Zoology. — *The influence of concentration, duration of treatment and stage of development in the lithium-effect upon the development of Limnaea stagnalis.* By CHR. P. RAVEN, J. C. KLOEK *), E. J. KUIPER and D. J. DE JONG. (From the Zoological Laboratory, University of Utrecht).

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In 1942, one of the authors discovered the remarkable effects of a treatment with LiCl upon the eggs of *Limnaea stagnalis* (RAVEN, 1942). These effects are of two kinds. First, the embryos may develop into large, thin-walled vesicles, resembling the "exogastrulae" of sea urchins. As a matter of fact, the study of these structures in sections has shown that their wall consists partly of ectodermal, partly of entodermal cells; hence, we are justified in considering them as exogastrulae. In other instances, development proceeds further, but a graded series of malformations is obtained; they affect especially the head region. Dorsal displacement of the eyes, leading to synophthalmic or cyclopic embryos, splitting of one of the eyes, reduction of one or both eyes and tentacles are common malformations in these series; in extreme cases, the head is nearly entirely absent. In all these instances, merely the head region is affected; the foot and trunk are normal or only slightly disturbed. Only in a small percentage of cases, highly abnormal, teratomorphic forms appear, with serious disturbance of the relation between different parts of the body. For the sake of brevity, we shall further refer to all these cases as "head malformations".

As to the physiological aspects of the Li-action, a great variability was noted. The effects of the treatment depended on the concentration of lithium, the length of exposure time and the stage of development at which the eggs were exposed. However, the combination of these factors did not suffice to explain the diversity of the results. In some instances, where portions of different egg masses at the same stage of development were exposed simultaneously to the same solution during the same length of time, some of them showed a normal development, while in others all eggs became exogastrulae. It was clear, therefore, that individual differences in susceptibility of the egg masses played a part. Moreover, it was suspected that the procedure of the experiments, in which whole egg masses or portions of them were exposed to the solutions, might be responsible in part for the variability of the results.

Therefore, the experiments were continued in 1943 by J. C. KLOEK,

*) J. C. KLOEK died 16.IV.1947. This paper is dedicated to his memory by the other authors.

with the purpose to work out a method allowing to improve the reproducibility of the results. Although KLOEK made a great number of experiments, he did not succeed in this; the individual differences in susceptibility are, evidently, so great as to be subversive of all regulations of treatment. It became clear, therefore, that only by means of a great number of experiments, the results of which could be treated statistically, the regularities underlying the diversity of the Li-effects could be disclosed.

The experiments of KLOEK gave a key to one of these regularities which had not been detected before. They indicated that the results of the Li-treatment do not only depend on the stage at which the eggs are transferred to the Li-solution, but still more so on the stage at which the treatment is ended and the eggs are returned to tap water; with respect to this, a regular alternation of periods of increased and lessened susceptibility seemed to exist. In order to check this presumption, a number of experiments were undertaken in 1945 by F. J. KUIPER. At the same time, D. J. DE JONG examined the effects of Li-treatment in advanced stages of cleavage which had not been investigated before.

Taken together, the experiments of the 4 authors give a sufficient basis admitting a statistical treatment of the results, in order to bring to light the regularities of the Li-effect.

Material and methods.

4 Concentrations of LiCl have been used: 0.01 %, 0.005 %, 0.002 % and 0.001 % (= 0.00236 M, 0.00118 M, 0.00047 M and 0.00024 M.) The solutions were made from a stock solution of 1 % LiCl in distilled water by diluting with tap water.

Together, 437 experimental series, including a total of 9482 eggs, have been performed. Table I gives their distribution over the diverse concentrations.

TABLE I.

Concentration of LiCl-solution	Number of series	Number of eggs.
0.01 %	74	2258
0.005 %	173	3592
0.002 %	8	287
0.001 %	182	3345
	437	9482

With 0.002 % LiCl, only a small number of experiments have been made; they will not be discussed below. With the other concentrations, the number of experiments is sufficient to permit a statistical treatment.

In the first experiments, whole egg masses or portions of them were exposed to the solutions. To avoid the complications caused by the external jelly, in later experiments the latter was removed and the isolated egg

capsules were transferred to the solution. However, here a new difficulty arose: in control egg capsules, cultured in tap water, the development of the eggs is seldom entirely normal; often they show considerable abnormalities and die at the trochophore or "veliger" stage. Evidently, tap water is an inappropriate culture medium for single egg capsules. Therefore, KLOEK made a number of experiments in order to find a suitable medium.

First, the influence of the standard salt solutions according to RINGER, HOLTFRETER or LEHMANN (1937) in various dilutions was tested. They gave no improvement, as compared with tap water: in single egg capsules exposed to the solutions, the development of the eggs was often very abnormal, seldom entirely normal. The best results were obtained with LEHMANN solution diluted 4 to 32 times; HOLTFRETER solution proved to be very unsuitable.

A remarkable result was obtained, when single egg capsules were put into distilled water: the embryos developed nearly normally till a stage with pigmented eyes, then suddenly they died.

In order to test a possible protective effect of the jelly, single egg capsules were put into tap water, to which mucous substance of the tunica interna of the egg mass was added. However, this proved to have a very detrimental effect: the eggs died within 4 days. It is possible that the mucus promoted bacterial growth which was injurious to the eggs.

PH-measurements with a glass electrode (executed in coöperation with the Laboratory of Comparative Physiology of the Utrecht University [1]) showed that the pH in one day old egg masses in tap water lies between 5.49 and 5.62. The measurements were carried out in the following way: the egg mass was cut open lengthwise, then the glass electrode was put on the cut surface of the jelly and read off immediately. Since the pH of Utrecht tap water and of the above-mentioned salt solutions is much higher (7.14—8.84), that of old distilled water still slightly higher (5.88—6.09) than that of the jelly, it is possible that this accounts for the deficient development of the eggs in isolated egg capsules in these media.

These considerations led to the attempt to adjust the pH of the environment by means of a substrate of agar-agar. The pH of a layer of 2 % agar-agar, as determined with the glass electrode, was 5.65; hence, it agrees quite well with that of the jelly. Egg capsules in tap water or distilled water on a bottom of agar-agar showed a highly abnormal development, however. At last the following culture method proved to give satisfactory results: The egg capsules were placed on a bottom of agar-agar in a Petri dish, without water; this dish was closed by a cover and put in a larger one, containing water, to prevent drying up. By this method, the eggs develop normally till hatching in a large percentage of cases.

This method was employed in the later experiments. In general, an experimental series took the following course: The egg capsules of an egg

[1] It is a pleasure to thank Dr. H. J. VONK for his assistance in these determinations.

mass were freed from the common jelly. A number of these capsules served as controls; they were put immediately into a tube with tap water. The remaining capsules were, after determination of the stage of development of the eggs, distributed over some tubes with LiCl solutions. After a definite exposure time, these solutions were replaced by tap water; the stage of development was noted again. The tap water was renewed frequently. 24 Hours after the end of the Li-treatment, the experimental eggs and the controls were put on the agar-bottom in a Petri dish, where they continued their development. The eggs were arranged in rows on the agar and could be followed in their development individually by placing the Petri dish under a binocular microscope.

Stages of development.

In order to indicate exactly the stage of development of the eggs at the beginning and the end of the Li-treatment, a series of normal stages, covering the first part of the cleavage period, was composed. The stages 1—19 have been described and pictured by RAVEN (1946). In short, they are the following:

Stage 1:	First cleavage.	Beginning of cleavage furrow at animal pole.
2.	,, ,,	Beginning of cl. f. at vegetative pole.
3:	,, ,,	Cl. furrow cut half way through egg.
4:	,, ,,	Cl. f. cut entirely through egg. Cells rounded.
5:	2-cell stage.	Beginning flattening of blastomeres.
6:	,, ,,	Further flattening of blastomeres.
7:	,, ,,	Flattening of blastomeres completed.
8:	,, ,,	Lenticular cleavage cavity.
9:	,, ,,	Wide cleavage cavity.
10:	Second cleavage.	Beginning of cleavage furrow.
11:	,, ,,	Cells rounded.
12:	4-cell stage.	Beginning flattening of blastomeres.
13:	,, ,,	Beginning of cleavage cavity.
14: }		Wide central cleavage cavity with periodic
15: }	,, ,,	expulsion of fluid.
16:	Third cleavage.	Formation of 1st micromeres.
17:	,, ,,	Cells rounded.
18:	8-cell stage.	Flattening of blastomeres.
19:	,, ,,	Wide cleavage cavity.

For the purpose of the present investigation, the following stages have been added:

Stage 20:	Formation of 2d micromeres; 12 cells.
21:	Division of 1st micromeres; 16 cells.
22:	16-cell stage. Flattening of cells.
23:	,, ,, Wide cleavage cavity.
24:	Formation of 3d micromeres; 20 cells.
25:	Division of 2d micromeres; 24 cells.

In many instances, the regularities come out more distinctly in combining these stages into groups; then, stages 1—9 are taken together as 2-cell stage; stages 10—15 as 4-cell stage; stages 16—19 as 8-cell stage, the following stages are indicated as "morula".

Results.

1. *Exogastrulation.*

On an average, in the experimental series treated with 0.01 % LiCl 41 % of the eggs developed into exogastrulae; with 0.005 % LiCl, only 16 %, with 0.001 % LiCl 35 % of exogastrulae developed.

With 0.01 % LiCl, an exposure time of 1 hour suffices to give the maximum percentage of exogastrulae. With 0.005 % LiCl, a treatment of 1 hour gives a low percentage of exogastrulae; the number of exogastrulae rises with increasing length of exposure time; a maximum is reached at 7 hours of treatment. When the duration of the treatment exceeds 12 hours, the percentage of exogastrulae drops; at the same time, the number of embryos showing unspecific abnormalities or early death increases. With 0.001 % LiCl, as a rule no exogastrulae develop when the exposure time is less than 6 hours; the maximum is reached with 24 hours of treatment.

As to the influence of the stage of development, this is most evident in the series with 0.01 % LiCl. Fig. 1 summarizes the relation between stages of a. beginning b. ending of treatment and percentage of exogastrulae. Both graphs show a distinct maximum with a value of nearly 100 % exogastrulae;

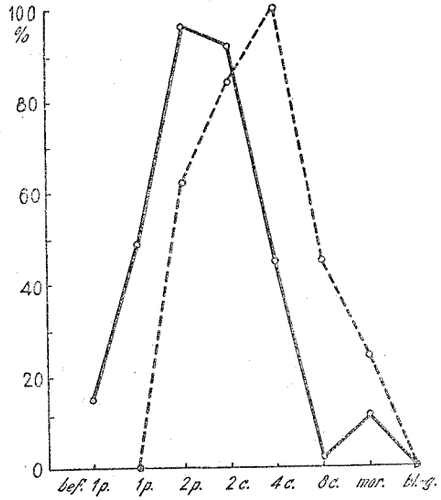

Fig. 1. *Limnaea stagnalis.* 0.01 % LiCl. Percentage of exogastrulae (ordinates) in dependence of stage of development (abscissae) at which treatment is begun (full line) and ended (broken line). *bef. 1 p* = before formation of 1st polar body; *1 p, 2 p* = 1 or 2 polar bodies present; *2c, 4c* etc. = 2-cell, 4-cell stage etc.; *mor* = morula; *bl—g* = blastula and gastrula stages.

for the beginning of treatment it lies at the stages: 2 polar bodies to 2-cell stage; for the ending of treatment there is an even sharper maximum (100 % exogastrulation) at the 4-cell stage.

These graphs point to the presence of a distinct maximum of sensibility for the production of exogastrulae at the 2- to 4-cell stage. Additional evidence for this is furnished by the other series which permit at the same time to delimit more sharply this period of sensibility.

Both in 0.005 % and 0.001 % LiCl, no exogastrulae developed when the Li-treatment was ended in stages 1—6; in 0.001 % LiCl, no exogastrulae developed when treatment was begun after stage 22; in 0.005 % LiCl, with suboptimal exposure times (1—2½ hours) no exogastrulae developed when the eggs were exposed after stage 12—13. We may conclude from these figures that there is a maximum of sensibility for the production of exogastrulae between stages 7—12 (i.e. shortly before and during the second cleavage) and a decreased sensibility until stage 22 (16 cell stage) or, making allowance for the fact that the effective concentration of Li in the eggs will be reached in 0.001 % LiCl only after at least 6 hours, somewhat later; after this stage, in no case exogastrulae have been produced.

2. *Head malformations.*

Head malformations occur less frequently than exogastrulae. On an average, the series with 0.001 % LiCl yielded 7 % of head malformations, those with 0.005 % and 0.01 % LiCl only 4 %.

With 0.01 % LiCl, a treatment of 1 hour produced the maximum percentage of head malformations; longer exposure times gave lower values. With 0.005 %, a treatment of 1 hour suffices to give some head malformations; the percentage rises till an exposure time of 6 hours, then drops; with exposure times exceeding 8 hours no head malformations were produced. With 0.001 %, a treatment during at least 5 hours is needed to produce head malformations.

As is also the case with the exogastrulae, the influence of the stage of development is most evident in the 0.01 % LiCl series. Fig. 2 shows that

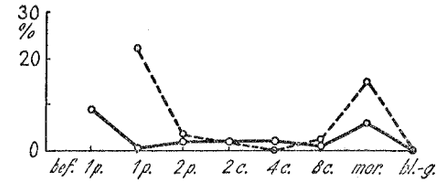

Fig. 2. *Limnaea stagnalis.* 0.01 % LiCl. Percentage of head malformations in dependence of stage of development at which treatment is begun and ended. Explanation cf. fig. 1.

there are 2 periods of maximum sensibility: 1⁰. immediately after laying (beginning of treatment before 1st polar body, ending between 1st and 2d polar body formation). 2⁰. at late cleavage stages (beginning and ending

of treatment at "morula" stage, i.e. after the 8-cell stage). Between the maxima, there is a period of low sensibility, in which nearly no head malformations are produced.

In the series with 0.005 % and 0.001 % LiCl, the first of these maxima does not appear. Evidently, when the eggs are put into these solutions immediately after laying, the effective concentration of Li within the eggs is not reached before the first period of sensibility has already ended. On the other hand, the second period of sensibility also in these series is clearly visible. Fig. 3 shows this for 0.001 % LiCl with regard to the stage of beginning of treatment. Up to the 2-cell stage, head malformations are extremely rare; at the 8-cell stage, there is a distinct rise, which continues in the next stages and leads to a maximum at stages 23—25, with 42 % of head malformations; then the line drops to zero at still older stages. No head malformations have been obtained as yet when the eggs were put into LiCl-solutions after stage 25. On the other hand, when the treatment was ended at stages 1—7, also no head malformations developed. Making allowance for the time needed to reach the effective concentration of Li in the eggs (5 hours in 0.001 % LiCl), we may conclude that there is a first period of sensibility immediately after laying, which is of short duration; it is followed by a period of minimum sensibility. From stage 7 on sensibility rises again slowly to a maximum lying at least 5 hours after stages 23—25, then there is a rather sudden drop.

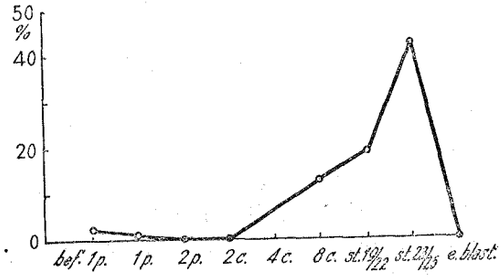

Fig. 3. *Limnaea stagnalis*. 0.001 % LiCl. Percentage of head malformations in dependence of stage of beginning of treatment. *St. 19/22* = stage 19—22; *e. blast* = early blastula. Further explanation cf. fig. 1.

Periodicity.

As has been mentioned above, the results of KLOEK's experiments pointed to a regular alternation of periods of increased and lessened susceptibility of the eggs. In his series with 0.005 % LiCl, head malformations occurred when the eggs had been taken from the LiCl solution and transferred to tap water at stages 7—10, 14—15 or 19, whereas no head malformations developed when the treatment was ended in stages 1—6, 11—13 and 16—18. Since the stages 7—10, 14—15 and 19 represent comparable phases

of the 2-, 4- and 8-cell stage, respectively, namely, the stages with wide cleavage cavity preceding the next divisions, it was suspected that this regularity had a real significance. As the number of cases was rather small (69 series including 771 eggs, yielding 42 head malformations), the experiments of KUIPER were intended to check this possibility. Their results did not corroborate, however, those of KLOEK, and so the experiments seemed to prove that these findings had been only accidental.

In summarizing the results of the series with 0.001 % LiCl of the 4 authors, however, a same regularity appeared. As Table II shows, it does not only concern the head malformations, but the exogastrulae as well.

TABLE II.

Number of exogastrulae and head malformations in dependence of stage of ending of treatment with 0.001 % LiCl.

Stage	Number of series	Number of eggs	Exogastrulae		Head malformations	
			Number	%	Number	%
2	1	10	—	—	—	—
3	3	31	—	—	—	—
4	3	33	—	—	—	—
5	3	32	—	—	—	—
6	4	46	—	—	—	—
7	3	32	—	—	—	—
8	8	88	—	—	1	1
9	3	28	5	18	1	4
10	10	104	4	4	3	3
11	—	—	—	—	—	—
12	3	30	—	—	—	—
13	1	16	—	—	—	—
14	3	36	—	—	—	—
15	3	26	22	85	—	—
16	1	16	2	13	—	—
17	3	26	8	31	2	8
18	5	65	—	—	—	—
19	9	209	20	10	1	0.5
	66	828				

The total number of exogastrulae and head malformations produced is only small; this may be due to a suboptimal exposure time, and, as regards the head malformations, to the general reduction of sensibility during these stages (cf. fig. 3). However, the table seems to indicate an increased susceptibility of the eggs at stages 8—10, 15—17 and 19. In general, this corresponds to the above-mentioned results, though there is a slight shift in the stages as compared with these. However, still the numbers of cases in the various groups are small, and it is difficult to come at a definite conclusion. We can only say that the alternation of more and less sensible periods in correspondence to the phases of cleavage is indicated without being definitely proved.

Discussion.

The Li-effects in *Limnaea* are of 2 kinds; in some cases the eggs develop to exogastrulae, in other cases various head malformations are produced; in many series both abnormalities may arise side by side. The question arises as to what factors decide which of these abnormalities appears. Since the disturbance of development in the case of exogastrulae is much more serious and shows itself earlier in development, it seemed reasonable to suppose that the disturbing influence has been stronger in this case; one might expect that exogastrulae would develop especially with higher concentrations of LiCl and longer exposure times. As a matter of fact, the series with 0.01 % LiCl yielded, on an average, the highest percentage of exogastrulae, whereas head malformations were most numerous in the series with 0.001 % LiCl. On the other hand, within each of these groups the duration of treatment seemed to have no differential effect. Both with regard to exogastrulae and head malformations with 0.01 % LiCl 1 hour of treatment suffices to give the maximum percentage; with 0.005 %, the maximum is reached at 6—7 hours exposure time; and with 0.001 %, 5—6 hours of treatment at least are needed to obtain the Li-effects.

It is evident, therefore, that the length of the exposure time is no factor in deciding between the 2 divergent ways of development. Another circumstance proves to be of paramount importance, however: the stages during which the eggs are exposed to the Li-solutions. For exogastrulation, on the one hand, and head malformations, on the other, there appear to exist different periods of sensibility. The maximum of sensibility for the production of exogastrulae lies shortly before and during the second cleavage. It must be emphasized that this phase of development appears to be a "critical" stage in many respects: in eggs developing in distilled water or urea solutions, development comes to a standstill in just this phase (RAVEN & KLOMP, 1946). It is possible that the permeability of the eggs is increased at this moment which would explain the high susceptibility of the eggs to the composition of the medium.

On the other hand, for the production of head malformations there are 2 periods of increased sensibility: one immediately after laying, the other reaching its maximum at least 5 hours after the 24-cell stage; between these maxima, there is a period of minimum sensibility.

During the latter period, another regularity is indicated though not definitely proved: the alternation of phases with increased and lessened sensibility in correspondence to the phases of cleavage. When the eggs are taken from the LiCl solution and transferred to tap water at stages with wide cleavage cavity preceding the next division, both exogastrulae and head malformations are produced, whereas this is not the case when the treatment is ended in the intermediate phases of cleavage. At first, it seems rather queer that the moment of ending of the Li-treatment should have such an important effect. One might think that the Li-treatment would act in preparing the way for some reaction occurring only at the moment of

transfer to tap water, comparable to the activation of the sea urchin egg upon transfer to sea water after treatment with butyric acid according to LOEB. On second thought, the explanation is, probably, much more simple. When the eggs with their capsules are put into the Li solution, Li-ions will permeate through the capsule membrane; in his way, the concentration of Li in the capsule fluid will gradually rise. The egg will take up these ions from the capsule fluid; in view of the low external concentrations of LiCl which are effective in bringing about serious disturbances of development, it seems probable that an elective accumulation of these ions in the eggs takes place. It will take some time before the really "effective" concentration of Li is reached within the egg. Upon returning to tap water, the reversed phenomena will occur; the Li is slowly washed out of the egg capsule fluid, the egg will thereupon give off the accumulated Li-ions partly or entirely. Hence, it is clear that, especially with suboptimal exposure times, the highest concentration of Li within the eggs will be attained immediately before or even some time after the moment of return of the egg capsules to tap water. Especially during periods of generally reduced sensibility (corresponding, probably, to a raised threshold value of the effective concentration) this moment will, therefore, be of much importance; slight differences in threshold value with the stages of development will influence the results markedly. Hence, if the observed effect is real, it points to differences in sensibility coinciding with the phases of cleavage.

The fact that head malformations are induced most easily at the 24-cell stage and later is very important. As has been emphasized previously (RAVEN, 1942), these malformations are not due to local losses of cells, but to an alteration in the pattern of determination of the embryo. It is, therefore, highly significant that these alterations can be induced still at so late a stage in these "mosaic" eggs; evidently, the pattern of determination has not yet been laid down irrevocably at this stage. This approaches these Mollusks to the forms with "regulative" development, and raises the suspicion that the determination of development proceeds in both groups along the same lines.

Summary.

1. Eggs of *Limnaea stagnalis* have been exposed to solutions of LiCl in 4 concentrations, varying between 0.01 % and 0.001 % (0.00236 M and 0.00024 M). The results of 437 experimental series, including 9482 eggs, are taken together.

2. There is a distinct maximum of sensibility for the production of exogastrulae shortly before and during the second cleavage.

3. With regard to the production of head malformations, a first period of sensibility exists immediately after laying; it is followed by a period of minimum sensibility; then, the sensibility rises slowly to a maximum lying some hours after the 24-cell stage.

4. The results indicate that in the period of generally reduced sen-

sibility during the first 3 cleavages the eggs show an alternation of phases of increased and lessened sensibility corresponding to definite phases of cleavage.

5. It is evident that the pattern of determination of the organs of the head has not yet been laid down irrevocably at the 24-cell stage in *Limnaea*.

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Physics. — *Recovery and recrystallization viewed as processes of dissolution and movement of dislocations.* II. By W. G. BURGERS. (Laboratorium voor Physische Scheikunde der Technische Hoogeschool, Delft.) (Communicated by Prof. J. M. BURGERS.)

(Communicated at the meeting of March 29, 1947.)

II *Block-structure of the crystalline state.*

Starting from the assumption [LENNARD JONES (13)] that an "ideal" lattice represents the condition of minimal free energy for a crystal, we must conclude that even in the case of a pure element or compound, every crystalline testpiece, independently of its being uni- or polycrystalline, undeformed or coldworked, represents a "metastable" state of thermodynamical equilibrium. The structural differences between these states are merely gradual and not essential. In what follows we shall consider this point somewhat more in detail.

II, 1. *Single crystal.*

According to numerous observations, every "real" crystal, apart from "macroscopic" irregularities ["lineage structure" of BUERGER (14) [5])], has a certain "mosaic" structure, consisting of ideally regular lattice blocks [or lamellae, according to GRAF (16)] with dimensions of the order of magnitude of 0.1—1 micron, the blocks, however, including angles varying from perhaps seconds to minutes of arc. Their presence follows partly from measurements of the intensity of diffracted X-rays [DARWIN (17); EWALD and RENNINGER (18); DEHLINGER and GISEN (19)], partly from microscopic observations of the natural or etched surface of crystals [see in particular GRAF (16)]. Also the "structure-sensitive" character of many physical and mechanical properties [SMEKAL (20)] leads to the same conclusion. Finally the often considerable influence of minute quantities of foreign atoms on the properties of pure metals seems to find a plausible explanation on the assumption that such atoms are preferably "adsorbed" at the boundaries of the lattice blocks and in some way or other exert here their remarkable influence [BRAGG (21)].

As to the "structure" of the block-boundaries, suggestions have been made by various authors [J. M. BURGERS (22); BRAGG (23)]. It is now generally assumed that the deviations of the atoms from their normal positions in these transition layers, which necessarily must occur with regard to the positions of the atoms in both adjoining blocks, are as small as possible. Fig. 4 shows a schematic picture given by J. M. BURGERS (22): here the "fit" between two blocks which include a small angle α, is brought about by a succession of "edge-dislocations" (TAYLOR-dislocations), *all of the same sign,* lying at equal distances h, determined by $tg\ \alpha = \lambda_0/h$, where

[5]) In this connection recent observations by LACOMBE and BEAUJARD (15) of corrosion patterns on aluminium crystals, prepared by recrystallisation, are of great interest.