Effects of L-phenylalanine on somite formation in the early chick embryo

By K. PALÉN¹ & L. THÖRNEBY¹

From the Department of Zoophysiology, University of Lund, Sweden

SUMMARY

Chick embryos were treated in ovo and in vitro with L-phenylalanine from the intermediate streak stage (Hamburger & Hamilton stage 3, 12–13 h of incubation) to the 7-somite stage (H & H stage 9, 29–33 h of incubation).

Treatment in ovo resulted in a large number of embryos developing somite blocks, i.e. imperfectly segmented somites. In embryos treated at an early developmental stage (12–21 h of incubation), the blocks of unsegmented somite mesoderm occurred mostly in the somite pairs 1–5, whereas treatment that began at a later stage (24–30 h of incubation) caused blocks in the somite pairs 5–10, i.e. the appearance of blocks of unsegmented somite mesoderm is correlated in time with the onset of the treatment. No difference regarding mitotic indices could be distinguished between normally segmented somites and blocks of unsegmented somite mesoderm. Autoradiography based on tritiated L-phenylalanine showed no regional differences in labelling of the chick embryo body. Electronmicroscopical observations indicate a slightly suppressed formation of microvilli in the cells of the unsegmented mesoderm blocks compared with cells in normally segmented somites. The observed disturbances are probably caused by a suppressed yolk granule decomposition in the developing somite cells.

The experiments in vitro support the findings in the in ovo material; at the same time, they reveal an unexpectedly slow diffusion of L-phenylalanine through the vitelline membrane.

INTRODUCTION

Previous work (Emanuelsson & Palén, 1975) showed that L-tryptophan supplied to chick embryos in ovo produced typical retardation of brain formation and somitogenesis. This might partly be due to a blocking of gluconeogenesis from L-tryptophan, but the principal disturbance was thought to be impairment of intracellular yolk-granule decomposition. L-tryptophan supplied to the embryo is thought to be transformed into serotonin, which in turn, by feed-back inhibition, prevents yolk-granule degradation at which serotonin is actually liberated. There is reason to believe that morphogenesis is controlled by the orderly degradation of the intracellular yolk granules, and that the degradation process in turn is influenced by the released serotonin. Serotonin and some substances known to interfere with its formation induced malformations which could be traced to delayed yolk degradation, impaired formation and function of microvilli, and impaired ability of the embryo cells to change shape.

¹ Author’s address: Department of Zoophysiology, University of Lund, S-22362 Lund, Sweden.
(Palén, Thörneby & Emanuelsson, 1979). It was also suggested that, in morphogenesis, serotonin primarily promotes the activity of microtubules and microfilaments.

Because aromatic amino acids – i.e. not only tryptophan but also phenylalanine and tyrosine – constitute a significant component in yolk granules (Romanoff & Romanoff, 1949; tenCate, 1952), it was of interest to see whether changes in the phenylalanine level, achieved by administration of L-phenylalanine, produced any morphogenetic effects on the early chick embryo.

The prospects of obtaining obvious effects might seem small, as labelled phenylalanine is widely used in protein synthesis in different tissues and organs in many kind of animals, without any side-effects apparently. Interestingly, however, morphogenetic disturbances were indeed obtained, and as will be seen, they proved to be of a very particular kind.

MATERIALS AND METHODS

Fertilized hen eggs were incubated at 37.5 °C ± 0.05 until they reached various stages of development ranging from the intermediate-streak stage (stage 3, 12–13 h of incubation) to the 7-somite stage (stage 9, 29–33 h of incubation) (Hamburger & Hamilton, 1951). At this point, L-phenylalanine was supplied to the embryo, and incubation was continued either in ovo or in vitro. For all embryos, the initial incubation + continued incubation time was 48 h.

Experiments in ovo. A window was made in the eggshell just over the blastoderm, and L-phenylalanine (0.1–10.0 mg/ml; 0.1 ml) in 0.93% NaCl was injected immediately beneath the embryo area by means of a bent cannula that penetrated the vitelline membrane about 10 mm outside the ridge of the blastoderm. Controls were given the same volume of saline. The eggshell was then sealed with tape, and incubation of the egg was continued for another 18 to 36 h. The blastoderms were then transferred to slides and fixed in absolute alcohol-glacial acetic acid (3:1) and stained in Gomori haematoxylin. The blastoderms were usually examined under the microscope as whole mounts. Selected blastoderms were also sectioned at 5 μm for closer examination. About 500 blastoderms were used in the in ovo experiments.

Experiments in vitro. (1) In part of the in vitro experiments, the embryos were cultivated from the primitive-streak stage (stage 4, 18–19 h of incubation) for about 30 h according to the beaker method of Palén & Thörneby (1976). This method consists of the transfer of the intact yolk into a beaker containing a modified Pannett–Compton medium + 45% homogenized albumen to which was added, in the present case, L-phenylalanine (0.01–15.0 mg/ml). During incubation, the beaker is sealed with PVC-foil. Care is taken to ensure that medium covers the yolk by about 1 mm. If the yolk is more than 3 mm below the surface, typical malformations are formed, probably owing to lack of oxygen. The beaker method allows the medium to be changed at any time.
Effects of L-phenylalanine on the early chick embryo

(2) Another part of the material was cultivated according to New's method (1955), in which the blastoderm is removed from the yolk and transferred to a watch glass kept in a Petri dish for further cultivation. In our experiments, the watch glass contained a modified Pannett–Compton medium with L-phenylalanine (0.05–10.0 mg/ml) + 25 % homogenized albumen.

When using New's method, we noted that the vitelline membrane would sometimes curve and place the embryo well below the surface of the medium. Such embryos become malformed probably due to lack of oxygen. This might explain why embryos treated with a low concentration of the amino acid (0.1–0.5 mg/ml) sometimes showed abnormal morphogenesis. On the other hand, if the membrane bulges, as is more common, the medium 'flows off' it. As embryos treated with concentrations of the amino acid as high as 5.0 mg/ml often became normal, we suspect that the amino acid did not immediately penetrate the membrane from the medium below (as discussed later). To overcome these obstacles, we adopted a modified New's method using a plastic net (same diameter as the ring and pore size 0.6 x 0.6 mm) under the membrane when mounting the embryo in order to keep the membrane fixed and horizontal. Before use, the net was conditioned in saline for 24 h. Using this device, we could adjust the amount of medium lying over the embryo (0.05–0.1 mm) thus ensuring that the amino acid was present throughout the cultivation period.

To measure rapidity of diffusion of L-phenylalanine through the vitelline membrane, we used an Ussing apparatus (Ussing, 1948) in which the membrane to be tested forms the partitioning wall between two liquid-filled chambers. We mounted the free-dissected membrane from an egg incubated for 20 h, and then subjected the outside of the membrane to a concentration of 5.0 mg/ml L-phenylalanine in 0.93 % NaCl and the inside to 0.93 % NaCl alone. The concentration of amino acid was then continually recorded in both chambers for 24 h. During the experiment, the apparatus was kept in an incubator at 37 °C.

We found that, after the first 4 h, the amino acid which had diffused through the membrane had reached 1 mg/ml; complete equilibrium was not attained until after 24 h. The experiment accordingly verifies the suspicion that diffusion of L-phenylalanine through the membrane is too slow to allow the rapid build-up of an effective concentration in the in vitro experiments.

After in vitro cultivation, the blastoderms were fixed and stained as above. The in vitro material included about 200 blastoderms.

Electron microscopy. The ultrastructural examination is based on blastoderms injected in ovo with L-phenylalanine (5.0 mg/ml; 0.1 ml) either at the primitive-streak stage (stage 4, 18–19 h of incubation) or at the 4- to 7-somite stage (stages 8–9, 26–33 h of incubation). The examination was focused on two stages during the treatment: (1) embryos treated at the 4- to 7-somites stages and examined 8 h after the injection; (2) embryos treated at the primitive-streak stage and examined about 30 h after the injection.

Fixation was with 2.5 % glutaraldehyde in phosphate buffer according to
Millonig, followed by osmification. They were dehydrated and stained in 1% phosphotungstic acid + 0.5% uranyl acetate and finally embedded in Vestopal W and sectioned on an LBK ultratome. They were examined in a Philips EM 300 electron microscope, at the Zoological Institute, Lund.

**Autoradiography.** The autoradiographic analysis of the distribution of L-phenylalanine in treated blastoderms is based on embryo material injected *in ovo* at the primitive-streak stage. $[^{3}H]$L-phenylalanine (The Radiochemical Centre, Amersham. Spec. act. 29 Ci/mmol) was added to the injected solution (5.0 mg/ml; 0.1 ml) to a final activity of 125 Ci/ml. After 6 h, the blastoderms were fixed for electronmicroscopy as described above and then sectioned at 1 μm on an LKB ultratome. The autoradiographs were made according to the dipping method with Ilford Liquid nuclear emulsion (K2). They were exposed for 1–2 weeks, developed in Kodak D19 (5 min, 18°C), briefly rinsed in distilled water, fixed in Kodak F24 (6 min, 18°C), and finally stained in Richardson’s azure II and methylene blue.

**RESULTS**

*Experiments in ovo*

The blastoderms were given 0.1 ml injections of L-phenylalanine at concentrations of 0.5, 5.0, and 10.0 mg/ml at different developmental stages; thereafter, incubation continued for various periods from 18 to 36 h. The concentration of 0.5 mg/ml did not affect morphogenesis of the developing embryo, whereas the highest concentration of 10.0 mg/ml produced strong morphogenetic disturbances; even stopping the development of some embryos. The concentration of 5.0 mg/ml affected the embryos in various ways, which involved embryos that, besides somite formation disturbances showed other strong morphogenetic disturbances, and in addition embryos that showed disturbances of somite formation only. Moreover, a group was obtained which, although treated with L-phenylalanine, did not show any visible disturbances, here termed visibly unaffected embryos. The following account of the *in ovo* experiments refers, unless otherwise stated, to the concentration of 5.0 mg/ml.

In the experiments, the age of the embryos is given as 12, 16, 21, 24 and 30 h, the time that the eggs were kept in the incubator before treatment.

Embryos given 0.1 ml injections of 0.93% NaCl were used as controls. Of 103 embryos treated as controls 95 (92%) developed normally. The remaining 8 abnormal embryos (8%) showed disturbances in the brain; in the closure of the neural tube, which was often zigzag shaped and in the formation of the heart and of the somites. None of the disturbed somites showed the typical somite blocks.

As seen in Fig. 1, embryos that showed somite-formation disturbances and also strong morphogenetic disorders represent a small percentage of the material. They were abnormal in shape and development, but in common with the other treated embryos, the treatment did not affect the diameter of the area opaca and area pellucida. The embryos were apparently most sensitive at a 21-hour
Effects of L-phenylalanine on the early chick embryo

Fig. 1. Morphological effects on chick embryos in ovo from L-phenylalanine injected beneath the blastoderm. For details, see text. ○, Visibly unaffected embryos; ●, embryos showing disturbances of somite formation only; △, embryos showing strong morphogenetic disorders in brain only; ▲, the whole embryo is showing strong morphogenetic disorders.

dvelopment stage; treatment from this stage showed the highest frequency of disturbances, such as an apical heart and/or an absent or reversed brain. Other abnormalities occurring in this group of disturbed embryos were: a malformed brain and a neural tube partly or not closed. The somites were missing or existed as a very diffuse strand and were often small and irregular in shape. Moreover, the whole embryo was very small.

In embryos showing disturbances of somite formation only, the typical disturbance was irregularity in the formation of the somites manifested as an inability of the somite mesoderm to segment properly. Thus, in the somite row, there appeared isolated blocks of unsegmented somite mesoderm which normally should have developed into two to six separate somites (Fig. 2A, B).

The largest frequency of embryos with unsegmented somite mesoderm was obtained among the 24-hour embryos; namely, 50% (Fig. 1). Otherwise, compared with controls, the treatment did not influence the diameter of the area opaca and the area pellucida, the length of the embryo, the brain, the neural tube, and the heart. The somite blocks, as a rule, did not have a symmetrical localization, i.e. somites of both sides of the neural tube did not usually appear as blocks in the same level of the axis of the embryo, although this might occur in some cases. Sometimes more than one block of somites existed along the somite row. The level in the somite strand where these somite blocks appeared proved to be dependent on the stage of treatment; thus, when the treatment began at an early stage of development, they were more frequently found proximally, and similarly, treatment given at a later stage produced blocks of somites peripherally in the embryo.
Effects of L-phenylalanine on the early chick embryo

Figure 3 A, B record how embryos, reacting towards L-phenylalanine with only a disturbed somite formation, display the disturbances at varying somite levels, depending on the stage that the L-phenylalanine is given. The somites are numbered in the cranial-caudal direction and the percentage distribution of somite blocks regarding somite pairs 1–12 are given. A block of the somite means that at least two somites are involved, and if, for instance, somite number 4 is denoted as a block, somite number 3 or 5 or both must be included in that block. It might also be that somites 2 and 6 in this case belong to the same block.

When treatment was started at 12–21 h, the largest frequency of somite blocks was found around the fourth somite (Fig. 3 A). On the other hand, treatment starting at 24 h results in somite blocks around the sixth somite and at 30 h around the tenth somite (Fig. 3 B). At the primitive-streak stage, which is about 20 h of incubation in the incubator used in these experiments, the embryos were most susceptible to the treatment: 50 % of the material showed block of somites (Fig. 1).

The visibly unaffected embryos is a group showing no disturbances in the morphogenesis. They form a varying part of the material, depending on the developmental stage when treatment started: from 70–75 % at 12 and 30 h to 40–45 % at 21 and 24 h. These embryos visibly develop normally, and no morphological or structural differences were observed in this group compared with the control embryos.

To reveal differences between the number of somites developed in embryos treated with L-phenylalanine and controls, embryos were placed into four groups: (I) less than 10 somites, (II) 10–15 somites, (III) 16–20 somites, (IV) more than 20 somites. Each group was further subdivided according to the stage treated into an earlier subgroup treated at 12–21 h, and later subgroup treated at 24–30 h (Fig. 4).

As the figure shows, the visibly unaffected embryos have developed slightly fewer somites than the controls. The number of somites is thus intermediate.

Fig. 2. (A, B) Chick embryo supplied in ovo with L-phenylalanine (5-0 mg/ml; 0-1 ml) at 30 h of incubation and then further incubated for 18 h. Note incomplete somite segmentation but otherwise normal development. The bar represents in A 0-5 mm and in B 0-2 mm. (C, D) Chick embryo supplied in vitro with L-phenylalanine (5-0 mg/ml) at 20 h of incubation and then subjected to further incubation for 28 h. Note incomplete somite segmentation and the inability of the somites to segment after the somite blocks. In other respects, a normally developed embryo. The bar represents in C, 0-5 mm and in D, 0-2 mm. (E) Somite block in a chick embryo supplied in ovo with L-phenylalanine (5-0 mg/ml; 0-1 ml) at 24 h of incubation and then subjected to a further 24 h incubation. Longitudinally sectioned at 1 μm. Note the loosely packed and rounded cells in the attachment zone between the separate units of the somite blocks. The bar in E represents 0-02 mm. (F) Chick embryo supplied in vitro with L-phenylalanine (7-5 mg/ml) at 20 h of incubation and then further incubated for 28 h. Note the poor development of the somites and also the detrimental effect on the nervous system. The bar in F represents 0-2 mm.
Fig. 3. A, B. The distribution of imperfectly segmented somites (somite blocks) among developing somites in chick embryos treated *in ovo* with L-phenylalanine injection at the following developmental stages (h of incubation): O——O, 12; •——•, 16; •——•, 21; □——□, 24; ■——■, 30. The somites are numbered in cranio-caudal sequence. A fusion involves a minimum of two consecutive somites. The proportion of files with an abnormal first somite within a class of embryos is expressed as a percentage. Similar percentages are obtained for each of the next eleven somites. The percentages are plotted against somite number to provide a curve for each class.

between the controls and the embryos showing disturbances of somite formation only; the latter have a markedly reduced somite number.

Whereas the control embryos show an even distribution of somites between Groups II and III (Fig. 4), there is in 12- to 21-hour embryos a shifting in the number of somites from Group III (16- to 20-somites) to Group II (10- to 15-somites). This is most pronounced among the embryos showing disturbances of somite formation only (C). However, also among visibly unaffected embryos (B), there is a marked reduction of somites compared with the control embryos (A).
Effects of L-phenylalanine on the early chick embryo

Age of embryos at onset of treatment: 12–21 hrs

Fig. 4. Number of somite pairs appearing in chick embryos treated with L-phenylalanine in ovo compared with controls. Number of somites: I = < 10, II = 10–15, III = 16–20, IV = > 20. A, Embryos treated with NaCl (controls). B, Visibly unaffected embryos. C, Embryos showing disturbances of somite formation only.

In the group of 24- to 30-hour embryos, the differences are less pronounced. For embryos showing disturbances of somite formation only (C), most somites are usually found in Group II (10–15 somites), whereas both the visibly unaffected embryos (B) and the control embryos (A) have most somites usually in Group III (16–20 somites) (Fig. 4).

Autoradiography. The analysed embryos represent those incubated for 21 h and then injected with tritiated L-phenylalanine. As the treatment lasted for only 6 h, morphogenetic disturbances were not yet apparent at fixation. Silver-grain counts over somites, neural tube, and notochord in embryos treated with tritiated L-phenylalanine in ovo did not reveal localization of label in any particular tissue.

Frequency of mitoses (Table 1). Mitoses were counted in embryos treated in ovo with 5.0 mg/ml L-phenylalanine that showed segmented somites and unsegmented somite mesoderm. These were compared with control embryos. The number of the somite cells, i.e. the number of cell nuclei, was approximately the same in somites in different parts of the somite file. This was confirmed by measuring cells from somites chosen at random. Counting was done in a fixed optical field of about the same magnitude as the examined somites. The process was: all mitoses in the field were counted (= number of mitotic figures) and the number of mitotic figures thus can represent the mitotic index. In each group, an average of 200 somites in ten embryos from different stages of development were examined.

No significant differences, as far as the mitotic figures are concerned, exist between treated but visibly unaffected embryos, embryos showing disturbances of somite formation only, and control embryos.

Observations on the somite cells. In the light microscope, the cells of the attachment zone between the separate units in a somite block of treated embryos did not show the typical triangular shape of the somite cell, i.e. with the base of the
Table 1. Number of mitotic figures per field (mitotic index)

Mean value ± s.d. are given in table

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<th></th>
<th>Segmented somites</th>
<th>Unsegmented somites</th>
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<tr>
<td>Visibly unaffected embryos</td>
<td>7·6 ± 3·9</td>
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<tr>
<td>Embryos showing disturbances of somite formation only</td>
<td>7·6 ± 3·9</td>
<td>7·1 ± 3·7</td>
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<td>Control embryos</td>
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<td>8·5 ± 4·6</td>
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cells situated outwards and the apical part towards a central cavity in the somite. Instead, this polarity seems to have been lost in the phenylalanine-treated embryos: moreover, the cells are more loosely packed and round in shape (Fig. 2 E).

Ultrastructural analysis of the somite cells in phenylalanine-treated embryos was made both at the estimated time for the onset of somite-block formation, i.e. 7–10 h after injection of primitive-streak-stage embryos, and after completion of the treatment. So far, it has been impossible to demonstrate any qualitative differences between the somite cells in treated and those in untreated embryos that could provide a rational explanation of the somite-block phenomenon. Compared with the somite cells in untreated embryos the cells in somite blocks appear to contain slightly more decomposing yolk granules. There are also indications of fewer cell projections from the somite cells in the treated embryos but this difference is slight (Fig. 5 A, B).

Experiments in vitro

We used the following methods: (1) the beaker method of Palén & Thörneby, (2) New’s method.

(1) When we used the beaker method, we obtained normally developed embryos even when L-phenylalanine was used at the highest concentration (15 mg/ml). The treatment started at the primitive-streak stage and lasted for 28 h. Thus the embryos at the time of fixation are always age 48 h.

Normal development of embryos under present culture conditions is probably due to incomplete diffusion of the amino acid through the vitelline membrane. (See Materials and Methods.)

Fig. 5. Electronmicrographs showing the appearance of the cells situated at the posterior margin of (A) a somite in a control embryo treated with saline, (B) a somite in an embryo treated with L-phenylalanine. The latter somite has fused with an adjacent somite (to the right) into a somite block. In both cases, the treatment started at the primitive-streak stage and lasted for 24 hours. Note: the cells in B are more loosely packed than those in A and also contain yolk granules that are less degraded than those in the cells in A. Y, yolk; N, nucleus; M, mitochondria. The bar represents 2 μm.
In experiments based on New's method, normally developed embryos were obtained when using concentrations up to 5.0 mg/ml L-phenylalanine. When using the concentration 5.0 mg/ml and the modified New's method, the somite mesoderm in about half of the treated embryos failed to segment properly into somites, but somite blocks occurred (Fig. 2C, D). However, the impaired segmentation pattern did not show exactly the same picture as in the in ovo experiments; thus, after a few somites had developed, blocks occurred and following somites were poorly developed in that the somite mesoderm was unable to continue a normal segmentation process (Fig. 2D). In the in vitro experiments, moreover, the somite blocks are not very distinct laterally.

In a few instances, the concentration 7.5 mg/ml was used, producing mostly embryos malformed in a more general way (Fig. 2F). These malformations were more pronounced when using 10.0 mg/ml L-phenylalanine: an open neural tube, disturbed brain formation, and irregular somites; and not as in the in ovo experiments with exclusive disturbances of somite formation only.

**DISCUSSION**

L-phenylalanine treatment of chick embryos in ovo led to a distinct abnormality, i.e., impaired segmentation of the somite mesoderm into somites (Fig. 2A, B). Essentially the same result was obtained in vitro but with some modifications (Fig. 2C, D).

The differences that exist seem to be due to the different methods of administering the phenylalanine.

In the in ovo method, the substance is injected directly beneath the embryo, thus giving the substance direct access to the embryo. An initial maximum concentration of the substance is obtained. This after some time, is diluted by diffusion in the yolk and sub-blastodermic fluid. In this way, a ‘pulse’ of the substance is received causing the somite mesoderm to produce the somite blocks in one or other way. Possibly in the in ovo method, this ‘pulse’ is just short enough to influence the formation of the somites, leaving other structures uninfluenced. This dependence of the time the treatment starts is supported by the fact that the whole somite file is not affected but only two to about six somites (Fig. 3A, B). Abnormalities occurred only in the somites and never in more than half of the treated embryos (Fig. 1). The ‘visibly unaffected embryos’ developed fewer somites than in the ‘controls’ but more than in ‘embryos showing disturbances of somite formation only’ (Fig. 4), probably because here a lower concentration reaches the embryo. On the other hand, a bigger concentration, 10.0 mg/ml, produces strong malformations of the whole embryo; for instance, a distorted brain and an often unclosed neural tube, and irregularly formed somites. When L-phenylalanine is administered to the chick embryo in vitro, the vitelline membrane apparently forms a barrier, as the diffusion of L-phenylalanine through the vitelline membrane was slow: full equilibrium was attained after 24 h.
Effects of L-phenylalanine on the early chick embryo

Only the modified New's methods produced a satisfactory result, i.e. somite blocks were obtained. As the L-phenylalanine was present during the whole cultivation period, it seems natural that the somite disturbances are not focused on a few somites, but include also the somite mesoderm distally to the recognized somite blocks (Fig. 2C, D).

Earlier authors have assigned the segmentation process of the somites to: (1) somite-forming centres (Spratt, 1955, 1957a, b), (2) Hensen’s node and notochord (Nicolet, 1970), (3) the regression movement of the primitive streak (Bellairs, 1963), and (4) the neural plate (Waddington, 1935; Fraser, 1960; Butros, 1967). Lipton & Jacobson (1974a, b) showed that the process of segmentation was dependent on the successive splitting of the mesoderm caused by the regression of Hensen’s node. In their opinion, a pre-pattern of the segmentation is laid down in the somite mesoderm before it splits. This pre-pattern, however, cannot be expressed until the regression movement has split the mesoderm.

Cellular and non-cellular projections play a prominent part in the formation of somites. Backhouse (1974) in SEM could show that many fine processes projected from the somites towards the neural plate and also towards sequential somites. Most of these processes are believed to be non-cellular, usually of fibrous materials. It was suggested that this extra-cellular matrix, especially the collagen component, is an ‘inductive factor’ during spinal cord–somite interaction (Cohen & Hay, 1971). There are also many microvilli projections from the somite cells. The mesodermal cell projections are obviously made use of when attaching to the ecto- and endoderm, using these cell layers as a foundation for movement (Trelstad, Hay & Revel, 1967; England & Wakely, 1977). As demonstrated by Bellairs (1979) and Bellairs, Curtis & Sanders (1978) somite formation is also characterized by increased cell adhesiveness between the cells within the developing somite, which in turn leads to somite separation at the region of lower adhesiveness. In the present case the EM analyses give no clear indication of a solution, although there was a slight tendency to reduced formation of microvilli in the cells of the somite blocks compared with the controls. The fact that the cells in the attachment zone between fused somites are loosely packed and round in shape indicates, however, that the normal increase in cell adhesiveness is suppressed.

The literature reports incomplete segmentation of the somite mesoderm in the chick embryo after treatment with various amino-acid analogues. Herrmann (1953), using a technique described by Spratt (1948), which implies cultivation of embryos on the surface of a mixture of agar and egg extract, investigated the effect of analogues of phenylalanine, leucine, and valine on embryos from the late head-fold stage to the 6-somite stage. Analogues from phenylalanine produced retardation of growth, which was partly reversed when phenylalanine and the analogue were added simultaneously to the medium. Besides the retarded growth, the analogues produced a zigzag neural tube and an irregularly formed brain, and the somites were often small and irregular in shape. The leucine
analogue produced a characteristic malformation in the somites: the somite mesoderm did not segment: 2- to 4-hour somites remained fused in what Herrmann called a ‘somite block’. Except for the retardation of growth and in a few instances disturbances of the central nervous system, he found no disturbances other than the ‘somite blocks’ in the treated embryos. Summarizing, he argues that it is probable that in many instances the analogues interfere more or less directly with the formation of new proteins, and also that different analogues do not inhibit the formation of various proteins in the same way.

Rothfels (1954), using a modified technique of Spratt (1947), found that analogues to iso-leucine and valine produce ‘somite blocks’ of various sizes. The level at which this malformation occurred could be correlated with the stage at explantation. She also found a time lag before the effect of the analogue was apparent. The transient effect of the analogues was suggested to be due to their instability. When the embryos were subjected to L-leucine and L-valine, they developed normally. When analogues to phenylalanine were used, ‘somite blocks’ were obtained, among other malformations. Moreover, phenylalanine alone, at a concentration of 5-0 ml/mg, was toxic to the embryos, whereas a lower concentration has no effect. Unfortunately, Rothfels does not give any details of the toxic effect produced by phenylalanine alone!

From this, it is clear that analogues to various amino acids produce the same abnormalities as are demonstrated in the present paper when chick embryos were treated with L-phenylalanine. It is also obvious that a certain time lag elapses between the start of treatment and the observation of the malformation. Evidently, a concentration has to be built up in the reacting tissue before the effect is expressed. Both Herrmann and Rothfels used an in vitro method, i.e. the embryos were cultivated on agar medium, whereas the present study used an in ovo method. The suspicion that a mechanical injury from the injection moment in the in ovo method produced the somite blocks can thus be ruled out.

In the present case, the results from the autoradiographic investigations of L-phenylalanine-labelled embryos showed no regional differences in silver-grain distribution within the embryo body; therefore tissue-specific effects from phenylalanine are unlikely to be the cause of the observed abnormality.

Instead, we suggest that, by a feed-back mechanism, exogenously administered phenylalanine influences the normal balance of amino acids in the embryo cells, and results in the somite blocks. At this time of development, the embryo depends on intracellular yolk granules for material for its protein synthesis. Flickinger (1949) and Mastrangelo (1966) pointed out the detrimental effects of phenylalanine on the breakdown of the intracellular yolk granules in amphibian and sea-urchin embryos; therefore a possible action of phenylalanine in the chick embryo could be its effect on the yolk granules. The reason that such a general effect of phenylalanine results in disturbed somite formation could be that this breakdown process represents such a rapid and decisive event that temporary disturbances of it cannot be compensated for in a short time. The observation
that the cells of somite blocks had a tendency to contain less microvilli than had cells of normally segmented somites could be a result of suppressed yolk-granule decomposition. The latter phenomenon might also have provoked the apparent suppression of normal increase in cell adhesiveness between the somite cells, e.g. through withholding necessary building material contained in the yolk granules.

Moreover, the observations by Bruce (1976) who treated chick embryos in agar culture with antiserum against intracellular yolk granules agree with our view. Bruce found many more granules present in treated embryos than in the controls, which indicates a reduced decomposition of the yolk granules. When the treatment was started at stages 3 to 4+, it often induced somites 'connected to each other'.

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