EXPERIMENTAL LATHYRISM

IN VIVO AND IN VITRO

by

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CONTENTS

P R E F A C E

Disease of the aortic media, at one time predominantly
syphilitic, is still not infrequently seen despite the fact
that advanced syphilis is now uncommon, but in the great majority
of cases its aetiology is unknown.   Investigation of the disease
in man is difficult, for although histological methods most
often reveal the picture of Erdheim's Medial Degeneration they
are by their nature unsuitable for demonstrating the disordered
physiology that leads to the condition; in addition neither
experimental reproduction nor observation of the progress of
the disease in life is possible.

The alternative approach seemed to be to find in animals
a model in which to investigate disease of the aortic media.
Several naturally occurring diseases and experimental lesions
have been described, but the condition which seemed closest in
its histology to the human disease was the aortic lesion which
has often been found in young rats suffering from experimental
lathyrism.   It was therefore decided to use this type of
lathyrism as a model, though fully realising that it was probably
not causally or biochemically the same as the human disease.

Mice presented some obvious advantages over rats in the availability of an inbred strain, cheapness and ease of handling, and this made them my first choice of experimental animal, even although there was, at the time the experiments were started (1956) no evidence that mice could in fact develop lathyrism. Preliminary experiments to produce lathyrism in mice were however so successful that mice were used for all animal experiments.

The early experiments with mice and some of the tissue culture experiments have already been published, the latter in collaboration with Dr. John Paul of the Biochemistry Department, Glasgow University. In this collaboration Dr. Paul taught me many of the specialised techniques required and supplied the facilities of his laboratory. The devising of the experiments, the ideas underlying their planning and the actual execution were, however, my own.

My thanks are due to Dr. G. W. Crosbie of the Biochemistry Department, Glasgow University who prepared the β-aminopropionitrile and aminodipropionitrile, to Dr. A. Szewczuk, of the Institute of Immunology, Wroclaw, Poland, who supplied several of the other nitrile, to Dr. I.R.W. Lominski of the Bacteriology Department, Glasgow University for his constant encouragement and helpful criticism and finally to Professor D.F. Cappell who first suggested that I should work on lathyrism and who has allowed me the facilities of his department for my research.

INTRODUCTION

It has been known for over 2000 years that when the seeds of certain leguminous plants constitute a large proportion of the human diet, neurological symptoms develop of which the most constant is a spastic paraplegia, often with an acute onset. Outbreaks of poisoning of this type have been reported from North Africa, India, Spain and parts of Italy, and the peas most commonly implicated are the vetchlings - Lathyrus sativus, L. cicera and L. clymenum. These vetchlings are very hardy and are widely grown for cattle fodder and normally to a lesser extent for human consumption. When however other crops fail, they may become the staple diet and symptoms of poisoning occur. The name lathyrism was introduced to describe this condition by Cantani (Naples) in 1873, and the symptoms in man are fully described by McCombie - Young (1927); it has, however, been suggested that some of the symptoms seen in these cases are the result of avitaminosis B, which is likely to be present in the same patients. An interesting point in reports of the human disease is that males are said to be 10 to 12 times more frequently affected than females.

Susceptibility to lathyrus peas varies widely from species to species. Pigeons, hens and partridges are insusceptible, while ducks, geese and peacocks are highly susceptible. Similarly cattle, pigs and sheep can safely be given 20% of L. sativus in the diet, and, although death from acute poisoning is possible, have on occasion eaten a much higher proportion without ill effects.

Experimentally, Stockman (1931) was able to produce convulsions and paretic symptoms in monkeys given extracts of L. sativus, L. cicera, Ervum lens (lentils), Pisum sativum (edible pea), soya bean, Vicia sativa (tares), and Ervum ervilia (bitter vetch), though whether these symptoms were the same as those which occur in human lathyrism is open to doubt. The majority of laboratory animals are however quite unaffected by any of these legumes.

From 1933 it became clear that certain species of lathyrus peas contained toxic factors capable of affecting systems other than the nervous system. Geiger, Steenbock and Parsons (1933), while attempting to produce nervous symptoms in rats, found that bony lesions and in particular kyphosis accompanied the hind-limb paralysis which developed following feeding with L. odoratus (the garden sweet pea). Lewis et al. (1948) and Lee (1950) reported similar effects with L. odoratus, L. hisutus and L. tingitanus; L. sativus and L. cicera, were

non-toxic in this species.   Lewis believed the kyphosis to be
the primary condition, with resultant cord-compression and para-
plegia; he thought there was no evidence of a direct effect on
the nervous system.   This view has more recently been revised
by Selye (1957) who has termed the primarily nervous type of
disease neurolathyrism, and the type of disease with bony lesions
(and only secondary paraplegia) osteolathyrism.   He and most
other writers feel that these are two quite distinct conditions.
Vivanco and Jiménez Díaz (1951) take the opposing view; they
performed laminectomies on their rats before feeding them with
sweet peas and paraplegia developed in the same way as in
unoperated control rats.   They therefore concluded that there
was, in addition, to the bony lesions, a direct effect on the
nervous system.

        These descriptions of bony lesions in lathyrism led
Ponseti and Baird (1952) to feed weanling rats with a diet
containing 50% sweet pea seeds in the hope of producing a
condition comparable with juvenile kyphoscoliosis.   Unexpectedly
a high proportion of their rats died of aortic rupture, and the
histological picture of the aorta showed a disruption of the
elastic fibres and a marked increase of metachromatic ground
substance.   This report immediately attracted attention because
of the striking similarity to Erdheim's medial degeneration, and
its publication has greatly accelerated the research on lathyrism
over the past ten years.

In 1954 Dupuy and Lee isolated from Lathyrus pusillus seeds a crystalline substance capable of producing the bony lesions of experimental lathyrism, and later in the same year Schilling and Strong (1954) identified the crystalline material isolated from Lathyrus odoratus as β(-L-glutamyl) - aminopropionitrile, and showed that it was capable of producing lathyrism. It was soon clear that the active part of the molecule was β-aminopropionitrile (BAPN) and that both bony and aortic lesions could be produced using this latter compound (Bachhuber et al. 1955, Lalich 1956).

With the aim of determining the structure and chemical grouping necessary for lathyrogenic activity a number of workers investigated a great many chemically related compounds either for their ability to produce bony or aortic lesions in rats, or by a variety of assay methods believed to give parallel results; the latter methods include teratogenesis in toads (Chang et al. 1955) and loss of tensile strength and increased solubility of collagen in chicks (Gross, Levene and Orloff, 1960). The different methods used did not in fact always give identical results, and it seems wisest to consider as lathyrogenic only those which give either bone or aortic symptoms of the type seen with BAPN. These are:- aminoacetonitrile (AAN) (Wawzonek et al. 1955), aminodipropionitrile (Bachhuber et al. 1955), β mercapto-ethylamine (Dasler 1955), cystamine (Dasler and Milliser 1958) and methylene aminoacetonitrile (Ponseti et al.

1956).   Aminodipropionitrile was found to produce, in addition, severe nervous symptoms but Selye (1957) failed to confirm Bachhuber et al's findings that symptoms of osteolathyrism were present at all, and suggested that the latter might be due to impurities in their material.

The following are among those compounds which were non-lathyrogenic:-

Cyanamide	(Ramamurti and Taylor 1959)
γ aminobutyronitrile	(Ramamurti and Taylor 1959)
α aminopropionitrile	(Wawzonek et al. 1955)
Propionitrile	(Lalich 1958)
Ethylamine	(Lalich 1958)

The formulae of these active and inactive compounds are shown in fig. 1.   From this it appears that the active compounds have in common a basic structure of a short straight hydrocarbon chain (or two short chains joined) with no more than 2 CH_2 groups in the chain.   One end of the chain must be an amino group, and the other end may be either a nitrile group or a sulphydryl or $S = S$ grouping.

While all the above substances are chemically related in 1958 Dasler found that semicarbazide, a substance of quite different chemical structure, produced in rats both skeletal and aortic lesions indistinguishable from those of osteolathyrism; subsequently Dasler and Stoner (1959) and Milliser and Dasler (1959) have shown that a number of compounds related to semi-
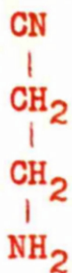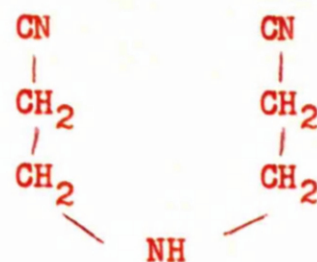
$$CN - CH_2 - CH_2 - NH_2$$

β-aminopropionitrile

$$CN - CH_2 - NH_2$$

Aminoacetonitrile

$$CN - CH_2 - CH_2 - NH - CH_2 - CH_2 - CN$$

Aminodipropionitrile

$$SH - CH_2 - CH_2 - NH_2$$

β mercapto ethylamine

$$S = S$$ with $CH_2 - CH_2 - NH_2$ on each sulfur

Cystamine

$$CN - NH_2$$

Cyanamide

$$CN - CH_2 - CH_2 - CH_2 - NH_2$$

γ aminobutyronitrile

$$CN - CH - NH_2,\ CH_3$$

∝ aminopropionitrile

$$CH_3 - CH_2 - NH_2$$

Ethylamine

$$CN - CH_2 - CH_3$$
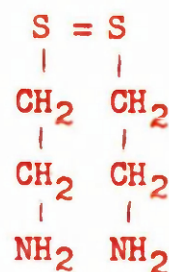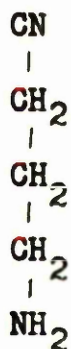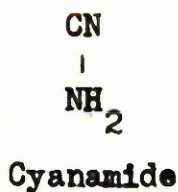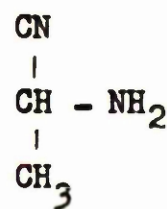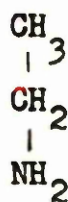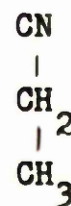
Propionitrile

Lathyrogenic Substances are shown in red.

Inactive Substances are in black.

Fig. I

carbazide have a similar effect. These are acetone semi-
carbazide, parahydrozinobenzoic acid, 4, 4 diphenyl semicarbazide,
1, 5 diphenyl carbazide, 1, 3 diethylthiourea, and thiosemi-
carbazide. Semicarbazide and its related compounds are highly
toxic to the nervous system and rapidly lethal when given
parenterally and can be tested only when given by mouth. It
seems clear however that though both semicarbazide and amino-
dipropionitrile produce nervous symptoms, these are either
convulsions or physical disorientation and are of quite a
different nature from the paraplegia of human lathyrism.

Lathyrus odoratus and the various lathyrogenic chemicals
have now been shown to produce symptoms of lathyrism not only in
rats but in a variety of animal and avian species; mice
(Dasler and Milliser 1957, McCallum 1958), rabbits (Castellani
and Castellani-Bisi 1958), chicks (Neuman 1956), turkeys
(Barnett et al. 1957) and toads (Chang et al. 1955) have all
shown changes in bones or aorta or both. The rat has however
been by far the most thoroughly investigated, and the morphology
of the lesions in this species has been described in detail many
times, notably by Ponseti and Shepard (1954) and Menzies and
Mills (1957). While the descriptions in general agree, there
is disagreement as to the pathogenesis of the condition.
Ponseti and Shepard considered that all the lesions were due to
defective formation or excessive destruction of the chondroitin
sulphate of ground substance. Menzies and Mills more

cautiously concluded that there was "an important connection between the lathyrus factor and sulphated mucopolysaccharides". They also observed changes in the elastic laminae of the aorta, and thought that the elastin broke down into amorphous granules, and reconstituted itself along the lines of stress. Walker (1957) considered that there was a decrease in the amount of elastic tissue throughout the body and thought that the aortic lesions could be explained by this. Van den Hooff, Levene and Gross (1959) who used chick embryos, thought that the lesion was due to an abnormality in the collagen. Another view was suggested by the similarities between lathyrism and Marfan's syndrome (Bean and Ponseti 1955, Annotation, The Lancet 1955) for this hereditory condition in man is thought to be the result of a defect involving all mesenchymal tissues (McKusick 1955). Many writers believed that lathyrism was or could be a degeneration, but Gillman and Hathorn (1958) insisted that it was a disturbance of the moulding of both bone and aorta which occurs in normal growth.

Biochemical analyses of epiphyseal cartilage of lathyritic animals by Castellani and Castellani-Bisi (1958) and Follis and Tousimie (1958) showed a marked fall in hexosamine indicating a fall in mucopolysaccharide. This was later shown by Castellani, Castellani-Bisi and Frigerio (1959) to be mainly a fall in galactosamine, and therefore the mucopolysaccharide

involved is presumably chondroitin sulphate. There was no fall in hydroxyproline and therefore no decrease in collagen, but Follis and Tousimis were able to show a marked decrease in the number of collagen fibrils and concluded that the defect appeared to be a failure of the tropocollagen molecule to form collagen fibres. To investigate further the fall in the hexosamine level, Pedrini and Pedrini-Mille (1959) studied the enzymatic synthesis of hexosamine in normal, and lathyritic epiphyseal plates. They found there was a marked fall in the amount of hexosamine synthesised in a standard time. The vessels of lathyritic animals have not, so far, been analysed biochemically.

Studies of epiphyseal cartilage using radioactive isotopes (Kennedy and Kennedy 1962) confirm the biochemical findings in that they show a decrease in sulphated mucopolysaccharide but no alteration in protein metabolism. This is interpreted by the authors as signifying a failure of chondroitin sulphates A and C to combine with non-collagenous protein and a consequent failure by fibrillogenesis. Similar studies on lathyritic aortas showed no variation from normal (Ponseti et al. 1956b, Kennedy and Kennedy 1962).

Experimental osteo-lathyrism is thus seen to be a condition which can be produced in a variety of mammalian and avian species, by any one of a number of different chemical compounds; its symptomatology is diverse, but appears to depend on some abnormality of connective tissue, though whether of all

connective tissues, or of one specific component of connective tissue is not clear; neither is it certain whether it is a degeneration or a defective formation. The present study was confined almost entirely to the aortic lesions and was designed to demonstrate the nature of the fault in the vessel wall and to reproduce this _in vitro_.

The first stage in investigating this condition was to produce aortic lesions in a suitable laboratory animal. Most other workers had used rats, and when these experiments were started the available information seemed to suggest that mice were insusceptible (Lewis and Schulert 1949, Delay _et al_. 1952), however the advantages of cheapness, ease of handling and the possession of a highly inbred strain suggested that the mouse, if susceptible, would be a more suitable animal, and investigations were begun in this animal; when I had demonstrated the inherent susceptibility of mice they were used exclusively. The results are contained in the section on Animal Experiments (pages 15 - 59)

These experiments led to the conclusion that the basic lesion is not damage to, but a failure of formation of elastica. Since elastica is believed to be formed by the action of cells - probably fibroblasts (Lansing 1959) - an attempt was made to study in tissue culture the action of lathyrogenic substances on the cells producing elastica. Accordingly an attempt was made to obtain elastic fibres in tissue cultures, and to test the

effect of the addition of lathyrogenic substances to the culture medium.   The section on Tissue Culture Experiments (pages 60 to 102) describes the successful production of elastic fibres in cultures of embryo mouse and chick tissue, and also that the formation of elastic fibres is inhibited by known lathyrogenic substances, but not by related non-lathyrogenic compounds.

ANIMAL EXPERIMENTS

Materials and Methods

The mice used were Porton pure bred mice originally obtained from Porton and kept in boxes of 6 to 12 mice. Pregnant mice or mice with young litters were kept in boxes individually. Mice referred to as weanling mice were aged 21 to 23 days, and weighed between 17 and 23 gms. Pregnant mice weighed 25 to 30 gms. at the time of mating. Water and food were supplied ad lib; the stock diet was diet 86 in the earlier experiments, and thereafter diet 41. (UFAW 1957)

In some experiments the mice were fed with special diets, in others lathyrogenic substances were given by injection. The special diets were made up as follows:- Stock diet (86 or 41) and sweet pea seeds were ground separately in a coffee mill. To make the sweet pea diets 20 - 50% w/v of sweet pea meal was mixed with the ground stock diet and bound with water into a mash. Where chemical compounds were given by mouth these diets were made up by adding a known weight (or in the case of liquids, volume) of the chemical to tap water to form a solution or suspension. A weighed amount of ground cake was then added to form a mash as before. The diets were renewed daily.

Chemicals used in injections were dissolved in sterile distilled water and given subcutaneously.   Before giving the injection, its pH was, if necessary, adjusted with HCl or NaOH to approximately 7.2 - 7.6 as judged by B.D.H. narrow range indicator papers.

All mice which were killed, and the majority of those which died during the course of the experiments were dissected post-mortem, but in a few cases this was not possible when mice which died were eaten or partially eaten before being discovered. Whenever possible, however, autopsy was performed and histological sections cut of the aorta at a number of levels.   Transverse sections seemed easiest to interpret and were used in most cases. In addition to vascular preparations, the knee joint of a proportion of mice was examined after decalcification.   In the case of infant mice transverse and occasionally sagittal sections were cut through the whole mouse.   Fixation was by formalin in all cases, but the infant mice were post-fixed in Heidenhain's SUSA fixative.   All the preparations were paraffin embedded, sections were cut at 5 to 7 μ. and the stains used were haemalum and eosin, Weigert's elastica counterstained neutral red, Verhoeff's elastica counterstained van Gieson, Orcein counter-stained haemalum, and in a few cases the silver impregnation methods for reticulin (Gordon and Sweet, 1936 and Slidders, Fraser and Lendrum 1958), phosphotungstic acid haematoxylin (PTAH) (Lieb's 1948 and Mallory's 1960), picro-Mallory (Lendrum, 1949), periodic acid Schiff (P.A.S.) (Pearse, 1949), and

toluidine blue.

The presence of lesions of lathyrism and their severity were judged by the gross and histological appearances of the aorta and bones.   In the aorta the lesions were graded according to severity into:-

Grade I - in which abnormalities of elastic laminae are present, but there is no apparent rupture or dilatation of the wall.

Grade II - saccular or fusiform aneurysm or healed dissecting aneurysm.

Grade III - rupture or acute dissecting aneurysm of the aorta.

The aortic lesions are described fully on page 35.

The bone lesions were assessed merely as present or absent, and were of one of two types.

(1) In adults a lesion of the cartilage plates of the knee joint with irregularity of the cartilage columns, tears in the cartilage, and the presence of linear streaks of PAS positive material (fig. 3).   This appearance is identical with that seen in the knee joint of mildly lathyritic rats (fig. 4).

(2) In neonatal mice one or more typical sharp kyphoses in the upper thoracic spine (fig. 5).
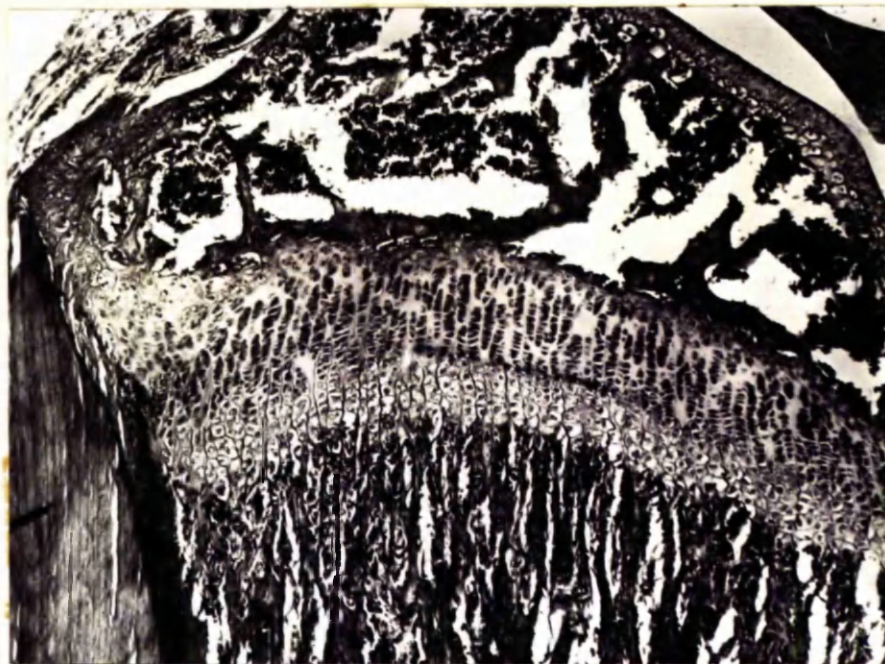
Fig. 2 Epiphyseal plate from upper end of tibia of a normal
young adult mouse to show regular columns of cartilage
cells. (X 60 H. & E.)

Fig. 3 Epiphyseal plate from upper end of tibia of female
mouse which died of aortic rupture aged 40 days, after
18 days of 10% BAPN.HCl diet, to show disorganisation
of cartilage columns. (X 60 H. & E.)
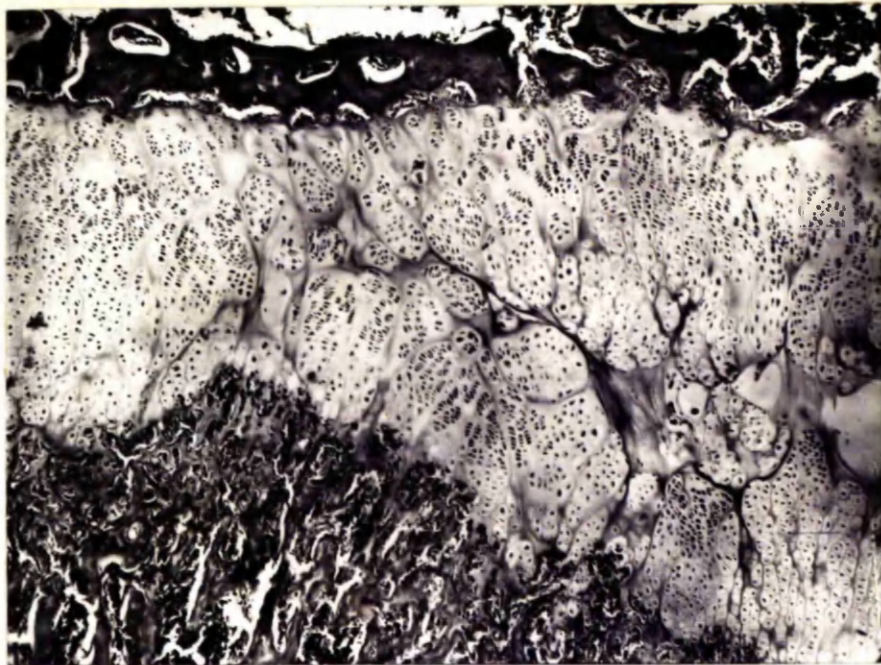
Fig. 4   Epiphyseal plate from upper end of tibia of a male rat
          killed aged 12 weeks after 9 weeks on 20% sweet pea
          diet.   To show disorganisation of the cartilage
          similar to that in fig. 3.   (X 40 H. & E.)
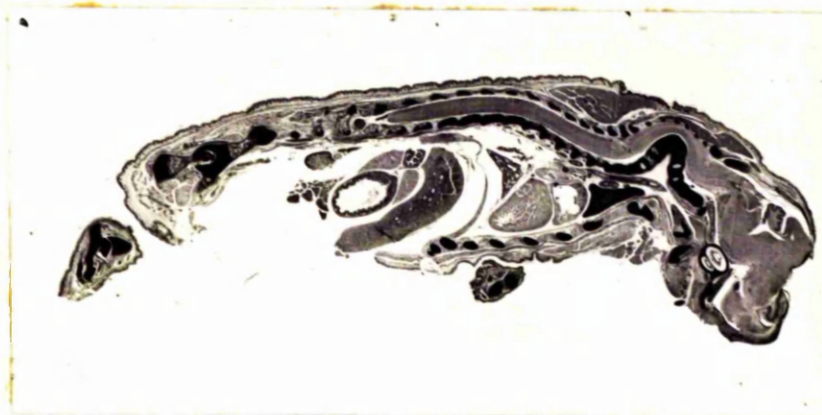
Fig. 5   Sagital section through newborn mouse.   The mother
          received 0.3 ml. of a 15% solution of BAPN.HCl
          subcutaneously 4 times in the 6 days preceding delivery.
          The typical upper thoracic kyphosis is clearly seen.
          (X 3½ Weigert's elastica counterstained neutral red.)

Experiments to show the effect of a known Lathyrogenic Agent in Mice

The first group of experiments was designed to investigate whether lathyrogenic agents can produce disease in the mouse, and if so whether it differs significantly from the picture seen in the rat in which the two main features are bony lesions and aortic dissection or rupture.

Experiment I   In order to determine whether lathyrism could be produced in mice, 31 weanling mice (20 male and 11 female) were fed on the 50% sweet pea diet.   Three male mice died, and 10 (7 male and 3 female) were killed during the course of the experiment.   Of the three which died, two, dying after $4\frac{1}{2}$ and 8 weeks respectively, had ruptured aortas.   In one mouse which died after $6\frac{1}{2}$ weeks, no obvious cause of death was found. The diet was discontinued after 18 weeks, and the remaining mice were killed at intervals from 3 to 12 weeks thereafter.

The histological appearances of the aorta were graded as follows:-

TABLE I

	Normal	Grade I	Grade II	Grade III	Total
Male	10	6	2	2	20
Female	8	3	0	0	11
Total	18	9	2	2	31

From these results it appears that with sweet pea feeding aortic lesions similar to those known to occur in the rat can be produced in the mouse.

Experiment II   To show whether, in mice, DAPN has a similar effect to that of sweet peas, 66 weanling mice (41 male and 25 female) were treated with DAPN.HCl.   In 37 (21 male and 16 female) it was given by daily injection of 0.1 ml. of a 1 in 10 w/v solution.   In the remaining 29 mice the DAPN.HCl was added to the diet in concentrations of from 0.75% to 1.0%.   Thirty-one mice (24 male and 7 female) died during the course of the experiment.   Of these 22 (18 male and 4 female) had aortic rupture.   In the other 9 no cause of death was found.   Six male and 5 female mice were eaten and unfit for examination. The state of the aorta in the 55 animals examined is shown in table II.

<div align="center">TABLE II</div>

	Normal	Grade I	Grade II	Grade III	Total
Male	2	14	1	18	35
Female	5	11	0	4	20
Total	7	25	1	22	55

These results show that aortic lesions of lathyrism can be produced with DAPN, and that with the doses used the lesions are both more frequent and more severe than with sweet pea diet.

Experiment III   The effective dose range which will produce symptoms of lathyrism was then investigated.   Nineteen weanling mice were given either 50% sweet pea diet plus an injection of BAPN.HCl, or a 1% BAPN.HCl diet.   Twelve of these (7 male and 5 female) died within 39 days and were found to have aortic rupture.   The remaining 7 were killed after 20 weeks.   In this group typical bony lesions of lathyrism (fig.3) were found in the knee joints of 2 mice (1 male and 1 female).   In addition 5 mice (all male) had very mild bony lesions.   The histological grading of the aortic appearances is summarised in table III.

<p align="center">TABLE III</p>

	Normal	Grade I	Grade II	Grade III	Total
Male	-	2	1	7	10
Female	1	2	1	5	9
Total	1	4	2	12	19

It appears from these results that with a higher dose of lathyrogenic agent there is a higher proportion of mice with damaged aortas.   There is also a higher proportion of mice with the more serious grades of damage.

Experiment IV   Twenty-seven weanling mice were fed on 40% or 20% sweet pea diet to discover the minimal effective dose.

With smaller doses fewer mice developed aortic lesions, and still fewer the severe grade II or III lesions, but even with only 20% diet one male mouse died of aortic rupture.

It is clear from the results of experiments I and II that when a moderate dose of a lathyrogenic substance is given to weanling mice, males are more frequently and more seriously affected than females - 18 out of 61 males developed rupture and only 4 out of 31 females. This sex difference is not apparent with the high doses used in experiment III. It was thought that the difference might be the result of the males eating more than the females, so that when given a lathyrism producing diet the male would absorb more of the BAPN. The following experiment was designed to test this theory.

Experiment V   Fifteen male and 5 female weanling mice were fed with a diet containing 0.75% BAPN.HCl. The mice were divided into 4 groups of 5 mice, each group being housed in a single box. Box 1 contained 5 female mice fed ad lib. Box 2 contained 5 male mice whose diet was restricted to equal that eaten by the mice in box 1. Box 3 contained 5 male mice fed ad lib. Box 4 contained 5 male mice with diet restricted as in box 2, but supplimented by ordinary stock diet ad lib. so that the intake of BAPN.HCl equals that of boxes 1 and 2, but the caloric intake was higher and presumed equal to box 3. (This was confirmed by the fact that the weights in boxes 3 and 4 increased equally and more rapidly than those in box 2.)

The results of this experiment are shown in table IV.

<u>TABLE IV</u>

	Deaths	Grade III	Alive at end of experiment
Box 1 5 F. diet <u>ad lib.</u>	3	0	2
Box 2 5 M. pair fed with box 1	5	1	0
Box 3 5 M. diet <u>ad lib.</u>	5	4	0
Box 4 5 M. pair fed with box 1 + stock diet <u>ad lib.</u>	5	4	0

It is seen that the greatest proportion of aortic ruptures occurred in the two boxes where the animals were given the higher caloric diet, and where in consequence the weight gain was greater. In the conditions of this experiment the more rapid rate of growth appears to be of more consequence in producing aortic rupture than does the difference in dosage. In 9 mice no cause of death was found; this may indicate that BAPN. has a general toxic effect in addition to its specific action on aorta and bones.

It is known that in rats it is difficult to produce the aortic lesions of lathyrism in animals over 2 months of age (Ponseti and Shepard, 1954, Gillman and Hathorn, 1959) no matter how high a dose of lathyrogenic substance is given. Certain experiments were therefore designed to discover the age limits

within which aortic lathyrism could be produced in the mouse.

Experiment VI   In an attempt to discover if there was, in
mice, an upper age limit for the production of lathyrism, 15
male mice aged 8 months and weighing 38 - 58 gms. were fed on
the 50% sweet pea diet for 10 weeks.   Two mice died of inter-
current disease at the end of the 10 weeks, the remainder were
killed 5½ months after cessation of diet.   None developed
grade III lesions, a single mouse had a grade II lesion and 12
had grade I lesions.   This last figure is probably not
significant as grade I lesions are common in mice over 10 months
of age.   (See section on histology of the normal mouse aorta
page 36.)   It is apparent that in mice, as in rats, it is
much more difficult to produce aortic lesions in older animals.

Experiment VII   To find the lower age limit it was decided to
test the effect of lathyrogenic substances on foetal mice by
feeding or injecting the mothers during pregnancy.   Twenty-
two female mice were treated for from 2 to 21 days during
pregnancy, the age of the pregnancy at the time of treatment was
estimated in retrospect from the date of delivery or in some
cases the period of mating was limited to 24 hours.   Of these
22, 8 were given the sweet pea diet (4 were given 40% and 4 50%)
and 14 were treated with BAPN or BAPN.HCl (by daily injection of
from 0.15 ml. to 0.2 ml. of 1 in 10 BAPN, or from 0.2 ml. to
0.4 ml. of 1 in 10 BAPN.HCl).   All mice were allowed to
continue till spontaneous delivery or abortion, and the young

were then examined. Infant mice which were still-born or died within 48 hours were examined histologically. In addition all bruised mice and a proportion of apparently normal mice were killed during the first 48 hours and examined histologically.

The results obtained were similar whether the mothers were given the sweet pea diet or injection of BAPN or BAPN.HCl. A definite variation was evident depending on the stage of development of the foetuses at the time they were treated — thus treatment confined to the first 11 days of pregnancy was ineffective, while treatment during the last 2 - 8 days of pregnancy was in itself sufficient to produce typical vascular lesions. The results of this experiment are shown in Table V.

Table V showing the effect on infant mice of treatment of the mother during all or part of pregnancy.

TABLE V

Days of gestation on which treatment was given.	5th-11th	11th-18th	During 3rd week only	1st-21st
No. of mothers	1	3	14	6
No. of young	8	16	97	24 (3 litters eaten)
No. of young ruptured	Nil	1	16	5
No. of young with aortic damage histologically	Nil (2 examined)	3 (3 examined)	10 (10 examined)	8 (8 examined)

The dosage used also influenced the frequency and type of lesion produced.   Thus when a high dose, i.e. 40 - 50% sweet pea diet for the last 6 days or more of pregnancy, or 4 or more daily injections of 0.4 ml. of 1 in 10 BAPN.HCl in the last days of pregnancy, was given to 8 mice, 4 aborted.   The remaining 4 produced full time litters containing a total of 38 young of which 26 were born dead, and died immediately after birth. Although abnormal aortic laminae were always present, in only 12 of the infant mice was there any aortic rupture, and whenever longitudinal sections were cut through the mouse there was found to be one or more sharp, upper thoracic kyphoses (fig. 5). Other findings were small capillary haemorrhages and generalised oedema.

A moderate total dose - e.g. 30% sweet pea diet or only 3 injections of 0.4 ml. 1. in 10 BAPN.HCl in the last 3 - 6 days of pregnancy - was given to 11 pregnant mice.   None aborted and the 11 litters contained 68 mice.   Of these 8 only were still-born, 12 died during the first 48 hours of life, and 7 of these had aortic rupture.   No kyphosis was present.

Low dosage - e.g. 0.2 ml. 1 in 10 BAPN.HCl daily for the last 3 days or 20% sweet pea diet for less than 4 days - was given to 3 mice.   The three litters born contained 29 young, all of which looked normal, but in those examined histologically, mild abnormalities were present in the aorta.   There was never any aortic rupture.   The effects of these different doses are

seen in Table VI.

Table VI.   Effect of dosage on infant mice both of
mothers treated during later part of pregnancy.

TABLE VI

Dosage	High	Medium	Low
No. of mothers	8	11	3
No. of mothers aborting	4	Nil	Nil
No. of full-time young	38	68	29
No. of stillbirths	26	8	Nil
Kyphosis	present	absent	absent
No. of young with aortic rupture	12	7	Nil

Experiments in Mice to test New Compounds

for Lathyrogenic Activity

The fact that it is possible to produce severe lesions of lathyrism in as little as three days in mice during the last days of pregnancy suggested that this might be a convenient way of testing compounds for their ability to produce lathyrism.

The method used was essentially the same as that described in Experiment VII. Female mice were given the substance to be tested during the last week of pregnancy, and the young were examined the same day if born during the day, or the next morning if born at night. The mode of administration was usually by subcutaneous injection of an aqueous solution. α glutamyloaminobutyronitrile and α glutamyloaminopropionitrile were both poorly soluble and the dose mentioned was injected as a suspension. Semicarbazide and its related compounds and γ aminobutyronitrile, which all proved to be rapidly lethal to the mother when given parenterally, were given mixed with the food as described on page 15.

For each substance tested a number of pregnant mice was used. The first one or two were given a dose which was approximately the mole-equivalent of that found effective with BAPN. Where no evidence of lathyrism was obtained the dose was increased to the maximum that could be tolerated by the mother before it was reported as negative in its lathyrogenic effects. Where the initial dose produced absorption of the

foetus, abortion or other non-specific effects, the dose to
subsequent test animals was lowered until the typical bony or
aortic lesions of lathyrism were obtained.

Table VII shows the chemicals tested with their
formulae, the range of dose used, and the result in terms of
aortic rupture and of spinal kyphosis.

It can be seen that DAPN.HCl, AAN.SO$_4$ and semi-
carbazide HCl produce both aortic rupture and kyphosis; in
higher dosage (>40 mg.) AAN.SO$_4$ caused absorbtion of the embryos.
Glutamylo-AAN and aminodipropionitrile produce aortic rupture
without kyphosis, but with both only a small dose could be given.
In the case of glutamylo-AAN only a limited amount of material
was available. In the case of aminodipropionitrile the toxicity
to the mother limited the dose. β-mercaptoethylamino and
γ-aminobutyronitrile were also given in low dosage because of
toxicity to the mother. In addition β-mercaptoethylamine
decomposed readily and this may account for the fact that although
aortic rupture was produced in one test, this result could not
be reproduced. Other substances produced neither vascular nor
skeletal changes at doses which were compatible with the life of
the mother.

TABLE VII

Name of chemical	Formula	Preparation used	Mol. Wt. of preparation used	Range of dose tested, stated as total dose to mother in last week.	Result	
					Dose to produce aortic rupture	Dose to produce kyphosis
Propylamine	CH_3 CH_2 CH_2-NH_2	-	59	0.1 - 0.15 ml sub-cut	negative	negative
γ-amino-butyronitrile	NH_2 CH_2 CH_2 CH_2 CN	sulphate	180	150 - 250 mg orally	negative	negative
Amino-acetamide	CH_2-NH_2 $CO NH_2$	hydro-chloride	110.5	240 - 320 mg sub-cut	negative	negative

TABLE VII (continued)

Name of chemical	Formula	Preparation used	Mol. Wt. of preparation used	Range of dose tested, stated as total dose to mother in last week	Result	
					Dose to produce aortic rupture kyphosis	Dose to produce kyphosis
α(glutamylo) amino-butyronitrile	glutamylo CH_2 CH_3 CH_2-NH_2 CN	-	213	350 - 540 mg sub-cut	negative	negative
α(glutamylo) amino-propionitrile	glutamylo CH_2 $CH-NH_2$ CN	-	199	320 - 420 mg sub-cut	negative	negative
Aminodipropio-nitrile	CH_2-CH_2-CN NH CH_2-CH_2-CN	-	123	0.12-0.2 ml sub-cut	0.12 ml	negative
β aminopropio-nitrile (BAPN)	NH_2 CH_2 CH_2 CN	BAPN.HCl	106.5	40 - 280 mg sub-cut	120 mg	280 mg

TABLE VII (continued)

Name of chemical	Formula	Preparation used	Mol. wt. of preparation used	Range of dose tested, stated as total dose to mother in last week	Result	
					Dose to produce aortic rupture kyphosis	Dose to produce kyphosis
Amino-acetonitrile (AAN)	NH_2 CH_2 CN	AAN. H_2SO_4	154	10 – 80 mg sub-cut	15 mg	30 mg
Glutamylo-aminoaceto-nitrile	glutamylo NH_2 CH_2 CN	–	185	80 – 90 mg sub-cut	80 mg	negative
β mercapto-ethylamine	NH_2 CH_2 CH_2 SH	β mercapto-ethylamine HCl	113.5	20 – 60 mg sub-cut	30 mg +	negative
Semicarbazide	NH_2 NH $CO-NH_2$	semicarbazide HCl	115.5	150 – 180 mg orally	180 mg orally	150 mg orally

+ In one test only.

TABLE VII (continued)

Name of chemical	Formula	Preparation used	Mol. wt. of preparation used	Range of dose tested, stated as total dose to mother in last week	Result	
					Dose to produce aortic rupture	Dose to produce kyphosis
Ethanolamine	NH_2 CH_2 $CH_2 \cdot OH$	-	61	60 - 90 mg sub-cut 200 mg orally	negative	negative
Sodium propionate	CH_3 CH_2 $COONa$	-	96	150 - 400 mg sub-cut	negative	negative

The Morphology of the Vascular Lesions of Lathyrism

Gross Appearances

The vascular lesions produced in lathyrism in the mouse have been described as of three grades (page 17) - aortic rupture (fig. 21), aortic aneurysm (fig. 19), and lesions recognisable only on histological examination (figs. 14 and 15). It was noted that aortic aneurysms were confined to the ascending aorta and arch of the aorta and were never seen in the descending aorta.   Aortic rupture might occur in any part of the aorta, but was also commonest in the ascending aorta and around the arch, the majority of mice being found at post mortem to have one or both pleural cavities filled with blood.   This preponderance of thoracic lesions was not so marked in the neonatal mice where the numbers with lesions at the arch and in the abdominal region were more evenly divided.

In a very few animals, a lesion was seen in at least one artery other than the aorta.   The vessel involved was either innominate, left subclavian or carotid, superior mesenteric or a renal artery, and the lesion was always an extension from the aorta itself.   No smaller arteries or veins were ever involved, though capillary haemorrhage was not uncommon.

Before describing the histological appearance of the vessels in lathyrism the histology of the normal mouse aorta will be considered.

Histological Appearances of the Normal Mouse Aorta

The intima consists of a single layer of flattened endothelium lying directly on the innermost elastic lamina. A fibro-muscular sub-endothelial layer such as is seen in man, never develops in a normal mouse. The media has essentially the same structure as in man, with alternating elastic laminae and layers of smooth muscle, the cells of which run from an origin in one lamina diagonally round the aorta to an attachment in the next. The elastic laminae can first be demonstrated histologically at the 14th day of intra-uterine life, when they appear as a network of interlacing elastic fibres which lie in the planes between the muscle layers (fig. 8). These sheets of fibres become progressively denser with increasing age and the fibres appear to fuse to form sheets of elastic tissue with occasional spaces - the fenestrated membranes.

In mice of increasing ages, histological appearances in transverse sections of aortas stained by Weigert's elastic tissue stain, reflect the development of the structure of the elastic laminae. In the foetus the laminae are seen to be thin, often with a beaded appearance, and thrown into many small irregular folds (fig. 9). By the time the mouse is born, however, the elastica is thicker, more even in thickness and the pull of the contracted muscle cells throws it into regular and rather fewer folds (fig. 10). In the young adult mouse the elastica is still even, and regular folds are seen on cross-

section. In addition fine Weigert positive fibres can now be seen extending from the main laminae at an angle of about 30° (fig. 12). These fibres seem to end abruptly among the muscle cells. In a few cases small lakes of Weigert positive material are present in association with these fibres. In the older mouse (over 10 months) irregularities are again seen; these take the form of occasional interruptions in the elastic laminae (fig. 13) with, in some cases, an apparent attempt at repair in the form of many fine Weigert positive fibres bridging the gap from one broken end to the other.

Although the diameter and thickness of the elastic laminae increase with age the number of laminae seems to be constant. In the ascending aorta there are from 6 to 7 laminae, while in the abdominal aorta the number is from 4 to 5 regardless of age.

A fibro-elastic adventitial coat is present at all ages, although becoming thicker with increasing age.

Histology of the Mouse Aorta in Lathyrism

The changes in the mouse aorta in lathyrism are confined at first to the media; the intima and adventitia are only secondarily involved. In young adult mice given lathyrogenic substances the changes have for convenience been divided according to severity into 3 grades. In the mildest form (Grade I) there is no naked eye alternation in the aortic wall, but there is recognisable histological change characterised by

interruptions of individual elastic laminae (fig. 14), an increase in the amount of metachromatic ground substance, and often an increase in the size and number of the lakes of Weigert positive material (fig. 15) which can often be seen in small amounts in normal vessels (page 37). Some laminae, while not necessarily interrupted, are thicker and stain more deeply with Weigert's stain and with orcein than do their neighbours, and these abnormal laminae also fail to take a purple colour with Mallory's PTAH stain, but are stained orange as are the lakes of Weigert positive material which lie between the laminae (fig. 17). (It was found that only certain batches of Mallory's PTAH stain gave a purple colour to normal elastic laminae and strict use of control sections is necessary before interpreting this stain.)

The interruptions in the laminae may be of one of two types. In some cases the lamina appears to have ruptured and the two ends to have retracted, for a short distance (fig. 14). In others a length of elastic lamina is seen to be irregular in thickness and a piece may be missing entirely (fig. 18). This is especially common in the innermost elastic layer and may involve a large part of the circumference. Where this happens there is proliferation of endothelial cells and fibroblasts and new "elastic" fibres are laid down. These take the form of a rather thin and irregular elastic lamina similar to that seen in foetal mice, and many irregular fine elastic fibres lying

parallel to the surface. All this new elastic tissue stains orange and not purple with Mallory's PTAH. The formation occurs even though treatment with lathyrogenic agents is continued up to the time of death.

A lesion is graded as **II** when the damage is so severe as to result in the formation of an aneurysm. In the mouse this is almost always a saccular aneurysm (fig. 7) and is most commonly seen around the arch of the aorta. These aneurysms are interpreted as being the result of a healed incomplete rupture, at least in the majority of cases. In a section through a typical aneurysm (fig. 19) there can be seen to be loss of continuity of the elastic laminae which appear to have ruptured and retracted. Many fine Weigert positive fibres can always be seen in the gap between the ends of the normal laminae. There is never the very markedly cellular medial repair which occurs in rats. There is however compensatory hyperplasia of the adventitia in relation to the defective media. This adventitia contains both collagen and Weigert positive fibres.

Grade III lesions involve rupture of the aorta (fig. 20). All the laminae rupture and there is haemorrhage which may extend for a short distance within the bounds of the adventitia, but usually bursts through this barrier close to the elastic rupture. Dissection between the layers of the media is rare, and no dissection of more than a few hours' duration has been recognised.

In neonatal mice where the lathyrogenic agent has been given to the mother, the appearances are slightly different. In all lathyrus treated neonatal mice there is an alteration in the appearance of the elastica which is more irregular than is normal in a full term mouse (compare figs. 10 and 11), and the folds in a transverse section of the aorta are more frequent and more uneven than in controls. The overall appearance approximates closely to the aorta of a normal mouse of 17 days gestation (fig. 9). There is no noticeable increase in ground substance. In a proportion of the damaged mice the aorta ruptures, usually bursting directly into the surrounding tissues but in some instances dissecting for a short distance either between the media and the adventitia, or less frequently between the layers of the media.

Animals which died weeks or even months after lathyrogenic agents had been withdrawn still showed abnormalities in the aorta, mainly in the form of interruption of aortic laminae. This applied not only where a grade II lesion was present but also followed grade I lesions. It was seen in a proportion of animals treated as weanlings and also in those treated in utero when the dosage had been sufficient to cause aortic rupture in the siblings.
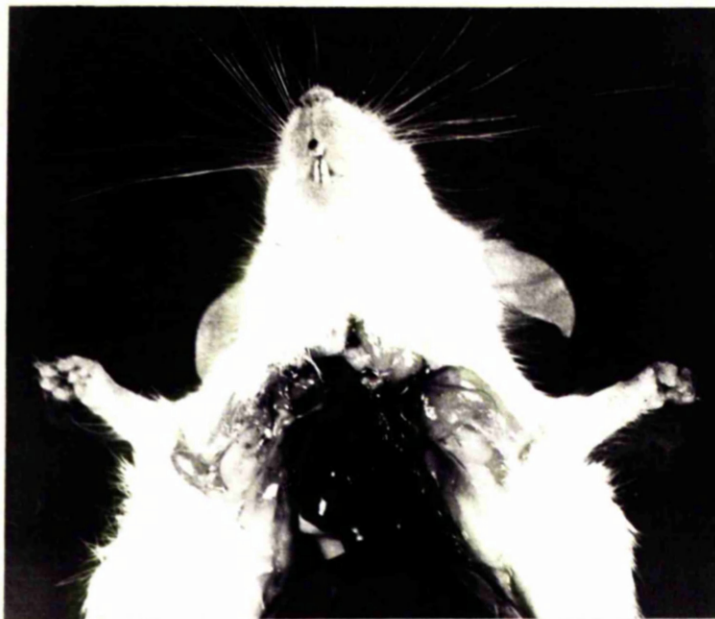
Fig. 6 Male mouse which died of rupture of the aortic arch.
Both pleural cavities contained blood clot, but this
has been removed from the right side to show the
collapsed lung.   This animal was fed on a 50% sweet
pea diet for $4\frac{1}{2}$ weeks from the age of 12 days.

Fig. 7 Aneurysm of the arch of the aorta (A) in a male mouse which received the 50% sweet pea diet for 12 weeks. There were no obvious symptoms from this condition.
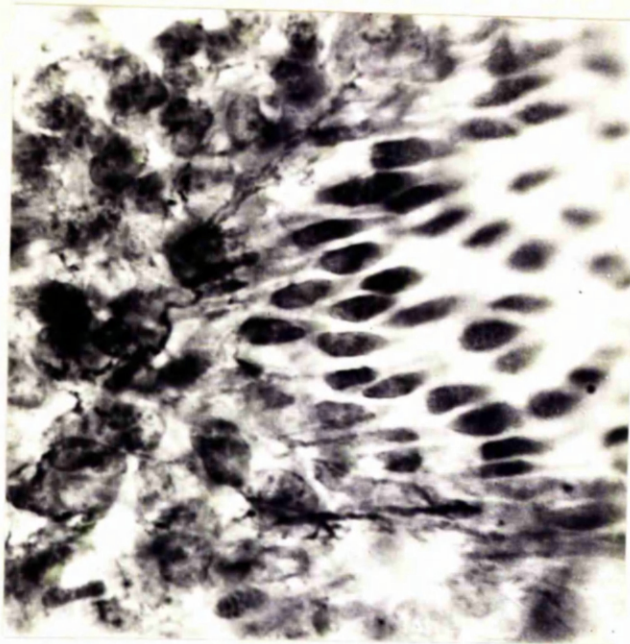
Fig. 8 Tangential section through endothelium (right) and
innermost elastic lamina in the aorta of a 17 day
mouse foetus, showing the early development of an
elastic lamina from a mass of elastic fibres running
in all directions in the one plane.
(X 800 Weigert's elastica)
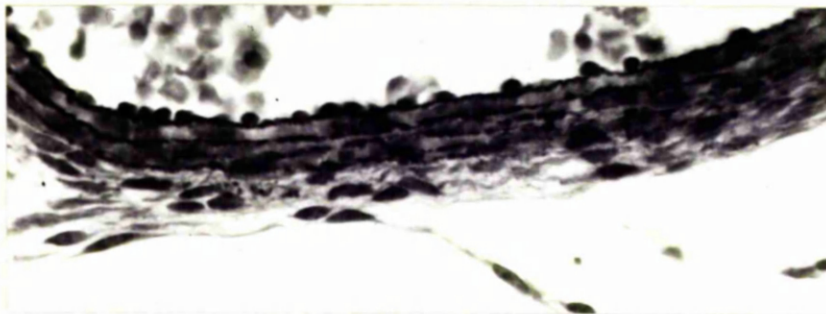
Fig. 9 Part of aorta of normal 17 day mouse foetus to show
the finely irregular folds and uneven thickness of
the elastica laminae.
(X 315 Weigert's elastica)

Fig. 10 Transverse section through the upper abdominal aorta
of a normal one day old mouse.   The uniform
thickness and regular folds of the elastic laminae
are clearly shown.   (X 170 Weigert's elastica)
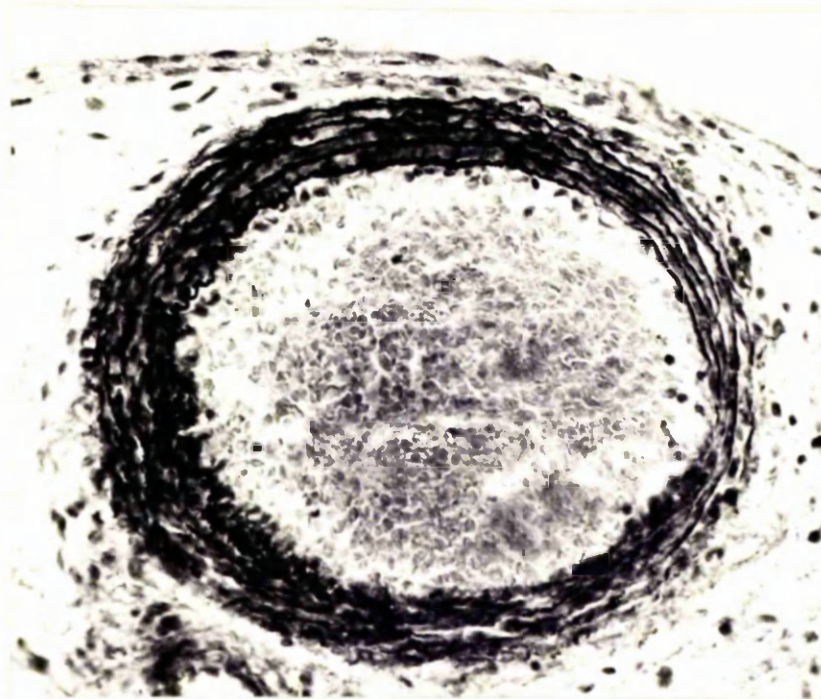
Fig. 11 Transverse section through the upper abdominal aorta
of a day old mouse.   The mother was fed on a 40%
sweet pea diet for the last 5 days of pregnancy.   The
elastic laminae are thinned and their folds are small
and irregular.   There is also interruption and in
places absence of elastic laminae.
(X 170 Weigert's elastica)   Compare with fig. 10.
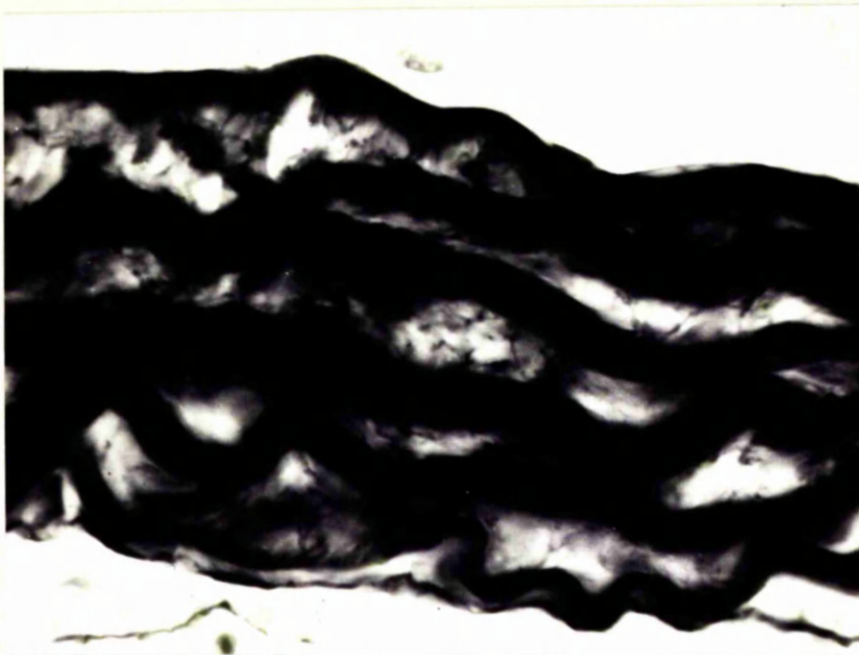
Fig. 12   Part of wall of aorta of a normal young male mouse
          showing fine elastic fibres between the elastic
          laminae.   (X 1050 Weigert's elastica)
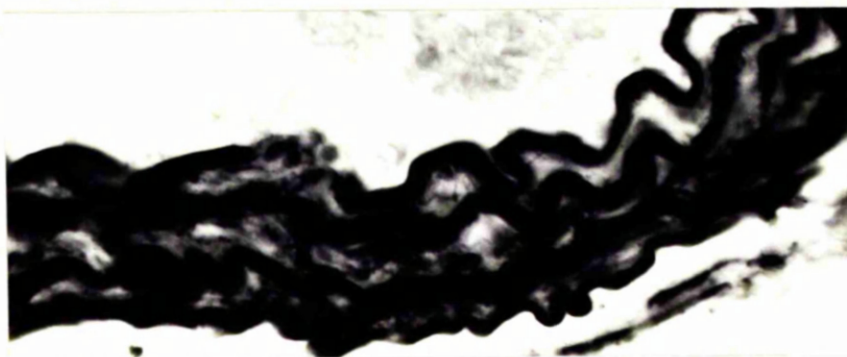
Fig. 13   Part of the wall of the aorta of an 11 month old
          mouse showing rupture of the innermost elastic
          lamina and hyperplasia of the endothelial cells.
          (X 590 Weigert's elastica)
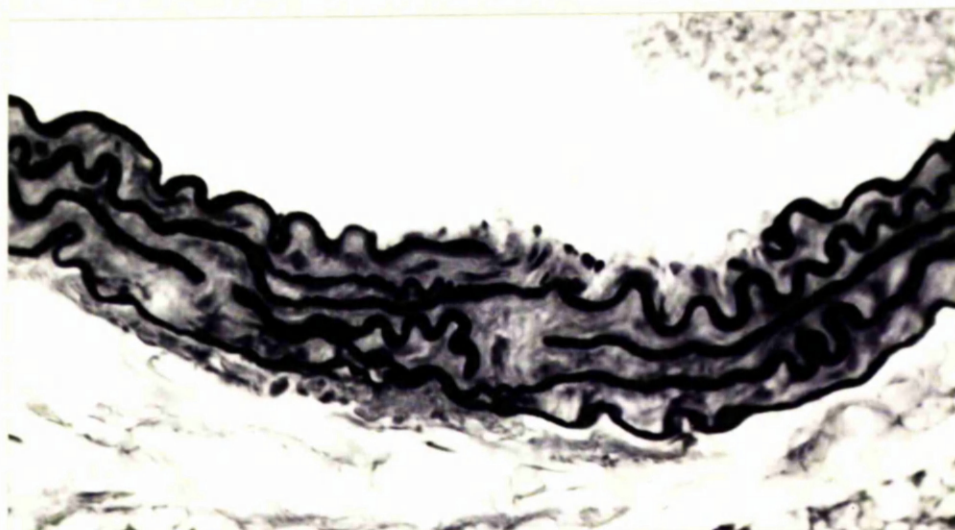
Fig. 14   Example of a Grade I lesion.   There is interruption
and retraction of elastic laminae with hyperplasia of
the endothelium.   This male mouse was fed on the
50% sweet pea diet for $7\frac{1}{2}$ weeks from the age of 3 weeks.
It was then killed.
(X 455 Verhoeff's elastica counterstained van Giessen)

Fig. 15   Another example of a Grade I lesion.   Section of the
aorta of a 28 day male mouse which had 10 mg. of
BAPN.HCl daily for 14 days preceding death.   The large
lakes of Weigert positive material between the laminae
are well seen.   (X 660 Weigert's elastica)
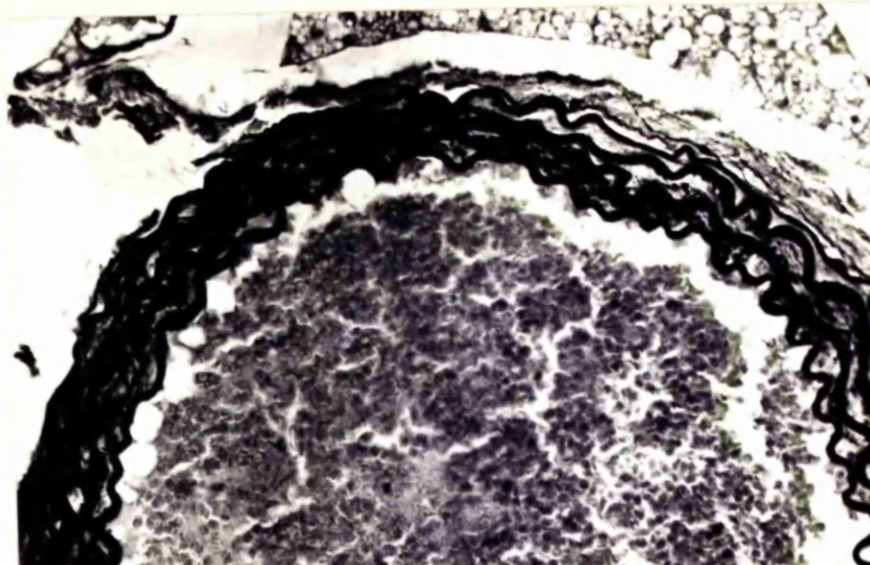
Fig. 16 Section through aorta of a 28 day male mouse which
had 10 mg. of BAPN.HCl daily for 14 days preceding
death (litter brother of that in fig. 14).
Stained with orcein to show the presence of 5 elastic
laminae at the left side. Weigert's elastica gave a
similar picture. (X 225)
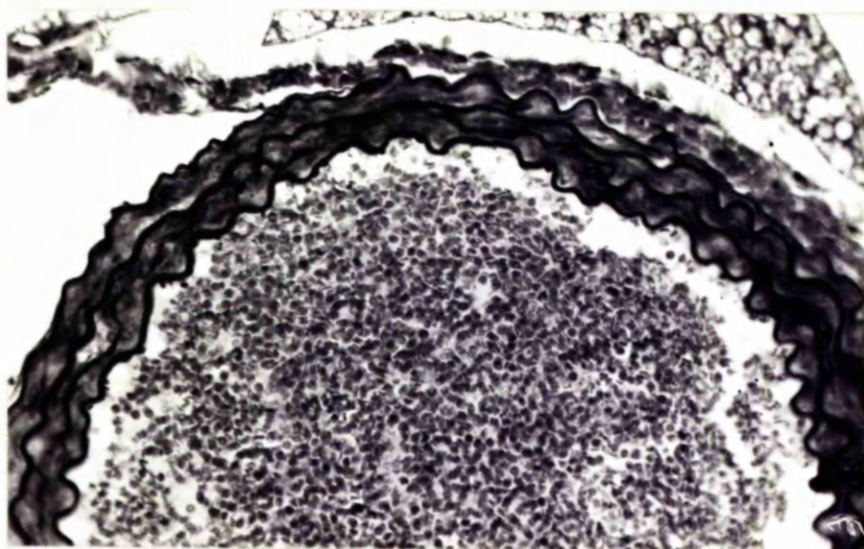
Fig. 17 Adjacent section to that in fig. 15, stained by
Mallory's PTAH. Much of the "elastic tissue" fails
to stain by this method. (X 225)

Fig. 18   Section through the aorta of a 28 day female mouse
which had 10 mg. of BAPN.HCl daily for 14 days
preceding death (litter mate of those in figs. 14,
16 and 17).   To show absence of a length of the
innermost elastic lamina with formation of irregular
new elastic tissue.
(X 550 Weigert's elastica)
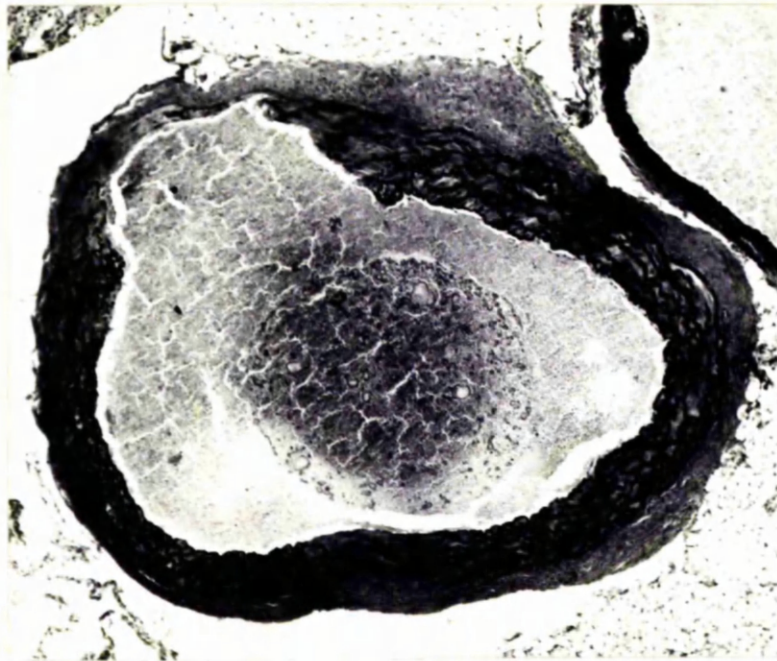
Fig. 19 Example of a Grade II lesion showing disintegration of
         the elastica, aneurysm formation, and hypertrophy of
         the adventitia.   Same mouse as fig. 7.
         (X 50 Verhoeff counterstained van Giessen)

Fig. 20 Another smaller Grade II lesion showing formation of
         new elastic fibres in the gap left by the retracted
         elastic laminae.   From an 8 month old mouse which
         received a 14 week 50% sweet pea diet from 3 weeks of
         age.   (X 550 Weigert's elastica)

Fig. 21 Example of a Grade III lesion. There is dissection
between the two outermost layers of the elastica
with rupture and retraction of the inner layers. The
outer layer has ruptured at another level and blood
can be seen in the surrounding tissues. This male
mouse was fed on 50% sweet pea diet for one month
before its spontaneous death. (X 65 Verhoeff
counterstained van Giessen)
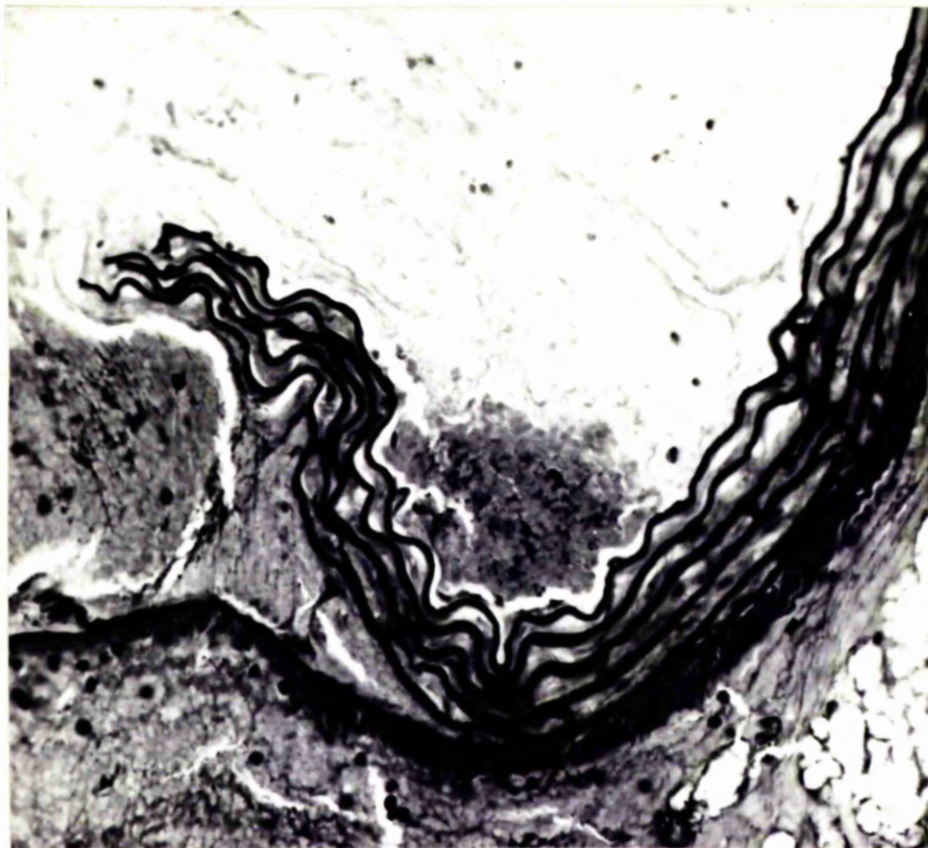
Fig. 22 Enlargement of part of previous figure to show
ruptured laminae, dissection between outer two layers,
and slight increase in interlaminar ground substance.
(X 315)

Discussion

In order that the results in mice may be considered in conjunction with other workers' results in rats, it would seem necessary first to show that lathyrogenic compounds produce the same disease in the two species.

Comparison with Lathyrism in the Rat

At the time this investigation was begun there seemed to be an unexpected discrepancy in the results reported, in that while lathyrism of the rat, both of the bones and of the aorta, was easily produced and well documented (Geiger et al. 1933, Ponseti and Baird 1950 and Lewis et al. 1948) the few reported attempts to produce lathyrism in mice were unsuccessful (Lewis and Schulert 1948, Delay et al. 1952). That there should be such a difference between two species so similar in other respects seemed surprising and if confirmed appeared to offer a fruitful line of investigation.

It has been demonstrated in experiments I and II that the aortic features of lathyrism can be produced in mice. The absence of bony lesions in these experiments probably explains the previous failure to produce lathyrism in mice since the reported attempts took place before Ponseti and Baird reported aortic rupture as a feature of osteo-lathyrism. Lewis and Schulert fed 50% sweet pea meal to young male mice for 17 weeks but they used, as their criterion for lathyrism, the

presence of bony lesions which do not occur with this dose in the mouse (Dasler and Milliser 1957 and Experiments I and II above). Delay et al. used BAPN injected into mice but killed them after only 4 days. Their purpose was to test the compound for neurotoxic effects. Since the minimum time taken to develop aortic rupture in my series was 14 days it is not surprising that they did not observe any aortic or bony lesions.

Experiments I and II show that mice do develop one aspect of lathyrism and in order to produce in the mouse the other major feature of lathyrism - the bony lesions - all that was found necessary was to increase the dose as in experiment III.

From these experiments it would seem that the only difference in the reactions of mice and rats to lathyrogenic substances is one of degree of susceptibility, and that mice when given a higher dose will react exactly like rats. This is farther shown by comparing the in utero experiments in mice with those reported by other workers using rats. In my mice it is clear that only with the highest dose of the most potent lathyrogenic substance - AAN.SO$_4$ - is absorption of the embryos the result, yet Walker and Wirtschafter (1956) using only a 50% sweet pea diet found that the result in rats was uniformly absorption. Stamler (1955), to obtain rats which proceeded to term and were still-born or died of aortic rupture, used a diet containing 20% sweet pea seed. This result corresponds with that in my mice which received 40 - 50% sweet pea diet.

There remains one point on which there might still seem to be a discrepancy between the results in rats and those in mice, and this is in the sex ratio. It has already been pointed out (page 23) that there is a much higher mortality from aortic rupture in males than in females, and this has never been reported in the rat. In experiment III, however, where the dose is higher and the lesion is therefore more comparable with that usually reported in the rat, the sex difference is not so marked. In addition in a small experiment 5 male and 3 female weanling rats were given 40% instead of 50% sweet peas (50% kills regardless of sex); a sex difference became apparent - after 10½ weeks all males were dead of aortic rupture, and all females were alive; one female died of aortic rupture after 12 weeks and the remaining 2 remained alive until killed.

It seems therefore that there is no qualitative difference between the disease produce by sweet peas or BAPN.HCl in the mouse and in the rat, only quantitative: for the sake of drawing conclusions it will be assumed that these are one and the same disease.

Importance of Blood Pressure

The most arresting feature of vascular lathyrism is the aortic rupture, which may be supposed to depend on a combination of the strength of the vessel wall and the pressure of the blood within it, and it is the case that a number of the observed facts can most easily be explained on the hypothesis that a rise or

fall in D.P. will determine whether or not a slightly imperfect aorta will rupture. The following facts support this conception.

1) The majority of lesions are in the ascending aorta between the aortic cusps and the origin of the innominate artery; the part of the aorta which has of necessity the highest pulse pressure.

2) The lesions are almost entirely confined to the aorta, (only a few extend from the aorta for a short distance into a major branch). It is probable that the higher D.P. in the aorta makes it more liable to rupture (though not necessarily to the other lesions of lathyrism) than the lesser elastic vessels.

3) Rupture of the aorta in infant mice seems to occur only at or around the time of birth; an impression similar to that of Stamler (1955). Rupture is never seen where the mother aborts and the infant is either found lying within the membranes or, when it is delivered by Caesarian section and killed immediately. Also if a mouse survives the first twenty-four hours without visible bruising, rupture does not develop thereafter, unless the mother is given lathyrogenic agents during lactation.

That alterations in foetal circulation occurring at birth lead to changes in blood pressure is evident from the work of Dawes et al. (1953) who found that in foetal lambs, while establishment of breathing has little effect on the systemic D.P. or slightly lowers it, there is a distinct rise, when,

thereafter the umbilical cord is tied, and they assume that this results from increase in peripheral resistance. It may be supposed that a similar rise in systemic blood pressure occurs in the mouse with the result that a weakened aorta though able to withstand the foetal blood pressure bursts when the normal pressure rise occurs at birth.

This hypothesis would also explain why Selye and Bois (1956) were able to produce aortic rupture in lathyrus treated adult rats by preliminary unilateral nephrectomy and DOCA, well known to raise the B.P. (Selye, Hall and Rawley 1943).

The Weakness in the Aortic Wall

In considering the nature of the aortic lesion in lathyrism, two outstanding facts must be noted. Firstly the vascular lesions are confined to elastic vessels, and almost always to the aorta itself. Now while the main strength of a muscular artery is in the smooth muscle, the strength of an elastic vessel is in the elastic laminae, and it is even possible to observe at postmortem necrosis of all the muscle cells in an elastic artery without any apparent weakening of the wall in the form of aneurysm, dissection or rupture. It is therefore not in the muscle that we must expect to find the lesion, but in the elastic laminae and possibly also in the other "connective tissues" (reticulin, collagen and ground substance) which bind the aorta together. This impression is confirmed by the histological appearances, for in lathyrism no lesion of the muscle can be recognised, and, though changes in the reticulin and in the ground

substance are undoubtedly present in many cases, the most
constant feature in the aortas examined, especially in the
neonatal mice, is in the elastica (see page 40).   It is
obvious that a defect involving the aortic laminae would be
itself enough to cause aortic rupture if it took place too
rapidly to allow time for compensatory fibrosis, and it was
therefore decided to pursue farther the nature of the elastic
tissue defect as being probably the chief, though perhaps not
the only cause of the aortic weakness.

A second fact which emerges both from my own work with
mice, and from the reports of other workers (Ponseti and Shepard,
1954) on rats, is that only in young animals is it possible to
produce aortic rupture in lathyrism, although there is no limit
to the age at which the bony lesions may be produced.   The
period at which it is easiest to produce aortic rupture is in
the last few days of foetal life (Experiment VII).   At this
time it is possible to produce aortic rupture in as little as 48
hours whereas when the lathyrogenic treatment is started at 21
days it takes a minimum of 14 days before the aorta ruptures.
Kajee (quoted by Gillman and Hathorn 1958) has shown that, in
rats, the rate of increase of growth of the aorta is very rapid
immediately after birth, decreases gradually until the 26th day,
and becomes very much less thereafter.   If it can be assumed
that this post-natal curve is merely a continuation of the pre-
natal growth curve, then the rate _in utero_ must be more rapid yet.

Certainly the rate of growth of the whole foetus is very rapid during the last few days in utero (Walker and Wirtschafter 1956) and presumably the growth of the aorta keeps pace with it.   It seems clear then that the ease with which aortic rupture can be produced is proportional to the rate of growth of the aorta at the time.

Another example of this is to be found in the sex difference already noted in the response of mice to lathyrism (page 23).   It is clear from the results of experiment VII that there is no difference in the incidence of aortic rupture in male and female neonatal mice born of mothers given BAPN.HCl during pregnancy.   On the other hand in the weanling mice used in experiments I and V the difference is marked - 35% of males and only 14% of females developing grade II and III lesions. This difference is statistically significant ($x^2 = 6.77$, p = <0.001).   If only those with the lower dosage are considered the proportion of females drops to almost 0%.   A possible explanation is offered by experiment V from which it can be seen that the incidence of aortic rupture depends not on the amount of BAPN consumed, but on the weight of food consumed.   Those animals which ate most were also shown to be the heaviest and so this would suggest that the reason that males are more prone to aortic rupture is that they grow more rapidly.   It has been shown in rats that while at birth the males weigh the same as the females, by 26 days they weigh slightly more (Kajee).

From this it appears that the sex difference is merely a result
of the more rapid growth of the male - and part of the general
observation that the frequency of aortic rupture is proportional
to the rate of growth.   From this it follows that the fault in
the aorta is not a degeneration for this might occur in the
absence of growth, but is an error in formation - a fault which
would be much more disastrous during a period of rapid growth
than when the only synthesis is the slow "wear and tear"
replacement of tissue which may be presumed to occur in the adult.

If this deduction is correct, that the aortic defect
is due to imperfect formation and if, as has been argued above,
the weakness in the aorta responsible for the rupture is mainly
that of the elastic laminae, then it follows that the main cause
of that aortic weakness is defective formation of elastic laminae.

TISSUE CULTURE EXPERIMENTS

The purpose of these experiments was to test _in
vitro_ the hypothesis which had arisen from the _in vivo_ work;
that, in lathyrism, there is a fault in the formation of elastic
tissue. For this reason a search was made for some form of
tissue culture which would form elastic fibres. Almost all
reports of connective tissue fibres in tissue culture refer to
either collagen or reticulin, but in 1929, Bloom described the
production in tissue culture of fibres having the staining
properties of elastic tissue. These he produced by growing
guinea-pig embryo heart in plasma clot in a culture medium
consisting almost entirely of Tyrode fluid. After 10 - 16
days distinct fibres could be seen which stained with orcein and
Weigert's elastica stain, and these he believed to be elastica.
Bloom thought that his success in growing elastica was due to
the low nutritive value of his culture medium which, by slowing
the rate of multiplication of the cells might be expected to
encourage their differentiation. This belief was shared by
Odiette (1932) who claimed to have produced by this method,
elastic fibres in tissue culture of "all tissues containing
elastica".

Since the only tissue used successfully by both workers
was embryo heart, and since it was my intention to use the

technique as a model for vascular elastic tissue, the embryo heart was used as the source of explants in the great majority of experiments but, in a few, explants were taken from the great vessels.   The findings of Bloom that elastic fibres can be produced _in vitro_ have been confirmed in the present experiments. Earlier experiments were further based on Bloom's and Odiette's work in that they used media of poor nutritive value, but this was later found to be of no advantage.

A brief account of these investigations has already been published (McCallum and Paul 1961).   I am indebted to my co-author for advice and guidance on tissue culture techniques and for the facilities of his department for some of the experiments, but the actual explantation and examination of all the tissues was carried out by me alone.

Materials and Methods

<u>Glassware:</u>   Either 7/8 square or 1½ by 7/8 "Chance" cover-slips were used for all cultures grown in plasma clot.   The Petri dishes used were Monax 50 mm.   The same type of cover-slips and dishes were also used for some of the trypsinised cultures, but in addition 4 x ½ inch soft glass tubes were used for many of the experiments.

<u>Fluid Media:</u>   Various different media of which the composition is given by Paul (1960) were used.

(1)   Waymouth's medium + 10% calf serum.

(2)   Hank's B.S.S. + 10% calf serum.

(3)   Eagle's medium + 10% calf serum.

(4)   T.C. 199 (Glaxo) + 10% calf serum.

The volume used depended on the type of vessel.   With the Petri dishes it was 4 ml., while with the test tubes 1.6 or 2 ml. was used.   The pH of the medium was adjusted using 10% w/v $NaHCO_3$, to give a rather alkaline initial reaction – usually around 7.8; this level fell during incubation.   In a few cases a lower initial pH was used but this was found to be less satisfactory.

<u>Temperature:</u>   Most chick cultures were grown at 39°C but in a few the temperature was 37°C, though this was later found to be less satisfactory.

<u>Atmosphere:</u>   Many of the cultures were grown in stoppered

glass tubes, but where Petri dishes were used some means of
controlling the atmosphere was required in order to maintain the
pH of the medium.   This was achieved either by placing the
dishes in an incubator with an atmosphere saturated with water
and containing 5% CO_2, or by incubating the dishes in a closed
atmosphere in a plastic box in an ordinary incubator.

Preparation of Tissue: For most experiments hearts from chick
embryos were used; there were a few experiments using mouse
embryo tissue, but no significant difference was observed between
the two, therefore since chick embryo hearts are technically
more convenient because of the larger size, they were preferred.
The chick embryos were at about the 10th. to 12th. day of
development; hearts from embryos of over 14 days were generally
unsatisfactory especially in trypsinised cultures, since they
often failed to adhere to the glass.   The great vessels and
auricles were removed and discarded, and thereafter one of the
following techniques was used.

(1) Cultures using plasma clot.   The ventricles were cut
between two knives into explants of uniform size (approximately
1 mm. cube).   The explants were washed with Hank's B.S.S. and
arranged in a clot of chick plasma on a coverslip.   The cover-
slips were then placed in Petri dishes and covered with a layer
of fluid medium.

(2) Culture grown directly on glass.   The ventricles were cut
into explants of rather less uniform size, but again to

approximately 1 mm. cubed, and placed in 10 times their volume
of 0.25% trypsin solution at pH 7.6 for a half to two hours at
37°C.   The explants were then washed twice in Hank's B.S.S.,
suspended in the fluid medium, and allowed to settle onto the
surface of the dish or tube, or onto a coverslip on the bottom
of the dish.

Explants of great vessels were taken from 14 day old
chick embryos.   The preparation was the same as for the heart
explants grown directly on glass, but the great vessel explants
showed little inclination to adhere to the glass and were
discouraged from doing so by occasional agitation .

In a single experiment the trypsinisation was replaced
by incubation with 2X crystalline elastase (Nutritional Biochemical
Co.) in sterile B.S.S. at pH 7.8 for 45 minutes at 37°C.

<u>Length of Incubation:</u>   Initially heart cultures were fixed
for histological examination after 2,3,4,7,10 and 14 days.   The
most satisfactory length of incubation was found to be 10 days
and most later cultures were grown for this period.   All great
vessel cultures were examined after either 3 or 10 days.

<u>Addition of Test Substances to the Cultures:</u>   It was desirable
that, when testing unknown compounds, a single constant technique
should be adhered to, and from the comparison (see page 68) of
the different methods tested it was possible to choose the most
reliable in producing elastica.   The following technique was
therefore used in all experiments involving test substances.

(1) Fluid medium - Waymouth 's medium + 10% calf serum.

(2) Trypsinisation of explants.

(3) Temperature of 39°C.

(4) A pH of approximately 7.8 in the initial culture medium.

(5) Incubation for, and examination after 10 days for heart cultures and three days for great vessel cultures.

There remained a single variable in that both tube and coverslip cultures were used, but the results obtained by these two methods were entirely comparable.

The substances to be tested were made up with B.S.S. to form a stock solution the strength of which depended on the molecular weight of the test substance.   A strength of 20 mg/ml was chosen empirically for the stock solution of BAPN.HCl, and thereafter other stock solutions were made up on a mole-equivalent basis.   Table VIII shows a list of the substances tested together with the molecular weights and strengths of the stock solutions.   The stock solution was added to the medium of the first culture to a final dilution of 1 in 10, thereafter five-fold dilutions were made in culture medium to a total of 6 to 8 dilutions.   The dilutions were made immediately before the explants were added to the medium.

TABLE VIII

Substance	Mol. Wt.	Stock Solution
β aminopropionitrile HCl	106.5	20.0 mg/ml.
Aminoacetonitrile H_2SO_4	154.0	29.5 mg/ml.
β mercaptoethylamine HCl	113.5	21.3 mg/ml
Aminodipropionitrile	123.0	24.1 mg/ml
Semicarbazide HCl	111.5	20.9 mg/ml.
Ethanolamine	61.0	11.5 mg/ml
Sodium propionate	96.0	18.2 mg/ml.
Propylamine	59.0	11.1 mg/ml
$CH_3CO. NHCH_2. CN$	98.0	18.5 mg/ml.
$HCl. H_2N-CH_2-CONH_2$	109.5	20.5 mg/ml.

Method of Examination of Cultures   Healthy growth of living

cultures was assessed at intervals using an inverted microscope,

but in addition all satisfactory cultures were fixed, and either

sectioned histologically, or examined as stained surface

preparations.   In the case of the test-tube cultures fixation

and staining took place in the test-tube, and the cleared

preparation was examined through xylol under an inverted micro-

scope.   The fixatives used were 10% formol saline and Carnoy's

fluid, and stains included Weigert's method for elastica, Orcein,

Gomori's aldehyde-fuchsin, PTAH, H. & E., silver methods for

reticulin, PAS (with and without diastase), toluidine blue and

Sudan III and IV. In addition some preparations were treated overnight with elastase, (Nutritional Biochemical Co.) in Tris (trimethyl amino methane) buffer at pH 8.8 stained by Weigert's method and compared with serial control sections similarly stained.

Identification of Elastic Tissue   The usual methods for identifying elastic tissue are by means of empirical stains. It is possible that these stains give a positive result with more than one substance.   For the purposes of this thesis the term elastic tissue has been used to describe any material which is in the form of fibres, and stains with Weigert's elastica stain. The further properties of the elastic fibres observed are described on page 74.

Production of Elastica in Tissue Culture

Preliminary experiments established that elastica could be produced in tissue cultures of chick embryo hearts, but its formation was irregular, occurring in only a proportion of cultures, and even then not necessarily in every explant in a given culture.   In an attempt to discover a method which could be relied upon to give elastic tissue in all cultures experiments were set up to provide a comparison of results by different techniques.

Comparison of Different Techniques using Chick Embryo Heart

The factors were compared in groups:-

(1)   Plasma clot preparations were compared with trypsinised preparations.   Elastic tissue could be produced in both, but as tho presence of the plasma clot tended to interfere with some staining techniques the trypsinisation method was preferred.

(2)   Four Media - Waymouth's, Hanks B.S.S., Eagle's and T.C. 199 (Glaxo), each with 10% added calf serum were compared.   In each case elastic fibres were grown , but in the cultures with Waymouth's medium the growth was more rapid, the production of elastica was more constant, and the amounts produced seemed to be larger.

(3)   pH - cultures set up with a pH of 7.8, 7.4 and 7.0 were compared after 10 days.   At pH 7.0 growth was poor, and the cultures failed to produce elastica.   The two more alkaline

groups grew well, and elastic fibres were formed in both.

(4) Period of Incubation. Until 7 days no elastic fibres were seen. After 7 days a few elastic fibres could be seen in a small proportion of cultures. By 10 days these fibres were much more prominent and were present in many more cultures. Thereafter the amount of elastica did not appear to increase to any marked extent, and since in addition some of the cultures tended to become detached from the glass after this time, it was felt that 10 days was the most satisfactory period of incubation.

(5) Temperature. This seems to be a most important factor. Cultures grown at 39°C produce good elastic tissue fibres in 10 days. Similar cultures grown at 37°C have the same morphology after 10 days, but no elastic fibres are seen. If the 37°C cultures are grown for a further 2 days, a few explants produce fine elastic fibres. It appears that although cellular growth is unaffected synthesis of elastica has a higher temperature optimum.

Although elastic fibres could be obtained by the methods described, their production was never constant with any method and there must therefore be other factors involved which are not understood.

Histological appearance of chick embryo heart explants before culture.

Sections stained with H. & E. (fig. 23) show the explants to

consist mostly of heart muscle cells, with a few fibroblasts,
and, where the epi- or endocardial surface is present, endo-
thelium. There are also a few blood vessels most of which
are capillaries with no smooth muscle in their walls, but there
are sometimes present a few larger vessels which can be
recognised as well formed arteries or veins.

An attempt was made to identify various types of
connective tissue fibres in suitably stained sections. The van
Gieson stain shows very little collagen especially in the 10
day chick hearts, but in the older ones up to 14 days fine
fibres can be seen which give the red staining reaction of
collagen. These are most common immediately under the epi-
and endocardial surfaces. Silver impregnation technique shows
relatively little reticulin. What is present is mainly in
the form of short branching fibres which run between the heart
muscle cells. In addition finer less well defined fibres can
be seen in the sub-epicardial tissue and around the larger
coronary vessels.

Elastic tissue as judged by Weigert's stain, is almost
completely absent. It is seen only in the walls of the larger
coronary arteries. There is no elastic tissue in the endo-
cardium at this age.

Microscopical appearance of heart cultures

Cells were seen growing out from the explants usually
within the first 24 hours, and always in successful cultures,

within the first 48 hours, whether the culture was grown in fibrin clot or directly on the glass.   If no outgrowth was seen within 48 hours growth was never satisfactory.   The source of these spreading cells is not clear, but they have the morphological characteristics of fibroblasts.   At first the cells spread outwards in a radial manner, but after 3 days the fibroblasts nearer the explants alter shape and become elongated tangentially instead of radially and so come to form a circle round, and a short distance from, the original explant (fig. 24). The cells outside this circle continue to grow radially and the ring itself enlarges by proliferation within.   Smaller explants frequently spread completely on the glass so that the ring of fibroblasts surrounds a monolayer of cells with no recognisable original explant.   In the smallest explants no ring formation may be recognisable.   Sections of the growing explants cut at 90° to the glass surface show that within 48 hours a single layer of flattened endothelium-like cells has formed on the surface of the explant at the farthest point from the glass.   The endothelial cells are continuous with the fibroblasts, the difference in morphology being apparently related to the presence or absence of overlying cells.

The extracellular elements were examined in similar cultures.   In sections of 48 hour cultures it is possible to see that the total reticulin has increased and that the distribution is changed.   Instead of short fibres running in

various directions, layers of reticulin can be seen lying between layers of cells. By 3 days there can be seen in surface preparations typical curving sheaves of reticulin fibres (fig. 27), and these increase in amount as the cultures age. The reticulin fibres are mainly seen over the original explant and their site and time of appearance have no apparent relation to those of the fibroblast ring. Elastic fibres by contrast, do not appear before the 7th day, and are not clearly seen with any regularity until the 10th day. Their site in larger explants is typically in the fibroblast ring, the fibres lying between the cells of this ring (fig. 26). In small explants where no ring has formed, the fibres are arranged haphazardly. The fibres are quite unlike reticulin fibres in that they are both finer, and (except in the smallest explants) straight or arc shaped and do not form sinuously curving skeins. In sections the elastic fibres are seen to be confined to a layer immediately beneath the surface endothelium (fig. 28) - a position corresponding to that of the internal elastic lamina of a small vessel.

Histological Appearance of Great Vessel Explants Before

Culture

A number of cultures of explants out from great vessels were examined. All cultures were examined as paraffin sections. Explants were examined both before and after trypsinisation. Before trypsinisation the appearance was that of a short length

of elastic artery (see fig. 30) with concentric layers of elastica alternating with smooth muscle.   After trypsinisation (fig. 31) the elastic tissue nearest the periphery of the explant becomes non-staining, while the reticulin, collagen and cells are unchanged.   The presence of an elastase as an impurity in the trypsin preparation is thought to be the cause.

In the single experiment where elastase was used instead of trypsin the histology of the explants after elastase treatment was the same as after trypsinisation.

Histological Appearance of Great Vessel Explants After
Culture

The appearances after culture were the same whether the explants were trypsinised or treated with elastase.   After three days the explants can be seen to have rounded off and to be surrounded by 2 or 3 layers of fibroblast-like cells with a surface endothelial layer.   In all cultures a layer of new reticulin (fig. 34) is present just deep to the endothelium. The old reticulin in the centre of the explant is tending to break up.   Very little collagen seems to be present and it is difficult to distinguish definite collagen fibres.   With Weigert's elastica stain the central part of the explant can be seen to contain the unchanged elastic laminae while in the outer part which was cleared of Weigert positive material by the trypsin, spots of Weigert positive material can now be seen between the cells, often in rows, and sometimes coalescing to

form what appears to be a membrane (Fig. 32).   This Weigert
positive material is most prominent in the media and is seldom
seen in the adventitia.   It may be seen in the new fibro-
elastic material if this is contiguous with the media but not
if it is separated from the media by a layer of adventitia.   No
marked difference is noted between 3 and 10 days cultures.

Identity of New Elastic Material

There is considerable evidence that the structure of
elastica varies in different sites and in the same site at
different times (Lansing 1959).   It has also been suggested
that there exist materials midway between collagen and elastica
and having some properties of both (Hall 1961);   these have been
termed pseudo-elastica.   It therefore seems necessary to
examine more closely the properties of the elastica formed in
vitro and to compare it with accepted elastic tissue.   This
comparison may be made under three headings:- morphology,
staining reactions and reaction to elastase.

Morphology   The morphology can best be studied in surface
preparations of heart, though if aortic explants are allowed to
adhere to the glass, a rather similar picture can be seen.   In
the surface preparations the new material is in the form of
straight or slightly curved fibres which are parallel to the
longitudinal axis of the cells in the ring which typically forms
in larger heart explants.   Where, as in smaller explants, no
such ring has formed, fine Weigert positive fibres can be seen

lying in a haphazard arrangement.   Each of these morphological arrangements is found to have a close parallel in the formation of vascular elastic tissue in vivo.

The formation of a ring of cells round the original explant has been noted in a number of tissue cultures from different sources, notably fibroblasts either in primary or in pure line culture, and skeletal muscle.   While the reason for this organisation is uncertain the most widely held view is that of Weiss (1959) who contends that it is the result of physical stresses set up within the culture.   In this respect the growth in tissue culture may be compared with the behaviour of fibroblasts in wound healing (Muir's Pathology, 7th edition) in that in both a healing wound and in a tissue culture the original longitudinal axis of the fibroblasts changes completely and often through 90°.   In vivo this allows the fibroblasts to lie at right angles to the line of the wound, and is presumably a response to the stress placed on the fibroblasts by the lack of support at this point.   By analogy it seems reasonable to suppose that the reorientation of the fibroblasts in tissue culture is also a response to physical stress.   It would then seem that the Weigert positive fibres are being laid down along lines of stress, and this has already been noted to happen in lathyritic aortas where, as a response to the rupture of the laminae, new elastic fibres can be seen bridging the gap between the old (page   ).

The haphazard arrangement of Weigert positive fibres seen in smaller explants may be compared with the original formation of an elastic lamina in vitro. If a developing aorta is cut tangentially the earliest elastic tissue is seen to be in the form of many elastic fibres running in all directions but in one plane. Although the smallest explants have not been satisfactorily sectioned, examination of sections of larger explants shows that the elastic tissue in these, forms in a single plane beneath the endothelial surface. In addition the small explants are frequently only two cells thick at the most and therefore it seems that here, as in the developing aortic laminae the fibres run in all directions but in one plane.

As well as suggesting similarities to elastic tissue in vivo, the in vitro morphology of the Weigert positive fibres differentiates them sharply from the reticulin fibres which may be demonstrated by silver methods. The reticulin can be seen to be much coarser, and to be arranged in twisted skeins which are not related to the cellular ring.

Staining: The staining properties of the new elastic material were examined in sections and surface preparations of both heart and great vessel cultures. In the great vessel cultures comparison was made between the reactions of the remaining normal elastica and the newly formed material.

It was found that the new elastic fibres, whether in heart or great vessel cultures, stain with Weigert's elastica,

orcein and Gomori's aldehyde-fuchsin, that is to say they stain
with the traditionally accepted stains for elastica. Moreover
they do not stain with the silver stains for reticulin. The
PAS reaction is probably positive, but the cultures contain so
much PAS positive material, even after treatment with diastase,
that identification is difficult. Mallory's trichrome and van
Gieson are also unhelpful. Mallory's PTAH has been recommended
as distinguishing between true and pseudo-elastica (Gillman
et al. 1955), but in my experience does not show any difference
between old and new elastica in tissue cultures.

Reaction to Elastase   It has been demonstrated by Balo and
Banga (1949) that purified pancreatic elastase will destroy
elastic fibres;   unfortunately they have since shown that it
will also destroy denatured collagen (1955). The enzyme can
be used on paraffin sections, and comparison of elastase-treated
sections with control sections shows that the Weigert positive
material has been removed. In addition in the aortic explants
it was possible to see that the original elastic laminae were
removed at the same time.

From the above evidence we may conclude that there is
formation of fibres having the morphology, staining reactions and
reaction to elastase identical with elastica. There seems
therefore no reason at present to assume that they are not
elastic fibres.

<u>Mouse Heart Explants</u>   A few experiments were carried out using hearts from 19 or 20 day mouse embryos as the source of explants. The histology of the explants before culture was the same as for chick embryo heart.   The techniques used were similar but a temperature of $37^{\circ}C$ was found to be as effective as $39^{\circ}C$.   Growth tended to be more exuberant than in the chick cultures, and the cells are larger more densely staining and have a more bizarre appearance (fig. 36).   A fibroblast ring usually forms though sometimes with very few cells within it, and elastica is produced in a number of cases (fig. 37).   The elastic tissue has the same properities as that formed in chick cultures.

Demonstration of Specific Inhibition of Elastica Production

by Lathyrogenic Compounds

A series of experiments was next set up to test the effect of known lathyrogenic and related non-lathyrogenic substances on the production of elastic tissue in tissue culture. Each experiment comprised a number of control cultures with no added chemical, plus graded test cultures. In some experiments no elastica was produced in any of the controls and these experiments have been omitted. In addition a number of individual cultures died or were contaminated, and these also have been omitted. The cultures were assessed as positive or negative; positive signifies that elastic fibres could be seen in Weigert stained preparations, and negative that after careful microscopic search no such fibres could be found.

Experiment 1   Effect of BAPN.HCl.

In order to demonstrate whether the production of elastica could be inhibited by a known lathyrogenic substance, cultures of chick embryo heart were set up with BAPN.HCl added to the medium in graded five-fold dilutions descending from 2000 μg/ml.

In all there were 109 successful cultures - 46 controls and 63 test cultures. There was only one live culture with the highest concentration used (2000 μg/ml) because this concentration of BAPN.HCl is almost always lethal to the
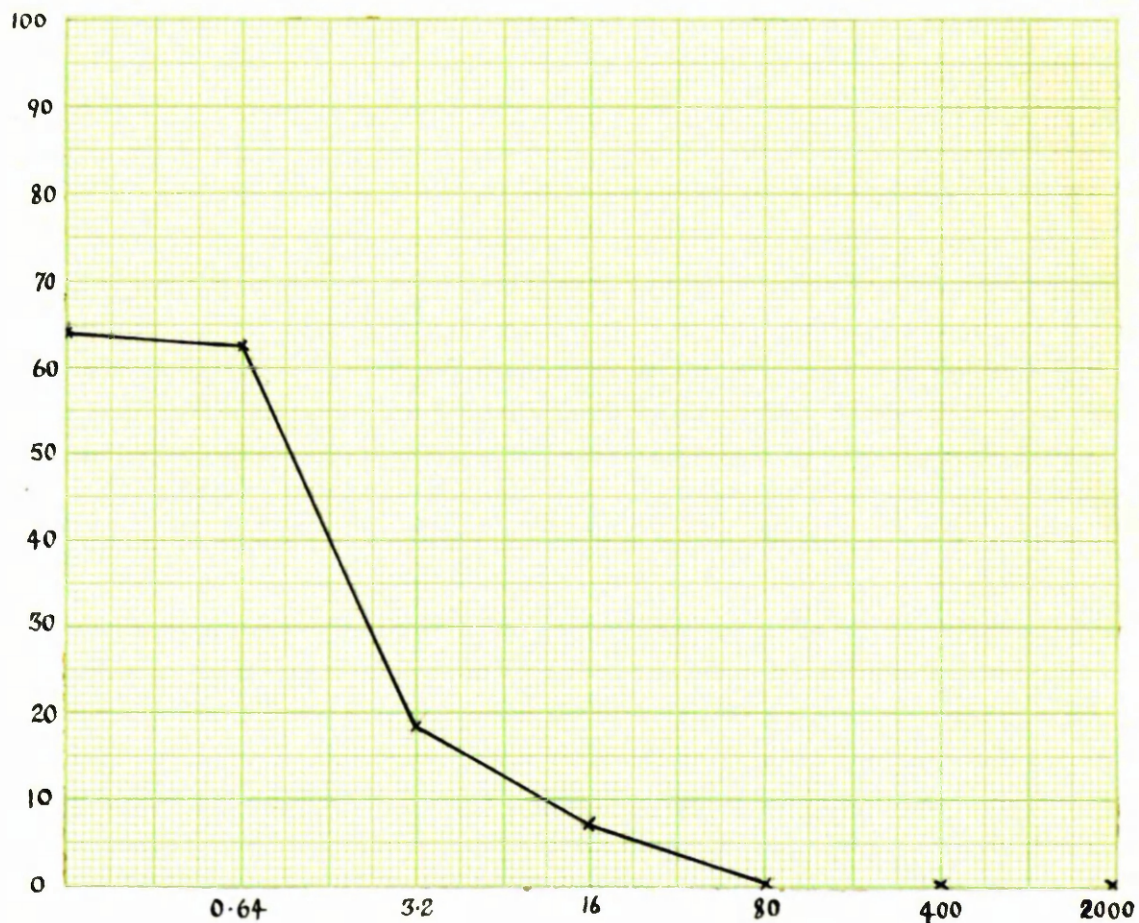
Fig. 38 To show the percentage of cultures producing elastica
in the presence of graded concentrations of BAPN.HCl.

explants. In the one live culture there was some inhibition
in the growth and spreading of the explants. This appearance
was also present in some cultures with the second dilution
(400 µg/ml), but at lower concentrations there was no difference
from the controls in the morphology of the explants as judged
by either their gross appearance or the appearance in cover-
slip preparations (fig. 29) or in paraffin sections.

The results are shown in table IX and the percentage
of positive cultures are graphed in fig. 38 against the
concentration of BAPN.HCl.

Table IX. The effect on elastica production in
chick embryo heart tissue cultures of the addition of graded
doses of BAPN.HCl to the medium.

<p style="text-align:center;">TABLE IX</p>

Concentration in µg/ml	2000	400	80	16	3.2	0.64	Control	Total
Elastica Positive	Nil	Nil	Nil	1	2	10	32	45
Elastica Negative	1	10	17	13	9	6	17	73
Total	1	10	17	14	11	16	49	118
Percentage Positive	0%	0%	0%	7%	18%	62.5%	64%	-

It is clear that with chick embryo heart cultures the presence
of BAPN.HCl in the culture medium inhibits the production of

elastic fibres completely in the higher concentration and with
decreasing frequency as the concentration is reduced.

Experiment II   Effect of BAPN.HCl on mouse heart cultures.

A few cultures of mouse embryo heart were tested by
the addition of BAPN.HCl to the medium to see whether in this
species elastica formation was inhibited in vitro.   The
technique was as for chick test cultures, but the incubation
temperature was 37°C.

There was a total of 20 live cultures - 8 controls and
12 test cultures.   The highest concentration (2000 µg/ml)
killed the explants.   The histological appearance of the
other test cultures was similar to that of the controls.   The
results are given in table X.

Table X.   The effect on elastica production in mouse
heart tissue cultures of the addition of graded doses of BAPN.
HCl to the medium.

TABLE X

Concentration of BAPN.HCl in µg/ml	2000	400	80	16	Control nil	Total
Elastica Positive	Nil	Nil	Nil	1	5	6
Elastica Negative	Nil	9	2	Nil	3	14
Total	Nil	9	2	1	8	20

It will be seen that the higher doses of BAPN.HCl inhibit elastic tissue production, but that a dose of less than 60 µg/ml is not effective.

Experiment III Effect of a non-lathyrogenic substance (n-propylamine).

To test whether the effect of inhibiting the formation of elastic tissue in tissue culture was specific to lathyrogenic compounds or was shared by other chemically related but non-lathyrogenic compounds, such a substance - n-propylamine - was tested in chick tissue cultures and its effect compared with those of BAPN.HCl. n-Propylamine had already been shown to have no lathyrogenic action in mice in vivo (see table VII).

All cultures containing the highest concentration (1100 ug/ml) died. There were 51 live cultures - 18 control cultures and 33 test cultures. In the three live cultures with the second concentration (220 µg/ml) there was some alteration in cellular morphology but in the remaining cultures the morphology was similar to that seen in the controls.

Table XI. Effect of graded concentrations of n-propylamine on elastica production in chick embryo heart cultures.

TABLE XI

Concentration in μg/ml	1100	220	44	8.8	1.76	0.35	Control	Total
Elastica positive	nil	nil	2	7	3	3	11	26
Elastica negative	nil	3	8	3	2	2	7	25
Total	nil	3	10	10	5	5	18	51
Percentage positive	0%	0%	20%	70%	60%	60%	61%	-

The results are given in table XI. It is seen from comparison with table VIII that while the cells are killed by n-propylamine in the same molar concentration as by BAPN.HCl, elastic tissue production is possible with a substantially higher relative concentration of a n-propylamine in the culture medium.

In contrast with lathyrogenic substances where apart from concentrations which inhibit growth there is a wide zone of concentrations compatible with growth but incompatible with elastica formation, n-propanylamine either prevents growth or allows both growth and the synthesis of elastic tissue. It is an all or nothing response without specific inhibition of elastica synthesis.

Experiment IV   Effect of other lathyrogenic and non-lathyrogenic substances.

In order to show whether the difference between BAPN.HCl and n-propylamine in inhibiting elastic production

in vitro is a function of their lathyrogenic activity, a number
of other substances whose lathyrogenic activity in vivo was
already known, was tested to assess the minimum concentration
at which they inhibited formation of elastic tissue in 100% of
cultures.   The results are shown in table XII along with
the figures for BAPN.HCl and n-propylamine for comparison.

Table XII.   To show the minimum concentrations of
various substances which will inhibit elastica production in
chick embryo heart cultures.

TABLE XII

Test substance	Minimum concentration to give 100% inhibition of elastogenesis
AAN. SO$_4$	0.19 µg/ml
Semicarbazide HCl	0.66 µg/ml
β-mercapto ethylamine HCl	17.0 µg/ml
BAPN.HCl	80.0 µg/ml
n-propylamine	220.0 µg/ml
Sodium propionate	1820.0 µg/ml

It will be seen that the substances in the upper part of the
table inhibit the formation of elastica at a much lower
concentration than those in the lower part.   It has already

been shown (see page 31) that those in the upper part are lathy-
rogenic *in vivo* and those in the lower part are not. Of the
lathyrogenic substances the relative toxicity is roughly
parallel to that seen *in vivo* although since semcarbazide has
been given only by mouth it is difficult to produce a comparative
figure (see page 29, table VII).

Effect of test substances on Great Vessel Cultures

In order to test a larger number of substances more
quickly, cultures of great vessels were used. In these elastica
is formed in only three days and its production is more constant
both between different batches of cultures and between different
individual cultures. Only a single concentration was used and
the cultures were set up in duplicate, along with control
cultures to which no test substances had been added. The culture
technique, and preparation and staining of the explants were
the same as had been described for great vessel cultures on
page

Experiment V   Effect of DAPN.HCl.

In the first cultures DAPN.HCl was added to test
whether it would inhibit elastica formation in great vessel
cultures as it had already been shown to do in heart cultures.
The concentration used was 80 μg/ml i.e. a dose which in heart
cultures caused inhibition of elastica production but did not
affect cellular growth.

Results:- It appears that BAPN.HCl does prevent elastica
formation in cultures of great vessels. It does not have any
elastolytic activity, and it does not prevent the formation of
reticulin at this concentration. The cellular morphology is
the same as in the controls. Weigert staining showed that
while the central elastic laminae which were not affected by
trypsinisation are still strongly stained no new elastica has
formed around it as is the case in the controls (figs. 32 and
33). The layer of new reticulin described in the control
cultures (page 73) is also present in these test cultures (fig.
35).

Experiment VI   Effect of other compounds.

Once the effect of BAPN.HCl on these cultures was
known a number of other compounds were tested under the same
conditions. In each case the dose used was the mole equivalent
of the effective dose of BAPN.HCl i.e of 80 μg/ml. The
concentrations used and the effect on elastica formation are
shown in table XIII. In no case was there any obvious elasto-
lytic activity, nor was there inhibition of reticulin formation
except when the culture was killed by the test substance.

Table XIII   Effect of mole equivalent concentration
of a number of substances in inhibiting the formation of new
Weigert positive material in chick embryo and great vessel
cultures.

TABLE XIII

Compound	Concentration in Medium	New Weigert positive material
BAPN.HCl	80.0 µg/ml	absent
AAN. SO$_4$	118.0 µg/ml	absent
Semicarbazide HCl	80.3 µg/ml	absent
CH$_3$ CO. NHCH$_2$ OH	74.0 µg/ml	present
HCl H$_2$N - CH$_2$ CONH$_2$	80.2 µg/ml	present
Chloracetamide	70.8 µg/ml	Explant killed

It will be seen that those substances above the dotted line inhibit elastica formation without disturbing cellular growth. These substances have all been shown to have lathyrogenic activity _in vivo_ and have also been shown to inhibit elastica formation in heart tissue cultures. None of the substances below the line inhibits elastica formation; chloracetamide is cytotoxic. None of these latter compounds has been shown to have any lathyrogenic activity _in vivo_.

Comparison of in vivo and in vitro effects

Active Compounds:- It has now been shown that elastic tissue can be found in tissue cultures and that this formation can be inhibited by the presence in the medium of substances known to produce lathyrism _in vivo_ but not by corresponding concentrations of chemically related non-lathyrogenic substances. Comparison of tables VII, XII and XIII which give the effect of lathyrogenic agents in mice, in cardiac tissue cultures and in aortic tissue

cultures respectively shows a very close agreement between _in vivo_ and _in vitro_ activity.   A few compounds tested _in vivo_ were not tested _in vitro_ because there was no material left, conversely a few substances appear in the _in vitro_ tables which could not be tested _in vivo_ because they are powerful neuro-toxins.   Excluding these however, it will be seen that those substances which produce lathyrism, also constantly inhibit elastica formation.

Concentration:-   If the mechanism of inhibition of elastica formation _in vitro_ is the same as that occurring in the aorta in mice with lathyrism then it would be reasonable to except that the required concentration of lathyrogenic agent _in vitro_ would correspond approximately with the tissue concentrations, of the same substances in the mouse.   An attempt was therefore made to calculate the tissue concentration which occurs in lathyritic mice.   For this the animals used were the pregnant mice, and the calculation was made from the minimum dose which produced aortic rupture in the young.   The total dose was then divided by the number of days that the treatment lasted since the high excretion rate, at least with AAN and BAPN (Ponseti et al. 1956a, Lalich 1958) means there can be no cumulative effect.   The total weight of the mouse was taken as 60 gms. - an average weight for female mice during the last two days of pregnancy - and the concentration expressed per gram of body weight assuming

a uniform distribution throughout the tissues. The figure for semicarbazide HCl is of course less accurate than the others because it was given orally. These figures are shown in table XIV for comparison with the effective doses in chick and mouse tissue cultures.

Table XIV. Comparison of the estimated minimum effective concentration of various compounds in the mouse with the minimum effective concentrations in chick and mouse heart cultures.

TABLE XIV

Compound	Estimated Min. Effective Tissue Concentration in Mouse Cultures	Min. Effective Conc. in Chick Cultures	Min. Effective Conc. in Mouse Cultures
BAPN.HCl	600 µg/gm	80.0 µg/ml	80 µg/ml
β-mercapto-ethylamine	166 µg/gm	17.0 µg/ml	-
Semicarbazide HCl	500 µg/gm	0.66µg/ml	-
AAN. SO$_4$	83 µg/gm	0.19µg/ml	-

It will be seen that the _in vivo_ figure exceeds the _in vitro_ one by at least 7½ times and in most cases by over 10 times. Since at least 80 - 95% of AAN and BAPN is excreted in 24 hours (Ponseti _et al_. 1956a, Lalich 1958) the figure calculated will only be achieved immediately following injection, and will fall to approximately a tenth of that figure before the next injection, on the other hand _in vitro_ the explant is exposed to the full

concentration throughout.   It does not seem therefore that the
figures are incompatible and there seems no reason to suppose
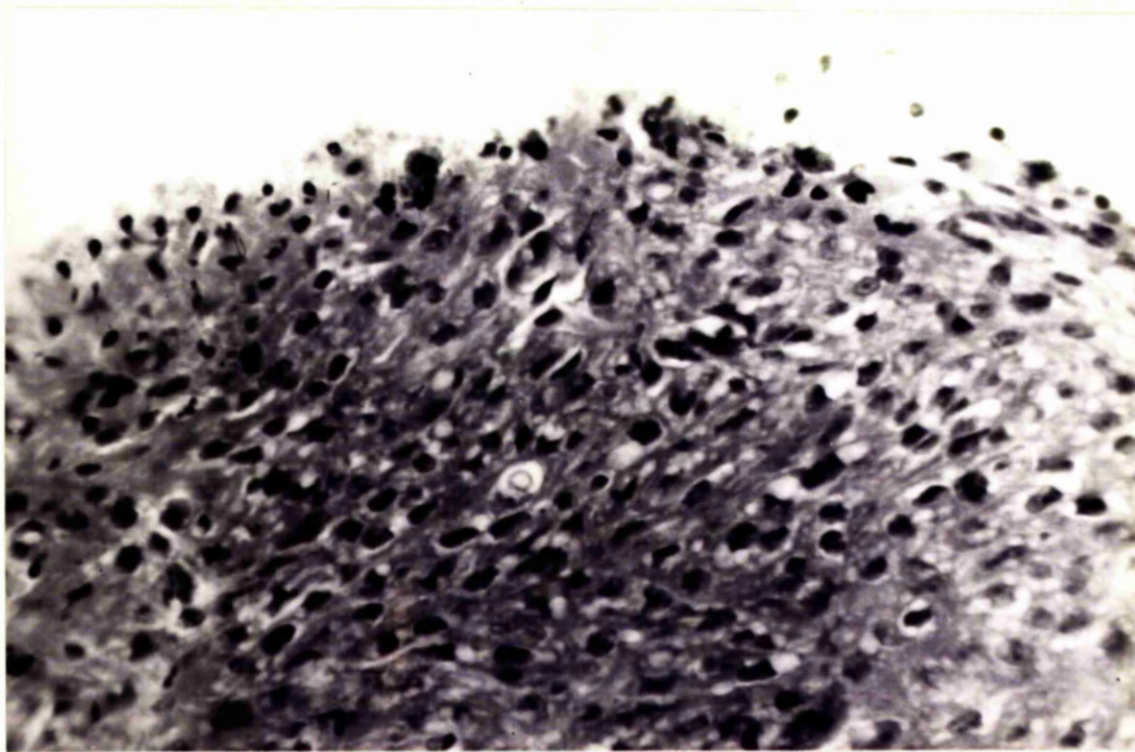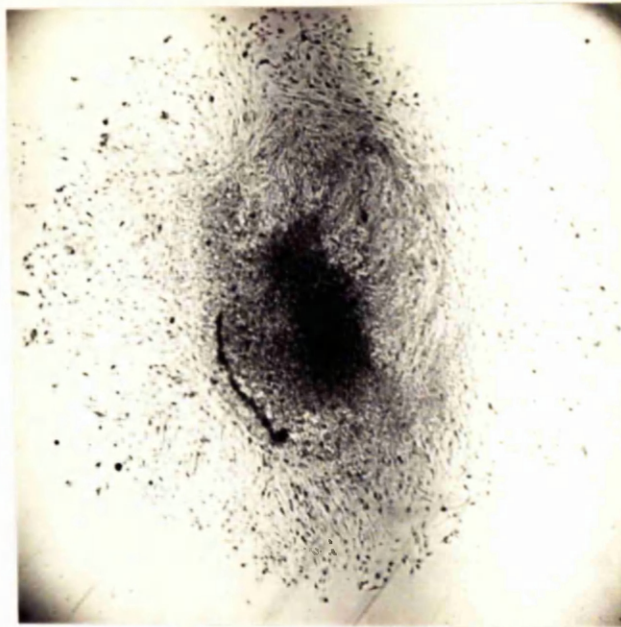that the same mechanism cannot be involved both _in vivo_ and _in
vitro_.

Fig. 23   Section of explant from heart of 12 day chick embryo.
          Most of the cells present are muscle cells.
          (X 630   H. & E.)

Fig. 24   The same chick heart culture photographed after 7 and
9 days culture respectively.   In both a fibroblast
ring can be seen with radially arranged fibroblasts
outside it.   Spreading continues inside the ring which
has a greater diameter in B. than in A.
(Unstained, both X 25)

Fig. 25   A chick heart explant grown on glass without any
          plasma clot for 10 days.   There is some
          detatchment from the glass at the lower part of the
          picture.   The fibroblast ring is well seen.
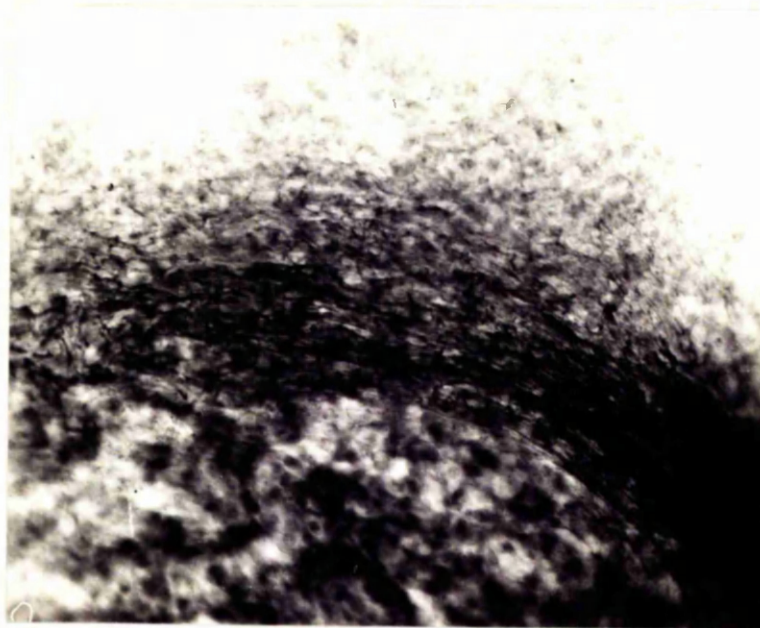   (X 30 Weigert's elastica)

Fig. 26   Detail of fig. 25 showing elastic fibres running in
          the fibroblast ring.   (X 200)

Fig. 27 Part of a chick heart explant grown directly on glass
in Waymouth's medium for 10 days.   The curving skeins
of reticulin fibres are seen to be much coarser than
the elastic fibres in fig.26 taken at the same
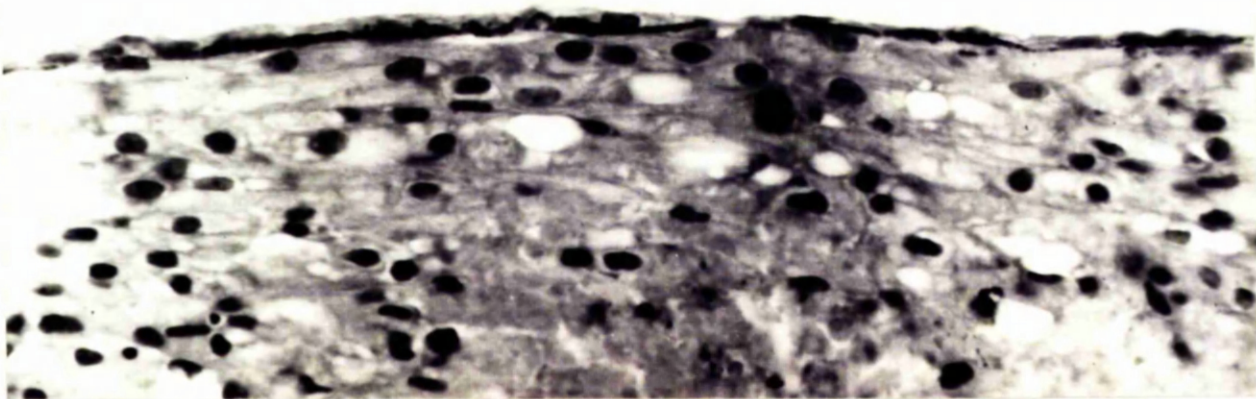magnification. (X 200 Silver stain for reticulin)

Fig. 28 Section through explant of chick embryo heart grown
for 10 days in plasma clot with BSS + 10% calf serum
as the fluid phase.   A layer of elastic tissue can
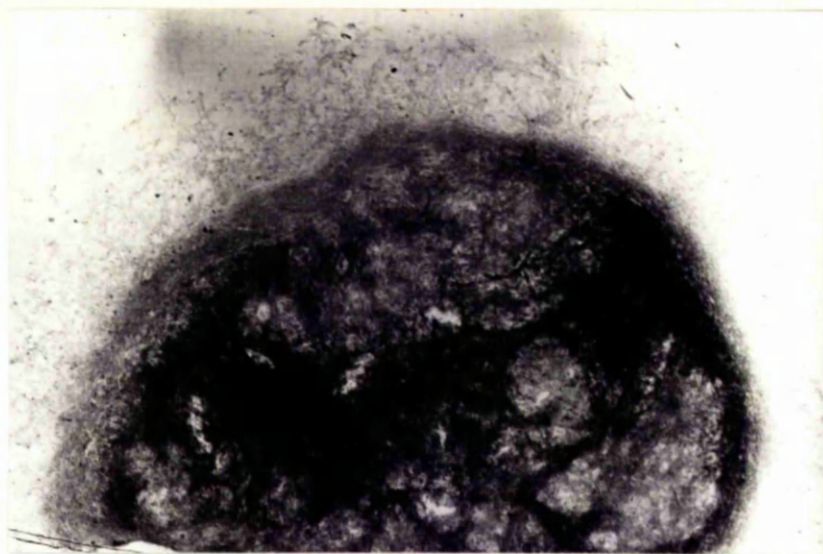be seen immediately beneath the surface cells.
(X 630 Orcein)

Fig. 29 Surface preparation of chick embryo heart culture
grown for 10 days in Waymouth's medium with added
BAPN.HCl (80 µg/ml).   The cellular morphology is
similar to that in the control cultures (figs. 22 and
23) but no elastic fibres are present.
(X 22 Weigert's elastica)

Fig. 30 Explant of great vessel from 14 day chick embryo
         before trypsinisation.   The elastic laminae are
         clearly seen.
         (X 210 Weigert's elastica)

A.
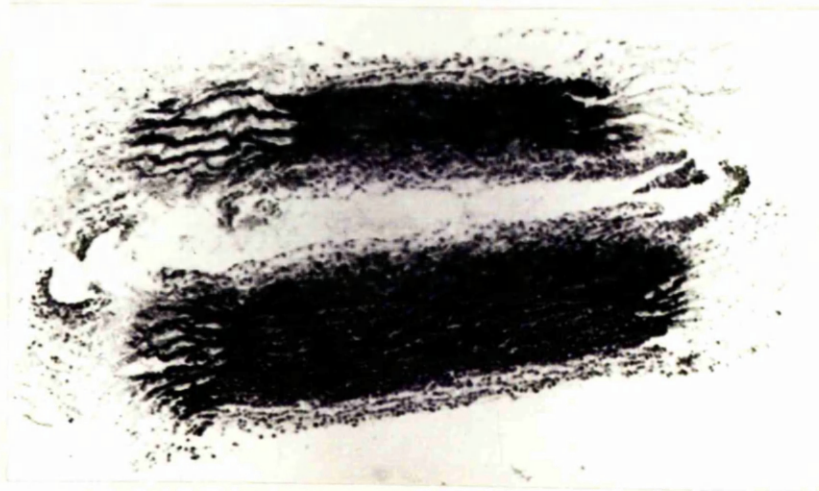
B.

Fig. 31   Explants of great vessel of 14 day chick embryo after
          trypsinisation, the one cut transversely and the other
          longitudinally.   The periphery no longer stains for
          elastica.
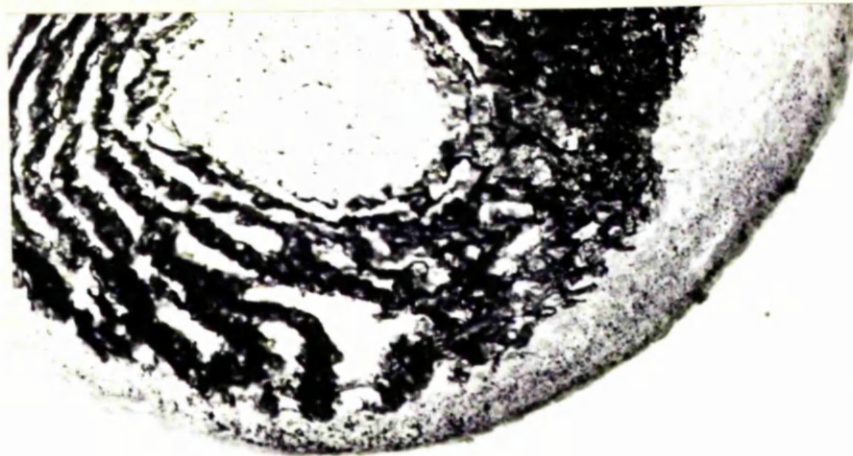   (X 360 Weigert's elastica)

Fig. 32 Section of great vessel explant grown for 3 days in
Waymouth's medium. The original elastic laminae
have not been altered, and in the peripheral zone
spots of Weigert positive material have appeared, most
dense immediately beneath the surface cells.
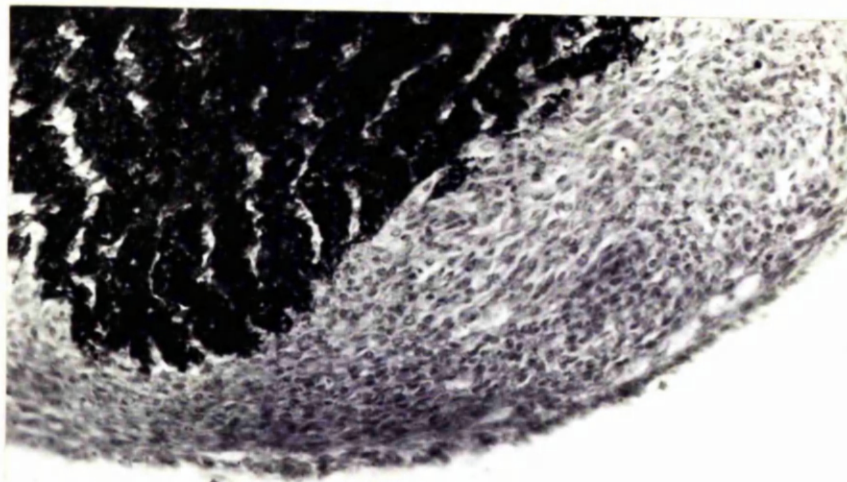(X 270 Weigert's elastica)

Fig. 33 Section of great vessel explant grown for 3 days in
Waymouth's medium with added BAPN.HCl. The original
elastic laminae are still present, but no new Weigert
positive material is present in the peripheral zone.
(X 270 Weigert's elastica)
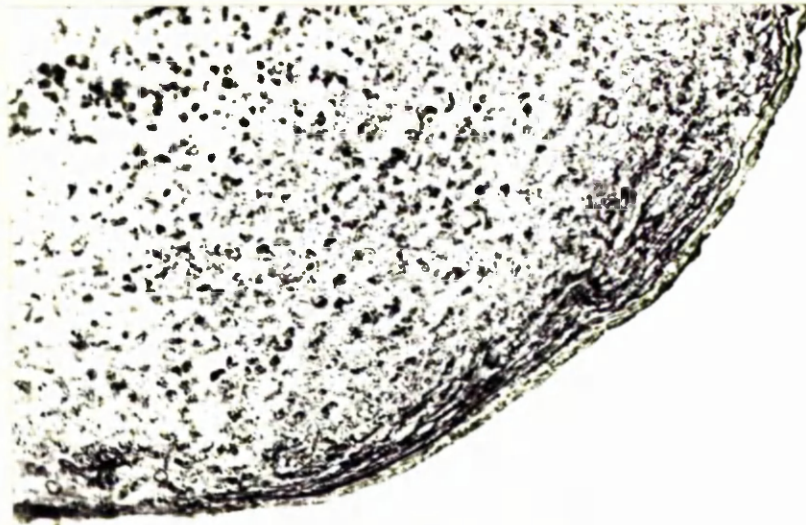
Fig. 34 Section through same explant as fig. 32 stained for
reticulin. Layers of newly formed reticulin can be
seen immediately beneath the surface.
(X 270)

Fig. 35 Section through same explant as fig. 33 stained for
reticulin. There is no evidence that the presence
of BAPN.HCl in the culture medium has inhibited the
formation of reticulin which is similar to that seen
in the control section (fig. 34).
(X 270)

Fig. 36   Periphery of mouse embryo heart tissue culture to
show the bizarre appearance of the cells.
(X 160 Weigert's elastica)
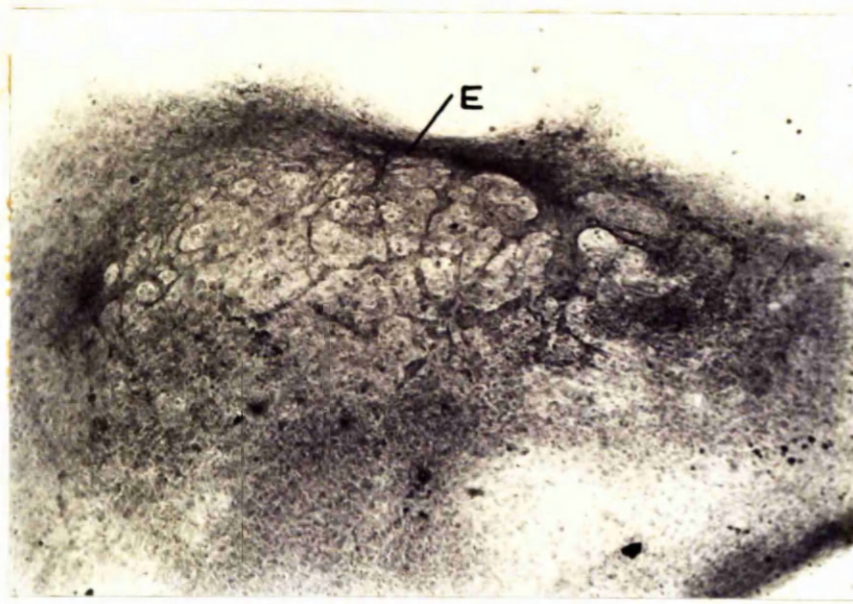
Fig. 37   Chick embryo tissue culture to show attempt at ring
formation.   Elastic fibres are present at part
marked E.
(X 55 Weigert's elastica)

Discussion

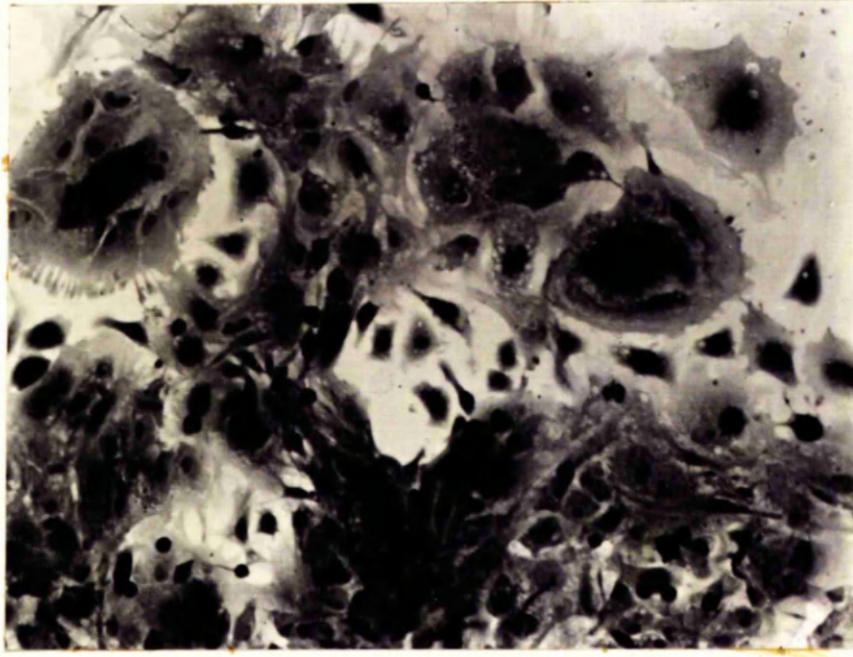It is clear from these results that material closely resembling elastic tissue can be formed in tissue culture, and that its formation is inhibited by lathyrogenic substances but not by chemically related non-lathyrogenic substances. From this it follows that lathyrogenic substances can, (at least in the tissues tested) act on cells outwith the complex interplay of the organism. The action can be either by inhibiting the synthesis of an elastica precursor in the cell or on an extra-cellular intermediate element.

Lalich (1958) has suggested on the basis of studies with C^{14} labelled BAPN, that the active principle might not be the original compound but one of its metabolites. While my findings do not exclude this possibility they show that such a metabolite would have to be capable of being formed not only at some distant site e.g. the liver, but also locally in the affected cells.

GENERAL DISCUSSION

In this section I intend to discuss (1) the _structure_ of active lathyrogenic compounds in the hope that the recognition of a common grouping may suggest a possible common mode of action, and (2) the identification of the _site_ or sites of action in the production of lathyrism (a) in the aorta and (b) in the entire syndrome of bony lathyrism.

Structure of Lathyrogenic Compounds

Since most of the compounds tested for lathyrogenic activity in mice were the same as those tested for their ability to inhibit elastic tissue formation in tissue culture and since it has been shown (page 90) that activity _in vitro_ is closely parallel to activity _in vivo_ the results of these two sets of experiments may now be discussed together. The chemicals known to produce lathyrism fall conveniently into two groups.

1. BAPN and related compounds.

2. Semicarbazide and related compounds.

These two groups are so different chemically that it seemed possible that some difference might be observed in their action; no such difference has however been detected in these experiments. In spite of this the chemical difference is such that even if the effects produced are identical the mode of action may still be different, and the two sets of compounds will therefore be discussed separately.

BAPN and related compounds (see fig. 1)   The fact
that BAPN and AAN, which contain 2 and 1 CH_2 groups respectively
are positive while γ aminobutyronitrile with 3 CH_2 groups, is
negative suggests that the chain length in a straight chain
must not exceed 2 CH_2 groups.   This is borne out by the
experience of other investigators who have failed to produce
lathyrism with any compound containing more than 2 CH_2 groups
in a straight chain.   Cyanamide with only one carbon atom has
also been shown to be ineffective (Ramamurti and Taylor 1959).

The positive result obtained with aminodipropionitrile
shows that two short chains may be present.   My result here
confirms that of Bachhuber et al. (1955) and is in opposition to
Selye's (1957) finding.   The possibility, suggested by Selye,
that the material might be impure and contain some BAPN has been
considered, but will be seen to be an unsatisfactory explanation.
If the lathyrogenic activity is to be attributed solely to the
presence of BAPN, and if, as is my finding (page 32) a minimum
of 120 mg. of BAPN.HCl must be given to the mother to produce
aortic rupture in the young, and 0.12 ml. of aminodipropionitrile
also produces rupture, then there must be approximately 120 mg.
of BAPN present as a contaminant in 0.12 ml. of aminodipropio-
nitrile (page 32) which is clearly untenable.   It is thus clear
that aminodipropionitrile is active when given parenterally, but
it is still probable that the effective compounds are breakdown

products derived from it.   The two very different actions
possessed by this drug would be adequately explained if it
could be assumed that the neural symptoms result from the amino-
dipropionitrile itself, and the osteo-lathyrogenic action from
DAPN. or AAN. derived from it.

It would appear however that a straight chain or a
combination of two straight chains is essential, since nitriles
having an amino-group attached laterally in the α position as
in α aminobutyronitrile and α aminopropionitrile are inactive.
This is shown both in my own experiments where these compounds
were used attached to a glutamylo radical, and in the work of
Wawzonek et al. (1955).

It is thus established that these lathyrogenic
materials are short straight-chain compounds, and their
constituent grouping must now be considered.   The chain is
basically a hydrocarbon chain with at one end a nitrile (CN)
and at the other an amino (NH_2) group.   If the CN group is
replaced by an SH group, as in βmercapto ethylamine, it retains
its lathyrogenic activity.   If the CN group is replaced by any
other grouping (e.g. CH_3 in propylamine (page 31) or CH_2OH in
ethanolamine (page 34)) the activity is lost.   Although this
has only been shown using two substitutes for the nitrile
grouping, many others have been tested in rats by other workers
and only when the SH group is used is the specific activity of the

compound retained. As with the CN groups in aminodipropionitrile, the presence of two short chains does not diminish activity of the SH grouping since Dasler has shown that cystamine is lathyrogenic. This compound may be expected to break down in the body to form two molecules of β-mercapto-ethylamine. It should be noted however, that there is some difference of opinion as to whether β-mercapto-ethylamine is indeed effective - Levene (1961) could find no evidence of lathyrogenic activity. This question has not been clarified by the present investigation since a positive result was obtained in one test only (Table VII). It seems possible that the tendency of this compound to decompose rapidly may account for the contradictory results.

The testing of nitriles having a short straight chain, and some grouping other than NH_2 at the other end is more difficult since many of these are fast acting neural poisons. Methyline AAN in which the NH_2 group is replaced by a $CH_2 = N$ grouping has been reported by Ponseti et al. (1956) as being lathyrogenic. I have so far been unable to confirm this. If it is indeed active it seems probable that it is converted into AAN in the body. Succinonitrile where the NH_2 group of AAN has been replaced by a CN has been shown to be inactive (Ramamurti and Taylor 1959).

The criteria of structure of an active compound of the amino-nitrile type would thus seem to be a short straight carbon

chain having 1 or 2, but neither fewer nor more, CH_2 groups, an
amino-group at one end and a cyanide or possibly a mercapto-
group at the other. Aminodipropionitrile and methyline AAN
are probably coverted into either BAPN or AAN and cystamine
into βmercapto ethylamine, and so need not confuse the picture.

Semicarbazide and Related Compounds (see fig. 39) It
is difficult to make a similar assessment of the chemical
structure required in order that a compound may act like semi-
carbazide. In my investigations only semicarbazide HCl has
been used and this has been shown to be effective in producing
both aortic and bony lesions. There is however a long list
of compounds related to semicarbazide which have been tested by
other workers. Those listed by Dasler and Stoner (1959) and
Milliser and Dasler (1959) as producing kyphosis and histological
aortic lesions when fed to rats can be compared with the list
of compounds tested and found ineffective by Ramamurti and
Taylor (1959).

It would appear from these results that much more
substitution is possible with the semicarbazide group of substances
than with the BAPN group. It seems possibly to replace or
partially replace either terminal NH_2 group and CO group
without altering the activity and only when the NH in the 2
position is replaced as in Glycineamide, is the compound
detoxicated.

Semicarbazide HCl 0.3% $NH_2CONHNH_2$ +++

Parahydrazino benzoic acid 0.1% $COOH\langle\rangle NHNH_2$ ++

Acetone semicarbazone 0.1% $NH_2CO.NH N:C (CH_3)_2$ ++

4, 4 diphenyl semicarbazide 1.0% $\langle\rangle N.CO.NH.NH_2$ +

1, 5 diphenylcarbazide 0.2% $CO(NH.NH.\langle\rangle)_2$ +

1, 3 diethyl 2 thiourea 0.1% $CS(NH C_2H_5)_2$ +

Thiosemicarbazide 0.1% $NH_2 CS NH. NH_2$ +

Glycineamide 2.0% $NH_2 CO CH_2 NH_2$ −

Guanylurea 2.0% $NH_2 CO NH_2 CN NH_2$ −

Aminoguanidine 0.5% $NH_2 CNH NH NH_2$ −

Hydrazine 0.2% $H_2 N NH_2$ −

Hydroxylamine 2.0% $H_2 N OH$ −

Phenylhydrazine 0.3% $H_2N NH \langle\rangle$ −

Methylamine 2.0% $H_2 N CH_3$ −

Fig. 39 Semicarbazide and related compounds.   To show
formula, percentage in diet and reported lathyrogenic
activity.

It is clear then that several substances are capable
of producing lathyrism. These may be divided into two major
sections having in common only the presence of one or more amino-
groups. The first section is characterised by having in
addition a short chain length and the presence of a cyanide or
mercapto-group. The second group appears to depend for its
activity on the possession of a NH_2 NH - grouping.

Identification of Site of Action in Aorta

It has already been shown that the main cause of
aortic rupture in lathyrism is a failure in the formation of ela-
stic laminae (page 59). Elastic tissue is generally agreed
to consist of two parts, one a mucopolysaccharide and the other
a protein - probably elastin (Thomas and Partridge 1960). It
follows that failure in the formation of either of these
components would result in weakness of the lamina. Since one
of the components is carbohydrate and the other protein it
seems that they must be synthesised by two different mechanisms;
it also seems that the two syntheses could occur simultaneously
or successively.

This hypothesis would explain the apparent contra-
diction between the observed facts that while in heart cultures
which previously contained no elastic tissue 10 days is
required before elastic fibres are formed, yet in great vessel
cultures from which some of the elastica has been removed by

elastase, well formed elastic tissue can be seen in 3 days.
It is well known that in, for example, healing wounds, mucopoly-
saccharide is deposited very rapidly; it is also well known that
elastic fibres take much longer to appear (Gillman, Hathorn and
Penn, 1957).   If it is assumed that in the great vessel cultures
elastase disrupts the elastic tissue, but that the component
parts remain in the tissues in non-staining form, then whenever
the rapidly synthesised mucopolysaccharide scaffolding is formed,
elastin will be available to be deposited on it.   In the heart
cultures, on the other hand, though mucopolysaccharide will
again be formed quickly, the specifically staining protein
fraction will have to be created de novo and this will account
for the 10 days delay before well formed elastic fibres can be
seen.   This hypothesis is in keeping with the work of Lansing
(195) and Adair et al. (1951) who found that the subcutaneous
insertion of plastic sponges containing elastin or elastolysate
encouraged the rapid development of new elastic tissue.

From this argument it follows that in lathyrism the
failure of formation of elastic fibres may be the result of an
inhibition of either the formation of mucopolysaccharide or the
union of mucopolysaccharide with elastin to form visible fibres.

This latter conception is comparable with that of
Kennedy and Kennedy (1962) who believe that in cartilage, lathyro-
genic factors act by blocking the combination of certain

sulphated mucopolysaccharides with non-collagenous protein, so
preventing fibrillogenesis.

Identification of Site of Action throughout the Body

The symptoms of bony lathyrism are at first sight
exceedingly diverse, but on closer inspection they are seen to
consist of lesions of elastica (aortic rupture, page 39) lesions
of collagen and possibly reticulin (increased solubility,
increased fragility of collagen, (Levene and Gross 1959) and
lesions of ground substance (alterations in cartilage, page
and in aorta, page 38).   It seems more likely that these are
all different aspects of one metabolic defect, than that
lathyrogenic substances act on each separately and differently:
a hypothesis which would explain all the changes of lathyrism
by the defective formation of a single group of substances is
now offered.

The Importance of the Mucopolysaccharide Component

It has already been deduced that the lesion of elastica
may be a fault in the union of elastin with mucopolysaccharide,
but may equally well be a fault in the formation of mucopoly-
saccharide itself.   The two other fibres possibly involved in
lathyrism also depend for their integrity on mucopolysaccharide.
Collagen occurs in the form of parallel bundles (which are thought
to be bound together by some cementing substance) in a matrix of
mucopolysaccharide (Randall 1948); reticulin fibres on the other

hand are embedded in sheets of amorphous carbohydrate material, the fibres running in every direction in one plane (Kramer and Little 1953). The positive reaction given by reticulin with PAS is presumably the result of this carbohydrate matrix, and strongly suggests that it contains mucopolysaccharide.

There is no evidence of failure of formation of either of these two fibres from my experimental results; no definite conclusion could be reached about the synthesis of collagen, and reticulin formation was not inhibited in vitro (page 87). There is however evidence from electron micrographic studies that epiphyseal cartilage of lathyritic rats is virtually devoid of the normal collagen fibrils (Follis and Tousimis 1958). The same authors, and Castellani and Castellani-Bisi (1958) have also shown that, paradoxically, there is no fall in the same cartilage in the level of hydroxyproline, an amino acid which does not occur to any appreciable extent in any normal tissue except collagen and reticulin. From this it seems that a demonstrated absence of collagen fibres does not necessarily mean an absence of the tropocollagen molecule.

The only alteration in reticulin which has been reported is of an increase and coarsening of the reticulin network in the aorta in lathyrism (Menzies and Mills 1957). This was estimated by examination of silver preparations which, like the PAS method may demonstrate the carbohydrate component (Glynn 1957).

There remains one substance which is strikingly
altered in lathyrism, that is the ground substance as it occurs
in both the aorta and the cartilage, in both of which sites it
is known to consist largely of chondroitin sulphate (Mörner,
1895).   The change which occurs may not, however, be the same,
for in the aorta there is generally agreed to be an increase in
metachromatic ground substance (Menzies and Mills 1957) and
probably also of a different PAS positive polysaccharide
(Churchill et al. 1955), while in the epiphyseal plate, though
the total ground substance present must be much increased, the
sulphated mucopolysaccharide content per unit weight appears to
be reduced (Bauer, Carlsson and Lindquist 1955).   The apparent
contradiction between this and the histological increase in
polysaccharide in the aortic wall may possibly be explained
when biochemical estimations of lathyritic aortas are available.

At the moment the only definite fact is that a change
occurs in the mucopolysaccharide ground substance in both the
sites where lathyrism is most damaging.

It seems then that since all the connective tissue
fibres probably depend for their integrity on mucopolysaccharide,
and since mucopolysaccharide forms a large part of ground subst-
ance, a single fault in the formation of mucopolysaccharide might
easily explain all the diverse symptomatology of lathyrism.

Such a fault has already been demonstrated in lathyritic epiphyseal cartilage by Pedrini and Pedrini-Mille (1959) who showed a dramatic fall in the _in vitro_ formation of hexosamine. Since hexosamine is a major and essential part of both acid and neutral mucopolysaccharide future investigations may show that it is by this single fault that all the symptoms of lathyrism are produced.

C O N C L U S I O N S

It is concluded that:-

1.   Experimental osteo-lathyrism can be produced in the mouse.

2.   The lesion in the aorta is a malformation and not a degeneration because it is most easily produced during the period of maximum growth.

3.   _In vitro_ the synthesis of elastica is inhibited by the same substances that produce lathyrism _in vivo_.

4.   This inhibition of synthesis agrees with the theory of malformation, and therefore the assumption is justified that the same mechanism which has been shown to operate _in vitro_ also operates _in vivo_.

S U M M A R Y

Experimental lathyrism has been studied _in vivo_ and
in vitro.

In vivo it has been produced in mice and has been
shown to be the same disease as in rats, though mice are slightly
less susceptible.   Histologically the main cause of the aortic
rupture is thought to be a defect in the elastica.   The
process leading to the defect is a malformation and not a
degeneration;   the period at which rupture can most readily be
induced coincides with the phase of maximum growth;   adult
animals are almost completely refractory.

In vitro the synthesis of elastica by tissue explants
was found to be inhibited by lathyrogenic substances.   In
blank controls and in the presence of compounds similar in
structure but known to be non-lathyrogenic _in vivo_, elastica was
formed.

The chemical structure of lathyrogenic substances is
discussed.

It is suggested that inhibition of synthesis of hexo-
samine may account for the defect in elastica and indeed may
explain the whole syndrome of lathyrism.

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