THE NATURE OF THE CONNECTIVE TISSUE DEFECT PRODUCED BY THE AMINONITRILES

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OBSERVATIONS on healing wounds (Enzinger and Warner, 1957), croton oil pouches (Mielke, Lalich and Angevine, 1957) and turpentine induced abscesses (Hurley, Storey and Ham, 1958) have shown that β-amino-propionitrile (BAPN) and related substances cause a delay in collagen maturation. To gain further information regarding this phenomenon the effects of BAPN and amino-aceto-nitrile (AAN) have been studied in two further experimental systems—the growth of granulation tissue into subcutaneously implanted polyvinyl sponges and the development of granulomata following the subcutaneous injection of carrageen.

MATERIALS AND METHODS

Sprague-Dawley and Wistar rats of either sex, both young (mean weight 50 g.) and adult (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*.

AAN was synthesized as its hydrosulphate by the method of Anslow and King (1941). BAPN was administered as its fumarate, obtained from Abbott Laboratories, Chicago. Each drug was injected subcutaneously daily.

Polyvinyl sponge (Prosthex, Raemer Chemical Co., London) pieces approximately 2 cm. square were implanted subcutaneously into the upper dorsal region by an aseptic technique.

Carrageen was prepared by boiling the dried seaweed, *Chondrus crispus*, with 100 times its weight of water for 30 min. and filtering twice through fine cotton gauze.

Material for histological examination was prepared and stained as previously described (Hurley *et al.*, 1958).

Material for estimation of hydroxyproline was weighed immediately after removal, cut into small pieces, defatted in 3 changes of acetone for a total period of 7 days and dried to a constant weight. A portion was hydrolyzed in a sealed tube with 4 N hydrochloric acid for 16 hr. at 110° (Levene and Gross, 1958). The hydroxyproline content was determined by the method of Neuman and Logan (1950) as modified by Martin and Axelrod (1953), using a " Unicam " spectrophotometer at a wave length of 558 mμ.

Because of the variation in different areas of the carrageen granulomata, 3 samples of approximately 50 mg. dry weight were taken from each granuloma.

RESULTS

Experiments with Polyvinyl Sponges

Sponges were implanted in 14 adult rats, half of which received 10 mg. of AAN per day. One injected and one control animal were sacrificed every week for 6 weeks. The remaining 2 rats received AAN for 5 weeks, injections were then discontinued, and the animals killed one week later—at the end of the 6th week.

Appearances in Control Animals

Macroscopic.—After one week the sponge was encapsulated by pink vascular tissue which required sharp dissection to separate the sponge without removing ingrowing material from its interstices. After 2 weeks the vascularity of the surrounding tissue had disappeared and over the succeeding 4 weeks little change in appearance was seen, but the mass of the sponge and its contents became progressively firmer.

Microscopic.—At the end of the first week ingrowth of tissue had filled most of the spaces in the sponge. This new tissue consisted of macrophages, occasional foreign body giant cells and numerous immature fibroblasts embedded in a ground substance containing fine argyrophil fibrils. The ground substance stained positively with Alcian blue, pale pink with PAS and green with the Picro Gomori stain. Numerous small blood vessels were present.

After 2 weeks (Fig. 1) macrophages had decreased in number, fibroblasts and ground substance were unchanged and numbers of coarse fibres with the staining reaction of collagen were present. Occasional fine argyrophil fibrils could still be seen.

From this time until observation ceased at the end of the 6th week (Fig. 3) collagen fibres appeared in increasing quantity. Fibroblasts decreased in number and altered in appearance to show small dark spindle nuclei and relatively little cytoplasm. Argyrophil fibrils disappeared, ground substance became less apparent and vascularity decreased. In addition, in specimens removed after 5 to 6 weeks, mature adipose tissue had replaced, in part, the developing fibrous tissue.

Appearances in Animals Receiving AAN

Macroscopic.—After one week there was no detectable difference from the control sponges. At all times thereafter, however, the sponges removed from animals receiving AAN showed greater vascularity than the corresponding control and even after 6 weeks the sponge from the AAN treated animal was significantly injected.

The sponges from the animals in which injections ceased one week before sacrifice were indistinguishable from the 6-week controls.

Microscopic.—After one week the reaction appeared identical with that seen in the control animal. Definite differences were seen at the end of the 2nd week (Fig. 2) and became progressively more obvious in later specimens. The macrophage reaction was not altered but the immature fibroblasts seen only in the first 2 weeks in the controls persisted until the 6th week (Fig. 4). Intercellular substance increased in amount, while retaining normal staining reactions. Argyrophil fibrils also persisted but very few collagen fibres appeared.

In the later stages fat was present as in the controls. The appearance of the sponges from the animals in which AAN administration ceased one week before sacrifice was indistinguishable from the 6-week control (Fig. 5).

Experiments with Carrageen

A volume of 1·5 ml. of 1 per cent carrageen extract and 1·5 ml. of air were injected subcutaneously into 24 adult rats, half of which received AAN 15 mg./day. Animals were sacrificed daily from the 3rd to 14th day.

Appearances in Control Animals

Macroscopic.—After injection the carrageen formed a firm oval swelling which was barely palpable by the following day. On the 4th day a semi-solid gelatinous mass, difficult to separate from the surrounding tissues, was found. From this time the size of the mass remained almost constant but became progressively firmer and more clearly defined. The early granulomata were pale pink and gradually lost colour with age.

Microscopic.—On the 3rd day the granuloma was composed of polymorphonuclear leucocytes and macrophages distributed in subcutaneous fat containing large blood vessels and scattered coarse collagen fibres. The macrophages contained abundant carrageen which stained deeply with Alcian blue, and a vivid red with PAS. By the 4th day a few immature fibroblasts had appeared and newly formed capillaries were first seen about the same time. Fine argyrophil fibrils appeared on the 6th day, were abundant by the 8th day, and joined in occasional areas to form fibres with the staining reactions of collagen. From the 9th to 14th day both the number of fibroblasts and the amount of collagen steadily increased (Fig. 7). The intercellular substance, staining positively with Alcian blue and pink with PAS was present only in small quantities. Carrageen-containing macrophages and occasional leucocytes were present at all stages.

Appearances in Animals Receiving AAN

Macroscopic.—For the first 9–10 days after carrageen injection the only differences detectable from the control granulomata were an increase in vascularity giving a pinkish colour and a somewhat softer consistence.

Over the next few days, however, a dramatic change occurred (Fig. 6). The mass decreased rapidly in size, to form a small soft area, occasionally with residual carrageen still visible in the centre, surrounded by a thin fibrous capsule. No coincident change in vascularity was seen.

During studies of several series of animals the decrease was first seen on either the 10th or 11th day and was of sudden appearance, the granuloma of the preceding day being of normal size.

Microscopic.—Over the first 6 days no difference from the controls was seen. By the 7th day argyrophil fibrils were present in approximately the same numbers as in the control, but the intercellular material appeared slightly increased.

By the 9th day a gross apparent increase in intercellular material was present and argyrophil fibrils were scattered through it (Fig. 8). The intercellular material showed its normal staining reactions. Fibroblasts were present in normal numbers but newly formed collagen fibres were not seen.

On the 11th day (after sudden decrease in size of the mass had occurred) the excess of intercellular substance was no longer seen and the dominant feature was masses of macrophages interspersed with coarse (preformed) collagen bundles and large blood vessels. By the 14th day very few fibroblasts were present, the mass consisting almost entirely of macrophages, densely packed together and still containing stainable carrageen.

Chemical

Two groups of animals were studied :

A volume of 1·5 ml. of 1 per cent solution of carrageen and 1·5 ml. of air were injected into 16 adult rats, half of which received, in addition, 15 mg./day

of AAN. Animals were sacrificed at intervals and the total wet and dry weights and the hydroxyproline content of their granulomata determined. The results obtained are contained in Table I.

TABLE I.—*Wet and Dry Weights, Hydroxyproline and Collagen Contents of Carrageen Granulomata from Control Rats and Rats Receiving AAN 15 mg./day*

Day	Granuloma weight in g. Wet	Granuloma weight in g. Dry	* Ratio dry/wet weight	Hydroxyproline mg./g. wet weight	† Total collagen in mg.	Comment
Control rats :						
2	2·465	0·181	0·073	0·59	10·8	
6	1·040	0·140	0·135	2·43	18·6	
8	2·840	0·315	0·111	2·26	47·3	An unusually large granuloma.
10	1·270	0·164	0·129	2·67	25·0	
11	1·225	0·231	0·188	4·38	39·4	
12	2·235	0·360	0·161	2·75	45·2	
13	2·370	0·434	0·183	4·66	81·3	
14	1·420	0·228	0·161	3·98	41·5	Granuloma smaller than usual.
Rats receiving AAN 15 mg./day :						
2	1·775	0·123	0·069	1·06	13·8	
6	1·005	0·121	0·120	2·12	15·7	
8	2·405	0·235	0·098	1·71	30·4	An unusually large granuloma.
10	0·555	0·102	0·184	3·68	15·0	
11	0·420	0·083	0·198	4·38	13·5	
12	1·105	0·191	0·173	4·65	37·6	" Shrinkage " less marked than usual. ? Very large granuloma initially.
13	0·530	0·114	0·216	5·40	21·0	
14	0·340	0·067	0·197	5·29	13·2	

* In both Tables I and II ratio dry/wet weight is given instead of water content as an unknown amount of fat was removed by acetone prior to dehydration.

† Total collagen $= \frac{\text{hydroxyproline}}{13{\cdot}6} \times 100 \times$ total wet weight.

Twenty-four adult rats received similar injections of carrageen and half were given 20 mg./day of BAPN. They were sacrificed at intervals as in the first group and the same determinations made. The results are contained in Table II.

Attempts to Define the Critical Period for the Macroscopic " Shrinkage " Effect

Two further groups of rats were studied :

A volume of 1·5 ml. of 1 per cent carrageen solution and 1·5 ml. of air were injected subcutaneously into 24 adult rats. The rats were divided into 8 equal groups.

One group received no further treatment. In the remaining 7, daily injections of 15 mg. of AAN were begun and administration discontinued at successive time intervals thereafter as indicated in Fig. 9.

All groups were sacrificed on the 14th day, the granulomata removed and weighed. All granulomata were of normal size except for the group receiving AAN for 11 days, in which definite " shrinkage " had occurred. The results are shown in Fig. 9.

TABLE II.—*Wet and Dry Weights, Hydroxyproline and Collagen Contents of Carrageen Granulomata in Control Rats and Rats Receiving BAPN 20 mg./day*

Day	Total granuloma weight (g.) Mean of 2 Wet	Dry	Ratio dry/wet weight	Hydroxyproline mg./g. wet weight	Total Collagen per granuloma (mg.)	Comment
Control rats :						
5	2·260	0·232	0·103	1·31	21·8	
7	2·040	0·248	0·122	1·74	26·1	
9	2·520	0·329	0·130	1·99	36·9	
11	2·745	0·311	0·113	1·52	30·7	
13	1·635	0·170	0·104	2·11	25·4	Granulomata smaller than usual.
15	2·243	0·358	0·160	2·99	49·3	
Rats receiving BAPN 20 mg/day :						
5	1·210	0·150	0·124	1·47	13·1	All granulomata a little smaller than usual, and than corresponding controls.
7	1·395	0·177	0·127	1·70	17·5	
9	1·542	0·196	0·127	1·76	19·9	
11	1·105	0·143	0·130	1·83	14·9	
13	0·735	0·131	0·178	2·98	16·1	
15	0·645	0·116	0·180	3·40	16·1	

EXPLANATION OF PLATES

FIGS. 1–5.—Each figure is a photomicrograph of a section of granulation tissue within an implanted polyvinyl sponge, stained with Van Gieson. × 180.

FIG. 1.—Control animal at 2 weeks. Note the large number of fibres present, mostly fine but some coarse and forming definite bundles.

FIG. 2.—Animal receiving AAN at 2 weeks. Note the presence of numerous fibroblasts and abundant intercellular substance in which are fine fibrils and large dilated blood vessels.

FIG. 3.—Control animal at 6 weeks. Bundles of coarse collagen fibres and areas of adipose tissue fill the interstices of the sponge, portions of which are present to the left and below.

FIG. 4.—Animal receiving AAN at 6 weeks. Some coarse collagen fibres are present, but most of the tissue closely resembles that in Fig. 2. Intercellular substance containing fine fibrils and dilated blood vessels is still prominent.

FIG. 5.—Animal killed one week after cessation of 5 weeks administration of AAN. Note the close resemblance to Fig. 3 ; coarse collagen fibres are present in large numbers and intercellular substance is scanty. Residual sponge is present on the right.

FIG. 6.—Each pair, A, B and C is a photograph, actual size, of two carrageen granulomata removed together with the overlying skin : to the left a granulomata from a control animal, to the right one from an animal receiving BAPN. A—treated animal (right) received 25 mg. BAPN per day. B and C—treated animal (right) received 20 mg. BAPN per day.

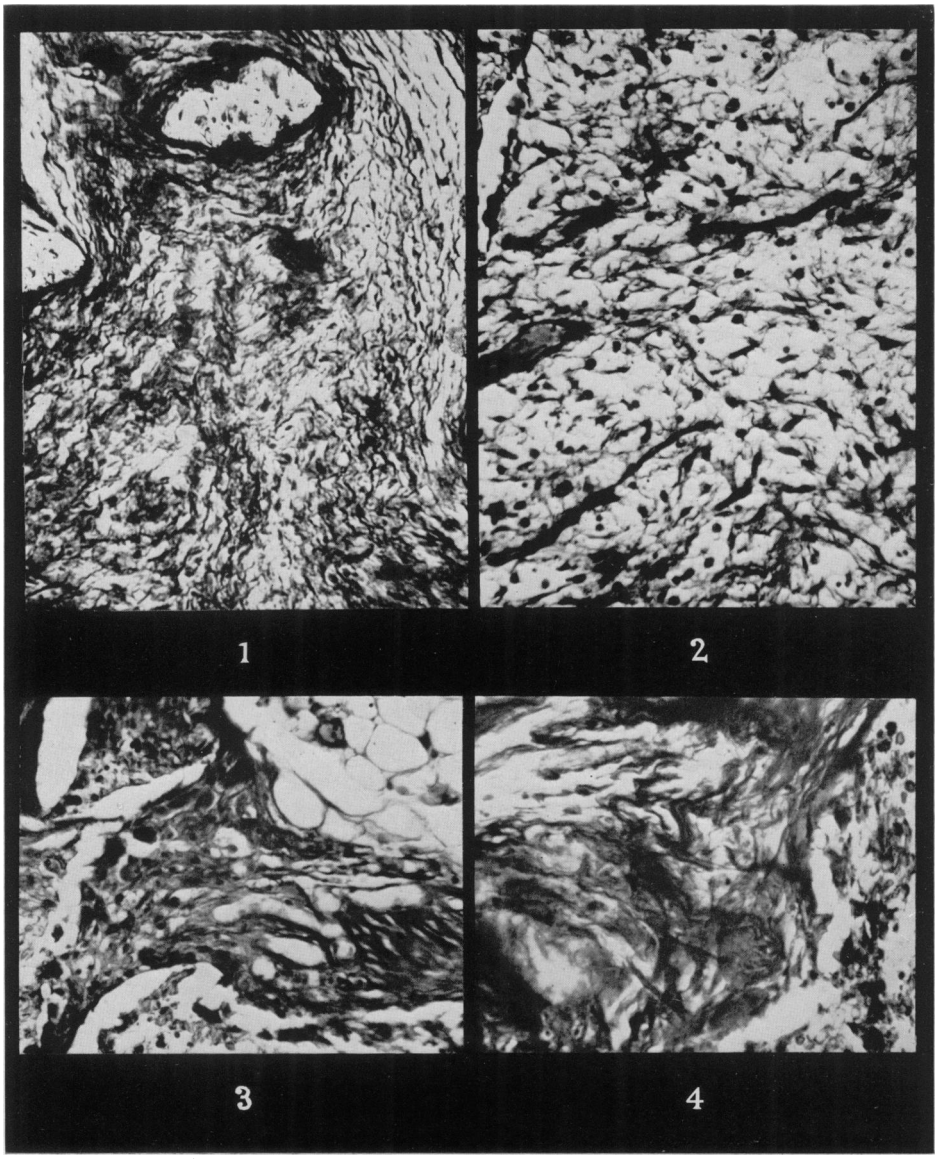
A—At 7 days. Note that well defined masses of similar size are present in both control and treated animals.

B—At 10 days. The granuloma from the control animal is similar to those seen in A, but more clearly defined : that in the treated animal is approximately half the size of the masses present at 7 days.

C—At 15 days. The appearance of the granuloma from the control is unchanged ; that from the treated animal is only a small fraction of the size of the granuloma from the 7-day treated animals seen in A.

FIG. 7.—Photomicrograph of a section of a carrageen granuloma from a control animal at 10 days. Note the large number of macrophages and fibroblasts and the moderate amount of intercellular material containing numerous fine fibrils. Stained with Van Gieson. × 180.

FIG. 8.—Photomicrograph of a section of a carrageen granuloma from an animal receiving AAN at 10 days. Many macrophages and fibroblasts are present embedded in abundant intercellular substance containing fine scattered fibrils. Dilated blood vessels are prominent. Stained with Van Gieson. × 180.



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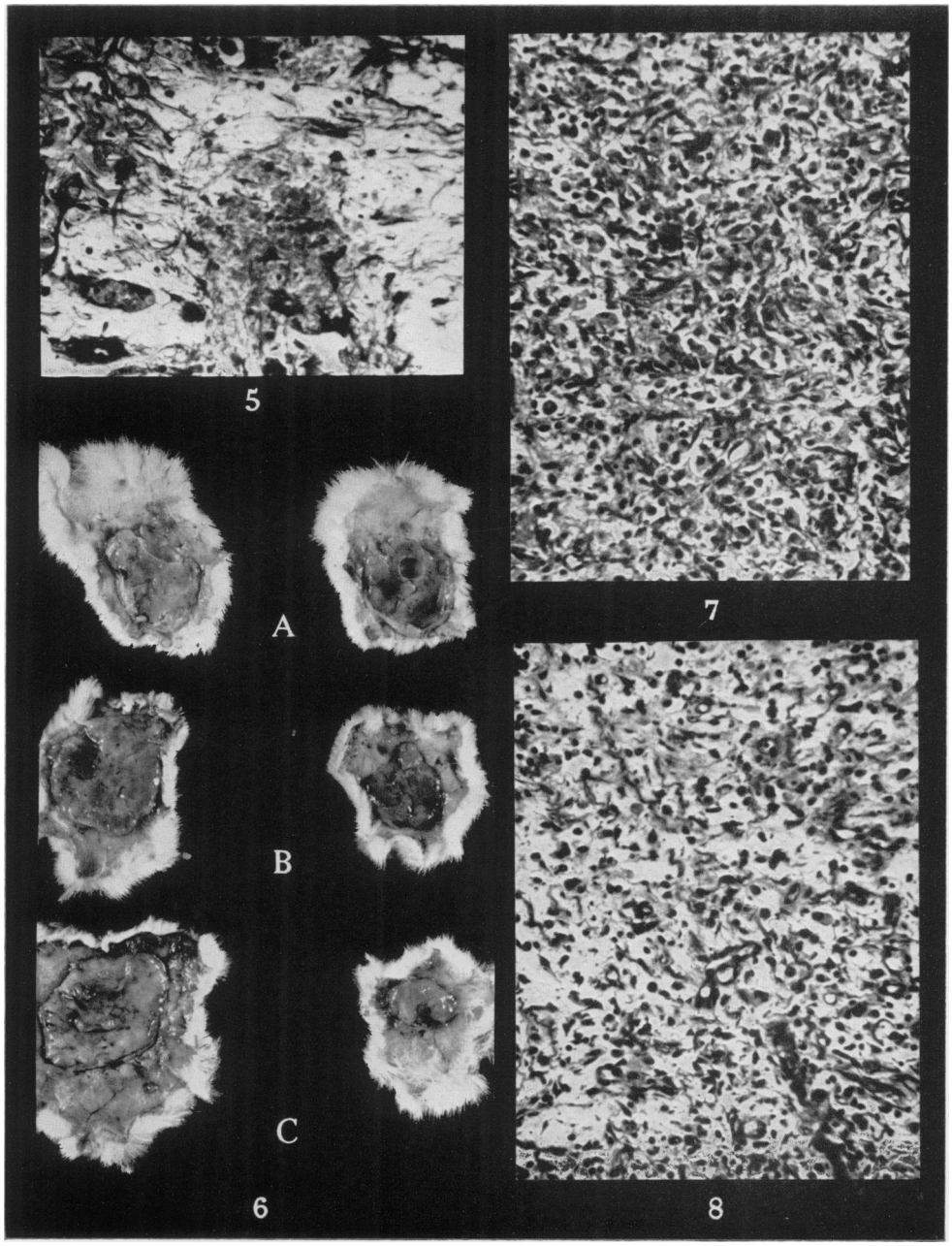

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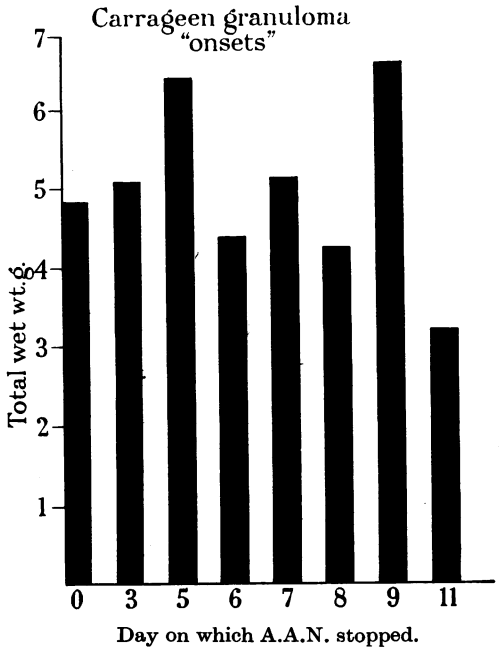

FIG. 9.—Wet weight of carrageen granulomata from animals in which AAN administration was started on the first day and stopped at various intervals. All animals killed on 14th day. Total weights are for groups of 3 animals in each case.

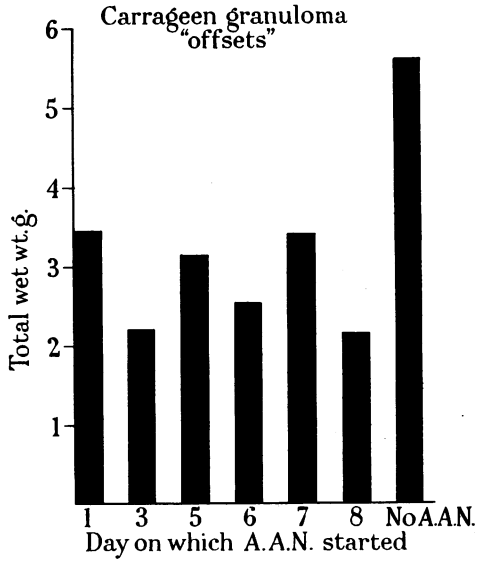

FIG. 10.—Wet weight of carrageen granulomata from animals in which commencement of AAN administration was delayed for various times. All animals killed on 14th day. Total weights are for groups of 3 animals in each case.

Twenty-four adult rats were similarly injected with carrageen and again divided into 8 groups.

Injections of AAN 15 mg./day were begun immediately in one group and at successive intervals thereafter in the other groups as shown in Fig. 10. All animals were sacrificed on the 14th day, the granulomata removed and weighed. All granulomata from animals receiving AAN showed " shrinkage ". The results are shown in Fig. 10.

DISCUSSION

The histological appearances in both polyvinyl sponge and carrageen granuloma preparations are essentially the same as the previously reported effects of aminonitriles on granulation tissue in both young and adult animals (Enzinger and Warner, 1957 ; Mielke *et al.*, 1957 ; Hurley *et al.*, 1958), namely : no change in the primary inflammatory response or in fibroblastic proliferation, an apparent increase in intercellular substance with unaltered staining properties and a delay in collagen formation. The increased vascularity noted in turpentine abscesses in lathyrous animals (Hurley *et al.*, 1958) is equally prominent in both polyvinyl sponge and carrageen preparations. The polyvinyl sponge experiments show that all these changes are reversible on cessation of aminonitrile administration.

If the percentage of hydroxyproline present be used as the index of collagen formation, Tables I and II demonstrate, at most, a slight retardation of collagen synthesis during administration of AAN and BAPN. Mielke *et al.* (1957) found that the collagen concentration in croton oil pouches of BAPN treated animals was slightly, but definitely, lower than that of the corresponding control pouches.

If, however, the total collagen present in the granuloma be used as the index of collagen formation, that is, the quantity of collagen formed in a given time in response to a uniform dose of carrageen, Tables I and II show that this figure increases two- to threefold between the 5th and 15th day in control animals, whilst in the animals receiving aminonitriles the total collagen present remains practically constant over the same period. This constant level must represent the preformed collagen present in the area of granuloma formation which can be clearly seen in the histological preparations, particularly those from the early specimens. These figures indicate that synthesis of new collagen practically ceases in the treated animals, whilst continuing actively in the controls.

If the results of Mielke *et al.* (1957) are rearranged as in Table III to show the total collagen present in the croton oil pouches of control and test animals, it is seen that here also the total collagen content remains constant from the 6th to 18th day in the test animals whilst a steady increase occurs in the controls over the same period.

In both the present carrageen experiments and in those of Mielke *et al.* (1957) occasional granulomata differ widely in total collagen content from the values anticipated by comparison with other specimens of similar duration. This variation is undoubtedly a reflection of the area over which the injection of carrageen or croton oil spread, and despite care to standardize technique as far as possible, represents an unavoidable source of error in the method used.

The experiment in which the beginning of AAN administration was delayed shows that commencement as late as the 9th day fails to inhibit the macroscopic " shrinkage " effect provided the drug be given during the period from the 10th to 15th day. If administration is begun on the first day and stopped at successive

TABLE III.—*Results of Mielke* et al. (1957) *Showing Wet Weights and Hydroxyproline Concentrations in Croton Oil Pouches from Normal and Test Rats fed BAPN, Rearranged to Demonstrate Total Collagen Content*

Day and rat type	Rat number	Wet weight of pouch in mg.	Weight of extracted protein in mg.	Hydroxyproline in extracted protein (per cent)	Total* collagen in extracted protein in mg.	Comment
6 controls	1	1810	138·6	2·70	27·5	
	2	1120	68·9	2·96	15·0	
	3	1130	106·2	2·97	23·2	
12 controls	4	1720	174·8	3·98	51·2	
	5	1700	159·4	3·80	44·7	
	6	1740	149·6	3·53	38·9	
18 controls	8	1410	124·2	4·26	39·0	
	9	1320	119·9	4·78	42·2	
	12	1340	97·1	4·16	29·8	
6 BAPN	13	540	44·6	1·89	6·2	
	14	650	33·9	1·65	4·1	
	15	1000	57·4	1·65	7·0	
12 BAPN	16	1450	122·5	3·66	33·0	Pouch unusually large
	17	670	37·8	2·48	6·9	
	18 & 24	810	62·5	2·94	13·5	
18 BAPN	25	580	42·5	1·88	5·9	
	29	530	26·8	3·52	6·9	
	33	810	35·9	3·52	9·3	

* Total collagen $= \frac{100 \times \text{hydroxyproline per cent} \times \text{weight of extracted protein mg.}}{13 \cdot 6}$

intervals thereafter, the animal can be given AAN for 9 days without subsequent shrinkage occurring provided no further drug is given. Thus the critical period for the drug's action is from the 10th to 15th day, and if AAN is given during this period, the " shrinkage " effect will occur whether or not the animal has previously been receiving the drug.

SUMMARY

Systemic administration of the aminonitriles, AAN and BAPN, to rats during the development of carrageen granulomata is associated with an apparently specific block in collagen maturation between the 10th and 15th day. At this time a sudden and extensive decrease in mass occurs, while control granulomata remain of constant size and histologically and chemically collagen formation continues actively.

The histological changes in granulation tissue in aminonitrile treated animals are similar in both carrageen granulomata and subcutaneously implanted polyvinyl sponges to those previously described in turpentine induced abscesses. In sponge preparations they are reversible on cessation of drug administration.

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