AN ANALYSIS OF CELLULAR AND SUBCELLULAR SYSTEMS WHICH TRANSFORM THE SPECIES CHARACTER OF ACID PHOSPHATASE IN *ACETABULARIA*

KONRAD KECK, Ph.D., and ELIZABETH A. CHOULES

From the Department of Biology, Johns Hopkins University, Baltimore

ABSTRACT

Several species-specific molecular forms of acid phosphatase are known to exist in the unicellular green alga *Acetabularia*. In graft combinations between cells of *Acetabularia mediterranea* (*med*) and *Acicularia Schenckii* (*acic*) the expression of the *med* phosphatase is dominant over *acic* phosphatase. There is good evidence that in such grafts the preexisting *acic* phosphatase is converted on the molecular level *via* an intermediate form to the *med* phosphatase. This conversion can be initiated by the transplantation of a *med* cell nucleus to an anucleate *acic* cell, but will also take place in grafts between anucleate *med* and anucleate *acic* cells, indicating that the direct participation of a cell nucleus is not required. An incomplete conversion of *acic* phosphatase, which terminates at the intermediate stage, is induced in *acic* cells by injection of a concentrated homogenate of *med* cytoplasm. A similar partial conversion occurs *in vitro* in a mixture of homogenates from *med* and *acic* cells. Subcellular particles, such as chloroplasts or mitochondria, can be removed from the homogenates by centrifugation without impairing the reactions leading to the intermediate phosphatase type. Experimental evidence suggests that the transformation of phosphatase types is enzymatically catalyzed and may involve the conjugation of small molecules with the phosphatase protein. It was shown, however, that sialic acid is not involved, since the incubation of *med* or *acic* homogenates with neuraminidase did not modify the electrophoretic mobility of either enzyme type. Another type of phosphatase, which occurs in *Acetabularia crenulata* (*cren*) and can be distinguished electrophoretically from the aforementioned types, is not subject to interaction. In various mono- and multi-nucleate graft combinations between *cren* cells on one hand, and *med* or *acic* cells on the other hand, the *cren* phosphatase is synthesized independently of the enzyme of the graft partner.

INTRODUCTION

The anucleate cytoplasm of the unicellular green alga *Acetabularia* retains a remarkable capacity to synthesize proteins (1–3). It has been reported that the activity of several enzymes increases at near normal rates over a period of 1 to 3 weeks after enucleation, probably sustained by a large cytoplasmic pool of genetic information which was originally supplied by the cell nucleus (4–6). The occurrence in *Acetabularia* of species-specific molecular forms of acid phosphatase, which can be separated conveniently by starch gel electrophoresis (7), permits the detection of short term

activities of the nucleus. Such control activities can be distinguished from preexisting synthetic capacities of the cytoplasm on the basis of the species label of this enzyme protein.

Transplantation experiments with nuclei from *Acetabularia mediterranea* (*med*) and *Acicularia Schenckii* (*acic*) revealed, however, that the expected additive relationship between both types of phosphatase did not exist (7, 8). Although short term effects of the implanted nucleus were observed, these did not comprise a complementation of the *acic*, host type phosphatase by the type which is characteristic for the nuclear donor species. Instead, one phosphatase type seemed to be dominant over the other type in that in a variety of cellular grafts, including hybrid multinucleate grafts, the presence of a *med* nucleus, or of *med* cytoplasm in any combination with *acic* components would invariably bring about or maintain the *med* character of this enzyme. Shortly after the transplantation of a *med* nucleus to *acic* anucleate cells, a new transient phosphatase type appeared in place of the *acic* enzyme (8). The electrophoretic mobility of the transient phosphatase was intermediate between the mobilities of both parental phosphatase types. The occurrence in such grafts of a stage in which all phosphatase activity could be recovered in the intermediate form suggested that a conversion of the enzyme protein on the molecular level had taken place rather than a substitution of one population of enzyme molecules by another. Hence, the phenomenon of dominance could be interpreted as an expression of a preferred direction of the molecular process. The reactions involved seemed to be under control of the *med* genome since *acic* phosphatase is changed specifically into a form which is electrophoretically indistinguishable from the *med* enzyme.

Although the original investigations with species-tagged phosphatase were carried out primarily for the purpose of demonstrating and characterizing short term *de novo* activities of an implanted cell nucleus, we have also attempted to gain more information about the interesting phenomenon of phosphatase conversion, particularly concerning the chemical nature of the conversion process and of the cytoplasmic systems which are involved in controlling protein structures.

Two different experimental approaches to these problems are reported in the present paper. The first part of the paper—Interacting Systems—deals with partially resolved cytoplasmic systems which are still capable of carrying out a modification of the *acic* phosphatase structure. The second part—Non-Interacting Systems—follows a preliminary report on a new phosphatase type in *Acetabularia crenulata*, which seems to be codominant in the corresponding graft combinations with *med* or *acic* phosphatase, and, therefore, not convertible into one of the other two forms.

METHODS

Acetabularia was grown in Erdschreiber Medium following the techniques which were described by Hämmerling (9) and Beth (10). Microsurgical operations and dissections were carried out with stainless steel instruments, such as De Wecker iridectomy scissors and Watchmakers' forceps.

Anucleate hybrid grafts between *med* and *acic* cells were prepared from full grown cells shortly before cap formation. Rhizoid-amputated stalks were joined at their basal ends in a telescope-like manner, and the grafts were then allowed to recover overnight in low intensity illumination.

The procedures for collecting concentrated preparations of cytoplasm are described in detail in the Results section of this paper. The cytoplasm of cells is sedimented by spinning in a special adapter (Fig. 1) at 7500 RPM for 15 minute at 5°C in the No. 856-head of the International refrigerated centrifuge, model PR-2. The glass micropipettes for the injections were hand-drawn from precision capillary tubing, 1.00 mm OD and 0.80 mm ID (Drummond Scientific Co., Philadelphia), and connected via 22-gauge stainless steel needle stock to a 50 μl No. 705 microliter syringe (Hamilton, California), which was operated by means of a micrometer drive. Freshly boiled distilled water was used as fluid in the all-hydraulic injection system. The material to be injected was separated from the hydraulic fluid by a seal of silicon oil. For guidance, the injection needle was attached to a Singer microdissector (Singer Instrument Co., Reading, England). After injection the cells were ligated, a short distance behind the cut, with No. 70 white cotton thread which had been previously soaked and sterilized by brief submersion in boiling water.

Homogenates for the *in vitro* experiments were obtained from whole cells which were freed of excessive culture medium by careful blotting on filter paper, and then ground without the addition of any liquid in a glass homogenizer. Broken cell walls were removed by filtration through a double layer of cheese cloth (Grade 90). Chloroplasts and mitochondria were removed from small aliquots of these homogenates by centrifugation in steel tubes for 20

minutes at 20,000 RPM (26,360 × *g*, average) in the No. 40 head of the Spinco Model L ultracentrifuge.

For the neuraminidase treatment the 26,360 *g* supernatants of homogenates from *med* and *acic* cells were neutralized to pH 6.9 by the addition of dilute NaOH. Then 0.4 ml of a concentrated preparation of neuraminidase (Behringwerke, A.G., Marburg, Germany) was added to each 1.0 ml of the neutralized supernatants together with a few drops of toluene. The samples were incubated for 5 days at 25°C along with control samples in which the neuraminidase solution was replaced by the same amount of water.

For the analysis of phosphatase types by starch gel electrophoresis, an individual cell or cell part was placed on a small Lucite block, the stalk cut open at one end and the content of the cell squeezed out. A small drop containing cytoplasm and cell sap was thus produced. The material was absorbed to a small piece of Whatman 3 mm filter paper, which was then inserted in the starch gel.

The gels were prepared with 0.05 M Tris(hydroxymethyl)aminomethane buffer pH 8.9 and 8.5. Electrophoresis was carried out at room temperature (23°C) for 5 hours in a voltage gradient of 4 v/cm. Then the starch blocks with the dimensions of 6 × 25 × 300 mm were cut horizontally into 4 slices, each 1½ mm thick. For the histochemical demonstration of phosphatase activity, the two center slices were incubated in the dark for 12 hours in a solution of 300 mg of Fast Blue BB and 30 mg of sodium alpha-naphthyl acid phosphate in 100 ml of 0.1 M acetate buffer pH 4.6.

A minimum of 20 grafts of each of the described combinations were prepared and tested individually in starch gel electrophoresis in the above-outlined manner.

RESULTS

Interacting Systems

The interaction of unknown *med* factors with *acic* phosphatase, which ultimately leads to the conversion of this enzyme into the *med* form, can be brought about, as previously reported, by the transplantation of a *med* cell nucleus to an *acic* cytoplasm (med_1-$acic_0$).[1] The responsible factors thus seem to be contained in, or synthesized under the control of, the *med* nucleus. The *med* determining factors, once present in the cytoplasm, will remain effective even after the inducing *med* nucleus has been removed, as has been demonstrated with reciprocal grafts ($acic_1$-med_0) and also with binucleate hybrid cells ($acic_1$-med_1) by subsequent removal of the *med* nucleus. All the above-described graft combinations contain at least one nucleus from either species, and we may, therefore, assume that in interspecific grafts the majority, if not all of the vital synthetic processes are permanently sustained under such conditions. It remained to be investigated, however, whether the *acic* phosphatase was still subject to structural modifications in the complete absence of a cell nucleus, *i.e.*, in combinations of the anucleate cytoplasm of both these species.

Experimentally, an anucleate hybrid system could be obtained by grafting together rhizoid-amputated *acic* and *med* stalks at their basal ends (med_0-$acic_0$). The yield of successful grafts of this kind was rather low, in the order of 10 to 20 per cent of the operated cells, which was probably due to disturbances in the cytoplasmic structures imposed by the opposite polarity of the graft partners. Well healed grafts were maintained in culture for a period of 2 to 3 weeks. After this time the grafts were examined microscopically for cytoplasmic continuity at the junction. The *acic* and *med* moieties were identified, cut apart, and analyzed individually by starch gel electrophoresis.

A survey of the resultant zymograms demonstrated beyond doubt that a conversion of phosphatase types could indeed take place in the absence of a cell nucleus. A high percentage (*ca.* 75 per cent) of the grafts contained *med* phosphatase as the sole component in both moieties. In some grafts an intermediate type phosphatase was detectable, usually in the *acic* stalk, with pure *med* enzyme in the other stalk. In rare cases a graft contained a mixture of the parental enzyme types in one or both moieties, indicating that an interaction had not taken place in spite of mixing of the cytoplasm from both stalks.

In our next step toward further resolution of the system, we have replaced the anucleate *med* cell by a concentrated preparation of *med* cytoplasm which was injected directly into rhizoid amputated *acic* cells. This system offered certain advantages over the graft combinations of the previously described experiments. One could, for instance, subject the cytoplasm to various physical and chemical treatments prior to injection and thus characterize the active principle. The host cytoplasm could, furthermore, serve as an assay

[1] The composition of a cellular graft is symbolized by setting in subscript after the abbreviated names the number of nuclei of each represented species. Subscript *zero* identifies the anucleate cytoplasm.

system for subcellular fractions of another species which carry genetic information.

The preparation of concentrated homogenates from *Acetabularia* cells requires unusual procedures since in full-grown cells all cytoplasm lines the inside of the cell wall as an extremely thin layer surrounding a large cell vacuole. Due to the large amount of cell sap in relation to cytoplasm, a homogenate of cells prepared by the standard technique would result, even without the addition of any liquid to the cells, in great dilution of the cytoplasm rendering it unsuitable for injection into other cells. A concentrated preparation of cytoplasm can be obtained, however, by centrifugation of cells. Rhizoid-amputated and, therefore, enucleated algae were fastened at the basal end to a specially prepared Lucite adapter, which fitted into a 40 ml plastic centrifuge tube (Fig. 1). The cells were submersed in sea water and were centrifuged with their tips pointing in the direction of the centrifugal force. Almost all of the cytoplasm of a cell sedimented in the tip of the stalk, filling the terminal 2 mm as a compact, dark-green mass and leaving behind a colorless stalk. The dark portion of the tips was cut off under sea water and the cytoplasm of several tips drawn up into a fine micropipette, which was attached to a microinjection apparatus. This process caused a structural disintegration of the cytoplasm quite comparable to that obtained by grinding in a glass homogenizer.

The *acic* host cells were cut open either at the basal end by amputation of the rhizoid, or at the tip by removing a small apical portion of the stalks. The latter operation permitted injections into nucleate cells. With sterile sea water as the surrounding medium, cytoplasm collected from 1½ to 2 *med* cells was injected into the open vacuole space of one *acic* cell. The injected material remained clearly visible inside the host cell as an opaque, dark-green string with the diameter of the pipette orifice. The injected cytoplasm usually adhered immediately to the peripheral cytoplasm of the host cell where it spread and was gradually absorbed. Several hours subsequent to the injection, the material could still be recognized by the appearance of dark-green areas in the host cell. Leakage of injected material was prevented by ligation of the open end of the stalk immediately on withdrawal of the pipette. The injected cells

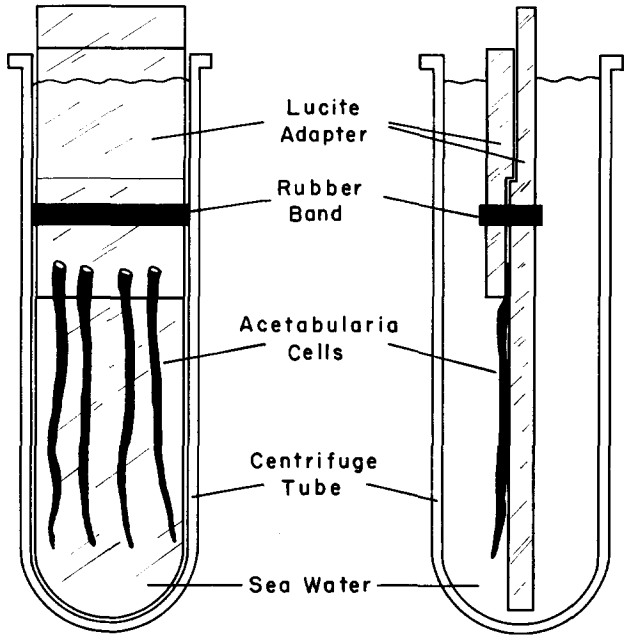


FIGURE 1 Front and side views of a Lucite centrifuge adapter holding *Acetabularia* cells in a position that permits centrifugation of the cytoplasm into the tips of the stalks.

recovered quite well. At regular intervals covering a period from 1 to 21 days after the operation, cells were tested individually by starch gel electrophoresis for the phosphatase types present.

Algae tested 1 day after the injection did not contain, as one would expect, *acic* and *med* phosphatase in approximately equal proportions. Instead, only very little *med* phosphatase was found, represented by a faint band in the starch gel in addition to an *acic* band of normal intensity. This result was somewhat surprising, since the amount of injected *med* cytoplasm slightly exceeded that of the host cytoplasm, and a subsequent loss of *med* cytoplasm from the stalks had been prevented by ligation. Therefore, a loss of enzyme protein could have occurred only during or after the structural disintegration of the *med* cytoplasm, either during its centrifugation or while the sedimented cytoplasm was slowly drawn into the micropipette. A degradation of *med* phosphatase after the injection into the vacuole space, perhaps due to its exposure to the acidic cell sap, can be ruled out on the basis of the *in vitro* experiments which are described below.

Approximately 1 to 2 weeks after the injection, an intermediate type phosphatase completely replaced the *acic* enzyme in the injected cells (Figs. 2 *a* and *b*). A further change from the intermediate type to pure *med* phosphatase could not be detected even after an additional 2 weeks. Nucleate *acic* cells, which were injected with an equal amount of *med* cytoplasm through a cut in the tips of the stalks, responded in the same way with only partial conversion of the host *acic* phosphatase. The apparent deficiency of this system, *i.e.*, its inability to carry out the complete conversion, must, therefore, be attributed to the *med* component. Since intact anucleate *med* cells are capable of inducing the complete process, it is most likely that the disintegration of the *med* cytoplasm was accompanied by destruction or loss of some of the responsible factors along with the previously mentioned loss of *med* phosphatase.

In another series of experiments, we injected cytoplasm of *med* cells, which had been kept in the dark for 3 weeks prior to the preparation of the homogenate. In *Acetabularia*, protein synthesis is inhibited in the dark; however, the amounts of total protein per cell, as well as the activity of acid phosphatase, remain almost constant under such conditions (3, 5). It was, therefore, surprising to find that the homogenates prepared from dark-treated *med* cells had lost to a great extent their capacity to induce a change in the *acic* phosphatase mobility. Even 4 weeks after the injection, the cells still contained *acic* phosphatase as major component. In some of the injected cells a slight trailing ahead of the *acic* band was noticeable, probably representing a very small amount of intermediate type enzyme in addition of the unchanged *acic* type.

The experiments with *med* homogenates do not allow us to draw any conclusions with respect to the role of the host cytoplasm in the partial conversion process. *Med* factors could, for instance, interact directly with the *acic* phosphatase molecules, or they might require for their expression the participation of certain synthetic processes. The similarity of the reactions which followed the injection of *med* cytoplasm into nucleate and into anucleate host cells does not necessarily support the first suggested mechanism, since many synthetic processes are known to continue in *Acetabularia* at almost normal rates for at least 1 week after enucleation. If, however, the *med* factors acted directly on *acic* phosphatase, maybe in the form of some enzymatic reactions, one would expect to find enzyme conversion occurring under *in vitro* conditions, *i.e.*, in a mixture of homogenates or their subfractions, from both species of *Acetabularia*.

Preliminary experiments in this direction have indicated that indeed some structural modification of *acic* phosphatase molecules did occur when *med* and *acic* homogenates were mixed and incubated at room temperature for several days. Each phosphatase type alone proved quite stable. In individual control homogenates the phosphatase activity did not decrease significantly over a period of 4 weeks at room temperature, provided that bacterial growth was retarded by the addition of a few drops of toluene to the preparation. Moreover, the species-specific electrophoretic mobilities remained unchanged during the incubation.

The cell homogenates for these experiments were prepared by grinding cells without the addition of any liquid in a glass homogenizer. *Acic* and *med* homogenates were mixed in a 1:1 ratio and incubated at 25°C for a total of 4 weeks. Changes in the electrophoretic mobility of the initially present 2 enzyme types were detected in samples which were taken after 2 days of incubation. In place of the 2 parental bands a very wide band was observed which almost occupied the space of the *acic* and *med* band plus their interspace. This band was also significantly wider than

the previously described intermediate band, its position with respect to the reference bands being slightly asymmetric, *i.e.*, the new band was slightly shifted toward the anode (Fig. 3, *f* and *g*). The phosphatase activity seemed evenly distributed throughout the band.

After 28 days of incubation essentially the same wide band was found, except that higher phosphatase activity was now encountered in the anodic half of the band. A changeover from this intermediate type enzyme to a characteristic *med* phosphatase, which was always found to comprise the final stage in cellular graft combinations, but not with injections of cytoplasm, could not be observed in mixtures of homogenates.

The reactions which are responsible for the appearance of an intermediate type enzyme in mixtures of homogenates are not dependent on the presence of larger subcellular particles. We have centrifuged the homogenates, prior to mixing, for 20 minutes at 20,000 RPM (26,360 *g*) and found that mixtures of the resultant clear supernatants, which are devoid of mitochondria and chloroplasts, but probably not of microsomes, gave very similar results. Some of the supernatants were neutralized and treated with the enzyme neuraminidase in order to test whether a part of the net electrical charge of the phosphatase molecules of either type was due to conjugated sialic acid residues. This test was negative in that even prolonged incubation of the phosphatase solutions with neuraminidase did not lead to changes in the electrophoretic mobilities of *med* or *acic* phosphatase.

Non-Interacting Systems

The terms "dominant" or "recessive" have been applied in this and earlier papers to characterize the relationship between *med* and *acic* phosphatase in divers graft combinations. As a simple explanation for the phenomenon of molecular dominance it was theorized that the recessive *acic* phosphatase might be a natural precursor of the *med* enzyme even in *med* cells, but not detectable as such due to the expected low concentrations of a transient form. The final steps during the biosynthesis of *med* phosphatase might involve, for instance, a conjugation of the "precursor" *acic* molecule with low molecular weight compounds which would thus add negative electric charges and increase the electrophoretic mobility, as detected in our assay. In this respect, the conversion of phosphatase types in hybrid grafts would actually comprise the completion of a biosynthetic process, which is somehow defective in its final stages in *acic* cells. This postulated relationship between both these enzyme types has not been thoroughly investigated from this angle. Particularly interesting seems to be the question, how phosphatase of hitherto untested species of *Acetabularia* would fit into the above-assumed pattern.

The phosphatase of our laboratory culture of *Acetabularia calyculus* and 2 varieties of *Acetabularia crenulata* were assayed in terms of activity per cell, activity per unit of protein in the homogenate, as well as for the electrophoretic mobilities in starch gel. The electrophoretic mobility of *A. calyculus* phosphatase could not be distinguished

FIGURE 2 In the starch gel zymograms illustrated in the Figs. 2 *a*, *b*, *d*, *e*, and *f*, individual cells were analyzed for acid phosphatase types. Serving as reference, a freshly prepared mixture of *med* and *acic* homogenates is included in every run and inserted in the outermost position to the right of each starch strip. In the reference insertion, the *acic* phosphatase type is represented by the upper, the *med* phosphatase by the lower of the two bands.

a, *b* Intermediate type phosphatase of five *acic* cells which were injected with *med* cytoplasm.

d, *e* Analysis of four binucleate *cren*-II_1-*med*$_1$ cells; both parental phosphatase types are present. The *cren*-II phosphatase shows slightly lower mobility than the *acic* reference enzyme on the right of the starch strips.

f Binucleate *cren*-II_1-*acic*$_1$ cells contain both phosphatase types; although not completely resolved, two overlapping bands demonstrate the presence of both enzyme types in each of the two analyzed cells.

c Full-grown cells with caps; *Acetabularia mediterranea* (cap on the left) and *Acetabularia crenulata*, variety II.

g Binucleate cells without caps, which have regenerated from grafts in which two rhizoids were joined.

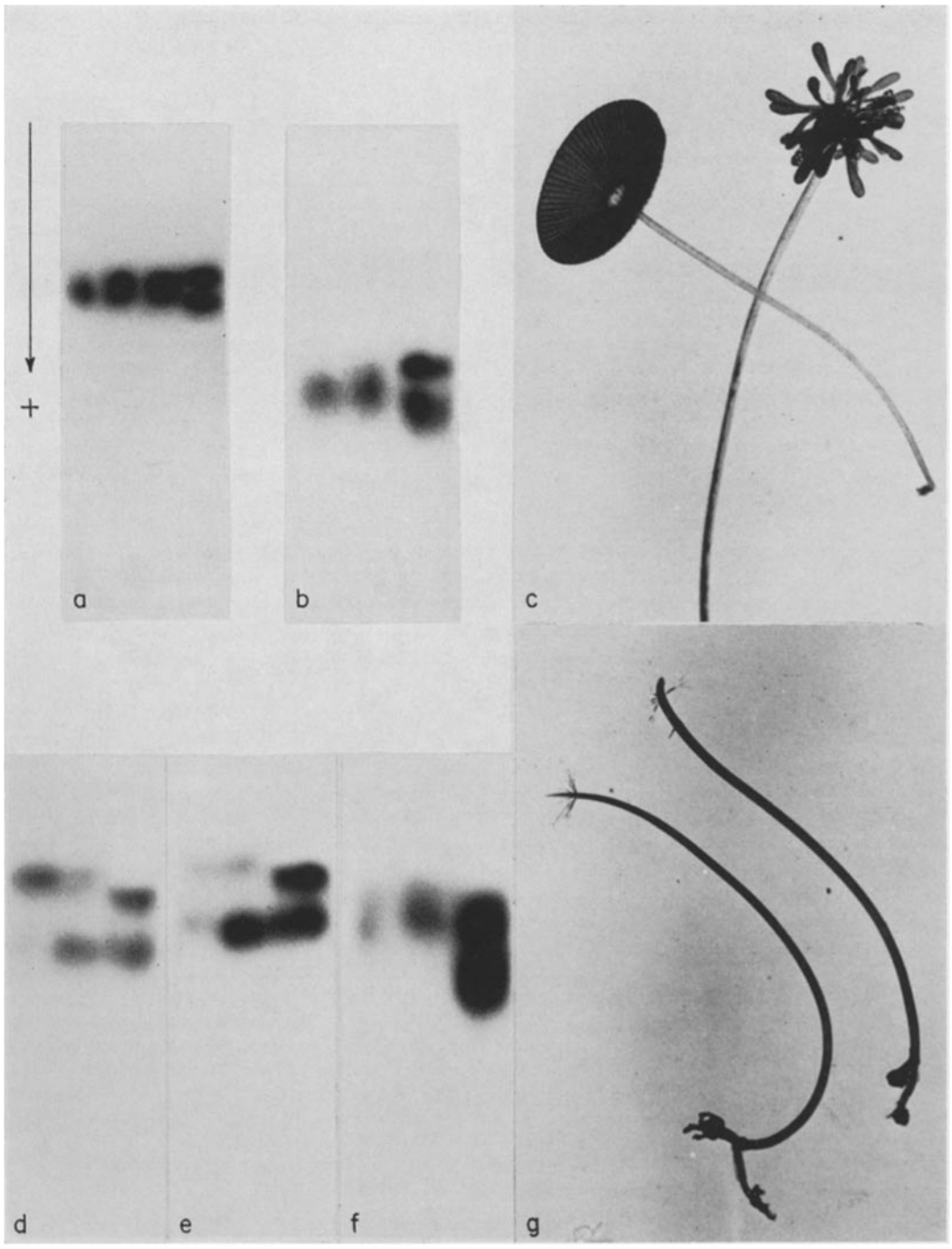


FIGURE 2

from that of *med* phosphatase, whereas the mobility of *A. crenulata*, variety I, phosphatase seemed to be the same as the mobility of *acic* phosphatase. Unfortunately, in both these newly tested species the activities of phosphatase in homogenates were so low that the electrophoretic analysis of individual cells was nearly impossible, which rendered these algae unsuitable for grafting experiments. The second variety of *A. crenulata* (*cren*-II), which was grown in culture from specimens which were originally collected in Bermuda, contained a new type of phosphatase. In starch gel of pH 8.9 the mobility of the new enzyme type was slightly lower than that of *acic* phosphatase. The difference became more pronounced when the pH of the gel was lowered to 8.5, although even at this pH a complete separation of *cren*-II phosphatase from *acic* phosphatase in mixture of homogenates could not be achieved.

We have prepared binucleate grafts as well as nuclear transplants between *cren*-II on one hand and *acic* or *med* cells on the other hand. Although the relatively low resolution of *cren*-II phosphatase in the presence of *acic* enzyme did not permit the unequivocal evaluation of the results obtained with grafts between these two species, especially with respect to the possible occurrence of intermediate forms, the general picture emerged from the experiments that interferences in the expression of the species-specific enzyme types of *cren*-II in combination with *acic* or *med* components did not take place. In both kinds of binucleate hybrid cells, *cren*-II_1-*med*$_1$ and *cren*-II_1-*acic*$_1$, both parental types of phosphatase were synthesized during the regeneration of the stalk (Fig. 2 *d* to *f*). In the case of all 4 nuclear transplant combinations, *cren*-II_1-*acic*$_0$, *cren*-II_0-*acic*$_1$, *cren*-II_1-*med*$_0$, and *cren*-II_0-*med*$_1$, the phosphatase type of the nuclear donor species appeared a few days after the operation in addition to the preexisting cytoplasmic phosphatase of the host. The *cren*-II enzyme type, therefore, should be considered to be codominant with the *med* or *acic* enzyme. In *cren*-II_1-*med*$_1$ binucleate cells, in which both types are synthesized simultaneously, the *med* phosphatase is generally, but not always, present in higher concentrations than the *cren*-II enzyme. The estimated ratio of activities, as judged from the histochemical reaction in starch gel, correlates very well with the specific enzyme activities, as determined in homogenates from either species and expressed as activity per cell or activity per unit of total protein in the homogenate.

DISCUSSION

The biological significance of the observed phenomena rests to a great extent upon the nature of the molecular modifications which underlie the conversion of one species-specific protein into another one. The most direct experimental approach to this problem, *i.e.*, isolation of the protein and its subsequent structural analysis, meets with great difficulties because of limitations in the amounts of available material which are imposed by the inherent slow growth of *Acetabularia* and the limited capacity of a laboratory culture of this alga.

The observed modifications of the electrophoretic mobility of *acic* phosphatase may be described in operational terms as a 2-step reaction, 1) formation of the intermediate type and 2) its change to the *med* phosphatase. No kinetic considerations involving two individual chemical reactions should be implied here. In fact, the observed variations in the width and position of the band which represents the intermediate type phosphatase in zone

FIGURE 3 *a* Zymogram in which the electrophoretic mobility of *acic* phosphatase and *med* phosphatase is compared to the mobility of these enzymes when contained in a mixture of homogenates from both species. *Acic* inserted on the left, the mixture in the center, and the *med* homogenate on the right side of the starch strip.

b Full-grown cells of *Acetabularia mediterranea* (left) and *Acicularia Schenckii* (right), both with caps.

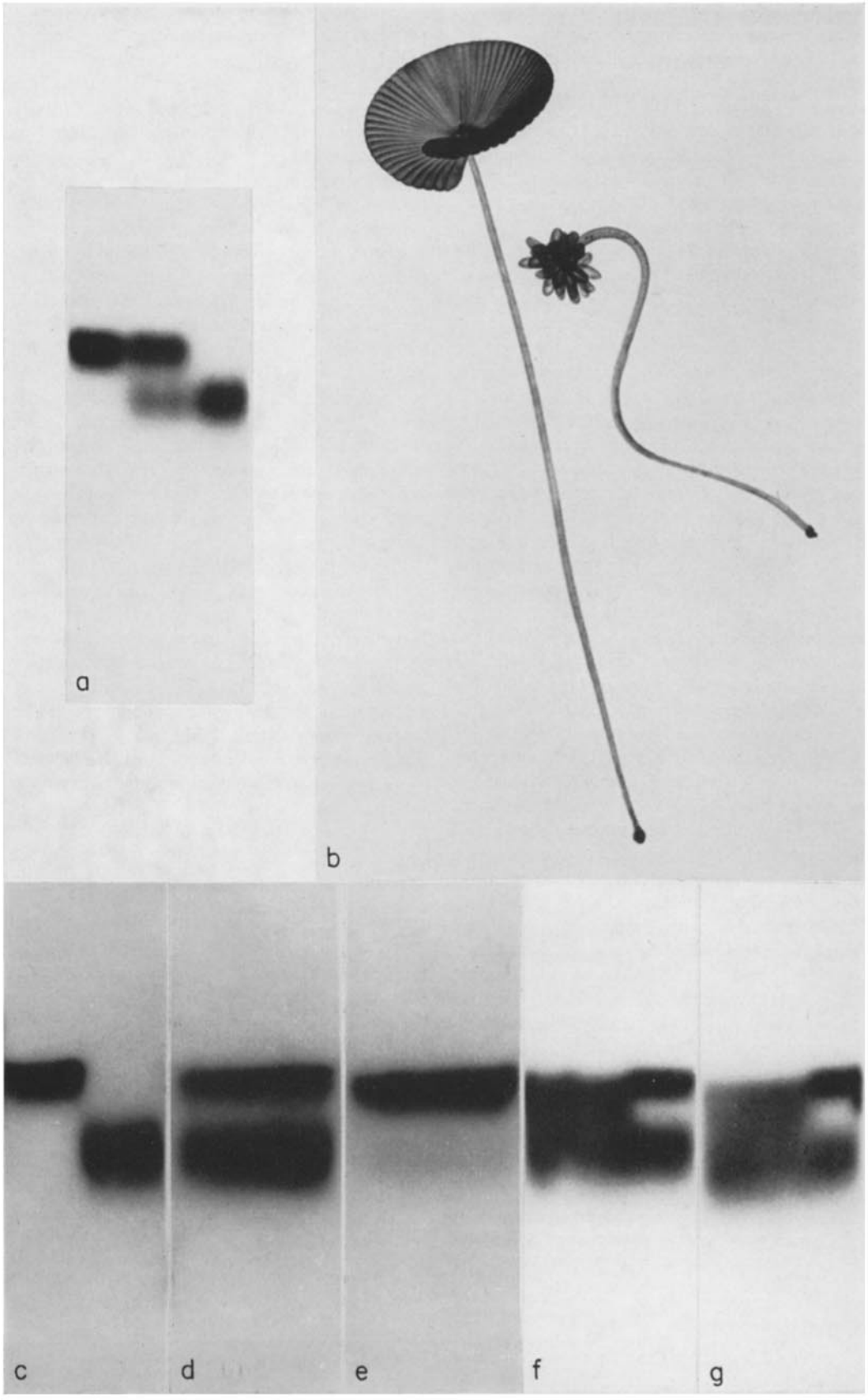
c to *g* Zymograms illustrating the *in vitro* transformation of phosphatase in mixtures of *med* and *acic* homogenates.

c Individual homogenates, *acic* left and *med* on the right side.

d A freshly prepared 1:1 mixture of homogenates.

e A freshly prepared 1:10 (*med*:*acic*) mixture of homogenates.

f, *g* Intermediate form (left), together with reference (right), as observed in 1:1 mixtures after 5 days of incubation.


a
b
c
d
e
f
g

electrophoresis indicate that this type might consist of a heterogeneous population of molecules, and that the process in its entirety probably comprises a multistep reaction.

Step I seems to be a prerequisite to step II, as is evidenced by the brief appearance of the intermediate enzyme type in all cases that finally lead to complete conversion. Under certain experimental conditions, however, the process ends with step I, as with homogenate injections and with *in vitro* mixtures of homogenates from both species. Subcellular particles, such as mitochondria and chloroplasts, can be removed from the homogenates by centrifugation without impairing the reactions to step I, indicating that the functioning of a complex metabolic system is not required.

Step II, in which the intermediate phosphatase is changed to, and becomes indistinguishable electrophoretically from, *med* phosphatase, has been observed so far only in graft combinations between cells of the two species of *Acetabularia*. The presence of a cell nucleus of either species is not required, but the well known metabolic capacities of the anucleate cytoplasm would be sufficient to provide synthetic activities that might be required for step II reactions to take place. At this time, it is not possible to decide whether the blocking of step II is caused by quantitative or qualitative deficiencies in the respective systems. Thus the complete conversion could depend on higher concentrations of the same factors that also produce the intermediate form. A possible instability of the evoking factors could, for instance, reduce their effective concentration under *in vitro* conditions, whereas in the intact nucleate or anucleate *med* cytoplasm a continued synthesis would replenish these factors.

A direct participation of the dominant *med* phosphatase, perhaps in the form of an exchange of protein subunits (11, 12) followed by the formation of hybrid molecules, seems unlikely but has not yet been ruled out entirely. Against this hypothesis speaks the fact that the loss of most of the phosphatase from injected *med* cytoplasm did not cause its ineffectiveness to induce step I. Furthermore, dark treatment of *med* cells hardly reduced the phosphatase content but strongly impaired the power of the homogenates prepared from these cells to provoke step I reactions after their injection into *acic* cells. But thus far a complete separation of the active principles from *med* phosphatase has not been achieved.

The finding of a non-interacting type of phosphatase in a laboratory culture of *Acetabularia crenulata* would also, at least to some extent, argue against a subunit exchange, unless one postulates an incompatibility between subunits from *acic* and *med* algae on one hand, and the subunits of *A. crenulata* phosphatase on the other hand, to form hybrid molecules. The term "non-interacting" has been used rather loosely and is based on the observations that, in hybrid grafts with *cren*-II, neither transformations nor intermediates were discovered. In lacking accurate quantitative assays we cannot exclude, of course, that an undetectable small amount of phosphatase of one graft partner was converted to the enzyme type of the other partner.

Although the test with neuraminidase for sialic acids as components of the enzyme protein was completely negative, there still exists the possibility that *Acetabularia* phosphatase molecules are conjugated with small non-protein molecules other than sialic acid, and that the conversion factor proper is an enzyme which, in analogy to the cholinesterase-neuraminidase system (13, 14), removes small molecules from, or couples them to, a basic phosphatase molecule and thus changes the net electrical charge.

This investigation was supported by a research grant (G-15948) of the National Science Foundation, Washington, D. C.

The authors are indebted to Dr. C. L. Markert for kindly making available to us specimens of *Acetabularia crenulata*, variety II, which were collected in Bermuda.

We also wish to express our gratitude to Dr. von Pölnitz and Dr. Dietzel, Behringwerke AG., Marburg, Germany, for providing us with a preparation of neuraminidase.

Received for publication, January 25, 1963.

REFERENCES

1. VANDERHAEGE, F., Les effets de l'énucléation sur la synthèse des protéines chez *Acetabularia mediterranea*, *Biochim. et Biophysica Acta*, 1954, **15**, 281.

2. CLAUSS, H., Ueber quantitative Veränderungen der Chloroplasten- und cytoplasmatischen Proteine in kernlosen Teilen von *Acetabularia mediterranea*, *Planta*, 1958, **52**, 334.

3. HÄMMERLING, J., CLAUSS, H., KECK, K., RICHTER, G., and WERZ, G., Growth and protein synthesis in nucleated and enucleated cells, *Exp. Cell Research*, 1958, suppl. **6**, 210.
4. BALTUS, E., Evolution de l'aldolase dans les fragments anucléés d'*Acetabularia*, *Biochim. et Biophysica Acta*, 1959, **33**, 337.
5. KECK, K., and CLAUSS, H., Nuclear control of enzyme synthesis in *Acetabularia*, *Bot. Gazette*, 1958, **120**, 43.
6. CLAUSS, H., Das Verhalten der Phosphorylase in kernhaltigen und kernlosen Teilen von *Acetabularia mediterranea*, *Planta*, 1959, **52**, 534.
7. KECK, K., Nucleo-cytoplasmic interactions in the synthesis of species-specific proteins in *Acetabularia*, *Biochem. and Biophys. Research Commun.*, 1960, **3**, 56.
8. KECK, K., Nuclear and cytoplasmic factors determining the species specificity of enzyme proteins in *Acetabularia*, *Annals New York Acad. Sc.*, 1961, **94**, 741.
9. HÄMMERLING, J., Zur Lebensweise, Fortpflanzung und Entwicklung verschiedener Dasycladaceen, *Arch. Protistenk.*, 1944, **97**, 7.
10. BETH, K., Experimentelle Untersuchungen über die Wirkung des Lichtes auf die Formbildung von kernhaltigen und kernlosen *Acetabularia*- Zellen, *Z. Naturforsch.*, 1953, **8b**, 334.
11. MARKERT, C. L., *in* Proceedings of the Thirteenth Annual Conference on the Kidney-Genetic, Development, and Immunological Aspects of Renal Diseases, (J. Metcoff, editor), Evanston, Illinois, Northwestern University Press, 1962.
12. LEVINTHAL, D., SIGNER, E. R., and FETHEROLF, K., Reactivation and hybridization of reduced alkaline phosphatase, *Proc. Nat. Acad. Sc.* 1962, **48**, 1230.
13. AUGUSTINSSON, K., and EKEDAHL, G., The properties of neuraminidase-treated serum cholinesterase, *Biochim. et Biophysica Acta*, 1962, **56**, 392.
14. SVENSMARK, O., Human serum cholinesterase as a sialo-protein, *Acta Physiol. Scand.*, 1961, **52**, 267.