STUDIES ON THE EFFECTS OF ADULT ANIMAL TISSUE EXTRACTS ON WOUND HEALING*

A PRELIMINARY REPORT OF THE FACTORS RESPONSIBLE

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IN NORMAL HEALTHY INDIVIDUALS who have sustained injuries to tissue or organs, the process of repair proceeds through an apparently orderly sequence of biologic events to the point of healing, where the speeding-up of regenerative processes stops. The work here presented is based upon the assumption that it may be possible to influence this cycle directly in one or more of its phases, in order to accelerate the healing process.

This hypothesis is not new for, in 1925, Baker and Carrel[1] reported an apparent growth-accelerating effect of embryo-web porridge evident both *in vitro* and *in vivo* experiments. Other investigators also demonstrated an accelerating effect of embryonic extracts on wounds. However, during this period it was felt that if growth-promoting substances were present in tissues there should be more in embryonic cells.

In 1939, in a series of *in vitro* experiments it was shown by one of us (R. S. H.), and others,[16] that adult animal extract, has a greater stimulating effect than embryonic juices, and that this effect is not only present on standard chick fibroblast cultures but is noticeable on explanted human epithelium.[10] Furthermore, it was found that adult animal tissue extracts of sheep, beef, rabbit and dog were as effective on chicken fibroblasts as were homologous extracts.

It was logical, from the results of these *in vitro* experiments, to assume that clinical use might be found for the various extracts of tissue when applied to human wounds. From 1925 to 1932, many workers[2, 5, 19, 22, 24, 25, 28] used embryonic extracts of various tissues in an attempt to accelerate healing of so-called indolent wounds. However, the accompanying case reports are few and unimpressive. Furthermore, during this period less was known of the many systemic factors which influence final repair; hence, the criteria for refractivity were not sufficiently exacting. At the beginning of World War II interest in embryonic extracts was again revived when Waugh,[29] in 1940, reported beneficial results in refractory human wounds to which a desiccated embryonic extract prepared by Fischer[13] was applied. More recently, in 1945, reports from Russia, by Goldberg,[14] describe successful treatment of indolent wounds with an ointment containing embryonic extract.

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Objections to the embryonic extract however have been raised since it is difficult to prepare in quantity, expensive, relatively unstable, and, as pointed out, it has less stimulating effect than have adult extracts.

In a series of experiments and clinical trials we have used a tissue extract prepared as follows: Sterile adult sheep hearts are finely minced in a blender with Tyrode's solution. After standing in the refrigerator for 24 hours the mixture is centrifuged and the supernatant extract decanted into convenient sterile flasks. This extract when kept refrigerated maintains its activity for one month, while a lyophilized ether-extracted fraction is active for a year. Both types of extracts were used in these experiments and were tested for potency.

As previously reported by us,[17] in 1944, the effect on dogs was studied by using bilateral areas of skin excision, one of which was treated with extract and the other by saline packs. It was found that in every animal the treated side was healed before the control in an average of 40 per cent less time (Chart 1).

At the same time, a number of clinical wounds were being treated, particularly those which appeared to be indolent or refractive to other types of treatment. Friendly skeptics would rarely call us to treat a wound until all other more orthodox methods had apparently failed. It soon became apparent that there was a definitely beneficial effect, which was obvious to many clinical observers. Concurrently, investigators[26, 21, 18] abroad were also using adult extracts or fractions thereof on indolent wounds, with reported success. Kerr and Werner[18] in this group used extremely exacting criteria for their selection of cases, but again interpretation of results, however objective, was based on clinical impressions.

Carrel and Hartmann,[6] in 1916, showed that wounds followed a definite pattern in healing, which could be expressed in a graphic curve, and DuNouy[12] in the same year pointed out that this curve could be expressed in a mathematic formula.

$$S'=S\,[1-i(t+\sqrt{T+t})] \text{ or } S'=S\,[1-i(t+\sqrt{nt})] \text{ (If t is constant)}$$

Key: S' = area t days later.
S = wound area at a known time.
T = age of wound from time of first observation S.
t = time between measurements.
n = number of time-periods.
i = a fixed coefficient which depends on the size of the wound and the age of the patient.

These observations were made on many carefully studied wounds which were followed to final healing.

In an effort to find some type of control for our treated clinical wounds, their expected healing time was plotted by the use of the above formula, and contrasted with the actual healing time of the extract-treated wounds. The results (Charts 2 and 3) appear to validate the clinical impressions. Furthermore, in a few instances, we were fortunate enough to obtain patients with bilateral lesions in comparable body areas. Using one wound for a control and

treating the other with extract, again a favorable difference was noted (Chart 4).

In this connection, Kerr and Werner[18] have concluded that upon the application of their extract "to one of several coexistent indolent wounds the process of healing resulting in the treated wound has been accompanied by the commencement of healing in others." They suggest that the absorption of the growth-stimulating substance at the primary wound may be responsible or that, as Young, Fisher, and Young[30] suggest, a growth-stimulating substance is liberated in the course of healing. We have found that in cases of multiple lesions, one may be stimulated to healing, while others remain relatively refractory.

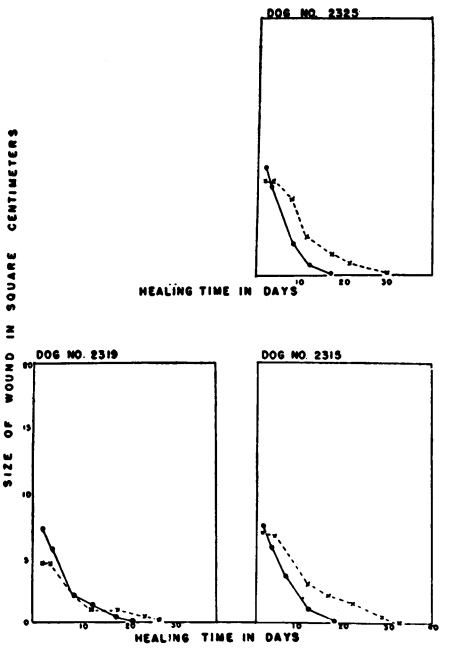


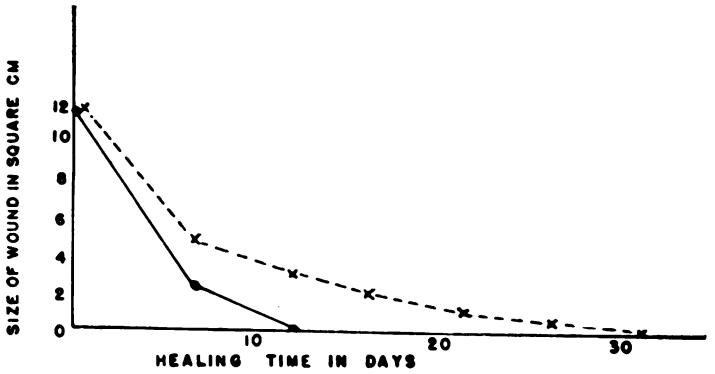
CHART I.—Graphs showing three of the animal experiments. The black line represents the treated wound and the dotted line the controls.

Finally in an effort to evaluate the assumed stimulating effect of adult animal tissue extract without relying on clinical impression, secondhand controls or animal experiments, a group of five human volunteers were obtained for study. These subjects ranged in ages from 30 to 50 years, were in excellent nutritional states, and were all at absolute bed rest. Under novocaine anesthesia, and complete sterile precautions, wounds were made on both anterior thighs of nearly equal size and depth. These varied from 3 to 4.5 sq. cm., and from simple complete skin excision, to removal of a block of tissue down to the deep fascia. After 48 hours the larger side was chosen for treatment and the contralateral lesion was used for a control method which consisted of constant dakinization. Dressings were done twice daily. The treated side was dakinized for 10 to 15 minutes and then flushed with saline. Following this an extract-saturated gauze square was placed in contact with the lesion and covered with paraffined gauze. The control lesion was treated by leaving a Dakin pack on the wound without washing with saline. It should be stated that under this therapy, there was no evidence of infection present in these wounds at any time. At regular intervals the surface circumference of these wounds was traced on a transparent plastic disk, the area measured with a planimeter and the wound sizes were marked on graphs (Charts 5, 6, 7, 8 and 9) so that the healing in both wounds in each individual could be followed. Obviously, by this method, no provision was made for volumetric measurement particularly in those subjects in whom deep blocks of tissue were removed.

Four of the five treated wounds were healed before the controls, and in

one case healing was simultaneous. In this subject (Chart 7), there appeared to be a mechanical impediment to epithelization. It had been noticed in observing the healing of some clinical lesions, particularly those which were deep and required filling in before epithelization, that the extract had a marked stimulating effect on granulation tissue growth. It was felt that the apparent accelerat-

CHART II



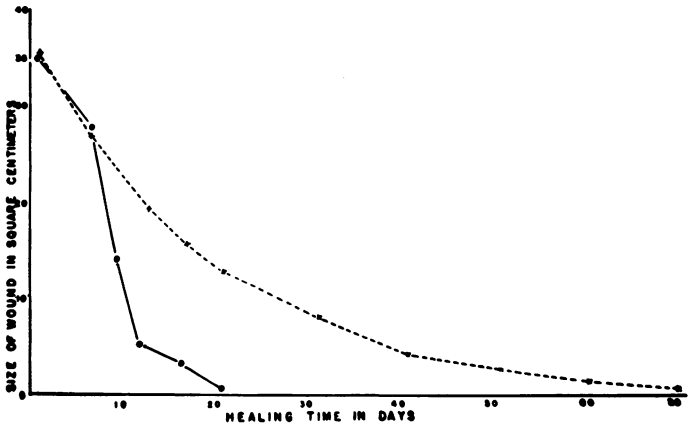


CHART III

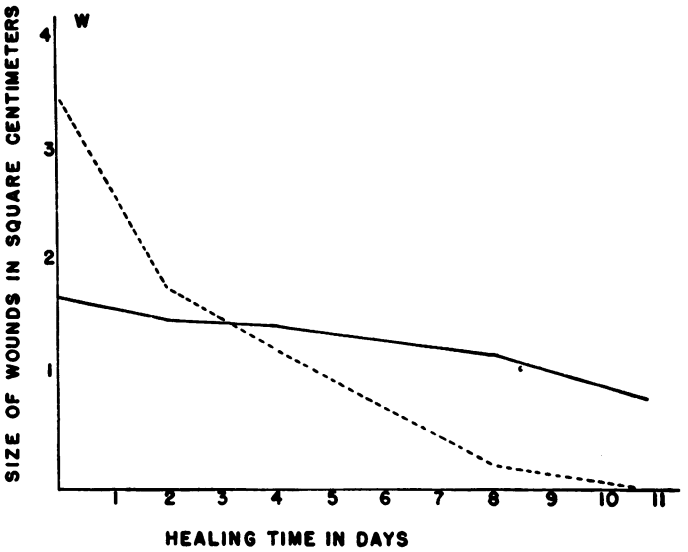
CHART II.—Case of E. K.: Solid line shows the actual healing time and the broken line represents the expected healing time. (DuNouy)

CHART III.—Case of A. W.: Solid line shows the actual healing time and the broken line the expected (DuNouy) healing curve.

ing effect of the extract in these wounds was due in part to the relative rapidity with which granulation tissue filled cavities. Accordingly, in the last two of the volunteers, blocks of tissue were excised to the deep fascia, and in these cases granulations were flush with the skin long before the control, and the epithelium had consequently crossed the denuded areas faster. In one case, where only full-thickness skin was excised, the excessive granulating effect was observed to impede the epithelization process, requiring actual excision of granulation tissue several times.

DISCUSSION.—From a survey of the literature it would seem that the

CHART IV



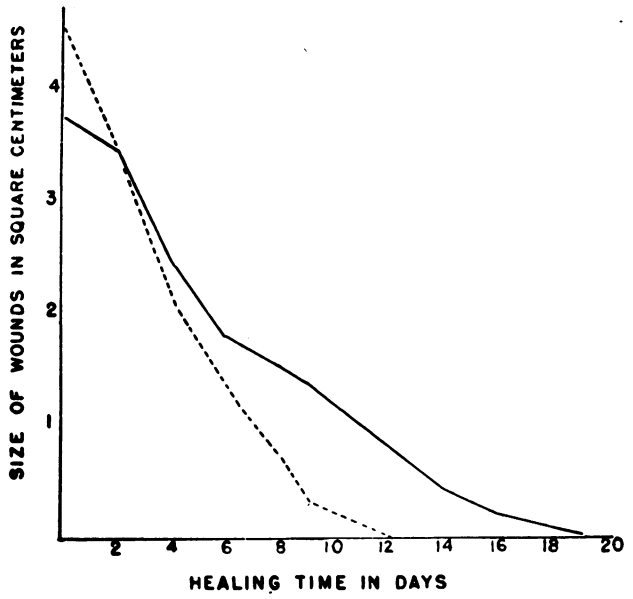


CHART V

CHART IV.—Case of W. (Patient with bilateral leg ulcers): The dotted line represents the healing curve of the treated lesion, and the solid line the control. The treated wound although over twice the size of the contralateral lesion was healed before the control showed much evidence of beginning healing. Notice that the untreated control side shows very little evidence of stimulation.

CHART V.—(Human Volunteer No. 1): Showing the decreased healing time of the treated wound (dotted line) as compared with the control (solid line).

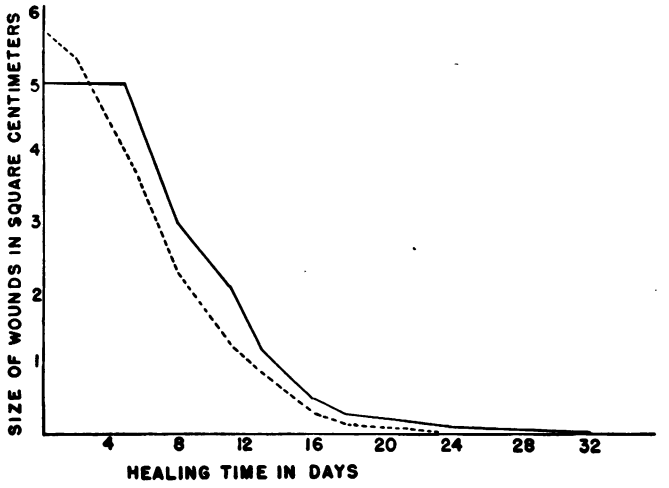
approach to the problem of wound healing has been largely an attempt to find and eliminate factors which can be shown to interfere with the healing of open wounds. It has been amply pointed out[4] that when a wound ceases to heal or remains stationary there may be a number of reversible conditions at play. The nutritional state of a person, with particular reference to protein and certain vitamins, must be maintained at a normal level and hematologic abnormalities corrected. Metabolic diseases such as diabetes and local circulatory disturbances play a part, and must be controlled before normal wound healing may take place. Finally, it is well known that the presence or absence of infection is of paramount importance, for the process of tissue repair will not take place when there is overwhelming local suppuration. Consequently, many of the medicaments which purport to accelerate wound healing are in fact agents which attempt to control local infection, thereby enabling the normal biologic healing sequence to intervene.

Certain other substances claimed to have growth-promoting properties are found in the literature. Some of these, such as chlorophyl,[27] allantoin,[15] preparations liberating sulfhydryl groups,[23] creatine[7] and, recently, human bone marrow antiserum[3] are presumed to have growth-stimulating properties acting in a positive manner on indolent wounds, rather than by neutralizing inhibiting factors. Many of these substances have not been proven to be active on cells growing *in vitro,* nor do some of the clinical cases treated meet the requirements necessary to draw conclusions.

As a working hypothesis, it may be stated that the process of repair begins with tissue damage. It would seem plausible, therefore, to assume that from injured cells a substance or substances are liberated which may initiate or influence the physical and chemical reactions of cellular proliferation. It has also been noticed both in cells growing *in vitro,* and wounds, that there is an initial quiescent or lag-period during which no cell growth takes place, normally of at least 48 hours' duration. Furthermore, it is certainly obvious that mature cells in an organism are subject to control by a local inhibitory mechanism which may oppose indiscriminate cell multiplication. Supportive evidence for this latter hypothesis may be found in the fact that malignant unlike normal cells exhibit no such latent period when growing *in vitro.*[11]

In other words, as an hypothesis, it may be assumed that tissue stability is a unique balance between local stimulating and inhibiting factors, with the latter normally in control. It may be that on injury stimulating substances are released locally, and become the preponderant force, thus, initiating the reparative process. Possibly, as a wound nears completion of healing the titer of stimulating substances falls off or the inhibiting factors assume more importance. Hence, in indolent wounds, it may be that the period of stimulation started by the release of local factors may cease and the healing process remains stationary either because the stimulating substances are used up, or inhibiting factors predominate. Evidence for this theory has been noted in the marked stimulation and improvement when indolent wounds are treated as compared to the lesser acceleration, possibly additive in effect, when freshly acquired wounds in healthy individuals are used.

CHART VI



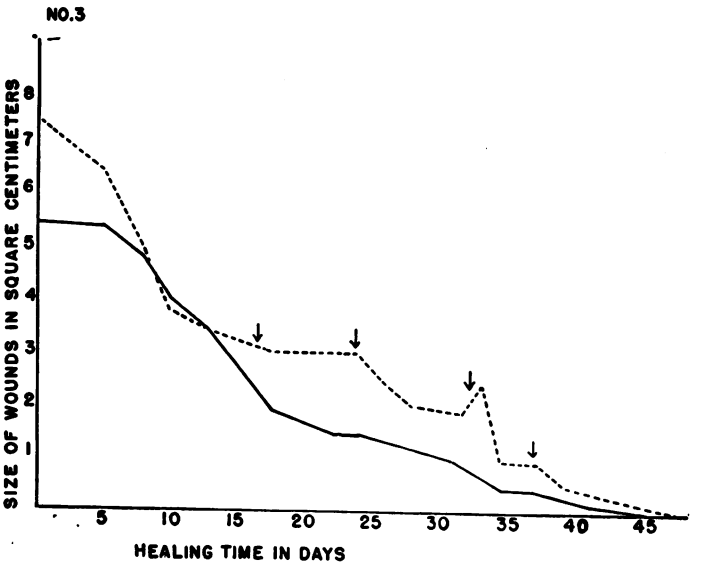


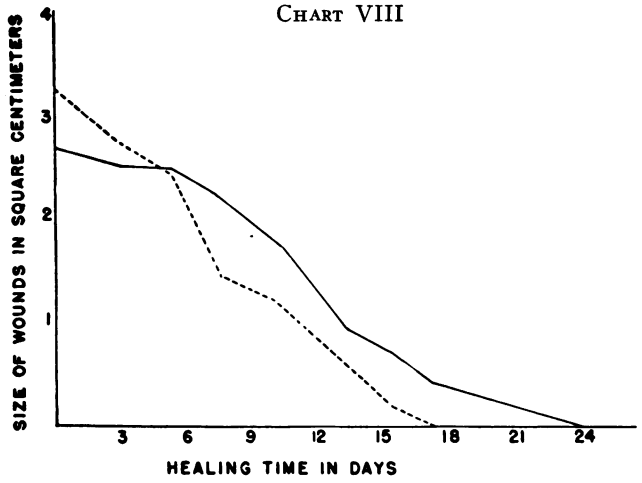
CHART VII

CHART VI.—(Human Volunteer No. 2): The broken line shows the healing time of the treated wound and the solid line that of the control.

CHART VII.—(Human Volunteer No. 3): The broken line represents the healing time of the treated wound and the solid line that of the control. The arrows point to periods of excessive granulation which mechanically impeded epithelization. Granulations were excised at these points.

Consequently, we feel that in adult animal tissue extract there is a substance (or substances) that exerts a growth-promoting effect when added to any heterologous tissue either *in vitro* or in human wounds. We further feel

CHART VIII



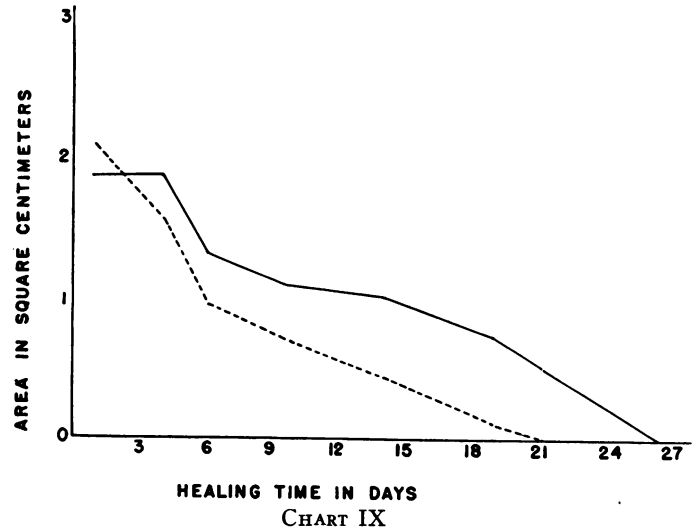


CHART IX

CHART VIII.—(Human Volunteer No. 4) : The broken line shows the healing time of the treated wound and the solid line that of the control.

CHART IX.—(Human Volunteer No. 5) : The broken line shows the healing time of the treated wound and the solid line that of the control.

that such substances act directly on the metabolic mechanism of cellular proliferation.

Many physical and chemical studies have been carried out by us in an attempt to isolate a presumed factor or factors responsible for the growth-

promoting effect of the total extract. The detailed reports of this work will be published elsewhere, but certain basic experiments warrant comment here. First of all, it should be mentioned that while Fischer,[13] Davidson,[9] and others who carried out extensive studies on the nature of embryonic tissue extracts, and Loofburow,[20] and Cook, *et al.*,[8] working with extracts of injured yeast cells, generally concluded that the active growth-promoting fraction was nucleoprotein; following our own studies we have concluded that this may not be the case.

In order to have a standard supply of material for laboratory and clinical investigation, a method was developed for preparation of extract on a large scale.* The initial extract was prepared in the manner described from fresh or frozen sheep heart which may have been stored for six months or more in a CO_2 ice box at $-70°C$. This extract was treated with ether which removed extraneous materials and also cleared-up slight contaminations. Following centrifugation the aqueous fraction, which was even somewhat more active than the original extract, was placed in small containers, frozen and dried *in vacuo,* the containers being sealed under sterile conditions. They contained extract in a powdered form which could be readily dissolved in distilled water. A standard strain of fibroblast cultures was used to test the material resulting from these experiments.

Heart extract is not thermostabile although heating to 60° for 30 minutes has no appreciable effect on activity. Heating-up to 70° C. for 5 to 10 minutes results in a 50% decrease in activity, and when carried out for a prolonged period of time results in a further diminution, but a certain degree of growth-promoting activity is still retained. A temperature of 100° C. does not produce complete inactivation but what remains is probably due to the ordinary nutrient materials which are present even in the heated extract rather than to any specific growth-promoting principle.

The extract is nondialyzable and loses its activity on passing through a Berkefeld filter over a wide range of pH from 3.8 to 8.0, though such changes in pH do not normally effect the growth-promoting activity of the extract. At this pH range about 50% loss in activity is observed in passage through a Seitz filter, but when a small amount of broth is first passed through a Seitz filter it is then possible to filter the extract at its usual pH 6:5 without any appreciable loss in activity.

The activity of the extract remains constant over a wide range of concentrations. Dilution of extract prepared by using one part cardiac muscle and two parts of Tyrode solution (1:2) with as much as four times the volume of Tyrode solution does not affect the activity. Further dilutions of this extract result in a gradual falling-off of activity. However, this extract diluted with 50 volumes of Tyrode solution still manifests some activity. Extracts more concentrated than 1:2 are injurious to cells.

Extracts prepared in the standard way contain about 30 to 130 mg.% of

*We wish to extend our appreciation to Lederle Laboratories Inc. for preparation of this extract in bulk.

protein, with a low protein content being usually less active. However, dilution of extract containing 130 mg.% protein with equal quantities of Tyrode solution did not result in a loss of activity; greater dilution of this extract did decrease activity but not in direct proportion to the diminished protein content, nor is the activity of the extract directly related to the general protein content, as may be seen after treatment with dilute hydrochloric acid. Here, lowering of pH from 6.5 to 4.8 causes precipitation, but, following centrifugation and readjustment of pH the supernatant fraction containing 0.5 mg. protein N. per cc. is quite as active as the original which contained 0.85 mg. protein N. per cc. Lowering of pH to 3.6 results in a reduction of protein N. content to 0.3 mg. per cc. without any loss of activity.

Extract was treated according to the method of Sevag, with an equal volume of chloroform and a small amount of octyl alcohol. This mixture was placed in a shaking apparatus overnight and then centrifuged for one hour. The entire procedure was carried out in the cold. Three fractions were obtained. The aqueous and interface fractions did not stimulate cells growing *in vitro*. The residue of the chloroform soluble fraction dissolved in Tyrode solution inhibited cell growth. No evidence was obtained of active nucleoprotein in the aqueous phase. Other proteins present in the interface fraction were denatured. Thus, absence of activity in this fraction does not preclude the possibility of the active factor being protein.

Twenty cubic centimeters of extract were adsorbed with 100 mg. of aluminum hydroxide in one case and with 20 mg. in the other. Both supernatant fractions actively promoted cell growth. The degree of activation was somewhat less than that of the original extract.

When the adult heart extract was centrifuged at 30,000 R.P.M. for 30 minutes, the supernatant fraction was found to be very active in promoting cell growth, while the macromolecular fraction showed only a slight degree of stimulating activity, such as might be due to the presence of nutrient material.

The total adult heart extract as well as the supernatant fraction obtained from ultracentrifugation were irradiated with ultraviolet light (2,640 A.°) for various periods of time up to 60 minutes. Following such irradiation, the extract did not lose any of its cell growth-promoting properties. Lyophilized heart extract may be redissolved in distilled water containing 50 mg. of sulfadiazine per 100 cc. without diminishing the activity of the extract or injuring the cells. Penicillin up to 300 Oxford units per cc. may also be added without detracting from the stimulating properties.

In view of recent ideas expressed, that growth stimulation may be produced by globulin, a brief study was made of the effect of the fractions isolated by Cohn, and his group, on cells growing *in vitro*. The gamma globulin fraction stimulates growth of cultures *in vitro* to a slight extent, but the degree of stimulation is much less than that of adult heart extract. Thrombin, also, has a slight stimulating effect, but even less than that of gamma globulin. Finally, fraction IV-I is somewhat more active than thrombin but its effect is not nearly so striking as that seen with adult tissue extract.

From the above data, it may be inferred that the active factor or factors involved in promotion of cell growth while protein in nature are not necessarily nucleoprotein. However, since it has been demonstrated that during cell proliferation or activity there is a large increase in the nucleoprotein content of cells it may well be that we are dealing here with an enzyme which plays a part in nucleoprotein formation.

CONCLUSIONS

(1) Experimental and clinical evidence is presented which indicates that there is an active growth-promoting factor present in adult sheep heart extract.

(2) A method is presented whereby adult sheep heart extract may be prepared in large quantities and, thus, be available in stable form for clinical use and experimental study.

(3) This extract appears to exert a markedly stimulating effect on indolent human wounds, and a lesser effect on wounds of normal healthy individuals.

(4) From the experimental data available the active factor would appear to be a protein having many characteristics of an enzyme.

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