

The cariostatic effect of calcium sucrose phosphate in a group of children aged 5-17 years. Part IV

R. Harris, R. G. Schamschula, J. Beveridge and G. Gregory

The cariostatic effect of calcium sucrose phosphate in a group of children aged 5-17 years. Part IV

R. Harris, R. G. Schamschula, J. Beveridge and G. Gregory

The cariostatic effect of calcium sucrose phosphate in a group of children aged 5-17 years.* Part IV

R. Harris,† R. G. Schamschula,†† J. Beveridge‡ and G. Gregory‡‡

Introduction

A preliminary report on the planning and establishment of a clinical trial of the efficacy of calcium sucrose phosphate, in the form of a food additive, as a cariostatic agent was published in 1967.⁽¹⁾ The results after one year indicated reductions in the dental caries increment for all children in the ages 5-17 years.

A further report published in 1968⁽²⁾ covering the results observed after two years showed an overall reduction of 25 per cent in caries increment which was mainly demonstrated in the proximal surfaces of posterior teeth in the order of 50 per cent.

Initially 1506 children were examined and participated in this clinical trial. Normal exodus of children from the Homes was

anticipated and it was hoped that approximately 800 of the original subjects would remain in the trial for at least two years. Based on the data presented in Barnard's survey⁽³⁾ these numbers would yield significant results if the difference were of the order of 20 per cent of the Control Group score.

The loss of subjects in the first two years was approximately as anticipated; their replacements participated in the project and observations of their dental caries experience have been recorded.

The circumstances which determined the initial selection of institutions, namely numbers of boys and girls in appropriate age groups, accessibility to the source of supply and control of treated foods, and agreement to participate in the project, restricted the location and numbers of children. Factors beyond the control of the institutions and of ourselves have brought about greater losses in the numbers of subjects over the three years.

Table 1 shows the distribution of the children between Control and Treatment Groups over the period 1965-1968. It will be seen that the rate of loss was somewhat greater than expected and only 527 children of the original groups remained for the final examination at the end of three years.

The mean ages at the time of the first examination (1965) of these children were:

Control	(361)	12.0 years
Treatment	(166)	10.8 years

* Financial support for the conduct of this trial was given by the Colonial Sugar Refining Co. Ltd., Sydney, and the processing of the data was carried out in the Computer and Operations Research Services Group of that company.

† Director, Institute of Dental Research, Sydney.

†† Research Fellow in Dental Science, Institute of Dental Research, Sydney.

‡ Professor of Pædiatrics and Head of the School of Pædiatrics, University of New South Wales.

‡‡ Formerly Reader, Department of Statistics, University of Melbourne. Presently at Department of Operational Research, University of Lancaster, England.

⁽¹⁾ Harris, R., Schamschula, R. G., Gregory, G., Roots, Miriam, and Beveridge, J.—Observations on the cariostatic effect of calcium sucrose phosphate in a group of children aged 5-17 years. Preliminary report. *Austral. D. J.*, 12:2, 105-113 (Apr.) 1967.

⁽²⁾ Harris, R., Schamschula, R. G., Beveridge, J., and Gregory, G.—The cariostatic effect of calcium sucrose phosphate in a group of children aged 5-17 years. *Austral. D. J.*, 13:1, 32-39 (Feb.) 1968.

⁽³⁾ Barnard, P. D.—Dental survey of State school children in New South Wales, Jan. 1954-June 1955. N.H.M.R.C. Sp. Rpt. Series No. 8, Canberra, 1956.

TABLE 1
Numbers of children included in observations for the three-year programme who were present at all examinations 1965-1968

Date of examination	Control	Treatment	Total
1965	885	621	1,506
1966	632	408	1,040
1967	479	242	721
1968	361	166	527

Between the 1965 and 1966 examinations 245 additional subjects (Control 123, Treatment 122) entered the trial and were present at the 1967 examination; of these, 161 (Control 83, Treatment 78) were also present at the 1968 examination. Between the 1966 and the 1967 examinations 274 additional subjects (Control 136, Treatment 138) entered the trial and were present at the 1968 examination.

Of the 361 subjects in the Control Group 195 were members of the one boys' boarding-school participating in the trial. The reason for this disparity in average ages of the Control and Treatment Groups developing since the earlier examinations is largely the lower turnover rate of students at that school.

This difference in ages means that direct unweighted comparisons between the two groups could be misleading.

The conditions of the trial and examinations (both dental and medical) and the dietetic supervision have remained as described in the previous reports.⁽¹⁾⁽²⁾⁽⁴⁾ Medical evidence shows that the physical status of both groups remains similar and this will be reported in detail elsewhere.

Results*

All subjects

Table 2 presents a summary of the mean DMF teeth per child for all subjects who were present at two or more examinations. The annual increment in mean DMF teeth for each age group present at all examinations (1965-1968) should allow measurement of any overall difference between Control and Treat-

* Extensive supplementary tables are available from the authors.

⁽⁴⁾Harris, R., Roots, Miriam, Gregory, G., and Beveridge, J.—Calcium sucrose phosphate as a cariostatic agent in children aged 5-17 years. III. Dietary factors. Austral. D. J., 13:5, 345-352 (Oct.) 1968.

TABLE 2
Mean DMF teeth per child at each annual examination (All subjects 1965-1968)

Date of first examination	Date of subsequent examination(s)	N	Average age in 1965	DMF teeth 1965	Difference 1966-1965	DMF teeth 1966	Difference 1967-1966	DMF teeth 1967	Difference 1968-1967	DMF teeth 1968
1965	1966, 1967, 1968									
	C ..	361	12.0	8.67	2.45	11.12	2.23	13.35	1.61	14.96
	T ..	166	10.8	5.75	1.64	7.39	1.95	9.34	2.35	11.69
1965	1966, 1967									
	C ..	479	11.9	8.53	2.45	10.98	2.12	13.10	—	—
	T ..	242	11.4	6.85	1.71	8.56	1.89	10.45	—	—
1965	1966									
	C ..	632	11.7	8.19	2.45	10.64	—	—	—	—
	T ..	408	11.9	7.75	1.93	9.68	—	—	—	—
1966	1967, 1968									
	C ..	83	8.6	—	—	5.82	1.94	7.76	1.77	9.53
	T ..	78	10.4	—	—	6.18	2.01	8.19	2.19	10.38
1966	1967									
	C ..	123	9.1	—	—	6.59	2.05	8.64	—	—
	T ..	122	10.5	—	—	7.25	1.78	9.03	—	—
1967	1968									
	C ..	136	8.1	—	—	—	—	6.19	1.90	8.09
	T ..	138	9.3	—	—	—	—	7.87	1.90	9.77

C=Control subjects. T=Treatment subjects. N=Number of subjects.

TABLE 3
The increment of dental caries expressed as mean DMF teeth and DMF surfaces in the Control (189) and Treatment (132) groups of children aged 9-13 years for the years 1965-1968

Age at first examination	Group	N	Increments in DMF teeth and DMF surfaces per child							
			1965-1966		1966-1967		1967-1968		1965-1968	
			Teeth	Sur-faces	Teeth	Sur-faces	Teeth	Sur-faces	Teeth	Sur-faces
9 years ..	C	22	1.54	4.87	2.23	3.73	2.23	7.00	6.00	15.60
	T	19	1.05	2.26	1.05	2.27	2.00	4.05	4.10	8.58
10 years ..	C	24	1.12	2.87	2.88	5.63	3.17	7.42	7.17	15.92
	T	20	0.85	3.25	2.35	5.90	3.85	8.80	7.05	17.95
11 years ..	C	29	4.07	7.34	3.59	9.73	1.72	9.45	9.38	26.52
	T	31	1.87	4.68	3.16	6.39	3.19	8.42	8.23	19.49
12 years ..	C	29	3.04	8.07	3.10	8.41	1.48	5.66	7.62	22.14
	T	37	2.73	5.60	2.57	6.94	2.62	9.33	7.92	21.87
13 years ..	C	85	3.06	7.79	2.65	8.40	1.73	6.89	7.44	23.08
	T	25	2.09	6.30	1.00	4.74	1.48	5.83	4.57	16.87
9-13 years	C	189	2.57	6.19	2.89	7.18	2.07	7.28	7.53	20.65
	T	132	1.72	4.42	2.03	5.25	2.63	7.29	6.38	16.96
Difference (per cent)	DMF teeth		33.1	—	29.8	—	-27.3	—	15.3	—
	DMF sur-faces ..		—	28.6	—	26.9	—	-0.1	—	17.9

Difference= $\frac{100 \text{ (Average Control - Average Treatment)}}{\text{Average Control}}$
C=Control subjects. T=Treatment subjects. N=Number of subjects.

TABLE 4
The increment of dental caries expressed as DMF surfaces in the Control (335) and Treatment (147) groups of children for the years 1965-1968. (Non-fluoridated areas)
Increments in DMF surfaces for boys and girls

Sex	Age (1965) (years)	N	1965-1966	1966-1967	1967-1968	1965-1968	Difference (per cent)
Boys ..	5-10	C 56	4.07	3.94	5.29	13.30	22.78
		T 24	2.91	3.13	4.29	10.33	
Boys ..	11-17	C 239	7.20	7.97	7.29	22.46	10.55
		T 76	5.66	6.10	8.33	20.09	
Girls ..	5-10	C 21	3.76	5.00	5.81	14.57	3.43
		T 28	2.71	4.50	6.86	14.07	
Girls ..	11-17	C 19	6.58	7.11	6.47	20.16	18.55
		T 19	5.63	4.63	6.16	16.42	

Difference= $\frac{100 \text{ (Average Control - Average Treatment)}}{\text{Average Control}}$
C=Control. T=Treatment. N=Number of subjects.

ment Groups. However, since depletion of subjects has greatly reduced numbers in some age groups, Table 3 shows these data restricted to the ages 9-13 years (1965) where the number in *both* groups is at least 19.

The mean increment of DMF teeth for the Control Group is 7.53 and for the Treatment Group 6.38, a difference of 15.3 per cent; and of DMF surfaces for the Control Group is 20.65 and for the Treatment Group 16.96, a difference of 17.9 per cent.

Table 4* shows the increments in all surfaces for boys and girls present at all examinations (1965-1968) in the age groups 5-10 years and 11-17 years. The difference is greatest in the boys aged 5-10 years (22.78 per cent) followed by the girls aged 11-17 years (18.55 per cent).

If the results for the proximal surfaces in the bicuspid and molar teeth in the age groups for all children 9-13 years (Table 5) and for the boys and girls in the age groups 5-10 and 11-17 years (Table 6*) are examined, it will be seen that for children aged 9-13 years there is a reduction in the dental caries increment of 29.5 per cent for the period 1965-1968 (Table 5). For all children there is a range in reduction from 3.95-36.06 per cent (Table 6). It should be noted that for the boys aged 11-17 years (the group in which the largest numbers remained) the reduction 25.18 is significant at the 1.0 per cent level.

Subjects from fluoridated area

Four institutions (2 Control, 2 Treatment) were located in an area where fluoridated water supplies existed since November, 1961. These institutions were treated in the trial exactly as the other institutions, but because of the fluoride factor the results of the dental examination have not been included where significance tests were carried out in the previous sections of this report. A summary of the results in terms of age and DMF teeth is given in Table 7. It will be noted that the subjects of the Treatment Group had a lower increment of caries (4.21 DMF teeth) than those of the Control Group (5.85 DMF teeth), a difference of 28 per cent.

* This age grouping in Tables 4 and 6 was used in the previous report.⁽²⁾ Also in these tables, subjects at institutions in fluoridated areas were omitted in order to present homogeneous groups for statistical significance testing.

The mean DMF surfaces (all and proximal) of the remaining 45 children in the fluoridated area are compared with those of the non-fluoride groups in Tables 8 and 9. The increment of DMF surfaces for children aged 5-17 years in the fluoride area ranges from 10.27-18.45 in the Control and from 8.08-17.00 in the Treatment Group. In the non-fluoride area the corresponding ranges are 13.65-22.29 for the Control Group and 12.35-19.36 for the Treatment Group.

Discussion

The problem of loss of subjects from the trial has been referred to above. Nevertheless, sufficient remained to enable observations to be made which show a general benefit in terms of a lower dental caries incidence for the Treatment Group. This can be demonstrated by statistical significance tests in a number of cases.

TABLE 5
The increment of dental caries on the proximal surfaces of the bicuspid and molar teeth of 321 children aged 9-13 years for the period 1965-1968

Age (1965) (years)	N	Differences in mean DMF proximal surfaces per subject			
		1965- 1966	1966- 1967	1967- 1968	1965- 1968
9	C 22	1.32	1.27	2.45	5.05
	T 19	0.26	0.16	1.47	1.89
10	C 24	0.75	1.67	3.17	5.58
	T 20	0.70	1.80	3.45	5.95
11	C 21	3.17	3.28	3.90	10.34
	T 31	1.35	1.16	4.39	6.90
12	C 29	3.03	3.24	3.21	9.48
	T 37	1.05	2.16	4.46	7.68
13	C 85	2.64	3.89	3.33	9.86
	T 25	1.22	1.04	3.70	5.96
9-13	C 189	2.18	2.67	3.21	8.06
	T 132	0.92	1.26	3.49	5.68
Difference (per cent)		57.8	52.8	-8.7	29.5

Difference=
100 (Average Control - Average Treatment).
Average Control
C=Control subjects. T=Treatment subjects.
N=Number of subjects.

TABLE 6

The increment of dental caries on the proximal surfaces of the bicuspid and molar teeth of the 482 children aged 5-17 years for the period 1965-1968. (Non-fluoridated areas)
 Increments in DMF surfaces for boys and girls

Sex	Age (1965) (years)	N	1965-1966	1966-1967	1967-1968	1965-1968	Difference (per cent)
Boys ..	5-10	C 56	0.90	1.23	1.73	3.86	32.12 N.S.
		T 24	0.12	0.92	1.58	2.62	
Boys ..	11-17	C 239	2.90	3.22	3.53	9.65	25.18**
		T 76	1.26	1.50	4.46	7.22	
Girls ..	5-10	C 21	0.53	1.23	2.29	4.05	3.95 N.S.
		T 28	0.78	0.97	2.14	3.89	
Girls ..	11-17	C 19	2.00	3.53	2.79	8.32	36.06 N.S.
		T 19	1.21	1.06	3.05	5.32	

$$\text{Difference} = \frac{100 (\text{Average Control} - \text{Average Treatment})}{\text{Average Control}}$$

** Significant at 1% level.

N.S. = Not significant. C = Control subjects. T = Treatment subjects. N = Number of subjects.

All subjects

The New South Wales survey of Barnard (1956)⁽³⁾ showed that although the annual increments in DMF teeth were almost equal in the years 6-15 there was a temporary increase in the rate around the age of 11 or 12 years. Such considerations make direct comparisons of these DMF rates invalid, except perhaps in cases where the distribution of ages is similar in the two groups.

In subjects who were present at all examinations it will be seen (Table 2) that some of the reduction in caries increment gained in 1967 has apparently been lost, since the difference in the mean DMF teeth per subject between the 1967 and 1968 examinations is greater in the Treatment than in the Control Group. It is suggested that this largely arises from the differences in age of the two groups which, because of losses of subjects, have become greater as the trial continued.

At the final examination in 1968 the average age of subjects in the Control Group was 15 years, whilst for those in the Treatment Group it was 13.8 years. In the Control Group 126 children were 17 years of age or over at the 1968 examination compared with only 10 children in the Treatment Group. Since it can be expected that children over 16 years of age have a lower annual increment of DMF teeth than younger children, the age difference referred to above favours the Control Group. Figures for all subjects are in fact weighted means of the different age rates, the weights being the numbers in the group of each age.

When allowance for age imbalance is made, the data in Table 2 for subjects present at all examinations suggest that the reduction in caries increment, significantly demonstrated⁽²⁾ in the data from the 1965-1967 examinations, was maintained in the 1965-1968 examination.

TABLE 7

Mean DMF teeth per child of 45 children of both Control and Treatment groups living in a fluoridated area (1965-1968).

	Number of children	Average age (years)	DMF teeth per child				Difference 1965-1968
			1965	1966	1967	1968	
Control ..	26	9.8	3.50	5.23	7.42	9.35	5.85
Treatment	19	10.1	4.42	5.58	6.84	8.63	4.21

A comparison of increments in DMF teeth and surfaces (Table 3) shows a reduction of approximately 30 per cent for each of the first two years in DMF teeth followed by an increase of 27 per cent in the third year. DMF surfaces show a similar reduction followed by approximately equal increments in the third year.

TABLE 8
The mean DMF surfaces increment of all children (527) aged 5-17 years living in both fluoride and non-fluoride areas, 1965-1968

	Age (years)	Mean DMF surfaces increment			
		Fluoride		Non-fluoride	
		No.	DMFs.	No.	DMFs.
Control ..	5-10	15	10.27	77	13.65
	11-17	11	18.45	258	22.29
Treatment	5-10	13	8.08	52	12.35
	11-17	6	17.00	95	19.36
Total		45		482	

As a tooth needs only one DMF surface to be classified as DMF, the above data suggest that further surfaces have been attacked in the Control Group without appreciably increasing the DMF teeth rate. This indicates, as does the data from Table 2, that the curve relating DMF surfaces to age for the Treatment Group may have shifted positively by a distance of two years on the age scale.

The data in Table 5 again support the results reported⁽²⁾ for the two-year period, namely that there is a reduction in caries increment on the proximal surfaces, although this has fallen to 29.5 per cent by the end of the third year.

The average effects for the age range (9-13 years) produced reductions for the Treatment Group of:

- DMF teeth 15.3 (Table 3)
- DMF surfaces 17.9 (Table 3)
- DMF proximal surfaces 29.5 (Table 5)

Examination of data for DMF surfaces grouped on an age and sex basis for subjects present at institutions in the non-fluoridated areas (Tables 4 and 6) will show reductions in increments both for all surfaces and for proximal surfaces in the Treatment Group.

For the 11-17 years age group containing the largest numbers of boys (Table 6) statistical significance was found at the 1 per cent level. [Similar significance was found in the 1965-67 data (Table 4).⁽³⁾] In other cases where high reductions were obtained, for example 36.06 per cent for girls aged 11-17 years, the limited numbers in the group and the inherent variability in the data prevent the same degree of significance, as was found after two years, from being obtained.

For subjects present at the last two and three examinations only, DMF rates for both teeth and surfaces are shown in Table 2. Statistical significance is at the 5 per cent level for boys and girls aged 11-17 years but the numbers of subjects are small.

The reduction gained by the Treatment Group for these two years is less than that reported for the first two years. This may be due to the extra variability caused by the subjects entering the trial at any time between the 1966 and 1967 examinations, whereas all original subjects entered the trial at the same time.

TABLE 9
The mean DMF increment for the proximal surfaces of the bicuspid and molar teeth of all children (527) aged 5-17 years living in both fluoride and non-fluoride areas, 1965-1968

	Age (years)	Mean DMF surfaces increment			
		Fluoride		Non-fluoride	
		No.	DMFs.	No.	DMFs.
Control ..	5-10	15	2.27	77	3.91
	11-17	11	7.18	258	9.55
Treatment	5-10	13	1.85	52	3.31
	11-17	6	4.33	95	6.84
Total		45		482	

Subjects from fluoridated area

Because of the reduced numbers, statistical significance should not be placed on the figures from the fluoridated area. However, they are consistent with an overall benefit for fluoride and an additional benefit for the calcium sucrose phosphate additive which is of the same order as it was in the non-fluoride institutions. This follows the pattern of the significant results found in the two years' data.⁽³⁾

Sixteen subjects joined these Homes in 1966 and of these only one joined a Control Group Home, and therefore reliable comparison could not be made between new Control and Treatment subjects. In subjects who were present from 1966-1968 in the Treatment Groups the average increments for all DMF surfaces and DMF proximal surfaces were 8.28 and 3.56 respectively and corresponding values in non-fluoride groups were 13.06 and 5.57. This does not in itself add anything to the purpose of the trial, except that it is consistent with the accepted results from fluoride trials and is evidence of the general reliability of the trial data.

It is true that in a large and complex trial in which subjects leave and enter the study it is possible to select and interpret data which apparently confirm a preconceived hypothesis. In our report for the first two years⁽²⁾ it was noted that the Groups initially had a difference in mean DMF teeth per subject but the initial erupted caries-free teeth per subject were almost identical (12.95 for Control Group and 12.96 for Treatment Group). Furthermore, at the end of two years the mean number of teeth per subject was 24.76 and 24.82 respectively and the difference in the proportion of new carious teeth was 40.4 per cent in favour of the Treatment Group.

TABLE 10
Comparison of DMF increments as a proportion of available surfaces and teeth for different populations in trial 1965-1968

Population	Average age at initial examination		Number of subjects		DMFS*/100 available surfaces				DMFT*/100 available teeth			
	C	T	C	T	C	T	C-T	$\frac{C-T}{C}$	C	T	C-T	$\frac{C-T}{C}$
1 1965-1968	12.0	10.8	361	166	17	14	3	17	34	27	7	20
1' 1965-1967	11.9	11.2	480	242	11	9	2	20	26	19	7	28
1'' 1965-1966	11.7	11.9	632	408	7	5	1	20	17	13	4	23
1''' 1966-1967	12.5	12.3	603	364	7	5	1	22	16	12	4	28
2 1966-1968	9.6	11.4	83	78	12	10	2	20	26	20	6	22
3 1967-1968	10.1	11.3	136	138	7	6	1	15	15	13	2	17

C=Control subjects. T=Treatment subjects. $\frac{C-T}{C}$ =Percentage reduction due to treatment.

* All figures of DMFS and DMFT rounded off to nearest whole numbers.

Population 1 consists of all children present at all four examinations.

1' consists of all children present at the first three examinations.

1'' consists of all children present at the first two examinations.

1''' consists of all children present at the second and third examinations.

Thus population 1 contains some of the children who would also have been in populations 1', 1'' and 1'''.

Population 2 consists of children who were present at the last three examinations but not the first.

Population 3 consists of children who were present at the last two examinations but not the first two.

Thus populations 2 and 3 are entirely different populations from one another and from population 1.

APPENDIX A

From the subjects present and examined throughout the trial two groups of 92 were selected from the Control and Treatment groups. Each of these two groups had a mean age of 10.6 years and a mean DMF surfaces per subject of 11.36 at the initial examinations. The caries incidence for these subjects during the trial was :

		Examina- tions	1965	1966	1967	1968	Difference (per cent)
DMF surfaces ..	Control ..		11.36	16.87	23.00	29.22	17.80
	Treatment		11.36	15.70	20.00	26.04	
DMF teeth ..	Control ..		4.90	7.36	9.80	11.62	16.60
	Treatment		5.35	6.97	8.75	10.95	
DMF surfaces (proximal bicuspids and molars)	Control ..		3.40	5.09	7.12	9.78	31.70
	Treatment		3.76	4.64	5.45	8.33	

It is important to reiterate that because of increased imbalance in age and the decreased numbers in the Groups after three years, the major analysis has been applied to the age groups 9-13 years.

However, an alternative to the method of analysis we have used is based on DMF teeth and surfaces as a proportion of available teeth and surfaces and which is less subject to variation with age and DMF per subject.

If the analysis is made on data based on caries-free teeth at the initial examination which became carious during the trial, the results (Table 10) closely approximate those which we have demonstrated above. This table summarizes data for the subjects present for different annual periods throughout the trial. It is submitted as further evidence. This analysis has been extended to the data on proximal surfaces of bicuspid and molar teeth and also supports our interpretation.

Because of loss of subjects from the original Groups and the creation of a certain imbalance in terms of mean age and DMF, a further analysis has been carried out on data from Control and Treatment subjects balanced for age and dental caries. On this basis it is possible to examine data for 92 subjects in each Group present throughout the trial, and these are presented in Appendix A.

This analysis supports the results we have demonstrated for all subjects present at the four examinations: a reduction for the Treatment Group of 17.8 per cent in DMF surfaces, 16.6 per cent in DMF teeth, and 31.7 per cent for DMF bicuspid and molar proximal surfaces.

Conclusions

1. One of the major difficulties of conducting a clinical trial of this nature lies in the loss of subjects originally examined.

2. The clinical trial of calcium sucrose phosphate used as a food additive for a period of three years in 527 children (Control 361, Treatment 166) aged 5-17 years demonstrated a lower incidence of dental caries for those children receiving the additive.

3. The results reported previously for the first two years of the trial have been confirmed after three years and this has been demonstrated by statistical significance in a number of cases.

4. There is also a consistent pattern of reduced incidence of dental caries in the Treatment Group in comparisons in which, due to the relatively small numbers involved, significance testing produces non-significant results.

5. In the age groups 9-13 years there are reductions for the Treatment Group of:

DMF teeth	15.3 per cent
DMF surfaces	17.9 per cent
DMF proximal surfaces	29.5 per cent

6. Strong evidence supports the earlier findings that most benefit is gained on proximal surfaces which accounted for approximately 40 per cent of all lesions found.

7. The medical investigations showed no differences in the physical status and general health between the children of the Control and Treatment Groups.

The Institute of Dental Research,
United Dental Hospital,
2 Chalmers Street,
Surry Hills, N.S.W. 2010

Calcium sucrose phosphate as a cariostatic agent in children aged 5-17 years.* Part III

Dietary factors

R. Harris,† Miriam Roots,†† G. Gregory,‡ and J. Beveridge‡‡

Introduction

Details of the basis on which the clinical trials in children were established have already been published⁽¹⁾⁽²⁾⁽³⁾ and a significant reduction in dental caries in the mouths of the children aged 5-17 years has been found.

In the planning of the clinical trial to observe the effect of calcium sucrose phosphate when incorporated in certain carbohydrate foods of the diet of children aged 5-17 years two things were essential:

- (1) an assessment of the dietary regime in each of the homes participating in the clinical trial, and
- (2) regular observations of the meal schedules and dietary regimes throughout the trial period.

To achieve the first of these objectives, interviews were held with the officers in charge of each institution in order to give them a clear statement on (a) the aims and objectives of the clinical trial; (b) the need to maintain the existing dietary regime throughout the trial; and (c) in the case of those institutions participating as the test group, the replacement of those foods selected for the inclusion of the additive with properly prepared and regularly supplied and treated food.

The responsibility for this supply and for the task of analysing the treated food to ensure that the food additive was present in proper amounts lay with the sponsors of this trial. The task of ensuring that the eating habits and the patterns of meal preparation and distribution were maintained as closely as possible to the original pattern was ours and was undertaken in detail by one of us (M.R.).

Subjects and methods

The trial commenced in February, 1965, with the participation of a total of 1,506 children aged 5-17 years living in 19 institutions. Three of these institutions have sections for both boys and girls and, for the purpose of analysing the data, such sections are considered separately. The institutions were placed into either Control or Treatment Groups.

Throughout the trial some children left the institutions and were replaced by newcomers and all the institutions boarded the children for the major portion of each year. During this time the food supplies to the Treatment

* Financial support for this project has been given by the Colonial Sugar Refining Company Ltd., Sydney.

† Director, Institute of Dental Research, Sydney.

†† Dietitian, part-time, United Dental Hospital, Sydney.

‡ Formerly Reader, Department of Statistics, University of Melbourne.

‡‡ Professor of Pædiatrics and Head of the School of Pædiatrics, University of New South Wales.

⁽¹⁾ Lillenthal, B., Bush, Elizabeth, Buckmaster, M., Gregory, G., Gagolski, J., Smythe, B. M., Curtin, J. H., and Napper, D. H.—The cariostatic effect of carbohydrate phosphates in the diet. *Austral. D. J.*, 11: 6, 388-395 (Dec.) 1966.

⁽²⁾ Harris, R., Schamschula, R. G., Gregory, G., Roots, Miriam, and Beveridge, J.—Observations on the cariostatic effect of calcium sucrose phosphate in a group of children aged 5-17 years. Preliminary report. *Austral. D. J.*, 12: 2, 105-113 (Apr.) 1967.

⁽³⁾ Harris, R., Schamschula, R. G., Beveridge, J., and Gregory, G.—The cariostatic effect of calcium sucrose phosphate in a group of children aged 5-17 years. *Austral. D. J.*, 13: 1, 32-39 (Feb.) 1968.

Group provided approximately 4.3 gm. of calcium sucrose phosphate per child per day.

The participating institutions were allocated to either Control or Treatment Group by the following considerations:

- (1) balance of numbers;
- (2) balance of ages;
- (3) balance of sexes;
- (4) willingness of the controlling authorities of an institution to participate in the Treatment Group;
- (5) random allocation.

the Dietary Allowances for Australian Children.⁽⁴⁾

Methods of evaluating the carbohydrate intake were:

- 1. The administrator or catering officer and the cook were interviewed and the following information was recorded:
 - (i) Daily menu for week-days and week-ends, with seasonal variation.
 - (ii) Food orders: daily, weekly, monthly, and annual, checked by reference to invoices.
 - (iii) Donations of food: quantities, raw or prepared.

TABLE I
Carbohydrate intake of children in the trial

Food	Average consumption lb./child/day	Percentage carbohydrate	Pounds carbohydrate child/day	Percentage of total carbohydrate (0.854 lb.)
Sugar, white	0.167	100	0.167	19.5
Sugar, brown	0.033	100	0.033	3.9
Flour, S.R.	0.054	76	0.041	4.8
Flour, plain	0.011	76	0.008	0.9
Golden syrup and/or honey	0.024	76	0.018	2.1
Jam	0.054	72	0.039	4.6
Weetbix and/or Cornflakes	0.053	75	0.040	4.7
Oatmeal	0.040	70	0.028	3.3
Tinned fruit	0.149	20*	0.030	3.5
Bread	0.776	48	0.372	43.5
Biscuits	0.103	76	0.078	9.1
Total	1.464	—	0.854	—

* Of this 20% carbohydrate about 11% or 12% comes from added sugar.

Four of the participating institutions were situated in a country town having a fluoridated water supply for approximately four years prior to the commencement of the trial.

To assist in the planning of the trial, one of us (M.R.) visited all the homes and institutions in 1964 and the programme of enquiry and recording of data follows:

- 1. Estimates of the average daily food intake of each child were made, with particular reference to the type and quantity of refined carbohydrate.
- 2. The estimates were made in relation to the children in the age groups: 5-7 years, 8-12 years, 12 and over years.
- 3. The estimated quantities were compared with the Australian Commonwealth figures for

- (iv) Home-produced food: vegetables and dairy products.
- (v) Wastage: in preparation, cooking, pantry and plate waste.
- (vi) Numbers of meals prepared: resident children, and both resident and casual staff.
- (vii) Number of days children resided in homes each year.

- 2. The dietitian spent a whole day in each organization observing the preparation and serving of foods and recording wastage for all meals for that day.

(4) Dietary allowances for Australians—1965 Revision. National Health and Medical Research Council, Sixtieth Session, Report, Canberra. Commonwealth Government Printer, Oct. 1965, Appendix 1, pp. 42-52.

- (i) Recipes were checked, ingredients weighed or measured and random samples of plate servings weighed.
- (ii) Variations in average quantities for each age group were recorded.

3. Daily consumption was recorded for the carbohydrates: sugar: white, brown, castor, icing; golden syrup; canned fruits; honey; jam; bread; biscuits.

Methods of control

1. Careful instructions were given by the dietitian to the catering and kitchen staff and to the administrative office to ensure that a constant pattern of ordering, preparation, cooking and serving was followed.

visits were reduced to summer and winter periods during the second and third years of the trial. In addition to these visits, two senior members of the team (R.H. and J.B.) visited each home or institution before the clinical examinations and at a varying interval during the succeeding six months in order to keep the authorities responsible for the welfare of the children fully informed on the progress of the trial and to ensure continued cooperation of the staffs.

Basis for distribution of calcium sucrose phosphate as a food additive

From the initial survey data were obtained for the average daily consumption of processed carbohydrate foods and these are shown in Table 1.

The estimated average intake per child of 4.3 gm. of calcium sucrose phosphate would mean about 500' mg. of calcium per day: an increase of about 50 per cent on the existing calcium intake of children in the institutions. Table 2 shows the amounts of calcium sucrose phosphate added to these foods.

Observations

The homes and institutions were divided into Treatment and Control Groups of approximately equal numbers of children and subsequently an adjustment was made by the addition of 292 boys aged 12-16 years to the Control Group. The numbers of children then were Treatment Group 619, Control Group 900.

The fluctuations in subjects which are inevitable in this environment did not appear to produce any marked variations in the dietary regimes in the homes.

Tables 3 and 4 show the average daily food intake of the children during the trial compared with the recommended daily intake. It should be noted that the data for calcium in the Treatment Group do not include that in the additive material.

Balance studies have been undertaken on boys and girls in both Control and Treatment Groups to determine actual intakes and excretions of calcium and phosphorus, and the results will be published in detail elsewhere. Balance studies on children in the field who are attending school are very difficult and could be inaccurate; none the less, the intakes of calcium and phosphorus by children in the Control homes are very similar to those in

TABLE 2

Levels of addition of calcium sucrose phosphate

Food	Percentage CaSP added on finished weight of food	Pounds CaSP/child/day
Sugar, white	1.0	0.00167
Sugar, brown	1.0	0.00033
Flour, S.R.	1.0	0.00054
Flour, plain	1.0	0.00011
Golden syrup and/or honey	1.0	0.00024
Jam	1.0	0.00054
Weetbix and/or Corn-flakes	0*	0
Oatmeal	0*	0
Tinned fruit	0.2†	0.00030
Bread	0.6‡	0.00466
Biscuits	1.0	0.00103
Total	—	0.00942

* Not treated, as normal use of treated sugar or syrups should result in the addition of approximately 3.0% CaSP to total carbohydrate.
† 1% on total carbohydrates.
‡ 1% on flour.
0.00942 lb.=4.3 g. CaSP/child/day.

2. Specific instructions were given to the staff of the institutions in the Treatment Group to ensure that only "treated" carbohydrate foods were used and a procedure was established whereby the central supplying agency would provide continuity of foods and control the amounts for each of the homes.

3. At various intervals throughout the trial visits were made to each home or institution to check on the procedure followed. During the first year of the trial this checking was made during summer, winter and spring. These

TABLE 3
Average daily food intake of boys aged 5-17 years

Institution number	Age	Calories	Protein gm.	Fat gm.	Carbohydrates		Calcium gm.
					Unrefined gm.	Refined gm.	
CONTROL	4-8	1980	66	80	90	152	1.15
	8-12	2270	78	85	105	192	1.13
	12-14	2350	85	90	105	218	1.13
	5-8	2000	76	70	122	172	1.17
	8-12	2330	72	80	122	210	1.17
	12-17	3390	106	130	178	272	1.63
	5-8	1860	60	80	60	165	1.05
	8-12	2110	70	90	60	195	1.06
	12-14	3200	74	95	54	226	0.89
	4-8	2040	55	60	58	260	0.72
TREATMENT	8-12	2400	65	65	69	320	0.74
	4-8	1830	46	65	74	192	0.61
	8-12	2200	54	80	83	232	0.56
	4-8	1975	55	55	88	227	0.74
	8-12	2185	60	63	92	253	0.76
	12-14	2200	60	60	87	268	0.6
	4-8	1920	57	75	85	185	0.99
	8-12	2550	71	92	100	260	0.98
	12-15	2850	76	96	70	350	0.68
	Dietary allowances for Australians—1965 ⁽⁴⁾						
TREATMENT	4-8	1700	35-51 av. 43	45	280		0.6-0.9
	8-12	2200	40-66 av. 53	55	400		0.6-1.1
	12-14	2900	52-87 av. 70	75	480		0.6-1.4
	9-17	2825	66	80	64	395	0.63
	10-12	2500	66	85	75	295	0.8
	12-17	2740	75	93	75	315	0.8
	6-8	2300	64	80	103	227	1.04
	8-12	2340	65	80	105	235	1.03
	12-17	2390	68	80	100	250	0.87
	6-8	1815	50	55	60	220	0.75
TREATMENT	8-12	2200	60	72	96	226	0.78
	12-15	2375	66	70	114	256	0.6

the recommended Dietary Allowances.⁽⁴⁾ Our studies have also shown that the intakes of calcium and phosphorus by children in the Treatment Group have not been as high as expected.

In addition to balance studies in a small number of children, the concentrations of calcium, nitrogen and creatinine have been estimated repeatedly in the urine of all children in both Groups; and it is interesting to note that the concentration of calcium in the

urine is no higher in children in the Test Group than in the Control Group.

Examples of daily food patterns from seven homes are given:

Institution No. 1. Boys and girls.

The organization of this home was somewhat different from the others, since the children, in groups of 12-20, live with "cottage parents" in semi-autonomous cottages under the supervision of the General Superintendent.

TABLE 4
Average daily food intake of girls aged 5-17 years

Institution number	Age	Calories	Protein gm.	Fat gm.	Carbohydrates		Calcium gm.
					Unrefined gm.	Refined gm.	
CONTROL	4- 7	1780	60	80	75	130	1.16
	7-12	1980	65	80	110	110	1.06
	12-15	2120	70	80	140	140	1.07
	5- 8	2080	76	70	122	172	1.17
	8-12	2330	72	80	122	210	1.17
	12-16	2250	69	80	125	187	1.17
	6- 8	1510	54	65	58	120	0.95
	8-12	1800	60	73	60	140	0.95
	12-15	1800	60	73	60	140	0.94
	4- 8	1850	60	80	83	172	0.92
	10-12	2200	69	90	90	193	0.92
	12-15	2100	64	86	90	180	0.75
	5- 8	1900	73	70	86	178	1.05
	8-12	2260	80	80	108	197	0.93
	12-15	2260	80	80	108	197	0.93
	4- 8	1920	57	75	85	185	0.99
	8-12	2270	67	80	90	230	0.98
	4- 8	1700	35-51 av. 43	45	280		0.6-0.9
Dietary allowances for Australians—1965 ⁽⁴⁾	8-12	2200	40-66 av. 53	55	400		0.6-1.1
	12-14	2500	52-75 av. 63	65	400		0.6-1.4
TREATMENT	5- 8	1800	60	70	81	154	1.2
	8-12	2250	65	75	72	256	1.0
	12-17	1990	60	70	62	218	0.87
	5- 8	1860	52	70	75	182	0.88
	5-12	2110	59	70	75	231	0.87
	12-17	2050	58	70	71	222	0.67
	4- 8	1940	52	57	82	224	0.87
	8-12	2350	60	75	96	264	0.88
	12-15	2275	56	70	91	264	0.88
	7- 8	1920	58	60	73	212	0.77
	8-12	2040	61	60	73	242	0.77
	12-17	1960	55	60	73	227	0.60

Breakfast: Cereal, bread, butter, milk, tea or cocoa.

Mid-morning: Fruit and, for junior school children, milk.

Mid-day lunch: Sandwich lunch with various fillings, cake and one piece of fruit.

After school: Bread and butter with various spreads. Occasionally a piece of fruit, tea or cake.

Dinner: Meat, two or three vegetables (grown on property), dessert, milk for young children.

Institution No. 9. Boys.

Breakfast: Cereal, bread and spreads, Aktavite made with milk and water.

Mid-morning: Biscuits and milk.

Lunch: Sandwich lunch and occasional fruit.

Dinner: Meat, two vegetables, dessert.

Institution No. 16. Girls.

Breakfast: Cereal, sweetened milk, toast with spreads, sweet tea.

Mid-morning: Biscuits or bread with spreads, milk for younger children.

Mid-day: Meat, two or three vegetables, dessert (five days per week), bread roll with spread, fruit (two days per week).

Dinner: Sausages and fried bread (two days per week), bread with spreads, sweet tea (five days per week).

Institution No. 12. Girls.

Breakfast: Cereal, hot dish (five days per week), bread with spreads, tea.

Mid-morning: Fruit.

Lunch: Sandwiches, occasional biscuits.

After school: Bread with jam or other spread.

Dinner: Meat, two vegetables, dessert.

Institution No. 14. Girls.

Breakfast: Cereal, sweetened milk, bread with spreads, tea.

Mid-morning: Milk, cake or scones, fruit.

Lunch: Meat, potato with one other vegetable, dessert.

After school: Bread with spreads or biscuits, fruit, tea.

Dinner: Soup, fried bread (in winter), bread with spreads, tea (milk for younger children).

Institution No. 2. Boys.

Breakfast: Cereal, sugar and dried milk added in cooking, bread with spreads, tea.

Mid-day: Meat, rice or spaghetti or macaroni. Chips or mashed potato (three days per week), lettuce or tomato (five days per week), cabbage or spinach (two days per week), bread, fruit, milk.

Dinner: Vegetable or cereal soup, bread with spreads, cake and custard, tea.

Institution No. 6. Boys.

Breakfast: Cereal, bread with spreads, milk.

Lunch: Sandwiches with variety of fillings.

Dinner: Meat, two or three vegetables, dessert, fresh fruit.

It should be noted that some homes varied the pattern of the meals by having in-between-meal snacks and some of the children have pocket-money which, although subject to control by the administrative staff, would allow them to buy some additional items from school tuck shops where they are not attending school on their own premises.

Spreads consist of peanut paste, vegetable extract, meat or fish paste, jam, honey, golden syrup. A different spread is provided at each meal. Some institutions provide one savoury and one sweet spread at each meal.

Discussion

Calcium sucrose phosphates are among the many sugar phosphates known to occur widely in natural products. First prepared in 1910 by Neuberg and Pollak, they were a mixture of various sucrose phosphates the nature and proportions of which, however, are constant and approximate to the formula of the mono-orthophosphate ester of sucrose— $C_{12}H_{22}O_{14}PCa_2H_2O$. The Colonial Sugar Refining Company has produced a substance which is a mixture of the same calcium sucrose phosphates as those produced by Neuberg and Pollak but it contains different proportions of the sucrose phosphates and more inorganic calcium phosphate.

The properties of calcium sucrose phosphates suggested their use as an additive to carbohydrate foods to restore calcium and phosphorus in a form and at a level comparable with those in natural carbohydrates. A study of the metabolism and excretion of calcium sucrose phosphates was carried out and showed that the material is a readily metabolized nutrient, the behaviour of which does not differ greatly from sucrose, and they are readily broken down in the gut to sugars and inorganic phosphates.⁽⁵⁾

The acceptable daily intake of phosphates for man has been set out in World Health Organization Technical Report No. 281, 1964, and for calcium in W.H.O. Technical Report No. 230, 1962. For communities with low calcium intake, the intake for phosphates is 30 mg./kg. body weight as a *total* dietary phosphate; where the calcium intake is high, the phosphate intake may be raised to 70 mg./kg. body weight. Daily intake of calcium can reach 2-3 gm. without detrimental effect (W.H.O. Technical Report No. 230, 1962) and there is no evidence that a daily intake as high as 1300 mg. is undesirable. The addition of 1 per cent calcium sucrose phosphate to the average weight of carbohydrate available per head per day (420 gm., Food and Nutrition,

⁽⁵⁾ Curtin, J. H., Gagolski, J., and Smythe, B. M.—A food additive for the control of dental caries. Food Technol. Aust., 19: 11, 508-513, (Aug.) 1967.

Notes and Reviews, Australia, 1964) would lead to calcium and phosphorus levels well within acceptable limits.

Balance studies which we have undertaken on boys and girls aged 8-11 years show an intake of about 900-1300 mg. of calcium and 800-1100 mg. of phosphorus. The expected daily increments for such children would be of the order of 300 mg. of calcium and 250 mg. of phosphorus and such increments are obviously well within acceptable limits.

On the basis of the animal studies on dental caries, the laboratory investigation on the inhibition of subsurface decalcification of enamel, the small but significant reduction in incidence of dental caries when calcium sucrose phosphate was incorporated in a dentifrice, the toxicity tests, the dietary allowances as set down by the World Health Organization and other authorities, it was considered that sufficient information was available to proceed with a clinical trial of the efficacy of calcium sucrose phosphate as a cariostatic agent in the diet of humans.

The role of phosphates as a means of reducing dental caries has been the subject of extensive studies following the initial suggestions of Klein and McCollum,⁽⁶⁾ Osborn and Noriskin⁽⁷⁾ and Osborn.⁽⁸⁾ More recent studies by Strålfors,⁽⁹⁾ Ship and Mickelsen,⁽¹⁰⁾ Averill and Bibby,⁽¹¹⁾ Averill, Friere and Bibby,⁽¹²⁾ Stookey, Carroll and Muhler,⁽¹³⁾ Finn and

Jamison⁽¹⁴⁾ and Finn⁽¹⁵⁾ have yielded variable results.

Strålfors used dibasic calcium phosphate in four ingredients of the school lucheon food—soft bread, hard bread, cooking flour and sugar, and Stookey, Carroll and Muhler used sodium dihydrogen phosphate in presweetened cereals. Finn and Jamison used dicalcium phosphate dihydrate in chewing gum. The advantages of placing the additives in a variety of carbohydrates ensures that the effects of likes and dislikes and habits are minimized, and there is a greater opportunity for the cariostatic action.

Some phosphates have unpleasant tastes, as Stookey, Carroll and Muhler⁽¹³⁾ found. The calcium sucrose phosphates used in this trial, made by a process developed for large scale production,⁽¹⁶⁾ are a fine white, non-hygroscopic powder which can be dissolved in water. It has a bland, neutral taste and consequently can be added to foods without any effect on flavour. It also contains as much as 20 per cent of inorganic phosphate as well as the calcium sucrose phosphates and yet remains readily soluble.

As expected in homes and institutions where financial factors are particularly important, there is a tendency to maintain a standard diet of monotonous pattern and a major source of calories is derived from carbohydrate foods. There are some variations between homes in terms of calories and in most instances the nutritional status is below the Australian Standard. In the case of institutions Nos. 2 and 9 there is a low level of calcium, especially in the older age groups.

It should be noted that the children live away from some of the institutions during the 13 weeks' school holidays each year and in some instances they are away during various week-ends. It is expected that at these times their diets will vary and their nutrition may show some improvement.

⁽⁶⁾ Klein, H., and McCollum, E. V. A.—A preliminary note on the significance of the phosphorus intake in the diet and blood phosphorus concentration in the experimental production of caries immunity and caries susceptibility in the rat. *Science*, 74: 662-664 (Dec. 25) 1931.

⁽⁷⁾ Osborn, T. W. G., and Noriskin, J. N.—The relation between diet and caries in the South African Bantu. *J. D. Res.*, 16: 5, 431-441 (Oct.) 1937.

⁽⁸⁾ Osborn, T. W. G.—Further studies on the *in vitro* decalcification of teeth. *J. D. Res.*, 20: 1, 59-69 (Feb.) 1941.

⁽⁹⁾ Strålfors, A.—The effect of calcium phosphate on dental caries in school children. *J. D. Res.*, 43: Suppl. 6, 1137-1143 (Nov.-Dec.) 1964.

⁽¹⁰⁾ Ship, I. I., and Mickelsen, O.—The effects of calcium acid phosphate on dental caries in children: a controlled clinical trial. *J. D. Res.*, 43: Suppl. 6, 1144-1149 (Nov.-Dec.) 1964.

⁽¹¹⁾ Averill, H. M., and Bibby, B. G.—A clinical test of additions of phosphate to the diet of children. *J. D. Res.*, 43: Suppl. 6, 1150-1155 (Nov.-Dec.) 1964.

⁽¹²⁾ Averill, H. M., Friere, P. S., and Bibby, B. G.—The effect of dietary phosphate supplements on dental caries incidence in tropical Brazil. *Arch. Oral Biol.*, 11: 3, 315-322 (Mar.) 1966.

⁽¹³⁾ Stookey, G. H., Carroll, R. H., and Muhler, J. C.—The clinical effectiveness of phosphate enriched breakfast cereals on the incidence of dental caries in children: results after two years. *J.A.D.A.*, 74: 4, 752-758 (Mar.) 1967.

⁽¹⁴⁾ Finn, S. B., and Jamison, H. C.—The effect of dicalcium phosphate chewing gum on caries incidence in children: 30 month results. *J.A.D.A.*, 74: 5, 987-995 (Apr.) 1967.

⁽¹⁵⁾ Finn, S. B.—The role of dicalcium phosphate chewing gum in the control of human dental caries. *Int. D. J.*, 17: 2, 339-352 (June) 1967.

⁽¹⁶⁾ Gagolski, J., and Curtin, J. H.—A process for the manufacture of phosphoric esters of carbohydrates and polyhydric alcohols. *Australian Pat.* 259, 269 (May) 1965.

The health of the children as determined by reference to predetermined medical criteria is to be reported in detail by one of us (J.B.), but in brief it can be said that no differences were noted between the data for the two groups of children nor between those of the children and accepted norms.

In order to reduce the advantage enjoyed by the administration in the provision of the treated carbohydrate foods in the Treatment Group institutions, annual per capita payments were made by the sponsors to the institutions of the Control Group.

Constant checking was found necessary in the early days of the trial to ensure that the ordering and supply of foodstuffs in the Treatment Group followed the previous patterns.

Every effort was made to maintain a complete liaison with the catering and cooking

staff, upon whom rested the major responsibility for cooperation in implementing our requirements.

Summary

1. The planning of the dietetic control in a trial of a food additive as a cariostatic agent is reported.
2. The dietary pattern and general nutritional state of the participating groups of children aged 5-17 years are recorded and show a general pattern at the lower levels as set out in the Dietary Allowances for Australians.
3. The trial covered a period of three years.

The Institute of Dental Research,
United Dental Hospital,
2 Chalmers Street,
Surry Hills, N.S.W. 2010.

Observations on the effect of topical sodium fluoride on caries incidence in children

Robert Harris*

Introduction

Many investigators have observed the effect of the topical application of sodium fluoride on the progress of dental caries. The initial work by Bibby (1942)⁽¹⁾ and Knutson (1943)⁽²⁾ is well known, and since then there has appeared a number of papers a few of which have presented the results of observations extending over more than three years. Amongst these are those by Syrrist and Karlsen⁽³⁾ who made observations for three years following sodium fluoride treatments carried out over two years. Syrrist,⁽⁴⁾ whose observations extended over seven years, applied 2 per cent sodium fluoride seven times during a two year period to one quadrant of the mouths of a group of 12-year-old children using another quadrant as a control. At the end of the treatment period there were 51 per cent less new carious surfaces on the treated quadrant. Re-examination five years after discontinuance of treatment showed a difference between control and treated quadrants of 15 per cent. Bergman⁽⁵⁾ surveyed results obtained over a three-year period. He, as

did many others,⁽⁶⁾ observed a 40 per cent reduction in the amount of caries developing after treatment with a 2 per cent aqueous sodium fluoride solution.

It was decided in 1952 to undertake a study observing the results of a minimum of five annual series of treatments to the teeth of children attending the Department of Preventive Dentistry, United Dental Hospital, Sydney.

Materials and Method

The groups selected for observation were in the ages 6-11 years at the time of the initial examination; they were born and lived in Sydney the water supply of which is fluoride-free. The greater numbers attending the Department came from the six-year-old children who had been receiving dental treatment during previous years and would be able to continue to do so for a number of years. This ensured longer co-operation, for as the study extended the older children became involved more and more in school activities and found attendance difficult. From these six-year-old children it was possible to use as a control group 69 who did not receive sodium fluoride treatment and who were available for comparison at the final examination. No comparable group was available from the older children, consequently the six-year-old children form the major part of the survey.

Because of the results of extensive dental caries, relatively small numbers of deciduous teeth remained, and only observations on the changes in the permanent teeth have been used.

⁽⁶⁾ Martin, N. D.—The scientific evaluation of fluoride as a means of preventing dental caries: *Austral. D. J.*, 1: 3, 141-150 (June) 1956.

* Assistant Superintendent, Head of the Department of Preventive Dentistry, United Dental Hospital of Sydney. Lecturer, Part-Time, Faculty of Dentistry, University of Sydney.

⁽¹⁾ Bibby, B. G.—Preliminary report on the use of sodium fluoride applications in caries prophylaxis: *J. D. Res.*, 21: 314 (Abstr.), (June) 1942.

⁽²⁾ Knutson, J. W. and Armstrong, W. D.—Public Health Rep., 58 1701 (Nov.) 1943.

⁽³⁾ Syrrist, A., and Karlsen, K.—A five-year report on the effect of topical applications of sodium fluoride on dental caries experience: *Brit. D. J.*, 87: 1, 1-6 (July) 1954.

⁽⁴⁾ Syrrist, A.—Seven-year report on the effect of topical applications of sodium fluoride on dental caries. *Odont. Revy*, 7: 386-396, No. 3, 1956.

⁽⁵⁾ Bergman, G.—Caries inhibiting action of sodium fluoride. *Experimental Studies. Acta Odont. Scandinavica*, 11: 9-112, suppl. 12, 1953.

The initial survey was made in 1952, all children being given a prophylaxis with pumice paste and rubber polishing wheels, a clinical examination supplemented by a radiographic examination, consisting of six anterior periapical films and four bite-wing films (except for some younger children, for whom two only were taken). This standard examination was repeated annually and recorded on the patient's chart. A different colour was used for recording each annual examination. The charts were used to keep a record of each application of sodium fluoride and, to facilitate sorting and filing, a distinguishing mark was placed on those for the control group. However, for the final examination records for all groups were made on fresh charts without any distinguishing mark. The data from the charts were transferred to a simple card index system which was used for processing and tabulation of the results.

Examination

The clinical examination was made in a good light and using a mirror and sharp probe each surface of each tooth was carefully examined. Compressed air was used to dry thoroughly the tooth surfaces. The explorers were sharpened from time to time, but were discarded immediately the length of the point was reduced to 1 mm. If the explorer could be forced into a fissure and the fissure was stained it was noted as carious and this decision was then confirmed or rejected after an examination of the bite-wing films. Obvious open carious lesions in the occlusal surfaces and on buccal, labial, lingual and palatal surfaces caused no difficulty in identification. Radiographic examination was found to be necessary to determine the status of many proximal surfaces and confirmed that proximal lesions began a considerable time before they were clinically evident.

Treatment

Following the prophylaxis the aqueous 2 per cent sodium fluoride solution, coloured for identification, was applied to the isolated and dried teeth by means of cotton wool applicator, cotton rolls and saliva ejector being used to prevent dilution from saliva. The lower jaw was treated in two quadrants and the upper jaw as one unit. The sodium fluoride solution was kept flooded over the

crowns of all teeth for three minutes. The treatment was repeated four times at intervals of one week or less. The child was given instruction in oral hygiene. A recall system ensured the repetition of this programme annually.

Some patients complained of a stinging feeling when the sodium fluoride solution touched the gingival tissues but no reaction, judged by change of colour, presence of oedema or desquamation of the epithelium, was observed.

Results

The dental caries prevalence of the 429 children who were initially examined is shown in Table I. It ranges from DMF of 2.05 at

TABLE 1.

Dental caries prevalence in 429 children aged 6-11 years, expressed as the mean number of DMF teeth.

Age in years.	Number in group.	Mean number of DMF teeth.	Standard deviation.
6	186*	2.05	0.43
7	58	2.94	1.82
8	31	4.09	1.98
9	55	4.76	2.02
10	52	5.82	2.18
11	47	8.27	2.28

* This is the total number of children examined initially and from which the control and treated groups were taken.

six years to DMF of 8.27 at eleven years. From these, 126 provided the material for the six-year-old group, namely 57 for the treated and 69 for the control group.

Table 2 shows a comparison of the dental caries prevalence between my survey groups at their initial examination and the children of New South Wales.⁽⁷⁾ It will be noted that children attending the Department of Preventive Dentistry had a lower standard of dental health than that observed by Barnard in his extensive survey in 1954-55, which shows a range in dental caries prevalence in the 6-11 years of 0.99-6.98 DMF teeth.

Table 3 shows the incidence of dental caries during five years for six groups of children of different ages who received treatment with sodium fluoride. It will be seen that there is a reduction in the incidence as the number

⁽⁷⁾ Barnard, P. G.—Dental Survey of State School Children in New South Wales. N.H.M.R.C. Special Reprint Series No. 8, Canberra, 1956.

TABLE 2.
Comparison of the dental caries prevalence of the children aged 6-11 years attending the Dental Hospital, and of those attending State schools in New South Wales.⁷

Age in years ..	6	7	8	9	10	11
DMF teeth, Dental Hospital children	2.05	2.94	4.09	4.76	5.82	8.27
Standard deviation	0.43	1.82	1.98	2.02	2.18	2.28
DMF teeth, State school children ..	0.99	2.31	3.22	4.44	5.28	6.98
Standard deviation	1.38	1.70	1.91	2.32	3.08	4.10

teeth compared with 6.98-13.91 DMF teeth for the State school children.

In Table 4 the comparison is shown between the dental caries prevalence of the control group and of the similarly aged treated group. There was a slight difference between the two groups in the initial examination, the control group having a mean 2.03 DMF teeth. At the end of the five-year period there was a difference in the mean DMF teeth rates for the two groups of 2.79, the mean number of DMF teeth for the control group being 8.37 com-

TABLE 3.
Dental caries incidence in permanent teeth following topical application of 2 per cent. aqueous sodium fluoride, in 165 children, during a five-year period. Data from the survey of New South Wales State school children⁷ for untreated groups of the same age is given in column 8 for comparison.

Age in years at each annual examination.	DMF teeth of each group.						State school children.
	Age in years at initial examination.						
	6	7	8	9	10	11	
6	2.05						0.99
7	2.77	2.94					2.31
8	3.44	3.50	4.09				3.22
9	3.39	3.86	5.12	4.76			4.44
10	4.40	4.95	5.90	5.58	5.82		5.28
11	5.58	5.29	6.51	7.18	7.33	8.27	6.98
12		6.92	8.81	9.53	9.63	10.34	9.32
13			9.85	11.20	11.50	11.38	10.70
14				11.70	11.84	12.58	12.78
15					12.66	13.30	13.91
16						15.88	—
Number of children	57	28	24	24	19	13	

of applications of sodium fluoride accumulate. In the six-year-old group the DMF range is from 2.05-5.58 over the five-year period and this is a lower range than is shown in the initial examinations (6-11 years) and it also does not reach the figure for the 11-year-old State school children.

The seven-year-old group has a range 2.94-6.92 DMF teeth, the final figure being below that of the similarly aged State school children and of the 11-year-old children at the initial examination. Similarly, the eight-year-old group when examined at the age of eleven years had a lower DMF than the similarly aged State school children and together with the nine and 10-year-old groups had lower DMF rates than the children aged 11 years at the initial examination. The range for the children of various ages at the final examinations was 5.58-12.66 DMF

pared with 5.58 for the treated group. This difference may be expressed as a 33.4 per cent reduction in dental caries in the group treated with 2 per cent aqueous solution of sodium fluoride.

TABLE 4.
Comparison of the dental caries prevalence of 57 children before and after treatment with that of 69 children in a control group.

Age in years.	DMF teeth.		
	Control group.	Treated group.	Difference.
First examination, 6 ..	2.03 (0.52)	2.05 (0.43)	0.02
Final examination, 11 ..	8.37 (4.67)	5.58 (3.19)	2.79
Number of children ..	69	57	

Standard deviation in brackets.
Variance $\sigma=4.123$, $t=3.8$ and $p=0.001$.

Discussion

In attempting to assess the efficacy of the application of 2 per cent sodium fluoride to the permanent teeth in preventing dental caries over a five-year period under the conditions existing in this particular clinic, certain points should be noted.

The children attending the clinic belonged mainly to the lower socio-economic levels in the community and the dental caries prevalence of the group was higher than a larger sample of a similar age group in New South Wales.

The difficulty of wastage of subjects has been noted and of the original 429 the largest group was the six-year-old children numbering 186 and of these 126 were available for the final examination including 69 used as a control group. Only 165 children of all ages receiving sodium fluoride throughout the five-year period were available at the final examination. In addition, by using the six-year-old children the possibility of the result being affected by subsequent dental treatment such as orthodontics and prosthodontics was eliminated.

Comparing Table 2 and Table 3 it will be seen that, despite the fact that the dental caries prevalence of the children in the State School Survey was lower than that of the Dental Hospital groups initially, as the number of treatments increased the six- and seven-year-old groups from their ninth year and the eight-year-old group from their eleventh year had less dental caries than a comparable age group in the general community and the six-year-old group had appreciably less dental caries than the control group and the State school children.

Considered on the basis of time, the group of six-year-old children required approximately 31 hours to achieve the reduction in dental caries which involved approximately 160 affected teeth. If we assume the treatment of each affected tooth would require 30 minutes, a reduction of 49 hours in chair-side time would have been achieved. However, it is wise to organize treatment so that the topical applications can be made as much as possible during appointments for other work, but any additional visits made by the child are not extravagantly time consuming since they serve as opportunities for improving patient-dental relations.

A final examination of as many children as possible will be made in 1962 and the results of this will make an interesting comparison with the present achievement and the pattern of dental caries in the untreated control group.

Summary

Groups of children were given a series of topical applications of aqueous 2 per cent sodium fluoride to their permanent teeth at annual intervals for five years. Observations showed that all groups had a reduction in caries incidence when compared with similarly aged untreated groups in the general population and one group of these children was found to have only 66.6 per cent of the amount of dental caries of that of a control group at the end of five years.

A reduction in dental caries incidence of 33.4 per cent was achieved.

United Dental Hospital,
2 Chalmers Street,
Sydney.

Observations on the effect of eight per cent stannous fluoride on dental caries in children

Robert Harris

Reprinted from the *Australian Dental Journal*, Vol. 8, No. 4, August, 1963, pp. 335-340.

Observations on the effect of eight per cent stannous fluoride on dental caries in children

Robert Harris*

Introduction

The history of investigations into the action of fluorides on teeth covers a long period. Volker⁽¹⁾ has stated that almost a century ago Magitot observed variations in the resistance of teeth to acid decalcification and called attention to the possibility that the more resistant enamel contained "a minute quantity of fluoride of calcium". Carnot in the late nineteenth century established the ability of calcified tissue to combine with substantial quantities of fluorides from aqueous solutions. These and other researches prior to the turn of this century stimulated an interest in the possible preparation of fluoride-containing mouthwashes, lozenges, tooth-powders, and toothpastes. For the past twenty years topical application of various fluoride solutions to teeth has been advocated, but reports concerning the efficacy of the method have varied considerably.

Following the initial work by Bibby⁽²⁾ the technique of topical application of sodium fluoride developed by Knutson and Armstrong,⁽³⁾ though somewhat time-consuming, has been found to reduce the incidence of dental caries in groups of children. Other workers have used variations of Knutson's technique, resulting in a reduction of dental caries of the order of 40 per cent when sodium

fluoride was used. Bergmann⁽⁴⁾ observed such a reduction in a clinical trial commenced in 1952 over a three-year period. Harris⁽⁵⁾ reported a reduction of 33.4 per cent in school children aged 6-11 years over a five-year period. The technique in the latter trial differed from Knutson's in that each year four applications of 2 per cent sodium fluoride were applied to the teeth at intervals of seven days. It has been shown⁽⁶⁾ that the fluoride concentrations in the surface enamel of teeth treated with 2 per cent sodium fluoride are approximately 50 parts per million greater than those found in the control teeth. Brudevold *et al.*⁽⁷⁾ have shown that intact enamel exposed to stannous fluoride takes up both tin and fluoride, although the amount of fluoride absorbed is less than from similar solutions of sodium fluoride. This result is in conflict with that of other investigators.⁽⁸⁾

In a study of the effects of 2 per cent unbuffered stannous fluoride applied four times at short intervals in each of two consecutive

⁽⁴⁾ Bergmann, G.—Caries inhibiting action of sodium fluoride. Experimental studies. *Acta Odont. Scandinavica*, 11: 9-112, Suppl. 12, 1953.

⁽⁵⁾ Harris, R.—Observations on the effect of topical sodium fluoride on caries incidence in children. *Austral. D. J.*, 4: 4, 257-260 (Aug.) 1959.

⁽⁶⁾ Simdval-Hagland, I., Brudevold, F., Armstrong, W. D., Gardner, D. E., and Smith, F. A.—A comparison of the increment of fluoride in enamel and the reduction of dental caries resulting from topical fluoride application. *Arch. oral Biol.*, 1: 1, 74-79 (Aug.) 1959.

⁽⁷⁾ Brudevold, F., Steadman, L. T., Gardner, D. E., Rowley, J., and Little, M. F.—Uptake of tin and fluoride by intact enamel. *J.A.D.A.*, 53: 2, 159-164 (Aug.) 1956.

⁽⁸⁾ Hatton, W. E., Nebergall, W. H., and Muhler, J. C.—The removal of fluorine from dilute solutions of sodium fluoride and stannous fluoride by powdered dental enamel. *J. D. Res.*, 34: 3, 350-357 (June) 1955.

* Director, Institute of Dental Research, Sydney.

⁽¹⁾ Volker, J. F.—Topical fluorides—historical developments. *J. D. Res.*, 39: 6, 1116-1117 (Nov.-Dec.) 1960.

⁽²⁾ Bibby, B. G.—Preliminary report on the use of sodium fluoride applications in caries prophylaxis. *J. D. Res.*, 21: 3, 314 (June) 1942.

⁽³⁾ Knutson, J. W., and Armstrong, W. D.—The effect of topically applied sodium fluoride on dental caries experience. iii. *Pub. Hlth. Rpt.*, 58: 11, 1701-1715 (Nov.) 1943. Also *Pub. Hlth Rpt.*, 61: 11, 1683-1689 (Nov.) 1946.

years to the teeth of school children, Howell *et al.*⁽⁹⁾ observed a reduction in dental caries of 58.8 per cent. More recently McDonald and Muhler⁽¹⁰⁾ pointed out the advantages of six-monthly intervals in the use of stannous fluoride applications. The apparent efficacy of stannous fluoride reported by overseas investigators warranted the making of a careful clinical study in this country. Consequently a group of children attending the Department of Preventive Dentistry, United Dental Hospital, Sydney, was selected and in September-December, 1959, a study commenced which was concluded in September-December, 1962.

Material and methods

The children selected were aged 7-12 years and at the commencement of the observations consisted of 230 boys and 257 girls. They were born and had lived all their lives in Sydney, the water supply of which is fluoride-free,⁽¹¹⁾ and were attending the United Dental Hospital of Sydney for continuation of previously planned treatments. By the time of the final examination only 126 boys and 152 girls remained in the study. The records of some of even these children had to be excluded from the data on which this paper has been prepared, because treatment, such as orthodontic therapy, had been instituted during the period of observation or the children had not received the full number of applications of stannous fluoride. Therefore the data for only 212 children, of the age-groups shown in Table 1 have been used in this survey. Furthermore, it was decided to use only the data obtained from those permanent teeth erupted at the commencement of the study. Lesions on the mesial surfaces of upper and lower centrals were not included in the record of the effect of the treatment (Table 1).

Once each year every child was examined clinically and radiologically. Six anterior peri-apical films and four posterior bitewing films were used for the radiological examination. The clinical examination was made in good

natural light supplemented by intra-oral illumination. After the teeth had been cleaned with a glycerine and pumice abrasive and the mouth thoroughly rinsed, compressed air was used to dry thoroughly the tooth surfaces and then, using a plain mirror and sharp probe, each surface of each tooth was carefully examined. The explorers were sharpened from time to time but were discarded when the length of the point was reduced to less than 1 mm. If the explorer could be forced into a stained occlusal fissure, it was noted as carious and this diagnosis was confirmed or rejected after an examination of the bite-wing films.

TABLE 1
Initial age-groups of 212 children used in the survey

Children	Age-group (yr.)						Totals
	7	8	9	10	11	12	
Boys ..	—	27	29	21	8	17	102
Girls ..	14	22	16	23	21	14	110
Totals ..	14	49	45	44	29	31	212

To the upper and lower teeth of one half of the mouth of each child an 8 per cent stannous fluoride solution* was applied at intervals of six months over a period of three years. The stannous fluoride solution was prepared for each child immediately prior to application and each segment of the mouth treated was isolated from saliva by means of cotton rolls and a saliva ejector. The stannous fluoride solution was applied carefully to the crowns of the teeth and allowed to dry for four minutes. The drying process was assisted by a gentle stream of compressed air directed across the crowns of the teeth. Treatment was given separately for the upper and lower quadrants of one side of the mouth and when completed the patient was instructed not to rinse the mouth. Where an abrasion of the mucosal surface existed, care was taken to avoid contact between the stannous fluoride solution and the abraded surface. Record charts were suitably identified to avoid confusion except for the final examination when a plain unidentified chart was used for

⁽⁹⁾ Howell, C. L., Gish, C. W., Smiley, R. D., Muhler, J. C.—Effect of topically applied stannous fluoride on dental caries experience in children. *J.A.D.A.*, 50: 1, 14-17 (Jan.) 1955.

⁽¹⁰⁾ McDonald, R. E., and Muhler, J. C.—The superiority of topical application of stannous fluoride on primary teeth. *J. Den. Children*, 24: 2, 84-86, 2nd quart., 1957.

⁽¹¹⁾ Jones, Pamela B.—The fluorine content and other chemical characteristics of potable waters in New South Wales. *D. J. Australia*, 21: 5, 231-257 (May) 1949.

* Stannous fluoride supplied by courtesy of Proctor & Gamble Co., Division of Dental Research, Cincinnati, Ohio, U.S.A. The stannous fluoride crystals were kept in an airtight container with silica gel, and gelatin capsules of 240 mg. were prepared for each day's treatment. When required, the material was placed in a medicament glass with 3 ml. distilled water to make an 8 per cent solution.

TABLE 2
The mean D.M.F. teeth in 212 children aged 10-15 years before and after receiving topical application of 8 per cent stannous fluoride compared with the caries prevalence in the untreated mouths of children in the State Schools of N.S.W.

Age (yrs.)	D.M.F. teeth		
	State school children, N.S.W. ⁽¹²⁾	United Dental Hospital	
		Before treatment	After treatment
7	2.31±0.062*	3.00±0.403*	—
8	3.22±0.070	3.14±0.234	—
9	4.44±0.086	4.26±0.236	—
10	5.28±0.115	5.09±0.348	5.00±0.38*
11	6.98±0.154	8.8 ±0.857	5.9 ±0.38
12	9.32±0.207	9.8 ±1.198	8.5 ±0.76
13	10.70±0.195	—	9.30±0.74
14	12.78±0.217	—	13.17±0.79
15	13.91±0.263	—	13.83±1.03

* Standard error of mean.

each patient. Subsequently this chart was identified appropriately to conform with the previous records.
A recall system operated to ensure return of patients at six-monthly intervals.

Table 2. It ranged from a D.M.F. of 3.00 teeth at 7 years to 9.8 teeth at 12 years at the initial examination, and from a D.M.F. of 5.00 teeth at 10 years to 13.83 teeth at 15 years at the final examination. These figures can be compared with the dental caries prevalence of State School children as reported by Barnard⁽¹²⁾ in 1956. It will be noted that the children of the present investigation before the topical application began had a similar standard of dental health to that of the State School children except that those 11 years of age had a mean D.M.F. of 8.8 whilst the figure for State school children was 6.98. This greater D.M.F. figure is reflected in the result three years later. After treatment, the mean D.M.F. for each age group, except the fourteen-year-olds, was lower than that of the State School children.
Since the study was designed to compare treated and untreated teeth on opposite sides of the mouth, tabulation has not been made of the annual dental caries incidence.
Table 3 sets out the total number of affected teeth at the commencement and completion of the observations. It will be noted that for

TABLE 3
D.M.F. teeth at initial and final examinations in treated and untreated teeth in 212 children

Teeth	Initial examination	Final examination			
	D.M.F. teeth	D.M.F. teeth	D.M.F. increase	Percentage of sound teeth affected	Critical ratio
Boys					
813 treated ..	238	354	116	20.17	2.93 (significant)
819 untreated ..	241	400	159	27.51	
Girls					
954 treated ..	344	474	130	21.31	2.24 (significant)
956 untreated ..	345	508	163	26.68	
Children					
1767 treated ..	582	828	246	20.76	3.61 (significant at 1 per cent level)
1775 untreated	586	908	322	27.08	

Results

The dental caries prevalence (as D.M.F., including treated and untreated teeth) of the children before treatment and at the conclusion of the observations is shown in

both boys and girls over the three years there is a smaller increase in the number of D.M.F. teeth for the treated side of the mouth than
⁽¹²⁾ Barnard, P. D.—Dental survey of State School Children in New South Wales. N.H.M.R.C. Sp. Rpt. Ser., no. 8, Canberra, 1956.

for the untreated side, that is teeth which were sound at the commencement of the study and were treated with stannous fluoride showed a smaller percentage of new caries at the end of the study. If the result is expressed as a percentage of sound teeth which became carious during the course of these observations it will be seen that for the 212 children only 20.8 per cent of sound teeth on the treated side of the mouth became affected compared with 27.0 per cent of sound teeth on the untreated side. This demonstrates a 23.3 per cent inhibition of caries on the treated side of the mouth as compared with that on the untreated side.

Discussion

Certain important limitations arise in the use of fluoride solutions for topical application. One is the necessity to repeat the treatment at regular intervals and where stannous salt is used *the solution must be freshly prepared immediately prior to each application*. It is essential to keep the tooth surface free from saliva during the application and it is also desirable that the patient should not rinse the mouth immediately after treatment. Many patients find this difficult to avoid because of the somewhat objectionably astringent taste of the stannous fluoride.

If the inhibitory effect from the use of stannous fluoride is considered satisfactory, one of the advantages of using this salt topically would be that the result can be achieved by a single application every six months—a time-interval which is widely accepted as that desirable for clinical examination of the dental and oral tissues of each patient in order to maintain a satisfactory measure of dental health. The procedure could therefore be of great value in areas where fluoridation of community drinking water is not operating.

After treatment, pigmentation varying from light to dark brown was observed on certain areas of some of the teeth. Some authorities⁽¹³⁾ consider this an indication that the carious process has been retarded and maintain that healthy surfaces are not discoloured, but others⁽¹⁴⁾ have noted the pigmentation on

intact surfaces. Muhler,⁽¹⁵⁾ who has done much of the research on stannous fluoride as a preventive agent, gives his opinion that the brown pigmentation is an indication of current caries in decalcified zones. If this view is correct, topical application will enable the dentist to demonstrate to his patient those areas of the tooth surfaces with initial carious lesions and also gain a measure of control. Some patients complain of the astringent taste when the cotton rolls are removed but there are no immediately harmful effects observed in the gingivæ and patients have not reported any changes in the gingivæ similar to those recorded by Swieterman *et al.*⁽¹⁶⁾

As the results obtained by various investigators are not consistent, they should be assessed carefully. It is important when comparing results of different studies to note the conditions under which they have been made. It should be remembered that differences in standards may exist between those made as part of a service programme and those carried out meticulously as a research project of limited scope. There is also the limitation imposed on a study when left and right sides of the mouth participate as treated and untreated areas of observation. It is not possible to leave one half of each mouth as a perfect control entirely free of treatment. Also it is a clinical observation that, according to the arch position of the teeth, variations exist in the probability of caries developing, but in the present study the initial difference between treated and control sides was extremely small—0.08 per cent (Table 3). Patients also differ from one another in their probability of developing caries.

The greatest reductions in caries have been observed in the primary dentition and whether the same degree of reduction can be achieved in the permanent dentition is not certain. McDonald and Muhler⁽¹⁰⁾ noted a 57 per cent reduction for the primary dentition. Slack⁽¹⁷⁾ over a two-year period, using one side of the mouth as a control, recorded the increment

⁽¹³⁾ Dunning, J. M.—Principles of dental public health. Harvard Univ. Press, Cambridge, Mass., p. 219, 1962.

⁽¹⁴⁾ Von Ehrenheim, V.—Tannfluorarpenslingar. Odont. Revy., 10: 1, 71-75 (Jan.) 1959 (English summary).

⁽¹⁵⁾ Muhler, J. C.—Stannous fluoride enamel pigmentation—evidence of caries arrestment. J. Den. Children, 27: 3, 157-161 (3rd quart.) 1960.

⁽¹⁶⁾ Swieterman, R. P., Muhler, J. C., and Swenson, H. M.—The effect of highly concentrated solutions of stannous fluoride on human gingival tissue. J. Periodont., 32: 2, 131-138 (Apr.) 1961.

⁽¹⁷⁾ Slack, G. L.—The effect of topical application of stannous fluoride on the incidence of dental caries in six-year-old children. Brit. D. J., 101: 1, 7-11 (July) 1956.

of caries in originally caries-free first permanent molars and found on the treated side an average reduction of 0.24 D.F. (21.1 per cent). Jordan⁽¹⁸⁾ observed at the end of two years the effect of a single application of 8 per cent stannous fluoride on 420 children aged 12-13 years and found a caries reduction of 37.85 per cent. In another study⁽¹⁹⁾ on 472 students aged 12-13 years, where again one application of stannous fluoride was made, the group (234) that received stannous fluoride had 20 per cent less caries than the untreated group (238) at the end of 12 months. However, if these results are interpreted in terms of surfaces, the reduction was only 14 per cent and the authors did note that bucco-lingual surfaces showed 30 per cent more caries among the treated subjects. Laws *et al.*,⁽²⁰⁾ when comparing one application of 8 per cent stannous fluoride solution with that of a 2 per cent solution applied four times, found at the end of 12 months approximately twice the reduction in dental caries with the four treatments. That study covered approximately the same age-groups and numbers of children as used by the present writer.

In this investigation restorative treatment was given to the children during the period of the observations but any effect so caused operated similarly on both control and test sides of the mouth and therefore any bias arising from such effect would be at a minimum. Variables that would arise from addition to the number of teeth by eruption have been eliminated by recording observations only on the teeth erupted at the time of the initial examination. Over the period, reduction in the mean D.M.F. teeth per child for the full mouth for each age-group is small because the figures include both treated and untreated teeth. However, the reduction is clear in all age-groups even among the 11-year-old children who had an average of 8.8 D.M.F. teeth at the commencement, which was 1.82 D.M.F. greater than that recorded for the State School children. It is not surprising

therefore that at the final examination this group still had more caries than the average for the State School children but even here the stannous fluoride effect is shown, the difference being reduced to 0.39 D.M.F.

To eliminate another variable, 112 children received the stannous fluoride application on the left side of the mouth and 100 children on the right side. At the end of three years there were 939 non-carious teeth in the treated quadrants and 867 in the untreated. The difference of 23.34 per cent can be compared with similar studies reported elsewhere. This is a relatively small reduction which applies to the whole group of children and may not be observable in all individuals of the group.

The results of the present study are supported by Slack's⁽¹⁷⁾ observations and the findings of Jordan,⁽¹⁸⁾ Jordan *et al.*,⁽¹⁹⁾ Muhler,⁽²¹⁾ and Peterson and Williamson,⁽²²⁾ although their techniques were restricted in the number of applications of stannous fluoride. Muhler found a reduction in dental caries of 24 per cent in 207 subjects aged 17-38 years and Peterson and Williamson found 26 per cent less caries in a group of children 9-13 years of age. Both results differ little from those of Jordan *et al.* More recently Gish *et al.*,⁽²³⁾ in a study using one application of 8 per cent stannous fluoride each year for five years observed 30 per cent reduction in D.M.F. teeth.

The value of topical application of stannous fluoride as opposed to other fluoride therapy must be assessed against the results obtained and the time, effort, and cost required for the applications. In practice, the recall of the patient at six-monthly intervals and the observation of any decalcified and brown-stained carious lesions could be a valuable aid in preventive dentistry. The technique of topical application is not time-consuming and the efficacy of topical fluorides has been demonstrated to be similar whether sodium or stannous fluoride is used.

(18) Jordan, W. A.—Study of a single application of eight per cent stannous fluoride. *J. Den. Children*, 26: 4, 355-359 (4th quart.) 1959.

(19) Jordan, W. A., Snyder, J. R., and Wilson, V.—Stannous fluoride clinical study in Olmstead County, Minnesota. *Pub. Hlth. Rpt.*, 73: 11, 1010-1014 (Nov.) 1958.

(20) Laws, F. E., Jeffrey, Margaret H., and Sheary, Helen C.—Topical application of fluoride solutions in dental caries control. *Pub. Hlth. Rpt.*, 76: 4, 287-290 (Apr.) 1961.

(21) Muhler, J. C.—The effect of a single topical application of stannous fluoride on the incidence of dental caries in adults. *J. D. Res.*, 37: 3, 415-416 (June) 1958.

(22) Peterson, J. K., and Williamson, Lois—Effectiveness of topical applications of eight per cent stannous fluoride. *Pub. Hlth. Rpt.*, 77: 1, 39-40 (Jan.) 1962.

(23) Gish, C. W., Muhler, J. C., and Howell, C. L.—A new approach to the topical application of fluorides for the reduction of dental caries in children. Results at the end of five years. *J. Den. Children*, 29: 2, 65-71 (2nd quart.) 1962.

Summary

1. Observations were made on the effect of applications of 8 per cent stannous fluoride in aqueous solution on the dental caries-experience in permanent teeth of 212 children aged 7-12 years.

2. The stannous fluoride was applied at six-monthly intervals to the upper and lower teeth on one side of the mouth for a period of three years. The upper and lower permanent teeth on the opposite side were used as controls.

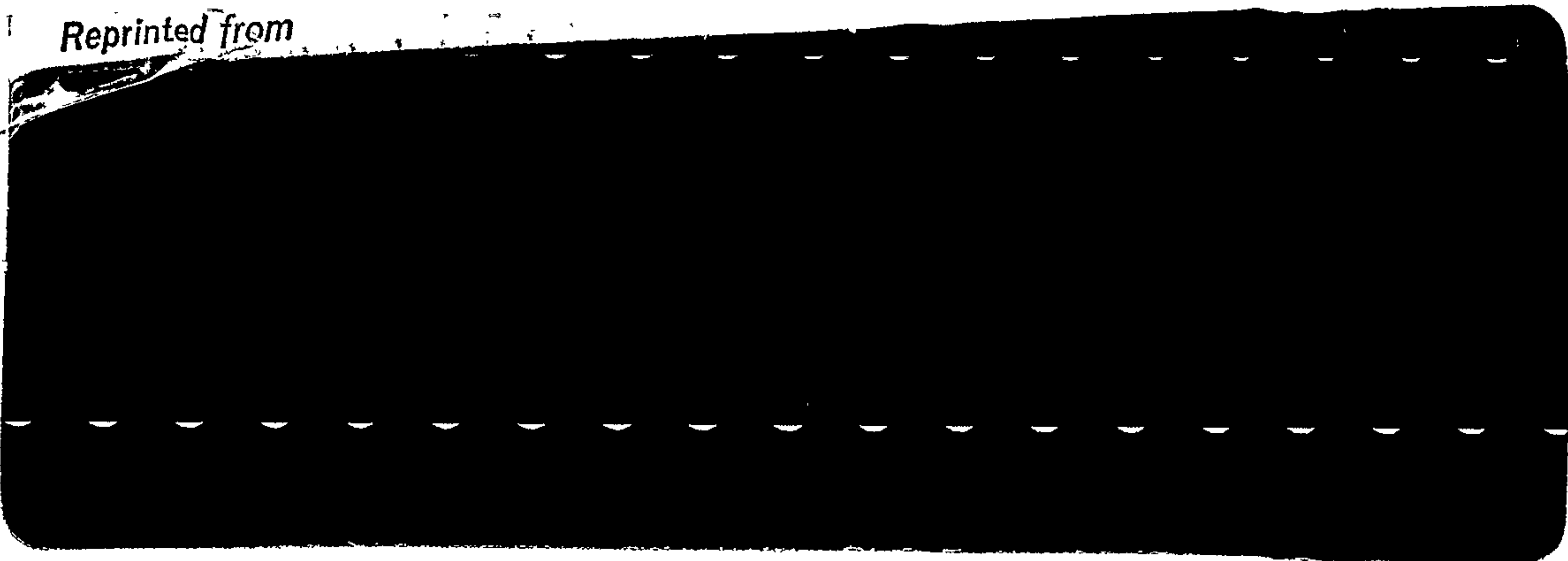
3. Approximately the same number of permanent teeth with almost similar caries-

experience were used in both the treated and control areas at the commencement of the study and observations made on these teeth are the basis of this report. Teeth erupting during the three years were excluded from the observations.

4. The reduction in dental caries was 23.34 per cent.

Institute of Dental Research,
United Dental Hospital,
2 Chalmers Street,
Sydney.

Reprinted from



PERGAMON PRESS
OXFORD NEW YORK LONDON PARIS



ALDEHYDE FUCHSIN-HALMI REACTION IN FIBRES OF THE HUMAN DENTAL PULP

R. HARRIS

Institute of Dental Research, United Dental Hospital, Sydney

and C. J. GRIFFIN

Department of Histology and Embryology, University of Sydney, Australia

Summary—The pulps of sixty-one human teeth in various stages of development from twenty-one males and forty females, aged 7–33 years, were subjected to aldehyde fuchsin-Halmi staining.

The staining reaction was noted in the following conditions: (i) without oxidation with peracetic acid, (ii) after oxidation with peracetic acid, (iii) with enzyme digestion, using β -glucuronidase, hyaluronidase or lysozyme before oxidation with peracetic acid, and (iv) with the same three enzymes after oxidation with peracetic acid.

Staining of fibres and ground substance was noted in the subodontoblastic, central and apical zones of the pulp. The intensity of the staining was most marked in the apical zone of the developing tooth. In the mature tooth the central zone frequently did not react. Digestion in β -glucuronidase, hyaluronidase or lysozyme prior to or after oxidation removed varying amounts of aldehyde fuchsin reactive material. Hyaluronidase was most effective and lysozyme was ineffective.

In sixteen teeth the pulps were removed before fixation. In three areas in each of the five conditions where oxidation was employed the staining reactions were less intense than observed in the pulps of decalcified teeth.

It is suggested that the staining reaction is restricted to areas where collagen synthesis occurs.

INTRODUCTION

FULLMER (1958) described a differential staining for fibres in connective tissues and FULLMER and LILLIE (1958) using histochemical methods described a fibre component in the periodontal tissues which they termed the oxytalan fibre. The histochemical basis for the identification of these fibres was that they stained purple with aldehyde fuchsin if the tissue was pretreated with peracetic acid. To demonstrate this reaction more usefully these authors used Halmi counter stain after the aldehyde fuchsin peracetic acid treatment in order to differentiate collagen fibres, which stain brown, from oxytalan fibres.

The fibres appear in the oral mucosa of embryos at 6 months and FULLMER (1959) suggested that they develop by condensation of the mucopolysaccharide lying between bundles of collagen fibres.

In a more recent publication FULLMER (1960) described the effect of enzymic digestion on mucopolysaccharides in developing tooth pulps, using aldehyde fuchsin-Halmi staining and the periodic acid-Schiff reaction. He noted that β -glucuronidase digestion reversed the action of aldehyde fuchsin and diminished the periodic acid-Schiff reaction and that hyaluronidase had no effect on the aldehyde fuchsin but

reduced the periodic acid-Schiff reaction. He also noted that lysozyme appeared to have no effect on either unless sections made from frozen material were used. In 1962 Dr. FULLMER visited Australia and in lectures pointed out that the exact nature of oxytalan fibres is uncertain. He emphasized that there existed a polysaccharide moiety associated with the fibres. It was decided therefore to make observations in developing and mature teeth on the effect of enzymic digestion, before and after peracetic acid oxidation, with aldehyde fuchsin-Halmi stain.

MATERIALS AND METHODS

The material for this study was obtained from fully-formed erupted and partially-developed permanent teeth of patients receiving orthodontic therapy or having multiple extractions for dentures. Observations were made on sixty-one teeth from twenty-one males and forty females, aged 7–33 years.

Immediately after the tooth extraction and prior to fixation one of two procedures was adopted: (1) the tooth was cut transversely under water spray at the cervical region with an air-rotor driven fissure-bur and the two halves placed immediately in fixative; or (2) a superficial cut was made in an axial plane through crown and root (without damage to the pulp), the tooth split open with a pair of bone cutters and the pulp extracted and placed in fixative.

Of the sixty-one teeth, forty-four pulps were fixed in Bouin's solution and seventeen in 10% neutral buffered formol saline solution. Fixation in most cases did not exceed 24 hr. Where required, decalcification was carried out with 5% nitric acid, completion of decalcification being determined by X-ray examinations. The material was embedded in paraffin and sectioned at 6μ .

Staining methods

Five staining sequences were used:

1. Aldehyde fuchsin-Halmi (FULLMER and LILLIE, 1958).
2. Peracetic acid (GREENSPAN, 1954) followed by aldehyde fuchsin-Halmi.
3. Peracetic acid, enzyme, aldehyde fuchsin-Halmi.
4. Enzyme, peracetic acid, aldehyde fuchsin-Halmi.
5. Peracetic acid, enzyme plus enzyme inhibitor, aldehyde fuchsin-Halmi.

The oxidizing agent, peracetic acid, was prepared according to the method of GREENSPAN (1954) and kept refrigerated when not in use. Oxidation of sections was carried out for 30 min at room temperature.

The aldehyde fuchsin was prepared according to GOMORI (1954). The dye was aged for 72 hr before use and discarded after 1 week.

Enzymic digestion

The methods of FULLMER (1960) were applied with the following enzymes:

1. β -Glucuronidase (Nutritional Biochemicals Corp.): 15 mg in 50 ml of 0.1M acetate buffer, pH 4.5, for 48 hr at 37°C. Glucuronidase inhibitor (saccharic acid) was used at 0.01–0.1M concentration.

2. Testicular hyaluronidase (450 I.U./mg, California Corporation for Biochemical Research): 1 mg per ml of 0.1M phosphate buffer, pH 6, for 48 hr at 37°C. Hyaluronidase inhibitor (heparin): 1 mg per mg of hyaluronidase used.

3. Lysozyme (8000 U/mg, California Corporation for Biochemical Research): 5 mg in 30 ml of 0.2M phosphate buffer, pH 5.3, for 48 hr at 37°C. Lysozyme inhibitor (Lugol's iodine): 1:300 buffer.

RESULTS

The teeth were classified into two groups according to their degree of development, as shown in Table 1.

TABLE 1. NUMBER OF TEETH AND AGE-GROUPS OF PATIENTS FROM WHOM SPECIMENS WERE EXAMINED

Stage of development	Age-groups (years)				Total
	7-10	11-14	15-18	19-33	
Developing tooth	1	19	15	—	35
Mature tooth	4	12	5	5	26
Totals	5	31	20	5	61

Staining reactions

After oxidation with peracetic acid had been carried out, fibres and ground substances in well-defined areas of the pulp reacted to aldehyde fuchsin. In young pulps this reaction was intense and diffuse in the ground substance and the cytoplasm of some cells. In older pulps the reaction was found only in fibres. Where further maturation of the pulp and increments of dentine had occurred, a well-defined and discrete purple-staining subodontoblastic zone was observed. The fibres in this zone had a predominant orientation at right angles to the dentinal tubules. The zone was quite discrete and averaged about 15μ in width.

The number and intensity of staining reactions for pulps from developing and mature teeth and for decalcified and undecalcified specimens for the three zones are shown in Table 2.

In the subodontoblastic zone, 25 (43.16%) of the pulps showed strong or intense staining whilst 10 (16.4%) and 35 (87.5%) showed similar reaction in the central and apical zones respectively, when aldehyde fuchsin-Halmi stain was used after oxidation. This assessment of staining reactions is based on results derived from both decalcified and undecalcified tissue.

The central zone of the coronal and root pulp was almost completely devoid of aldehyde fuchsin reactive material: 85.7% of negative reactions came from this zone. It was also extremely rare to observe reactive areas in the pulp without oxidation with peracetic acid prior to staining. It was observed in only nine instances. Table 3 shows that 44.6% of all pulps showed strong or intense reaction when staining followed oxidation.

TABLE 2. DEGREE OF STAINING REACTIONS AFTER β -GLUCURONIDASE AND HYALURONIDASE DIGESTION OF UNOXIDIZED AND PERACETIC ACID OXIDIZED TISSUE SECTIONS OF MATURE AND DEVELOPING HUMAN DENTAL PULPS

Zones of teeth	Enzymic Digestion																Controls							
	PA + AF				β-Glucuronidase + PA + AF				PA + β-glucuron- idase + AF				Hyaluronidase + PA + AF								PA + hyaluronidase + AF			
	—	+	++	+++	—	+	++	+++	—	+	++	+++	—	+	++	+++	—	+	++	+++	—	+	++	+++
Developing tooth:																								
subodontoblastic	3	18	13	—	5	12	15	1	18	10	5	2	2	12	18	1	17	10	8	—	2	14	17	2
central	23	3	4	3	20	8	4	3	27	4	4	1	22	4	6	2	28	3	5	—	22	5	3	6
apical	3	—	10	17	1	1	4	24	2	6	10	13	—	1	9	19	10	2	7	11	—	1	1	29
Totals	29	21	27	20	26	21	23	28	47	20	19	16	24	17	33	22	55	15	20	11	24	20	21	37
Mature tooth:																								
subodontoblastic	1	11	8	4	1	9	11	8	12	6	9	3	1	9	13	2	19	6	5	—	—	9	9	11
central	19	4	1	2	21	4	3	1	23	5	1	—	20	3	—	1	29	—	—	—	21	4	4	1
apical	—	2	6	2	1	1	4	5	3	3	1	4	—	2	4	4	5	1	3	3	—	1	1	9
Totals	20	17	15	8	23	14	18	14	38	14	11	7	21	14	17	7	53	7	8	3	21	14	14	21
Decalcified:																								
subodontoblastic	2	24	12	4	3	18	20	8	26	12	9	4	2	17	24	2	27	14	10	—	2	22	15	12
central	30	6	4	5	29	11	6	3	39	8	4	1	30	7	6	2	44	3	5	—	31	7	7	7
apical	—	2	13	12	1	1	7	18	3	8	9	9	—	1	9	16	10	1	9	8	—	1	2	26
Totals	32	32	29	21	33	30	33	28	68	28	22	14	32	25	39	20	81	18	24	8	33	30	24	45
Undecalcified:																								
subodontoblastic	2	5	9	—	2	4	6	1	4	4	5	1	1	4	7	1	9	3	3	—	—	1	11	1
central	12	1	1	—	12	1	1	1	12	1	1	—	12	—	—	1	13	—	—	—	12	2	—	—
apical	3	—	3	7	2	—	1	11	2	—	2	8	—	2	4	7	5	1	1	6	—	1	—	12
Totals	17	6	13	7	16	5	8	13	18	5	8	9	13	6	11	9	27	4	4	6	12	4	11	13

PA, peracetic acid; AF, aldehyde fuchsin-Halmi.
 —, no stain; +, sparsely stained; ++, strongly stained; +++, intensely stained.
 The results of the aldehyde fuchsin-Halmi stain without oxidation are not included in the Table.
 From 163 specimens 9 had some positive reaction (subodontoblastic 3, central 3, apical 3).
 There is a numerical difference between the various groups. Some specimens could not be included because of manipulation damage during staining procedures.
 The control sections were used in each batch of sections subjected to enzyme digestion.

TABLE 3: PERCENTAGES OF PULPS SHOWING STAINING AT TWO DEGREES OF INTENSITY FOR MATURE AND DEVELOPING TEETH AND FOR DECALCIFIED AND UNDECALCIFIED SPECIMENS

Pulps	Oxidation + aldehyde fuchsin-Halmi		Enzymic digestion								Controls	
	β-Glucuronidase + oxidation + aldehyde fuchsin-Halmi	Oxidation + β-glucuronidase + aldehyde fuchsin-Halmi	Hyaluronidase + oxidation + aldehyde fuchsin-Halmi	Oxidation + hyaluronidase + aldehyde fuchsin-Halmi								
All	—	+	+	+	—	+	+	+	—	+	—	+
	31.2	24.2	44.6	29.3	21.0	49.7	49.4	19.8	30.8	27.3	18.8	53.9
	62.8	12.8	24.4	62.8	12.8	24.4	62.8	12.8	24.4	62.8	12.8	24.4
Developing Mature	29.9	21.6	48.5	26.5	21.4	52.1	46.1	19.6	34.3	25.0	17.7	57.3
	33.3	38.3	38.4	33.3	20.3	46.4	54.3	20.0	25.7	35.6	23.7	40.7
	74.7	9.8	15.5	74.7	9.8	15.5	74.7	9.8	15.5	74.7	9.8	15.5
Decalcified Undecalcified	29.3	27.6	43.1	25.6	24.0	50.4	51.9	21.4	26.7	27.6	21.6	50.8
	41.9	9.3	48.8	40.0	12.5	47.5	43.9	12.2	43.9	34.2	15.8	50.0
	24.6	23.1	52.3	24.6	23.1	52.3	24.6	23.1	52.3	24.6	23.1	52.3
	30.9	9.5	59.6	30.9	9.5	59.6	30.9	9.5	59.6	30.9	9.5	59.6

—, no stain; +, sparsely stained; ++, strongly stained; ++++, intensely stained.

TABLE 4. PERCENTAGES OF STAINING REACTION* IN THE THREE ZONES OF DEVELOPING AND MATURE PULPS AND IN DECALCIFIED AND UNDECALCIFIED SPECIMENS

Test	Developing tooth			Mature tooth			Decalcified pulp			Undecalcified pulp		
	Sub- odonto- blastic	Central	Apical	Sub- odonto- blastic	Central	Apical	Sub- odonto- blastic	Central	Apical	Sub- odonto- blastic	Central	Apical
Oxidation + aldehyde fuchsin-Halmi	38.2	21.2	90.0	50.0	11.5	80.0	42.2	19.6	92.3	56.3	7.1	83.3
Enzymic digestion: β-Glucuronidase + oxidation + aldehyde fuchsin-Halmi	48.5	20.0	93.3	65.5	13.8	81.8	57.1	24.5	92.6	53.8	13.3	85.7
Oxidation + β-glucuronidase + aldehyde fuchsin-Halmi	15.5	13.9	74.2	40.0	3.4	45.5	25.5	9.6	62.1	42.9	7.1	83.3
Hyaluronidase + oxidation + aldehyde fuchsin-Halmi	57.6	23.5	96.5	60.0	4.2	80.0	57.7	17.8	96.1	61.5	7.7	84.6
Oxidation + hyaluronidase + aldehyde fuchsin-Halmi	22.9	13.9	60.0	16.7	0.0	54.5	19.6	10.2	60.7	20.0	0.0	50.0
Controls	54.3	25.0	96.8	69.0	16.7	90.9	52.9	26.9	96.6	92.3	0.0	92.3

*For these calculations the figures for sparsely stained specimens were included in negative reactions.

Enzymic digestion

Enzymic digestion removed more positive reactive material after the pulps had been pretreated with peracetic acid than when digestion was applied before oxidation (Tables 2 and 3). Sections treated with enzyme plus inhibitor were found to be identical in their reactivity to sections treated for the same time with physiological saline so that in this study control sections were prepared with physiological saline.

Although seldom causing complete loss of reactivity, β -glucuronidase and hyaluronidase after peracetic acid oxidation both removed varying amounts of reactive material from all types of pulp. From Table 2 it will be seen that, when β -glucuronidase was used, the number of strong and intense stained pulps for subodontoblastic, central and apical zones was 19 (29.2%), 6 (10.5%) and 28 (66.7%) respectively. When hyaluronidase was used, the numbers were 13 (20.0%), 5 (7.6%) and 24 (47.1%) respectively. Figure 1 is prepared from Table 2 and shows a comparison between the percentage of strong and intense staining and combined negative and sparse staining. Because of uncertainty and possible error, the category of sparse staining reactions has in Fig. 1 been combined with that of negative reactions.

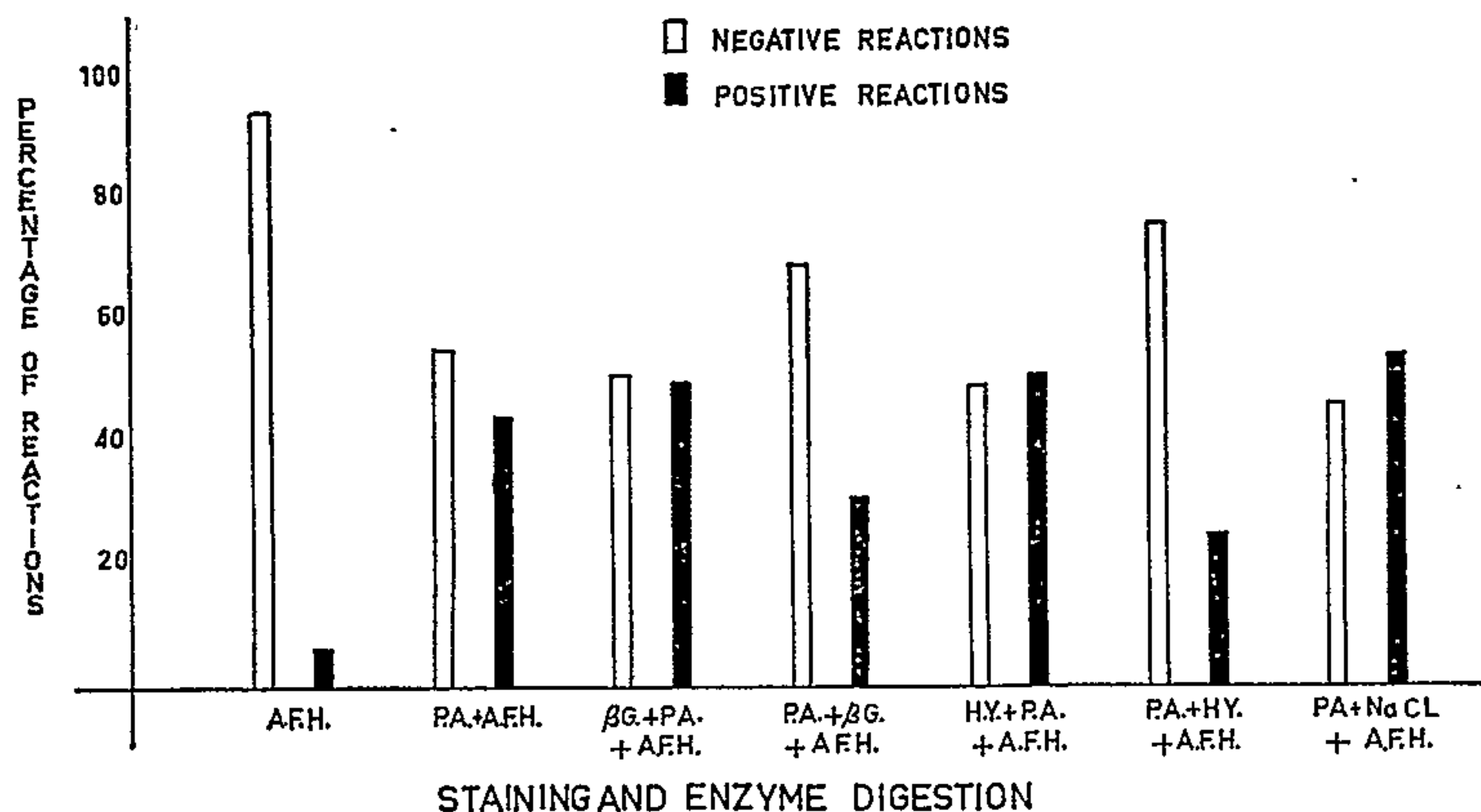


FIG. 1. Histograms showing percentage of positive and negative reactions to aldehyde fuchsin-Halmi staining with and without oxidation with peracetic acid and to digestion with β -glucuronidase and hyaluronidase. (Negative reactions include sparsely stained tissue.)

Table 3 shows that β -glucuronidase digestion reduced the strong and intense reactions from 44.6% to 30.8% and, when hyaluronidase was used, the number is further reduced to 24.4%. Control sections processed at the same time showed 54.0% of reactions in these categories.

The intensity of staining under any one set of conditions varied in each zone and this also was true in pulps removed from the tooth prior to fixation (i.e. undecalcified, Tables 2 and 3). In all teeth the positive staining of the central zones was less than that

in the other zones. By either method of preparation, enzyme digestion caused approximately the same reduction in intensity of the staining reactions and also in the numbers of zones staining (Tables 2 and 4).

In developing teeth staining reactions were more intense in the two zones where dentine was forming (subodontoblastic and apical) than in the central zone. In mature teeth a similar distribution but of lesser extent in the central and apical zones was observed (Table 4). It is realized that with the methods of fixation employed, weakly-bound sulphated acid mucopolysaccharides are lost (ZUGIBE, 1963).

Examples of the various staining reactions are shown in Figs. 2-7. The pulp fibres stain green with aldehyde fuchsin-Halmi stain and the aldehyde fuchsin positive-staining fibres and ground substance can be demonstrated only after peracetic acid oxidation (Figs. 2 and 3). In the young pulp a wide area reacts to the aldehyde fuchsin stain after peracetic acid oxidation. For example, in Figs. 3, 6 and 7 wide areas of the apical region show a positive reaction in which fibres and ground substance are included. These are specimens obtained from young developing pulps and can be compared directly with those shown in Fig. 5 which was prepared from the pulp of a patient 33 years of age. In the older specimen there is no positive reaction to the stain either in the central zone or subodontoblastic zone.

Variations in intensity of staining of the tissue were noted and this is illustrated in Figs. 3, 6 and 7.

The effect of hyaluronidase digestion is apparent when this is interposed between the oxidation and staining processes and this is shown in Figs. 4 and 7 where reacting material (fibres and ground substance) has been removed. Some of the reacting material is also removed by the enzyme digestion before oxidation.

Digestion with lysozyme, whether prior to or subsequent to oxidation with peracetic acid, had no effect on the aldehyde fuchsin-reactive material.

DISCUSSION

The observation made by FULLMER (1960) that β -glucuronidase digestion of tissue from dental pulps pretreated with peracetic acid reversed the aldehyde fuchsin staining and that hyaluronidase on similarly treated tissue did not remove the aldehyde fuchsin staining material is not entirely confirmed.

Enzymic digestion after oxidation reduced the amount of material reacting to the aldehyde fuchsin and also the intensity of the reaction (Tables 2-4 and Figs. 4 and 7). It can be seen from Table 4 that, if sparsely staining material is excluded, hyaluronidase digestion removes more than 50 per cent of the positively stained material and β -glucuronidase is slightly less effective. It is notable that, as far as the central zone is concerned, hyaluronidase removed all the reactive material in the mature pulp.

We have observed that the staining reactions in teeth from all age groups have certain similarities in as much as reaction occurs where collagen fibres are elaborated prior to inclusion in the dentine matrix. The less reactive zone in all teeth was the central zone (Tables 2 and 4).

In the case of pulps removed from the teeth before fixation (non-decalcified), the

intensity of the reaction appeared to remain proportionately the same as for the decalcified material, but more ground substance was stained, so that the fibres appeared to be less clearly defined.

The observations lead to the conclusion that the material reactive to aldehyde fuchsin after peracetic acid oxidation is a component of newly elaborated ground substance and further may be associated with collagen synthesis, at least as far as the dental pulp is concerned. This conclusion is based on the fact that the growing parts of the pulp always exhibit ground substance and fibres which are reactive to aldehyde fuchsin following peracetic acid oxidation.

The fact that this material is on occasions liable to enzymic digestion with β -glucuronidase and hyaluronidase suggests its polysaccharide nature. The restriction of the stained material in later developmental stages to areas of collagen formation indicates that it could be the mucopolysaccharide-cementing substance claimed to be responsible for the argyrophilia of young collagen fibres (SCHWARZ, 1957). Alternatively the reaction may be due to the presence of protein polysaccharide filaments similar to those described by FITTON JACKSON (1964).

Acknowledgements—We wish to record our appreciation to Professor K. W. CLELAND for his advice, to Mrs. C. LOSSIN for technical assistance in the preparation of the material and to the National Health and Medical Research Council of Australia for financial assistance for part of this work.

Résumé—Les pulpes de 61 dents humaines, à différents stades de développement, provenant de 21 hommes et 40 femmes, âgés de 7 à 33 ans, ont été soumises à la coloration de l'aldéhyde fuchsiq et d'Halmi.

Les réactions colorées ont été observées dans les conditions suivantes: (1) sans oxydation avec l'acide peracétique, (2) après oxydation avec l'acide peracétique, (3) avec digestion enzymatique en utilisant la β -glucuronidase, l'hyaluronidase ou le lysozyme avant oxydation avec l'acide peracétique et (4) avec les trois mêmes enzymes après oxydation avec l'acide peracétique.

Les fibres et la substance fondamentale se colorent dans les zones pulpaire subodontoblastiques, centrales et apicales. L'intensité de la coloration est la plus marquée dans la zone apicale des dents, en voie de développement. Dans la dent adulte, la zone centrale ne réagit souvent pas. La digestion dans la β -glucuronidase, l'hyaluronidase ou le lysozyme, avant ou après oxydation, fait disparaître des quantités variables de matériel, réagissant avec l'aldéhyde fuchsiq. L'hyaluronidase est très actif: par contre le lysozyme est inactif.

Le pulpes de 16 dents ont été prélevées avant fixation. Dans les trois régions, au cours de chacune des cinq modalités expérimentales, où l'oxydation a été utilisée, les réactions colorées ont été moins intenses que celles observées au niveau de pulpes de dents décalcifiées.

Il semble que les réactions colorées soient limitées aux régions où se déroule la synthèse du collagène.

Zusammenfassung—Die Pulpen von 61 menschlichen Zähnen in verschiedenen Entwicklungsstadien, von 21 männlichen und 40 weiblichen Personen zwischen 7 und 33 Jahren herkommend, wurden der Aldehyd-Fuchsin-Halmi-Färbung unterworfen.

Unter folgenden Umständen wurde die Farbreaktion beobachtet: (1) ohne Oxydation mit Peressigsäure, (2) nach Oxydation mit Peressigsäure, (3) mit enzymatischem

Abbau unter Verwendung von β -Glukuronidase, Hyaluronidase oder Lysozym vor der Oxydation mit Peressigsäure, und (4) mit denselben drei Fermenten nach Oxydation mit Peressigsäure.

Die Anfärbung von Fasern und Grundsubstanz wurde in den Subodontoblasten-, den zentralen und den apikalen Zonen der Pulpa festgestellt. Die Intensität der Färbung war im apikalen Bereich des sich entwickelnden Zahnes am ausgeprägtesten. Im ausgereiften Zahn reagierte die zentrale Zone häufig nicht. Der fermentative Abbau durch β -Glukuronidase, Hyaluronidase oder Lysozym vor oder nach Oxydation beseitigte unterschiedliche Mengen des mit Aldehyd-Fuchsin reagierende Materials. Am wirkungsvollsten war Hyaluronidase, Lysozym war wirkungslos.

Bei 16 Zähnen wurden die Pulpen vor der Fixation entfernt. In drei Bezirken unter jeder der fünf Bedingungen, bei denen die Oxydation angewandt wurde, waren die Farbreaktionen geringer als in Pulpen entkalkter Zähne.

Daraus wird der Schluss gezogen, dass die Farbreaktion auf die Bezirke begrenzt ist, wo Collagen synthetisiert wird.

REFERENCES

- FITTON JACKSON, S. 1964. Connective tissue cells. In: *The Cell* (Edited by BRACHET, J. and MIRSKY, A. E.) Vol. 6, pp. 405-423. Academic Press, New York.
- FULLMER, H. M. 1958. Differential staining of connective tissue fibres in areas of stress. *Science*, N.Y. 127, 1240.
- FULLMER, H. M. and LILLIE, R. D. 1958. The oxytalan fibre: a previously undescribed connective tissue fibre. *J. Histochem. Cytochem.* 6, 425-430.
- FULLMER, H. M. 1959. Observations on the development of oxytalan fibres in the periodontium of man. *J. dent. Res.* 38, 510-518.
- FULLMER, H. M. 1960. Effect of peracetic acid on the enzymatic digestion of various mucopolysaccharides: reversal of the PAS staining reaction of mucin. *J. Histochem. Cytochem.* 8, 113-121.
- GOMORI, G. 1954. The histochemistry of mucopolysaccharides. *Br. J. exp. Path.* 35, 377-380.
- GREENSPAN, F. P., cited by LILLIE, R. D. 1954. *Histopathologic Technic and Practical Histochemistry*. Blakiston, New York.
- SCHWARZ, W. 1957. Morphology and differentiation of the connective tissue fibre. In: *Connective Tissue* (Edited by TUNBRIDGE, R. E.) p.153. Blackwell Scientific, Oxford.
- ZUGIBE, F. T. 1963. Mucopolysaccharides of the arterial wall. *J. Histochem. Cytochem.* 11, 35-39.

PLATE 1

FIG. 2. Aldehyde fuchsin-Halmi staining of apical region in axial section of the pulp of the unerupted lower first molar. Male 8 years. $\times 90$.

FIG. 3. Peracetic acid oxidation + aldehyde fuchsin-Halmi staining of section from same specimen as for Fig. 2. Note distribution of purple staining fibres. $\times 90$.

FIG. 4. Hyaluronidase digestion after peracetic acid oxidation followed by aldehyde fuchsin-Halmi staining of section from same specimen as for Figs. 2 and 3. Note the complete absence of purple staining. $\times 90$.

FIG. 5. Peracetic acid oxidation + aldehyde fuchsin-Halmi staining in transverse section of the pulp of an upper premolar. Note absence of reactive zone. Female 33 years. $\times 90$.

FIG. 6. Hyaluronidase digestion prior to peracetic acid oxidation + aldehyde fuchsin-Halmi staining of apical region in transverse section of the undecalcified pulp of a lower premolar. Male 12½ years. $\times 90$.

FIG. 7. Hyaluronidase digestion after peracetic acid oxidation followed by aldehyde fuchsin-Halmi staining of section from same specimen as for Fig. 6. Note the loss of intensity of staining. $\times 90$.

ALDEHYDE FUCHSIN REACTION IN DEVELOPING PULP

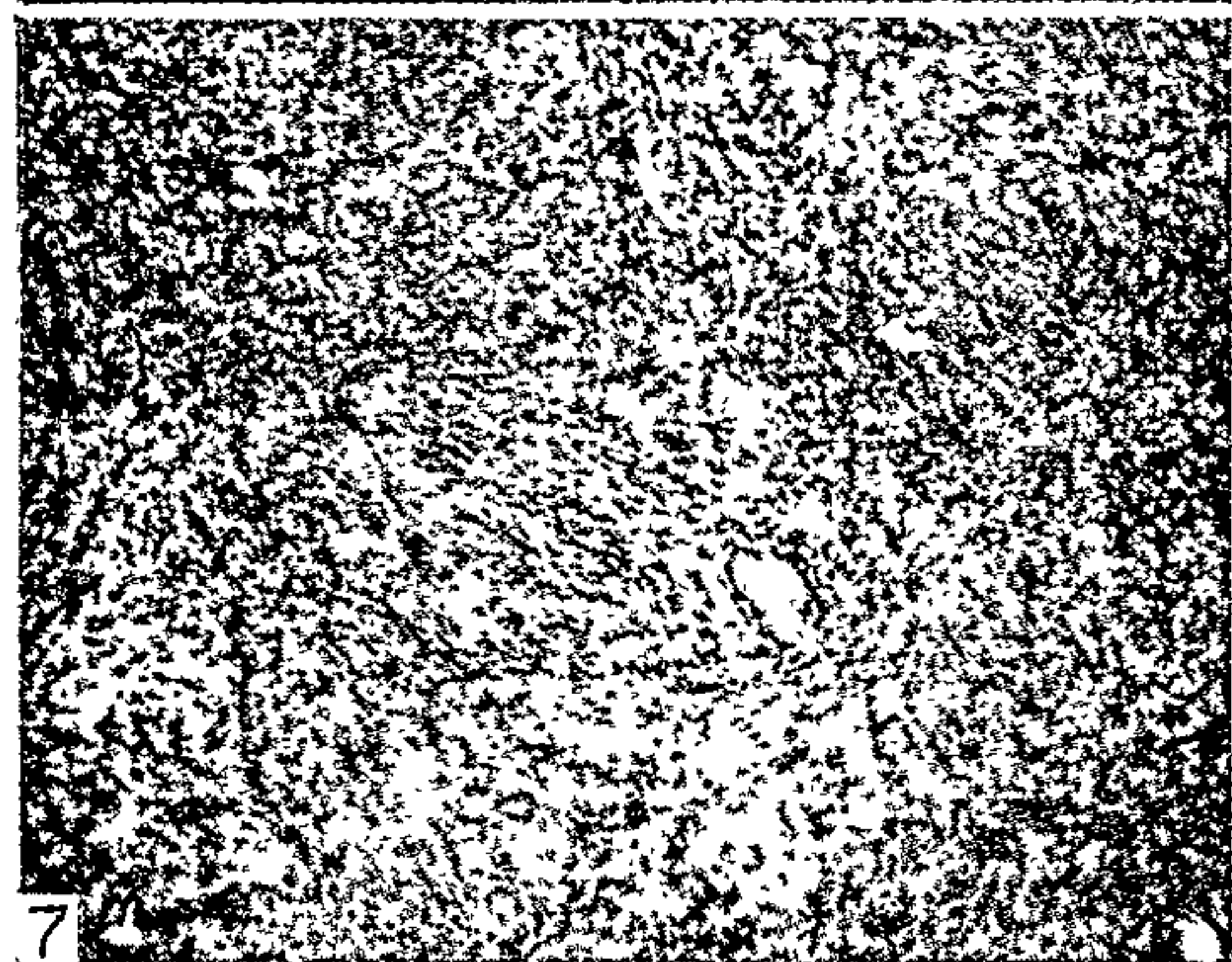
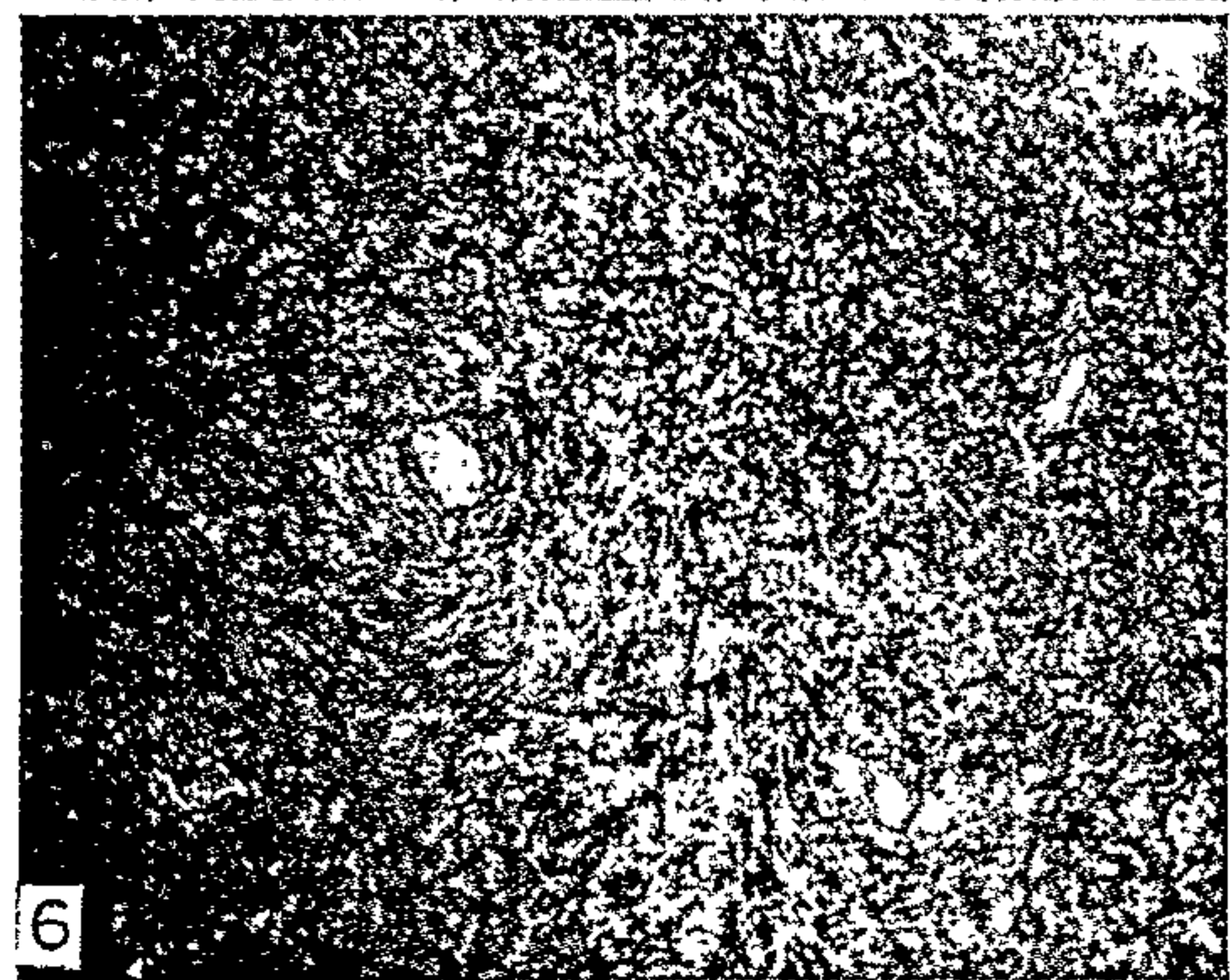
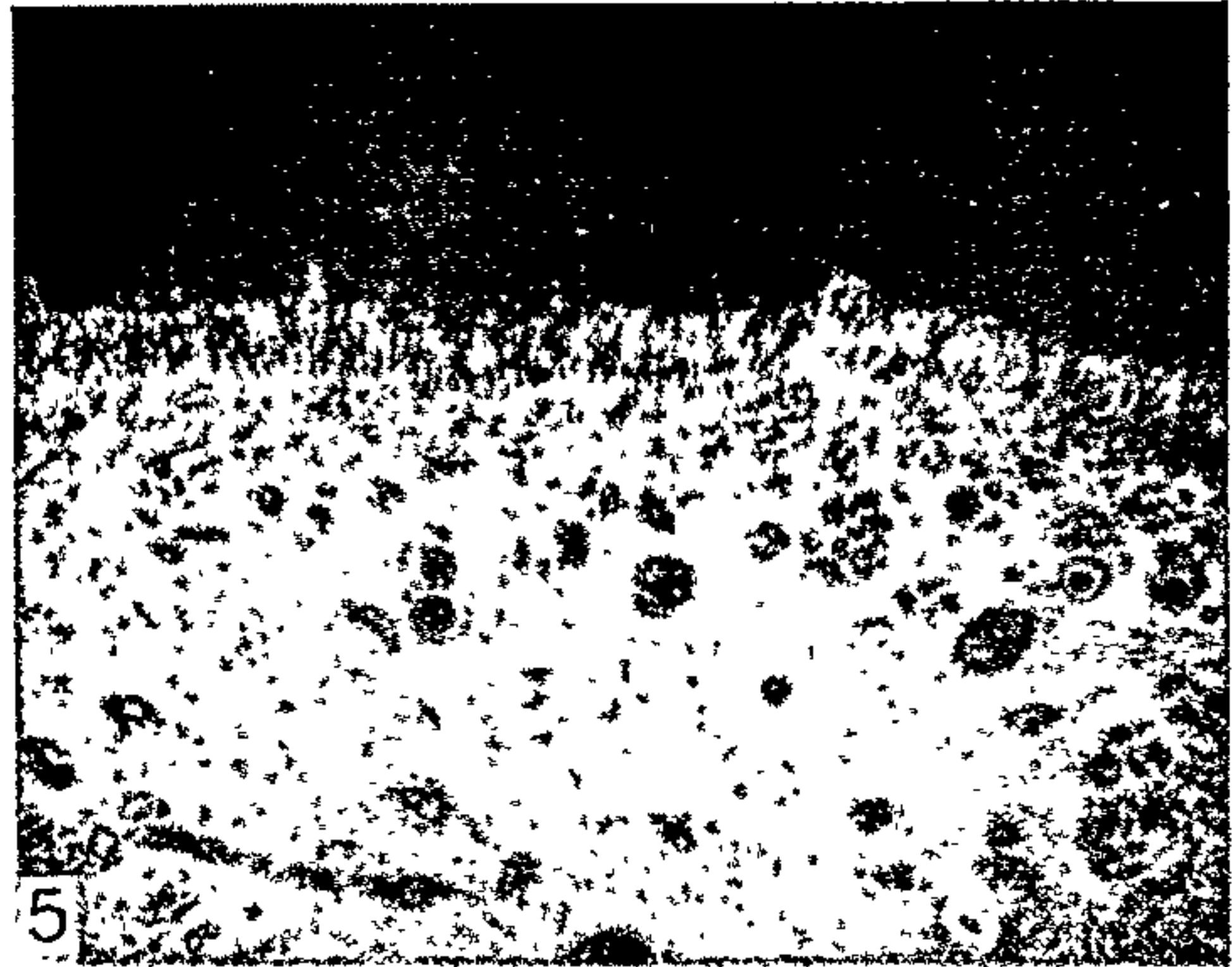
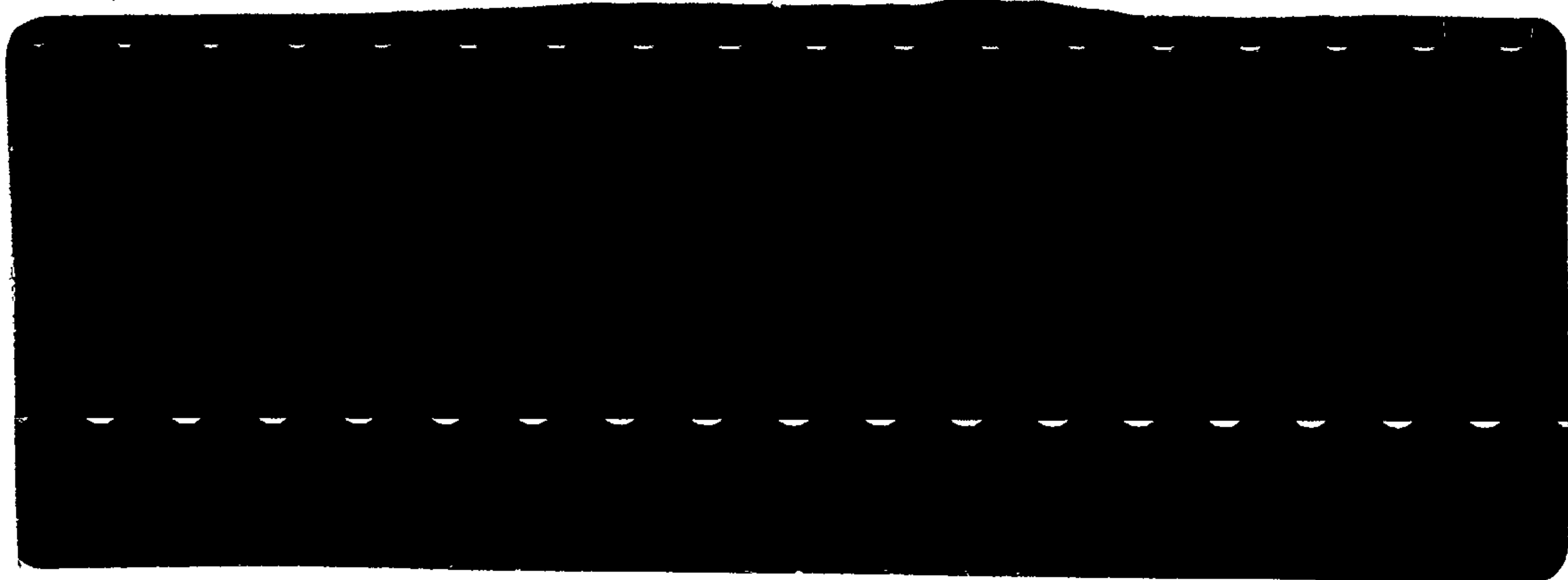


PLATE 1

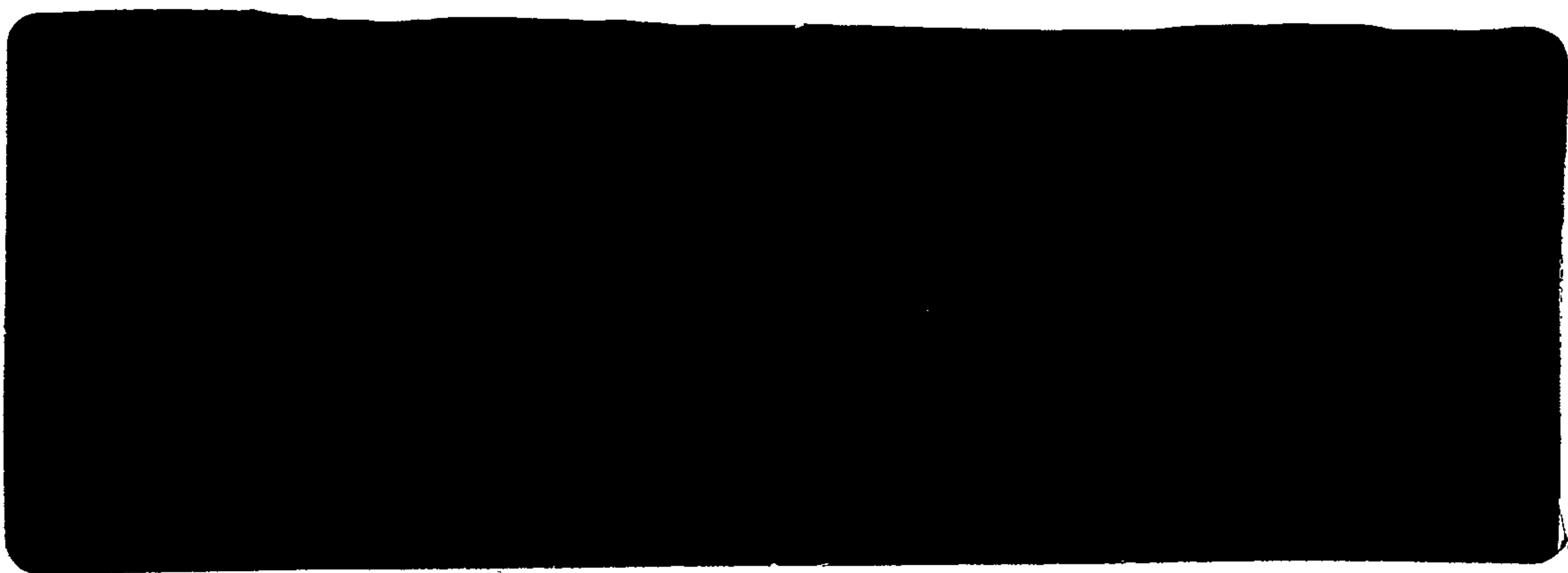
A.O.B. f.p. 658

Reprinted from



PERGAMON PRESS

OXFORD NEW YORK LONDON PARIS



ULTRASTRUCTURE OF COLLAGEN FIBRILS AND FIBROBLASTS OF THE DEVELOPING HUMAN DENTAL PULP

C. J. GRIFFIN

Department of Histology and Embryology, University of Sydney

and R. HARRIS

Institute of Dental Research, United Dental Hospital, Sydney, Australia.

Summary—Synthetizing fibroblasts and adjacent extracellular substance of twelve developing human dental pulps were examined by electron microscopy. The appearance of the cells suggested that collagen synthesis occurred by the formation of collagen precursors in the dilated cisternae of the rough-surfaced endoplasmic reticulum. This precursor substance probably reaches the exterior by secretory vesicles derived from the Golgi apparatus. Two types of collagen fibrils of different electron density were observed in the extracellular milieu. It is suggested that the more electron-dense fibril is the younger of the two types and that the electron density is caused by an associated rather specialized mucopolysaccharide cementing material. A coiled, irregularly beaded extracellular filament, about 100Å in diameter, is also described.

INTRODUCTION

IN A PREVIOUS paper (HARRIS and GRIFFIN, 1966) it was noted that fibres of developing dental pulp reacted strongly with aldehyde fuchsin after the tissue had previously been oxidized with peracetic acid. The reactive material was occasionally β -glucuronidase and hyaluronidase labile. As the dental pulp matured, the reactive substance decreased in amount, indicating that it might be a substance associated with young collagen fibres of the pulp.

Usually the only reactive zones in the mature pulp were discrete subodontoblastic and apical zones and occasionally discrete areas in the central part of the pulp. Areas of the developing dental pulp were therefore examined with the electron microscope and the results were correlated with histochemical and light microscopical observations (HARRIS and GRIFFIN, 1966).

MATERIAL AND METHODS

Twelve partially developed, unerupted teeth were removed from patients receiving orthodontic therapy or having multiple extractions. A superficial cut was made in an axial plane through crown and roots and the teeth were split open with a pair of bone-cutting forceps. The pulps were removed and cut into small pieces (approximately 0.5 × 1.0 mm) which were placed into Palade's osmium tetroxide fixative. Fixation was carried out at 0°C for 2 hr. The time elapsed between interruption of the blood supply and immersion in fixative in no case exceeded 90 sec. Following fixation the pieces were dehydrated in increasing concentrations of acetone (25%, 50% and 75%

for 15 min each) and, after rinsing in three changes of 100% acetone, embedded in Araldite. Polymerization was effected at 60°C for 24 hr.

Sections were cut on an L.K.B. ultramicrotome at 500–1000Å and stained with uranyl acetate on the grid for 1 hr. Electron micrographs and observations were made on a Hitachi H.S. 7 electron microscope.

RESULTS

Our observations were restricted to areas where active collagen synthesis was occurring. These areas could be identified by the presence of synthesizing (active) fibroblasts. These cells were recognized by the dilated profiles of the rough-surfaced endoplasmic reticulum, intracellular filaments, swollen mitochondria with irregular cristae, marginal condensations and occasional amorphous dense bodies (WEISS and FERRIS, 1956; PORTER and PAPPAS, 1959; POLICARD, COLLET and PREGERMAN, 1960; CHAPMAN, 1961; ROSS and BENDITT, 1961; CASTOR and MUIRDEN, 1964; FITTON JACKSON, 1964; GOLDBERG and GREEN, 1964). Synthesizing fibroblasts in the developing dental pulp of the hamster have been described by AVERY and HAN SEONG (1961).

The most conspicuous feature of the active fibroblasts of the developing human dental pulp was the extensive development of the rough-surfaced endoplasmic reticulum in the form of dilated cisternae. These cisternae contained an amorphous irregularly beaded electron-dense substance and sometimes filaments about 40Å in diameter (Figs. 1–3). In tangential sections of the cisternal walls ribosomes were often seen to be in rows (Fig. 1) but were not infrequently found in clusters only partly covering the cisternal membrane (Fig. 3). The plasma membranes in some sections were indistinct without this being ascribable to oblique sectioning of the membrane. Some vesicles, presumably secretory and usually containing an amorphous substance, were seen in the Golgi zone, throughout the cytoplasm, and near or attached to plasma membranes (Figs. 1, 2, 4 and 5). Intracellular filaments, with a diameter of about 70Å and with a rather irregular and indefinite periodicity were aligned more or less parallel with the plasma membrane (Fig. 5).

An extensive Golgi zone was usually seen near the nucleus and numerous vesicles of the type described above were present. Dilated cisternae in the vicinity of the Golgi zone sometimes contained an amorphous substance comparable in electron density to the substance within the secretory vesicles (Fig. 2). Cytoplasmic bodies containing substances of varying electron density and enlarged mitochondria with irregular cristae were seen around the Golgi zone (Fig. 2).

Plasma membranes of the synthesizing fibroblasts, when seen in vertical sections, were approximately 75Å thick (Fig. 3). Interruptions were frequently observed in these membranes and we believe that these interruptions were due to manipulation of the tissue, as suggested by ROSS and BENDITT (1961) and GOLDBERG and GREEN (1964).

Three types of vesicles were seen. The first type corresponded to pinocytotic vesicles, as described by GOLDBERG and GREEN (1964), and were seen only within the cell. They have a dense limiting membrane about 80Å thick surrounded by layers of amorphous material approximately 140Å wide (Figs. 1 and 4). This type of vesicle

appears to arise from an invagination of the plasma membrane (Fig. 11) and subsequently the invaginated material is cut off from the plasma membrane across the invagination (Fig. 6). No examples of this pinocytotic vesicle were seen in the extracellular milieu. The vesicles described as pinocytotic by GOLDBERG and GREEN (1964) were apparently being formed in this fashion in rat tissue fibroblast cultures. One of the reasons they designated the vesicles as pinocytotic was the presence of extracellular substances including what appeared to be collagen fibrils within them. In Fig. 4 an oval structure which may be a collagen fibril about 500Å in diameter can be seen within what we have designated a pinocytotic vesicle. However, similar structures are seen within the cytoplasm of the same cell. There is no certainty from our material that substances inside the vesicle are of extracellular origin.

In Fig. 1 two vesicles can be seen containing a moderately electron-dense substance comparable to similar material seen extracellularly (Fig. 6). Nevertheless substance of the same electron density can be seen within the cell. The possibility cannot therefore be excluded that these vesicles may be of a secretory type.

The second type of vesicle is the secretory vesicle which is smaller than the pinocytotic vesicles and appears to be derived from the Golgi apparatus (Fig. 2). These vesicles contain amorphous moderately electron-dense material and can be seen adjacent to the plasma membrane externally (Fig. 3) and intracellularly in Figs. 2 and 4.

The third type we have called the cisternal vesicle and this is probably derived from the dilated sacs of the rough-surfaced endoplasmic reticulum. These vesicles contain the coiled, irregularly beaded filaments similar to those in the dilated sacs of the rough-surfaced endoplasmic reticulum (Figs. 1, 3 and 12).

Occasionally large dense bodies 0.3–1.0μ in diameter, possibly lysosomes, were seen near the plasma membrane (Fig. 7).

Collagen fibrils were seen extracellularly both singly and in bundles, the latter presumably collagen fibres in the process of formation. The diameters of the individual collagen fibrils were in the range of from 500 to 800Å and two groups of differing electron density, c_1 and c_2 (Fig. 8), were discernible. All the collagen fibrils in any one group usually had the same high or low electron density. Occasionally heterogeneous populations of fibrils, c_3 , were seen (Figs. 5, 8 and 9). In these latter populations the more electron-dense fibrils were usually located at the periphery of the bundle. The difference in electron density was apparent whether the fibrils were observed in transverse or longitudinal sections (Figs. 8 and 10). The electron density of the darker fibrils appeared to be partially due to the concentration of an amorphous substance around the fibrils and this material contributed towards the masking of the periodicity which is normally seen in collagen fibrils. Mature collagen fibrils of the pulp, approximately 750Å in diameter, are seen in Fig. 10.

Two types of filaments were recognizable in the extracellular milieu. One type, approximately 200Å in diameter, exhibited a periodicity of about 200Å and had a relatively straight contour. The other type, about 100Å in diameter, was a coiled, branched and irregularly beaded element (Figs. 11 a and b). These latter filaments appeared to form a complex pattern throughout the ground substance, whereas the former filaments were more often associated with collagen fibrils.

DISCUSSION

Intracellular filaments and collagen synthesis

Intracellular filaments in synthesizing fibroblasts have been described by many observers. ROSS and BENDITT (1961) noted filaments 20–80Å in diameter and CHAPMAN (1961) observed intracellular filaments 50Å in diameter, which in some instances were aligned parallel to plasma membranes, especially in the elongated processes of fibroblasts. WASSERMAN (1954) described filaments (primary fibrils) of irregular nodular structure, 150–200Å in diameter, of limited length, and with tapered ends that lay within the marginal zone of the cytoplasm. WASSERMAN and KUBOTA (1956) observed that groups of filaments could be seen in the cytoplasm of connective tissue cells from 10-day old chick embryos. GOLDBERG and GREEN (1964) observed intracellular filaments of approximately 50Å diameter in the 'log-phase' of rat fibroblasts cultured *in vitro*; this was confirmed in human fibroblasts by CASTOR and MUIRDEN (1964).

GOLDBERG and GREEN (1964) did not consider these filaments to be aggregates of collagen molecules since hydroxyproline could not be recovered from hydrolysates of the cells. They were unable to find any morphological evidence relating the intracellular aggregation of filaments to the synthesized products in the rough-surfaced endoplasmic reticulum and the Golgi system. They further state that it is impossible to demonstrate by electron microscopy any continuity between the intracellular filaments and those outside the cell. Discontinuity of plasma membranes, which might afford a path for extrusion of these filaments, according to ROSS and BENDITT (1961) and GOLDBERG and GREEN (1964) is due to manipulation of the tissue and this may be the reason for the frequent discontinuities of plasma membranes seen in our material. Nevertheless plasma membranes of other cell types in our preparations did not exhibit discontinuity (Fig. 9), which suggests that the plasma membranes of synthesizing fibroblasts are particularly fragile.

FITTON JACKSON (1964) has expressed the opinion that collagen fibrils might be formed in two ways: in one instance small groups of filaments formed within the cytoplasm and in the other filaments formed extracellularly in close association with the cell surface. In dental pulp fibroblasts we were not able to relate intracellular filaments to collagen synthesis.

Collagen synthesis by fibroblasts of the human dental pulp appeared to be similar to collagen synthesis in tissue culture, as interpreted by GOLDBERG and GREEN (1964) and CASTOR and MUIRDEN (1964). Secretory vesicles with an amorphous electron-dense substance were found in the Golgi zone and accumulated in proximity to the plasma membrane (Fig. 12). They appeared to attach themselves to the plasma membrane and then either to discharge their contents into the extracellular milieu or to pass out of the cell as complete vesicles, presumably to break down later.

The filamentous material not infrequently seen in the dilated cisternae resembles the branched, beaded, extracellular filament more than it does the second filament which we regard as a collagen filament because the latter is usually straight and has a definite periodicity (PORTER, 1952). We have no positive evidence of how the coiled filaments of the dilated cisternae (Fig. 1) reach the exterior. However in Fig. 3

may be seen a cisternal vesicle, apparently derived from the dilated sacs of the rough-surfaced endoplasmic reticulum, approximating the plasma membrane and others external to it. These cisternal vesicles may have passed through the plasma membrane intact and then subsequently broken down, releasing their contents. They differ in appearance from the other vesicles (vp in Figs. 1 and 6) seen at the cell surface. The dense cytoplasmic granules (Fig. 7) previously referred to may be lysozomal in nature (FITTON JACKSON, 1964).

The fibroblast of the developing dental pulp appears to differ somewhat from the developing fibroblasts of the newborn hamsters. AVERY and HAN SEONG (1961) found in this animal that the major cell organelles were oval mitochondria and to a lesser extent endoplasmic reticulum which was not highly organized and was scattered in tubular form throughout the cell. They were unable with potassium permanganate fixation to observe ribosomes attached to the endoplasmic reticulum. The human dental pulp fibroblast on the other hand shows an extensive development of the rough-surfaced endoplasmic reticulum and ribosomes are plentiful both in association with it and in the cytoplasm (Fig. 1).

Extracellular fibrils

Collagen fibrils of differing electron density were noted by CHAPMAN (1961) in tissues stained with 1% phosphotungstic acid. He reported the presence of densely stained clumps of partly amorphous and partly filamentous material in which short lengths of striated collagen fibrils could be recognized. In Fig. 3, what appears to be collagen fibrils forming in a filamentous electron-dense material can be seen. CASTOR and MUIRDEN (1964) noted in tissue cultures the presence of extracellular dense amorphous material in which it was possible to distinguish fibrils. FITTON JACKSON (1964) stated that, as the diameter of the fibrils increased with age, there was a relative reduction of the perifibrillar material which surrounded each fibril.

In our preparations, as well as two distinct populations of collagen fibrils differing in electron density, there was present a third heterogeneous population. It was noted that in this latter population the more electron-dense fibrils were located at the periphery of the population (Figs. 8 and 9). Apart from this, ungrouped fibrils were usually markedly electron-dense (Fig. 11) and their periodicity was obscured.

It can be noted in Fig. 3 that single collagen fibrils are surrounded by an amorphous, moderately electron-dense substance in which filaments can be observed. Similarly this amorphous material can be noted between collagen fibrils which appear to be coming into register (Fig. 10). In Fig. 6 this amorphous material can be seen surrounding a group of fibrils and there are also filaments evident.

Because of this distribution, it seems reasonable to suggest that the more electron-dense fibrils are younger than those with a lower electron density and represent fibrils which are just aggregating to form a collagen fibre or are fibrils which are being added to an already existing fibre. There have been suggestions that the electron density of young collagen fibrils is due to the presence of an investing and possibly permeating mucopolysaccharide substance. For example, SCHWARZ (1957) proposed that the argyrophilia of young collagen fibrils was due to the presence of a polysaccharide

cementing substance which, as the fibrils became older, decreased in amount. More recently CASTOR and MUIRDEN (1964) noted that collagen fibres, derived from tissue culture of human synovioblasts and therefore presumably young fibres, stained red with the peracetic acid Schiff-alcian blue stain and they were able to recover a carbohydrate substance from the interfibrillar material. We should like to suggest that the amorphous electron-dense substance associated with collagen fibrils in the developing dental pulp corresponds to the mucopolysaccharide cementing substance of young fibrils observed elsewhere. If this were so, the substance demonstrated in developing dental pulp, which after peracetic acid oxidation was aldehyde fuchsin positive and at times β -glucuronidase and hyaluronidase labile (HARRIS and GRIFFIN, 1966) may correspond to the amorphous material causing the increased electron density of certain groups of fibrils. This view is consistent with the fact that the reactive material diminishes as the pulp matures and in the aged pulp is present only in discrete zones (HARRIS and GRIFFIN, 1966).

Extracellular filaments

Two types of extracellular filaments were recognized in the developing pulp tissue. The relatively straight types with a 200Å periodicity are most probably collagen filaments. They were not infrequently seen to be rather intimately associated with young collagen fibrils (Fig. 11). FITTON JACKSON (1964) and ROSS and BENDITT (1961), in developing tissue, and PORTER (1952) in tissue culture, noted the presence of extracellular filaments which had a periodicity of about 200Å rather than 640Å. As the material matured the filaments enlarged in diameter until they reached the size of collagen fibrils typical of the tissue of origin. During this maturation the typical periodicity of 640Å became evident.

The second type of filament present in the developing pulp appeared to form a complex network in the ground substance. These filaments were coiled, branched and irregularly beaded. They differed from the filaments (microfibrils) described by Low (1962) in that they were not concentrated around basement membranes. Low also found microfibrils around elastic fibres but no evidence for this type of fibre in dental pulp has been found by either light or electron microscopy. However, the diameter of these filaments, 100Å, approximates the diameter of the filaments described by Low (1962) in connective tissue and the diameter of the filaments described by CASTOR and MUIRDEN (1964) in tissue derived from culture of human synovioblasts. They are morphologically different from the protein polysaccharide filaments described by FITTON JACKSON (1964) in chick cartilage but may well be of a similar nature.

Acknowledgement—We wish to thank Professor K. W. CLELAND, Department of Histology and Embryology, University of Sydney, for help in the preparation of the electron micrographs and for constructive criticism of them, and the National Health and Medical Research Council of Australia for financial assistance for part of this work.

Résumé—Des fibroblastes actifs et la substance extracellulaire adjacente de douze pulpes humaines, en voie de développement, ont été étudiés à l'aide de la microscopie électronique. L'aspect des cellules suggère que la synthèse du collagène s'effectue par l'intermédiaire de précurseurs qui s'accumulent dans la lumière dilatée du réticulum endoplasmique, à surfaces rugueuses. Ces substances précurseurs sont vraisemblablement rejetées vers l'extérieur par l'intermédiaire de vésicules sécrétrices, dérivées de l'appareil de Golgi. Deux types de fibrilles collagènes, de densité électronique différente ont été observés dans le milieu extracellulaire. Il semble d'une part que la fibrille la plus dense aux électrons constitue la variété la plus jeune et d'autre part que la densité aux électrons est liée à la présence d'un matériel cimentant complexe de mucopolysaccharides. Un filament extracellulaire, spiralé et irrégulièrement moniliforme, d'environ 100Å de diamètre, est également décrit.

Zusammenfassung—Synthetisierende Fibroblasten und umgebende Extrazellularsubstanz von 12 sich entwickelnden menschlichen Zahnpulpen wurden elektronenmikroskopisch untersucht. Das Bild der Zellen legte nahe, dass die Kollagensynthese durch Bildung von Kollagenvorläufern in den erweiterten Zysternen des oberflächlich rauhen endoplasmatischen Reticulums vor sich ging. Diese Vorläufersubstanz erreicht wahrscheinlich die Umgebung durch vom Golgi-Apparat herstammende Bläschen. Im extrazellulären Milieu wurden zwei Arten von Kollagenfibrillen mit unterschiedlicher Elektronendichte beobachtet. Es wird angenommen, dass die elektronendichtere Fibrille die jüngere beider Typen ist und dass die Elektronendichte durch ein ziemlich spezialisiertes Mucopolysaccharid-Kittmaterial verursacht wird. Ausserdem wird ein verschlungenes, irregulär geperltes extrazelluläres Filament mit einem Durchmesser von etwa 100Å beschrieben.

REFERENCES

- AVERY, J. K. and HAN SEONG, S. 1961. The formation of collagen fibrils in the dental pulp. *J. dent. Res.* **40**, 1248-1261.
- CASTOR, C. W. and MUIRDEN, K. D. 1964. Collagen formation in monolayer cultures of human fibroblasts: the effects of hydrocortisone. *Lab. Invest.* **13**, 560-574.
- CHAPMAN, J. A. 1961. Morphological and chemical studies of collagen formation I. The fine structure of guinea pig granulomata. *J. biophys. biochem. Cytol.* **9**, 639-651.
- FITTON JACKSON, SYLVIA. 1964. Connective tissue cells. In: *The Cell* (Edited by BRACHET, J. and MIRSKY, A. E.) Vol. 6, pp. 387-509, Academic Press, New York.
- GOLDBERG, B. and GREEN, H. 1964. An analysis of collagen secretion by established mouse fibroblast lines. *J. Cell Biol.* **22**, 227-258.
- HARRIS, R. and GRIFFIN, C. J. 1966. Aldehyde fuchsin-Halmi reaction in fibres of the human dental pulp. *Archs oral Biol.* **11**, 649-658.
- LOW, F. N. 1962. Microfibrils: fine filamentous components of the tissue space. *Anat. Rec.* **142**, 131-137.
- POLICARD, A., COLLET, A. and PREGERMAIN, S. 1960. Étude au microscope électronique des dispositifs ergastoplasmiques de certaines cellules du granulome inflammatoire. *Proc. European Regional Conf. on Electron Microscopy*. Delft (Edited by HOUWINK, A. L. and SPIR, B. J.) **2**, 879-882. Der Nederlandse Vereniging voor Electronemicroscopie, Delft.
- PORTER, K. R. 1952. Repair Processes in Connective Tissue. *2nd Conf. Connective Tissue*, pp. 126-158. Josiah Macy Jr. Foundation, New York.
- PORTER, K. R. and PAPPAS, G. D. 1959. Collagen formation by fibroblasts of the chick embryo dermis. *J. biophys. biochem. Cytol.* **5**, 153-166.
- ROSS, R. and BENDITT, E. P. 1961. Wound healing and collagen formation—I. Sequential changes in components of guinea pig skin wounds observed in the electron microscope. *J. biophys. biochem. Cytol.* **11**, 677-700.
- SCHWARZ, W. 1957. Morphology and differentiation of the connective tissue fibre. In: *Connective Tissue*. (Edited by TUNBRIDGE, R. E.) p. 513. Blackwell Scientific, Oxford.
- WASSERMAN, F. 1944. Fibrillogenesis in the regenerating rat tendon with special reference to growth and composition of the collagen fibril. *Am. J. Anat.* **94**, 399-437.
- WASSERMAN, F. and KUBOTA, L. 1956. Observations in fibrillogenesis in the connective tissue of the chick embryo with the aid of silver impregnation. *J. biophys. biochem. Cytol.* **2**, Suppl. 4, 67-70.
- WEISS, P. and FERRIS, W. 1956. The basement lamella of amphibian skin. Its reconstruction after wounding. *J. biophys. biochem. Cytol.* **2**, Suppl. 4, 275-282.

PLATE 1

FIG. 1. Dilated cisternae bounded by rough-surfaced endoplasmic reticulum, eg, contain filaments (arrows). Channels of the rough-surfaced endoplasmic reticulum proximate the plasma membrane, cm, ribosomes, r, secretory vesicles, vs, and pinocytotic vesicles, vp, are present. The limiting membrane (80Å thick) of the pinocytotic vesicle can be seen. Extracellular substance, ecs, extracellular coiled filament, ef, and collagen fibrils, c, with amorphous substance at lower left, cisternal vesicle, vc. $\times 28,700$

FIG. 2. Golgi zone, G, secretory vesicles, vs, rough-surfaced endoplasmic reticulum, eg, cytoplasmic body, b, mitochondrion, m, nucleus, N. $\times 21,000$.

COLLAGEN FIBRILS AND FIBROBLASTS OF THE DENTAL PULP

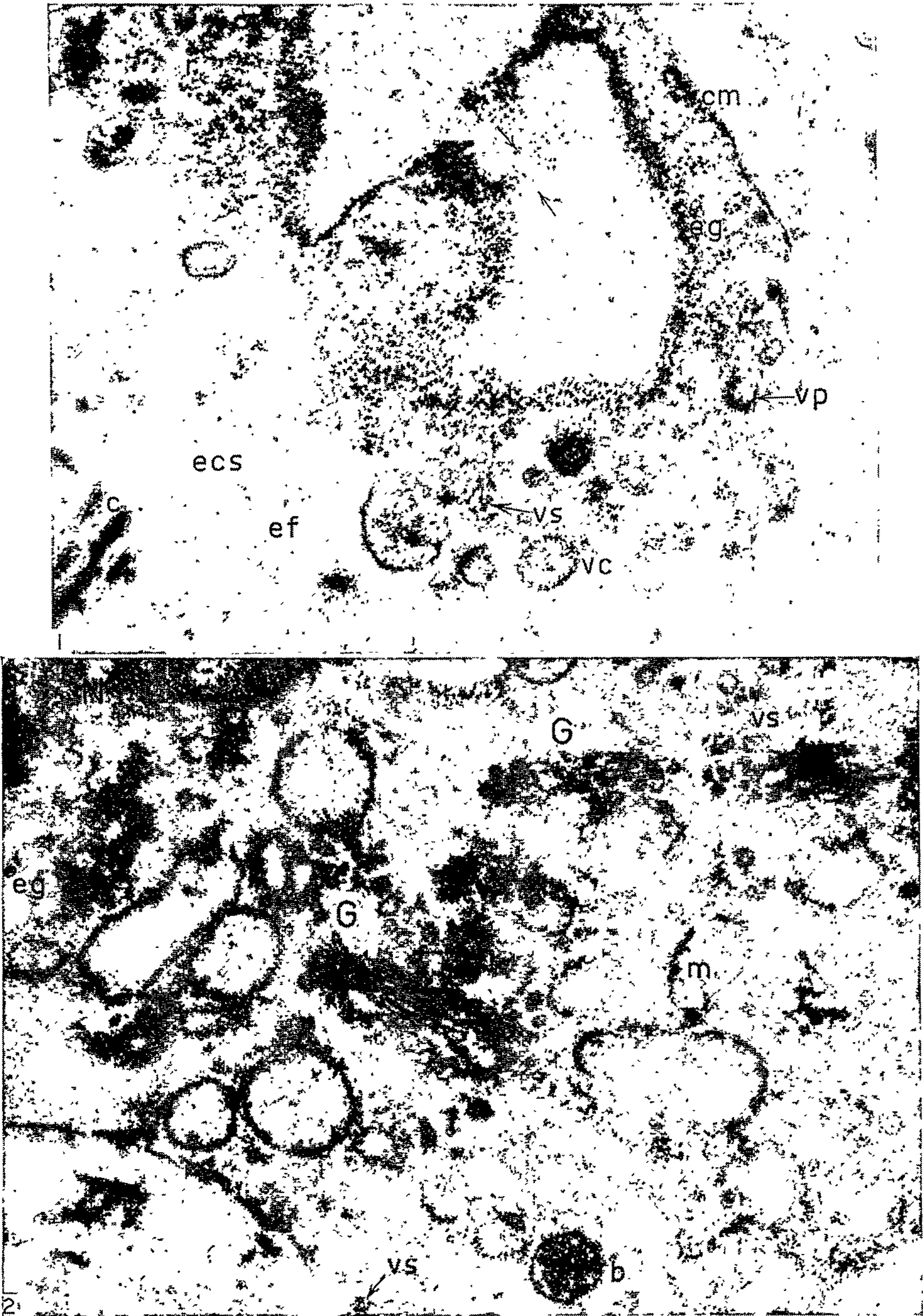


PLATE 1

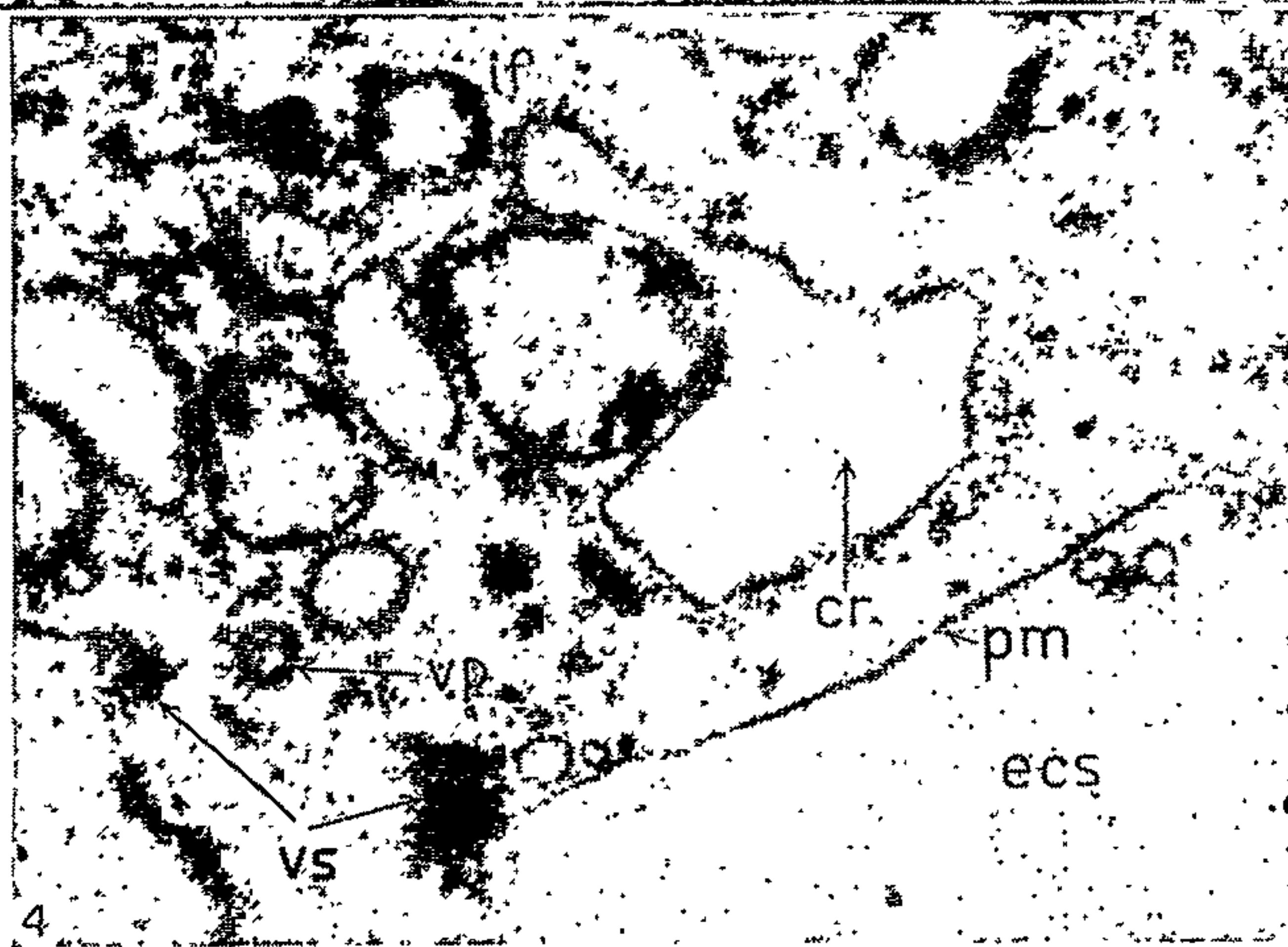
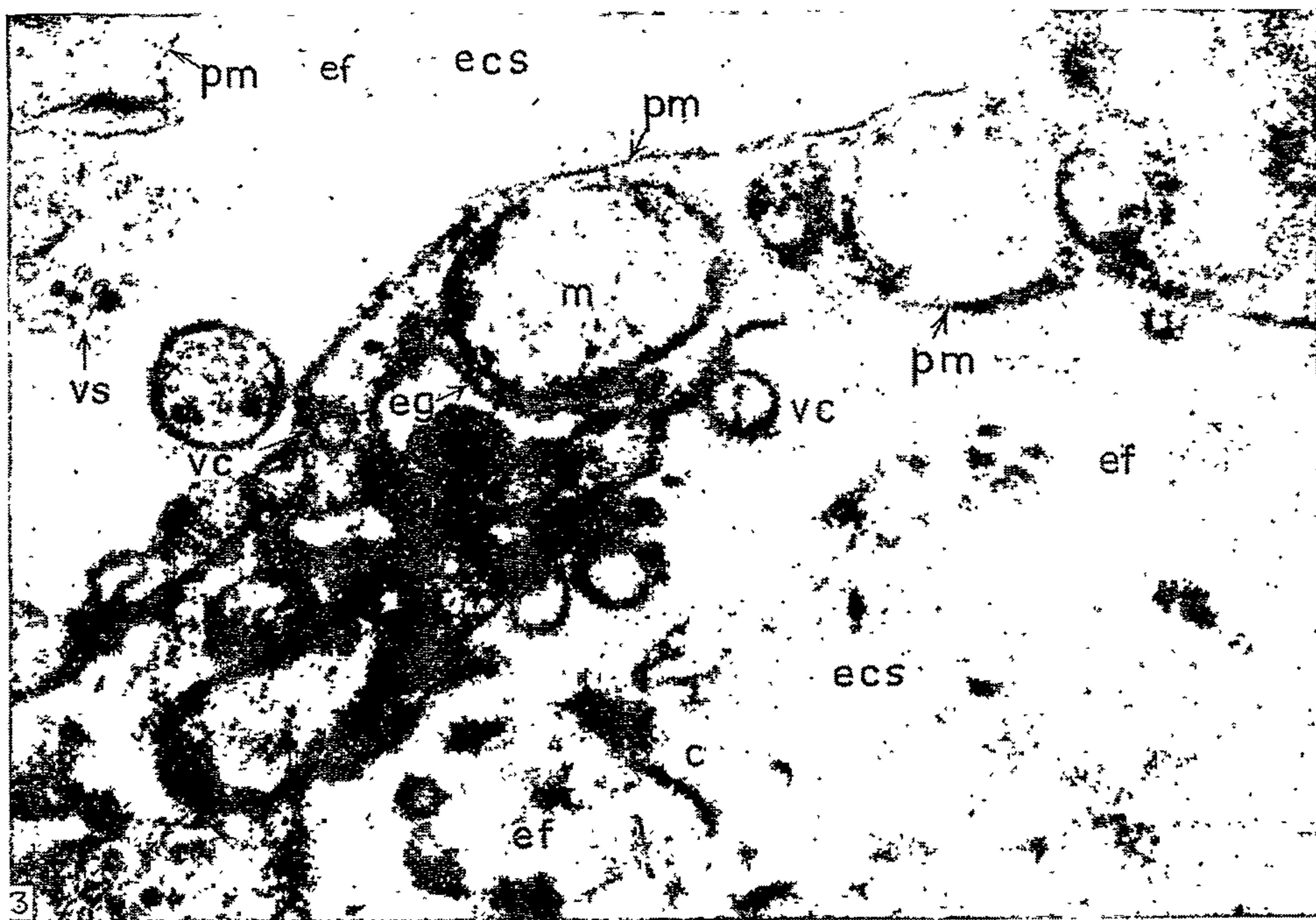


FIG. 3. Extracellular substance, ecs, collagen fibril, c, plasma membrane, pm, approximately 75Å thick. Mitochondrion, m, rough-surfaced endoplasmic reticulum, eg, extracellular vesicles, vs, extracellular coiled filaments, ef, and cisternal vesicle, vc. $\times 21,000$.

FIG. 4. Dilated cisternae, cr, pinocytotic vesicle, vp, intracellular filaments, if, amorphous material and secretory vesicles, vs, plasma membrane, pm, extracellular substance, ecs. $\times 21,000$.



FIG. 5. Fibroblast of dental pulp demonstrating intracellular filaments, if, aligned approximately parallel to the plasma membrane, pm, nucleus, N. A heterogeneous population of collagen fibrils, c_3 , can be noted. $\times 21,600$.

FIG. 6. Pinocytotic vesicles, vp, apparently originating from invagination of the plasma membrane, pm, amorphous material masking collagen fibrils, c, extracellular coiled, branched filaments, ef. $\times 60,000$.

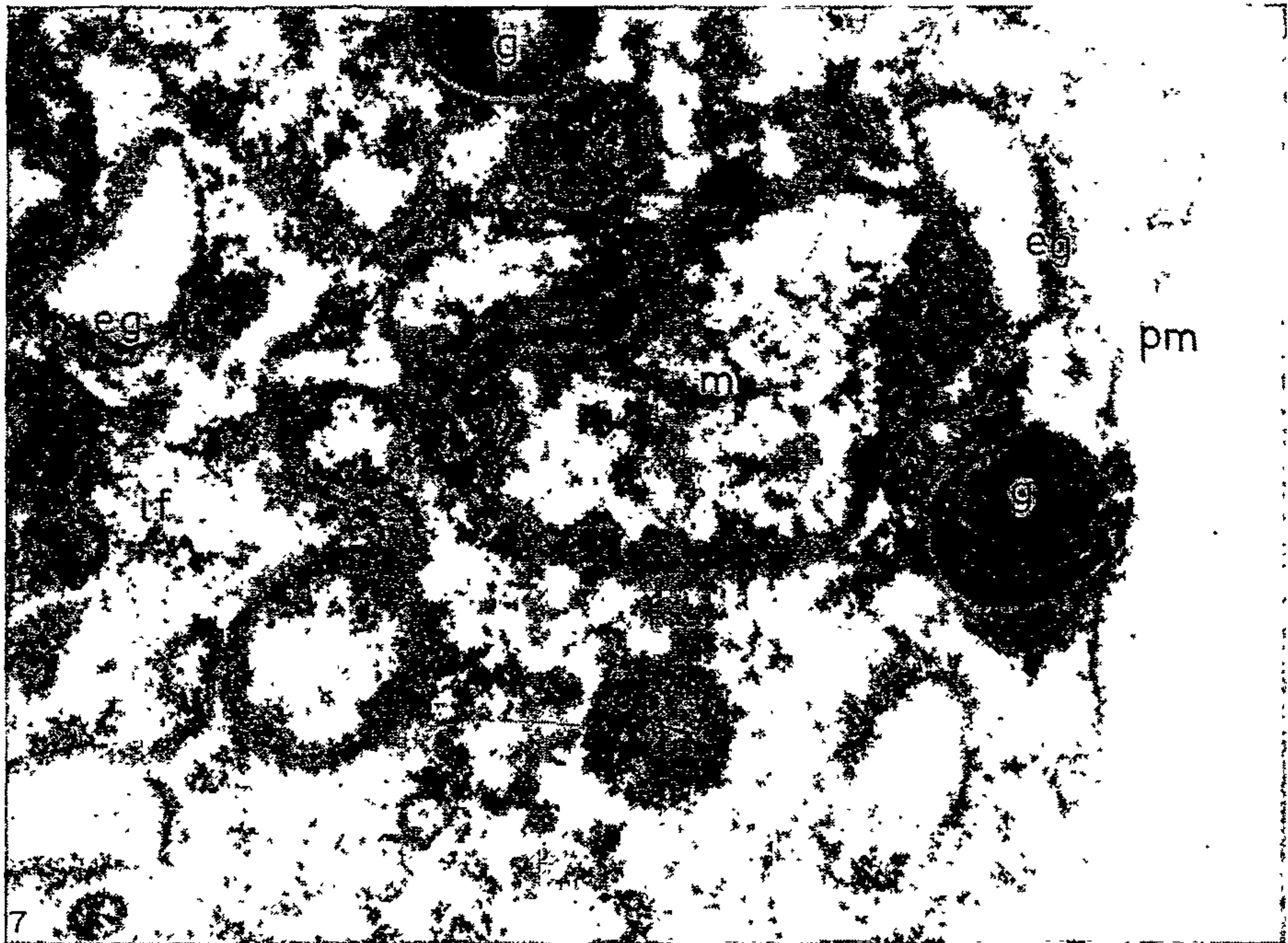


FIG. 7. Cytoplasm of fibroblast with cytoplasmic granules, g, mitochondrion, m, rough-surfaced endoplasmic reticulum, eg, intracellular filaments, if, plasma membrane, pm. $\times 42,000$.

FIG. 8. Fibroblast and extracellular substance with collagen fibrils, c_1 , c_2 , c_3 of differing electron density. Nucleus, N, Golgi zone, G. $\times 9,625$.

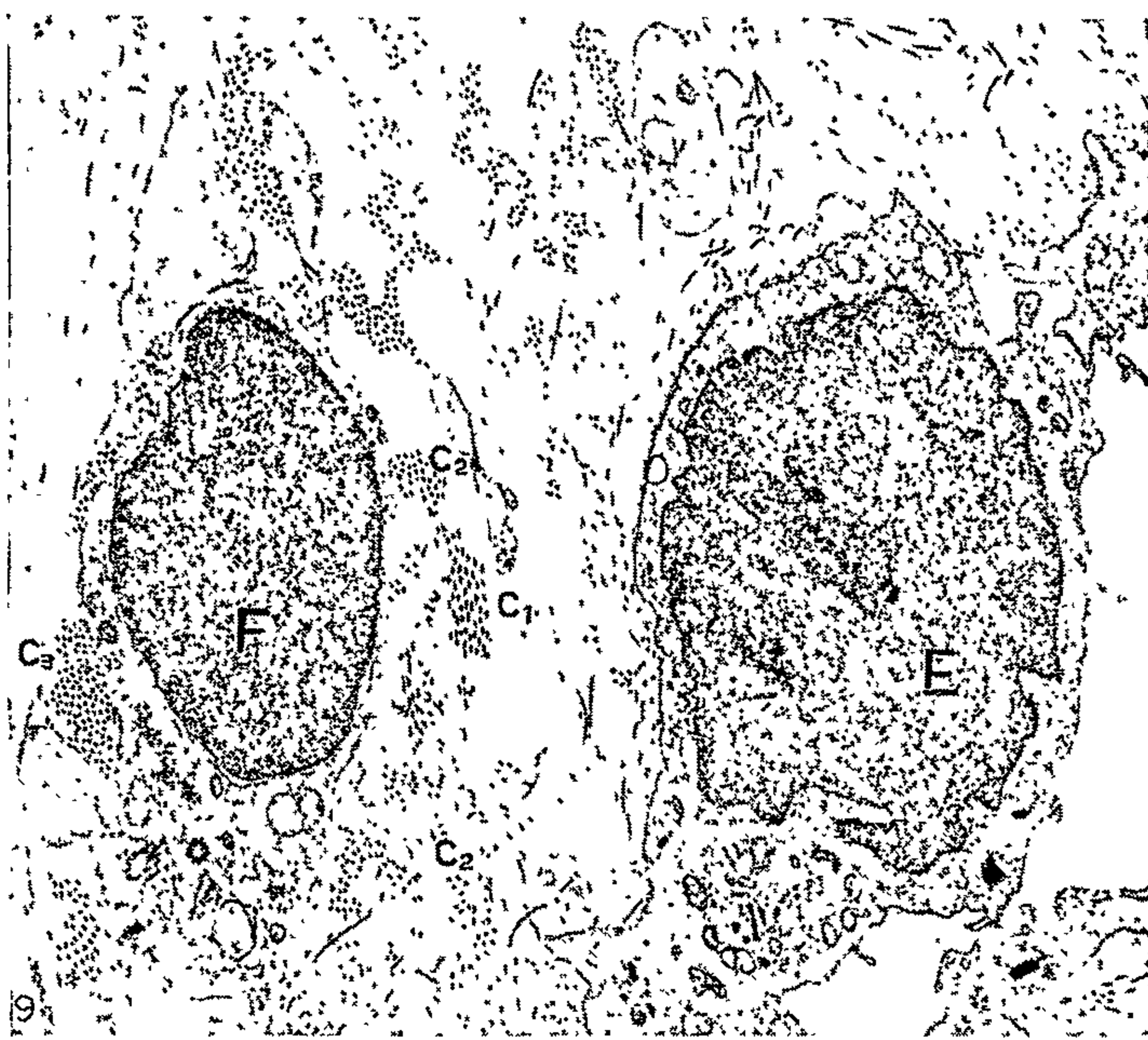


FIG. 9. Fibroblast, F, with frequent interruption of plasma membrane, endothelial cell, E, with intact cell membrane and collagen fibrils, c_1 dark, c_2 light and c_3 heterogeneous. $\times 6,300$.



FIG. 10. Longitudinal sections of an heterogeneous population of collagen fibrils. The intraperiodicity of most of the fibrils in the bottom half of the picture is masked compared with those at the top of the picture. Note that the fibrils, in the top of the picture, are in register. $\times 38,500$.

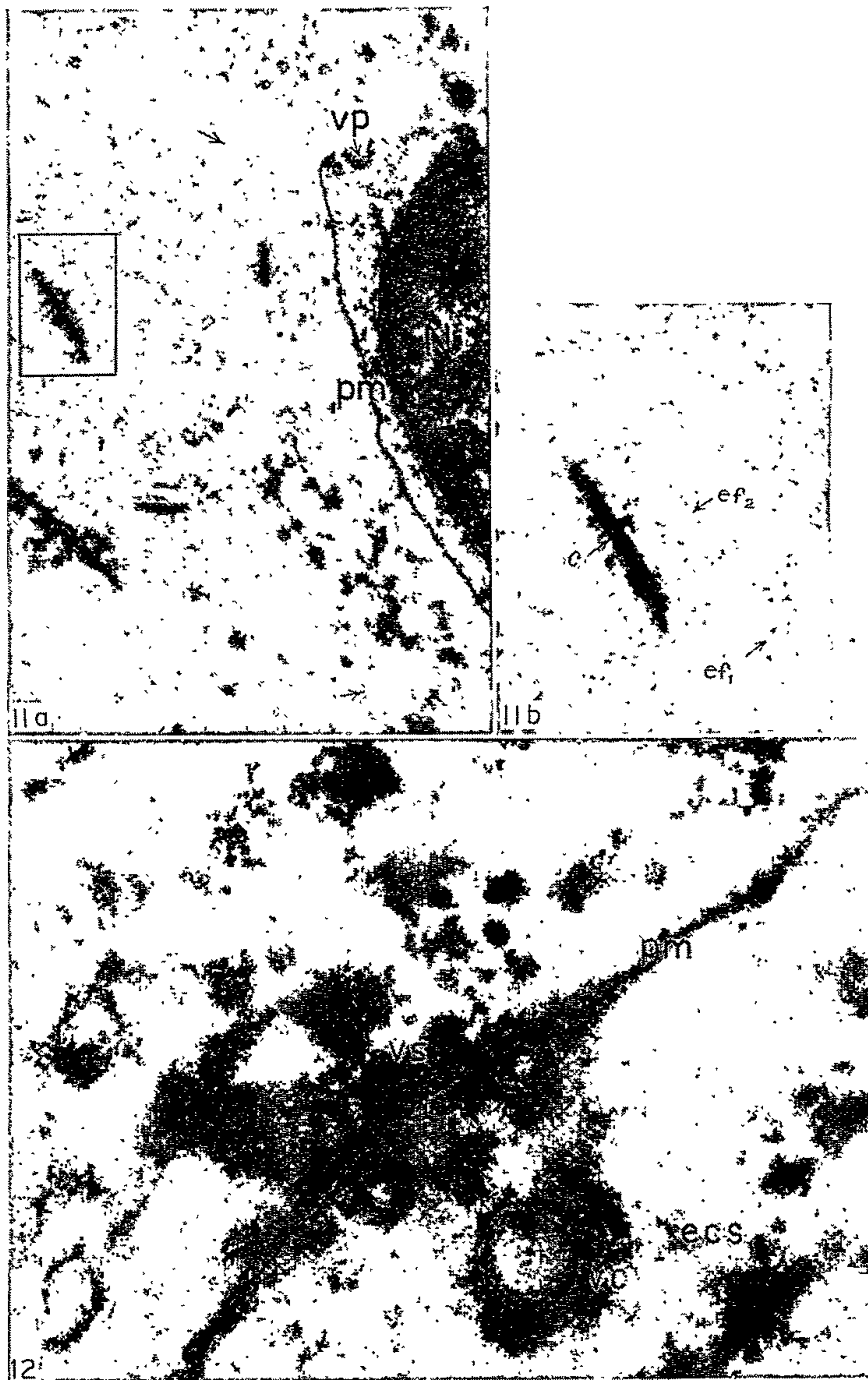
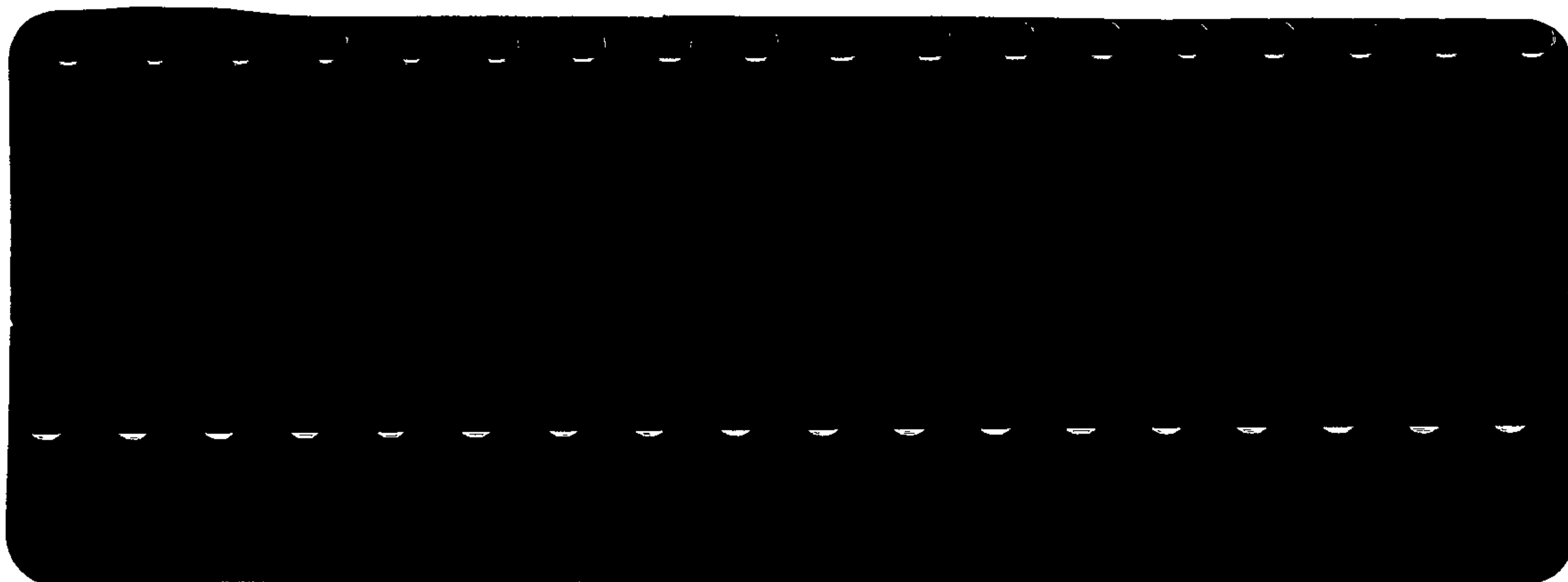


FIG. 11a. In the lower part of the figure extracellular filaments (arrow) of a fairly straight form with a $200\text{\AA}$ periodicity are seen. Extracellular filaments (arrow) of coiled, branched and intertwining types are seen in the top part of the figure. There is some amorphous material around collagen fibrils. Early stage of formation of pino-cytotic vesicle, vp, plasma membrane, pm, nucleus, N. $\times 14,350$.

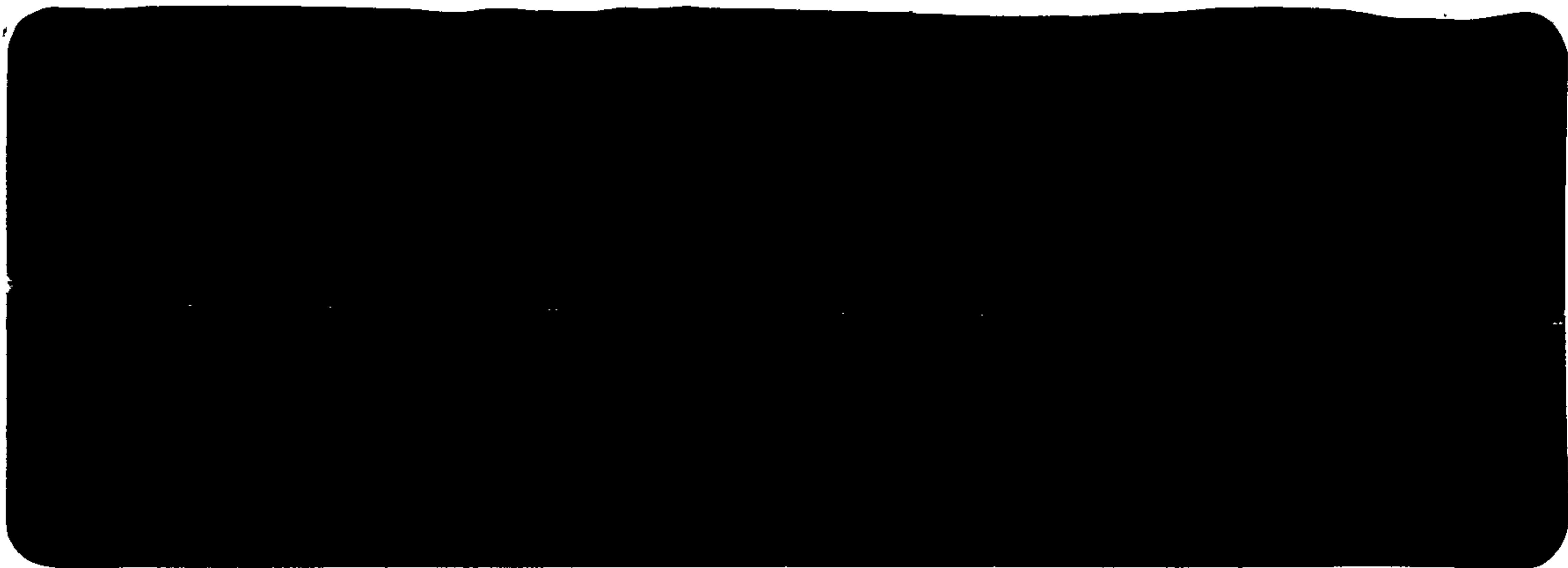
FIG. 11b. Enlargement of rectangular area in Fig. 11a. Note the striations of the straight filament, ef_2 , and coiled, irregularly beaded filament, ef_1 . $\times 28,700$.

FIG. 12. Cytoplasm of fibroblast and extracellular substance, ecs, secretory vesicles, vs, plasma membrane, pm, ribosomes, r, cisternal vesicle, vc. Note the body next to the cisternal vesicle which is similar to the cytoplasmic body seen in Fig. 2. $\times 42,000$.

Reprinted from



PERGAMON PRESS
OXFORD NEW YORK LONDON PARIS



HISTOGENESIS OF FIBROBLASTS IN THE HUMAN DENTAL PULP

R. HARRIS

Institute of Dental Research, United Dental Hospital, Sydney
and

C. J. GRIFFIN

Department of Histology and Embryology, University of Sydney, Australia

Summary—Electron microscopy has shown that two types of synthesizing fibroblasts, young and mature, could be differentiated in the developing human pulp. The mature cells tended to possess more organelles, aggregations of smooth-walled vesicles at the plasma membrane and an increase in the number of cytoplasmic ribosomes, whereas in the younger cell cisternal vesicles predominated. Mitochondria in the young fibroblast had a mean diameter of 4645Å compared with a mean diameter of 7030Å in the mature fibroblasts. In general, the younger pulp had a ground substance of irregularly orientated collagen fibrils (mean diameter 700.5Å) in a reticulum of coiled irregular filaments, whilst in the mature pulp collagen fibrils of larger mean diameter (778.2Å) predominated. In some cases the collagen fibrils formed bundles 2–3μ in diameter. The function of the young fibroblasts appeared to be the synthesis of irregularly beaded microfibrils whereas the mature cell was involved in the elaboration of soluble collagen.

INTRODUCTION

FIBRES in the embryonic pulp are said to be argyrophilic, and no mature collagenous fibres have been observed (ORBAN, 1962). Furthermore, the pulp matures over a long period of time—in the case of the third permanent molars this could be as much as 12–15 years—during which its cells proliferate and produce increasing amounts of ground substance and collagen fibrils. In the mature pulp collagenous fibres are seen. The pulp of the human tooth, therefore, is an excellent subject for the study of the histogenesis of fibroblasts and the observations to be described have been restricted to electron microscopy of these cells.

In a previous paper (GRIFFIN and HARRIS, 1966) the observations reported were restricted to synthesizing fibroblasts which were found in teeth mainly at the root formation stage of development. The authors were concerned in describing therein the characteristics of the actively secreting fibroblast. In the present paper the main information deals with the histodifferentiation of the fibroblast in the human dental pulp.

Differentiating fibroblasts in the pulp of the incisor of the guinea pig have been described by HAN, AVERY and HALE (1965). They found, in the younger type of cell, that the Golgi complex was small and inconspicuous; the mitochondria were small and variable in number and there were vesicles derived from the smooth-surfaced endoplasmic reticulum and occasional profiles of the rough-surfaced endoplasmic

reticulum. As the cell matured and became functional, the rough-surfaced endoplasmic reticulum became more elaborate and dilated cisternae appeared. They also noted flocculent material in the dilated cisternae similar to that seen extracellularly, and the "tight tubules and flattened sacs" contained intracisternal material much denser than that of the extracellular substance. Mesenchymal cells of the dental papilla of human fetuses and new-born cats were found to have an elaborate ergastoplasm and Golgi complex (FRANK, 1965). Fibroblasts in the apical third of the pulp, which had apparently regressed, resembled the more immature cells. HAN, AVERY and HALE (1965) also noted that mitochondria in the functional cell were elongated, larger in size and number and had more straight cristae mitochondriales.

NOBLE, CARMICHAEL and RANKINE (1962) noted somewhat similar changes in the odontoblasts of developing human pulps. AVERY and HAN (1961) noted that fibroblasts of the young pulp of new-born hamsters were different from fibroblasts of connective tissues of adult animals. There was less endoplasmic reticulum and the mitochondria of the young cells had fewer cristae mitochondriales.

The exact manner of collagen formation appears to be somewhat obscure. AVERY and HAN (1961) found that collagen fibrils appeared near, along or partly within the outer cell membranes. FITTON JACKSON (1964) is of the opinion that the appearance of fibrils within the outer cell membrane is possibly the result of oblique sectioning. QUIGLEY (1961) noted in the pulps of dogs and hamsters that collagen could not be demonstrated in the intercellular areas but small, randomly arranged, filamentous strands of the order of 25Å by 2000Å were seen.

MATERIALS AND METHODS

The material was obtained in the manner previously described (GRIFFIN and HARRIS, 1966) from twelve, partly developed, unerupted third molars. It was possible to divide these teeth into two broad categories. Four teeth from one individual were in the appositional stage (crown formation) of tooth development whilst the remainder, from eight individuals, had reached the stage of root formation. In the former, enamel and dentine were being laid down and the pulp could be considered to be relatively immature, whilst in the latter reduction of the pulp chamber was taking place.

Measurements were made of collagen fibrils and the mitochondria, and to avoid possible duplication the measurements were made from different blocks. Measurements of the mitochondria were taken at the smallest diameter and, because of their irregular shape and possible alterations during fixation, the data shown in Fig. 14 should be accepted with limitations.

Recognition of fibroblasts was based on the presence of the following features: dilated profiles of the rough-surfaced endoplasmic reticulum, intracytoplasmic filaments, mitochondria with irregular cristae, and occasional amorphous dense bodies (WEISS and FERRIS, 1956; PORTER and PAPPAS, 1959; POLICARD, COLLET and PREGERMAIN, 1960; CHAPMAN, 1961; ROSS and BENDITT, 1961; CASTOR and MUIRDEN, 1964; FITTON JACKSON, 1964; GOLDBERG and GREEN, 1964; GRIFFIN and HARRIS, 1966).

RESULTS

Appositional stage fibroblasts

Cells which could be identified as fibroblasts were seen. The main features of these cells were the presence of a rough-surfaced endoplasmic reticulum which was occasionally enlarged to form dilated cisternae (Figs. 1a, 2, 3, 6 and 7), intracytoplasmic filaments which had diameters of less than 70Å, usually arranged parallel to the plasma membrane (Fig. 3), peripheral condensations of amorphous material (Fig. 4), grossly dilated perinuclear cisternae which appeared to be continuous with the outer nuclear membrane (Figs. 4 and 5) and condensations of chromatin at the periphery of the nucleus (Figs. 2 and 5).

The rough-surfaced endoplasmic reticulum and perinuclear cisternae usually had a continuous array of attached ribosomes, but in some instances they were absent for short distances (Figs. 4 and 7). When the rough-surfaced endoplasmic reticulum was sectioned tangentially the ribosomes were seen to be arranged as polyribosomal strings (Fig. 6). Clusters of ribosomes were sometimes seen in the cytoplasm (Fig. 6) and attached to the outer nuclear membrane (Fig. 4).

The dilated sacs of the rough-surfaced endoplasmic reticulum usually contained coiled, irregularly beaded microfibrils approximately 40Å in diameter (Figs. 4 and 7).

The most conspicuous feature of the cells was the presence of grossly dilated perinuclear cisternae which appeared to be continuous with outer nuclear membranes (Figs. 4 and 5) and small mitochondria. Rough-surfaced vesicles, which we have termed cisternal vesicles (GRIFFIN and HARRIS, 1966), appeared to be derived from the rough-surfaced endoplasmic reticulum and were seen at the plasma membrane and extracellularly (Figs. 5 and 6). Extracellularly these vesicles appeared to have lost their ribosomal component but they could be recognized because they contained coiled, irregularly beaded microfibrils. Outside the cell they appeared to disrupt and lose their contents (Figs. 2 and 6).

These vesicles are difficult to distinguish, with certainty, from transverse sections of a cell process (Figs. 3, 8 and 13). However, these processes usually contain vesicles and intracytoplasmic filaments. We did not see coiled, irregularly beaded microfibrils in such processes. On the other hand, cisternal vesicles were seen extruded through the plasma membrane (Figs. 2 and 5) and extracellularly they always contained coiled, irregularly beaded microfibrils.

The Golgi complex consisted of aggregations of smooth-surfaced, double membranes arranged parallel to each other, numerous small, smooth-walled vesicles apparently empty (termed secretory vesicles: GOLDBERG and GREEN, 1964) and some dilated smooth-walled sacs, usually containing an amorphous, moderately electron-dense material (Figs. 2 and 7). These smooth-walled vesicles were seldom seen at the plasma membrane. The plasma membrane, when sectioned vertically, was 75Å thick (Fig. 3); discontinuities were seen and are presumed to be manipulation artifacts (ROSS and BENDITT, 1961; GOLDBERG and GREEN, 1964). The extracellular substance consisted of a reticulum composed of irregularly beaded microfibrils and sparse numbers of collagen fibrils not usually aggregated to form fibres (Figs. 2 and 5).

The mean diameter of 107 collagen fibrils was 700.5Å (standard deviation 18.9,

coefficient of variation 27.0%). The smallest diameters of forty-eight mitochondria were measured and the mean was found to be 4645Å (standard deviation 207.4; coefficient of variation 44.7%). The distribution of these measurements is shown in Fig. 14.

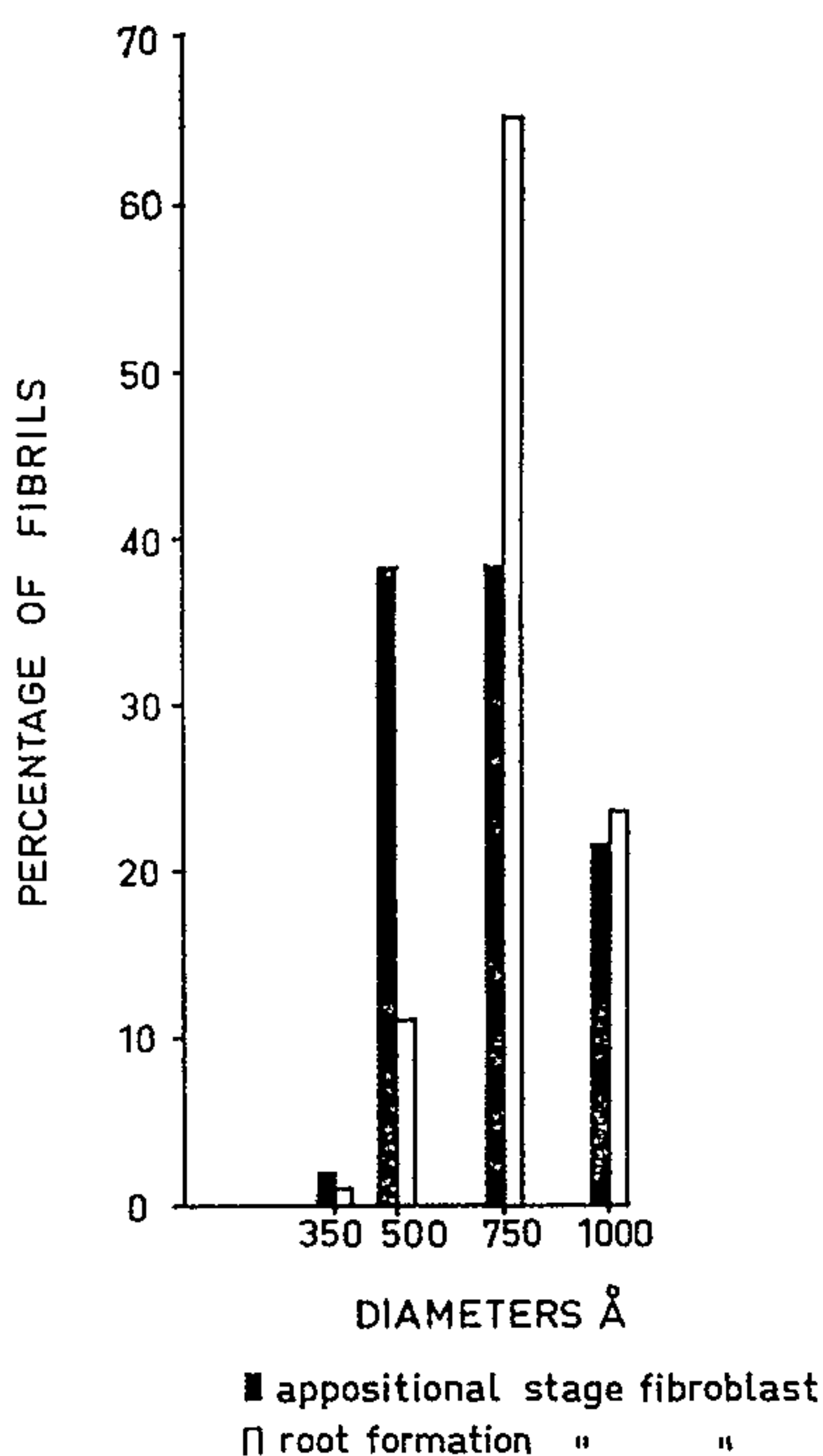
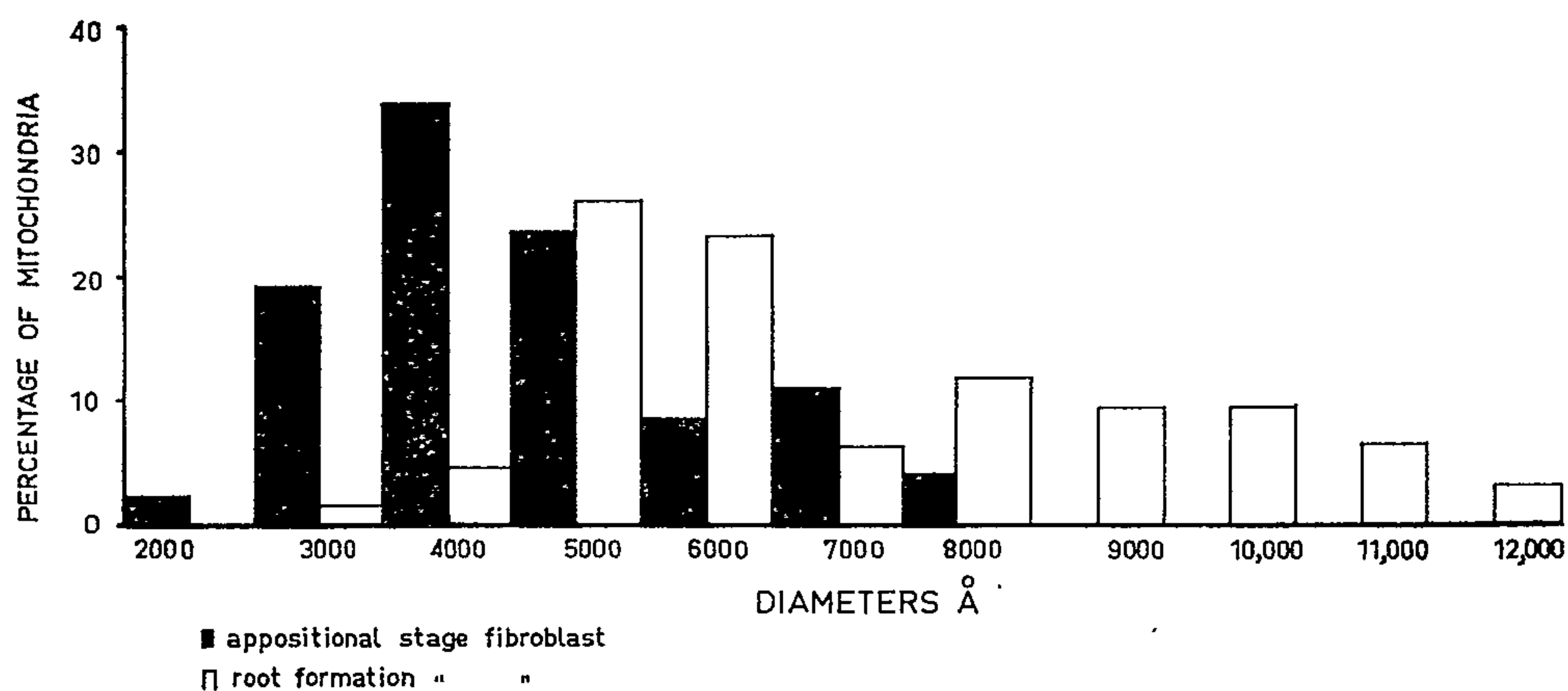


FIG. 14. Histograms showing differences in diameters of collagen fibrils and mitochondria in appositional and root formation stages of pulp development.

Fibroblasts at root formation stage

The synthesizing fibroblasts (Fig. 1b) of the more mature pulp were similar to those described in a previous paper (GRIFFIN and HARRIS, 1966). The dilated sacs of the rough-surfaced endoplasmic reticulum contained microfibrils similar to those of the appositional stage fibroblasts but there was an increase in the ribosome content of the cytoplasm (Fig. 8) and the mitochondria were enlarged (Figs. 8, 9, 10 and 12). The smallest diameters of sixty-six mitochondria were measured and the mean was found to be 7030Å (standard deviation 273.5; coefficient of variation 38.9%), see Fig. 14. The mitochondrial population of the appositional stage fibroblast was found to be very significantly different from the population of the root formation stage fibroblast (difference = 53.94 at the 5% level). Furthermore, it appears that the cisternal systems have been deranged by these organelles. The Golgi apparatus contained numerous smooth-walled vesicles, usually containing a moderately electron-dense substance (Fig. 10). Similar vesicles were frequently seen aggregated at the plasma membrane (Fig. 13) and these are presumed to contain tropocollagen (GOLDBERG and GREEN, 1964; ROSS and BENDITT, 1965). In contrast, appositional fibroblasts seldom showed an aggregation of these vesicles at the plasma membrane. The extracellular substance consisted of a reticulum (Figs. 1b and 11) of coiled, irregularly beaded microfibrils about 100Å in diameter and collagen fibrils, sometimes aggregated to form bundles of collagen fibrils approximately 20,000–30,000Å in diameter (Fig. 13). The mean diameter of 119 collagen fibrils was found to be 778.2Å (standard deviation 13.66, coefficient of variation 17.6%), see Fig. 14. The collagen fibril population of the appositional pulp was found to be very significantly different from the collagen fibril population of the root formation pulp (difference = 35.0 at the 5% level).

DISCUSSION

Appositional fibroblasts resemble log-phase fibroblasts, as described by GOLDBERG and GREEN (1964) and the differentiating fibroblasts as described by HAN, AVERY and HALE (1965). Usually free ribosomes are scarce in the cytoplasm and mitochondria are small. The most conspicuous feature of the appositional fibroblast was the presence of grossly dilated perinuclear cisternae, dilated cisternae and cisternal vesicles which often contained coiled microfibrils. These cisternal vesicles were seen aggregated at the plasma membrane and extracellularly where they appear to disrupt and discharge their contents. Secretory vesicles were usually confined to the Golgi apparatus and appeared rather empty.

In contrast, root formation fibroblasts had secretory vesicles containing a moderately electron-dense, amorphous material and these vesicles were frequently seen aggregated at the plasma membrane. The mitochondria appeared to be larger than those in the appositional fibroblasts and the cells corresponded to functional fibroblasts, as described by HAN, AVERY and HALE (1965) in the guinea pig and mesenchymal cells in the dental papillae of new-born cats and human foetuses, as described by FRANK (1965).

These observations suggest that the major activity of the appositional fibroblast is synthesis and secretion of ground substance microfibrils, whilst the root formation fibroblasts are possibly concerned with synthesis and secretion of soluble collagen (HAN, AVERY and HALE, 1965; FRANK, 1965).

There are two main theories of collagen secretion. It is thought by some (WASSERMAN, 1956; BRADBURY and MEEK, 1958; PORTER and PAPPAS, 1959; GIESEKING, 1960; YARDLEY *et al.*, 1960; CHAPMAN, 1961) that protein collagen is shed directly from the cytoplasm of the fibroblast into the extracellular milieu. Others hold the view that collagen leaves the cell as a soluble protein and is elaborated in the extracellular milieu under the influence of physico-chemical systems (FITTON JACKSON and SMITH, 1957; CARNEIRO and LEBLOND, 1959; YOUNG, 1962; LEBLOND, 1963; REVEL and HAY, 1963; YOUNG and GREULICH, 1963; GOLDBERG and GREEN, 1964; FRANK, 1965; ROSS and BENDITT, 1965). The periodic fibrils in Golgi vacuoles of chondroblasts, as seen by SHELDON and KIMBALL (1962), may be due to precipitation of collagen at this site during fixation or may be due to reversible polymerization and depolymerization within chondroblast vacuoles *in vivo* (REVEL and HAY, 1963). It is also possible that collagen fibrils may reach the interior of the cell by a pinocytotic mechanism (GOLDBERG and GREEN, 1964; GRIFFIN and HARRIS, 1966).

The production of collagen in the dental pulp appears to occur by synthesis of soluble collagen in the ergastoplasm of the fibroblast and its transference to the Golgi complex where it leaves the cell by secretory vesicles which appear at times to discharge their contents at the plasma membrane (reverse pinocytosis: REVEL and HAY, 1963), and subsequently to precipitate as fibrils in the ground substance. Alternatively, secretory vesicles may pass through the plasma membrane into the extracellular substance where they then discharge their contents (GRIFFIN and HARRIS, 1966). The elaboration of collagen in the ground substance appears to be associated with a microfibrillar reticulum.

If the above suggestions are valid, it would seem that the elaboration and secretion of a microfibrillar reticulum is the main activity of the appositional fibroblast of the human developing dental pulp. This reticulum is possibly capable of organizing collagen fibrils into collagen fibres. It has been suggested by WEISS and FERRIS (1956) that fibril orientation could possibly be determined by an underlying lattice system of the ground substance elements.

The diameter of the collagen fibrils in the human developing dental pulp appears to vary between 200Å (GRIFFIN and HARRIS, 1966) and 1000Å (Fig. 14). FRANK (1965) found that the diameter of collagen fibrils in new-born cats and human foetuses varies between 120–550Å. He also noted fine fibrils 70–80Å in diameter which he thought might correspond to aggregations of tropocollagen. However it is possible that these fibrils might correspond to those constituting the microfibrillar reticulum described in this paper.

Acknowledgement—We wish to thank Professor K. W. CLELAND, Department of Histology and Embryology, University of Sydney, for his assistance in the preparation of the electron micrographs and for his constructive criticism.

Résumé—La microscopie électronique montre deux types de fibroblastes (jeune et adultes) dans la pulpe humaine en voie de développement. Les cellules adultes semblent avoir plus d'organelles, plus de vésicules à parois lisses, accolées à la membrane cellulaire et plus de ribosomes, alors que les cellules plus jeunes présentent plus d'ergastoplasme. Les mitochondries du jeune fibroblaste ont un diamètre moyen de 4645Å, alors que celles du fibroblaste adulte ont un diamètre de 7030Å. En générale, la pulpe jeune présente une substance fondamentale remplie de fibrilles collagènes (diamètre moyen 700,5Å) orientées irrégulièrement dans un réticulum de filaments, alors que dans la pulpe adulte, des fibrilles collagènes d'un diamètre moyen plus large (778,2Å) prédominent. Dans certains cas, les fibrilles collagènes forment des faisceaux de 2–3µ de diamètre. Le rôle des jeunes fibroblastes semble être la synthèse de fibrilles irrégulièrement moniliformes alors que les cellules adultes semblent élaborer un collagène soluble.

Zusammenfassung—Elektronenmikroskopisch wurde gezeigt, daß zwei Typen synthetisierender Fibroblasten, junge und ausgereifte, in der sich entwickelnden menschlichen Pulpa unterschieden werden konnten. Die ausgereiften Zellen neigten dazu, mehr Organellen, Zusammenballungen glattwandiger Bläschen an der Plasmamembran und eine anwachsende Anzahl zytoplasmatischer Ribosomen zu besitzen, während in der jüngeren Zelle das Ergastoplasma vorherrschte. Die Mitochondrien in den jungen Fibroblasten besaßen einen mittleren Durchmesser von 4645Å im Vergleich zum mittleren Durchmesser von 7030Å in den ausgereiften Fibroblasten. Im allgemeinen basierte die jüngere Pulpa eine Grundsubstanz irregulär orientierter Kollagenfibrillen (mittlerer Durchmesser 700,5Å) in einem Retikulum verschlungener irregulärer Filamente, während in der ausgereiften Pulpa Kollagenfibrillen mit größerem mittlerem Durchmesser (778,2Å) vorherrschten. In einigen Fällen bildeten die Kollagenfibrillen Bündel im Durchmesser von 2–3µ. Funktionen der jungen Fibroblasten schien die Synthese irregulär angeordneter Mikrofibrillen zu sein, während die ausgereifte Zelle an der Entwicklung löslichen Kollagens beteiligt zu sein scheint.

REFERENCES

- AVERY, J. K. and HAN, S. S. 1961. The formation of collagen fibrils in the dental pulp. *J. dent. Res.* **40**, 1248–1261.
- BRADBURY, S. and MEEK, G. A. 1958. A study of fibrillogenesis in the leech, *Hirudo medicinalis*. *Q. J. microsc. Sci.* **99**, 143–148.
- CARNEIRO, J. and LEBLOND, C. P. 1959. Role of osteoblasts and odontoblasts in secreting collagen of bone and dentin, as shown by radioautography in mice, gibbon, tritium-labelled glycine. *Exp. Cell Res.* **18**, 291–300.
- CASTOR, C. W. and MURDEN, K. D. 1964. Collagen formation in monolayer cultures of human fibroblasts: the effect of hydrocortisone. *Lab. Invest.* **13**, 560–574.
- CHAPMAN, J. A. 1961. Morphological and chemical studies of collagen formation. I. The fine structure of guinea pig granulomata. *J. biophys. biochem. Cytol.* **9**, 639–651.
- FITTON JACKSON, S. and SMITH, R. H. 1957. Studies of the biosynthesis of collagen. I. The growth of fowl osteoblasts and the formation of collagen in tissue culture. *J. biophys. biochem. Cytol.* **3**, 897–912.
- FITTON JACKSON, S. 1964. Connective tissue cells, in: *The Cell* (Edited by BRACHET, J. and MIRSKY, A. E.), pp. 387–509. Academic Press, New York.
- FRANK, R. 1965. Microscopie électronique de la genèse du collagène dans la papille dentaire. *J. Microscopie* **4**, 43–56.
- GIESEKING, R. 1960. In: *Struktur und Stoffwechsel des Bindegewebes*. II. Symp. an der medizinischen Universitas-Klinik Münster (Westf.) (Edited by HAUSS, W. H. and LOSSE, H.), pp. 131–140. Georg Thieme, Stuttgart.
- GOLDBERG, B. and GREEN, J. 1964. An analysis of collagen secretion by established mouse fibroblast lines. *J. Cell Biol.* **22**, 227–258.
- GRIFFIN, C. J. and HARRIS, R. 1966. Ultrastructure of collagen fibrils and fibroblasts of the developing human dental pulp. *Archs oral Biol.* **11**, 659–666.
- HAN, S. S., AVERY, J. K. and HALE, L. E. 1965. The fine structure of differentiating fibroblasts in the incisor pulp of the guinea pig. *Anat. Rec.* **153**, 187–210.

- LEBLOND, C. P. 1963. Elaboration of dentinal collagen in odontoblasts as shown by radioautography after injection of labelled glycine and proline. *Annls Histochem.* 8, 43-50.
- NOBLE, H. W., CARMICHAEL, A. F. and RANKINE, D. M. 1962. Electron microscopy of human developing dentine. *Archs oral Biol.* 7, 395-397.
- ORBAN, B. 1962. *Orban's Oral Histology* (Edited by SICHER, H.) (5th ed.), p. 146. Mosby, St. Louis.
- POLICARD, A., COLLET, A. and PREGERMAIN, S. 1960. Étude au microscope électronique des dispositifs ergastoplasmiques de certaines cellules du granulome inflammatoire. *Proc. European Regional Conf. Electron Microscopy*, Delft (Edited by HOUWINK, A. L. and SPIT, B. J.), Vol. 2, pp. 879-882. Der Nederlandse Vereniging voor Electronemicroscopies, Delft.
- PORTER, K. R. and PAPPAS, G. D. 1959. Collagen formation by fibroblasts of the chick embryo dermis. *J. biophys. biochem. Cytol.* 5, 153-156.
- QUIGLEY, M. B. 1961. Electron microscopy of the dental pulp. *J. dent. Res.* 40, 756. Abst.
- REVEL, H. P. and HAY, E. 1963. An autoradiographic and electron microscopic study of collagen synthesis in differentiating cartilage. *Z. Zellforsch.* 61, 110-144.
- ROSS, R. and BENDITT, E. P. 1961. Wound healing and collagen formation. I. Sequential changes in components of guinea pig skin wounds observed in the electron microscope. *J. biophys. biochem. Cytol.* 11, 677-700.
- ROSS, R. and BENDITT, E. P. 1965. Wound healing and collagen formation. V. Quantitative electron-microscope radioautography observations of proline- H^3 utilization by fibroblasts. *J. Cell Biol.* 27, 86-106.
- SHELDON, H. and KIMBALL, F. B. 1962. Studies on cartilage. III. The occurrence of collagen within vacuoles of the Golgi apparatus. *J. Cell Biol.* 12, 599-613.
- WASSERMAN, F. 1956. Fibrillogenesis in the regenerating rat tendon with special reference to growth and composition of the collagenous fibrils. *Am. J. Anat.* 94, 399-437.
- WEISS, P. and FERRIS, W. 1956. The basement lamella of amphibian skin. Its reconstruction after wounding. *J. biophys. biochem. Cytol.* 2, Suppl. 4, 275-282.
- YARDLEY, J. H., HEATON, M. W., GAINS, L. M. and SHULMAN, L. E. 1960. Collagen formation by fibroblasts. Preliminary electron microscopic observations using thin sections of tissue cultures. *Bull. Johns Hopkins Hosp.* 106, 381-393.
- YOUNG, R. W. 1962. Autoradiographic studies on bone and Sharpey's matrix formation in young rats injected with glycine- H^3 . *Anat. Rec.* 142, 335.
- YOUNG, R. W. and GREULICH, R. C. 1963. Distinctive autoradiographic patterns of glycine incorporation in rat enamel and dentine matrices. *Archs oral Biol.* 8, 509-532.

PLATE 1

FIG. 1. Comparison of appositional fibroblasts and root formation fibroblasts. The appositional fibroblast (a) has very few organelles as compared with the root formation fibroblast (b). The ground substance of the young pulp is relatively afibrillar as compared with the ground substance of the more mature pulp. (a) $\times 3500$; (b) $\times 8400$.

HISTOGENESIS OF FIBROBLASTS IN THE HUMAN DENTAL PULP



PLATE I

A.O.B. f.p. 466

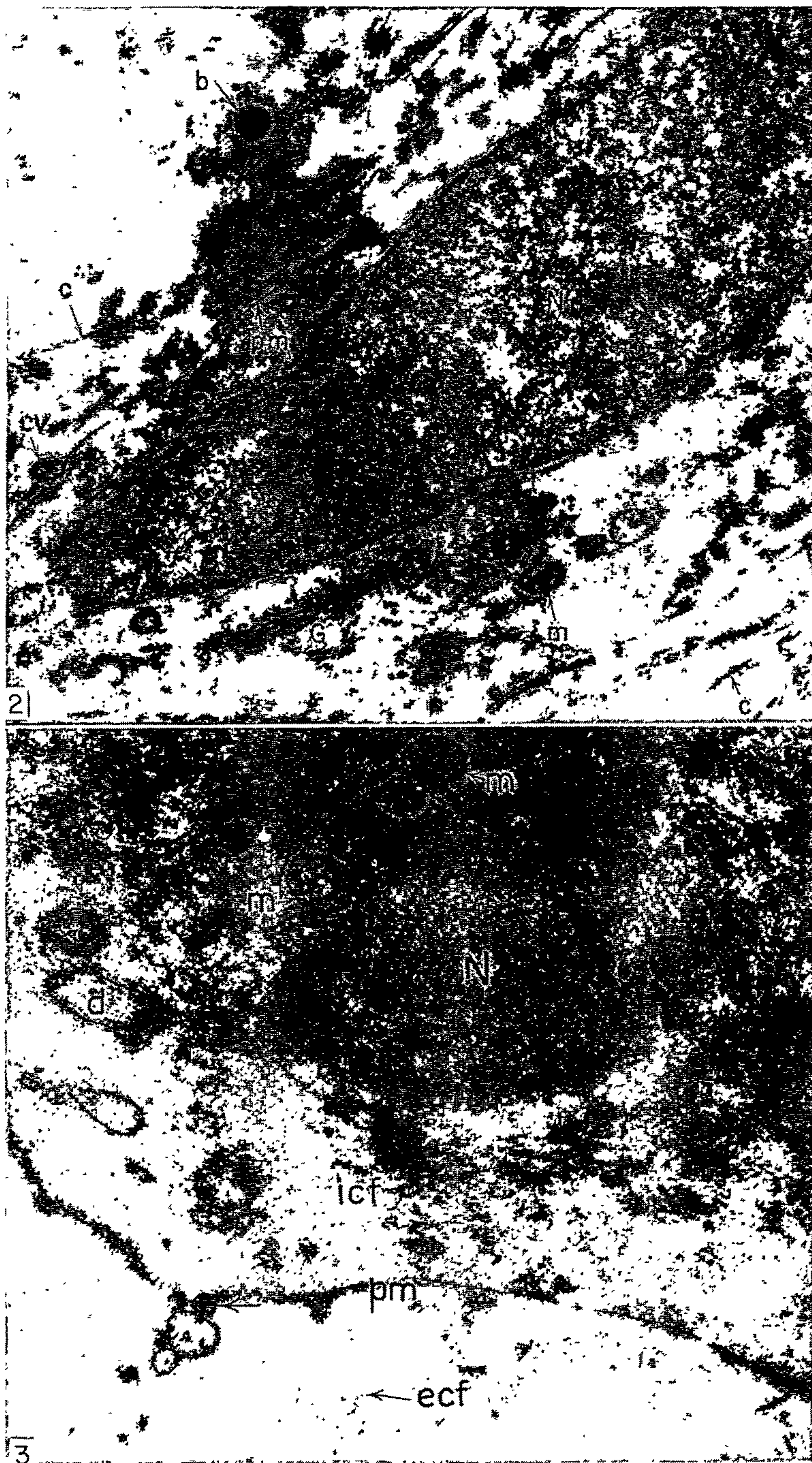


PLATE 2

FIG. 2. Appositional fibroblast. N, nucleus; G, Golgi apparatus; m, mitochondrion; pm, plasma membrane; cv, cisternal vesicle, one of which appears to be disrupting; b, extracellular body of varying density; c, collagen fibrils. $\times 8000$.

FIG. 3. Appositional fibroblast. N, nucleus; icf, intracytoplasmic filaments; m, mitochondria; d, dilated cisternae; pm, plasma membrane; ecf, extracellular microfibrils and cisternal vesicle (arrow) containing coiled microfibrils. $\times 8000$.

PLATE 3

FIG. 4. Appositional fibroblast. N, nucleus; pc, greatly dilated perinuclear cisternae; pm, plasma membrane; ecf, extracellular microfibrils; v, vesicles, a, amorphous material at the plasma membrane. $\times 27,600$.

FIG. 5. Appositional fibroblast. N, nucleus; pc, dilated perinuclear cisternae; d, dilated cisternae; m, mitochondria; pm, plasma membrane; ecf, extracellular microfibrils; vc, cisternal vesicle, appearing to pass through plasma membrane of the process of a fibroblast. $\times 8000$.

HISTOGENESIS OF FIBROBLASTS IN THE HUMAN DENTAL PULP



PLATE 3

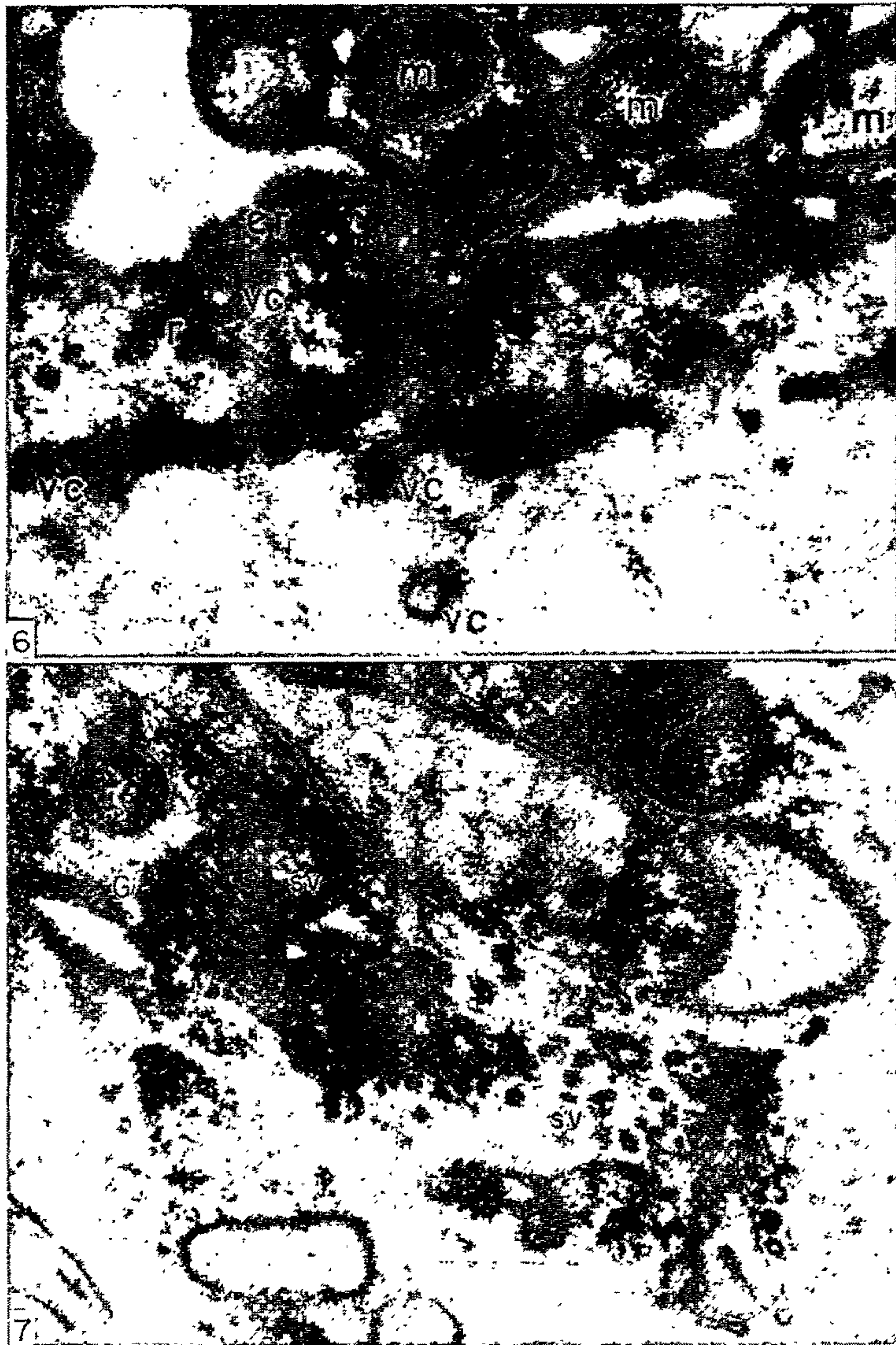


FIG. 6. Appositional fibroblast. m, mitochondria; er, rough-surfaced endoplasmic reticulum; vc, intra- and extracellular cisternal vesicles (one of the extracellular type appears to be disrupting). $\times 24,000$.

FIG. 7. Appositional fibroblast. G, Golgi apparatus; sv, secretory vesicles. $\times 24,000$.

HISTOGENESIS OF FIBROBLASTS IN THE HUMAN DENTAL PULP

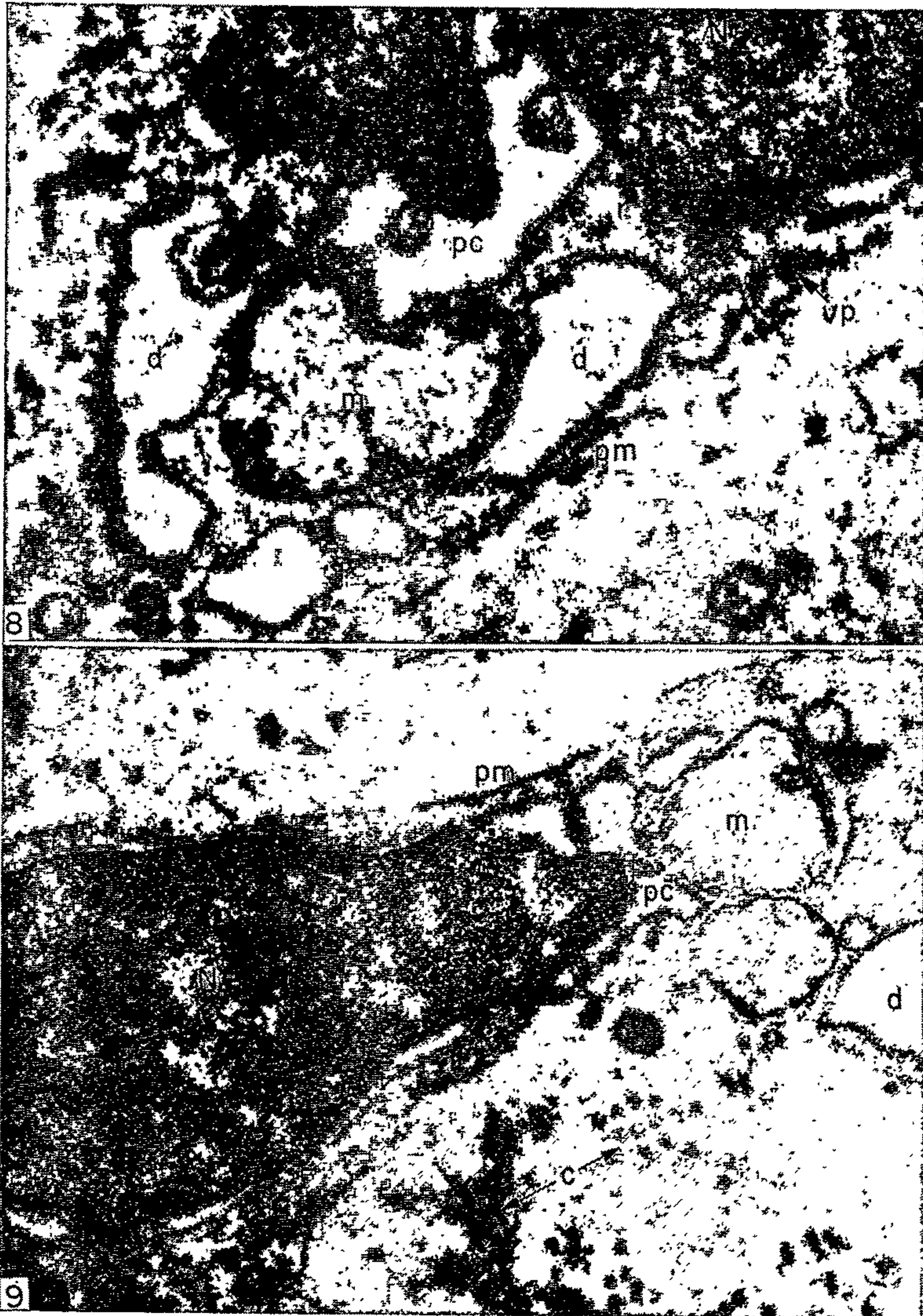


FIG. 8. Root formation stage fibroblast. N, nucleus; d, dilated sacs of the rough-surfaced endoplasmic reticulum; pc, dilated perinuclear cisternae; m, enlarged mitochondria; r, clusters of ribosomes; pm, plasma membrane; vp, pinocytotic vesicle. $\times 20,000$.

FIG. 9. Root formation stage fibroblast. N, nucleus; pc, dilated perinuclear cisternae; d, dilated cisternae; m, enlarged mitochondria; c, collagen fibrils; pm, plasma membrane. $\times 16,000$.



FIG. 10. Root formation stage fibroblast. G, Golgi apparatus; sv, secretory vesicles; pm, plasma membrane. $\times 24,000$.

FIG. 11. Extracellular substance of pulp at root formation stage, showing collagen fibrils and a complex network of coiled, irregularly beaded microfibrils. $\times 22,800$,

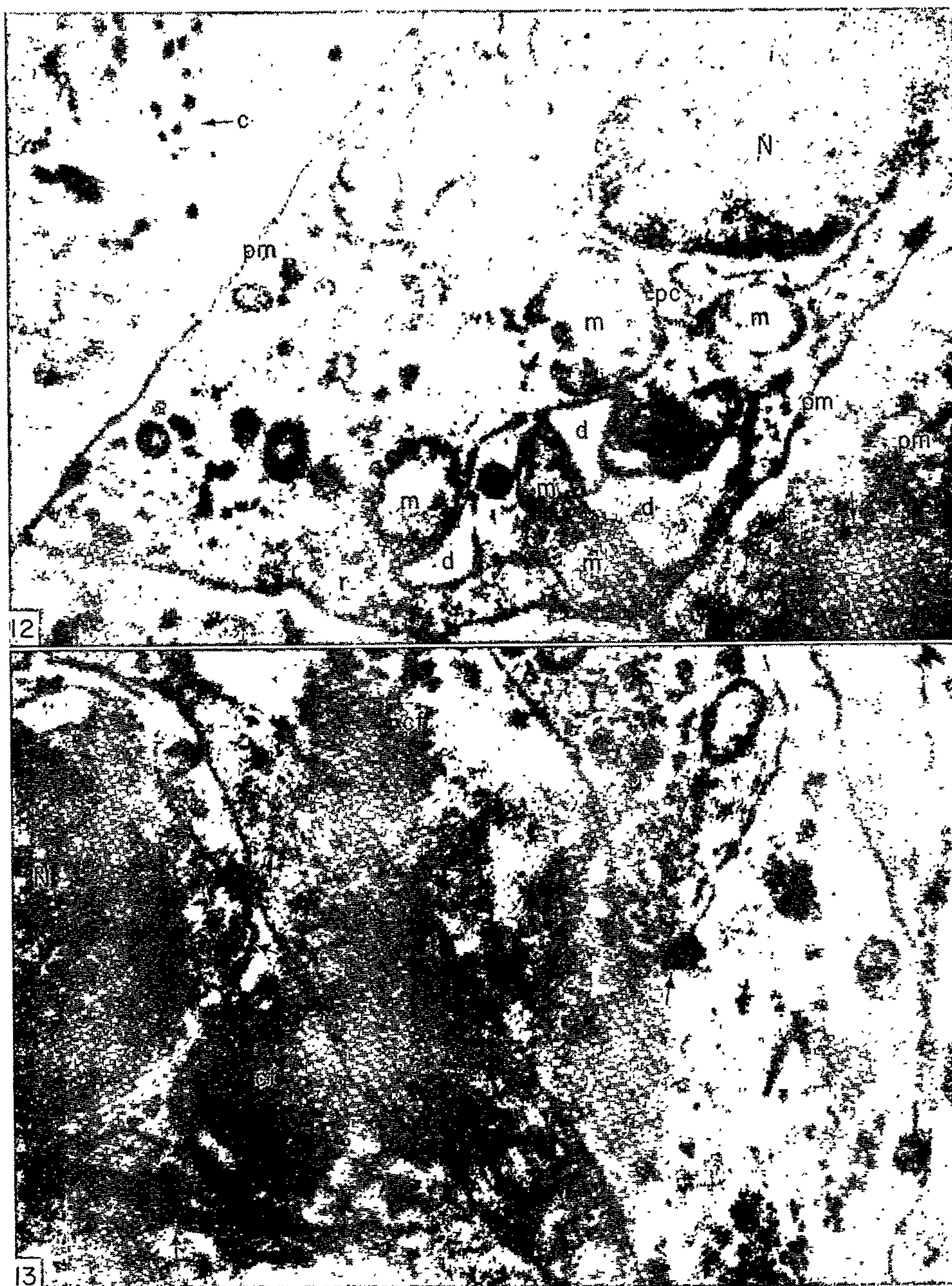


FIG. 12. Root formation stage fibroblast. N, nucleus; pc, perinuclear cisternae; d, dilated cisternae; m, mitochondria; pm, plasma membrane; invagination (arrow); c, collagen fibrils; r, ribosomes. $\times 19,680$.

FIG. 13. Root formation stage fibroblast and collagen fibres. N, nucleus; cf, bundles of collagen fibrils; plasma membrane (arrows) and secretory vesicles aggregated at plasma membrane. $\times 12,000$.

Reprinted from



PERGAMON PRESS
OXFORD NEW YORK LONDON PARIS



FINE STRUCTURE OF NERVE ENDINGS IN THE HUMAN DENTAL PULP

R. HARRIS

Institute of Dental Research, United Dental Hospital, Sydney

C. J. GRIFFIN

Department of Histology and Embryology, University of Sydney,
Sydney, Australia

Summary—Thick sections of the peripheral coronal areas of the mature dental pulp were examined under light microscopy and thin sections prepared for electron microscopy after fixation in Palade's osmium tetroxide. The area examined corresponded to the "plexus of Raschkow" and myelinated nerve fibres 2–5 μ dia. and unmyelinated nerve fibres 2500–16,000 Å dia. were seen.

Three types of nerve endings could be identified by the presence of vesicles and mitochondria within their structure. The first type, presumably derived from myelinated nerve fibres, displayed varicosities up to 4 μ dia. and constrictions of 1 μ diameter, and axonal swellings 5000–10,000 Å dia. The second type consisted of fine-diameter unmyelinated nerve fibres exposed freely in the ground substance, which presumably correspond to pain afferent terminals. The third type consisted of unmyelinated fibres related to blood vessels.

INTRODUCTION

THE "PLEXUS of Raschkow" has been referred to by MUMMERY (1919), FEARNHEAD (1961) and ARWILL (1963). FEARNHEAD (1961) stated that the nerve fibres of the plexus were 0.5–3.0 μ dia. and that they cross the cell-free zone of Weil to reach the odontoblasts, where they either pass between them or turn back towards the pulp. FEARNHEAD and LINDER (1956) identified the "plexus of Raschkow" and traced from it very thin branches that passed between odontoblasts; in addition, they described loops of fine nerve fibres in the predentine similar to those described by BRADLAW (1939) and they also described a marginal pulp plexus consisting of fine beaded fibres almost at the limit of optical resolution. According to FEARNHEAD (1963), terminal nerve fibres can be seen inside the odontoblastic tubules in the predentine or in the subodontoblastic zone. These terminal fibres are approximately 1.0 μ or less dia. and are beaded and branched. In reference to the predentine or dentine, FRANK (1966) demonstrated by electron microscopy terminal nerve fibres near the surface of odontoblastic processes within the dentinal tubules.

The present paper describes nerve endings in an area of the pulp that corresponds to the "plexus of Raschkow".

MATERIAL AND METHODS

The pulps of normal human teeth were removed, immediately after the teeth had been extracted, by cutting under saline with an air-rotor and splitting each tooth with bone cutting forceps. The time between removal of the teeth and placing the pulp tissue in fixative did not exceed 2 min in each instance.

Portions of the coronal pulp were cut into small pieces and fixed in PALADE's (1952) osmium tetroxide fixative at 0°C for 2 hr (2 per cent osmium tetroxide solution (5.0 ml), veronal-acetate buffer (2.0 ml), distilled water (1.0 ml), and 0.1 N HCl (2.0 ml), to bring the solution to pH 7.3–7.5). Following fixation, the pieces were dehydrated in increasing concentrations of acetone (15 min in each of 25, 50, and 75 per cent acetone) and, after rinsing in three changes of 100 per cent acetone, embedded in Araldite. Polymerization was effected at 60°C for 24 hr.

Sections 4.0 μ thick were cut and stained with toluidine blue. Examination of these sections showed nerve fibres 1–4 μ dia. From the blocks which showed these structures, sections 500–1000 Å thick (cut on an L.K.B. ultramicrotome and stained with uranyl acetate on the grid for 1 hr) were examined on an Hitachi H.S.7 electron microscope.

Eight teeth were examined; the observations reported here specifically refer to 2 teeth obtained from adults aged 29 and 35 years and are representative of the findings in all teeth.

OBSERVATIONS

The thick sections showed nerve fibres in longitudinal and cross section (Fig. 1 and 2). The electron micrographs showed myelinated nerve fibres 2–5 μ dia. (Fig. 3 and 4) and unmyelinated fibres 2500–16,000 Å dia. (Fig. 5 and 6). The unmyelinated nerve fibres were either enclosed by the plasma membrane of the Schwann cell or surrounded by its basement membrane (Fig. 5 and 6).

Fine-diameter unmyelinated nerve fibres

The unmyelinated nerve fibres, with the Schwann cell membrane exposed to the extracellular substance, correspond to the endings of unmyelinated nerve fibres described in other tissues (BISCOE and STEHBENS, 1966). Three types of vesicles were seen in transverse sections of the unmyelinated nerve endings (Fig. 5 and 6). The smaller type, referred to as microvesicles by BISCOE and STEHBENS, had a diameter of 300–500 Å: larger vesicles had a diameter of 800–1000 Å (Fig. 6). Both of these types contained a moderately electron-dense substance. A third type of vesicle—devoid of any definite structure and having a diameter of 900–1000 Å—called by us a macrovesicle, was also seen. Mitochondria of 3000–4000 Å dia. were occasionally seen (Fig. 5).

Within the axons of the unmyelinated nerve fibres two types of fine fibrils were seen, one approximately 100 Å dia. and the other in the form of microtubules 200–300 Å wide. Unmyelinated fibres lying in close relation to blood vessels were also seen (Fig. 7). The diameters of the endings of unmyelinated nerve fibres varied from 800–7000 Å (Fig. 6).

Beaded unmyelinated nerve endings

Nerve endings structurally different from those of unmyelinated fibres were also seen. They correspond to the beaded nerve endings arising from myelinated nerve fibres in the "plexus of Raschkow", described by FEARNHEAD and LINDER (1956) and FEARNHEAD (1961, 1963) in a light microscopy study, and can be recognized by the presence of varicosities up to $4\ \mu$ dia. and constrictions $1\ \mu$ in dia., both of which are surrounded by Schwann cell processes until approaching their termination. In the constricted zone, condensation of microfibrils was seen, whilst the varicosities contained mitochondria and vesicles of varying size similar to those described in the unmyelinated fibres (Fig. 8). These nerve endings appear to arise by divisions of axons, $2\text{--}5\ \mu$ dia., which have presumably lost their myelin sheaths (Fig. 4). Until near their termination the nerve endings are surrounded by the Schwann cells and then, as constricted axons without Schwann cell coverings, they emerge to end as axonal expansions (Fig. 8 and 9). The diameters of the smallest axonal expansions varied between $5000\text{--}10,000\ \text{\AA}$. There are two types of fibrils in the axons similar to those seen in the unmyelinated nerves, those about $100\ \text{\AA}$ dia. and microtubular forms. Numerous vesicles, ranging from $300\text{--}1000\ \text{\AA}$ dia., and small mitochondria approximately $5000\ \text{\AA}$ dia., were also seen.

Schwann cells

Myelinated and unmyelinated nerve fibres were surrounded, until near their termination, by the plasma membrane of the Schwann cells. In some instances the plasma membrane of the Schwann cell appeared to be fused to the plasma membrane of the unmyelinated axon (Fig. 6 and 8). Two types of fibrils were seen in the cytoplasm—some approximately $100\ \text{\AA}$ dia., and others in the form of microtubules $200\text{--}300\ \text{\AA}$ wide. Usually the basement membrane of the Schwann cell could be seen (Fig. 3) although in other sections it was indistinct (Fig. 8).

DISCUSSION

Three types of nerve endings were seen:

- (a) Fine-diameter unmyelinated nerve fibres,
- (b) Beaded nerve fibres with axonal expansions,
- (c) Perivascular unmyelinated nerve fibres.

These nerve endings exhibited common elements; vesicles of varying size, microfibrils, fibrils and microtubules. Similar elements in synaptic nerve endings in other tissues have been described by GRAY and GUILLERY (1966). The structure of myelinated and unmyelinated nerve fibres in the human dental pulp has been described by BERNICK (1948) and GRAF and BJÖRLIN (1951). MIYOSHI, NISHIJIMA and IMANISHI (1966) by electron microscopy found the diameters of the myelinated fibres to lie in the range $2.0\text{--}2.5\ \mu$ and $1.0\text{--}1.5\ \mu$ respectively.

FEARNHEAD (1961, 1963) has described beaded nerve endings derived from myelinated nerve fibres in the "plexus of Raschkow". The axonal expansions, constrictions,

tions and varicosities described here have been obtained from material found in the area described as the "plexus of Raschkow". Therefore, they are probably nerve endings of myelinated nerve fibres. It further appears that the axonal expansions in the present material are derived from myelinated nerve fibres because they are preceded structurally by varicosities and constrictions of the axons. We have shown that in these regions several branchings of the axons occur within a single Schwann cell and that the branches are surrounded by the processes of the Schwann cell. The terminal fibres leave the Schwann cell as a constricted axon and end as terminal swellings in the ground substance as illustrated in Fig. 10.

We have identified the swellings at the end of constricted axons as nerve endings because of the elements noted therein and because such elements indicate the termination of the nerve fibre (BISCOE and STEHBENS, 1966). These elements were small mitochondria, numerous microvesicles 400–500 Å in diameter and vesicles 800–1000 Å dia.

Studies on unencapsulated nerve endings (MUNGER, 1965) have shown that these endings are accompanied either by the Schwann cell or supporting cells almost up to their termination, when the terminal part of the nerve appears as an axon surrounded by basement membrane. We are unable to say whether or not the axonal expansions are surrounded by Schwann cell basement membrane because of lack of definition of this structure. Nevertheless the possibility exists that they may be sections through the varicose part of an axon and not a terminal structure.

The nerve endings of the unmyelinated nerve fibres are less complex and appear simply to lose their Schwann cells so that their axons become exposed to the ground substance (Fig. 5 and 6). Both types of nerve endings contain vesicles which may contain the transmitter substances (DE ROBERTIS, 1964). The vesicles seen in the nerve endings of the dental pulp appear to be identical with vesicles found in Meissner's and Pacinian corpuscles (PEASE and QUILLIAM, 1957). The vesicles are also quite similar to those seen in Type I and Type II cells in the carotid body (BISCOE and STEHBENS, 1966), except that vesicles with electron-opaque cores were not seen. It was noted that the number of vesicles and mitochondria seen in these nerve endings generally were not as numerous as those in other afferent nerve endings described by CAUNA and ROSS (1960) and BISCOE and STEHBENS (1966).

HATTYASY (1964) has reported the presence of unmyelinated nerve fibres in relationship with capillaries and fibroblasts in the dental pulp and the latter type might correspond to the free unmyelinated nerve endings described in this paper. FRANK (1966) has demonstrated unmyelinated nerve fibres lying in close relationship with the protoplasmic processes of the odontoblasts in dentine and predentine. Frank also refers to possible nerve endings between the basal portions of the odontoblasts. He was unable to state whether these nerve endings arose from myelinated or unmyelinated nerve fibres. BERNICK (1948) and FEARNHEAD and LINDER (1956) by light microscopy observed beaded nerve endings in the dental pulp.

Our observations suggest that there are two types of afferent nerve endings, presumably derived from myelinated nerve fibres, in the peripheral part of the dental pulp. The fine-diameter unmyelinated nerve endings and the axonal expansions are

apparently concerned with conduction of pain, and perhaps the differences in diameter reflect different velocities of conduction.

Physiological experiments of BROOKHART, LIVINGSTON and HAUGEN (1953) indicate that fibres from the tooth pulp are a classic pure pain source and have a conduction rate of 6–30 m/sec characteristic of Group III fibres (LLOYD, 1943). Group III fibres have a diameter in the range of 1–6 μ .

Perivascular nerve endings are probably post-ganglionic fibres concerned with neurovascular reflexes.

Acknowledgement—We wish to thank Professor K. W. CLELAND, Department of Histology and Embryology, University of Sydney, for his assistance in the preparation of the electron micrographs and for his constructive criticism.

Résumé—Des coupes épaisses des parties périphériques de la pulpe dentaire coronaire adulte sont étudiées par microscopie optique et par microscopie électronique après fixation à l'acide osmique selon Palade. Les régions étudiées correspondent au plexus de Raschkow, qui présente des fibres nerveuses myélinisées de 2–5 μ de diamètre et des fibres non myélinisées de 2500–16000 Å de diamètre.

Trois types de terminaisons nerveuses ont pu être identifiés grâce à la présence de vésicules et de mitochondries. Le premier type, probablement dérivé de fibres nerveuses myélinisées, présentent des dilatations allant jusqu'à 4 μ de diamètre et des rétrécissements allant jusqu'à 1 μ de diamètre, avec des dilatations axoniques de 5000 à 10.000 Å de diamètre. Le second type est constitué par des fibres nerveuses non-myélinisées de faible diamètre, situées librement dans la substance fondamentale et correspondant vraisemblablement à des terminaisons afférentes à la douleur. Le troisième type comporte des fibres non-myélinisées en rapport avec les vaisseaux sanguins.

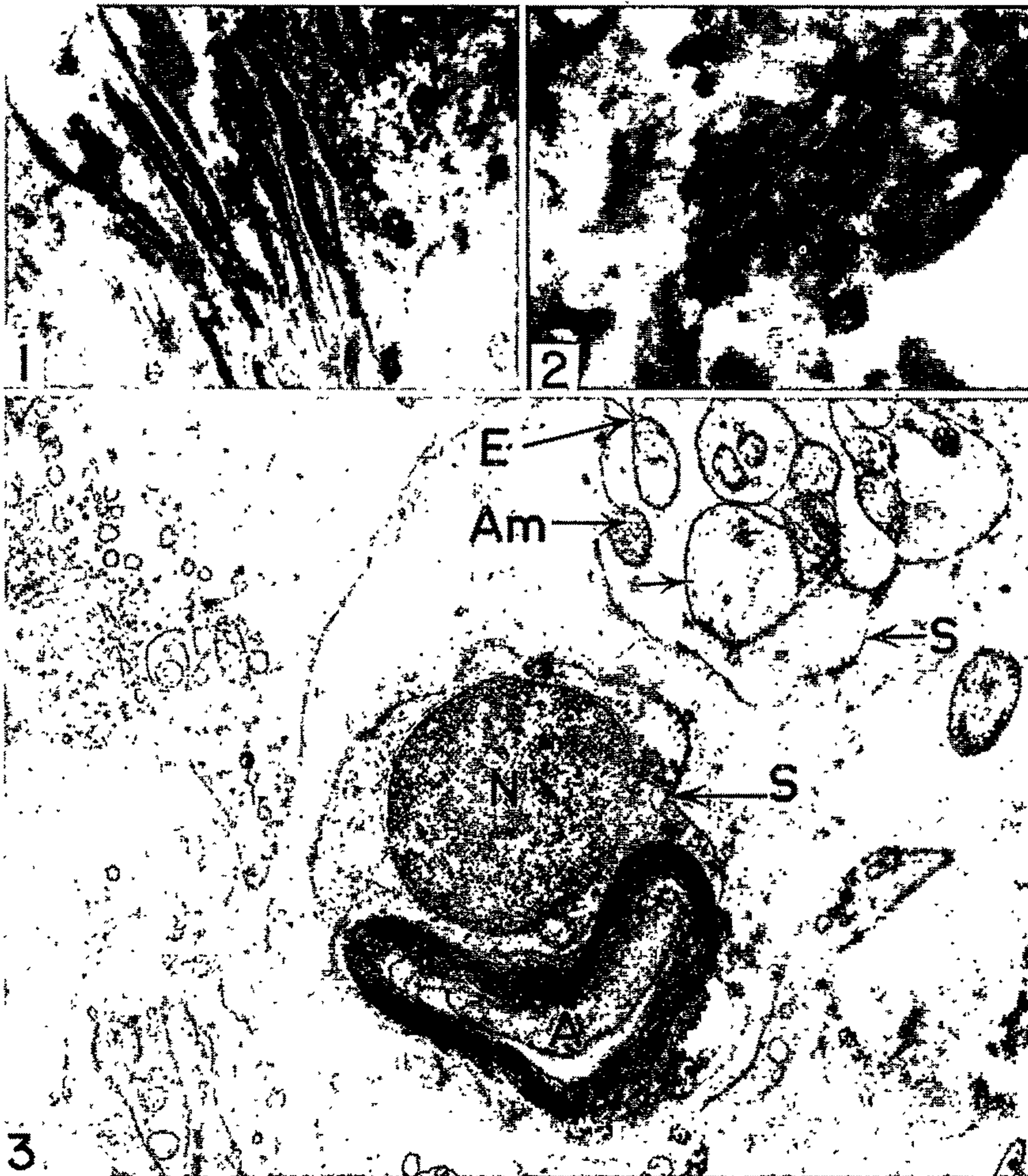
Zusammenfassung—Dicke Schnitte von peripheren, koronalen Bezirken der ausgereiften Zahnpulpa wurden lichtmikroskopisch untersucht, und es wurden nach Fixierung in Osmium-Tetroxid nach Palade Dünnschnitte für die Elektronenmikroskopie vorbereitet. Der untersuchte Bezirk entsprach dem "Raschkow'schen Plexus"; in ihm wurden markhaltige Nervenfasern mit einem Durchmesser von 2–5 μ und marklose Nervenfasern von 2500–1600 Å Durchmesser beobachtet.

An Hand der Existenz von Bläschen und Mitochondrien in der Struktur der Nervenendigungen konnten 3 Typen dieser Endorgane identifiziert werden. Der erste, vermutlich von markhaltigen Nervenfasern herstammende Typ zeigte Knotenbildungen bis zu 4 μ Durchmesser und Verengungen bis zu 1 μ Durchmesser, sowie Axon-Schwelungen mit einem Querschnitt von 5000–10 000 Å. Der zweite Typ bestand aus marklosen Nervenfasern mit kleinem Durchmesser, die frei in der Grundsubstanz endeten und vermutlich den Schmerzrezeptoren entsprechen. Der dritte Typ bestand aus marklosen Nervenfasern, die mit Blutgefäßen in Beziehung stehen.

REFERENCES

- ARWILL, T. 1963. Some morphological aspects of the dentinal innervation. In: *Sensory Mechanisms in Dentine* (Edited by ANDERSON, D. J.), p. 4. Pergamon Press, Oxford.
- BERNICK, S. 1948. Innervation of the human tooth. *Anat. Rec.* **101**, 81–107.
- BISCOE, T. J. and STEHBENS, W. E. 1966. Ultrastructure of the carotid body. *J. cell Biol.* **30**, 563–578.
- BRADLAW, R. 1939. The histology and histopathology of the dental innervation. *Proc. R. Soc. Med.* **32**, 1040–1053.
- BROOKHART, J. M., LIVINGSTON, W. K. and HAUGEN, F. P. 1953. Functional characteristics of afferent nerve fibres from tooth pulp of cat. *J. Neurophysiol.* **16**, 634–642.
- CAUNA, N. and ROSS, L. L. 1960. The fine structure of Meissner's touch corpuscles of the human fingers. *J. biophys. biochem. Cytol.* **8**, 467–482.

- FEARNHEAD, R. W. 1961. The neurohistology of human dentine. *Proc. R. Soc. Med.* **54**, 877-884.
- FEARNHEAD, R. W. 1963. The histological demonstration of nerve fibres in human dentine. In: *Sensory Mechanisms in Dentine* (Edited by ANDERSON, D. J.), p. 16. Pergamon Press, Oxford.
- FEARNHEAD, R. W. and LINDER, J. E. 1956. Observations on the silver impregnation of nerve fibres in teeth. *J. Anat., Lond.* **90**, 228-235.
- FRANK, R. M. 1966. Etude au microscope électronique de l'odontoblaste et du canalicule dentinaire humain. *Archs. oral Biol.* **11**, 179-199.
- GRAF, W. and BJÖRLIN, G. 1951. Diameters of nerve fibers in human tooth pulps. *J. Am. dent. Ass.* **43**, 186-193.
- GRAY, E. G. and GUILLERY, R. W. 1966. Synaptic morphology in the normal and degenerating nervous system. In: *International Review of Cytology* (Edited by BOURNE, G. H. and DANIELLI, J. F.), **19**, 111-182. Academic Press, New York.
- HATTYASY, D. 1964. Observations on the nerves of the human dental pulp. *Third European Regional Conference on Electron Microscopy, Symposium Czechoslovak Academy of Sciences, Prague*, pp. 425-426.
- LLOYD, D. P. C. 1943. Neuron patterns controlling transmission of ipsilateral hind limb reflexes in cat. *J. Neurophysiol.* **6**, 293-315.
- MIYOSHI, S., NISHIJIMA, S. and IMANISHI, I. 1966. Electron microscopy of myelinated and unmyelinated nerve fibres in human dental pulp. *Archs oral Biol.* **11**, 845-846.
- MUMMERY, J. H. 1919. *The Microscopic Anatomy of the Teeth*, p. 212. Oxford University Press, London.
- MUNGER, B. L. 1965. The intra-epidermal innervation of the snout skin of the opossum. A light and electron microscope study, with observations on the nature of Merkel's *Tastzellen*. *J. cell Biol.* **26**, 79-97.
- PALADE, G. E. 1952. A study of fixation for electron microscopy. *J. exp. Med.* **95**, 285-298.
- PEASE, D. C. and QUILLIAM, T. A. 1957. Electron microscopy of the Pacinian corpuscle. *J. biophys. biochem. Cytol.* **3**, 331-342.
- ROBERTIS, E. D. P. DE 1964. *Histophysiology of Synapses and Neurosecretion*. Macmillan, New York.



FIGS. 1 and 2. Thick sections of myelinated nerve fibres cut longitudinally and transversely at the periphery of the human dental pulp. Toluidine blue. $\times 800$.
 FIG. 3. Transverse section of myelinated and unmyelinated nerve fibres at the periphery of the human dental pulp. A, myelinated nerve fibre; S, plasma membrane of Schwann cell; N, nucleus of Schwann cell; E, mesaxon of unmyelinated nerve fibre; Am, exposed unmyelinated nerve fibre; axon of unmyelinated nerve fibre (arrow). Uranyl acetate. $\times 10,000$.

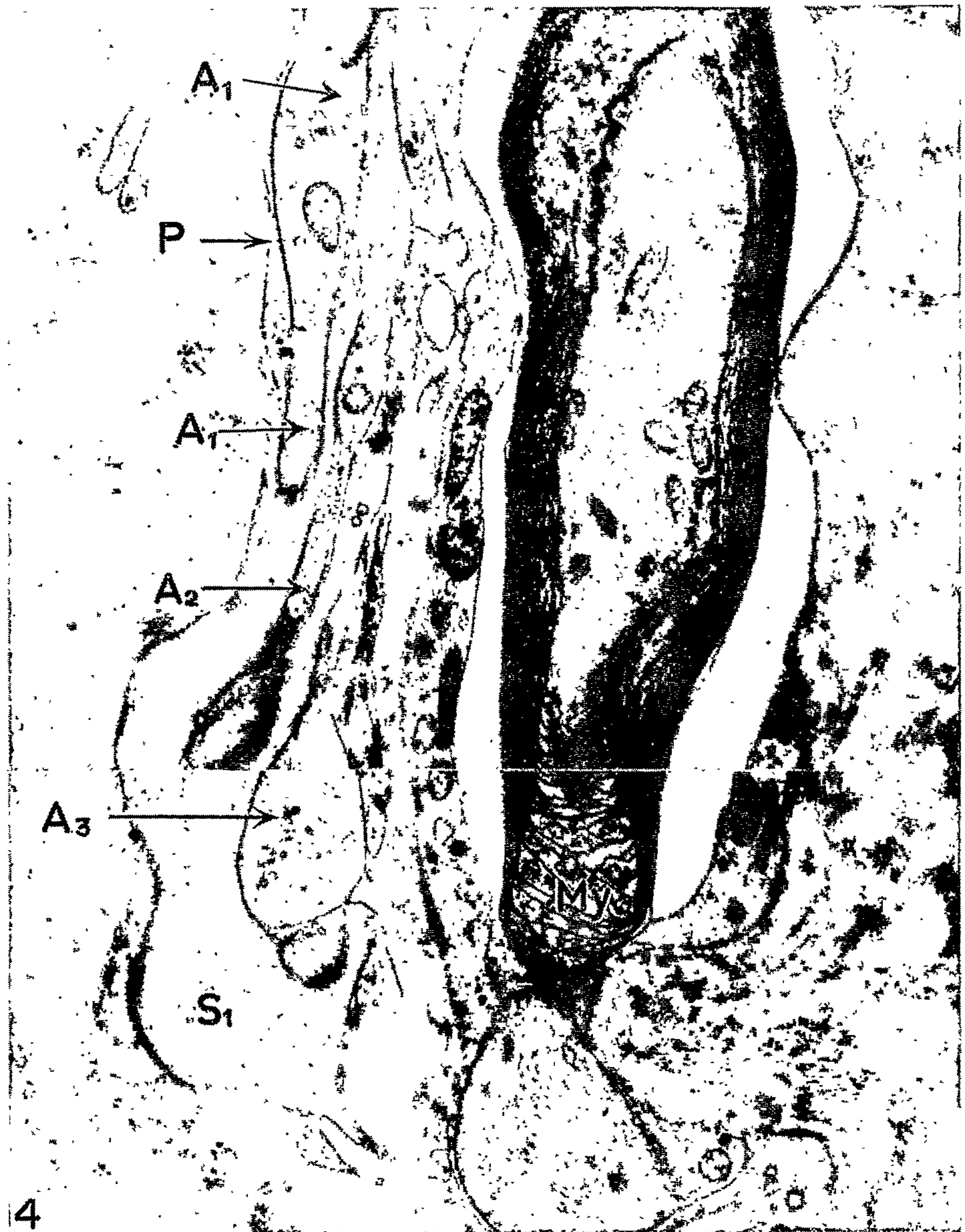


FIG. 4. Composite picture of myelinated nerve fibres. A₁, axon of myelinated nerve fibre; P, plasma membrane of Schwann cell; A₂, A₃, branching axon; S₁, cytoplasm of Schwann cell; My, node of Ranvier. Uranyl acetate. $\times 10,000$.

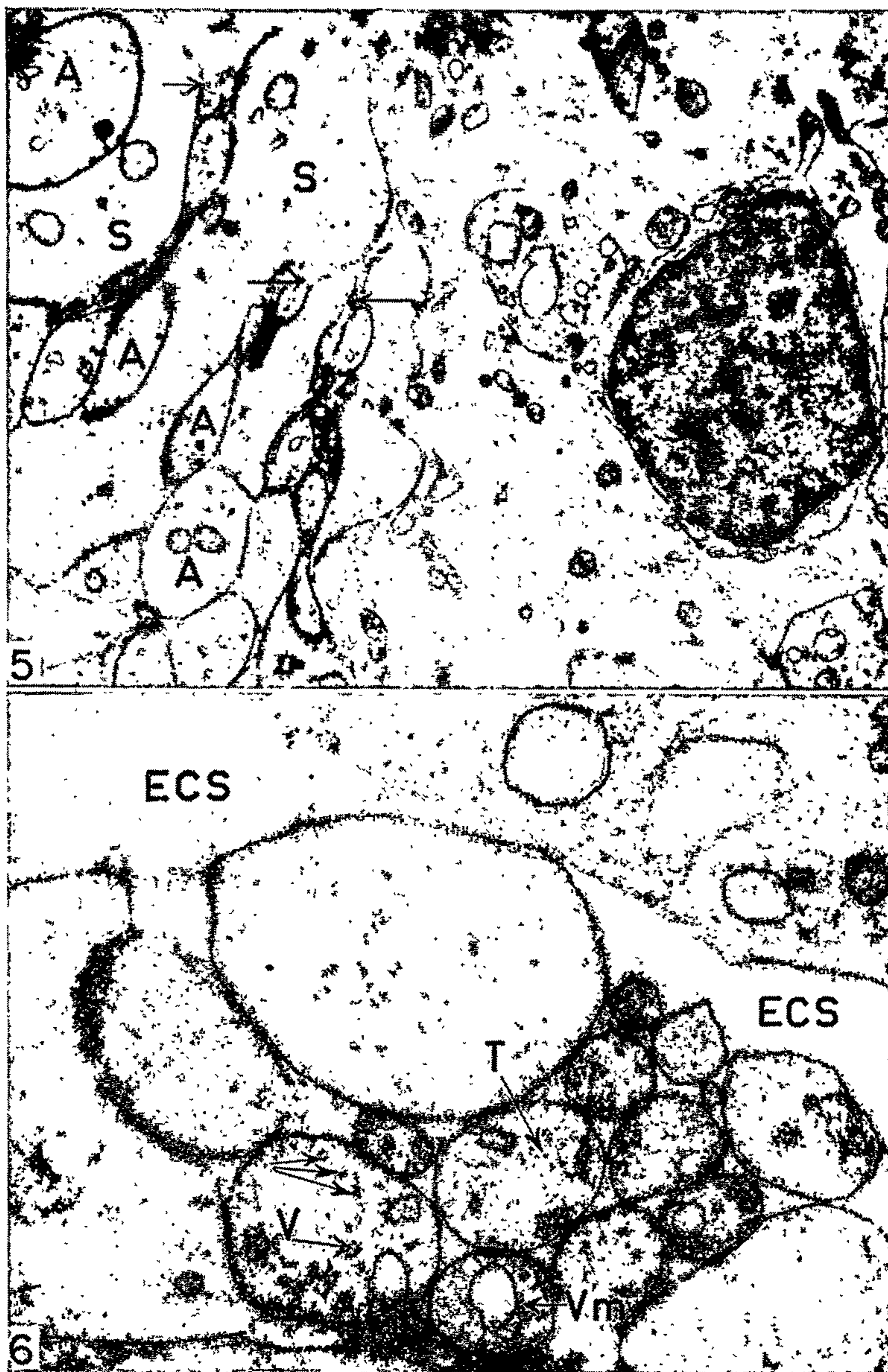


FIG. 5. Oblique section of unmyelinated nerve fibres. A, axon; S, Schwann cell; exposed axons (arrows). Uranyl acetate. $\times 8,750$.

FIG. 6. Exposed unmyelinated nerve fibres. V_m , macrovesicles; V, vesicles; ECS, extracellular substance; microvesicles (arrows); T, microtubular fibrils. N.B. Basement membrane of Schwann cell can be seen in most cases associated with the exposed axons. Uranyl acetate. $\times 35,000$.



PLATE 4

FIG. 7. Blood vessel at periphery of dental pulp. L, lumen; C, red blood corpuscles; N, nucleus of endothelial cell; Cm₁, internal plasma membrane; Cm₂, external plasma membrane with basement membrane; unmyelinated nerve fibres (arrow). Uranyl acetate. $\times 4200$.

FIG. 8. Nerve endings presumably of myelinated nerve fibres. V, varicosities; C, constricted axons surrounded by Schwann cell; A, naked axons; M, mitochondrion; S₁, S₂, cytoplasm of Schwann cell; Ne, axonal expansion; vesicles and microvesicles (arrows); ECS, extracellular substance. Uranyl acetate. $\times 19,100$.

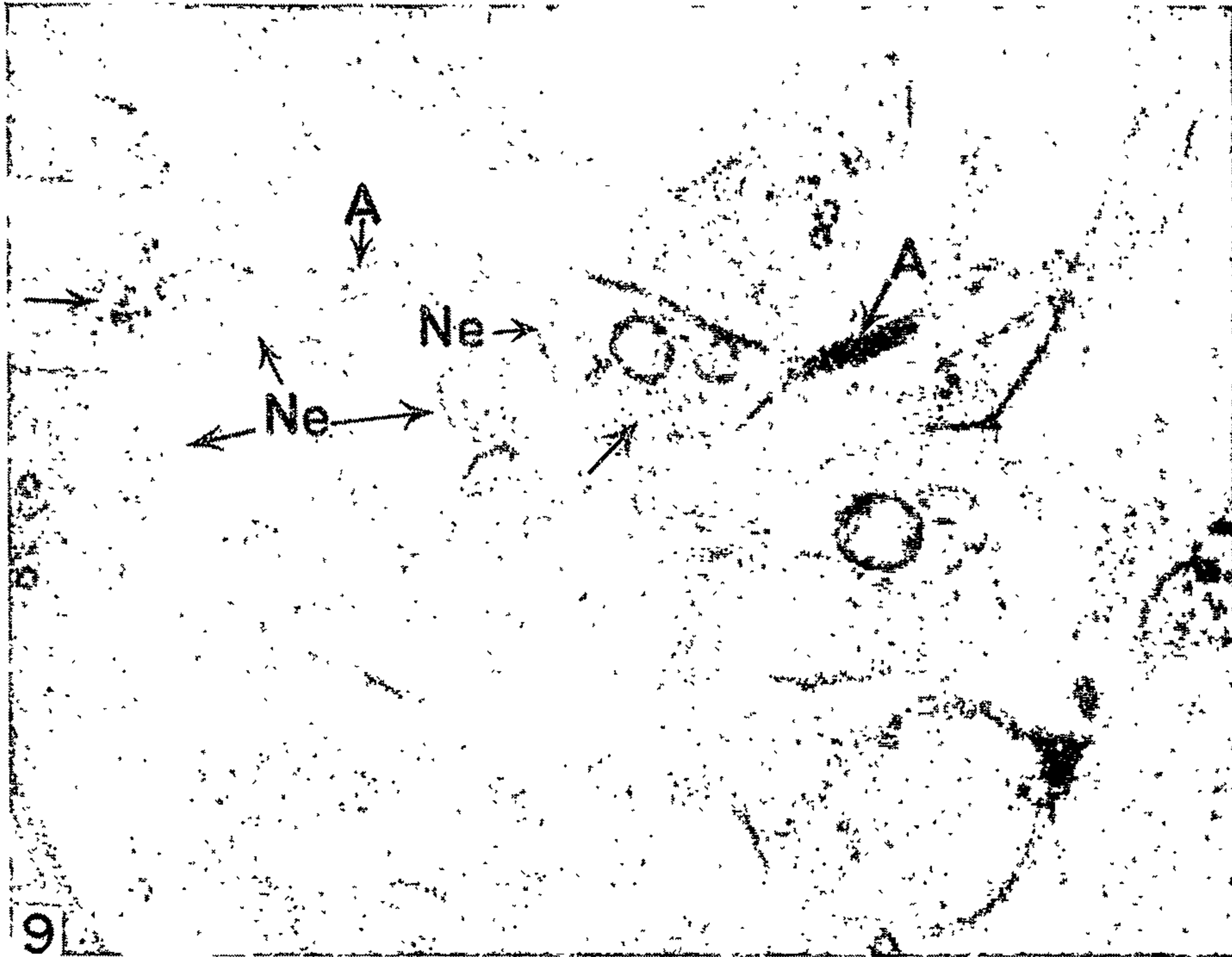


FIG. 9. Nerve endings at periphery of dental pulp. Ne, axonal expansions; A, naked axons; vesicles (arrows). Uranyl acetate. $\times 16,000$.

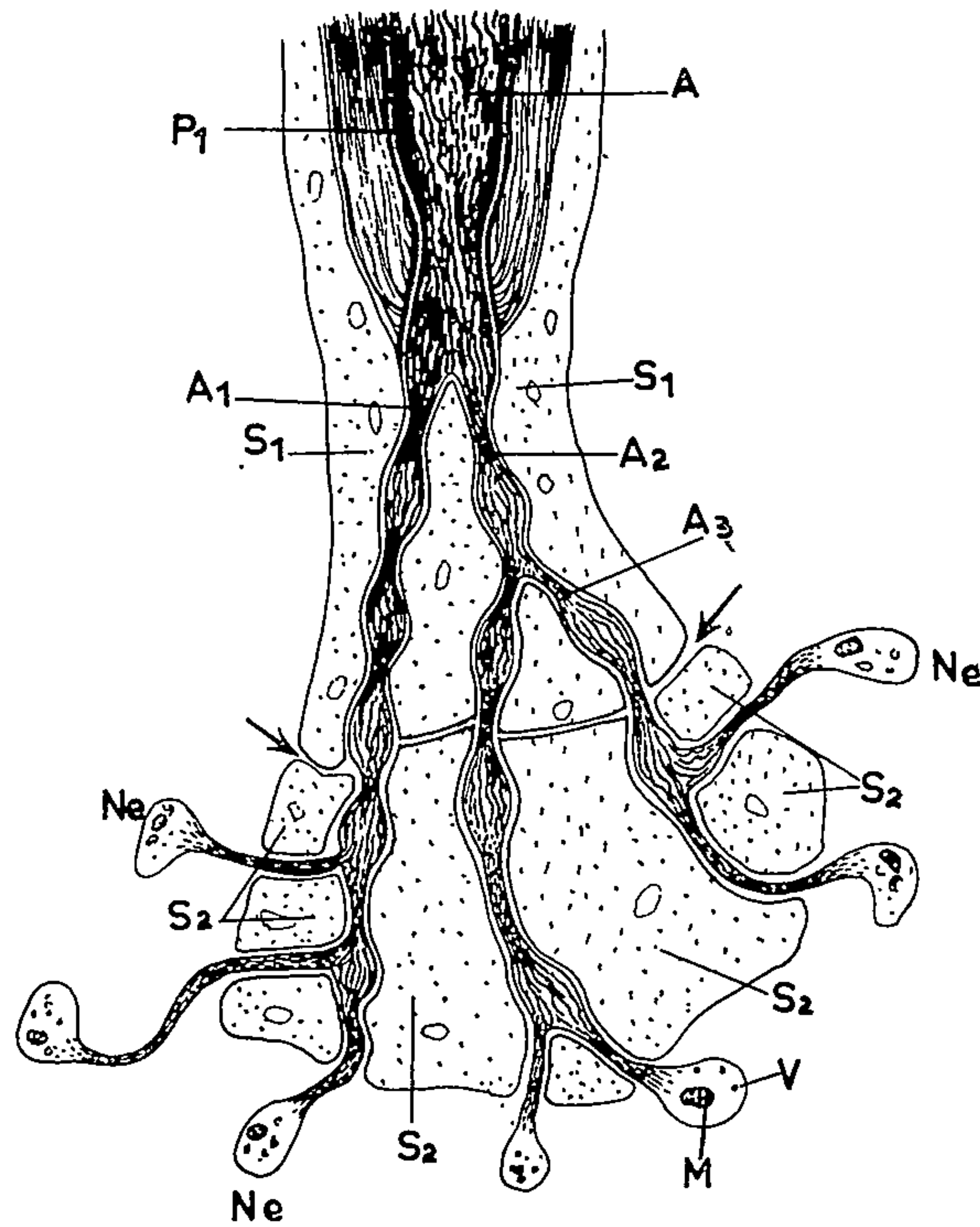


FIG. 10. Schematic reconstruction of nerve endings of myelinated nerve fibre. A, axon; P₁, plasma membrane of axon; S₁, S₂, cytoplasm of Schwann cell; A₁, A₂, A₃, branching axons; Ne, axonal expansions; M, mitochondrion; V, vesicles; junction between adjoining Schwann cells (arrows). This figure has been reconstructed from Figs. 4, 8 and 9.