irradiation.

III.2 Material and Methodology

Male mice of the NMRI stock of our own breed and 8 weeks old were used for the ivestigations. The mice were given total-body irradiation with an individual dose of 600 R. The conditions of irradiation corresponded to the data provided in section II.2.2. The skin wounds (SW) were

inflicted 7, 4 2 days before and 1 hour, 2, 4, 8, 14 and 21 days following irradiation. The technique for producing the skin wounds is provided in section II.2.3.

In predetermined time intervals, i.e., 1, 2, 4, 7, 10, 14 or 21 days following the last inflicted injury (irradiation or skin wound), five mice at a time were decapitated under ether narcosis and the blood flowing out collected individually in centrifuge capillary tubes. The product was allowed to stand for 2 hours and then was centrifuged for 10 . in. at 2000 rpm. The supernatant serum was siphoned off and again centrifuged for 5 min. at 3000 rpm. The sera were frozen and examined individually on the following day. Food was removed one day before decapitation whereas water was given as needed.

The electrophoretic separation was carried out on sheets of cellulose acetate in a Mikrozon chamber of the firm Beckman. The separation time lasted 25 min. using a constant voltage of 250 V. The veronal used (veronal-Na-buffer solution) had a pH of 8.6 and an ionic strength of 0.1. The protein-staining was done using amido black 10 B [8,9]. A mixture of dioxane and isobutanol 7: was used as transmittance liquid.

The transparent electrophorograms were plotted using a Zeiss integral /27
recorder. The values reflect the relative proportion of the separated proteins. This relative value is meant when "value" is mentioned in the section "Results". The height of the individual peak of the extinction curve is a measure for the absolute concentration of the separated components of the serum protein. Accordingly, statements can be made on the basis of a plane and elevation calculation of the peak concerning an increase or decrease of the absolute protein concentration of the individual fractions provided that the same sample quantities of the serum are used for the development of the electrophorogram. This is usually the case with a careful handling of the sampling plunger which has a capacity of 0.25 µl. The average values of the corresponding peaks are formed and the dispersion calculated from the five extinction curves determined per test.

Qualitative glycoproteide stainings as well as lipoproteide stainings on cellulose acetate sheets separated under the same electrophoretic conditions as above were used to specify the protein changes found.

III.3 Results

III.3.1 Recordings and Computations for Significance

Figures 7 to 12 show in the form of curves the percentage proportions of electrophoretically separated protein fractions of the serum at various times following irradiation, infliction of the wound and combined damage. At the same time, in addition to the curves of the values for the different combined injuries, the curves for the uncombined radiation injuries and skin wounds have also been drawn for purposes of comparison. It was calculated to what extent there exists a statistically significant difference between the values after combined and individual injury. At the same

time, the protein values after combined injury are always compared with the values of the morbific agent standing in second position with respect to time. Accordingly, in the case of combined injuries, in which irradiation takes place after the application of the wound, the comparison is undertaken with the values of the individual irradiation effect with reversed sequence of the tube morbific agents with the protein values after application of the wound. Student's t-distribution is used for calculations for the significance. On Figures 7 to 12 no symbol is given with differences which are not significant ($p \geq 0.05$) whereas significant differences ($p < 0.05$ to $p = 0.001$) are characterized by an (x) in the case of comparison with radiation effect. Highly significant differences ($p < 0.001$) are marked with two (xx), significant differences with respect to the values in the case of skin wounds with an (o) and, finally, highly significant with two (oo). /28

Table 5 shows the values of the highest and lowest point on the curves of the individual protein fractions for the various combined and individual injuries. It can already be seen that combined injuries lead to much more accentuated changes in the protein composition than is the case after individual wound application or irradiation. This is especially valid for the wound combinations in which the wounds are caused after irradiation. Figure 13 shows the examples of a few extinction curves. Figure 14 provides the electrophorogram of a number of sera.

III.3.2 Prealbumin (Figure 7a-k)

One skin wound (SW) causes (Figure 7a), as early as after one day, a drop in the prealbumin value which, however, after the fourth day again adjusts slowly to the normal value. After irradiation with 600 R, a clear reduction is visible by the tenth day which by the 14th day is further intensified (30% of the normal value). The normal value is almost achieved after 21 days and the changes are accordingly more well defined than after application of the wound.

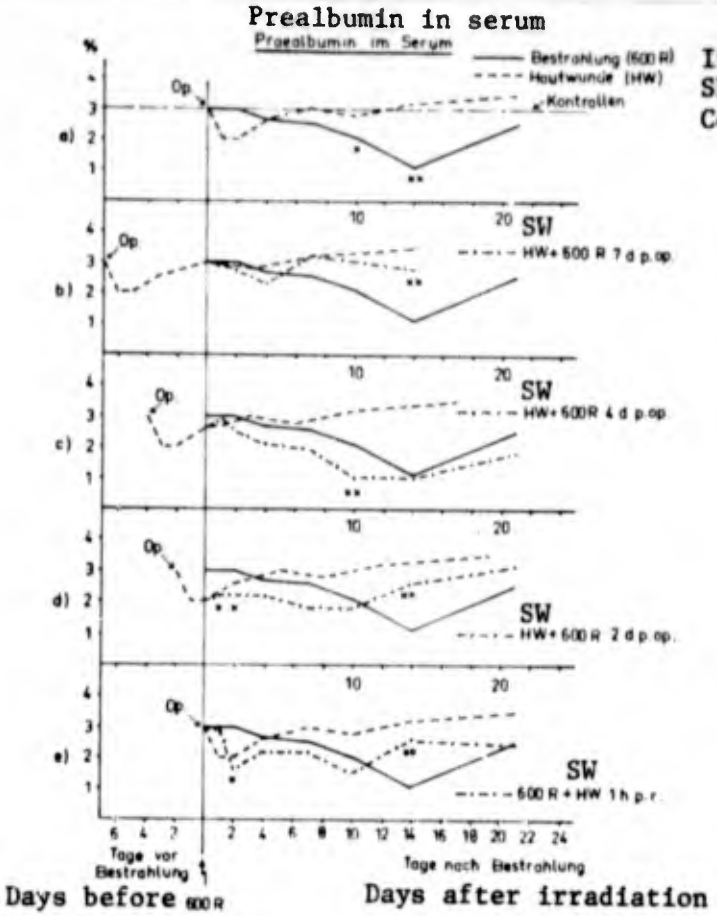
With combined injuries, when skin wounds are inflicted <u>before</u> or within 1 hour <u>after</u> irradiation (Figure 7b-4), there initially results a recognizable statistically insignificant drop in the values when compared to irradiation alone. It is remarkable, however, that the normal value is again achieved 14 days following irradiation when the skin wound is inflicted 7 days or 2 days before irradiation or 1 hour after irradiation (Figure 7b, d, e). This is a highly significant difference from the comparative value of the irradiation effect alone. /31

Skin wounds produced <u>after</u> irradiation (Figure 7f-k) cause a steady drop in prealbumin values until death of the animals. The prealbumin content drops to 0.33% in animals who have received a skin wound 4 days after irradiation (Figure 7g). The wound application on the 14th day after irradiation (Figure 7i) takes place at the time of the most severely changed prealbumin values in animals which had only been irradiated. There are further reductions in the prealbumin values which may be significantly differentiated from the corresponding values of the animals only inflicted

	Praealbumin		Albumin		α_1-Globulin	
	Max.	Min.	Max.	Min.	Max.	Min.
Control animals	3.0±0.5		50.0±2.7		16.4±1.5	
Skin wounds (SW)	3.5±0.5	2.0±0	45.8±2.2	39.3±1.9	16.9±0.9	13.0±2.1
600 R (½)	3.0±0.5	1.1±0.2	50.8±1.8	38.2±2.9	13.6±1.6	7.5±1.4
SW+½7d p.op.	3.2±0.8	2.3±0.4	43.1±3.9	39.2±6.4	14.1±0.6	7.2±2.1
SW+½4d p.op.	2.9±0.2	1.0±0	42.3±2.5	28.6±1.5	15.1±2.0	6.2±1.3
SW+½2d p.op.	3.1±0.7	1.8±0.8	50.6±1.9	34.8±2.0	17.1±2.5	8.2±0.8
600R+SW 1h p.r.	3.0±0	1.5±0.6	50.2±2.0	33.1±3.7	14.1±1.2	7.6±0.7
600R+SW 2d p.r.	2.1±0.2	0.8±0.5	39.6±1.1	26.0±2.7	16.0±0.7	6.7±0.6
600R+SW 4d p.r.	2.1±0.8	0.33±0.3	43.3±2.3	25.3±1.4	14.1±0.6	8.2±0.6
600R+SW 8d p.r.	1.6±0.3	1.0±0.5	40.6±2.1	33.1±0.8	10.1±0.5	8.7±1.6
600R+SW14d p.r.	1.0±0.5	0.8±0.3	36.5±4.4	28.6±0.8	7.6±0.3	5.8±1.6
600R+SW21d p.r.	2.8±0.3	0.5±0	44.6±2.1	31.6±3.6	10.8±2.8	7.0±0

	α_2-Globulin		β-Globulin		γ-Globulin	
	Max.	Min.	Max.	Min.	Max.	Min.
Control animals	13.0±1.3		15.2±0.7		3.0±0	
Skin wounds (SW)	18.1±1.8	15.3±1.3	21.6±1.4	16.5±0.9	4.2±0.3	2.0±0.4
600 R (½)	21.6±4.1	11.0±0	24.3±3.1	15.3±0.8	9.8±0.4	3.0±0
SW+½7d p.op.	22.0±1.7	15.7±1.5	24.5±3.5	20.0±3.3	5.3±2.1	2.3±0.8
SW+½4d p.op.	24.5±3.0	17.5±1.8	33.5±2.2	20.2±2.3	6.5±2.1	1.8±0.3
SW+½2d p.op.	24.2±2.5	13.1±1.7	26.2±2.5	17.9±2.0	6.6±1.0	1.0±0
600R+SW 1h pr.	25.7±1.2	12.7±0.8	26.9±3.4	15.1±1.2	6.6±1.3	1.5±0.5
600R+SW 2d p.r.	30.3±1.5	18.0±0.7	33.7±1.2	21.1±1.3	2.2±0.3	1.0±0
600R+SW 4d pr.	27.8±0.8	19.2±0.8	37.3±1.2	19.6±1.5	2.1±0.2	1.5±0.5
600R+SW 8d pr.	24.7±1.5	24.6±1.2	29.0±0.7	22.8±1.2	2.0±0	1.2±0.3
600R+SW14d pr.	28.0±1.8	22.9±1.7	31.6±1.3	27.8±2.5	4.8±0.3	2.3±0.6
600R+SW21d pr.	20.6±2.1	15.8±2.5	30.1±1.8	18.1±3.0	6.5±0.9	4.0±1.7

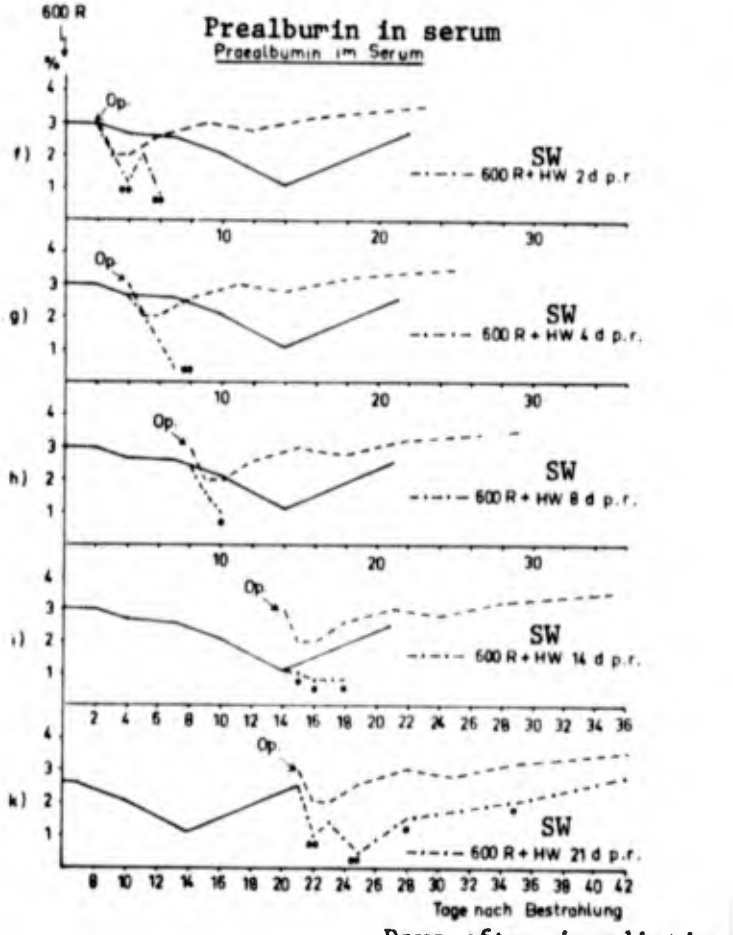
Table 5. Highest (max.) and lowest (min.) values of the protein fractions in the serum of mice which have been irradiated (600 R), inflicted with open skin wounds (SW) or exposed to various combinations of both types of injuries. (p.op. = before irradiation; p.r. = after irradiation)



Irradiation (600 R)
Skin wound (SW)
Control animals

Figure 7. Prealbumin (relative % in serum) in mice inflicted with an open skin wound (SW) at various times before or after irradiation (600 R).

(p.op. = time of wound application;
↳ = irradiation;
p.r. = after irradiation)



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with skin wounds. If the skin wound is inflicted 21 days after irradiation (Figure 7k), it follows that this clearly affects the recovery phase. A clear reduction of prealbumin is the consequence whereby the values lie under those of the animals who are only inflicted with skin wounds.

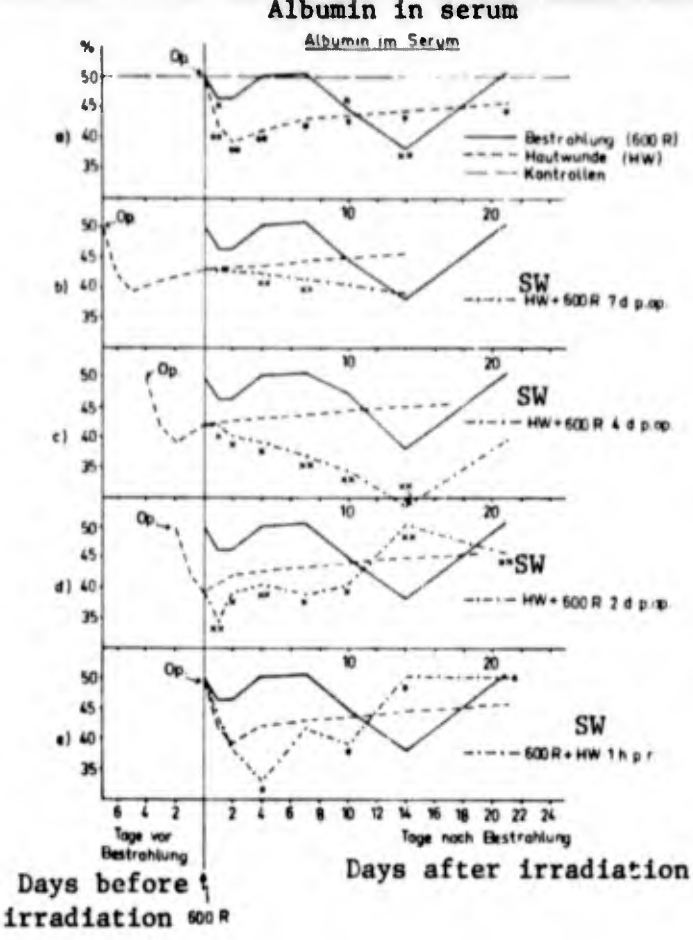
III.3.3 Albumin (Figure 8a-k)

The infliction of a skin wound leads after the first day to a severe reduction in albumin values which reach their lowest point on the second day. After this, the values slowly recover, not returning to the normal value until the 21st day. The albumin values after a "pure" exposure to radiation have their strongest reduction on the 14th day. They are approximately 25% under the normal value. The normal value is again achieved after 21 days (Figure 8a).

If the skin wounds are caused before irradiation, it follows that the albumin content is affected in various ways. In the case of wound application 7 days before irradiation, values are found which, as can be seen from Figure 8b, lie mostly far under the "pure" radiation and wound injuries. /33 Of the four values measured, those of the 4th and 7th day after irradiation are significantly different. The wound application 4 days before irradiation leads to values demonstrating highly significant differences from the 4th day on. The 14 day value is situated far under that of the "pure" radiation damage with an almost 50% decrease. If the operation is performed 2 days before irradiation, it follows that the albumin values are situated far under those of the radiation or wounding alone. Later on, the values highly significantly exceed the comparative values of the irradiated animals on the 14th day. If the application of the wound and irradiation take place at approximately the same time, it follows that the albumin values drop until the 4th day. After that, the recovery phase begins whereby the irradiation value is also far exceeded here on the 14th day. The 4 day value lies significantly under the skin wound value and highly significantly under the irradiation value. The 21 day value corresponds to the irradiation only value. If the skin wound is caused after the irradiation, it follows that all test series show a similar profile; i.e., a decrease in albumin values. According to the progress of the skin wound values, the values immediately decrease to a considerable extent. This decrease remains continuous until death of the animals. Values for this have been found situated almost 50% under the norm.

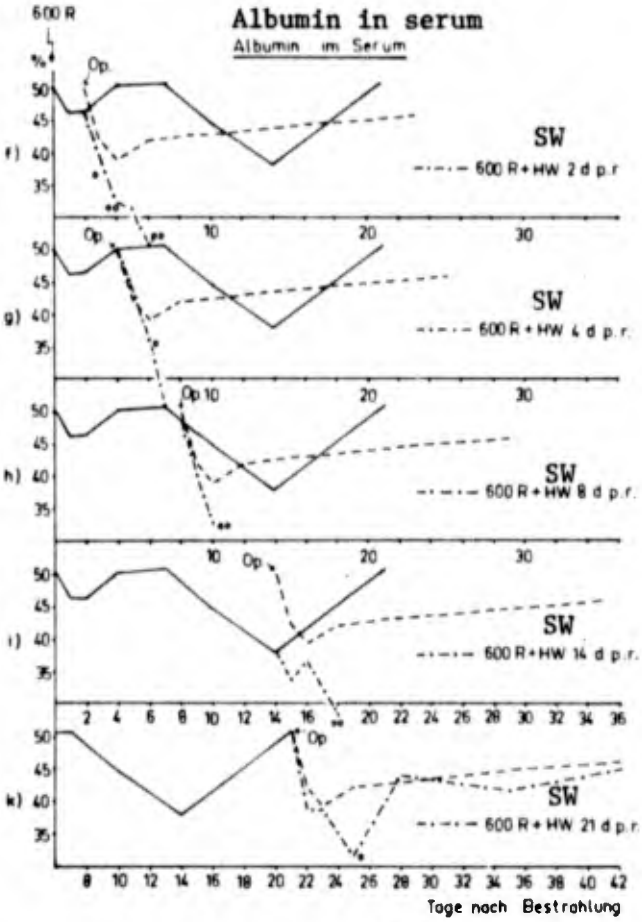
The progress of the albumin values in the case of operation 21 days following irradiation is differentiated from the skin wound values by a decrease extended up until the 4th day. The 4 day value is significantly lowered. Accordingly, the values again correspond (Figure 8k).

The extinction curve (Figure 13b, d, f) reveals in the albumin area, especially in the case of the lowest values, a severe reduction of the albumin peak whose area is up to 50% less in value. Accordingly, a considerable absolute reduction in albumin concentration can be concluded.



Irradiation (600 R)
Skin wound (SW)
Control animals

Figure 8. Albumin (relative % in serum) in mice which have been inflicted with an open skin wound (SW) at various times before or after on irradiation (600 R).



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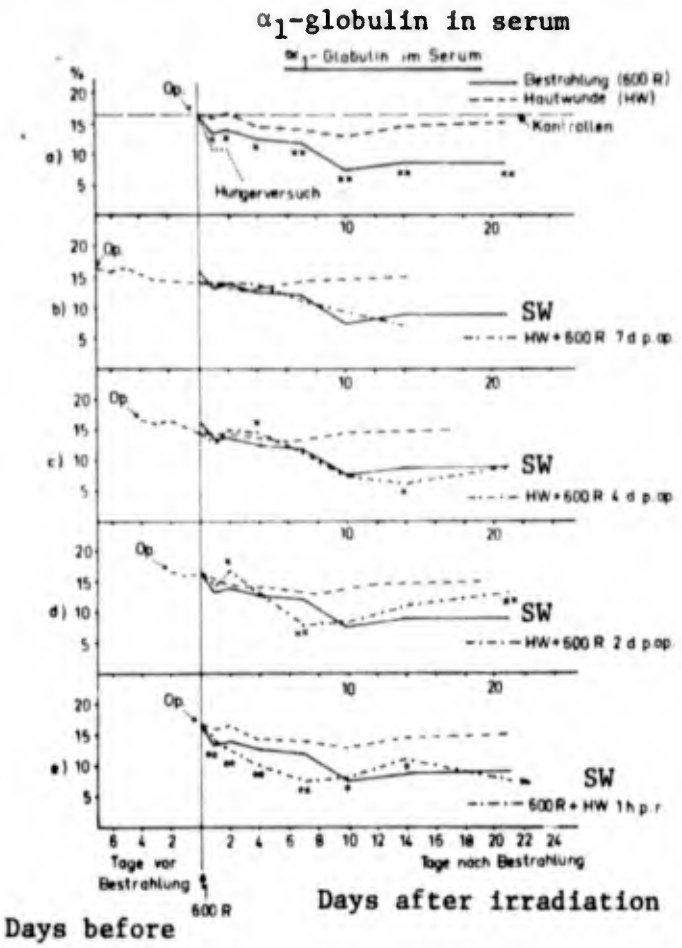

Irradiation (600 R)
Skin wound (SW)
Control animals

Figure 9. α_1-globulin (relative % in serum) in mice which have been inflicted with an open skin wound (SW) at various times before or after irradiation (600 R).

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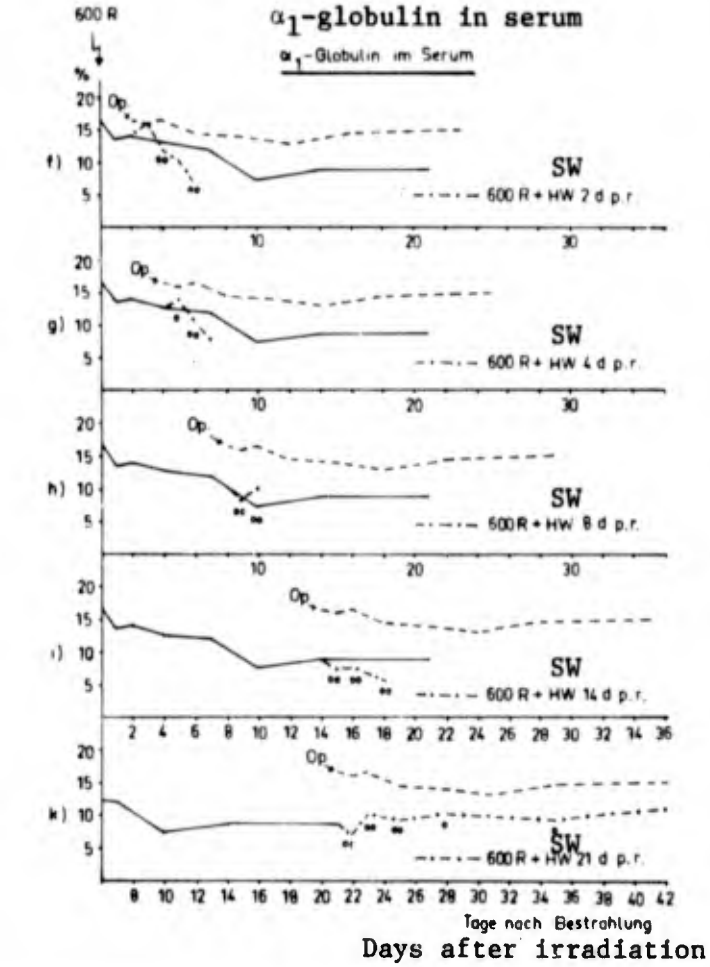


III.3.4 α_1-Globulin (Figure 9a-k) /35

A skin wound has only a slight effect in the direction of a decrease in α_1-globulin values. By irradiation, on the other hand, a lowest value is reached after 10 days which is situated 50% lower than that of the normal value. No essential change of this lowest value is observed even by the 21st day. Similar α_1-globulin values are obtained when inflicting a skin wound before irradiation as after pure irradiation. If the wound application is made 2 days before irradiation or at about the same time, it follows that the lowest values appear 3 days earlier than in the case of the irradiation-only values.

Skin wounds produced after irradiation lead as early as the very first days to a decrease in α_1-globulin values. The lowest value is reached after 4, 3, 2 or 4 days corresponding to the time intervals between irradiation and wound application.

If the skin wound is produced after 21 days, the α_1-globulin values are situated only slightly under the corresponding values after irradiation. The further values drop approximately to 33% under the normal value. Even the 21 day value is not significantly changed. All values are situated running almost parallel under the skin wound values.

It was possible to determine with a hunger test that the α_1-globulin values were most severely modified. They drop still further under the lowest value which is obtained after combined injuries.

The α_1-globulin fraction is formed by two peaks in the normal serum which, however, coincide in the sequence of radiation or combined injury and appear as one peak (Figure 13a, b).

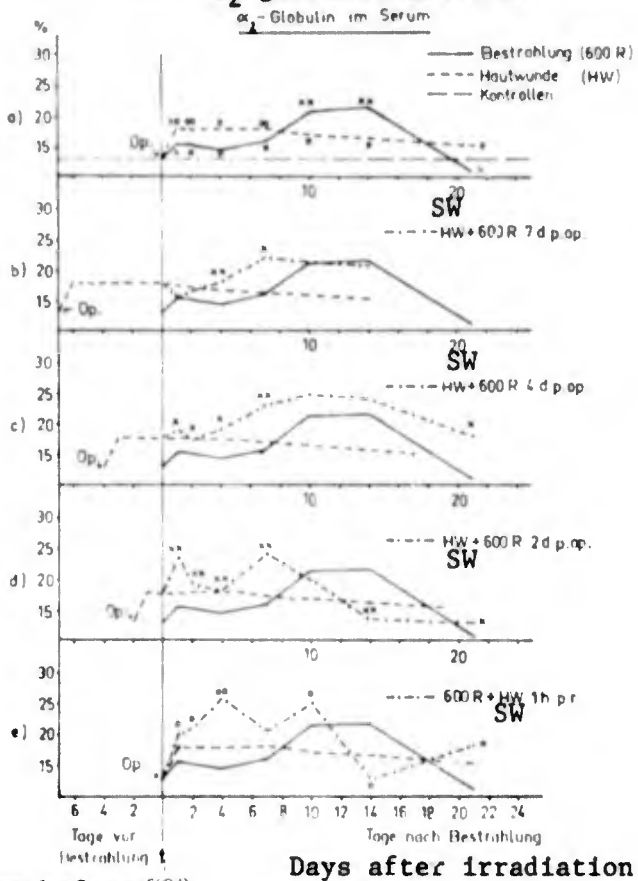
The investigation of the glycoproteides of the α_1-globulin fraction which are also separated into two peaks in the normal serum coincide in the sequence of radiation damage in a similar manner and then have a total glycoproteide content of 15% whereas its proportion in the normal serum amounts to 41%.

The comparison of the surface of the α_1-globulin peak of the normal /37
serum with one in the advanced combined injury indicates a severe decrease in absolute protein concentration of the α_1-globulin fraction.

III.3.5 α_2-Globulin (Figure 10a-k)

The wound application leads on the first day to an increase in the α_2-globulin value. The values gradually drop again without reaching the normal value after 21 days. After irradiation, the α_2-globulin values increase from the 7th day on and reach their highest value on the 14th day. On the 21st day, the value is situated considerably under the normal value. If the skin wound is inflicted before irradiation, the progress of the α_2-globulin values is similar to that of the pure irradiation

α_2-globulin in serum



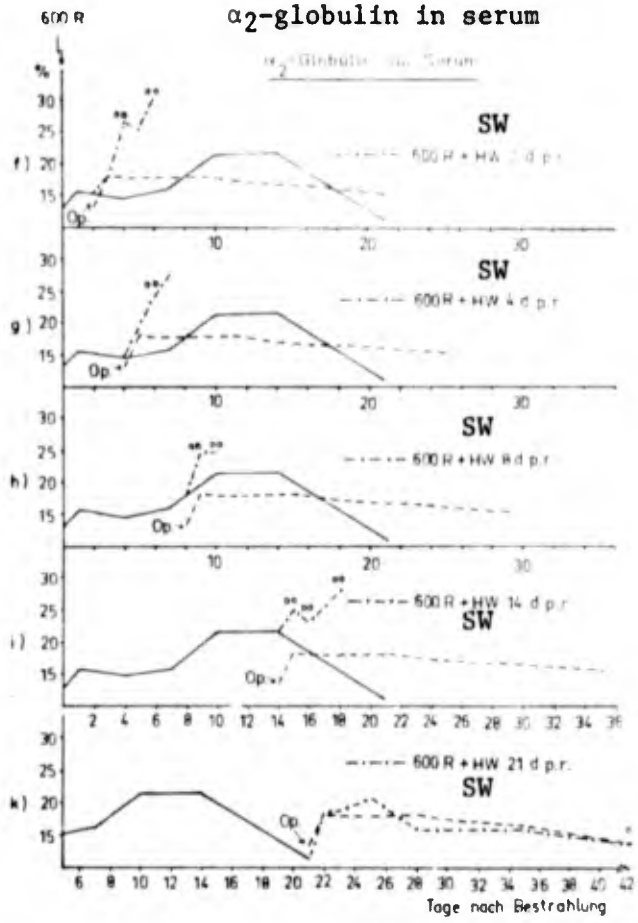
Irradiation (600 R)
Skin wound (SW)
Control animals

Days before irradiation

Days after irradiation

Figure 10. α_2-globulin (relative % in serum) in mice inflicted with an open skin wound (SW) at various times before or after an irradiation (600 R).

600 R α_2-globulin in serum



Days after irradiation

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values but shifted forward by 3 days. Skin wounds inflicted 4 days and 2 days before irradiation produce an additional α_2-globulin maximum value which, however, corresponds on the second day after irradiation again to the value corresponding to wound application alone.

If the skin wound is inflicted at approximately the same time as the irradiation, it follows that an early increase in the α_2-globulin values may be observed reaching the first maximum value on the 4th day and its increase may be considered highly significant in comparison to the skin wound value and to the irradiation-only value. The second maximum value is observed on the 10th day. In the case of this test, just as with the operation 2 days before the irradiation, the normal value is already achieved after 14 days. It is also significantly increased in comparison to the irradiation-only value.

Skin wounds inflicted after irradiation lead to a heavy increase in α_2-globulin values amounting occasionally to over 100% before onset of death. The increases are highly significant in the final stage. Skin wounds produced 21 days after irradiation lead to no noteworthy increases in comparison to the values after infliction of wound (without irradiation).

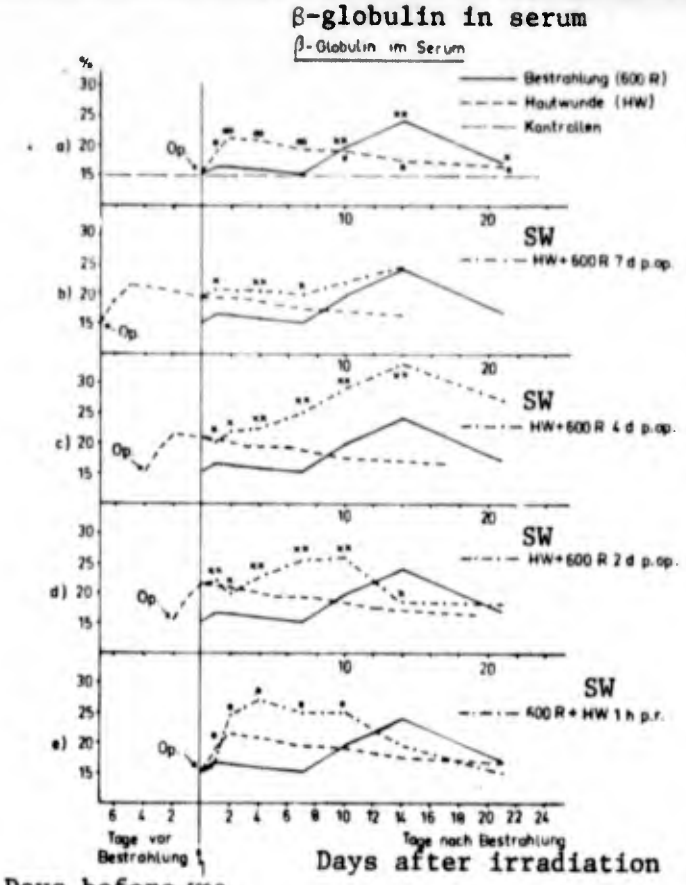
The peak surface of the α_2-globulin from the serum of the advanced combination or radiation injury has more than doubled with respect to the peak surface of the α_2-globulin from the normal serum (Figure 13a, b).

The extinction curve of the glycoproteides provides a surface magnified by two- or threefold in the α_2-globulin area (Figure 13c, d). The proportion of the α_2-glycoproteide in the total glycoproteide content in the normal serum amounts to 28%. This value rises to 50% with combined injuries. /39

III.3.6 β-Globulin (Figure 11a-k)

The β-globulin values also rise with wound application, the increase taking place rapidly up to the 2nd day. After this, they are close to the normal value without reaching this until the 21st day. The progress of the β-globulin values following irradiation is similar to that of the α_2-globulin values.

If the skin wound is inflicted before irradiation, it follows that the β-globulin values have a progress similar to that of α_2-globulin values. When the skin wound is caused 4 days before irradiation, even more pronounced changes are to be seen and all values are significantly increased. If the skin wound is inflicted after irradiation, or may likewise be found in the β-globulin component, a steep rise which can amount to over 100% before onset of death. If the skin wounds are caused after 21 days, it follows that they lead to a different profile when compared to the α_2-globulin values. The curve is always situated here over the skin wound value and is increased to a highly significant extent in many areas.



Irradiation (600 R)
Skin wound (SW)
Control animals

Figure 11. β-globulin (relative % in serum) in mice which were inflicted with an open skin wound (SW) at various times before or after an irradiation (600 R).

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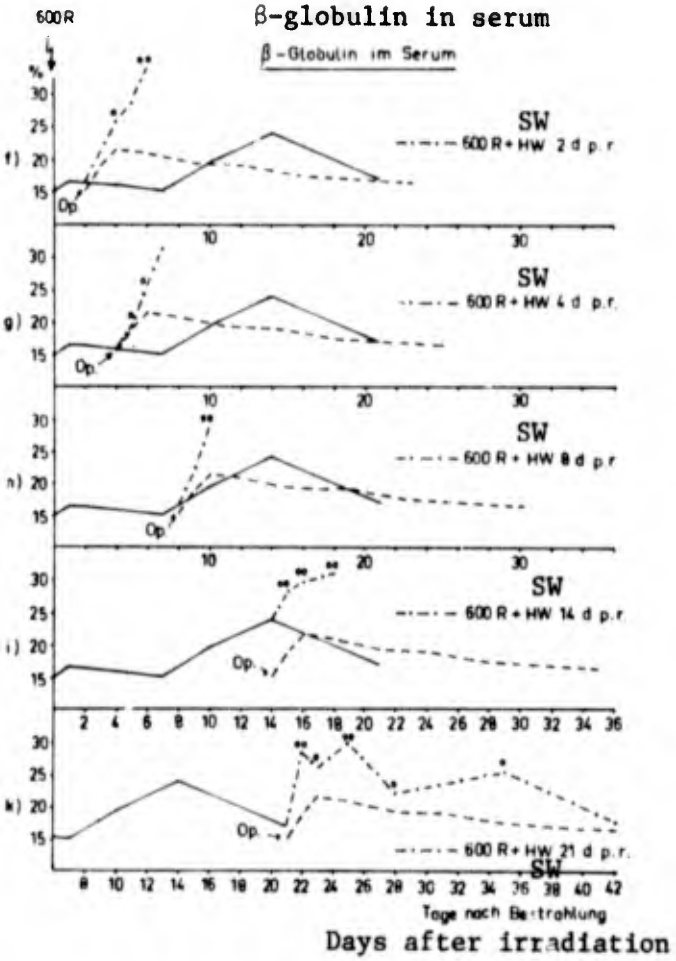

The peak surface of the β-globulins or β-glycoproteides is seen to become larger in the case of intense combined injuries. This has already been noted with the α_2-globulins or α_2-glycoproteides (Figure 13a, b). The proportion of the β-glycoproteides in the total glycoproteides amounts to 19% (Figure 13c, d). This value rises to 32% with combined injuries.

The lipoproteide staining of a normal serum results in two bands (Figure 13c, f) whereby the first band is situated in the α_1-α_2-globulin region of the second band located in the β-globulin region (β-lipoproteides). The quotient of the first and second band amounts to 1.7 in the normal serum whereas it decreases to 1.0 in the case of the combined injury (600 R + SW 2d p.r.). At the same time, it can be seen from the extinction curve that the quantity of lipoproteide has not decreased in absolute quantity in the α-globulin region whereby it can be concluded that there has been an absolute increase in the β-lipoproteide region.

III.3.7 γ-Globulin (Figure 12a-k) /41

The wound infliction initially causes a decrease in γ-globulin values. After the 7th day, the values again rise and stay from the 10th to the 21st day on a plateau situated above the normal value. Irradiation alone results in hardly a change in γ-globulin values within the first 7 days. From the 7th day on, the value rises and exceeds the normal value by 200% on the 21st day.

Skin wounds which are caused before or simultaneously with irradiation lead to γ-globulin values which are situated lower than values from irradiation alone. The value becomes increasingly low as the infliction of the wound and irradiation become more coincident in time. After the 8th day, the values increase considerably as in the case of irradiation alone.

Inflictions of wounds 2 and 4 days following irradiation lead to steadily decreasing γ-globulin values. In the tests involving creation of a skin wound 14 and 8 days following irradiation, the operation encounters a phase of increase of γ-globulin values as a consequence of radiation effect. Thereby it starts initially with a decrease in γ-globulin values and followed 1 or 2 days later by an increase. In the case of skin wounds which were produced 21 days after irradiation, a plateau is reached on the 7th day after an initial decrease in γ-globulin values. This plateau continues until the 21st day whereby the plateau line runs parallel to the pure skin wound values.

Two additional study groups of mice were used to determine the γ-globulin values for older individuals. Twenty-four-week-old mice show a γ-globulin content of 4.5%, 32-week-old animals reveal one of 6.5%. These values are situated significantly higher than the γ-globulin contents amounting to 3% in the 8-week-old mice investigated by us.

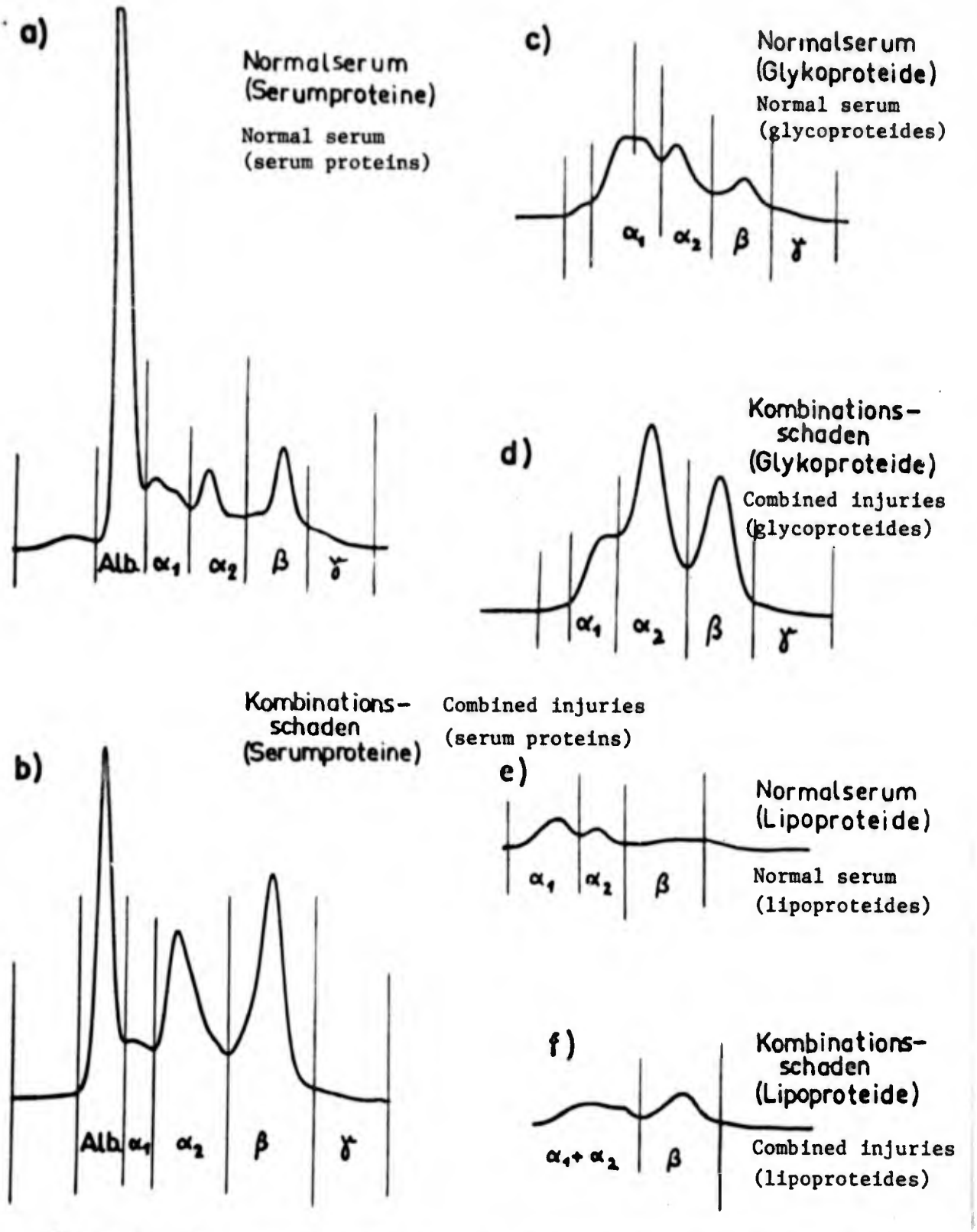

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Figure 13. Extinction curves of serum protein electrophorograms (a and b), glycoproteide electrophorograms (c and d), and lipoproteide electrophorograms (e and f), in each case of normal sera and combined injuries (600 R + SW 2d p.r.). Serum from the 4th day after operation.

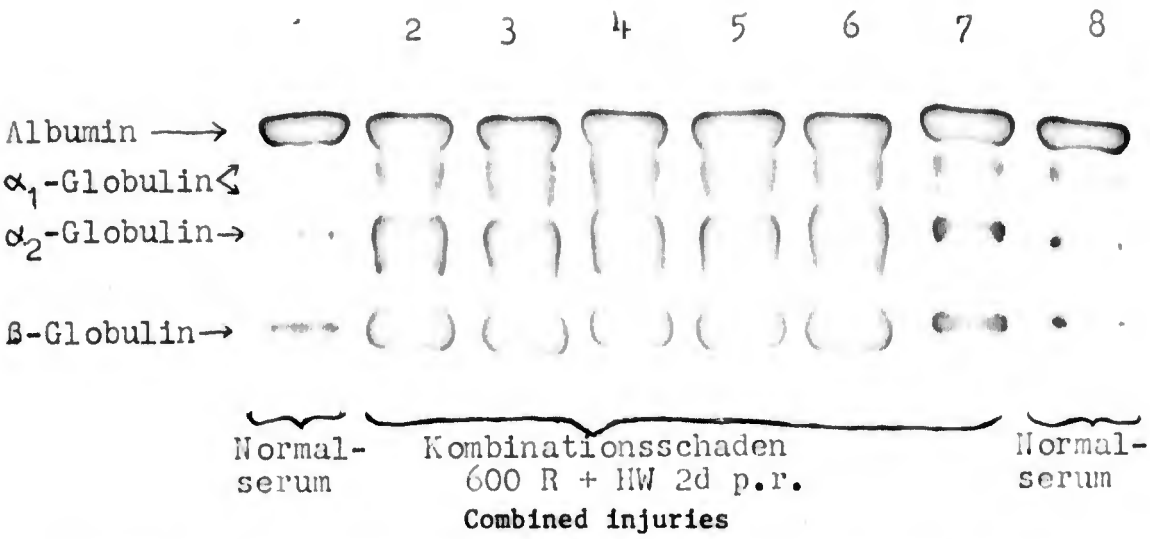

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Figure 14. Electrophorogram of a normal serum (1,8) and of a combined injury (600 R + SW 2d p.r.) (2 to 7).

The electrophorograms 2, 3, 4, 5 and 6 in Figure 14 were made from sera which were obtained after sacrificing the animals 2 days after infliction of the wound. Electrophorogram 7 was obtained after sacrificing 4 hours following the skin wound operation. The phorograms 2, 3, 4, 5 and 6 clearly show the increases of α_2 and β-globulins. The prealbumin and γ-globulin components could not be made visible by photographic means owing to the minimal content. Electrophorograms from the normal sera (1 and 8) may be used for purposes of comparison.

III.4 Discussion of Results

The serum protein changes which occur with irradiation alone or infliction of a skin wound are reinforced by the combined injury. If the skin wound is inflicted after irradiation, there then follows an especially intensive change in the protein profile which also has a parallel in the higher lethality of test animals. The irradiation leads to especially pronounced protein displacements in the period from 7 to the 14th day. A skin wound inflicted in this time interval can clearly no longer be taken care of by the organism with its natural mechanisms of adjustment. /44 When the skin wound is inflicted 2 days following irradiation, the average survival time amounts to 4 days. This is reduced to 3 days when the wound is inflicted 4 days following irradiation, and, finally, when the wound is inflicted 8 days after irradiation, the survival time is reduced to 2 days. When the wound is inflicted 14 days after irradiation, the survival time again increases and when the wound is inflicted 21 days after irradiation, the lethality of the animals is no longer increased.

It is worthy of note that in the case of a wound infliction 2 days before irradiation as well as almost simultaneously with the irradiation,

the normal values are reached after 14 days whereas this is only the case after 21 days with irradiation alone. It is possible that a prematurely activated accommodation through the skin wound is seen here.

Hardly any differences are found in the α_1-globulin region when a comparison is made of the protein profile of radiation injury and combined injury. The hunger test which revealed a heavy reduction in the same region suggests that this effect is attributable to the lasting loss of appetite with rejection of food occurring after irradiation.

The decrease in γ-globulin values only appeared with combined injury. In this respect, the excessive production of γ-globulins on the 21st day after irradiation is noteworthy. Reuter et al. [10] reported concerning a comparable increase in γ-globulins and other serum proteins with mice after irradiation and additional incorporation of phenylalanine and tryptophane. The γ-globulin values are quite low in the mice stock used for our investigations. The humoral immune bodies possibly appear in other protein fractions.

The albumin values decreased most spectacularly with combination injury. On the basis of our own investigations on rats [11], this circumstance was explained in that the disturbance in the permeability of the capillaries in the area of the skin wound is more pronounced with additional radiation exposure. The increased capillary permeability then is especially effective on the albumins which have the smallest molecular weight.

The increases in the α_2- and β-globulin area could originate from /45
tissue disturbances. It is well known that irradiation causes a massive cell destruction especially in the lymphatic and hematopoietic system. Further, protein deposits could be activated owing to the decrease in albumins. This loss of albumin could be compensated for by the release of proteins from the protein deposits into the circulation in order to maintain the flow properties of the serum.

The loss of prealbumin with combined injury corresponds quite closely to that of a pure radiation injury. Pectom et al. [12] characterized this loss as a sensitive effect of protein balance. Tests with lipoprotein breeds revealed in the lipo-prealbumin after irradiation a lesser decrease than in the prealbumin with a high concentration of trypotophane. Since trypotophane is an essential amino acid, it can be assumed that the rejection of food following radiation contributes to this reduction.

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IV. THE CONTENT OF CORTICOSTEROIDS AND CHOLESTEROL IN THE ADRENAL GLANDS AND SERUM OF MICE EXPOSED TO TOTAL-BODY IRRADIATION IN COMBINATION WITH OPEN SKIN WOUNDS /47

IV.1 Introduction

There are a good number of experimental findings available concerning the role of the adrenal cortex in biological radiation injury and the significance of this organ for the symptoms of radiation sickness. There are certainly connections between the hypophysis-adrenal cortex function and radiation injury or another "stress" such as cold, heat, mechanical trauma, etc. Accordingly, Selye [1] reports "that antiphlogistic hormones (ACTH, glucocorticoids) are formed in excess during differing stress conditions".

With animals which have undergone hypophysectomy, a change in the corticosteroid synthesis does not appear after total-body irradiation of rats. This finding indicates the participation of ACTH and the development of these radiation effects [2]. Winkler and Schorn [3] have shown in the hormone extraction rates of injected 4^{14} C-cortisol that an increase in cortisol production occurs up to 5 days after a supralethal radiation of guinea pigs and, accordingly, an increased content of corticosteroids in organs, blood and urine is to be expected.

This was likewise concluded from the decrease in the cholesterol and ascorbin acid content in the adrenal glands of the irradiated rats [Bacq, 1954], for the corticosteroid synthesis in the adrenal glands begins with cholesterol whereby ascorbic acid is consumed [4]. An early phase of the cholesterol consumption determinable after 2-3 hours was characterized as "first reaction" (nonspecific stress reaction) and the late phase determinable after 2-3 days was characterized as "second reaction" (specific stress reaction) [Bacq, 1957] [5,6].

This two-phase increase in activity was also later directly demonstrated through the increase in corticosteroid content in the adrenal glands and in the peripheral blood of rats [Flemming, 1964, 1967] [4] and was characterized as "first corticosteroid increase" and "second corticosteroid increase" whereby the first increase is maximally demonstrable soon after irradiation and holds up to six hours and the second increase begins /48
2 days after the radiation attack and reaches its maximum on the third day.

As our own earlier investigations have shown [7], an additional skin wound which is inflicted before or after a total-body irradiation changes the activity state of the adrenal glands as opposed to that of pure irradiation. By inflicting a wound on rats which have already been injured by irradiation, the increase in corticosteroid content is intensified in comparison to the values after irradiation alone and is maintained over a longer period of time. On the other hand, when the wound is inflicted before irradiation, no significant differences are determined when compared with the radiation alone [7].

This result agrees with lethality investigations. As already discussed in Chapter II, there is an increase in lethality with mice who have been inflicted with open wounds or other stress forms when the wounds are made following total-body irradiation. The radiation lethality is, nevertheless, not noticeably increased or even decreased when the wound precedes the irradiation [8,9,10].

The present investigations should show how the adrenal gland activity of mice behaves with pure irradiation and with combined injuries (irradiation + open skin wounds). In addition, the total corticosteroids will be determined directly in the adrenal glands. Further, the changes in cholesterol content in serum and adrenal glands of mice are investigated since it is assumed that a decrease in cholesterol suggests hyperactivity of the adrenal glands.

IV.2 Material and Methodology

IV.2.1 Test Animals and Their Processing

610 female mice of the NMRI stock of our own breed were used. The animals received standard feed (Altromin) and water as needed. During the X-irradiation, care was taken that the animals were kept as free as possible from excitement.

The skin wounds on the back were made under ether narcosis as already described in Chapter I. /49

The mice were sacrificed with ether in order to make the corticosteroid determination. Following adrenalectomy, the adrenal glands were removed from attached fatty tissue, immediately weighed and placed for safekeeping in the cooling chest until processing. In order to determine cholesterol in the serum and adrenal glands, the animals were decapitated following ether narcosis and the blood flow was carefully captured in centrifuge capillaries. It is then allowed to stand for 2 hours for coagulation purposes and centrifuged at 2000 rpm. The adrenal gland is then taken out as above and likewise kept for safekeeping like the serum in the cooling chest.

IV.2.2 Determination of Total Corticosteroids in the Adrenal Glands

For the plotting of a linear calibration curve (Figure 15), the corticosterone standard (approx. 10^{-6} molar) was prepared by the dissolving of 20 mg corticosterone in 5 ml ethanol abs. and diluting to 1 l with double distilled water. From this, dilutions of 0.1, 0.2, 0.3, 0.4 and 0.5 µg/ml were prepared. 4.0 ml of the solutions were extracted with 4.0 ml chloroform, 3.0 ml of the extract was agitated with 3.0 ml fluorescence generator (sulfuric acid/ethanol 3:1) and 2.0 ml of the acid phase measured in the Zeiss spectrum fluorometer.

Exciter wavelength: 470 mµ, gap 2.0 mm
Emitted wavelength: 530 mµ, gap approx. 0.18 mm

The fluorometric determination of total corticosteroids was carried out according to a modified method [11] of Moncloa et al. [12] and Silber et al. [13]. The adrenal glands were homogenized with a 2 ml of 33% ethanol. The homogenizate was diluted to 5.0 ml using double distilled water and then washed with approx. 7 ml light petroleum. Samples amounting to 1.0, 1.2 and 1.5 were taken from this and brought to 2.0 ml using 13% ethanol. The samples were then washed with ligroin (30-60°) and extracted with 4.0 ml of chloroform. 3.0 ml of the extract were washed with 1.0 ml of ice-cold 0.1 N NaOH and agitated with 3.0 ml of fluorescence generator. 2.0 ml of the acid phase was separated and after a 15 min. period dwell in the spectrum fluorometer measured against the corticosterone standard.

Extractions or washings: Agitate strongly for 1 min. and centrifuge for 5 min. at 2000 rpm.

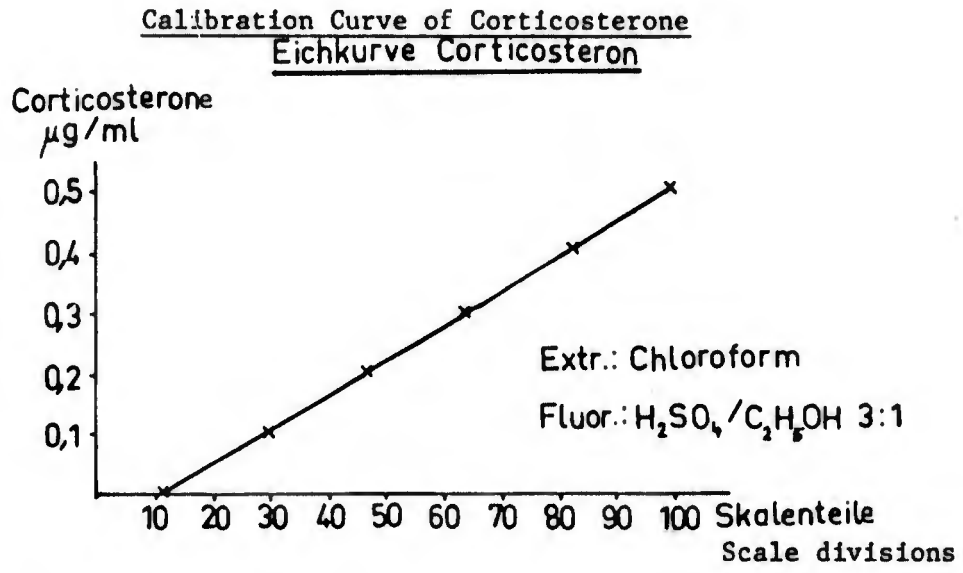

Figure 15. Calibration curve of corticosterone, extraction agent chloroform, fluorescence generator H_2SO_4/C_2H_5OH 3:1.

The mice were divided up for each experiment into groups of 4 animals each and the corticosteroid determination was carried out for every 2 animals (4 adrenal glands-AG) so that 4 values independent from each other would result per group. Since every one of these corticoid values consist of 2-3 determinations, the average values (µg corticosteroids/gAG) ± standard deviation represent the result of 4-6 individual determinations. The statistical analysis was performed according to the Student t test [14].

IV.2.3 Determination of Cholesterol in Serum and Adrenal Glands

The photometric determination of cholesterol was performed by means of the Boehringer test combination or the Merckotest. The adrenal glands were homogenized in 1.0 ml ethanol abs. and quickly filtered. From this, 0.1 ml filtrate or 0.1 ml serum were mixed in the test tube with 2.5 ml acetic acid/acetic acid hydride and allowed to stand for 5 minutes in a water bath (20-25° C). It was acidified with 0.5 ml conc. sulfuric acid and, after a 10 min. dwell in the water bath, measured in the Eppendorf spectrum photometer with 578 mμ against the cholesterol standard (200 mg/100 ml). /51

For this test, the mice were divided up into groups of 3 animals each and the resulting 6 adrenal glands were homogenized together and the serum was investigated as a pool. The average value (mg cholesterol/100 ml serum or mg cholesterol/gAG) are from 2-3 individual determinations per group.

IV.2.4 Test Groups

The corticosteroid content in the adrenal glands and the cholesterol content in the serum and adrenal glands was investigated at various times according to the following combinations of injury:

a. Total-body irradiation with 500 R

b. Producing a skin wound on back (SW)

c. Combined injury 500 R + SW 2d p.r.

d. Combined injury 500 R + SW 10 min. p.r.

e. Combined injury SW + 500 R 2d p.op.

Further, the cholesterol content in serum and adrenal glands of male NMRI mice was investigated at various times following total-body irradiation with 500 R, 600 R, 700 R, 800 R, 900 R and 1000 R.

IV.3 Results

IV.3.1 Total-Body Irradiation and Infliction of Wound

A maximum increase in corticosteroid content can already be determined 10 min. after irradiation ($p < 0.001$). This is now also true for the mice (Figure 16) just as with the earlier investigations on rats [4,7]. One hour after irradiation, a minimum value is reached. After two hours, the "first corticosteroid increase" ($p < 0.001$) begins, this being highly significant for the control value, the increase again dying out after 8 hours. The "second corticosteroid increase" begins one day after irradiation and reaches its maximum on the second day ($p < 0.001$). Three days after irradiation, this second increase likewise dies away and the corticosteroid values continue later to remain in the area of the control value. /53

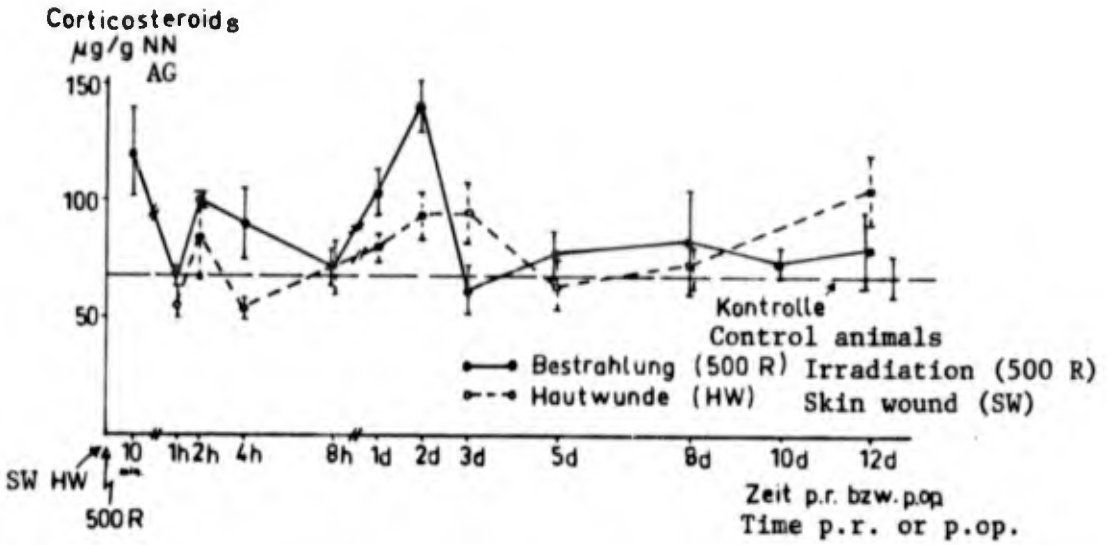

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Figure 16. Corticosteroid content in the adrenal glands of mice which have been exposed to irradiation (500 R) or inflicted with an open skin wound (SW).

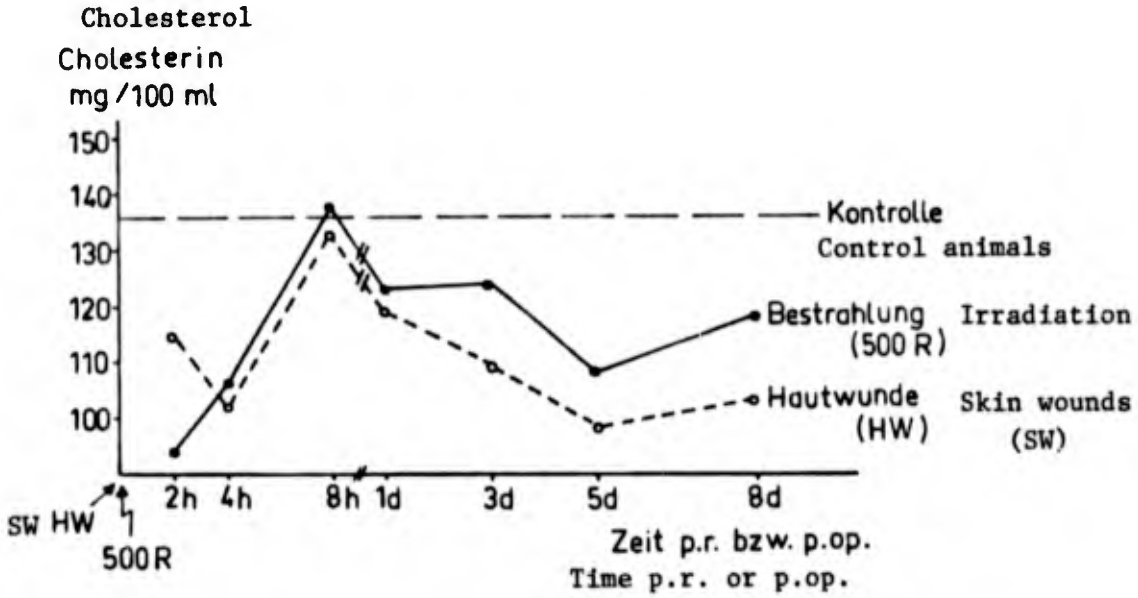

Figure 17. Cholesterol content in the serum of mice which have been exposed to irradiation (500 R) or inflicted with open skin wounds (SW).

The cholesterol contents vary considerably (Figure 17) in the serum and adrenal glands after "pure" irradiation. Whereas they are situated in the serum far under the control value 2 and 4 hours after irradiation ("first reaction"), after 8 hours they reach this value and then drop back again ("second reaction"). Still, the relations to the corticoid values in the case of the adrenal glands (Figure 18) are not so well defined. The values are indeed located under the control values up to 8 hours following irradiation. Later, however, rising and remaining above the control value to the end of the measuring period.

In general, it can be said, as shown by the cholesterol investigations on male mice irradiated with 500, 600, 700, 800, 900 and 1000 R, that the cholesterol contents in serum and adrenal glands preponderantly lie under the values of the control animals. This finding confirms that actually the substrate cholesterol is used in the case of increased corticosteroid synthesis in the adrenal glands.

For all investigations of the adrenal glands; i.e., determinations of corticosteroids and cholesterol with the various injury combinations, the absolute values of the total corticosteroids and cholesterols were calculated as the average values for one mouse. At the same time, the curves to which the relative values (corticosteroids or cholesterol per gAG) are so similar that a special representation can be dispensed with here. This also means that the weights of the adrenal glands do not change essentially after the damages.

A significantly less influence on the steroid production in the adrenal glands is caused by infliction of a wound (Figure 16). It is remarkable that similarly as with "pure" radiation exposure 2 hours and 2 days after making the skin wound, maximum values are achieved. The very first one is, nevertheless, not significant ($p < 0.1$) although the increase after 2 and 3 days is significant ($p < 0.005$). A highly significant increase then takes place again on the 12th day after infliction of the wound ($p < 0.001$). One hour, 4 hours and 5 days after the operation, the corticoid values are situated significantly under the control value and the decreases 1 hour p.op. ($p < 0.05$) and 4 hours p.op. ($p < 0.01$) are significant for control. /55

The cholesterol contents after wound infliction correspond in the serum as well (Figure 17). The values of the contents after irradiation whereby initially (2 and 4 hours p.op.) and after reaching a maximum (8 hours p.op.), are situated far under the control value. The cholesterol curve of the adrenal glands (Figure 18) is likewise similar to the curve after irradiation only the contents are higher here and always over the control value.

IV.3.2 Combined Injuries 500 R + SW 2d p.r.

In this investigation, the skin wound is made 2 days after irradiation, accordingly during the "second corticosteroid increase" (Figure 19) whereas, to begin with (1 day p.op.), the corticoid contents are situated highly significantly over the irradiation value ($p < 0.001$). They then

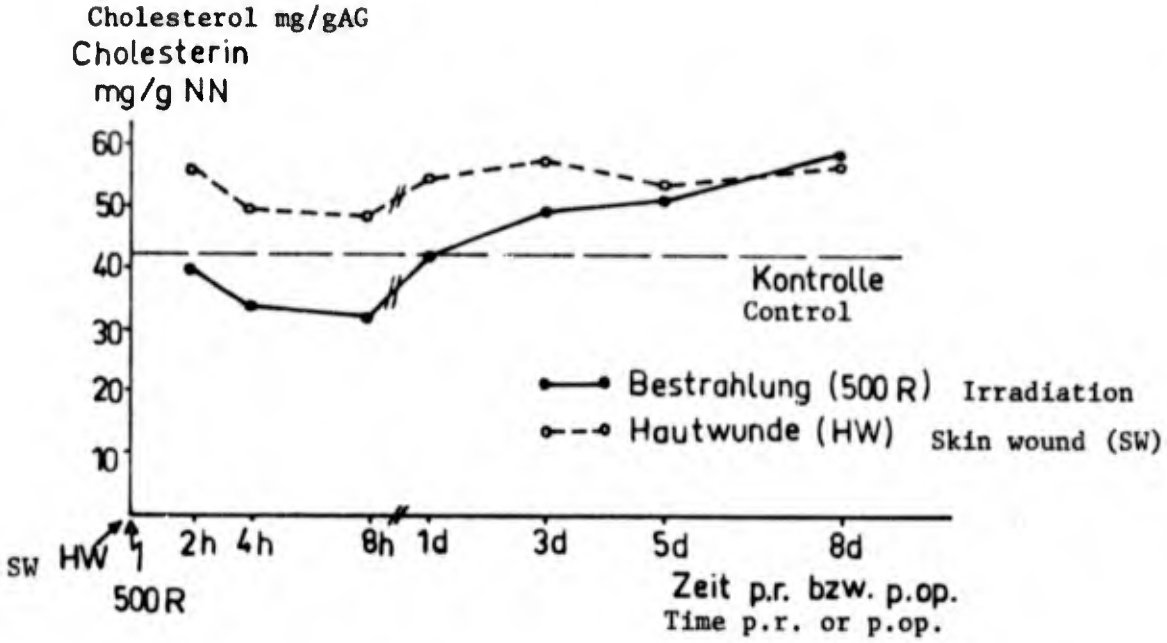

Figure 18. Cholesterol content in the adrenal glands of mice stressed through irradiation (500 R) or open skin wounds (SW).

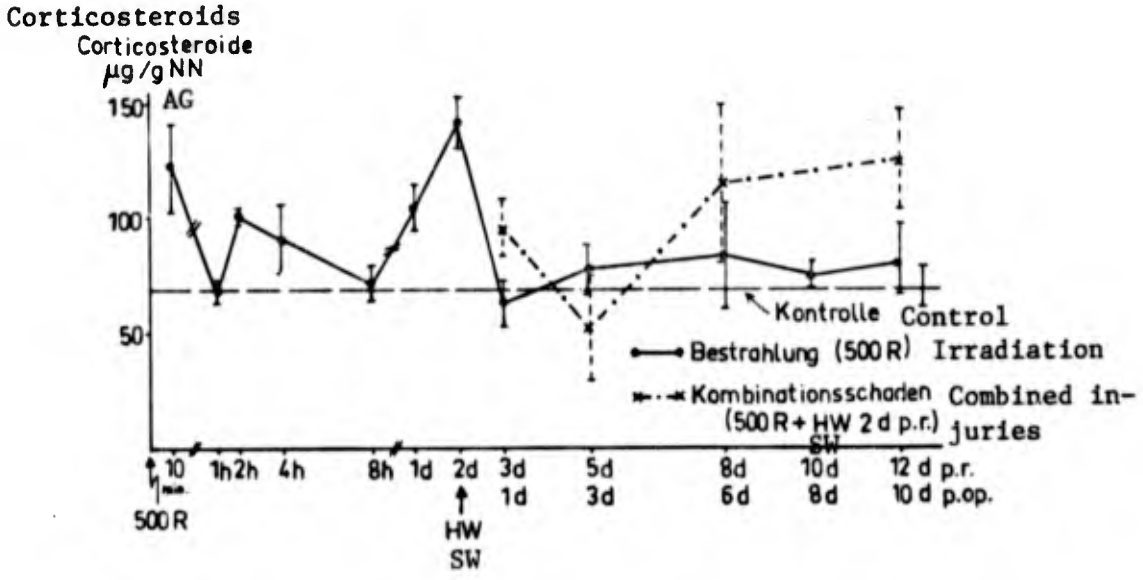

Figure 19. Corticosteroid content in the adrenal glands of mice stressed by combined injuries (500 R + SW 2d p.r.). Irradiation (500 R) for comparison.

drop 3 days p.op. under the control value ($p < 0.05$) increasing intensely again later on. The increase 6 days p.op. is not yet significant with respect to the irradiation value ($p < 0.2$) indeed as a consequence of the great standard deviation but then, however, becomes highly significant by the end of our investigations 10 days p.op. ($p < 0.005$). The high lethality of the mice is worthy of mention in this connection. Double the number of mice had to be used for the 6 days p.op. and 10 days p.op. tests in order to have enough surviving animals for the measurements.

The cholesterol curves with combined injuries are difficult to interpret. In the serum (Figure 20) the contents are situated here first of all (1 day p.op. and 3 days p.op.) under the control value and then increase (6 days p.op. and 10 days p.op.) beyond these values. The cholesterol values in the adrenal glands (Figure 20) are initially in the range of the control value and likewise increase at the end (10 days p.op.).

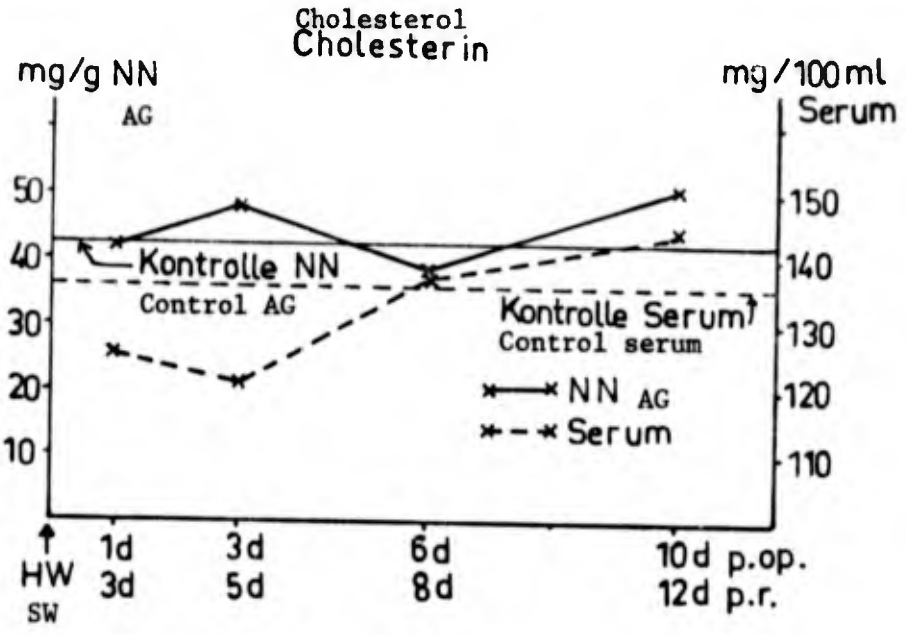

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Figure 20. Cholesterol content in serum and adrenal glands of mice exposed to combined injuries (500 R + SW 2d p.r.).

The skin wound is applied here during the first maximum point of the corticoid value after irradiation (Figure 21). One hour after operation, the corticosteroid content is increased in contrast to the "pure" irradiation ($p < 0.05$). It remains 2 hours p.op. in the range of the "first corticosteroid increase" and drops until 8 hours p.op. like the irradiation curve down to the control value. Whereas the "second corticosteroid increase" now begins after one day in the case of the "pure" radiation exposure, the corticoid contents remain chiefly with this combined injury

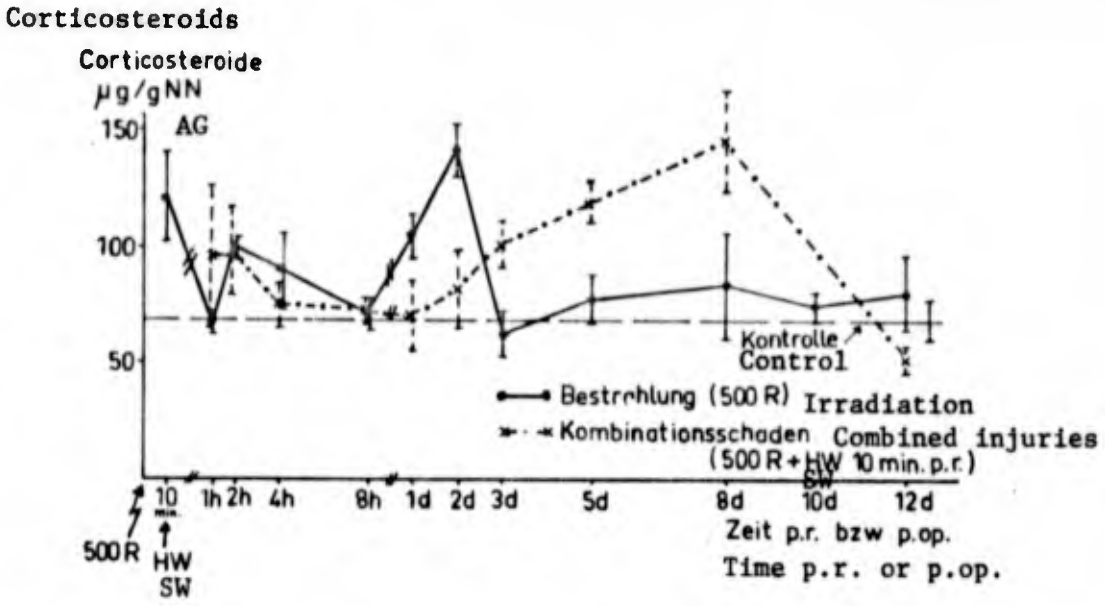

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Figure 21. Corticosteroid content in the adrenal glands of mice exposed to combined injuries (500 R + SW 10 min. p.r.). Irradiation (500 R) for comparison.

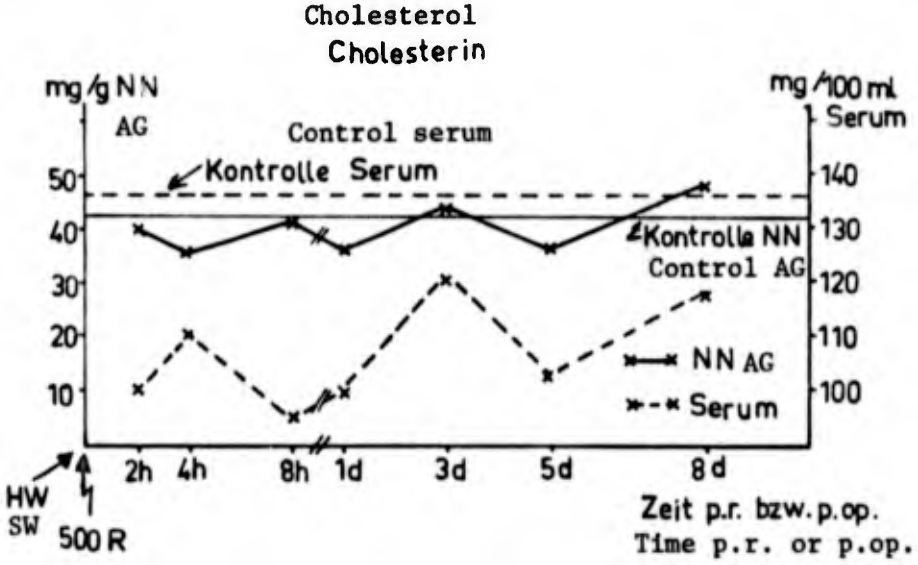

Figure 22. Cholesterol content in serum and adrenal glands of mice exposed to combined injuries (500 R + SW 10 min. p.r.).

in the area of the control value. However, they rise 3 days p.op. and 5 days p.op. quite intensely with respect to the irradiation curve ($p < 0.001$) and reach their maximum ($p < 0.01$) 8 days p.op. On the 12th day p.op., the hormone content then drops steeply until it comes under the control value ($p < 0.05$).

The cholesterol contents in the serum (Figure 22) are situated far under the control value during the entire period of investigation (higher consumption of cholesterol!). Minimum values can be seen 2 and 8 hours p.op. as well as 5 days p.op. The cholesterol in the adrenal glands (Figure 22) is not lowered as much but is also situated here up until 5 days p.op. under the control value and only rises 8 days p.op. above it. /58

IV.3.4 Combined Injuries SW + 500 R 2d p.op.

In this case, the irradiation occurs 2 days after application of the skin wound; i.e., at a time in which the corticosteroid content has just been increased in the adrenal glands (cf. Figure 16). In contrast to the combined injuries described previously, there already takes place one hour after the second trauma; i.e., the irradiation, a very heavy increase in the corticosteroid content over and beyond the radiation damage and is maintained until one day after the radiation effect (Figure 23). The difference to the "pure" irradiation is highly significant ($p < 0.001$) one hour, 2 hours, 4 hours, 8 hours, and one day p.r. Two days following irradiation, at the time of the "second corticosteroid increase", the values oscillate in the range of the irradiation curve and no longer increase significantly.

Even here, the cholesterol contents in the serum (Figure 24) are always situated under the control value and they are especially low 2 and 4 hours as well as 3 and 5 days following irradiation. The cholesterol values in the adrenal glands move in the area of the control value with a maximum on the first day after irradiation (Figure 24).

IV.4 Discussion of Results

With the total-body irradiated mice, it was possible as with the rats investigated earlier [4,7] to demonstrate a two-phase progress of the adrenal cortex activity. The corticosteroid increase shortly after irradiation is probably caused by a shedding of ACTH as a result of the excitation of the animals owing to the irradiation process. This concept is confirmed in that a significant decrease in ACTH content of the pituitary appears with simultaneously activation of the adrenal cortex as early as 30 minutes afterward in the case of X-irradiated rats [4].

Bacq et al. (1960) assume that the neuroendocrine "first reaction" occurring with rats 1.5 to 3 hours after irradiation as cholesterol and ascorbic acid loss comes about owing to an excitation of specific nerve cells in the hypothalamus. For this reason, the "first corticosteroid increase" 2 hours after irradiation which accordingly comes about owing to endocrine stimulation of the nervous system can be characterized as "neural corticosteroid increase". It appears to be irradiation-specific since it can be prevented by cysteamine [15]. /60

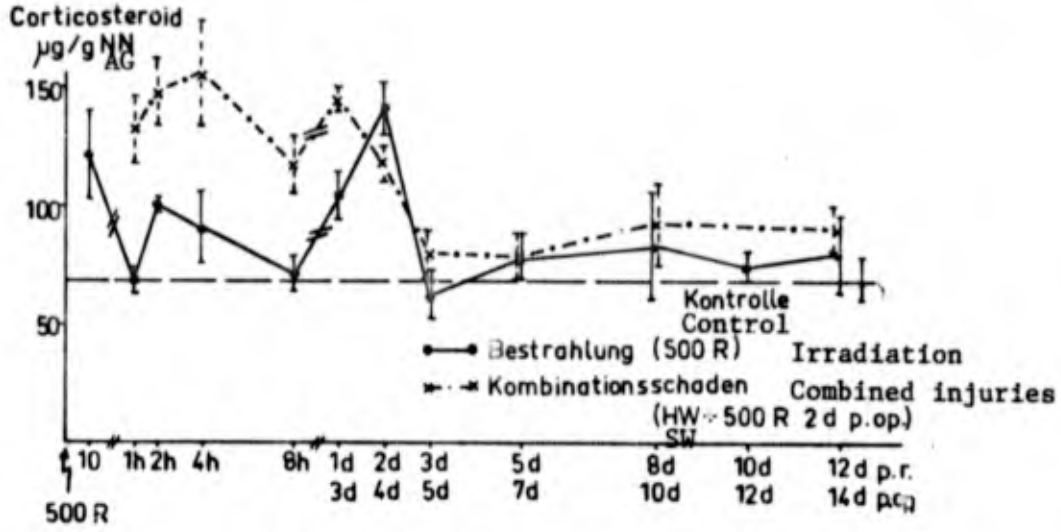

Figure 23. Corticosteroid content in the adrenal glands of mice exposed to combined injuries (SW + 500 R 2d p.op.). Irradiation (500 R) for comparison.

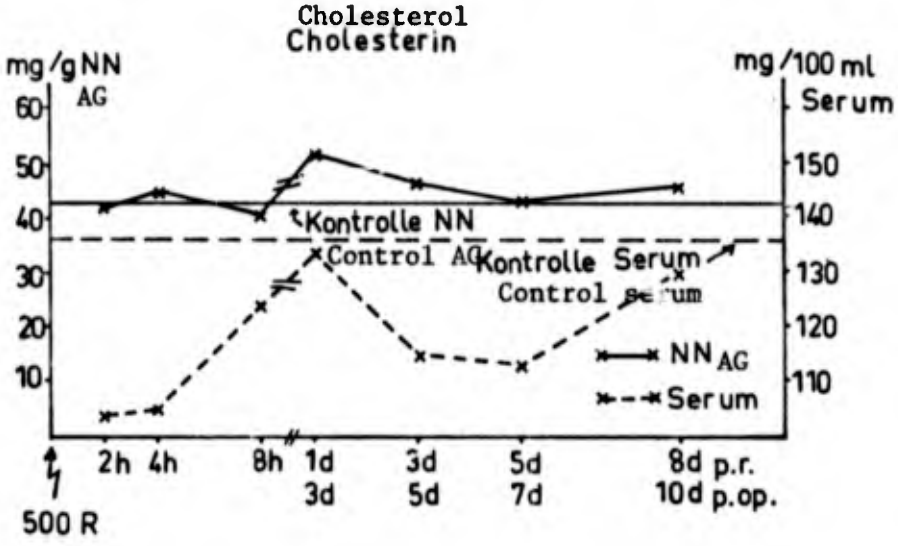

Figure 24. Cholesterol content in serum and adrenal glands of mice exposed to combined injuries (SW + 500 R 2d p.op.).

The "second corticosteroid increase" occurring 2 days after irradiation can be regarded as a stress reaction of the organism to radiation sickness (stressor) in accordance with Selye. It is probably induced by toxic substances coming from the damaged intestine which pass into the blood through the intestinal wall and activate the pituitary-adrenal cortex system. It is also termed "intestinal corticosteroid increase". The degree and duration of the "second corticosteroid increase" is a function of the dose. After 500 R to 1000 R, Flemming [15] found a reversible increase in rats becoming irreversible after 1200 R. The corticosteroid values then always reached a maximum on the third day after irradiation but did not, however, decrease again on the fourth day.

The glucocorticoids (cortisone, hydrocortisone) are more greatly increased after irradiation than the mineralocorticoids (aldosterone, desoxycorticosterone). This displacement in equilibrium probably contributes to the radiation-conditioned changes in the carbohydrate metabolism [2]. For this reason, we began to investigate the changes of the lactate and pyruvate budget in the blood of radiation damaged mice.

The skin wound alone likewise causes an activation of the pituitary-adrenal cortex system although of course not to the extent as in a total-body irradiation. The increase in corticosteroids which can be determined after 2 or 3 days is a stress reaction from the wound application whereas the increase on the 12th day following the operation appears to be certainly a consequence of the inflammation processes taking place in the wound area [7].

Whereas -- as also in the case of the "pure" irradiation -- the reductions in the cholesterol values in the serum approximately correspond to the increases in corticosteroids in the adrenal glands, the cholesterol contents of the adrenal glands do not run parallel. This means that the cholesterol contents are not reduced as much as are the serum values. Thus, we find that the cholesterol for the biosynthesis of the corticosteroids in the adrenal glands must mainly come from the blood. Flemming [4] as well comes to the conclusion that the changes in cholesterol and ascorbic acid in the adrenal glands and blood do not often correspond in time with the corticosteroid changes.

The effect of the adrenal cortex activity owing to a skin wound inflicted in addition to irradiation becomes clear with the combined injuries investigated. Insofar as the corticosteroid content is concerned, the time of infliction of the wound before or after irradiation is especially decisive. When the wound is inflicted 2 days after irradiation, the corticosteroid values are first increased (1 day p.op.) as a result of effects of stress, then dropping sharply (3 days p.op.). This dropping is probably to be seen in connection with the high lethality of the mice on the 3rd to the 4th day after the operation. Later on (6 days p.op.), the hormone values increase sharply until the end of our investigations (10 days p.op.). The surviving animals then accordingly show hyperactivity of the adrenal cortex which also could be the basis of their survival. When the skin wound is inflicted shortly after irradiation, the corticoid contents first approximately correspond to the "pure"

irradiation values. The "second corticosteroid increase" two days after irradiation or after operation appears 1 day later and a very high increase then occurs from the third to the 8th day after operation. It is reinforced with respect to the irradiation value as a result of 2 injuries and is maintained over a longer period. The values later drop considerably otherwise than in the case of the previously mentioned combined injury.

These results can be related to the lethality investigations which were described in Chapter II of this report. When the wound is inflicted 2 days after irradiation, the lethality of mice with respect to irradiation alone increases considerably.(dropping of the hormone values 3 days p.op.). Nevertheless, in the case of a simultaneous effect of both traumata, only a lesser increase in lethality is to be determined (the animals die in this case on the 12th day after the operation on which day the steroid content has dropped under the control value). /62

The conditions are otherwise when the wound is inflicted before irradiation. In this case, there is found just at the beginning of measurements an intense hormone increase with the highest values found. This condition continues until 2 days after irradiation. Then, however -- in contrast to the combined injuries where infliction of the wound followed irradiation -- the corticosteroid contents drop and remain until the end of the investigations within the range of the irradiation curve or in the range of the control value. With a combined injury of this kind, the lethality of the mice with respect to pure irradiation exposure is often found to be reduced probably as a response to the immediate very high pouring out of hormones [8,9]. A wound can cause increases in resistance when it is produced a few days before irradiation. These effects of operative interventions can be pointed out accordingly as an expression of an increased nonspecific resistance as a result of the activity increase of the pituitary-adrenal cortex system in accordance with Selye [16].

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V. CHANGES IN RADIATION SENSITIVITY OF BONE MARROW CELLS OF MICE EXPOSED TO TOTAL-BODY IRRADIATION IN COMBINATION WITH OPEN SKIN WOUNDS /64

V.1 Introduction

Earlier investigations of the blood cell formula of rats [1,2] have shown that significant differences can exist between cell counts of animals with "pure" radiation injuries and those with combined injuries. At the same time, an earlier regeneration of the red and white blood cell formula could be demonstrated after combinations of open skin wounds with total-body irradiations. A further investigation examined to what extent differences in the mitoserata can also be demonstrated in hematopoeitic colonies in the spleen of mice [3,4]. It was seen at this time that with a combination of total-body irradiation (510 R) and with a subsequent production of an open skin wound, the mitoserata increased much higher and at an earlier time than in the case of a pure radiation injury after exposure to the same dose.

These results are to be examined with other methods in the following investigations. Since the radiation death of test animals in the range of $LD_{50/30}$ is generally attributed to the destruction of the bone marrow [5], the investigations should be valid for the behavior of the bone marrow stem cells.

V.2 Material and Methodology

V.2.1 Preparation of the Animals

The investigations were carried out on 1220 7-9 week old female NMRI mice of our own breed. They were irradiated or irradiated and additionally stressed with open skin wounds on the back. The conditions under which the animals are kept are described in II.2.1, those of the irradiation in II.2.2 and, finally, the manner of inflicting the wound is described in II.2.3.

V.2.2 Application of the Endogenous CFU Technique /65

The determination of radiation sensitivity was made using the methods of the endogenous and exogenous CFU technique of Till and McCulloch [6,7]. With the endogenous CFU technique, 335 animals were irradiated for 2 days after a skin wound and 275 animals were irradiated without skin wound in 10 separate experiments using radiation doses between 400 and 700 R. As a rule, each irradiation group consisted of 10 animals. Nine days later, the animals were sacrificed in ether, the spleen removed and fixed in Bouin's solution. All nodules on the surface recognizable with a binocular microscope were counted as spleen colonies.

V.2.3 Application of the Exogenous CFU Technique

Using the exogenous methods, bone marrow cells from a total of 110 donor animals which had been irradiated with radiation doses of 200 R to 600 R immediately or 2 days after the skin wound, transplanted intravenously to a total of 500 donee animals. The donee animals were irradiated with 850 R 24 hours before the transplantation. The donor animals were sacrificed immediately after irradiation, both femora were removed and the epiphyses excised. The diaphysis was rinsed with an ice-cold physiological sodium chloride solution. The cell suspension produced in this way was counted in the Coulter counter and adjusted to the final concentration with physiological sodium chloride solution. 100,000 - 150,000 bone marrow cells suspended in 0.5 ml of physiological sodium chloride solution were injected into each donee animal in the control tests. The concentration of bone marrow cells from irradiated donor animals was adjusted corresponding to the expected survival rates. In this way, after 600 R, 10 million cells were injected into each donee animal. Nine days later, the donee animals were killed, the spleen removed, fixed and the colonies counted.

V.3 Results /66

The results of the tests for determination of radiation sensitivity of endogenous CFU (colony forming units) are shown in Figure 25. Each circle provides the arithmetical mean of the number of spleen colonies in a group of at least 10 animals as a function of the radiation dose 2 days after a skin wound. The dots show the corresponding values for animals which have only been irradiated. The lines were computed according to the method of least squares.*

* As a measure for the tendency of the dose effect curve, the dose is given which reduces the number of surviving units to about 37%. This dose is called D_0 (Kellerer and Hug [8]).

The slope of the dose effect curves of the control animals has a D_0 of 91 R whereas the slope of the dose effect curve of the animals previously inflicted with a skin wound has a D_0 of 110 R. In the case of a radiation dose of 600 R (which almost corresponds to the $LD_{50/30}$ for control animals) the normal animals show on the average 4.5 colonies whereas the animals inflicted with a skin wound show 14.7 colonies. Both curves have a point of intersection with the ordinate which are close together (the coefficient b of the regression straight lines amounts to 3.498 in the control animals and 3.485 in the wounded animals).

The results of the tests for determination of radiation sensitivity of the exogenous CFU are shown in Figure 26. Every circle and every dot provides the survival rate of the transplanted CFU derived from an independent test compared to the actual control animals whereby the circles characterize the tests in which the donors were irradiated after a skin wound whereas the dots designate the control irradiations. The regression

straight line which is plotted in Figure 26 is the dose effect curve calculated for only irradiated animals which can be characterized by the parameter D_o = 101 R and n = 1.2.*

* In order to characterize the deviation of a dose effect curve from a purely exponential function, the point of intersection of the extrapolated exponential function with higher doses is usually given with the ordinate. n is the extrapolation number (Kellerer and Hug [8]).

The regression straight line for the results of irradiation 2 days after wounding is provided by the parameter D_o = 123.5 R and n = 0.65.

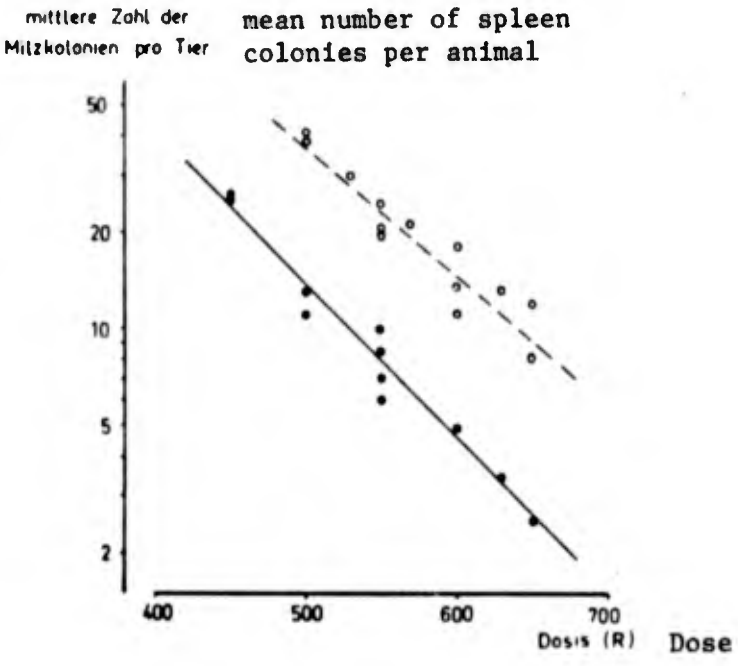

Figure 25. Mean number of spleen colonies in untreated mice (dots) and in operated (i.e., inflicted with skin wounds) mice (circles) 9 days after exposure to radiation doses between 450 and 650 R. Each dot or circle is the mean value of the spleen colony numbers of at least 10 animals of an independent test. The straight lines were calculated according to the method of least squares. /67

The relative and absolute number of the CFU in the normal femur and in the femur of wounded animals is not significantly different. In most cases, the number of spleen colonies was about 10% less with the same number of transplanted bone marrow cells. However, on the other side, an approximately 10% higher cell number was determined in the femur of wounded animals.

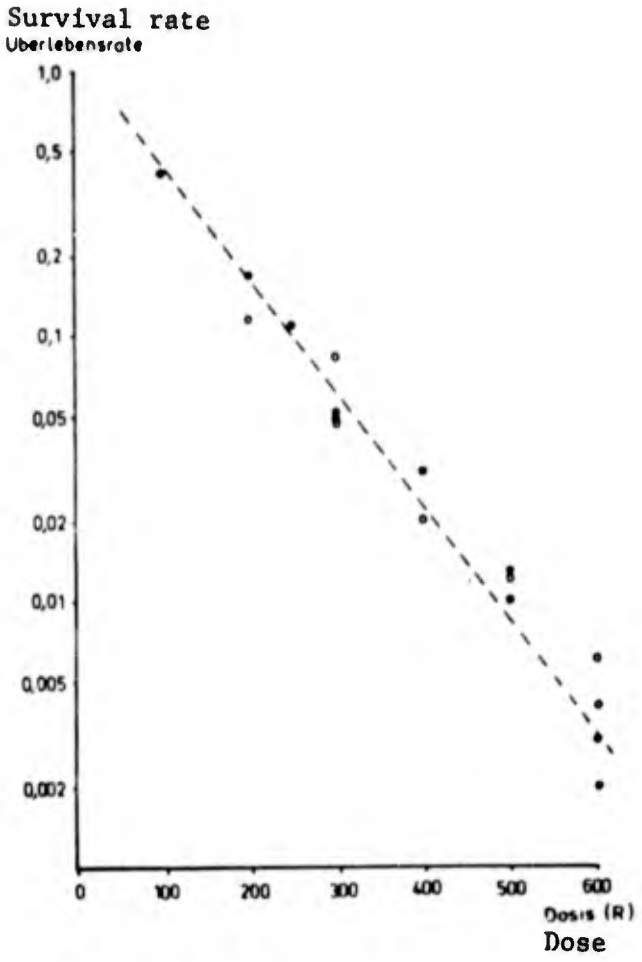

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Figure 26. The fraction of the colony formation rate of transplanted bone marrow cells of untreated mice (dots) and operated (i.e., inflicted with skin wounds) mice (circles) after radiation with 100 to 600 R. Each dot or circle is the mean value of the spleen colony number which can be counted in an independent transplantation test in contrast to the control value of the same day.

V.4 Discussion

Both methods enable determination of a lesser radiation sensitivity in the bone marrow stem cells in the animals which had been inflicted with a skin wound 2 days before irradiation. The slope of the dose effect curve is about 20% shallower in both cases. The D_0 of 91 R rose to 110 R in the endogenous test and in the exogenous test it rose from 101 R to 123.5 R.

There is a decisive difference between the two dose effect curves of Figure 25 and 26. Whereas the curves of the endogenous CFU proceed from about the same point on the ordinate (i.e., displaying equally great populations of differing radiation sensitivity) the extrapolation number in the exogenous test is significantly less than 1 and is situated at 0.65. A two-phase dose effect curve must therefore be assumed, representing two populations of differing radiation sensitivities. It can be concluded

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from the value of the extrapolation number that both subpopulations are approximately of equal size. Whereas in both test methods the changes in the slope of the dose effect curves are comparable, the extrapolation values suggest that in the endogenous test, the absolute number of stem cells which can be developed to colonies are twice as high in wounded animals as in normal animals. After, however, in the exogenous test such a relative or absolute increase in the number of transplantable CFU was not found in the bone marrow, it appears more appropriate to suggest differences in the proliferation behavior after irradiation. Corresponding preliminary testing has confirmed this hypothesis.

Changes in the radiation sensitivity or number of surviving bone marrow stem cells after irradiation were found according to the effect of various external influences.

The number of endogenous spleen colonies after irradiation is increased manyfold a number of days after injection of phenylhydrazine (Hodgson [9]), bacterial endotoxins (Smith et al. [10]), antilymphocyte serum (Chertkov [11]), foreign plasma (Boggs et al. [12]), heat-denatured erythrocytes (Morley et al. [13]). The cause of the increased colony number, in all these cases, however, not a change in the radiation sensitivity, measured on the slope of the dose effect curve, but a massive increase in the number of transplantable stem cells of the spleen as response to the stimulation.

Changes in the proliferation behavior after irradiation without an increase in the number or the radiation resistance of bone marrow stem cells are, after losses of blood (Sugahara et al. [14]) and chronic hypoxia (Beran and Tribukait [15]), the main cause of the observed radiation protective effect of the actual preliminary treatment on the survival of the animals.

When the bone marrow stem cells are stimulated by an excitation to extreme proliferation, this leads, according to the investigations of Hendry [16] to an increase in the radiation resistance by about 10% whereas Duplan and Feinendegen [17] did not find such a proliferation dependent modification of radiation sensitivity on another mouse stock. /70

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