Growth and Cellular Proliferation in the Early Rudiments of the Eye and the Lens

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Summary

1. The relation between growth, cellular proliferation, and morphogenetic movements was investigated in the case of lens formation in *Elephantulus myurus jamesoni* and *Xenopus laevis*.
2. For this purpose the volume of the eye cup and lens rudiments was estimated, counts of cells were made, and at the same time counts of cells in mitosis. The mitotic index was calculated, and the material wherever possible was treated statistically.
3. The lens rudiment grows at a greater rate than the eye cup rudiment during the stages in which the lens is being formed. The rate of cellular proliferation in the lens rudiment is also higher than in the eye cup rudiment. The size of the lens cells remains constant whilst the size of the eye cup cells diminishes during the period investigated (at least in *Xenopus*).
4. The mitotic index in the lens material is lower than in the eye cup material. This indicates that the duration of mitosis in relation to the interkinetic period is, in the eye cup rudiment, greater than in the lens rudiment.
5. The mitotic index in the lens material does not increase or decrease significantly during any stage of the lens development, nor were there found any other indications of an increased or decreased growth or proliferation of the lens material. It is therefore concluded that the formation of a visible lens rudiment is due to morphogenetic movement—contraction of a sheet of cells towards the centre of the future eye cup.

IT is well known that unequal growth is a major factor in establishing the proportions of the body in animals. It is not so clear what part unequal growth plays in the earlier phases of morphogenesis, that is, in stages when the organ rudiments are first formed and acquire their general shape. The growth of the embryo has been studied successfully in many animals, but not much has been made known concerning the growth processes in stages when the organ rudiments are being formed. In previous papers I have examined the growth and cellular proliferation in the earliest rudiments of the mammary glands (Balinsky, 1950*a*, 1950*b*). I have now found it desirable to investigate the same processes in organ rudiments of a different type. The organ selected was the lens of the eye throughout the period when it is being formed from cells of the embryonic epidermis. I have also studied the growth of the eye cup in the same stages for comparison.

Material and Methods

The investigation of the growth processes in early rudiments requires a special approach and a special technique. It is impossible to determine the

size of parts by weighing them after dissection as is done with organs of the older embryos and juvenile animals. The size must be estimated by some method of reconstruction from serial sections of the embryos. As an additional method of estimating growth, cell counts in serial sections may be used, and also the estimation of the mitotic index (percentage of cells in mitosis in relation to total number of cells) which can reasonably be supposed to be correlated with the rate of proliferation in a given tissue. As all these methods are very laborious, only a small number of embryos could be treated in this way.

Accordingly, two series of embryos were made available for this work. The first series comprises five embryos of the elephant shrew, *Elephantulus myurus jamesoni*, selected from the embryological collection of Prof. C. J. van der Horst at the Zoological Department of the Witwatersrand University. The uteri with embryos were preserved in Bouin and cut in serial sections. In the youngest embryo (25 pairs of somites) there is no trace of the lens thickening, in the oldest embryo (40 pairs of somites) the lens is rounded and completely separated from the epidermis, and its proximal wall is being thickened, owing to the beginning of the differentiation of the fibres. There was no means of estimating the actual age of the embryos and the time which is required for the embryo to pass from the 25-somite stage to the 40-somite stage.

The second series was that of embryos of the clawed toad, *Xenopus laevis*, specially preserved for the purpose of this work. A number of embryos were kept at room temperature, and groups of embryos were preserved over a period of 24 hours. The temperature range was 19° to 25° C. The series (19 embryos) covers the period from stage 15— to stage 19+ (stages after Weisz, 1945). The embryos were preserved in Bouin, stained in block with borax carmine, and embedded for sectioning in celloidin-paraffin wax.

The *Xenopus* series was later supplemented by a small additional one to cover a gap in the original series where the groups of embryos seemed to have been taken too far apart. The embryos of the additional series (9 embryos), unfortunately, are not in all respects comparable with the main series, as the eggs of this batch turned out to be larger, and correspondingly the eyes and lenses were larger than in comparable stages of the first series.

The morphological changes taking place in the eye rudiment during the stages covered by my investigation are shown in a series of drawings representing typical transverse sections through the eye and lens rudiments of *Xenopus* (fig. 1).

In stages 15— to 17 there is no thickening of the epidermis to form the lens, and the outer surface of the eye vesicle is uniformly convex. The epidermis consists of two distinct layers: the outer or covering layer, and the inner or 'sensory' layer. Only the latter is concerned in lens development.

In stage 17+ the outer surface of the eye vesicle begins to be invaginated, starting with the upper rim of the future eye cup (fig. 1, D). At the same spot the sensory layer of the epidermis becomes thickened whilst the other parts of the future lens rudiment do not seem to be affected.

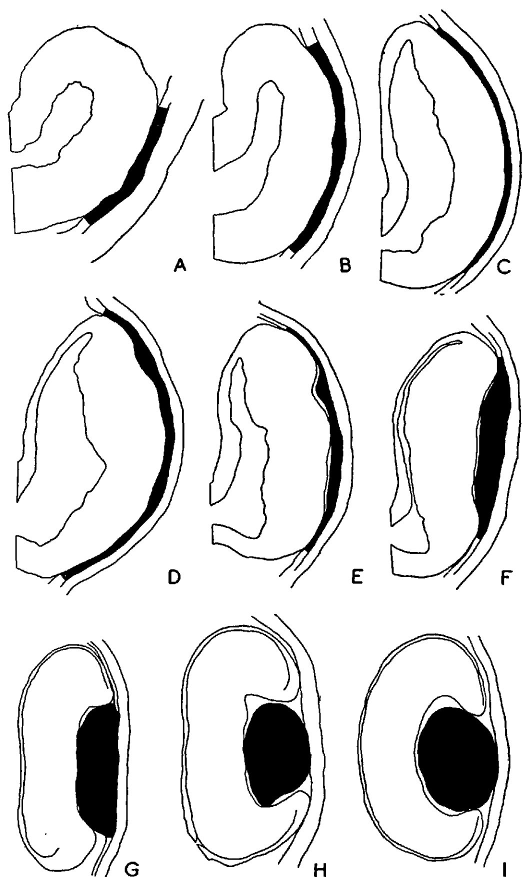

FIG. 1. Typical transverse sections through the eye rudiment in *Xenopus*. A, stage 15−. B, stage 16−. C, stage 17. D, stage 17+. E, stage 17++. F, stage 18−. G, stage 18. H, stage 19−. I, stage 19+.

In stages 17++ and 18— the invagination of the eye cup progresses further (fig. 1, E, F) and at the same time the portion of epidermis forming the lens becomes distinctly thickened throughout its whole area.

In stage 18 I found the lens already a solid mass of cells lying in the opening of the eye cup, but still not separated from the epidermis (fig. 1, G).

In stages 19— and 19+ the lens is completely separated from the epidermis and the fibre differentiation has started.

The above is a description of the development as it could be reconstructed from the more typical specimens. Although the embryos selected for preservation in each group seemed to be in the same stage of development as regards the external features, I found later that the degree of development of the eye and lens in embryos of the same stage varied considerably. These variations caused some overlapping between the groups of embryos belonging to different stages in so far as the eye and lens development were concerned. So as not to bias the results of the investigation, I did not discard the aberrant specimens. Fortunately, however, I found that there are three distinct natural periods of development which could be clearly discerned, and between which there is no overlapping. The periods are:

From stage 15— to stage 17.
From stage 17+ to stage 18.
From stage 19— to stage 19+.

I will have occasion to make use of these three periods in my further analysis of the data.

I estimated the volumes of the rudiments (eye and lens) as in my previous investigations (Balinsky, loc. cit.) by drawing sections of the rudiment with a camera lucida, and then measuring the surface of the drawings with a planimeter. In some cases all the sections were drawn and measured (lens in *Xenopus*). In other cases only every second or every third section was drawn, and the total volume was estimated by 'graphical reconstruction' (Dornfeld, Slater, and Scheffe, 1942). In all cases the volume was estimated in arbitrary units. It would have been possible to calculate the volumes in absolute units from these data, but I did not find this necessary as only the relative increases were of interest in this investigation. Before the formation of the lens thickening, that part of the sensory layer of the epidermis (in *Xenopus*) or of the whole epidermis (in *Elephantulus*) with which the outer surface of the eye vesicle is in immediate contact, was measured as the equivalent of the later lens rudiment.

In the case of the lens rudiment all cells were counted in every section and the number of cells in mitosis was noted separately. As 'mitoses' were counted cells beginning with the metaphase (nuclear membrane dissolved), and ending with the early telophase (contour of the nucleus still irregular). It is inevitable that some cells should be counted twice if they are halved between two consecutive sections. The total number of cells counted must therefore be in

excess of the actual number of cells present in the rudiment. A uniform procedure of counting was adopted in every case, however, and the relative increases in the number of cells should therefore not be appreciably distorted. The same applies to the estimation of the mitotic index, as mitotic cells would also sometimes be counted twice in two consecutive sections.

By dividing the volume of the lens rudiment by the number of cells in the same lens I calculated an 'index of cell size'. Although the index is presumably proportional to the average size of the cell in the rudiment, the cell volume cannot be directly calculated in absolute units for both reasons mentioned above: (1) because the volume was determined in arbitrary units, and (2) because the cell counts give an exaggerated estimate of the number of cells present. In the case of the eye vesicle and eye cup the cells were counted only in selected representative sections. These counts were used for estimation of the mitotic index. Special counts of cells in selected sections have been made, to correlate cell number with volume of the eye rudiment. (This part of the work was performed by Miss V. Gubbay.) The sections were drawn as before with a camera lucida, and the number of cells counted in the section was divided by the surface of the section. This gave the number of cells per arbitrary unit of volume.

In *Elephantulus* I used both eyes of each embryo for measurements and counts. In *Xenopus* embryos I used only one eye in each (usually the left). The mitotic index was calculated for each rudiment separately, and I also calculated average mitotic indexes for groups of embryos by pooling the data for all rudiments investigated.

The cell and mitosis counts were treated statistically for the purpose of testing (1) whether counts made on the same tissue in different embryos might be considered as representing one homogeneous population of cells, and (2) whether there are significant differences between data obtained for different tissues or for the same tissues in embryos of different stages of development. The tests applied were: (1) the χ^2 test of homogeneity (or test for independence) (Snedecor, 1946, pp. 188 ff.); (2) analysis of variance, with angular transformation of percentages, and partial weighting (after Cochran, 1943); (3) in comparing the mitotic index in the eye cup and lens of *Elephantulus* I made use of a method suggested to me by Dr. A. Robertson and employed by myself in a previous investigation (Balinsky, 1950*a*), that is, the treatment of weighted differences between pairs of counts made in one and the same embryo.

The Data

(*For Tables 1–3 see pp.* 362, 363, 364).

Analysis of the Data

The first conclusion which can be reached by studying the data presented in this work is that the lens-forming material has a higher rate of growth

TABLE 1. *Volume Measurements (in arbitrary units) and Cell Counts in* Elephantulus

Stage	*Volume, eye*	*Increase by factor*	*Volume, lens*	*Increase by factor*	*Cell counts, eye, mitoses in brackets*	*Eye, mitotic index*	*Cell counts, lens, mitoses in brackets*	*Lens, mitotic index*	*Lens, cell size index*
25 somites	1,020		90		1,357 (41)	3·02	936 (22)	2·35	0·096
	999		57		1,249 (57)	4·56	774 (19)	2·45	0·074
		1·55		2·06					
27 somites	1,485		54		1,474 (63)	4·27	468 (9)	1·92	0·115
	1,647		249		1,455 (48)	3·30	1,901 (38)	2·00	0·131
		1·23		2·57					
30 somites	1,953		372		1,270 (36)	2·83	3,294 (81)	2·46	0·113
	1,890		408		1,531 (23)	1·50	4,335 (98)	2·26	0·094
		1·95		1·90					
34 somites	3,930		696		1,247 (19)	1·52	6,077 (104)	1·71	0·115
	3,576		783		1,188 (18)	1·52	6,595 (103)	1·56	0·119
		1·00		1·18					
40 somites	3,792		885		1,821 (48)	2·64	7,700 (129)	1·68	0·115
	3,726		867		1,754 (32)	1·82	7,551 (115)	1·52	0·115
Average						2·68		1·81	

Interaction χ^2 for probability of mitosis = 13·33. d.f. = 9. *P* nearly 0·10.

TABLE 2. *Volume Measurements,* Xenopus (*basic series*)

Stage and time from beginning of experiment	*Eye*			*Lens*		
	Individuals	*Average for stage*	*Increase by factor*	*Individuals*	*Average for stage*	*Increase by factor*
15– 0 hours	4,832 3,264 3,760	3,952		299 80 153	177	
16– 2 hours	4,576 4,224 3,568	4,123	1·04	146 265 155	189	1·07
17 4 hours	5,136 4,016	4,576	1·11	352 139	245	1·30
18– 9 hours	4,992 5,616	5,304	1·16	535 412	473	1·93
18 14 hours	5,280 4,016 5,616	4,971	no increase	573 344 427	448	no increase
19– 19 hours	6,064 6,128 5,536	5,909	1·19	633 767 524	641	1·43
19+ 24 hours	6,784 6,688 7,648	7,040	1·19	780 861 932	858	1·34

It will be noted that there is no increase either in the size of the lens or of the eye from stage 18— to stage 18, there is even a slight decrease. This is obviously due to the variability in the size and degree of development of the embryos (overlapping of the adjacent stages).

(volume increase) than the material of the eye vesicle and eye cup. This is true in both *Elephantulus* and *Xenopus.*

The total increase is by a factor of:

	Eye	*Lens*
Xenopus . .	1·78	4·85
Elephantulus .	3·72	11·92

The increase of the lens volume is consistently higher than the increase of the eye when adjacent stages are compared, though such a comparison is in itself unreliable owing to variability of the specimens. It might have been suggested that the relative increase of the lens is not due to an intrinsically higher rate of growth, but to an aggregation of cells which are being added to the area measured from without (from the surrounding parts of the epidermis). This cannot apply to the later stages of lens development, after the lens rudiment has been segregated from the remainder of the epidermis. The lens, however, continues to grow faster than the eye cup even after this has happened (compare stages 19— and 19+ in *Xenopus*).

TABLE 3. *Cell Counts,* Xenopus *(basic and supplementary series)*

		Eye		*Lens*		
Periods	*Stage*	*Cell counts, mitoses in brackets*	*Mitotic index*	*Cell counts, mitoses in brackets*	*Mitotic index*	*Index of cell size*
First period	15−	649 (41)	6·32	143 (5)	3·50	2·09
		352 (19)	5·40	51 (0)	0·00	1·57
		571 (19)	3·33	94 (2)	2·13	1·63
	16−	593 (39)	6·58	108 (0)	0·00	1·35
		688 (2)	0·29	169 (0)	0·00	1·57
		452 (31)	6·86	79 (0)	0·00	1·96
	17	623 (49)	7·67	236 (1)	0·42	1·49
		467 (27)	5·78	135 (3)	2·22	1·03
		855 (64)	7·49	306 (7)	2·29	1·02
		1,043 (55)	5·27	343 (5)	1·46	1·54
Average for period			5·50		1·38	
Second period	17+	1,005 (73)	7·26	355 (4)	1·13	1·84
		936 (67)	7·16	419 (9)	2·15	1·61
	17++	1,064 (46)	4·32	410 (11)	2·68	1·54
		937 (48)	5·12	321 (4)	1·25	1·27
	18−	820 (34)	4·15	409 (9)	2·20	1·70
		860 (44)	5·12	334 (2)	0·60	1·62
		1,019 (61)	5·99	344 (9)	2·62	1·39
		669 (19)	2·84	237 (5)	2·11	2·26
		815 (40)	4·91	208 (3)	1·44	1·98
	18	856 (26)	3·04	338 (11)	3·25	1·70
		850 (57)	6·71	212 (4)	1·87	1·62
		971 (33)	3·40	330 (0)	0·00	1·29
Average for period			5·07		1·81	
Third period	19−	1,225 (48)	3·92	389 (11)	2·83	1·63
		1,080 (27)	2·50	428 (9)	2·10	1·79
		1,038 (38)	3·66	373 (5)	1·34	1·40
	19+	832 (14)	1·68	570 (7)	1·23	1·37
		1,041 (32)	3·07	665 (8)	1·20	1·29
		953 (23)	2·41	627 (7)	1·12	1·49
Average for period			2·95		1·54	

Interaction χ^2 for the whole material . . 40·91. d.f. 27. *P* nearly 0·05.
for the first period . . . 14·93. d.f. 9. *P* nearly 0·10.
for the second period . . 19·24. d.f. 11. *P* nearly 0·05.
for the third period . . . 6.85. d.f. 5. *P* between 0·30 and 0·20.

When analysing the data of the mitotic counts we are confronted with a very considerable variability in these counts. The test of homogeneity in the case of *Xenopus* lens cells (Table 3) shows that there is a significant interaction in the probability of mitosis. It is likely, therefore, that the samples are not drawn from a homogeneous population. The variability, however, may come from at least three different sources:

(1) Sampling errors due to the finite numbers of cells counted. In the case of frequencies this sampling error has the form of binomial variability.

(2) The fluctuations in true mitotic frequency due to differences between the embryos in one and the same phase of development.
(3) The variability due to the stage of development of the embryos.

The latter two sources of variability cause 'extraneous variation' in terms used by Cochran (1943). Extraneous variation is not dependent on the numbers of cells counted. For the purposes of this investigation it was necessary to differentiate between the three sources of variability. Cochran (loc. cit.) gives a method for evaluating the proportions of binomial and extraneous variation in the total variation. Applying his method I found that binomial variation constitutes the following percentage of total variation:

If equal weights are used	68 per cent.
If weights proportional to n are used . .	72 per cent.

Analysis of variance may be further applied for differentiating between individual variation and group variation. In the case where about 70 per cent. of variation is binomial and nearly 30 per cent. is extraneous, and where the percentages studied are based on unequal numbers of counts, Cochran (loc. cit.) recommends performing the analysis of variance with partial weighting, i.e. fixing an upper limit for weights, applicable to two-thirds of the total number of samples. The results are shown below.

Analysis of Variance of Mitotic Frequencies in the Lens of Xenopus *in the three Periods of Development*

	d.f.	*Sum of squares*	*Mean square*
Individuals within periods . .	25	46,516	1860·64
Period means	2	7,181	3590·50
Total	27	53,697	

Variance ratio $(F) = 1{\cdot}9297$. For $n_1 = 2$ and $n_2 = 25$, $F_{.05} = 3{\cdot}38$, $P > 0{\cdot}05$.

In the above analysis the variation of the individual embryos contains both sampling variation and 'extraneous variation'. It is obvious that there is no significant difference of mitotic frequency in the lens material throughout the three periods of development. I also performed the analysis of variance for the nine stages used with essentially the same result.

The results of the homogeneity test applied to mitosis counts in *Elephantulus* are presented in Table 1. The interaction in this case falls short of the 5 per cent. probability limit. Therefore the assumption that all counts represent one population need not be rejected. These results are thus in agreement with the conclusion drawn from the data on *Xenopus*. This is an important conclusion because it allows us to make the inference that the formation of the lens rudiment is not connected with significant modifications of the mitotic activity in the lens material.

In the case of *Xenopus* the mitotic frequency in the eye cup material remains without significant change throughout periods 1 and 2 (i.e. whilst the eye vesicle is being transformed into an eye cup), but there is a sharp decline in the frequency of mitoses in period 3. The difference between periods 2 and 3 may be shown to be fully significant. The figures in *Elephantulus* also show a decrease in the percentage of mitoses in the older embryos.

Between the percentages of mitoses in the lens material and the eye cup material there is, in *Xenopus*, a very significant difference, the figures for the eye cup being very much higher than those for the lens. When comparing the mitotic index for the eye cup and the lens in *Elephantulus*, one finds that the preponderance for the eye cup is by no means so clear. The average mitotic index of the eye cup is higher than in the lens (2·68 and 1·81 respectively) but in some counts the relations are reversed. Analysing the difference between the mitotic index of the eye cup and the lens by the Robertson method (p. 361) I found that the mean weighted difference is 0·4731, standard error of the mean is 0·2853, $t = 1{\cdot}65$, with 9 degrees of freedom P_t lies between 0·2 and 0·1. The existence of a difference between the mitotic frequency of the eye cup and the lens is not proved. The ratio between the mitotic frequency of the eye cup and the lens is, however, not stable, as is shown by the presence of a highly significant interaction: P for interaction $\chi^2 = 0{\cdot}01$. This must be mainly due to the variability of the mitotic frequency in the eye cup, since the counts for the lens have not shown a significant departure from homogeneity.

Some of the data presented in this paper can be used to analyse the relationship between the proliferation of cells (increase of cell numbers) and the growth (increase of volume) of tissues. Both should be equal if the volume of each cell is doubled between two successive cell divisions. This, however, need not necessarily be the case. Tables 1 and 3 show the values of the 'index of cell size' for lens cells in *Elephantulus* and *Xenopus*. The figures show that the lens cells do not appreciably change in size as development proceeds. To test the significance of the figures, I have applied the analysis of variance to the data for *Xenopus*. I grouped the data according to stage of development from 15— to 19+, and made the assumption that all subsamples represent one population.

Analysis of variance of the 'index of cell size' in Xenopus

	d.f.	*Sum of squares*	*Mean square*
Individuals within stages	19	1·30	0·068
Stage means	8	0·93	0·116
Total	27	2·23	

Variance ratio (F) = 1·70. For $n_1 = 8$ and $n_2 = 19$, $F_{.05} = 2{\cdot}48$, $P > 0{\cdot}05$.

The variance ratio falls short of the 0·05 level. This justifies the assumption that the cell volume of the lens material does not change significantly during the period investigated.

In the case of the eye cup the results are quite different. An estimate of the cell size made by the method described on p. 361 showed that the average cell size in stage 19+ is only 0·55 of the cell size in stage 15—. The proliferation of cells proceeds with a higher rate than the rate of volume increase. Between stage 15— and stage 19+ the total volume increases 1·78 times, but the increase in cell numbers, calculated from the above data, is by a factor of 3·26.

I can now discuss the actual significance of the mitotic index as an indicator of cell proliferation in tissues. In the *Xenopus* series the embryos were preserved at known time-intervals, and the actual increase of the cell number during these time intervals has been estimated. It is therefore possible to calculate what actual rates of increase of cell numbers correspond to certain values of the mitotic index. I have calculated how often, on the average, each cell should divide between stage 15— and stage 19+ to account for the increase in the number of cells in the lens rudiment and the eye cup rudiment. The formula used for this calculation is the following:

$$\frac{N_2}{N_1} = 2^{(t_1-t_2)/T} \quad \text{(Woodard 1948)}$$

where N_1 and N_2 are the numbers of cells at the beginning and at the end of the period, t_1 and t_2 is the corresponding time, and T is the average interval between two consecutive cell divisions. The actual time interval (t_2-t_1), it will be remembered, was 24 hours.

The average interval between two cell divisions calculated from the above formula is:

In the lens material . . . 8 hours and 54 minutes.
In the eye cup material . . . 14 hours and 5 minutes.

These data should be compared with the average mitotic index, which is over the whole period 1·63 for the lens and 4·62 for the eye cup. It is obvious that the higher mitotic index for the eye cup does not indicate a higher rate of cellular proliferation. The reverse is true of the lens material. This can only mean that the duration of the actual mitosis in relation to the interkinetic period (interphase) is, in the eye cup cells, greater than in the lens cells.

It has been suggested by Woodard (1948) that the mitotic index is not a reliable indication of the rate of proliferation in tissues. Woodard found that in the neural tube of the 18–72-hour chick embryo the rate of increase of cell numbers remains constant whilst the mitotic index decreases. My data do not directly confirm Woodard's results, as the discrepancies between the mitotic index and the rate of proliferation have been found in comparing different tissues, and not the same tissue in successive stages of development. It is conceivable that in different tissues, having a different mechanism of growth, the relation between mitosis and interphase need not always be the same, and this of course would greatly change the actual value of the mitotic index as an indicator of cell proliferation. On the other hand, in parts of the same tissue, or in tissues having the same type of differentiation and the same

mechanism of growth, the duration of mitosis may remain unchanged, and in this case the mitotic index would still be valid as an indicator of proliferation. It is conceivable, however, that the mechanism of growth may change with incipient differentiation, and perhaps change quickly. It seems obvious that there is still much to be learned about the relation between the mitotic rate and growth, and further investigations in this field are very desirable.

In recent investigations P. Weiss and McKeehan (Weiss, 1950; McKeehan, 1951), found that the formation of the lens rudiment is due to change in shape of the cells of the future lens which orient themselves perpendicularly to the surface of the eye cup rudiment, and become columnar instead of flat. This conclusion is supported by my own observations in so far as (1) I could not discover any trace of an increased proliferation of cells during the period when the lens rudiment is being formed; (2) I could not discover any increase in the volume of cells during the period when, according to Weiss, they become columnar. I have also found no reason to suspect that additional cellular material is attracted to the site of lens formation: any increase in the size of the lens rudiment can be accounted for by the rate of growth intrinsic to the cells originally situated between the eye vesicle and the outer layer of the epidermis. The actual formation of the lens is carried out by means of a concentration of its cellular material, i.e. by a morphogenetic movement in the sense of W. Vogt: the diameter of the lens when it is formed is considerably smaller than the diameter of the sheet of cells which are used for its development (see fig. 1, especially the drawings of stages 18—, 18, 19—).

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