THE URODELE NEUROEPITHELIUM

III. THE PRESENTATION OF PHENYLALANINE TO THE NEURAL CREST BY ARCHENTERON ROOF MESODERM

CHARLES E. WILDE, JR.
Department of Pathology, School of Dentistry, University of Pennsylvania

EIGHTEEN FIGURES

INTRODUCTION

Recent experiments with metabolite analogues have established that the differentiation of both pigment cells and ectomesenchyme from the urodele embryonic neuroepithelium is dependent upon metabolic activities in which phenylalanine is a necessary substrate. This causal relationship was further confirmed by the elicitation, in vitro, of pigment cells and a strange ectomesenchyme from competent ventral ectoderm by low concentrations of phenylalanine (Wilde, '55a, b).

Not only was phenylalanine shown to be essential for the normal differentiation of pigment cells and ectomesenchyme, but portions of the molecule proved to be particularly necessary to the differentiation of specific cell types. The α-amino group is essential for normal pigment cell differentiation and melanogenesis. The phenyl group is required in the metabolism concerned with ectomesenchyme differentiation.

The urodele neuroepithelium is a system well suited to precise studies on cell differentiation and the causal relations thereof. It can be explanted to nutrient medium, where, under favorable conditions, its differentiation is comparable to its differentiation in vivo. The types of cells which differentiate can be characterized distinctly and are strikingly different from their precursors (DuShane, '35; Hörstadius, '50; Wilde, '55c). The neuroepithelium can be excised with surgical

THE JOURNAL OF EXPERIMENTAL ZOOLOGY, VOL. 133, NO. 3
DECEMBER 1956

precision and freed completely from presumptive tissues of different fates. This is, therefore, a standardized experimental system where repeatability is excellent.

With the establishment of the fact of substrate control of cellular differentiation, by phenylalanine in the urodele neural crest and by vitamin A in fowl keratinizing epithelium (Fell and Mellanby, '53; Weiss and James, '54), it is important to elucidate further the mechanism of such control. Markert ('55), studying melanoblasts of embryonic chick skin, has reported that tyrosine labelled with C_{14} in the alanine side chain is not preferentially segregated in the differentiating melanoblasts, whereas randomly labelled C_{14} tyrosine is. This evidence indicates that the chick melanoblast may only be able to use fully formed tyrosine in melanogenesis. It is important to discover whether the urodele neural crest requires fully formed phenylalanine for normal differentiation of pigment cells and ectomesenchyme, or whether exogenous materials can be used by the differentiating cells to synthesize phenylalanine. Further studies of the rôle of phenylalanine in neural crest differentiation are also indicated. What is its source in the embryo? What is the rôle of the alanine side chain in differentiation? What part is played by the β-carbon configuration of the molecule? Experiments described below were designed to answer these questions.

MATERIALS AND METHODS

The experiments reported here have been carried out by means of well established techniques of short term tissue culture in nutrient media. The methods used to obtain precise tissue samples, the structure of the medium, and means of sterilization of originally unsterile tissues have been published at length (Wilde, '48, '50, '52, '55a, '55b).

The protein-free medium has been supplemented with 1.0 millimoles/L. phenylalanine in control series of the current report. All experimental series were compared with contemporaneous controls of identical tissue source. Other types of controls, i.e. comparative, differentiation controls were car-

ried out where necessary. Supplementation of the nutrient medium in experimental series was by compounds chosen for particular analytical reasons. These were intended to form a step-wise experimental analysis of the mechanism of utilization of phenylalanine by the differentiating neural crest cells. The following compounds or groups of compounds were used.

(a) 1.0 millimoles/L. phenyllactic acid and 1.0 millimoles/L. β-2 thienylalanine (both compounds used at 2.0 millimoles/L. levels for ventral ectoderm experiments)
(b) 1.0 millimoles/L. phenylglycine
(c) 1.0 millimoles/L. phenylserine
(d) 0.5 millimoles/L. o-fluorophenylalanine, 0.5 millimoles/L. phenylacetic acid and 0.5 millimoles/L. glycine
(e) 0.5 millimoles/L. o-fluorophenylalanine, 0.5 millimoles/L. phenylacetic acid and 0.5 millimoles/L. α-alanine

Explants were made from embryos of Ambystoma maculatum (Shaw) of stages 11–14 (Harrison). The embryos were obtained from North Carolina, Princeton, New Jersey and Hanover, New Hampshire. Cultures were prepared as flat hanging drops over flat bottomed well-slides. They were observed daily by phase contrast microscopy and photographed when desired. Examples of the photographs serve as figures illustrating the data. Cultures at term, usually 12 to 15 days, were fixed in sublimate-acetic-formol (Gregg and Puckett, '43) and stained with haematoxylin and fast green for further study.

It is convenient to adopt an equation system in order to summarize briefly the results and deductions from the experimental series which appear in the body of this report. These conventions will facilitate review of the experiments by the reader and will avoid much repetition, since each experiment is pertinent to the others in the series.

The following equation for example

$$11\text{–}14 \left[\frac{\text{cran. neuroepith.}}{\text{basal} + \text{DLPA}} \right] \dashrightarrow \text{normal diff.} \qquad (1)$$

indicates that at stages 11 to 14 (Harrison), cranial neuroepithelium explanted to basal nutrient medium supplemented with 1.0 millimoles/L. phenylalanine differentiates normally

into pigment cells, neurons, and ectomesenchyme (Schwann cells, fibroblasts, melanophages). The numbers at the left indicate Harrison's stages, inclusive, at which explantation was carried out. The numerator on the left specifies the tissue explanted (e.g., cran. neuroepith., cranial neuroepithelium; meso., archenteron roof mesoderm; ventr. ecto., ventral ectoderm; ventr. ecto. + "meso.," ventral ectoderm and internal yolky cells). The left denominator specifies the medium used and its supplementation (basal refers to nutrient medium without supplementation (Wilde, '48, '55c); DLPA is phenylalanine; OFPA, o-fluorophenylalanine; PGL, phenylglycine; PSE, phenylserine; β-2 THA, β-2 thienylalanine; Phen., phenyllactic acid; GLY, glycine; AAL, α-alanine; PAA, phenylacetic acid).

Thus:

$$11\text{–}14\left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{DLPA}}\right] \dashrightarrow \text{normal diff.} \quad (2)$$

$$11\text{–}14\left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{Phen}}\right] \dashrightarrow \text{specif. inhib. pigment e.} \quad (3)$$

$$11\text{–}14\left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \beta\text{-2 THA}}\right] \dashrightarrow \text{specif. inhib. ectomes.} \quad (4)$$

Equations 2, 3 and 4 summarize pertinent data from Wilde, '55a, '55b.

EXPERIMENTAL

General statement

Data to be presented below indicate that the neural crest component of the urodele cranial neuroepithelium requires for its cellular differentiation metabolism, preformed molecular phenylalanine. Tests show that the amino acid cannot be synthesized from exogenous materials supplied in the nutrient when pure neuroepithelium is cultured in vitro. At least, neuroepithelial cells fail to differentiate when cultivated with materials from which phenylalanine can be synthesized. The failure of differentiation provides a sensitive test in this

system for the unavailability of phenylalanine, or the inhibition of its utilization.

Compounds similar in structure to phenylalanine which differ slightly at the β-carbon atom (e.g. phenylglycine and phenylserine), do not satisfy the phenylalanine requirement of neuroepithelium. They act instead as inhibitors of its cellular differentiation. This inhibition can be relieved by inclusion of equimolar amounts of phenylalanine in the nutrient medium containing the explants.

The urodele archenteron roof (somite and prechordal plate mesoderm) appears to synthesize phenylalanine from exogenous materials supplied to it in these experiments. The events in this synthesis will be reported in detail. They appear to include: cleavage and later condensation at the β-carbon atom, transamination and transmethylation. The neuroepithelium is dependent upon the archenteron roof for both synthesis of phenylalanine and its supply. The processes involved are probably not those of detoxication, but rather constitute a morphogenetic system through which a metabolite causally concerned with a particular set of cellular differentiations is supplied to the cells which require it. These statements will be supported below.

The activities of the cultured cells

The data are derived from experiments in which cells either differentiated or failed in differentiation to some degree. It is, therefore, germane to describe briefly normal differentiation of neuroepithelium in vitro, and failure of differentiation in vitro. These conditions have been previously described in greater detail (Wilde, '55a, '55b), but are here reviewed for convenience and to eliminate the necessity of excessive description with the analysis of each experimental series.

Explanted cells of the urodele embryo of stages 11 to 14 migrate from the explant along the glass-medium interface as free ameboid cells. Except for size differences (viz. dorsal cells are smaller than ventral cells), all are morphologically

and kinetically similar at the time of explantation. The cells are rounded and move by means of large lobopodia which appear to burst out of the cell body (Holtfreter, '46). The movement is random (fig. 1) and the cells do not tend to form aggregates once they have left the explant. These states of homogeneity of cell form and cell movement have been selected in these and in previous experiments as readily identifiable criteria of the undifferentiated state of urodele embryonic cells. This is, therefore, a convenient starting point for studies of differentiation. In this report, classification of cells as "undifferentiated" refers to this condition. When explanted cells remain in this state for excessive lengths of time, i.e., significantly longer than the controls, they are considered to have failed to differentiate and to be inhibited. Such cells are shown in figure 2, after 12 days in vitro following explantation at stage 12½. Inspection of figures 1 and 2 indicates the similarity of "undifferentiated" (fig. 1) and inhibited (fig. 2) cells.

The normal differentiation in vitro of pigment cells is illustrated in figure 3. These cells have differentiated during the 12 days in vitro following explantation at stage 12½. They should be compared with the inhibited cells of figure 2 and with those of figure 4. Figure 4 represents the normal differentiation of pigment cells in explants cultured under conditions where an inhibition to differentiation has been relieved. The

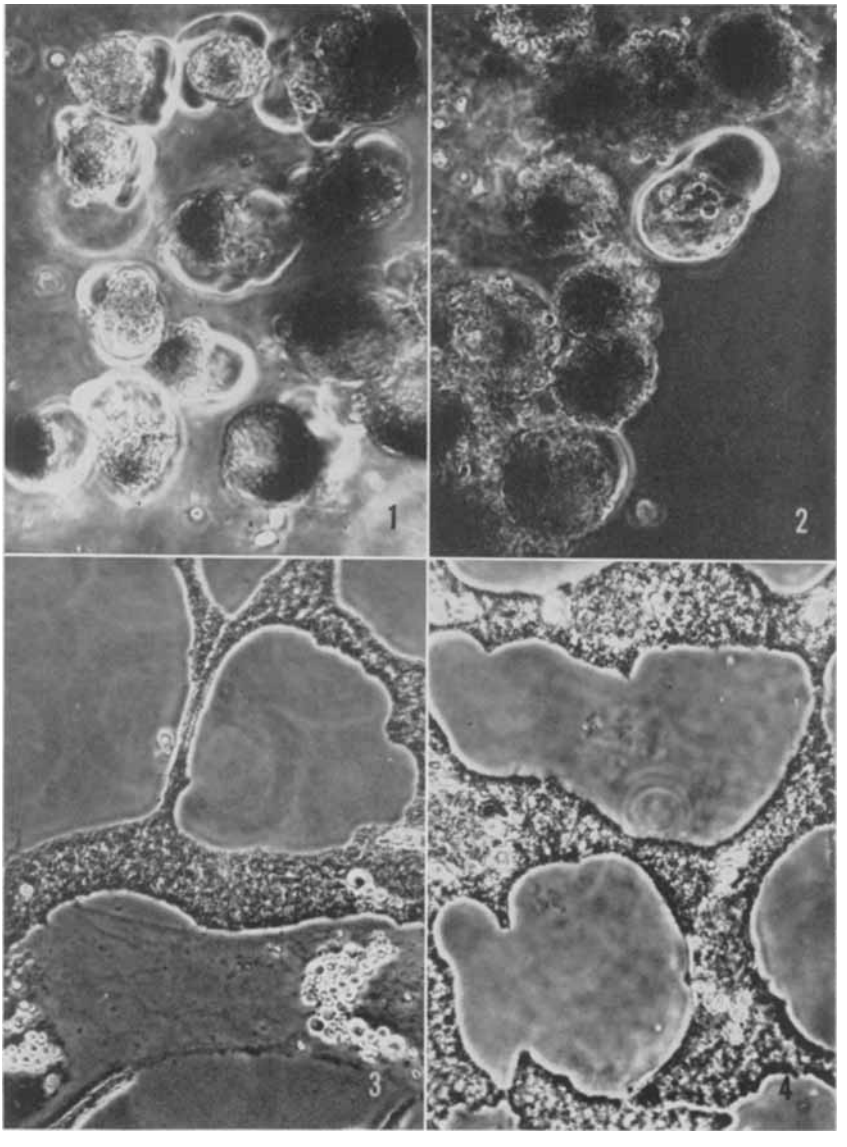
Fig. 1 Explant of cranial neuroepithelium with archenteron roof mesoderm, cultivated for three days in basal medium with 1.0 mM/L. phenylalanine. Control series, explanted at stage 12½. Note undifferentiated ameboid cells. × 300.

Fig. 2 Explant of cranial neuroepithelium without mesoderm, cultivated for 12 days in basal medium supplemented with 1.0 mM/L. phenylglycine. Explanted at stage 12½. Complete failure of differentiation, ameboid cells only. × 300.

Fig. 3 Explant of cranial neuroepithelium with archenteron roof mesoderm, cultivated for 12 days in basal medium supplemented with 1.0 mM/L. phenylalanine. Control series explanted at stage 12½. Normal differentiation of dendritic pigment cells and ectomesenchyme. × 300.

Fig. 4 Explant of cranial neuroepithelium and archenteron roof mesoderm cultivated for 11 days in basal medium supplemented with 1.0 mM/L. of both phenyllactic acid and β-2 thienylalanine. The inhibition has been relieved and normal differentiation of dendritic pigment cells with heavy melanogenesis has occurred. Explanted at stage 13. × 300.

inhibition has been previously established by the introduction into the culture medium of a phenylalanine analogue. Relief of inhibition was accomplished in these experiments either by the activity of archenteron roof mesoderm (fig. 4), or by an



Figures 1 to 4

exogenous supply of phenylalanine added to the nutrient medium containing the inhibitor (fig. 5). The degree of melanogenesis of the pigment cells in figures 3, 4 and 5 is similar. So also is the degree to which the cells have become dendritic. The contrast between figures 1 and 2; and 3, 4 and 5 is a measure of the degree of morphological change from the starting point as these changes occur in the experiments. Relief of inhibition therefore results in differentiation similar to that seen in the controls.

Figures 6, 7 and 8 illustrate ectomesenchyme differentiation under control (fig. 6) and experimental conditions (figs. 7 and 8). Figure 6 represents the normal differentiation of ectomesenchyme cells. After 8 days in vitro under control conditions, these cells are colorless, spindle or triangular in shape with slender filopodia. More mature ectomesenchyme is seen in figure 7 where differentiated Schwann cells, (S); fibroblasts, (F); and melanophages, (M) lie in a field of nerve axons. In figure 8 a group of fibroblasts with vacuoles of melanin is shown. In figure 7 inhibition of differentiation has been relieved by archenteron roof mesoderm, while in figure 8, relief of inhibition has been brought about by the addition of an equimolar amount of phenylalanine to the nutrient medium containing the inhibitor.

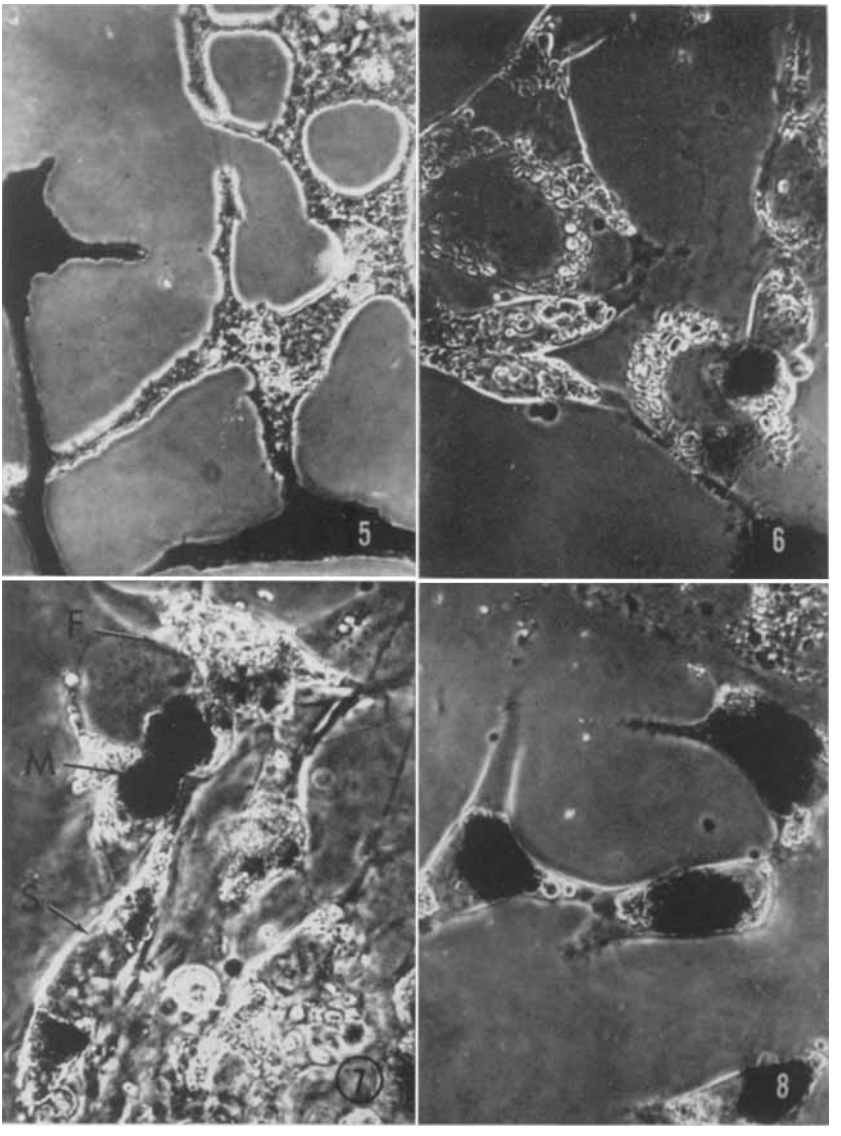
Fig. 5 Explant of cranial neuroepithelium without mesoderm, cultivated for 12 days in basal medium supplemented with 1.0 mM/L. of both phenylalanine and phenylglycine. Inhibition relief and normal differentiation of dendritic pigment cells. Explanted at stage 11½. × 300.

Fig. 6 Explant of cranial neuroepithelium and archenteron roof mesoderm, cultivated for 8 days in basal medium supplemented with 1.0 mM/L. phenylalanine. Control series, normal differentiation of ectomesenchyme. Explanted at stage 13. × 300.

Fig. 7 Explant of cranial neuroepithelium and archenteron roof mesoderm, cultivated for 10 days in basal medium supplemented with 1.0 mM/L. of both phenyllactic acid and β-2 thienylalanine. Inhibition relief and normal differentiation of ectomesenchyme. F, fibroblast; M, melanophage; S, Schwann cell. Explanted at stage 13. × 300.

Fig. 8 Explant of cranial neuroepithelium without mesoderm, cultivated for 12 days in basal medium supplemented with 1.0 mM/L. of both phenylglycine and phenylalanine. Inhibition relief and normal differentiation of ectomesenchyme. Explanted at stage 11½. × 300.

The presence of archenteron roof mesoderm (presumptive somite) in an explant results in the self-differentiations seen in figures 9 and 10. Both illustrate well-differentiated striated muscle. Figure 9 represents striated muscle which developed



Figures 5 to 8

from explants placed in control medium. Figure 10 is a photograph of striated muscle which differentiated from archenteron roof in an experiment where the included mesoderm has relieved an inhibition to neuroepithelium differentiation, as well as undergoing its own differentiation. The similarities between control cultures and inhibition-relief cultures are obvious. These experiments deal with the differentiation of three classes of cells: pigment cells, ectomesenchyme cells, and striated muscle cells. Depending upon the experimental conditions, the cells are either undifferentiated as at the beginning of the experiment (fig. 1), or through the activities of the inhibitor (fig. 2); or differentiated as under control conditions (fig. 3), or through relief of an established inhibition. This relief has been brought about either by the activity of added normal metabolite (fig. 5) or by the synthetic activity of added mesoderm cells (fig. 4). The figures just described have been chosen as characteristic of these different states which are important in the analysis of the experiments. To treat each experimental series *seriatim* by means of extensive photographs would be redundant, since many of the photographs are similar. Consequently, each photograph has been identified in the captions of the figures, but will be used as a repre-

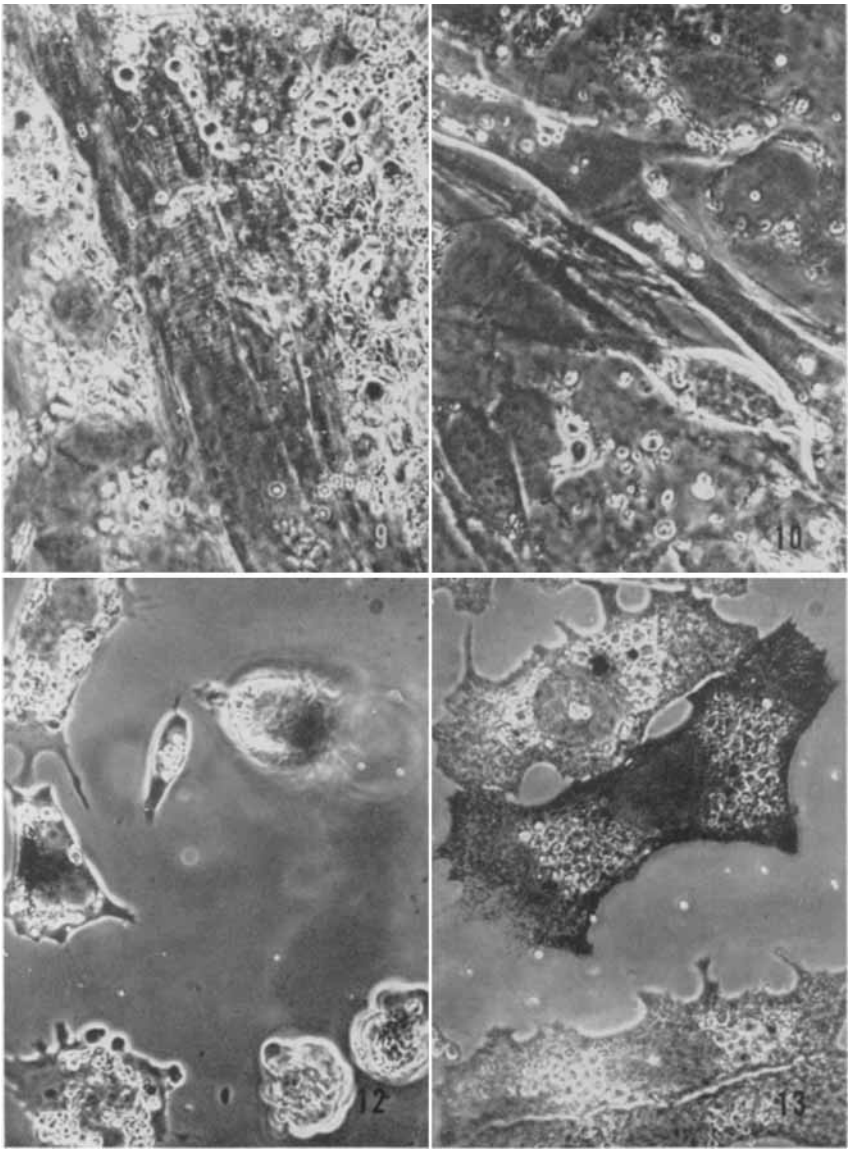
Fig. 9 Explant of cranial neurocpithelium and archenteron roof mesoderm, cultivated for 12 days in basal medium supplemented with 1.0 mM/L. phenylalanine. Control series. Normal differentiation of striated muscle from archenteron roof mesoderm. Explanted at stage 12. × 300.

Fig. 10 Explant of cranial neuroepithelium and archenteron roof mesoderm, cultivated for 11 days in basal medium supplemented with 1.0 mM/L. phenylserine. Inhibition relief and self-differentiation of striated muscle from mesoderm cells. Explanted at stage 12½. × 300.

Fig. 12 Explant of cranial neuroepithelium without mesoderm, cultivated for 6 days in basal medium supplemented with 0.5 mM/L. of o-fluorophenylalanine, phenylacetic acid and glycine. Attempted inhibition relief has not occurred and no cellular differentiation has taken place. The cells remain ameboid. Explanted at stage 12. × 300.

Fig. 13 Explant of cranial neuroepithelium and archenteron roof mesoderm, cultivated for 6 days in basal medium supplemented with 0.5 mM/L. of o-fluorophenylalanine, phenylacetic acid and glycine. Inhibition relief and normal differentiation of dendritic pigment cells with heavy melanin load. Explanted at stage 12½. × 300.

sentative figure in reporting experiments when the cellular conditions pertinent to a particular experimental series are illustrated by that figure.



Figures 9, 10, 12 and 13

Does the differentiation metabolism of cranial neural crest require preformed molecular phenylalanine?

If the differentiation of the neural crest components does *not* require fully formed phenylalanine, and if the crest itself can synthesize phenylalanine from precursors, then explants of pure cranial neuroepithelium could differentiate normally in nutrient medium supplemented with exogenous materials from which phenylalanine could be made.

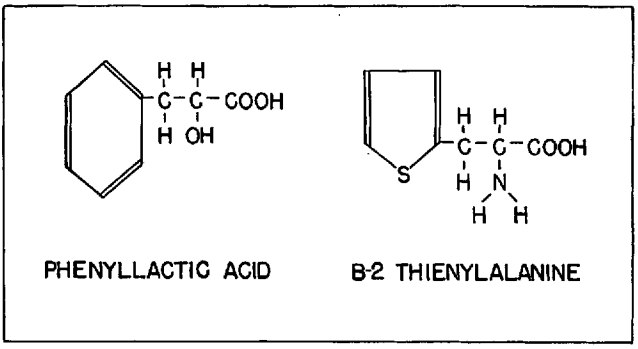


Figure 11

Inspection of the structural formulae of phenyllactic acid and β-2 thienylalanine (fig. 11) shows that, if a mechanism existed which could judiciously exchange portions of the two molecules, the necessary chemical configurations for the synthesis of phenylalanine would be available. These particular compounds were chosen because earlier work had demonstrated that: (Wilde, '55a, '55b)

$$11\text{–}14\left[\frac{\text{cran. neuroepith.}}{\text{basal} + \text{Phen}}\right] \dashrightarrow \text{inhib. of pigment c.} \quad (5)$$

$$11\text{–}14\left[\frac{\text{cran. neuroepith.}}{\text{basal} + \beta\text{-2 THA}}\right] \dashrightarrow \text{inhib. of ectomes.} \quad (6)$$

If phenylalanine can be synthesized by pure neural crest cells from these substances, then differentiation should be normal in a medium supplemented with both phenyllactic acid and β-2 thienylalanine. Contrariwise, if phenylalanine cannot

be so synthesized, then total inhibition of differentiation would be expected since the specific inhibitions of the single compounds together cover the range of cellular differentiations normally seen in control cultures (cf. equations 3, 4, 5 and 6 and table 1).

TABLE 1

MEDIUM AND SUPPLEMENT	TYPE OF EXPLANT AND NUMBER OF EXPERIMENTS	
	Pure cranial neuroepithelium	Cranial neuroepith. with mesoderm
Basal medium with 1.0 millimoles/L. phenylalanine	4	16
Basal medium with 1.0 millimoles/L. phenyllactic acid and 1.0 millimoles/L. β-2 thienylalanine	16	16

When explants consisting of pure cranial neuroepithelium are cultured in basal medium supplemented with 1.0 millimoles/L. of both phenyllactic acid and β-2 thienylalanine, no cellular differentiation takes place. The complementary specific inhibitions characteristic of the two compounds (equations 5 and 6) summate and no differentiated cells appear in the culture. The vast majority of cells remain ameboid with large lobopodia. Rarely, abnormal, somewhat bluntly stellate cells without pigment are seen. Figure 2 is representative of such an inhibited culture. There is failure of cell differentiation but the cells are still actively ameboid.

$$11\text{–}14\left[\frac{\text{cran. neuroepith.}}{\text{basal} + \text{Phen} + \beta\text{-2 THA}}\right] - - - - - \rightarrow \text{total inhib.} \quad (7)$$

It seems highly probable that the previously demonstrated metabolic pathway of cellular differentiation concerned with phenylalanine has been interfered with and that no mechanism exists within the cranial neuroepithelium to overcome the resulting inhibition. Presumably this mechanism would act in the synthesis of phenylalanine from the molecular configurations available in the exogenous substrates supplied.

When archenteron roof mesoderm is explanted with neuroepithelium (specificity control, Wilde, '55a, '55b) to basal

medium supplemented with 1.0 millimoles/L. of both phenyllactic acid and β-2 thienylalanine, both the neural crest component and the mesodermal component differentiate normally. Figures 4, 7 and 10 describe the situation. The mature cultures contain well-differentiated dendritic pigment cells with heavy melanin load (fig. 4), ectomesenchyme with fibroblasts, Schwann cells and melanophages (fig. 7), and striated muscle (fig. 10).

The results indicate that activities inherent in the archenteron roof mesoderm are capable of overcoming the inhibition to pure neuroepithelium recorded in equation 7. Since it has already been established that the differentiation of the urodele neural crest is causally related to special metabolisms involving phenylalanine as a substrate, it seems justifiable to conclude that synthesis of phenylalanine from phenyllactic acid and β-2 thienylalanine has taken place in the archenteron roof mesoderm.

Therefore:

$$11\text{–}14\left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{Phen} + \beta\text{-2 THA}}\right] \dashrightarrow \text{normal diff.} \quad (8)$$

If the foregoing data are used as a basis for extrapolation to the intact embryo, it is probable that the normal differentiation of the neural crest is dependent upon synthetic activities in the archenteron roof, among which are synthesis of phenylalanine and its presentation to the environment of the overlying neuroepithelium. The neural crest responds to this activity by differentiating into pigment cells and ectomesenchyme. Such a dependent system has many of the attributes of induction systems of classical experimental embryology (Spemann, '38).

Since the archenteron roof mesoderm appears to be capable of synthesizing phenylalanine from exogenous phenyllactic acid and β-2 thienylalanine, the mechanisms of this synthesis should be elucidated. This phenomenon requires a chemical change in the inhibitory substances. Inspection of figure 11 indicates that the most likely mechanisms (direct, enzyme-

mediated reactions for which there is evidence in other biochemical systems) are: transfer of the α-amino group of β-2 thienylalanine to replace the α-hydroxyl group of phenyllactic acid (transamination), or cleavage of both compounds between the α and β carbon atoms, and the condensation of the glycine-like acid with the phenylmethyl (benzyl) moiety. The latter mechanism, cleavage followed by condensation of two-carbon fragments, recalls β-oxidation of fatty acids and reactions involving coenzyme A. Transaminations require specific transaminases which insert amino groups into the α-position only of molecules in which enol-keto tautomerism is present at the α-β carbon bond (White et al., '54). Therefore, since phenyllactic acid does not exhibit enol-keto tautomerism, direct transamination is unlikely. Direct exchange of phenyl groups is extremely unlikely also. Since both analogues must be supplied from the environment simultaneously in order for mesoderm cells to act to relieve the inhibitions, it is clear that neither endogenous materials nor the mechanism to overcome the specific inhibition of either single compound exists in the archenteron roof. This evidence reinforces the concept that the system is a synthetic one which functions only when proper substrate materials are available to it (equations 3, 4, 5, 6, 7, and 8).

Inhibition relief of neuroepithelium differentiation by mesodermal synthesis

It has been previously established that o-fluorophenylalanine is a general inhibitor of the differentiation of the cells of both neuroepithelium and archenteron roof mesoderm. This inhibition can be relieved by the presence of equimolar amounts of phenylalanine in the culture medium (Wilde, '55a, '55b).

$$11\text{–}14 \left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{OFPA}}\right] \dashrightarrow \text{total inhib.} \quad (9)$$

$$11\text{–}14 \left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{OFPA} + \text{DLPA}}\right] \dashrightarrow \text{normal diff.} \quad (10)$$

If the archenteron roof mesoderm is active in the synthesis of phenylalanine and in presenting the finished material to the environment of the crest, and further, if the synthesis is, in fact, by means of cleavage and condensation across the α-β carbon bond, then other compounds which could serve as substrates in these events should also be active.

When phenylacetic acid and glycine, or phenylacetic acid and α-alanine are introduced into media made inhibitory by the addition of 0.5 millimoles/L. o-fluorophenylalanine, the

TABLE 2

TYPE OF MEDIUM	TYPE OF EXPLANT AND NO. OF CASES	
	Cranial neuroepith.	Cranial neuroepith. and mesoderm
Basal medium with 1.0 mM/L. phenylalanine (Control)	6	6
Basal medium with 0.5 mM/L. o-fluorophenylalanine, 0.5 mM/L. phenylacetic acid, and 0.5 mM/L. glycine	12	12
Basal medium with 0.5 mM/L. o-fluorophenylalanine, 0.5 mM/L. phenylacetic acid, and 0.5 mM/L. α-alanine	12	12

inhibition might be relieved. A series of experiments to test this hypothesis was designed and carried out. A striking result was obtained (figs. 12 and 13).

When pure cranial neuroepithelium is explanted and placed in basal medium supplemented with 0.5 millimoles/L. each of o-fluorophenylalanine, phenylacetic acid and glycine, no relief of the inhibition due to the o-fluorophenylalanine is obtained regardless of the length of time the culture is carried (up to 13 days). This result is shown in figure 12 (cf. figs. 1 and 2). The cells of the explant remain for the most part as ameboid cells which move with broad lobopodia. Later (after 12 days in vitro), a few colorless stellate cells are seen. This indicates that a mechanism to overcome the inhibition due to

o-fluorophenylalanine through use of these compounds is not present in explants of pure cranial neural crest. Therefore:

$$11\text{–}14\left[\frac{\text{cran. neuroepith.}}{\text{basal} + \text{OFPA} + \text{PAA} + \text{GLY}}\right] \dashrightarrow \text{total inhib.} \qquad (11)$$

Complete differentiation of all cell types is obtained when cranial neuroepithelium and archenteron roof mesoderm are placed in nutrient medium supplemented with 0.5 millimoles/L. each of o-fluorophenylalanine, phenylacetic acid and glycine. Pigment cells which differentiated under these circumstances are illustrated in figure 13. Ectomesenchyme indistinguishable from that seen in control cultures differentiated. Striated muscle and myoblasts developed from the included somitic cells. Figure 13 should be compared with figures 3, 4 and 5. The inhibition to cellular differentiation brought about by o-fluorophenylalanine has been relieved by phenylacetic acid and glycine of equimolar concentration in the presence of *mesoderm*. Thus:

$$11\text{–}14\left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{OFPA} + \text{PAA} + \text{GLY}}\right] \dashrightarrow \text{normal diff.} \qquad (12)$$

The substitution of α-alanine for glycine in this experimental series did not strikingly affect the results (equations 13 and 14). Unfortunately, in the α-alanine series, supplies of o-fluorophenylalanine, a rare and expensive compound, became exhausted and the strict 0.5 millimolarity per liter could not be maintained. However, the cultures lacking mesoderm were inhibited strongly and there was no evidence of inhibition relief in the absence of mesoderm. Cultures which contained both mesoderm somitic cells and neural crest cells differentiated normally. This result, again, emphasizes that the mechanism whereby the inhibition to differentiation has been relieved is most probably by synthesis of phenylalanine in the explanted mesoderm.

$$11\text{–}14\left[\frac{\text{cran. neuroepith.}}{\text{basal} + \text{OFPA} + \text{PAA} + \text{AAL}}\right] \dashrightarrow \text{total inhib.} \qquad (13)$$

$$11\text{–}14\left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{OFPA} + \text{PAA} + \text{AAL}}\right] \dashrightarrow \text{normal diff.} \qquad (14)$$

In analyzing the results of these experiments, the data summarized by equations 9 and 10 must be borne in mind. The archenteron roof does not have the machinery to relieve the inhibition brought about by o-fluorophenylalanine in the absence of exogenous materials necessary to the synthesis of phenylalanine. Furthermore, inspection of the structural formulae of these exogenous compounds (fig. 14) indicates that the most probable synthetic pathway lies through condensation across an α-β carbon bond following the decarboxylation of phenylacetic acid.

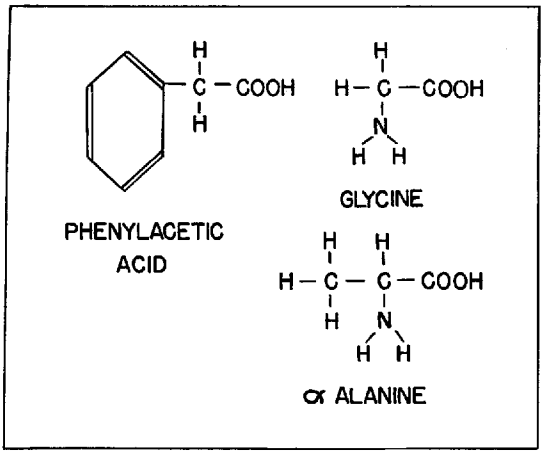


Figure 14

The utilization of α-alanine in such a synthetic sequence would require the removal of its methyl group.

It can be concluded from the experiments, summarized by equations 5, 6, 9, 10, 11 and 12, that relief of the inhibition of differentiation of the neural crest cells has been brought about through synthesis of phenylalanine by the archenteron roof mesoderm, and the presentation of this compound, causally associated with crest differentiation, to the environment of the explanted neural crest. The most probable method of synthesis lies through chemical pathways involving, as a major step, condensation of glycine with phenyl-methyl (benzyl) across the α-β carbon bond to form an alanine side chain.

The effect of alterations at the β-carbon atom

In view of the importance in differentiation metabolism of an ultimate condensation between the α and β carbon atoms to yield an alanine side chain of phenylalanine, it was important to test the role of this portion of the phenylalanine molecule in the differentiation of the neural crest. It had previously been established that the α-amino group was essential in pigment cell differentiation, while the phenyl group was essential for the differentiation of ectomesenchyme (Wilde,

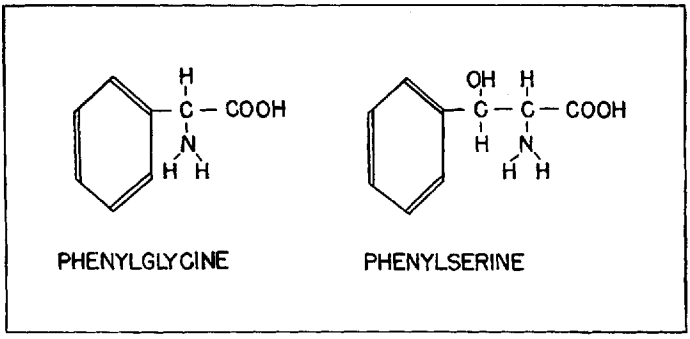


Figure 15

TABLE 3

MEDIUM AND SUPPLEMENT	NUMBER OF EXPERIMENTS	
	Pure cranial neuroepith.	Cranial neuroepith. and mesoderm
Basal medium with 1.0 mM/L. phenylalanine (Control)	12	12
Basal medium with 1.0 mM/L. phenylglycine	12	12
Basal medium with 1.0 mM/L. phenylglycine and 1.0 mM/L. phenylalanine	12	12
Basal medium with 1.0 mM/L. phenylserine	12	12
Basal medium with 1.0 mM/L. phenylserine and 1.0 mM/L. phenylalanine	12	12

'55a, '55b). Information concerning the role of the β-carbon atom would aid in understanding the functional aspects of the whole molecule.

Compounds varying from phenylalanine at the β-carbon atom were tested for their effects on the cellular differentiation of the neural crest. The compounds tested were: Phenylglycine, where the β-carbon atom is absent, and phenylserine, where the β-carbon carries an hydroxyl group rather than hydrogen (fig. 15).

When pure cranial neuroepithelium is explanted to nutrient medium supplemented with 1.0 millimoles/L. phenylglycine, no cellular differentiation occurs regardless of the length of time in vitro (up to 13 days). The cells remain ameboid and morphologically resemble those seen in figures 1 and 2. The result is total unrelieved inhibition of differentiation. Rarely a clear colorless cell with blunt stellate processes is seen. The normal differentiation seen in control specimens (fig. 3) does not take place. It would appear, therefore, that phenylglycine acts as an inhibitor of cellular differentiation of the neural crest. In equation form:

$$11\text{–}14\left[\frac{\text{cran. neuroepith.}}{\text{basal} + \text{PGL}}\right] \dashrightarrow \text{total inhib.} \qquad (15)$$

When archenteron roof mesoderm and cranial neuroepithelium are explanted together to nutrient medium supplemented with 1.0 millimoles/L. phenylglycine, the inhibition summarized in equation 15 is relieved. Normal differentiation, similar in all respects to the controls, takes place (figs. 4 and 7; compare with figs. 3 and 6, examples of the controls).

$$11\text{–}14\left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{PGL}}\right] \dashrightarrow \text{normal diff.} \qquad (16)$$

The inhibition to cellular differentiation brought about by cultivation of explants of cranial neuroepithelium in medium supplemented with phenylglycine is relieved by the presence in the culture of cells of archenteron roof mesoderm. These

mesodermal cells, in addition to carrying out their own self-differentiation (fig. 10), appear to provide the proper chemical environment for neural crest cell differentiation. Since phenylglycine lacks a β-carbon atom, it seems probable that the mechanism of inhibition relief found in the mesoderm lies in the metabolic conversion of phenylglycine to phenylalanine. Without prejudice toward the unknown intermediate pathways of such a process, the end result would be the insertion of a β-CH_2 group in the molecule, or the replacement of the glycine side chain by alanine. It should be noted that an exogenous source of the β-CH_2 group cannot be specified in these experiments in contrast to those reported above.

When pure cranial neuroepithelium is explanted to nutrient medium supplemented with 1.0 millimoles/L. phenylglycine and 1.0 millimoles/L. phenylalanine, differentiation of the explant is in all respects similar to the controls (figs. 5 and 8). Therefore, an equimolar amount of phenylalanine acts to relieve the inhibition to cellular differentiation of the neural crest components brought on by phenylglycine as efficiently as does the archenteron roof mesoderm.

$$11\text{–}14\left[\frac{\text{cran. neuroepith.}}{\text{basal} + \text{PGL} + \text{DLPA}}\right] - - - - - \rightarrow \text{normal diff.} \quad (17)$$

When both cranial neural crest and archenteron roof mesoderm are explanted together and cultivated in nutrient medium supplemented with 1.0 millimoles/L. phenylglycine and 1.0 millimoles/L. phenylalanine, differentiation remains perfectly normal and is strictly comparable to that seen in the controls.

$$11\text{–}14\left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{PGL} + \text{DLPA}}\right] - - - - - \rightarrow \text{normal diff.} \quad (18)$$

If phenylserine is substituted for phenylglycine, the situation with regard to inhibition of differentiation of the neural crest is fundamentally similar. Thus, the inhibition of differentiation of the cells of the neural crest brought on by inclusion in the medium of 1.0 millimoles/L. phenylserine is relieved

equally well be equimolar phenylalanine or by archenteron roof mesoderm. Therefore:

$$11\text{–}14\left[\frac{\text{cran. neuroepith.}}{\text{basal} + \text{PSE}}\right] \text{- - - - - -} \rightarrow \text{total inhib.} \quad (19)$$

$$11\text{–}14\left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{PSE}}\right] \text{- - - - - -} \rightarrow \text{normal diff.} \quad (20)$$

$$11\text{–}14\left[\frac{\text{cran. neuroepith.}}{\text{basal} + \text{PSE} + \text{DLPA}}\right] \text{- - - - - -} \rightarrow \text{normal diff.} \quad (21)$$

$$11\text{–}14\left[\frac{\text{cran. neuroepith.} + \text{meso.}}{\text{basal} + \text{PSE} + \text{DLPA}}\right] \text{- - - - - -} \rightarrow \text{normal diff.} \quad (22)$$

In one of the twelve experiments where pure cranial neuroepithelium was explanted and cultivated in nutrient medium supplemented with 1.0 millimoles/L phenylserine, differentiation of pigment cells was nearly normal. This was the only anomalous case in all of the experiments reported here. It was probably due to accidental dilution of the inhibitory medium during transfer of the explant.

The inhibitory effects of phenylglycine and phenylserine upon the differentiation of neural crest cells are similar and differ from those inhibitions by other phenylalanine analogues previously obtained in that the inhibitory effects are relieved by archenteron roof mesoderm as well as by phenylalanine. Structurally, both compounds vary from phenylalanine by differences at the β-carbon atom (fig. 15).

The β-carbon is absent in phenylglycine and it bears an hydroxyl group in place of hydrogen in phenylserine. Conversion of phenylserine to phenylalanine requires the removal of the β-carbon hydroxyl and its replacement by hydrogen. The ability to carry out this reaction (or one similar) is inherent in the cells of the archenteron roof mesoderm. Phenylglycine conversion to phenylalanine has been discussed previously.

The conclusion to be drawn from the foregoing experiments is that a β-carbon atom configuration is an important molecular locus in the chemical reactants by means of which the cranial neural crest undergoes its normal differentiation. Further-

more, the β-group must be CH_2. Other groupings, for instance HCOH, do not satisfy the requirements of the neural crest cells' differentiation metabolism. The presence of the β-CH_2 group may well be important in the spatial distribution of the other reactive groups of molecular phenylalanine in differentiation reactions.

Elicitation of pigment cells from ventral ectoderm through "mesodermal" synthesis of phenylalanine

When urodele ventral ectoderm is explanted to nutrient medium, its differentiation is limited to the formation of epithelial sheets. If, however, the nutrient medium is supplemented with from 2.0 to 8.0 millimoles/L. phenylalanine, many pigment cells indistinguishable from pigment cells of neural crest origin differentiate. This phenomenon has been called elicitation (Wilde, '55a, '55b). Elicitation takes place under these circumstances in the presence or in the absence of ventral "mesoderm."

$$11\text{–}14\left[\frac{\text{ventr. ecto.}}{\text{basal}}\right] \longrightarrow \text{normal diff. (epith.)} \qquad (23)$$

$$11\text{–}14\left[\frac{\text{ventr. ecto.}}{\text{basal} + \text{DLPA}}\right] \longrightarrow \text{elicitation pigment c.} \qquad (24)$$

If the ability to control the environment of overlying cells by synthesis of selected substrates is a general property of mesoderm, then supplying materials from which phenylalanine might be synthesized by mesodermal cells could lead to the elicitation of pigment cells from ventral ectoderm. Experiments were designed to test this possibility using phenyllactic acid and β-2 thienylalanine (see equations 3, 4, 5, 6, 7 and 8).

TABLE 4

MEDIUM AND SUPPLEMENT	NUMBER OF EXPERIMENTS	
	Pure ventral ectoderm	Ventral ectoderm and "mesoderm"
Basal medium with 2.0 mM/L. of both phenyllactic acid and β-2 thienylalanine	16	12
Basal medium	8	

When pure ventral ectoderm is explanted to nutrient medium supplemented with 2.0 millimoles/L. of both phenyllactic acid and β-2 thienylalanine, there is no elicitation of pigment cells, anomalous pigment cells (Wilde, '55a, '55b), or any deviation from the differentiation into epithelial sheets seen in the controls.

$$11\text{–}14\left[\frac{\text{ventr. ecto.}}{\text{basal} + \text{Phen} + \beta\text{-2 THA}}\right] \longrightarrow \text{normal diff. (epith.)} \qquad (25)$$

Conversely, when ventral ectoderm and internal cells ("mesoderm") are explanted and cultured in nutrient medium supplemented with 2.0 millimoles/L. of both phenyllactic acid and β-2 thienylalanine, a moderate number of abnormal or anomalous pigment cells differentiates (figs. 16, 17, 18). In a few cases, some of the free cells which migrate from the explant become somewhat dendritic. In these cells there is a moderate amount of melanin synthesis with granules which are homogenous in size and dispersed throughout the cytoplasm of the cells (figs. 16 and 18). The rounded anomalous pigment cells show excellent melanin synthesis and become very dark. Their cytoplasm becomes filled with melanin granules (fig. 17). It may be concluded that the internal ventral cells of the urodele embryo can synthesize phenylalanine from exogenous precursors supplied in the environment, although not as well as cells of the dorsal mesoderm (archenteron roof). This is reflected in the elicitation of pigment cells, although here the response is weaker than that seen with added molecular phenylalanine itself.

$$11\text{–}14\left[\frac{\text{ventr. ecto.} + \text{"meso."}}{\text{basal} + \text{Phen} + \beta\text{-2 THA}}\right] \longrightarrow \begin{matrix}\text{elicitation anomalous}\\ \text{pigment c.}\end{matrix} \qquad (26)$$

The data just reported make it more probable that the elicitation reaction in ventral ectoderm is specifically a property of phenylalaline concentration in the environment of the ventral ectoderm. It should be noted that both phenyllactic acid and β-2 thienylalanine are compounds structurally similar to phenylalanine itself, yet they have very specific inhibitory activities against particular types of neural crest

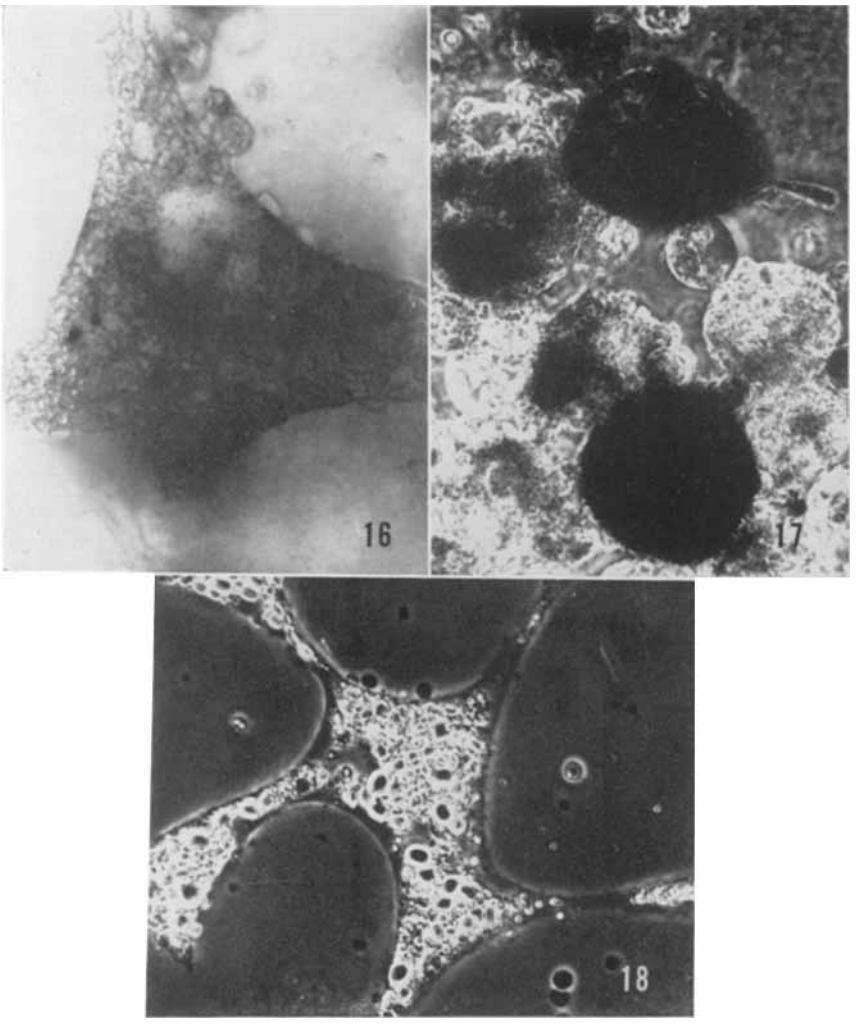


Fig. 16 Explant of ventral ectoderm with internal ventral cells (''mesoderm''), cultivated for 6 days in basal medium supplemented with 2.0 mM/L. of both phenyllactic acid and β-2 thienylalanine. A free stellate cell with beginning dendritic processes and mild melanin synthesis has been induced. Explanted at stage 12. × 300.

Fig. 17 Explant of ventral ectoderm with internal ventral cells (''mesoderm''), cultivated for 10 days in basal medium supplemented with 2.0 mM/L. of both phenyllactic acid and β-2 thienylalanine. Heavy melanogenesis and free anomalous pigment cells induced. Explanted at stage 11. × 300.

Fig. 18 Explant of ventral ectoderm with internal ventral cells (''mesoderm''), cultivated for 12 days in basal medium supplemented with 2.0 mM/L. of both phenyllactic acid and β-2 thienylalanine. Free stellate cell and very slight amount of pigment. Explanted at stage 11. × 300.

cellular differentiations (see equations 3 and 4). The compounds together do not elicit even anomalous pigment cells from pure ventral ectoderm in the absence of mesoderm.

The data presented may be readily summarized by reading serially equations 1 to 26. These lead to the following general conclusions: A metabolism in which phenylalanine is concerned is an essential mechanism in the differentiation of pigment cells and ectomesenchyme from the cranial neuroepithelium of the urodele embryo. The whole molecule is essential and (at least early in the period of cell differentiation) it is not present in sufficient amounts in the cells themselves, or if present its use in cellular differentiation is readily interfered with. Phenylalanine is apparently synthesized and transferred to the environment of the competent cells from underlying mesoderm. This embryonic region has kinetic properties, probably enzymatic, for the synthesis and transfer of phenylalanine to the environment of the neural crest. The mechanism must involve the transfer of two-carbon fragments and their condensation directly or indirectly at an α-β bond with phenylmethyl groups. Furthermore, the materials for this synthesis appear to be supplied exogenously in in vitro experiments.

DISCUSSION

These experiments have confirmed the causal relation between a particular metabolism of phenylalanine and the biochemistry and morphology of differentiation of the cells of the urodele neural crest. When considered along with the previously reported experiments in this series (Wilde, '55a, '55b), with those of Markert ('55), those of Fell and Mellanby ('53) and of Weiss and James ('54) it becomes evident that a profound control over both process and type of cellular differentiation is exerted by the metabolic environment within which an embryonic cell exists.

A preliminary insight into the mechanisms of cellular differentiation has also been obtained and certain statements concerning these mechanisms can now be made. It would be naïve to assume that a particular concentration of phenylalanine

alone was the cause of a type of cellular differentiation. A more logical conception is that a specific ratio of metabolites provides the materials from which the synthetic machinery of a particular embryonic cell may refine its structure. Included in this ratio there is a precise level of molecular phenylalanine. Other ratios would presumably define the response to be expected of cells in other types of differentiation. This reasoning leads to the conclusion that cellular differentiation is an adaptive process.

If cellular differentiation is in fact an adaptive process, then some regulating system is necessary to ensure that the proper cells are provided with their proper metabolic environment in time and space. It is in such regulatory processes that the chemical mechanisms elucidated in the present experiments find their closest relationship to events established in classical experimental embryology. The similarity between mesodermal relief of inhibition, elicitation of pigment cells from ventral ectoderm by phenylalanine, or by mesodermal synthesis of specific metabolites and embryonic inductions has already been noted.

Inspection of the present experimental data indicates that the provision of the proper environment to the neuroepithelium is a property of the archenteron roof mesoderm. This control appears to be carried out by chemical reactions which provide phenylalanine in the environment within which the crest component will differentiate. It has also been established that phenylalanine in fully synthesized form is a necessary environmental agent, and that pure neural crest does not have enzyme systems for the synthesis of phenylalanine from exogenous precursors. This statement is, however, valid only within the parameters set by the present experiments. The archenteron roof is the cellular derivative of the dorsal lip of the blastopore, the "organizer" of Spemann ('38). As an organizer or inductor, the function of the archenteron roof is to induce an overlying medullary plate and neural crest. A feasible chemical mechanism for such an induction lies in the establishment of suitable substrate ratios in the environment

of the target cells. The cells of the presumptive neuroepithelium respond by adapting their metabolism to the substrates provided, and this, in turn, defines their future biochemical and morphological differentiations. Important in all studies of embryonic induction (and equally important in the present experiments), is the ability of subject cells to respond to the inductive stimulus or biochemical environment. This has been defined as competence (Waddington, '32).

Competence is a term which sums the ability of the enzyme complement of the embryonic cell to adapt to a particular ratio of metabolites. The adapted enzymes, the substrates used, and the synthetic products would in turn define the structure of the differentiated cell. It must be borne in mind that without competence, induction and inductors would have remained unknown. From this analysis and the experiments presented, it is probable that no unitary substance or inductor exists (Grobstein, '54). Induction, to have meaning, must refer to the constellations of enzymes which provide metabolites in the proper ratio to which the enzyme sequences of competent cells may adapt. Singularity in an inductor, as an eye-inductor, can only refer to a limiting enzyme concentration or reaction, in which case the factor which limits the enzyme itself is the inductor. For the purpose of retaining the value of the classical concept of induction, singularity must be abandoned, or the concept tends to absurdity.

Examination of some penetrating modern experiments on induction (Grobstein, '53; Niu and Twitty, '53) shows that the biochemical interpretation given above is valid for the data. In fact, the experiment of Niu and Twitty in which inductions of neurons and pigment cells were obtained in competent ectoderm placed in culture medium previously "toned" by the cultivation of archenteron roof mesoderm, finds a direct interpretation in the experiments reported here. The archenteron roof released into the medium the proper metabolite concentrations to which small fragments of competent tissues could respond by particular differentiations.

When the data from previous experiments (Wilde, '55a, '55b) and those in the present report are surveyed (equations 1 through 26), the degree of specificity of the molecule phenylalanine for the processes of cell differentiation in the neural crest becomes increasingly apparent. Not only is the intact molecule essential for neural crest differentiation, but configurationally most portions of the molecule cannot be changed. The phenyl group is particularly important in the chemical reactions whereby ectomesenchyme differentiates. The α-amino group plays a similarly important role in pigment cell differentiation. The β-carbon atom must be present (equations 15–18) and must bind two hydrogen atoms by covalent bonds. Where this does not occur the resulting compound acts as an inhibitor. HCOH as a group in the β-position does not suffice; such a compound is inhibitory (equations 19–22). Although chemical reactions which are concerned with these cell differentiations have not been further clarified, it should be pointed out that the bond strengths and angles peculiar to phenylalanine can be altered only with detriment to the metabolic system of neural crest differentiation.

The α-β carbon bond is also of importance in the archenteron roof synthesis of phenylalanine as part of the induction process. Synthesis of an α-β carbon bond between glycine-like fragments and phenylmethyl fragments to form phenylalanine is the common chemical denominator in all experiments where the archenteron roof mesoderm acted to relieve the inhibition to neural crest differentiation caused by a phenylalanine analogue.

It remains for future studies to discover whether phenylalanine is itself the only active compound in this system or whether some intermediate compounds are more directly concerned. Since information about such compounds is not presently available, the principle of paucity of hypotheses must obtain.

CONCLUSION

An extended analysis of the causal relations between the differentiation of cells of the urodele neural crest and a spe-

cific series of metabolic reactions involving phenylalanine has been carried out. Short term tissue cultures of precise anlagen explants were made and cultivated in protein-free nutrient medium with varying supplementations.

The experiments indicate that molecular phenylalanine is required by cells of the early neural crest for their specific differentiations. This has been demonstrated by three series of experiments in which three different groups of possible precursor molecules would *not* support differentiation of explanted neural crest cells. These combinations were:

(a) Phenyllactic acid and β-2 thienylalanine
(b) Phenylacetic acid and glycine
(c) Phenylacetic acid and α-alanine

Further experiments with the same precursor molecules indicate that the archenteron roof mesoderm cells of embryos of stages 11 to 14 (Harrison) are capable of overcoming inhibitions to cellular differentiation of the neural crest cells, provided materials from which phenylalanine could be synthesized are made available. It is probable that the mechanism of inhibition relief is through synthesis of phenylalanine. This is apparently brought about by enzymatic activity of archenteron roof cells whereby ultimately a phenylmethyl moiety is condensed with a glycine-like moiety across an α-β bond to form phenylalanine.

The type of chemical activity expressed by archenteron roof cells and its effect upon the co-cultured neural crest cells is similar to embryonic induction.

Investigation of the β-carbon position of phenylalanine indicates that this molecular locus cannot be altered without inhibiting the cellular differentiation of cultured neural crest cells (phenylglycine; phenylserine). Inhibition by analogues which differ from phenylalanine at the β-carbon atom is reversible by equimolar phenylalanine or by activity of archenteron roof mesoderm cells.

Differentiation of archenteron roof mesoderm cells into striated muscle is *not* affected in these experiments.

Anomalous pigment cells have been induced from competent ventral ectoderm by mesoderm (internal ventral cells) using environmentally supplied phenyllactic acid and β-2 thienylalanine (2.0 mM/L.). Since these compounds singly are specific inhibitors of certain types of crest cell differentiations, and further, since together they may be used by co-cultured archenteron roof for inhibition relief via phenylalanine synthesis, this induction confirms mesodermal production of phenylalanine and environmental control of cellular differentiation.

LITERATURE CITED

DuShane, G. P. 1935 An experimental study of the origin of pigment cells in amphibia. J. Exp. Zool., *72:* 1–31.

Fell, H. B., and E. Mellanby 1953 Metaplasia produced in cultures of chick ectoderm by high vitamin A. J. Physiol., *119:* 470–488.

Gregg, V. R., and W. O. Puckett 1943 A corrosive sublimate fixing solution for yolk-laden amphibian eggs. Stain Tech., *18:* 179–180.

Grobstein, C. 1953 Morphogenetic interaction between mouse tissues separated by a membrane filter. Nature, *172:* 869–870.

———— 1954 Tissue interaction in the morphogenesis of mouse embryonic rudiments in vitro. Aspects of Synthesis and Order in Growth. Thirteenth Growth Symposium, 233–256. Princeton Univ. Press.

Holtfreter, J. 1946 Structure, motility and locomotion in isolated amphibian cells. J. Morph., *79:* 27–62.

Hörstadius, S. 1950 The Neural Crest. Oxford Univ. Press.

Markert, C. L. 1955 Substrate utilization in cell differentiation. Ann. N. Y. Acad. Sci., *60:* 1003–1014.

Niu, M. C., and V. C. Twitty 1953 The differentiation of gastrula ectoderm in medium conditioned by axial mesoderm. Proc. Nat. Acad. Sci., *39:* 985–989.

Spemann, H. 1938 Embryonic development and induction. Yale Univ. Press.

Waddington, C. H. 1932 Experiments on the development of chick and duck embryos, cultivated in vitro. Phil. Trans. Roy. Soc. B., *221:* 179–230.

Weiss, P., and R. James 1954 Vitamin A and skin keratinization in vitro; experimental dissociation of induction and realization phases in cytodifferentiation. Science, *119:* 587.

White, A., P. Handler, E. L. Smith and D. Stetten, Jr. 1954 Principles of Biochemistry. McGraw-Hill Book Company.

Wilde, C. E., Jr. 1948 Technical procedures for the study of organogenesis in vitro in Amblystoma. Proc. Soc. Exp. Biol. Med., *69:* 374–376.

———— 1950 Studies on the organogenesis in vitro of the urodele limb bud. J. Morph., *86:* 73–114.

WILDE, C. E., JR. 1952 Studies on the organogenesis in vitro of the urodele limb bud. II. The effect of size of the explant and the interrelation between gill and limb. J. Morph., *90:* 119–148.

———— 1955a The urodele neuroepithelium. II. The relationship between phenylalanine metabolism and the differentiation of neural crest cells. J. Morph., *97:* 313–344.

———— 1955b The role of phenylalanine in the differentiation of neural crest cells. Ann. N. Y. Acad. Sci., *60:* 1015–1025.

———— 1955c The urodele neuroepithelium. I. The differentiation in vitro of the cranial neural crest. J. Exp. Zool., *130:* 573–596.