THE EFFECTS OF AMINO-ACETONITRILE AND CORTISONE ON THE HEALING OF TURPENTINE-INDUCED ABSCESSES IN THE RAT

J. V. HURLEY, E. STOREY AND K. N. HAM

From the Department of Pathology, University of Melbourne

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SINCE Ponsetti and Baird (1952) demonstrated that diets containing large amounts of the garden sweet pea (*Lathyrus odoratus*), in addition to their previously known effects on bone (Geiger, Steenboch and Parsons, 1933), produced gross damage to the aortic wall of the rat, many papers have appeared on various aspects of this subject. This work has been reviewed by Selye (1957). The toxic factor has been isolated and identified as N-γ-L-glutamyl-β-amino-propionitrile, and it has been shown that a series of related compounds, chiefly nitriles, have a similar action. However, the mechanism of their action which appears to be a widespread one on developing connective tissues is still obscure. In an attempt to gain further information of this action, it was decided to study the developing wall of an experimentally induced abscess in the rat and the changes produced in it by administration of the most potent of known lathyrogens, amino-acetonitrile (A.A.N.) and, further, to compare any changes observed with the known effects of cortisone on such abscess walls (Taubenhaus and Amromin, 1950).

MATERIALS AND METHODS

Sprague-Dawley and Wistar rats of either sex, both young (mean weight 50 g.) and adult (mean weight 150–200 g.) were used.

The animals were fed a diet of Baristoc—a local proprietary food pellet—supplemented by fresh green vegetables. This diet and water were supplied *ad libitum*.

Abscesses were induced by the injection of 0·25 ml. of turpentine and 0·75 ml. of air subcutaneously into the scapular region posteriorly.

A.A.N. was synthetized as its hydrosulphate by the method of Anslow and King (1941).

Both A.A.N. and cortisone acetate were injected daily subcutaneously into the inner side of the thigh: A.A.N. dissolved in distilled water at a concentration of 100 mg. per 1 ml., and cortisone acetate as a suspension in a concentration of 50 mg. per 1 ml. Injections were begun on the same day as the turpentine was administered.

At intervals varying from 2 to 19 days after the injection of turpentine the animals were killed by a blow on the head or by ether anaesthesia and the area of the abscess and overlying skin dissected from the deeper tissues and fixed immediately in 10 per cent formol saline. After fixation the abscess was dissected from the skin and strips cut from the deep aspect of its wall in a plane vertical to the skin surface. These were embedded in paraffin in the usual way and sections cut at 5–7 μ in thickness.

The following stains were used:

1. Haematoxylin and eosin—the eosin used is made in this Department and the details have been recently published by Menzies and Mills (1957). It differentiates Alcian blue very efficiently.
2. Van Gieson.
3. Gomori's modification of Masson's trichrome.
4. Alcian blue (Steedman, 1950).
5. Periodic acid Schiff (P.A.S.) (McManus, 1948), without the acid-reducing rinse of Hotchkiss (1948).

6. Ammoniacal silver for reticulin (Gomori as modified by Lillie, 1948).
7. Hale's colloidal iron as modified by Rinehart and Abul-Haj (1951).

Contraction temperatures were determined by cutting a strip approximately 3 × 0·2 cm. from the wall of the unfixed abscess, suspending it with a small weight attached in a beaker of water and applying heat gradually. The end point was quite sharp, the strip contracting to half to two-thirds of its initial length over a temperature range of 1–2°.

Capillary permeability was estimated by intravenous injection of 0·5 ml. of a 1 per cent solution of Evan's blue into a rat, followed immediately by the intradermal injection of 0·1 ml. of the substance to be tested into the previously shaved abdominal skin. The changes in the area of intradermal injection were followed for up to one and a half hours after injection.

RESULTS

Four groups of experiments were undertaken :

I. Control animals ;

II. animals receiving A.A.N.—10 mg. per day to young rats and 20 mg. per day to adult animals ;

III. animals receiving cortisone acetate—5 mg. per day to both young and adult rats ;

IV. animals receiving both A.A.N. and cortisone simultaneously in the same dosage as groups II and III.

In all groups an attempt was made to study the abscess over as long a period as possible but it was found that only occasionally could observations be extended beyond fourteen days as the abscesses discharged through the overlying skin at or before this time and study of the residual collapsed wall was not satisfactory. An impression was gained that such discharge occurred earlier and more frequently in animals receiving A.A.N., but the results were too variable to allow of certainty on this point.

In all the young animals treated with either A.A.N. or cortisone, and especially with the two drugs simultaneously, gross retardation of growth occurred. In addition, the typical skeletal deformities of lathyrism developed rapidly in young animals of groups II and IV, a number being grossly deformed within fourteen days. No case of aortic rupture occurred.

Adult animals did not show either of these changes but a loss of coat lustre and generally apathetic behaviour were seen in animals receiving A.A.N. for seven days or more.

A small number of young animals from each of group II, III and IV died during the period of experiment—some from demonstrable secondary infection, others without obvious cause.

The number of animals surviving the experiment and available for histological examination is shown in Table I.

TABLE.—*Number of Animals Used Subdivided According to Age and Drugs Administered*

Group	Drug administered	Number of animals		
		Young	Adult	Total
I	None (control)	3	9	12
II	A.A.N.	8	9	17
III	Cortisone	6	5	11
IV	A.A.N. + Cortisone	6	4	10

No difference in the development of the abscess was seen between young and adult animals in either control or injected groups, and the findings will be treated together. It is convenient to describe the changes seen in the four groups in order.

Abscesses in control animals

Macroscopic.—After two days the abscess was well defined from the surrounding tissue, having a thin transparent wall through which yellow pus could be clearly seen. By the fourth day a firm white opaque wall was present, and this progressively thickened until on removal all the later abscesses appeared as firm, roughly spherical, white masses adherent to the deeper surface of the skin (Fig. 1A).

Microscopic.—At two days the wall consisted of necrotic material centrally, surrounded by a thick layer of polymorphonuclear leucocytes, showing degeneration and necrosis adjacent to the central necrotic area. Occasional macrophages were seen in the outer part of this leucocytic layer, which externally abutted directly on skeletal muscle.

By the fourth day the wall had increased considerably in total thickness, and from within out the following layers were seen :

i. Necrotic material ;

ii. a zone of polymorphonuclear leucocytes, considerably thinner than that seen on day 2 ;

iii. a layer of granulation tissue composed of macrophages, capillary loops and fibroblasts, with some polymorphs especially in its inner portion. The axis of the capillary loops and fibroblasts was perpendicular to the surface of the abscess.

Some fine fibres, staining black by silver impregnation, pink with P.A.S. and pink with van Gieson, were seen in the outer half of this layer, embedded in material staining pink with eosin or P.A.S. ;

iv. a layer of active fibroblasts with clearly visible fibres between them, both cells and fibres regularly orientated parallel to the abscess cavity—that is, at right angles to the preceding layer.

Fibres in this layer stained pale pink with P.A.S., black by silver impregnation and deep red with van Gieson, and large P.A.S.-positive granules were present in the fibroblasts and macrophages.

The relative thickness of layers ii, iii and iv was as 1 : 2 : 1.

By the seventh day the same four layers were still present, but their proportions had altered considerably, now appearing as 1 : 1 : 2 from within out (Fig. 1B).

The fibrillar content of the two outer layers had increased considerably, but their staining characteristics had not altered except that the fibres in the outermost part of the wall had thickened and stained brown rather than black with silver impregnation.

On the ninth day only a few polymorphonuclear leucocytes were present in the inner part of the wall, the granulation tissue layer was relatively thinner and the greater part of the wall was composed of concentrically arranged fibroblasts and fibres, thin in the inner portion and thicker externally (Fig. 1C). These fibres all stained pink with P.A.S. but the reaction to silver changed from black to brown and the intensity of staining with van Gieson increased from within out.

From this time until observation ceased on the fourteenth day these changes steadily progressed—the outer concentric layer occupying an increasing proportion of the whole wall and its fibres becoming thicker and assuming the staining properties of collagen rather than of reticulum (Fig. 5). The intervening cells concurrently became less active in appearance—the nuclei decreasing in size and staining more deeply and the cytoplasm, with its P.A.S.-positive granules, becoming less apparent (Fig. 6). Some mature fibroblasts were present in the outer part of the wall as early as the seventh day (Fig. 1C).

At all stages after the fourth day the intercellular substance in the two outer layers, relatively small in amount, stained positively to both Alcian blue and Hale's colloidal iron and a pale pink with P.A.S.

Abscesses in animals receiving A.A.N.

Macroscopic.—During the first four days the abscesses showed no significant differences from the controls. After this period the wall became opaque and white in the usual way, but the thickening of the wall seemed slower to develop than in the controls so that at all stages the wall appeared relatively looser and less opaque and had a softer consistence to touch.

In addition, from the fourth day onwards a prominent red margin appeared in the outer part of the wall (Fig. 2A) and this persisted until at least the fourteenth day. No corresponding structure was seen in the controls.

Microscopic.—For the first two days the reaction appeared identical with that seen in the control animals, except that in some areas polymorphonuclear leucocytes appeared to penetrate more deeply into the surrounding tissues.

Definite differences first became apparent about the fourth day, and were seen in the outer part of the wall—layer (iv) as described in the control.

At first the changes were slight but with time they became steadily more apparent. At all stages the changes were similar in type.

The layer of forming fibrous tissue was of normal or slightly increased thickness and fibroblasts were present in not less than their normal numbers (Fig. 2B). They were normal in appearance and contained obvious P.A.S.-positive granules, but their maturation was delayed so that at all stages the proportion of mature cells was relatively decreased (Fig. 2C) and as late as the tenth day the majority still possessed active nuclei and abundant cytoplasm containing P.A.S.-positive granules. Their regular arrangement, concentric with the cavity of the abscess, was lost and they appeared to follow no regular pattern (Fig. 7 and 8).

Between the cells was an apparent increase of intercellular substance of considerable degree—this material stained pale pink with P.A.S., positive to Alcian blue and to colloidal iron. The fibres embedded in it had also lost their concentric arrangement and whilst silver-positive reticular fibres were seen in normal numbers in the inner part of the zone, their usual aggregation into dense concentric bundles in the outer layers was not apparent in many areas, fine fibres of irregular arrangement persisting to the periphery of the abscess. These stained positively with van Gieson but still gave a black reaction after silver impregnation.

Capillaries were more prominent than usual in the full thickness of the fibroblast layer, accounting for the red margin seen macroscopically (Fig. 2B). No abnormality in the macrophages was detected.

Abscesses in animals receiving cortisone

Macroscopic.—By contrast with the preceding two groups localization of the abscess was very poor. Instead of small white spherical masses, large flat areas were seen (Fig. 3A), surrounded as late as the eleventh day by a thin transparent wall through which yellow pus was clearly visible.

Microscopic.—At four days the wall consisted of a layer of polymorphonuclear leucocytes of similar thickness to that of controls of the same period, with only scattered macrophages between it and underlying muscle.

By the seventh day the wall was approximately one half the thickness of the control and consisted of polymorphs and macrophages surrounded by a thin layer of fibroblasts and relatively mature fibrous tissue (Fig. 3B and C). A few dilated capillaries were seen in this layer.

Over the next few days the fibrous tissue layer gradually thickened, but by the eleventh day when observation ceased, it was not more than a small fraction of the thickness of the wall of the control.

At no time did a definite layer of granulation tissue develop internal to the fibrous layer, which appeared to be derived in part from compression of pre-existing collagen fibres in the area.

A few dilated capillaries remained in this layer on the eleventh day.

Abscesses in animals receiving A.A.N. and cortisone

Macroscopic.—These closely resembled those in group III, differing only in spreading even more widely and having still thinner walls and, more obviously, in the presence of a red line of dilated capillaries in the outer part of their wall (Fig. 4A). This was clearly seen in all abscesses of the group.

Microscopic.—The changes seen closely resembled those in animals receiving cortisone alone. The fibrous layer external to the inflammatory cell zone was thinner than in the preceding group (Fig. 4B) and in some areas consisted of fine irregular fibrils staining black with silver impregnation and positively with van Gieson in place of the thicker, more regular bundles of group III, which showed the typical staining reactions of collagen.

Slight increase of the intercellular substance, staining as in the animals receiving A.A.N. alone, was seen in these areas.

Dilated blood vessels were a prominent feature of the outer part of the wall in every case (Fig. 4C).

Contraction temperatures of abscess walls

Strips were tested from the wall of abscesses in all four groups on the fourth, seventh, ninth and eleventh day after turpentine injection. In all cases contraction occurred in the range 65–68°, with no detectable difference between the four groups.

Local Effect on Capillary Permeability

A solution of A.A.N. sulphate in the concentration used for subcutaneous injection, and 10- and 100-fold dilutions of this solution, were tested concurrently with a control solution of normal saline.

In the two higher concentrations a spreading weal developed rapidly and reached a diameter of 2 cm. within thirty minutes ; a well-defined band of blue

was present around the weal. The 1/100 dilution showed a similar but smaller response and the saline control produced neither weal nor blue band.

Estimation of the pH of the A.A.N./sulphate solution gave a value of 1·5–2·0 ; the experiment was repeated with the pH of the A.A.N. solution adjusted to 6·5–7·0 by addition of a veronal buffer. After this alteration of the pH neither weal nor blue band was apparent with any of the A.A.N. solutions.

DISCUSSION

From the experiments described there is no doubt that A.A.N. has a demonstrable effect on the developing abscess wall.

This seems not to be one affecting cellular proliferation, and in particular not one involving the multiplication of fibroblasts. The effect seems rather to lie in the maturation of these cells and in the formation of collagen and is accompanied by a loss of the normal regular orientation of cells and fibres in the abscess wall.

This is in agreement with several known observations regarding the action of A.A.N. and related compounds on connective tissue. Enzinger and Warner (1957) found considerable reduction in the tensile strength of healing incised wounds in the abdominal wall of the rat, most clearly demonstrable on the fourth and fifth day of healing. In a histological study of these wounds they found no delay in fibroblastic proliferation, but weakness and irregularity in collagen fibre formation accompanied by unduly long persistence of P.A.S.-positive intercellular substance normally present in the earlier stages of wound healing.

Mielke, Lalich and Angevine (1957), in a study of the effect of β-amino-propionitrile on croton oil induced subcutaneous pouches in the rat, reported poor formation and collapse of the pouches and were able to demonstrate decreased production of hydroxyproline in the pouch wall, hexosamine production being apparently normal. In an accompanying histological study, not completely satisfactory apparently due to the necessary chemical treatment of the wall before fixation, they reported increase in fibroblast numbers, decrease in collagen fibres, a general loss of orientation in the wall and an apparent increase in intercellular substance. They concluded that β-amino-propionitrile in some way interferes with collagen synthesis.

Evidence that fibroblast proliferation is not impaired, but is rather stimulated was provided by Yeager and Hamre (1957), who found definite proliferation of fibroblasts in exostoses of the adult rat femur within twenty-four hr. of commencing a diet containing 50 per cent *Lathyrus odoratus* meal.

Indirect evidence that connective tissue formation is the site of action rather than any change in formed connective tissue is provided by the observation that gross skeletal deformities, aortic rupture and other features of the experimental lathyrous syndrome only occur in growing animals, adults being relatively insusceptible to even high dosage of A.A.N. and allied compounds. By contrast the growing tissue in a healing wound or abscess wall appears equally susceptible in young and mature animals. Whilst all the above work leads to the same general conclusion regarding the essential nature of the lathyrous lesion, the study of the development of the bony and aortic lesions has not led to similar clarity. Menzies and Mills (1957) concluded that the basic change was " an accumulation of chondroitin sulphate together with a general breakdown of supporting structures such

as collagen fibres ". By contrast, Churchill, Gelfant, Lalich and Angevine (1955), while finding a similar chondroitin sulphate increase also described a primary degeneration of elastic fibres, in which finding they are supported by Walker and Wirtschafter (1956) whose illustrations however are not convincing, as in some, pre-existing smooth muscle cells appear to have been confused with " fibroblastic proliferation ".

The technical difficulties associated with study of the rat aorta, allied to the circumstance that, with the exception of the work of Yeager and Hamre (1957) quoted above, all the work on bone has been done on relatively chronic lesions, make it probable that the conclusions drawn from studies on wounds and abscesses indicate more accurately the basic change, but more work on both is needed to resolve the apparent anomalies.

The changes seen in granulation tissue are somewhat similar to those described in scurvy by Penny and Balfour (1949) and Bunting and White (1950). However, vitamin C is most unlikely to be involved in the mode of action of A.A.N. as Enzinger and Warner (1957) found administration of vitamin C made no difference to the delay in wound healing caused by A.A.N., and heavy dosage of vitamin C fails to prevent the bony and aortic lesions of lathyrism (Lewis, Fajans, Esterer, Shen and Oliphant, 1948). This finding was confirmed in work carried out in this Department during 1955. Direct comparison of the two lesions in the rat is not possible as scurvy cannot be produced experimentally in this species.

In view of the doubt regarding the histochemical specificity of the various stains for mucopolysaccharides and related compounds (Meyer, 1953), and the incompleteness of our knowledge of the intimate structure of collagen fibres and their relation to fibroblasts (Jackson, 1956), a discussion of the nature of the change on a histochemical rather than a histological descriptive plane is of little value.

The evidence from our experiments on the local effect of A.A.N. on capillary permeability suggests that there is no significant local action on small blood vessels and the striking zone of capillary dilatation seen around abscesses in animals receiving A.A.N., either alone or with cortisone, may be a consequence of the delay in fibrous tissue maturation rather than a direct effect of the drug. The local haemorrhage at the site of injection reported by Selye (1957), and repeatedly confirmed by us, is almost certainly due to the high degree of acidity of the solution administered and not to the drug itself.

The changes observed in this series following the administration of cortisone are in general agreement with the known effects of this substance applied either locally or systemically to granulation tissue (Ragan, Howes, Plotz, Meyer and Blunt, 1949; Taubenhaus and Amromin, 1950). The changes seen are even more gross than those observed by these authors; but a higher dose of cortisone was used.

The type of change is quite distinct from that produced by A.A.N. and consists of a gross decrease in the rate of multiplication of fibroblasts with a consequent diminution in the amount of granulation tissue and collagen formed. We saw no evidence of the abnormal accumulation of macrophages described by Taubenhaus and Amromin, but the primary polymorphonuclear response appeared unimpaired.

In the animals who received both A.A.N. and cortisone the dominant effect observed was that due to cortisone. However, the changes described above as characteristic of A.A.N. administration were seen in what little fibrous wall

developed in the abscesses of this group, and the peripheral red zone of vascular dilatation was very obvious in these animals in striking contrast to the reduced vascularity of the abscesses in animals receiving cortisone alone.

It would appear that the two drugs produce effects on granulation tissue independently of one another.

The failure to detect any change in contraction temperature of the abscess wall in animals receiving either A.A.N. or cortisone indicates that what collagen does form is in each case normal, in this respect at least, as it can be assumed that heat contraction of the wall is due to its collagen content.

The factors influencing such contraction have been recently reviewed by Gustavson (1956).

SUMMARY

The effect of systemic administration of A.A.N. and cortisone, alone and together, on the healing of turpentine-induced abscesses in the rat is described.

The effect of A.A.N. is to cause a delay in the laying down of mature collagen in the wall, accompanied by abnormal persistence of amorphous intercellular substance. Proliferation of fibroblasts is not impaired, but their maturation is delayed.

A.A.N. appears to have no local dilator effect on blood capillaries, nor does it increase capillary permeability.

The known effect of cortisone in grossly delaying fibroblastic proliferation is confirmed.

The effects of A.A.N. and cortisone on developing connective tissue appear to be independent of each other.

EXPLANATION OF PLATES

In each figure (A) is a photograph, actual size, of the opened abscess. (B) is a low power photomicrograph of a section of the whole thickness of the abscess wall, stained with haematoxylin and eosin. × 40. (C) is a photomicrograph of a section of the outer part of the wall in Fig. 1 and 2, and its whole thickness in Fig. 3 and 4, stained with haematoxylin and eosin. × 180.

FIG. 1.—Abscess in control animal at 7 days.

FIG. 2.—Abscess in animal receiving A.A.N. at 7 days. Note its approximately normal size and thickness of wall in (A) and (B), with prominent congested margin in (A), and also separation and loss of orientation of cells in outer part of the wall in (C).

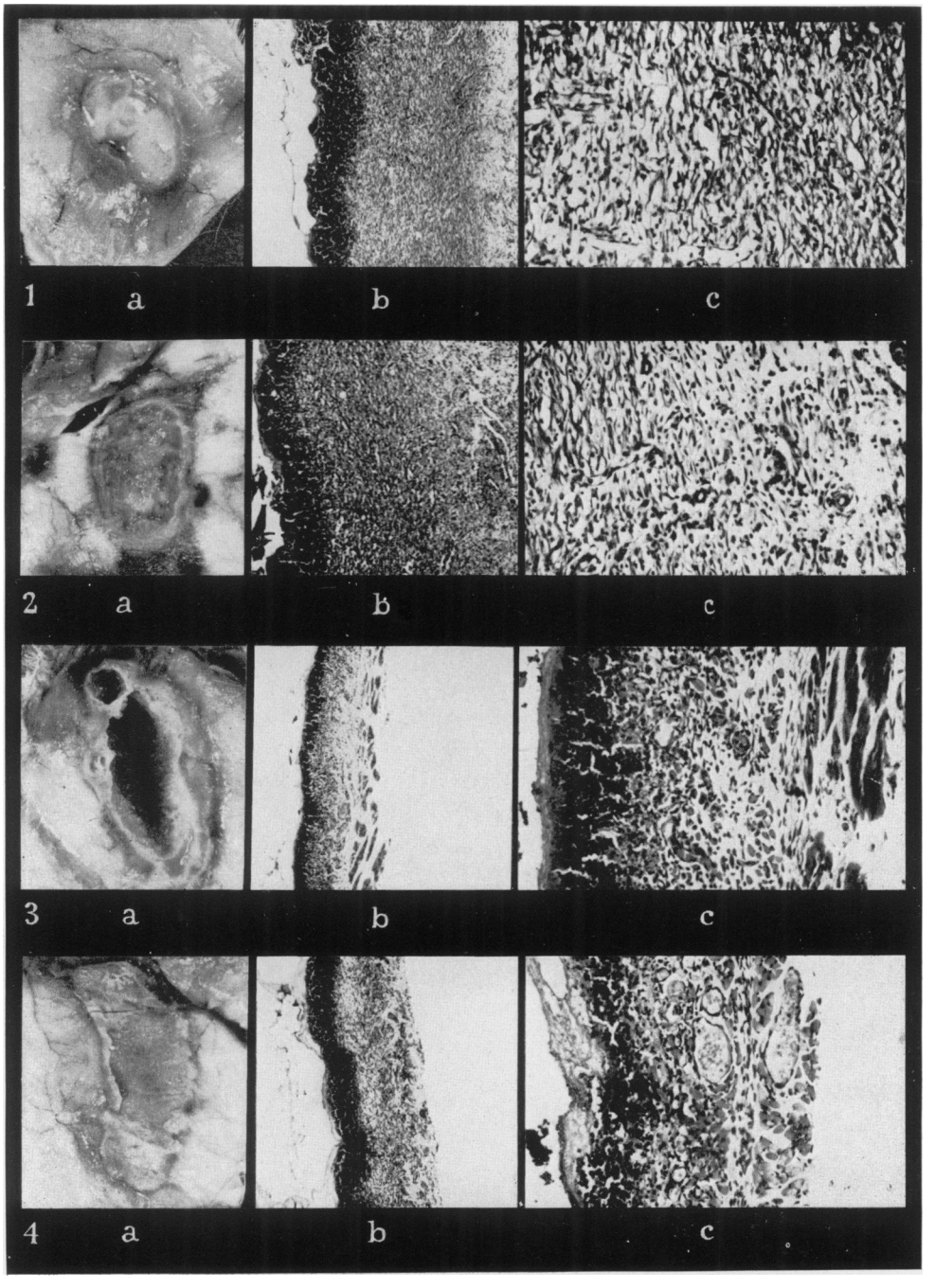
FIG. 3.—Abscess in animal receiving cortisone at 7 days. Note increase in size and the thinness of the wall in (A) and (B), and gross decrease of granulation tissue in outer part of wall in (C).

FIG. 4.—Abscess in animal receiving both A.A.N. and cortisone at 7 days. Note increase in size and extreme thinness of wall visible in (A), (B) and (C), almost complete absence of granulation tissue and presence of large dilated blood vessels in outer part of the wall in (C).

FIG. 5 and 6.—Photomicrographs of portion of a section of the wall of abscess in control animal at 12 days, stained with haematoxylin and eosin. Fig. 5 × 180 Fig. 6 × 960.

FIG. 7 and 8.—Photomicrographs of portion of a section of the wall of abscess in an animal receiving A.A.N. at 12 days, stained with haematoxylin and eosin. Fig. 7 × 180. Fig. 8 × 960.

Note absence of the normal concentric arrangement of fibroblasts and fibrils seen in Fig. 5 and 6, with relative immaturity of many of the cells and great increase of eosinophilic intercellular material.



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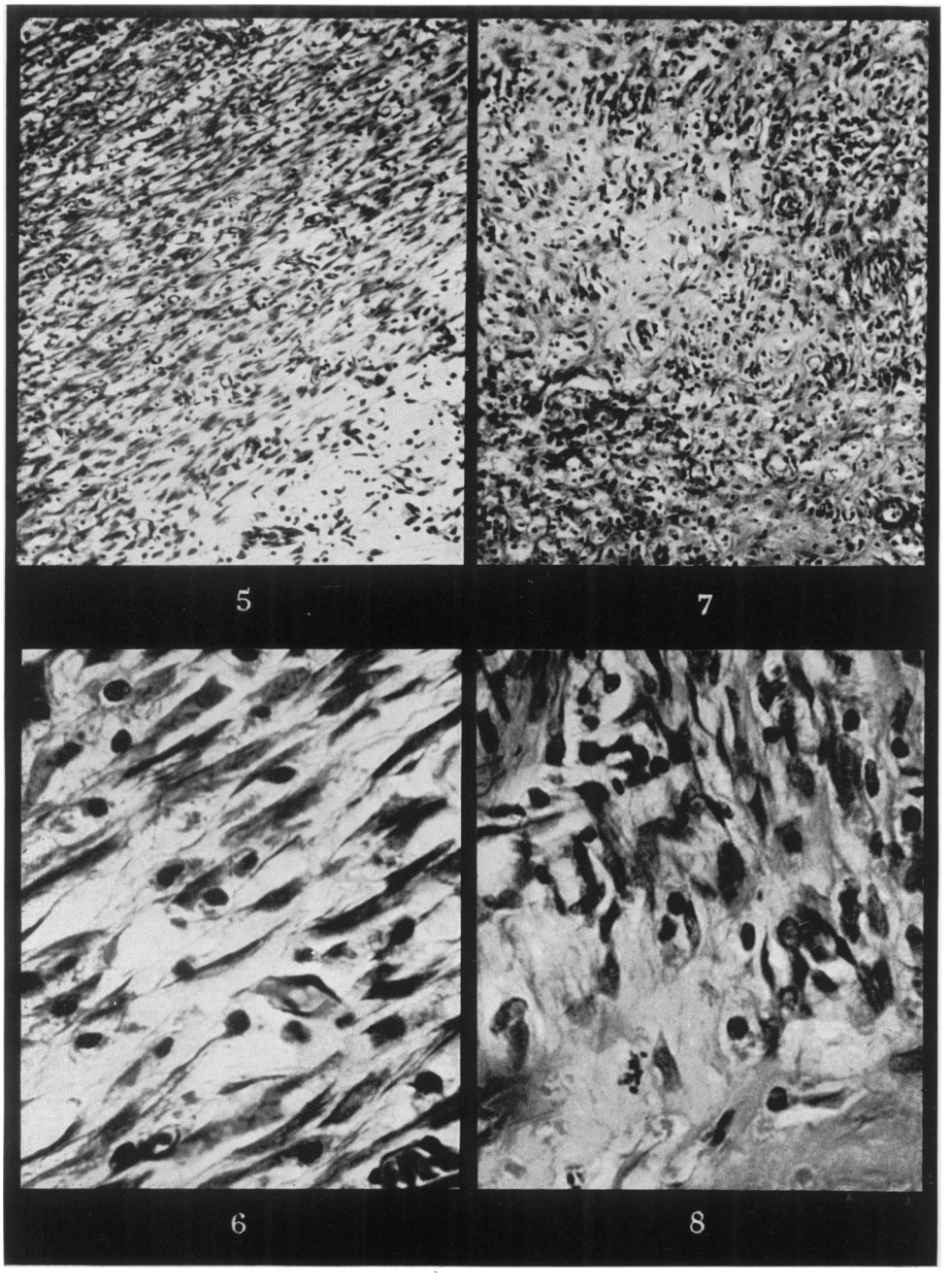

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Failure to detect any alteration of the contraction temperature of the wall of any of the abscesses studied suggests that what collagen is formed in the presence of either A.A.N. or cortisone is normal.

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