

CLXXIII. THE SILVER NITRATE STAINING REACTION FOR ASCORBIC ACID IN THE ADRENAL, PITUITARY AND OVARY OF VARIOUS SPECIES OF ANIMALS.

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SZENT-GYÖRGI [1928] has demonstrated that when a section of the adrenal of the ox is immersed in 0.4 % AgNO_3 solution, its cortex darkens owing to the deposition of metallic silver. The reduction, he concluded, was due to the presence of ascorbic acid (hexuronic acid). In view of the recent observations on the high and persistent antiscorbutic activity of ascorbic acid it became obvious that the above observation could be utilised in studying the function of ascorbic acid in the development of scurvy. The experiments to be described here were originally undertaken by Zilva with the hope of obtaining information concerning the problem of the identity of ascorbic acid with vitamin C. In this respect suggestive but not conclusive evidence has so far been obtained from this investigation, but some facts have emerged which we consider of sufficient interest to be put on record.

In preliminary experiments guinea-pigs on a scorbutic diet receiving various doses of decitrated lemon juice and others subsisting on a diet containing cabbage *ad lib.* were examined. It transpired that although the cortex of the adrenals from the animals receiving cabbage *ad lib.* darkened markedly on immersion in the AgNO_3 , this was not so in the case of the adrenal glands from animals which were receiving daily doses of as much as 10 cc. of decitrated lemon juice, although the guinea-pigs were found at the *post mortem* examination to be in excellent condition and free from scurvy. Even the microscopical examination of sections of the glands treated with AgNO_3 did not reveal any deposition of Ag in the adrenal cortex of the guinea-pigs protected with high doses of decitrated lemon juice. Yet histological sections of the adrenals from these animals did not show any abnormality.

The search when extended to other organs from various species of animals, irrespective of their susceptibility to scurvy, revealed that certain other organs showed the AgNO_3 staining reaction macroscopically, especially the anterior and intermediate lobes of the pituitary gland, which in some species reduced the reagent even more markedly than the cortex of the adrenal. The disparity in the intensity of staining in these two glands was particularly marked in the human subject, where with very few exceptions the anterior lobe of the pituitary

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was found to stain more intensely than the adrenal cortex. In the case of the ox the anterior lobe of the pituitary was moreover found to be antiscorbutically very active. As had been found in the guinea-pig, in many of the human cases examined the adrenal cortex did not stain, although the patients were free at least from clinical symptoms of scurvy.

EXPERIMENTAL.

Technique.

The scorbutic diet employed in these experiments was similar to that already described [Bracewell, Hoyle and Zilva, 1930] and was complete in all essentials apart from vitamin C. Freshly prepared decitrated lemon juice was administered daily, except during the week-end, allowance being made for this shortage during the remaining days of the week. The guinea-pigs on the mixed diet received oats, bran and cabbage *ad lib.*

The silver staining was carried out in the dark since the gland reticulum stains in the light independently of the presence of ascorbic acid. The slices were placed in 0.4 % AgNO_3 solution for 15 minutes, after which time they were fixed in a 5 % solution of sodium thiosulphate for 10 minutes. They were then washed in several changes of distilled water and stored in 50 % alcohol. The assessment of the intensity of the staining was carried out with the help of a hand lens. For the microscopical examination the material after treatment with absolute alcohol for 3 hours was cleared and embedded in paraffin, and serial sections were made without further staining.

For general histology, sections fixed in formalin and embedded in paraffin were stained with haemalum and eosin. Fat was examined in frozen sections stained with Sudan III and haemalum. The chrome reaction was carried out by Ogata's method [1917], and Gough and Fulton's mitochondrial method [1929] was used in the cytological examination.

The silver nitrate staining reaction of the organs of various species of animals.

Man. Sometimes there was some staining in the cortex of the adrenals but it was in most cases comparatively slight. In several cases there was distinct staining of the medulla even when the cortex showed none at all. The anterior lobe of the pituitary stained more markedly than the adrenal. Staining sometimes occurred in the ovary, and in one or two cases it was confined to the linings of the larger follicles. The corpus luteum of pregnancy in two cases showed uniform but slight staining.

Ox. The cortex of the adrenals stained markedly. In fresh glands there was very little staining of the medulla but in glands from animals killed about 3 to 6 hours previously the medulla also stained appreciably. The anterior lobe, pars intermedia and cone of Wulzen of the pituitary stained very darkly. In cases in which the adrenals and pituitary from the same animal were compared, the latter almost invariably stained the more intensely. In the ovary (two heifers) there was slight staining of the corpora lutea, but not in the rest of the gland. In the testicle (one animal) there was slight staining.

Dog (5 animals). In the adrenal the cortex and the medulla stained deeply. The anterior lobe and the pars intermedia of the pituitary stained very intensely. The corpora lutea of the ovaries also stained quite darkly.

Cat (2 animals). Staining of the adrenal cortex and of the anterior lobe of the pituitary was well marked. In one animal the staining of the two organs

was of about equal intensity, and in the other the pituitary was slightly darker than the adrenal. The adrenal medulla showed only slight staining in each case.

Guinea-pig. The adrenal stained quite darkly in most instances. The anterior lobe of the pituitary also showed definite staining which in some instances was equal to that of the adrenal but in others was somewhat less. The ovaries showed a slight diffuse staining. The staining reaction in this animal will be dealt with more fully in the next section.

Rat. The cortex of the adrenal stained very intensely. In only one out of five animals there was slight staining found in the medulla. Staining of the adrenals was also found in rats which had subsisted for about 8 weeks on a diet free from vitamin C [cf. Moore and Ray, 1932]. The pituitary also showed distinct staining but it was never quite as dark as that of the adrenal. The ovary showed slight diffuse staining.

It is to be pointed out that, contrary to expectations, in no single case did the livers from the above species of animals stain with silver nitrate.

The silver nitrate staining properties of the adrenal glands of guinea-pigs protected from scurvy with decitrated lemon juice.

A few representative cases will be described in this connection. In the earlier experiments (B 24, B 44 and B 23) the adrenal glands were not examined microscopically for silver staining but only by means of a lens. They are included here because some of these guinea-pigs were on their respective diets for a number of months. Moreover the assessment by the macroscopical method was found to be as reliable as the microscopical examination of sections. Although the medulla of the adrenal also stained in the animals receiving cabbage *ad lib.* the staining never approached that of the cortex in intensity.

B 24. This animal subsisted on a scorbutic diet and a daily dose of 1.5 cc. of decitrated lemon juice from December 8th, 1931, to December 31st, 1932, when it was killed by chloroform: initial weight 290 g.; final weight 515 g. At the autopsy, apart from slight ridging at one of the costochondral junctions, no macroscopic signs of scurvy were to be observed. The adrenal glands did not stain with AgNO_3 . The histological examination of sections stained with haemalum and eosin revealed that there was deep congestion but no other abnormality. These glands were however somewhat enlarged.

B 44. This animal subsisted on a scorbutic diet and a daily dose of 10 cc. of decitrated lemon juice from March 3rd, 1932, to September 21st, 1932, when it was killed by chloroform: initial weight 260 g.; final weight 660 g. No macroscopic signs of scurvy were observed at the autopsy. The adrenals did not stain with AgNO_3 . The histological examination revealed nothing abnormal.

B 23. This animal, which subsisted on a mixed diet containing cabbage *ad lib.*, weighed 650 g. when chloroformed, about the same weight as B 44. Its adrenal cortex however stained very darkly with AgNO_3 . The histological appearance of the glands was quite normal.

B 33. This animal subsisted on a scorbutic diet and a daily dose of 10 cc. of decitrated lemon juice from April 15th, 1932, to March 1st, 1933, when it was chloroformed: initial weight 265 g.; final weight 600 g. There were no macroscopic signs of scurvy at the autopsy. The staining of the adrenals with AgNO_3 was so slight that it could not be perceived with the naked eye although a trace could be discerned with a lens. Histological and cytological appearances and the chrome reaction were normal. The pituitary did not stain with AgNO_3 .

B 34. This animal subsisted on a scorbutic diet and a daily dose of 10 cc. of decitrated lemon juice from June 6th, 1932, to March 1st, 1933, when it was

chloroformed: initial weight 375 g.; final weight 560 g. There were no macroscopic signs of scurvy at the autopsy. The adrenals did not stain with AgNO_3 . Histological and cytological appearances and the chrome reaction were normal. AgNO_3 staining of the pituitary could only be observed with the help of a lens.

B 58. This animal subsisted on a scorbutic diet and a daily dose of 10 cc. of decitrated lemon juice from January 3rd, 1933, to March 1st, 1933: initial weight 300 g.; final weight 340 g. There was a check in the growth of the animal during the first month, which was apparently due to an intercurrent disease from which the guinea-pig recovered. There were no macroscopic signs of scurvy at the autopsy. The adrenals did not stain with AgNO_3 . Histological and cytological appearances and the chrome reaction were normal. The AgNO_3 staining of the pituitary could only be observed with the help of a lens.

B 59. The dietetic history of this animal is the same as that of B 58: initial weight 285 g.; final weight 470 g. There were no macroscopic signs of scurvy at the autopsy. As in B 34 the AgNO_3 staining was so slight that a trace could be discerned only with the aid of a lens. Histological and cytological appearances and the chrome reaction were normal. The AgNO_3 staining of the pituitary could only be observed with the help of a lens.

B 17. This animal, which subsisted on a mixed diet with cabbage *ad lib.* and weighed 720 g. when chloroformed, was used as a control for B 33, B 34, B 58 and B 59. The cortex of the adrenal gland and the anterior lobe of the pituitary from this animal, in contradistinction to the aforementioned animals, stained very darkly. The histological and cytological appearances and the chrome reaction were normal as in the case of the guinea-pigs which received decitrated lemon juice.

The adrenals from guinea-pigs in advanced stages of scurvy did not stain with silver nitrate [*cf.* Moore and Ray, 1932; Miller, Siehrs and Brazda, 1933; Siehrs and Miller, 1933] but, unlike the above specimens, they deviated from the normal macroscopically and especially histologically, showing gross haemorrhages and foci of necrosis.

The absolute diameter of the fat zone of the adrenals showed no appreciable differences in the above animals (Table I). In B 17, B 33 and B 34 there was

Table I.

No. of specimen	Diam. cortex mm.	Diam. fat zone mm.	Fat content of cortex %
B 17	1.9	0.86	45
B 33	1.2	0.83	70
B 34	1.4	0.82	59
B 58	0.96	0.7	73
B 59	0.99	0.7	71

no abnormality in the appearance of the sections stained with Sudan III. In B 58 and B 59 the number of large fat droplets was greater than in the control glands of comparable size. The cells, however, were not distended, and the total amount of fat was only slightly greater than in the normal.

The antiscorbutic activity of the anterior lobe of the pituitary of the ox.

The very marked silver nitrate staining capacity of the anterior lobe of the pituitary suggested the necessity of assessing the antiscorbutic potency of this gland. The fresh pituitary from the ox which was always found to stain very darkly was therefore tested. Fig. 1 gives the growth curves and clinical signs

of guinea-pigs which received daily 0.25 g. of the anterior lobe of the gland, and for comparison those of guinea-pigs which received daily doses of 0.25 g. and 0.5 g. respectively of adrenal cortex from the ox [Zilva, 1932]. It will be seen that the condition of the animals on the 0.25 g. dose of the pituitary was as good as that of the guinea-pigs on the 0.5 g. dose of the adrenal cortex. The *post mortem* findings in these two groups were also similar. The pituitary was therefore found to be twice as active as the adrenal cortex. As the glands originated from different animals of unknown dietetic history it is impossible

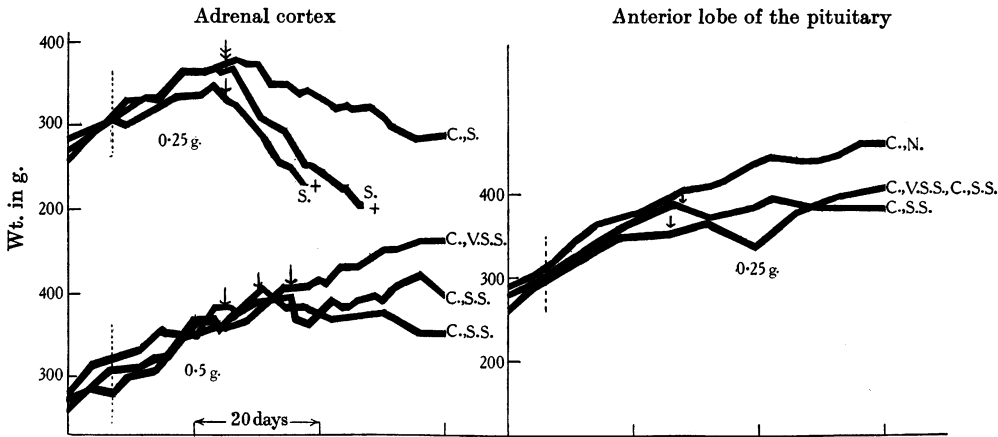


Fig. 1.

| = Beginning of dosing.
 C. = Chloroformed.
 + = Died.
 S. = Scurvy.
 ↓ = Onset of clinical symptoms of scurvy.
 S.S. = Slight scurvy.
 V.S.S. = Very slight scurvy.
 N. = Normal.

to say whether this relative antiscorbutic activity holds true always in the case of the ox. It is, however, definitely established that the anterior lobe of the pituitary, which when compared with decitrated lemon juice contains 40 to 50 international units of vitamin C per g. of fresh tissue, can be as potent or rather more potent than either of the two most active natural sources on record, namely, the Alphonso variety of mango [Perry and Zilva, 1932] and paprika [Svirbely and Szent-Györgyi, 1933]. It may be mentioned here that Agnoli [1932] did not find any antiscorbutic activity in desiccated pituitary glands.

The relative silver nitrate staining capacity of the adrenal and pituitary glands in man.

Reference was made in an earlier section to the fact that the anterior lobe of the pituitary in man stains more intensely than the adrenal cortex. This observation is brought out in detail in Table II. The dietetic history of the patients is rather vague since it was difficult to gauge the patients' daily food intake from the details that could be obtained, especially as regards vitamin C during the few months preceding death. Nevertheless it is striking that out of 42 patients, none of whom manifested clinical symptoms of scurvy, 23 showed hardly any silver nitrate staining (- or ±) in the adrenals and the highest score recorded for this gland was only ++. The anterior lobe of the pituitary on the other hand with very few exceptions always stained more intensely. In some

Table II.

No.	Age	Sex	Disease	Diet	Nutrition	Staining		Time of autopsy after death hours
						Pituitary	Adrenal	
37	55 years	F	Pernicious anaemia	Fluids	Fairly good	+	-	-
38	9 "	F	Cerebral abscess	Mixed diet	Good	+++	++	7
39	34 "	M	Chronic meningitis. Hydrocephalus	Mainly fluids	Good	++	±	14
40	71 "	M	Enlarged prostate. Pulmonary embolism	Light mixed	Very good	++	+	14½
41	5 days	F	Bronchitis	—	Good	++	±	24
42	5 years	F	Strangulation of intestine	Mixed diet	Fair	+++	-	57
43	55 "	M	Papilloma of bladder. Bronchopneumonia	Light mixed diet	Good	+	++	12
44	50 "	M	Carcinoma of pancreas	Mainly fluids	Very poor	±	-	3
45	45 "	F	Obstructive jaundice	Fluids	Very poor	±	-	38
46	57 "	M	Gastric ulcer. Pulmonary embolism	Fluids then light diet	Poor	±	-	19
47	45 "	M	Carcinoma of oesophagus	Fluids	Poor	+	-	18
48	67 "	F	Cerebral softening	Mixed diet	Good	++	-	10
49	1½ "	F	Tuberculous peritonitis and meningitis	Fluids	Very poor	-	-	9
50	12 "	M	Stenosis of oesophagus	Fluids	Very poor	±	++	5½
51	8 "	M	Mastoiditis. Meningitis	Fluids	Fair	++	±	38
52	56 "	M	Carcinoma of lung. Metastasis in brain	Light mixed diet	Good but had lost much weight	+	-	18
53	50 "	M	Ethmoiditis. Septic meningitis	Mixed diet then fluids	Poor	-	-	45
54	62 "	M	Carcinoma of stomach	Light mixed diet. Fluids for 1 week	Fairly good	++	-	16
55	16 hours	F	Laceration of tentorium cerebelli	—	Fairly good	++	±	28
56	43 years	F	Pneumonia. Strangulation of intestine	Fluids	Fair	+	Trace	33
57	0	F	Still born	—	Full term. Weight 8½ lbs.	++	+	24
59	1½ years	F	Tuberculous meningitis	Fluids	Good	+++	++	30
60	32 "	F	Abortion. Infection of uterus	—	Good	+++	++	15
61	42 "	F	Gummatous meningitis	Mixed diet	Obese	++++	±	10
62	14 "	F	Congenital hydrocephalus. Bronchopneumonia	Mixed diet	Fair	+	-	6
63	49 "	F	Myeloid leucaemia	Light mixed diet	Fairly good	++	-	22
64	1 day	F	Prematurity	—	Weight 5 lbs.	++	±	43
65	37 years	F	Acute myeloid leucaemia	—	Good	++++	+	6
66	33 "	M	Tuberculous pericarditis	Mixed diet	Good	+++	±	7½
67	42 "	M	Carcinoma of adrenals. Metastasis in brain	—	Fairly good	++	Involved in tumour	36
68	47 "	M	Cholecystitis. Duodenal ulcer	Light mixed diet	Very good	++	±	11
69	36 "	F	Gangrenous cystitis following pregnancy	—	Fairly good	+++	++	22
70	75 "	M	Carcinoma of colon	—	Very good	-	+	61
72	35 "	M	Malignant melanoma. Metastases in liver	—	Very poor	±	-	6
74	24 "	M	Cellulitis of face. Septic meningitis. Pyaemia	Mixed diet then fluids	Good	±	+	27

Table II (*contd.*).

No.	Age	Sex	Disease	Diet	Nutrition	Staining		Time of autopsy after death hours
						Pituitary	Adrenal	
75	5 months	F	Convulsions. Rickets	Condensed milk	Good	Trace	—	16
76	4 years	M	Pneumonia. Empyema	—	Fair	+	+	40
77	18 „	M	Abscess of lung. Empyema	—	Very poor	+	+	12
78	4 „	M	Sarcoma of jaw. Bronchopneumonia	Fluids	Poor	+	Trace	9
79	48 „	F	Obstructed labour. Uterine infection	Mixed diet	Obese	+++ at periphery + at centre	+	18
80	31 „	M	Abscess of liver. Peritonitis	Mixed diet then fluids	Fair	+++	+	—
81	25 „	F	Puerperal septicaemia	Mixed diet then fluids	Very good	++	+	36

— No staining.
 ± Slight staining barely perceptible to naked eye but recognised with lens.
 + Staining recognisable distinctly to naked eye giving greyish appearance.
 ++ Definite staining.
 +++ Marked staining.
 ++++ Extremely dark staining.

cases the disparity was extremely marked (42, 61, 65, 66, 79 and 80). It can hardly be explained by the fact that the adrenal is more perishable than the pituitary, since it occurs in some cases when the autopsy was carried out 6 and 10 hours after death (Nos. 61 and 65). Moreover, appreciable staining of the adrenal cortex was obtained in certain other cases when the period was longer (Nos. 59, 60, 69). It is also of interest to note that the reducing substance was present in the pituitary of a full term infant which failed to breathe (No. 57) and also in a premature (35 weeks) infant which lived 6 hours (No. 64).

CONCLUSIONS.

On the assumption that the substance reducing silver nitrate is ascorbic acid it would appear from the preceding experiments that it occurs in at least three tissues, namely, adrenal, pituitary and ovary of all animals whether they are susceptible to scurvy or not. It also transpires that the human subject and the guinea-pig may be free from any scorbutic symptoms and yet not show the presence of ascorbic acid in any of these organs by the method employed. In the case of the guinea-pig it has been demonstrated that even when the animals received a dose of decitrated lemon juice very much above the minimum protective dose for three months or longer no accumulation of ascorbic acid could be discovered by this method in the adrenal cortex or pituitary. If the absence of staining be an index of the total absence of ascorbic acid the experiments would suggest that its presence in these glands cannot be essential. The concentration of the reducing substance and of vitamin C in the adrenals and in the anterior lobe of the pituitary is rather striking since these two glands are physiologically related. Our present knowledge of this relationship is, however, too vague to justify us in attaching much significance at this stage of the inquiry to this apparently outstanding selective capacity for ascorbic acid. It is further shown that there is a rough parallelism between the intensity of staining and antiscorbutic activity in the anterior lobe of the pituitary gland

as well as in the adrenal cortex. On the other hand the liver, which contains vitamin C, although in smaller quantities than the above glands, does not stain with silver nitrate.

SUMMARY.

1. The adrenal cortex, anterior and intermediate lobes of the pituitary and the ovary from various animals, whether susceptible to scurvy or not, stain with silver nitrate in the dark. The liver, on the other hand, does not stain with this reagent.

2. The adrenals from guinea-pigs subsisting on a scorbutic diet *plus* high doses of decitrated lemon juice do not stain with silver nitrate although the animals are fully protected from scurvy and show no abnormalities macroscopically and microscopically at the autopsy.

3. The anterior lobe of the pituitary from the ox was found to be extremely potent antiscorbutically (40–50 international units of vitamin C per g. of fresh tissue).

4. The examination of the adrenals and pituitary glands from 42 human cases free from clinical scurvy revealed that, although in the majority of these cases the adrenals did not stain with silver nitrate, the anterior lobe of the pituitary did so in most cases; the intensity of staining being almost always more marked in the latter gland.

We should like to take this opportunity of expressing our indebtedness to Messrs Burroughs, Wellcome and Co. for the gift of the pituitary gland and to Miss O. E. V. Perry for helping with the tests.

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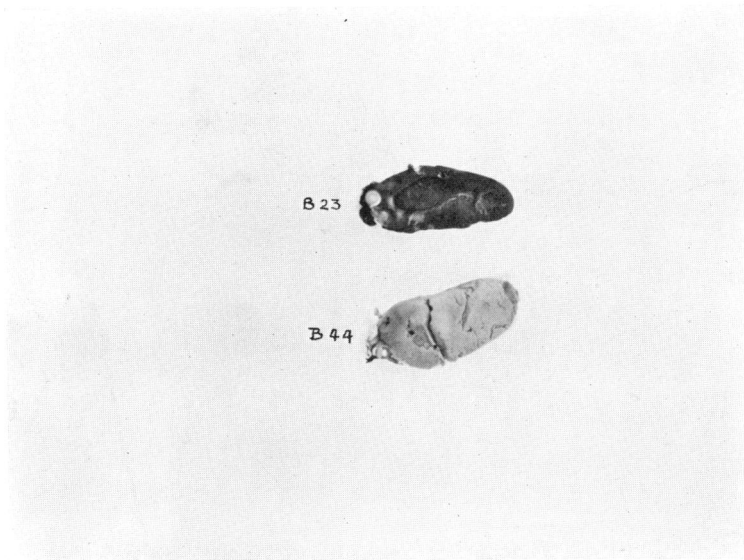


Fig. 1. B 23, B 44. Stained 15 mins. in 0.4 % AgNO_3 .

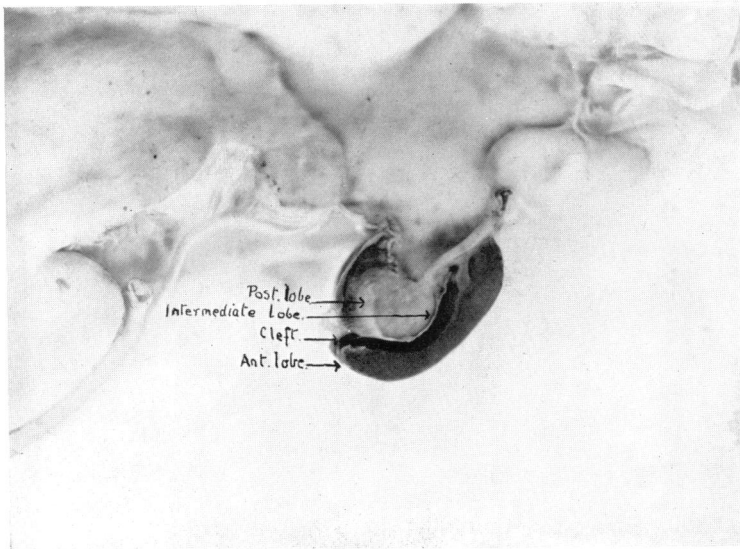


Fig. 2. Pituitary of dog. Stained 15 mins. in 0.4 % AgNO_3 . Showing intense staining of anterior lobe.