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COMPUTERIZED COMPARISON OF HISTOLOGICAL METHODS FOR THE EVALUATION OF CRANIO-FACIAL GROWTH

by
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INTRODUCTION

Since *Hales* in 1727 published his investigation into the growth of the long bones, many special techniques for the microscopic investigation of bone growth have been developed. The classical method of vital staining was used by *du Hamel* (1742), *Hunter* (1772) and *Brasch* (1934). Bone labelling with tetracyclines, which permits a quantitative analysis of the rate of growth, was introduced by *Milch et al.* (1957), and microradiography, which yields information on the degree of mineralization, by *Clark* (1947) and *Amprino & Engström* (1952). Attempts have also been made by means of conventional histological techniques to evaluate osseous activity by ascertaining the amounts of osteoid, osteoblasts, osteoclasts, etc. (*Reitan*, 1951; *Meyer*, 1956; *Moss*, 1958; *Löe*, 1959; *Frost*, 1961).

Since the nature of the information obtained differs according to the method employed, several methods can be combined to advantage. This has been demonstrated in particular for cortical bones (*Kelly et al.*, 1965, *Owen et al.*, 1955; *Jowsey et al.*, 1954). In histological studies of the growth of the facial skeleton individual workers have, however, mainly confined themselves to one or a few of the existing techniques (*Moss*, 1954; *Enlow*, 1968).

The aim of the present investigation has been to establish which combination out of five different histological techniques is most suitable for evaluating

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the various kinds of growth found in the cranio-facial skeleton, i.e. sutural growth, periosteal growth and endochondral ossification. The methods employed comprised 3 different stainings of decalcified sections: haematoxyline-osin (1), periodic acid-Schiff (2), Masson's trichrome (3), and 2 different techniques applied to undecalcified sections: microradiography (4), methylene blue staining (5). As a control fluorescence microscopy after tetracycline labelling (6) was utilized.

It is well known that the histological picture of a bone surface may change from one histological section to the next, but it seems clear that while an individual is growing, one must find a histological picture which differs from that of a grown person. It is thus of interest to find what would be the most characteristic feature of a certain bone surface in different age groups.

In order to obtain an objective evaluation, the results of the various combinations of methods were compared statistically in an electronic computer. Since the primary object of this investigation was to evaluate methods and method combinations, the information on the growth of the facial skeleton incidentally obtained was not subjected to further analysis.

MATERIAL AND METHODS

In any investigation which utilizes many histological methods for description of the same area, it is necessary to employ animals which are large enough to supply the necessary amount of tissue for histological study. In the present instance, rabbits were chosen, as even the youngest animals provide sufficient material for the necessary decalcified and undecalcified sections. To permit growth activity to be evaluated, the rabbits were grouped according to age based on periods of 3 weeks (Table I). The number of rabbits in each group was dependent on the size of the litter.

The bone labelling to be used in the fluorescence microscopy (method 6) was effected in all cases by the injection of tetracycline. Two intraperitoneal injections were given. The first of these employed oxytetracycline (20 mg/kg), which gives a yellow fluorescence, and the second chlortetracycline, which gives a greenish fluorescence.

In order to determine the optimal interval between injections of tetracycline a pilot investigation was necessary. The interval had to be long enough to permit measurement of the amount of new bone formed, but short enough to avoid appreciable remodelling during the observation period. 5 days proved to be appropriate at age 4—7 weeks, 10 days at age 2—4 months and 15 days in older rabbits.

The animals were killed by intraperitoneal injection of Nembutal. Imme-

Table I.

Distribution of animal material according to age and duration of experiment

Number	Age in weeks	Number of days between 1st and 2nd injection	Number of days between second injection and sacrifice
9	3	5	3
4	6	5	3
7	12	10	3
8	15	10	3
6	21	10	3
6	52	15	5

diately afterwards, blocks of tissue were removed from the following areas: 1. Sagittal suture. 2. Transverse palatine suture. 3. Internasal suture. 4. Spheno-occipital synchondrosis. 5. Anterior border of the foramen magnum. It was hereby possible to study 3 different types of sutures: serrate (1), limbous (2) and plane (3), and 2 kinds of ossification: endochondral (4) and periosteal (5).

The blocks were bisected in the mid-sagittal plane, one half being used for decalcified sections the other for undecalcified sections. The mid-sagittal sutures were bisected frontally.

The blocks were fixed in concentrated methanol. Decalcification was effected in EDTAC (*Warshawsky & Moore*, 1967) under X-ray control. When decalcification was complete, the block was double embedded according to the method described by *Kraus, Kitamura* and *Latham* (1966). Serial sections 12 μ thick were cut on a Leitz sledge microtome. Every tenth section was in rotation stained with haematoxylin-eosin, PAS and Masson's trichrome (*Kraus et al.*, 1966).

The undecalcified half of each block was embedded in methyl methacrylate as described by *Jowsey et al.* (1965) and sectioned to a thickness of 200 μ on a milling machine, resulting in approximately 2 sections per mm of the block. The sections were then ground and polished between glass to a thickness of about 70 μ. The plane-parallelity of the sections was checked by means of a micrometer screw. Contact microradiographic exposures were then made of each section using a Machlet X-ray tube AEG 50, with a film focus distance of 6.1 cm and an exposure of 20 min. at 12 kV and 12 mA. Kodak Maximum Resolution Spectoscopic plates were employed and developed for 5 min. in Kodak developer D-19b *Sissons* (1950).

The ground sections were examined for tetracycline labelling by fluorescence microscopy and finally stained with methylene blue at pH 4.8 with the object of demonstrating osteoid.

Registration

Analysis of histological findings has usually been based on verbal description of specimens. When numerous histological observations are to be compared and, as in this case, the results from several different methods evaluated, it is necessary to use a more precisely defined system of classification to permit a statistical processing of the observations. A special procedure of classification was therefore developed with a view to the subsequent coding of the observations for computer analysis. The observed features were recorded on the form shown in Table II.

Decalcified sections: In the haematoxylin-eosin stained sections (method 1), each bone surface was inspected for the following features:

1. Osteoblasts: Polyhedral basophilic active osteoblasts on the bone surface.
2. Osteoid: Visible osteoid seam.
3. Woven bone: Acellular bone surfaces without organization of collagen fibres.
4. Resorption: Resorption surface with osteoclasts.
5. Resting line: Lamellar bone with resting line on the surface.
6. No registration: Registration impossible.

The numbers refer to the rows in the registration form (Table II). The category »No registration» is employed when it is impossible to allocate the surface in question to any one of the five other groups.

The PAS stained sections (method 2) were classified in a similar manner and recorded in rows 7—12 in Table II.

In the Masson stained sections (method 3), the bone surfaces were recorded as:

13. Immature bone: Osteoid or immature (green-stained) bone.
14. Mature bone: (red-stained).
15. No registration: Registration impossible.

In most sutures the growth activity was the same on both surfaces, and therefore only one registration was carried out. In the transverse palatine suture, however, the activity in the two confronting surfaces was recorded separately, as it very often differed in the anterior and the posterior surface.

Table II.

Example of observed features as recorded on form of registration

SUBJECT No. 7 Age 15
SURFACE No. 5 Sex 1
No of sections

				1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Σn
subject no. surface no.	Method 1 H & E	1 2 3 4 5 6	osteoblasts osteoid seam woven bone resorption resting line no registration																
subject no. 5 surface no. 7	Method 2 PAS	7 8 9 10 11 12	osteoblasts osteoid seam woven bone resorption resting line no registration	x	x	x	x	x	x	x	x	x	x	x	x				6 3 3
subject no. surface no.	Method 3 Masson	13 14 15	immature bone mature bone no registration																
subject no. surface no.	Method 4 Microradio- graphy	16 17 18 19	growing surface resting surface resorption surface no registration																
subject no. surface no.	Method 5 Methylene blue	20 21 22	osteoid seam no osteoid seam no registration																
subject no. surface no.	Method 6 Tetracyc- line	23 24 25 26 27	2 lines 1st line 2nd line no lines no registration																

Undecalcified sections: In the microradiographs (method 4) the bone surfaces were classified as:

16. Growing surface: A slightly mineralized zone.
17. Resting surface: A highly mineralized even zone.
18. Resorption surface: A highly mineralized uneven zone.
19. No registration: Registration impossible.

The undecalcified sections were then examined by fluorescence microscopy (method 6) and the surfaces evaluated according to the number of fluorescent lines:

23. 2 lines: Separate lines visible.
24. 1st line: First line visible.
25. 2nd line: Second line visible.
26. No lines: No lines visible.
27. No registration: Registration impossible.

Finally, the undecalcified sections stained with methylene blue (method 5) were inspected for the presence of an osteoid zone:

20. Osteoid seam.
21. No osteoid seam.
22. No registration: Registration impossible.

For each animal a separate form was completed for each of the bone surfaces examined and for each method, thus ensuring an unbiased registration. As an example of the information to be found in a completed form, Table II shows that from animal no. 7, 12 decalcified sections stained with PAS were prepared of suture no. 5; 6 of these were recorded as exhibiting osteoblasts, 3 as being covered by osteoid, and 3 as consisting of woven bone. These totals were entered in the right-hand column after registration of the individual sections had been completed.

Coding

It was now desired on the basis of the registration form Table II to count the number of bone surfaces the growth activity of which was judged to be the same. A computer program developed earlier (*Björk, Krebs & Solow*, 1964; *Solow*, 1964; *Solow & Helm*, 1968) for tabulation of coded malocclusion data proved to be suitable for the purpose, on account of its flexibility with respect to tabulation. Code numbers were defined as shown in Table III and the tabular lay-out shown in Table V adopted.

Table III.

Code numbers for histological registration of craniofacial growth

	Methods		sut. sagit.	sut. transv. ant.	sut. transv. post.	sut. inter-nas.	synchondr. sphenoocc. ant.	synchondr. sphenoocc. post.	basion
Method 1 H & E	osteoblasts	1	200	227	254	281	308	335	362
	osteoid	2	201	228	255	282	309	336	363
	woven bone	3	202	229	256	283	310	337	364
	resorption	4	203	230	257	284	311	338	365
	resting line	5	204	231	258	285	312	339	366
	no registration	6	205	232	259	286	313	340	367
Method 2 PAS	osteoblasts	7	206	233	260	287	314	341	368
	osteoid	8	207	234	261	288	315	342	369
	woven bone	9	208	235	262	289	316	343	370
	resorption	10	209	236	263	290	317	344	371
	resting line	11	210	237	264	291	318	345	372
	no registration	12	211	238	265	292	319	346	373
Method 3 Masson	immature bone	13	212	239	266	293	320	347	374
	mature bone	14	213	240	267	294	321	348	375
	no registration	15	214	241	268	295	322	349	376
Method 4 Microradio-graphy	growing surface	16	215	242	269	296	323	350	377
	resting surface	17	216	243	270	297	324	351	378
	resorption surface	18	217	244	271	298	325	352	379
	no registration	19	218	245	272	299	326	353	380
Method 5 Methylene blue	osteoid seam	20	219	246	273	300	327	354	381
	no osteoid seam	21	220	247	274	301	328	355	382
	no registration	22	221	248	275	302	329	356	383
Method 6 Tetracyc-line	2 lines	23	222	249	276	303	330	357	384
	1st line	24	223	250	277	304	331	358	385
	2nd line	25	224	251	278	305	332	359	386
	no lines	26	225	252	279	306	333	360	387
	no registration	27	226	253	280	307	334	361	388

SEX	
M	1
F	2

AGE	
3 weeks	3
4 weeks	4
41 weeks	41
42 weeks	42
> 42 weeks	43

In practice, the coding was carried out in two phases. The first of these comprised a transfer of general information and the results of the visual inspection to punched cards. The general information consisted of identification number, and sex and age expressed in code numbers (1—43) as

indicated in Table III. As registration results, the totals (Σn) in the right-hand column of the registration form were recorded.

In the second phase of the coding the set of totals for each animal, surface and method was analysed and replaced by a code number. This process comprised a sorting of data so that all information from the same individual was collected. A further sorting was then carried out in order to collect the information appertaining to each particular bone surface. It was then analysed which feature had most frequently been observed with each method employed i.e. which of the corresponding rows in the summation column (Σn) of the registration form showed the largest number of registrations. This was expressed with the aid of the corresponding code number (Table III). In cases where two or more features occurred with equal frequency the lowest code number was recorded, representing the most active category. All code numbers were finally transferred to magnetic tape, which was used as input in the tabulation program.

Tabulation

The tabulation program requires a coded specification of criteria for each table (Fig. 1). For each of the 5 bone regions examined 3 tables were formulated. The first table characterizes bone surfaces undergoing growth, the second table bone surfaces consisting of newly formed bone, and the third table resting surfaces.

The positions in the tables comprised both individual methods and combinations of several methods, chosen to facilitate the comparison between methods applied to decalcified (methods 1—3) and undecalcified sections (methods 4—6) and to express the results obtained with all methods except vital labelling (Table V). The criteria employed for definition of the activity groups according to each method are given in Table IV.

The advantage of using a combination of methods to decide whether a bone surface exhibits growth is that it permits the exclusion of surfaces which only register growth with one or a few methods.

As an example of specification criteria for tabulation, Fig. 1 defines the internasal suture as characterized by growth.

The table specifications and coded observations were processed by the above-mentioned tabulation program on an IBM 7094 computer at Northern Europe University Computer Centre. An example of output is given in Table V.

Method 1	$\frac{281 \vee 282}{1 \vee 2}$
» 2	$\frac{287 \vee 288}{1 \vee 2}$
» 3	$\frac{293}{1 \vee 2}$
» 4	$\frac{296}{1 \vee 2}$
» 5	$\frac{300}{1 \vee 2}$
» 6	$\frac{303}{1 \vee 2}$
» 1, 2, 3	$\frac{(281 \vee 282) \wedge (287 \vee 288) \wedge 293}{1 \vee 2}$
» 4, 5, 6	$\frac{296 \wedge 300 \wedge 303}{1 \vee 2}$
» 1, 2, 3, 4, 5	$\frac{(281 \vee 282) \wedge (287 \vee 288) \wedge 293 \wedge 296 \wedge 300}{1 \vee 2}$
All Methods	$\frac{(281 \vee 282) \wedge (287 \vee 288) \wedge 293 \wedge 296 \wedge 300 \wedge 303}{1 \vee 2}$

Fig. 1. Example of criteria specification for Table V. (For detailed description see *Solow & Helm*, 1968).

Table IV

Criteria for definition of the activity groups

	Method 1 H & E	Method 2 PAS	Method 3 Masson	Method 4 Microradiography	Method 5 Methylene blue	Method 6 Tetracycline
growth surface	osteoblasts osteoid	osteoblasts osteoid	young bone	growing surface	osteoid seam	1 lines
new bone	woven bone	woven bone	young bone	growing surface	undefined	2 lines
mature bone	resting line	resting line	mature bone	resting surface	no osteoid seam	no lines

Table V.

Surfaces showing sutural growth in sutura internasalis

	N	PCT.	S.E.
Method 1	10	25.00	6.85
Method 2	11	27.50	7.06
Method 3	25	62.50	7.65
Method 4	32	80.00	6.32
Method 5	16	40.00	7.75
Method 6	28	70.00	7.25
Method 1, 2 and 3	10	25.00	6.85
Method 4, 5 and 6	16	40.00	7.75
Method 1, 2, 3, 4 and 5	10	25.00	6.85
all Methods	10	25.00	6.85

PCT based on number of surfaces recorded

RESULTS

The results from each of the regions investigated are shown in Tables VI—XI and Figs. 2—4. In the column on the extreme left in Tables VI—XI, bone surfaces are divided into 3 activity groups on the basis of their predominant character. The methods employed are given in column 2. In the next column, the frequency of bone surfaces registered by each method is given, expressed both in absolute figures (i.e. number of bone surfaces) and as a percentage of all observations carried out with the method in question. If the methods are divided into two groups according to whether they employ decalcified (haematoxylin-eosin, PAS and Masson stained) or undecalcified (microradiography, fluorescence microscopy and methylene blue stained) sections, information is gained as to how many surfaces were registered as belonging to the same activity group in all decalcified and undecalcified sections respectively (column 4). In column 5, all methods except fluorescence microscopy are combined. The reason for this division is that all methods except vital labelling can be applied to human tissue post mortem. In a large number of cases there was a discrepancy in the results, as seen in the decline in the number of bone surfaces in each group as one moves to the right in the tables. The last column gives the number of surfaces similarly evaluated by all methods.

As apparent from Table VI there was an essential difference in the frequencies within each activity group according to which staining technique

Table VI.

Sutural growth in sutura sagittalis

	Method	N.	PCT.	N.	PCT.	N.	PCT.	N.	PCT.
surfaces showing growth	H & E	10	25.0						
	PAS	9	22.5	9	22.5	9	22.5		
	Masson	22	55.0					9	22.5
	Microradiography	24	60.0						
	Methylene blue	16	40.0	16	40.0				
	Tetracycline	20	50.0			20	50.0		
surfaces consisting of new bone	H & E	13	32.5						
	PAS	12	30.5	10	25.0	10	25.0		
	Masson	22	55.0					10	25.0
	Microradiography	24	60.0						
	Methylene blue	40	100.0	20	50.0				
	Tetracycline	20	50.0			20	50.0		
surfaces consisting of mature bone	H & E	17	42.0						
	PAS	19	47.5	17	42.5	16	40.0		
	Masson	18	45.0					16	40.0
	Microradiography	16	40.0						
	Methylene blue	24	60.0	16	40.0				
	Tetracycline	17	42.5			17	42.5		

Table VII.

Sutural growth in sutura palatina transversa (ant.)

	Method	N.	PCT.	N.	PCT.	N.	PCT.	N.	PCT.
surfaces showing growth	H & E	5	12.5						
	PAS	5	12.5	5	12.5	5	12.5		
	Masson	28	70.0					5	12.5
	Microradiography	33	82.5						
	Methylene blue	20	50.0	20	50.0				
	Tetracycline	29	72.5			29	72.5		
surfaces consisting of new bone	H & E	24	60.0						
	PAS	25	62.5	22	55.0	22	55.0		
	Masson	28	70.0					22	55.0
	Microradiography	33	87.5						
	Methylene blue	40	100.0	29	72.5				
	Tetracycline	29	72.5			29	72.5		
surfaces consisting of mature bone	H & E	11	27.5						
	PAS	10	25.0	10	25.0	6	15.0		
	Masson	12	30.0					6	15.0
	Microradiography	7	17.5						
	Methylene blue	20	50.0	7	17.5				
	Tetracycline	7	17.5			7	17.5		

Table VIII.

Sutural growth in sutura palatina transversa (post.)

	Method	N.	PCT.	N.	PCT.	N.	PCT.	N.	PCT.
surfaces showing growth	H & E	15	37.5						
	PAS	16	40.0	15	37.5	15	37.5		
	Masson	24	60.0					15	37.5
	Microradiography	29	72.5						
	Methylene blue	25	62.5	25	62.5				
	Tetracycline	26	65.0			26	65.0		
surfaces consisting of new bone	H & E	12	30.0						
	PAS	11	27.5	11	27.5	9	22.5		
	Masson	24	60.0					9	22.5
	Microradiography	29	72.5						
	Methylene blue	40	100.0	26	65.0				
	Tetracycline	26	65.0			26	65.0		
surfaces consisting of mature bone	H & E	13	32.5						
	PAS	13	32.5	13	32.5	10	25.0		
	Masson	16	40.0					10	25.0
	Microradiography	11	27.5						
	Methylene blue	15	37.5	11	27.5				
	Tetracycline	11	27.5			11	27.0		

was used. It is for instance apparent from Tables VI—VIII, which all refer to different types of sutural growth (Figs. 2—3), that there was a great difference in the frequencies in the group pertaining to surfaces undergoing growth. In the undecalcified sections, a larger percentage of these surfaces was found than in decalcified sections stained with haematoxylin-eosin and

Fig. 2. The transverse palatine suture between the palatine process of the maxilla and the horizontal plate of the palatine bone.

A. General view of the nasal septum.
B. Detail corresponding to the inset in fig. 2A.
C. Method 1 Haematoxylin-eosin
D. » 2 PAS
E. » 3 Masson
F. » 4 Microradiography
G. » 5 Methylene blue
H. » 6 Tetracycline

It is apparent using all methods that there is a distinct difference between the two bone surfaces which border the suture. The anterior part consists of fully calcified bone (E and F). The cells on this surface are only slightly differentiated and occur sparsely (C and D). In contrast, the posterior surface is seen to consist of slightly calcified, immature bone (E and F) and to be covered with osteoblasts (C and D).

Tetracycline labelling reveals the same difference, since in the period between the two tetracycline injections no visible apposition on the maxillary part of the suture has occurred whereas the distance between the two tetracycline lines on the palatine bone testifies to appositional growth (H).

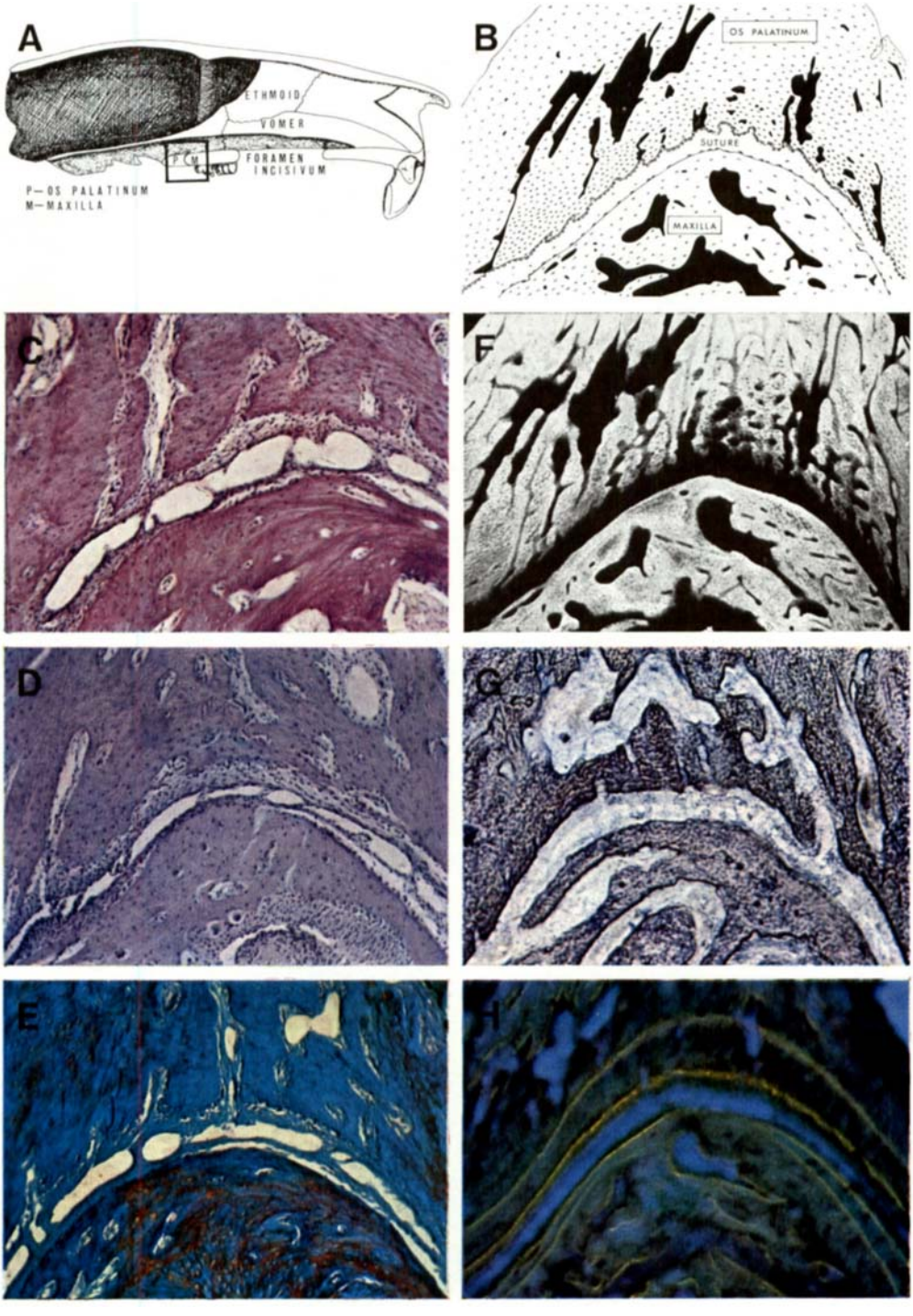

A
ETHMOID
VOMER
FORAMEN INCISIVUM
P—OS PALATINUM
M—MAXILLA
B
OS PALATINUM
SUTURE
MAXILLA
C
F
D
G
E
H

Table IX.

Sutural growth in sutura internasalis

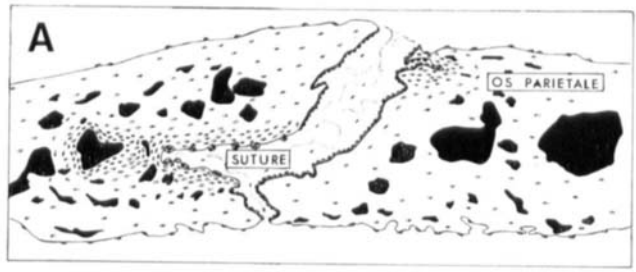
	Method	N.	PCT.	N.	PCT.	N.	PCT.	N.	PCT.
surfaces showing growth	H & E	10	25.0						
	PAS	11	27.5	10	25.0	10	25.0		
	Masson	25	62.5					10	25.0
	Microradiography	32	80.0						
	Methylene blue	16	40.0	16	40.0				
	Tetracycline	28	70.0			28	70.0		
surfaces consisting of new bone	H & E	21	52.5						
	PAS	18	45.0	18	45.0	16	40.0		
	Masson	25	62.5					16	40.0
	Microradiography	32	80.0						
	Methylene blue	40	100.0	28	70.0				
	Tetracycline	28	70.0			28	70.0		
surfaces consisting of mature bone	H & E	9	22.5						
	PAS	11	27.5	9	22.5	8	20.0		
	Masson	15	37.5					8	20.0
	Microradiography	8	20.0						
	Methylene blue	24	60.0	8	20.0				
	Tetracycline	10	25.0			10	25.0		

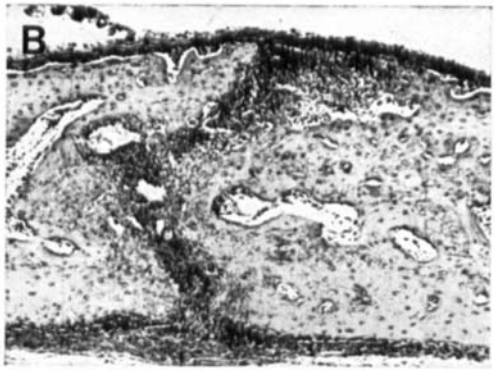
PAS. Likewise, in the group pertaining to bone surfaces consisting of young bone, a larger number was found when undecalcified sections were employed. This is not least due to the fact that it was not possible by means of microradiography and fluorescence microscopy to separate the groups with growing surfaces from those consisting of newly formed bone, for which reason the same surfaces feature, according to the definition of tabular criteria (Table IV and Fig. 1), in both groups. With methylene blue staining it was not possible to differentiate the sections in activity group 2. Therefore surfaces both with and without osteoid seams were included. In the group with resting surfaces,

Fig. 3. Sagittal suture.

A. Diagram showing the area concerned.
B. Method 1 Haematoxylin-eosin
C. » 2 PAS
D. » 3 Masson
E. » 4 Microradiography
F. » 5 Methylene blue
G. » 6 Tetracycline

It is apparent that in this suture even in the same bone surface there is decisive difference in activity and thereby in the amount of new bone formed, which can contribute to the changes in the morphology of the suture.


A
OS PARIETALE
SUTURE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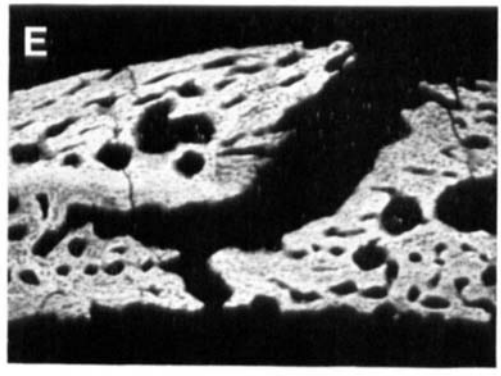
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D

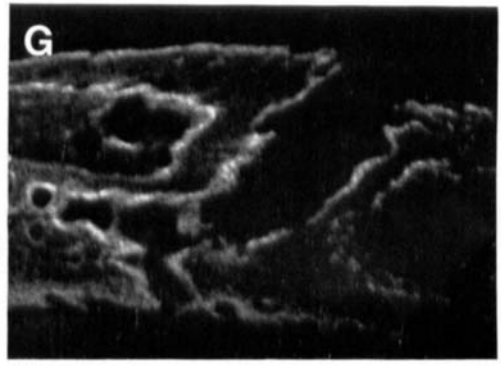

G

Table X.

Periosteal ossification in basion

	Method	N.	PCT.	N.	PCT.	N.	PCT.	N.	PCT.
surfaces showing growth	H & E	22	55.0						
	PAS	21	52.5	21	52.5	21	52.5		
	Masson	25	62.5					21	52.5
	Microradiography	32	80.0						
	Methylene blue	25	62.5	28	70.0				
	Tetracycline	28	70.0			28	70.0		
surfaces consisting of new bone	H & E	10	25.0						
	PAS	13	32.5	10	25.0	8	20.0		
	Masson	25	62.5					8	20.0
	Microradiography	32	80.0						
	Methylene blue	40	100.0	28	70.0				
	Tetracycline	28	70.0			28	70.0		
surfaces consisting of mature bone	H & E	8	20.0						
	PAS	6	15.0	6	15.0	8	20.0		
	Masson	15	37.5					8	20.0
	Microradiography	8	20.0						
	Methylene blue	15	37.5	8	20.0				
	Tetracycline	9	22.5			9	22.5		

the distribution was reversed, since more cases were recorded in this group when conventional methods were utilized.

In Table X, which records periosteal ossification, similar circumstances are seen to apply, whereas no systematic difference between the various methods was seen in the evaluation of endochondral ossification (Table XI, Fig. 4).

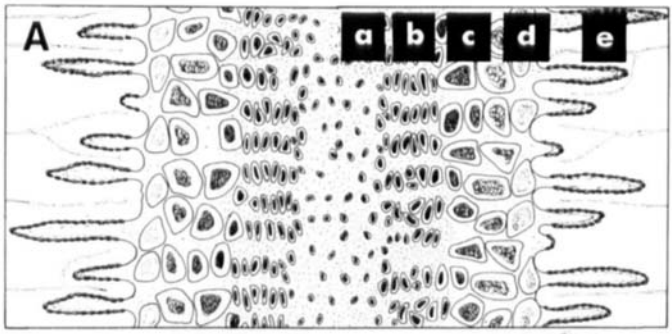
Fig. 4. Spheno-occipital synchondrosis.

A. Diagram indicating the different zones in the endochondral ossification.

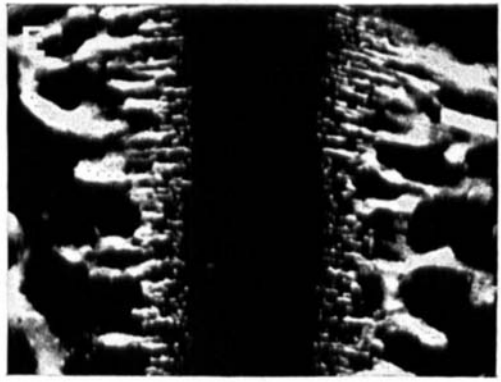
a. cartilage
b. columnar cartilage
c. fibrohyaline tissue
d. primary ossification
e. secondary ossification

B. Method 1 Haematoxylin-eosin
C. » 2 PAS
D. » 3 Masson
E. » 4 Microradiography
F. » 5 Methylene blue
G. » 6 Tetracycline

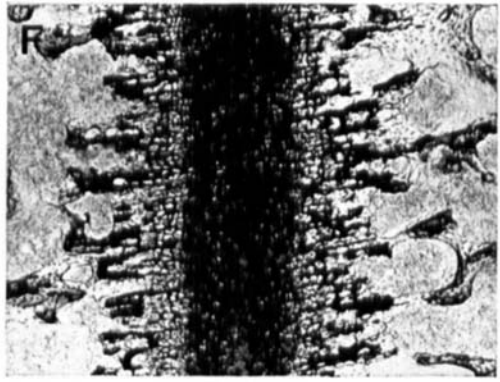
All the methods employed give a clear picture of endochondral ossification.

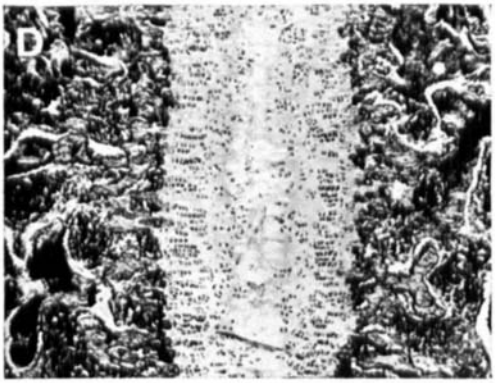

A
a
b
c
d
e


B


F


C


R


D

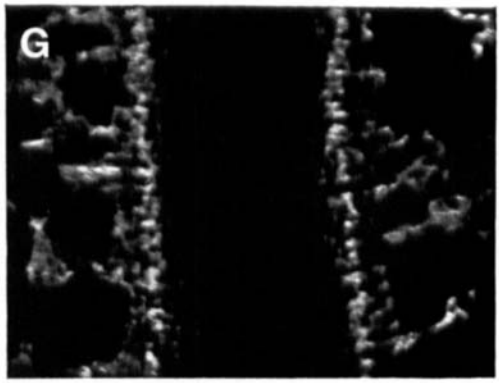

G

Table XI.

Endochondral ossification in synchondrosis sphenooccipitalis

	Method	N.	PCT.	N.	PCT.	N.	PCT.	N.	PCT.
surfaces showing growth	H & E	15	37.5						
	PAS	15	37.5	15	37.5	15	37.5		
	Masson	21	52.5					15	37.5
	Microradiography	21	52.5						
	Methylene blue	17	42.5	17	42.5				
	Tetracycline	19	47.5			19	47.5		
surfaces sisting of new bone	H & E	8	20.0						
	PAS	7	17.5	7	17.5	7	17.5		
	Masson	21	52.5					4	10.0
	Microradiography	21	52.5						
	Methylene blue	40	100.0	19	47.5				
	Tetracycline	19	47.5			19	47.5		
surfaces consisting of mature bone	H & E	17	42.5						
	PAS	18	45.0	18	45.0	18	45.0		
	Masson	19	47.5					18	45.0
	Microradiography	19	47.5						
	Methylene blue	23	57.5	19	47.5				
	Tetracycline	19	47.5			19	47.5		

DISCUSSION

The greatest variation between the results obtained with the different methods was found in activity group 1, where the difference between the results obtained by microradiography and those obtained by conventional histological methods was striking. One must, however, consider which factors are decisive in the allocation of a bone surface to the group in question with the methods employed. For haematoxylin-eosin and PAS stained sections, the presence of osteoblasts and/or osteoid was the deciding factor (Figs. 2—4), but these features in the formation of new bone are present only for a short time. In an investigation by *Frost* (1960), osteoblast activity in man has been stated to yield 0.9 μ osteoid per day irrespective of whether it occured in bone undergoing normal growth or in bone undergoing remodelling. The interval between the formation of osteoid and the onset of calcification has been stated on the basis of both light-microscopic (*Amprino & Engström* 1952) and electron-microscopic examination (*Robinson*, 1960) to be of a few hours' duration, so that the osteoid seam will be often so narrow that it will be difficult to discern under the optical microscope. As it has also been demonstrated, for instance by the presence of resting lines (*Harris*, 1933), bone formation is discon-

tinuous even in rapidly growing individuals, forming an explanation for the limited number of surfaces showing growth found in the decalcified sections of even young animals.

Microradiographic differentiation between growing and resting surfaces in bone is based on the degree of calcification of the bone surface. It has been demonstrated earlier (*Amprino & Engström*, 1952; *Engström et al.*, 1955) that about 70 % of the calcification occurs very rapidly (in the course of a few days), while the calcification of the remaining 20—30 % occupies a period of several months. Since bone surfaces which are in the last stages of mineralization are registered in microradiography as growing surfaces, the number of such surfaces registered with this method will be considerably larger than with the employment of conventional techniques. The slightly calcified surfaces undergoing growth were in all cases registered as growing surfaces, also with tetracycline labelling. The fact that there was nevertheless a greater incidence of growing surfaces with microradiography than with tetracycline fluorescence is due to the ability of the latter method to eliminate obliquely cut resting surfaces, which were wrongly registered in microradiography as growing surfaces.

In undecalcified sections, more osteoid surfaces were found with methylene blue staining than in decalcified sections. The explanation for this may be found in the fact that osteoid was stained to great contrast with this technique and was more easily demonstrated. With Masson trichrome staining it was of decisive importance in the evaluation of a bone surface whether it was red or green. Only mature bone, i.e. acidophilic, highly calcified bone, was stained red (*Bélanger et al.*, 1963), whereas the green-stained surfaces represented both osteoid and young bone, which were thus registered in both group 1 and group 2 (Figs. 2—3).

Group 2, which was defined as bone surfaces consisting of young, immature bone, was characterized in the case of haematoxylin-eosin and PAS stained sections by the presence of woven bone, which both in its structure and affinity to staining differs from mature lamellar bone. It is, however, also possible to allocate bone surfaces which have been passive for a long period to this group, especially in relation to the cranial sutures (*Pritchard et al.*, 1956), and it is difficult with this technique to differentiate between these and bone surfaces which have recently been undergoing growth. In this case too, both microradiography and bone labelling are of great value, since immature bone can be identified.

In group 3 with resting surfaces, the discrepancies between the evaluations by different methods were considerably less than in the two previous groups. In microradiography, however, as has been mentioned, fewer resting surfaces

were found than with the other methods, as obliquely sectioned surfaces were allocated to the two first groups.

In the evaluation of the duration of growth in a bone surface, the identification not only of growing bone, but of new bone is of interest, since bone deposition is intermittent. The registered surfaces must therefore comprise both growing surfaces and bone which has been formed during the last few months, but which is not fully calcified.

If one wishes to evaluate the advantages of the combination of methods employed here over single methods, one must establish how the results of the single methods have affected the results in the 3 groups. As a check on methods 1—5, tetracycline labelling was employed, as this technique gives a direct expression of bone formation in the distance between the two fluorescent lines. In order to eliminate bone surfaces where there was doubt as to whether new bone was formed, only surfaces with two separate lines or quite without tetracycline labelling were included. The number of sections was thereby reduced as early as column 3, since bone surfaces with one tetracycline line were eliminated. The question is then: How many of the growing or resting surfaces identified with the aid of tetracycline would one be able to identify by other methods and which methods would be decisive for the evaluation in the 3 groups? In group 1, comprising surfaces with active growth, the results obtained by conventional haematoxylin-eosin and PAS staining were decisive, in the combination of both the first five and all six methods, so that it was unnecessary in this group to utilize the other methods. The results of haematoxylin-eosin and PAS staining were almost identical, so that one of these methods will suffice. Since one has to take surfaces from group 2 into account when deciding whether growth in a given region is complete, it was important to analyse the value of the individual methods for the identification of surfaces in this group. Using haematoxylin-eosin and PAS, it was not possible to distinquish between newly formed and fully mature woven bone, and these methods must therefore be considered inadequate. By microradiography one was able to eliminate all the fully calcified surfaces with the exception of those few which in oblique section appeared as immature bone. Masson staining will contribute to a further sorting. In group 2 therefore, microradiography and Masson staining supplemented with either haematoxylin-eosin or PAS staining were called for.

In the group with resting surfaces, it was impossible either with conventional techniques or with microradiography to identify all the bone surfaces selected on the basis of tetracycline labelling. The number found with normal histological techniques was greater. This was due to the fact that in both haematoxylin-eosin, PAS and Masson stained sections, surfaces which had

Table XII.

Results of growth determination according to the method used

	Growth		No growth		Indeterminate		Total	
	no.	pct.	no.	pct.	no.	pct.	no.	pct.
Tetracycline	103	64.4%	45	28.1%	12	7.5%	160	100%
Combination of haematoxylin-eosin, Masson and microradiography	96	60.0%	—		22	13.8%	160	100%
Microradiography	—		42	26.2%				
Difference	—7	4.4%	—3	1.9%	+10	6.3%		

only been inactive for a short period were registered. Conversely, the number of bone surfaces identified by microradiography was reduced by the number of obliquely sectioned surfaces registered in group 2.

To sum up, the most effective differentiation between growing and resting bone surfaces was obtained by a combination of conventional methods and microradiography. The combination haematoxylin-eosin, Masson and microradiography was employed to identify growing surfaces, whereas microradiography sufficed for the identification of resting surfaces. A survey of the results obtained for the sutural growing surfaces is given in Table XII.

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SUMMARY

With an improved histological evaluation of growth activity of the bones of the face and the base of the skull in view, 6 different histological methods were compared. The material comprised 40 rabbits injected with bone-labelling tetracycline. After the animals had been killed, blocks of bone were removed to represent various kinds of sutural growth, appositional growth and endochondral ossification.

Paraffin sections of decalcified material stained with haematoxylin-eosin, PAS and Masson's trichrome stain were examined. Corresponding undecalci-

fied sections were stained with methylene blue and examined by microradiography. These sections were also examined by fluorescence microscopy for tetracycline labelling.

On the basis of the evaluation with each method according to predetermined criteria, the information on the bone surfaces was registered and processed. Data processing was carried out in 3 phases, first a sorting and counting of bone surfaces in different phases of activity, next a conversion of this information to code numbers, and finally a processing and writing out of these in tabular form.

Evaluation of the growth activity of a bone surface with any single method proved inadequate, whereas it was found that by combining methods, in particular by utilizing both decalcified and undecalcified sections, one could obtain an evaluation which was in close agreement with the direct evaluation of growth by means of tetracycline labelling for fluorescence microscopy.

The most effective method for identification of growth proved to be a combination of conventional histological technique and microradiography. In the identification of resting surfaces microradiography alone was sufficient.

RÉSUMÉ

COMPARAISON À L'ORDINATEUR DES MÉTHODES HISTOLOGIQUES DESTINÉES À L'ÉVALUATION DE LA CROISSANCE CRÂNIO-FACIALE.

Dans le but d'obtenir une meilleure évaluation histologique de l'activité de la croissance des os de la face et de la base du crâne, 6 méthodes histologiques différentes ont été comparées. Le matériel comprenait 40 lapins ayant recu des injections de tétracycline pour le marquage du tissu osseux. Après que les animaux aient été sacrifiés, des blocs de tissu osseux devant représenter différentes sortes de croissance suturale, de croissance par apposition et d'ossification endochondrale ont été prélevés.

Des coupes en paraffine de matériel décalcifié coloré par hématoxyline-éosine, PAS et trichromique de Masson ont été examinées. Des coupes non décalcifiées correspondantes ont été colorées au bleu de méthylène et examinées par microradiographie. Le marquage de ces coupes à la tétracycline a aussi été examiné par microfluoroscopie.

Sur la base de l'évaluation obtenue suivant des critères déterminés à l'avance avec chacune de ces méthodes, les informations sur les surfaces osseus ont été enregistrées et traitées. Le traitement des informations a été effectué en 3 phases, d'abord un triage et un comptage des surfaces osseuses dans leurs différentes phases d'activité, ensuite une conversion de ces in-

formations en nombres codes, et enfin le traitement et l'écriture de ceux-ci sous forme de tables.

L'évaluation de l'activité de la croissance d'une surface osseuse au moyen d'une seule méthode s'est révéleé inadéquate; par contre, en combinant plusieurs méthodes, et particulièrement en utilisant conjointement des coupes décalcifiées et des coupes non décalcifiées, on pouvait obtenir une évaluation concordant bien avec l'évaluation directe de la croissance par microfluoroscopie après marquage à la tétracycline.

La méthode la plus efficace pour l'identification de la croissance s'est révéleé être la combinaison d'une technique histologique classique et de la microradiographie. Pour l'identification des surfaces inactives, la microradiographie était suffisante à elle seule.

ZUSAMMENFASSUNG

HISTO-RADIOLOGISCHE UNTERSUCHUNG DES SUTURELLEN WACHSTUMSMECHANISMUS

Zwecks einer verbesserten histologischen Einschätzung von der Wachstumsaktivität in den Knochen des Gesichtsskelettes und der basis cranii wurden zum Vergleich 6 verschiedene Methoden herangezogen. Das Material bestand aus 40 Kaninchen, die mit Rücksicht auf die Knochenkennzeichnung mit Tetrazyklin injiziert worden waren. Nach dem Abtöten wurden Knochenblöcke herausgenommen zur Erhellung von verschiedenen Formen suturellen Wachstums sowie appositionellen Wachstums und enchondraler Ossifikation.

Es wurden Pariffinschnitte von entkalktem und mit Hämatoxylin-Eosin, PAS und Massons Trichrom-Methode gefärbtem Material untersucht. Entsprechende unentkalkte Schleifschnitte wurden mit Methylenblau gefärbt und mikroradiographisch sowie — mit Rücksicht auf die Tetrazyklinkennzeichnung — fluoreszenzmikroskopisch untersucht.

Auf Grund einer Beurteilung nach jeder einzelnen Methode nach im voraus festgelegten Kriterien wurden die Informationen über die Knochenflächen gebucht und datenverarbeitet. Die Datenverarbeitung wurde in 3 Stufen vorgenommen, zunächst eine Sortierung und Aufzählung von Knochenflächen in verschiedenen Aktivitätsphasen, danach eine Konvertierung der Informationen zu Kodenummern, und schliesslich eine Verarbeitung und Ausschreibung derselben in einer näher spezifizierten Tabellenform.

Es ergab sich, dass eine Beurteilung von der Wachstumsaktivität einer Knochenfläche mit je einer der erwähnten Methoden mangelhaft war, dass sich aber durch eine Kombination der Methoden, besonders bei gleichzeitiger

Anwendung entkalkter und unentkalkter Schnitte, eine Einschätzung erzielen liess, die eine treffende Übereinstimmung mit der unmittelbaren Wachstumsbeurteilung mittels der Tetrazyklin-Fluoreszenz (»Knochenkennzeichnung») erwies.

Am effektivsten zur Feststellung eines Wachstums war die Kombination von konventioneller histologischen Technik und Mikroradiographie, während die Mikroradiographie allein bei der Bestimmung der Ruheflächen massgebend war.

Die angewandten Methodenkombinationen sind als wohlgeeignet zu betrachten zur Beurteilung von der normalen Wachstumsaktivität der Schädelknochen bei laufender Untersuchung humanen Obduktionsmaterials.

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