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Purification and Characterization of the Hormone Initiating Sexual Morphogenesis in *Volvox carteri* f. *nagariensis* Iyengar

(glycoprotein)

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ABSTRACT **In *Volvox carteri* f. *nagariensis* male spheroids secrete into the medium a sexual hormone which controls the initiation of the developmental pathway leading to the formation of sexual embryos. In the absence of the hormone asexual embryos are formed. Analysis of the highly purified hormone indicates that it is a glycoprotein of 27,500–30,000 daltons, composed of one peptide chain, which has a typical composition except for a relatively low content of tryptophan. The glycosidic moiety, which accounts for about 45% of the weight of the molecule, consists of pentoses, hexoses, and amino hexoses. In the bioassay the highly purified hormone stimulates 100% formation of sexual embryos at a concentration of 10^{-10} g/liter and 14.4% sexual embryos at 10^{-11} g/liter (3×10^{-16} M). The activity is remarkably thermostable and resistant to different denaturing procedures.**

Although *Volvox* has been known to science since 1700 (1) the potential of the many species for studies in development, as suggested 65 years ago by Powers (2), has only recently been realized through the formulation of methods for cultivating it in the laboratory and for evoking at will its asexual and sexual phases. Cellular differentiation and development in a multicellular organism would appear to be in their simplest form in *Volvox*, for here the cells are arranged in a single layer on the periphery of a spheroid and consist of only two types, somatic and reproductive. The formation of new individuals is through a series of successive cleavages of an asexual reproductive cell, the gonidium. In certain species the morphological differentiation of the reproductive cells can be observed as unequal cleavages at specific stages of the embryonic development, the gonidia, the eggs, and the spermatogenous cells being delimited in the respective asexual, female, and male embryos at predictable positions and times.

The phenomenon of sexual induction in *Volvox* was first described by Darden (3) in a strain of *Volvox aureus*. In this species a substance was secreted by males which, when added to a population of asexual individuals, would result in the formation in the next generation of sexual embryos rather than asexual ones as would have occurred without this additive. Similar phenomena of sexual induction have since been described in a variety of species and forms of the genus (4, 5). In all species studied, the sexual hormone initiates the developmental pathway leading to the formation of a sexual individual rather than an asexual one, but the substance is not sex-specific. In species in which distinctive males and females are produced in separate clones, the same hormone initiates the formation of sexual embryos in both sexes. The hormone is, however, species-specific (6); it is usually produced only in populations of sexual males, but in one species both male and female populations produce active substances which are both self- and cross-inductive (7).

The sexual hormones from the various species which have been investigated are all inactivated by Pronase digestion (8), and so it has been assumed that they are proteinaceous. Ely and Darden (9) have attempted purification of the hormone in *V. aureus*, and though the yield was too small for analysis it was postulated that it might be a glycoprotein.

The present communication summarizes our efforts toward purification and characterization of the sexual hormone produced in male populations of *Volvox carteri* f. *nagariensis*. The morphology and embryonic development of this species, the methods of cultivation, the bioassay for the detection of the hormone, and accounts of genetic loci affecting the potency of the male as well as the developmental process of all types of embryos have been discussed in earlier papers (5, 6). Therefore the following paragraphs on the organism and its cultivation include only those details necessary for clarity in this communication.

The organism

The strains of *Volvox carteri* f. *nagariensis* used in this investigation were HK10 female and 69-1b male. The asexual spheroids of both strains are identical in appearance, having a maximum of 5000 small biflagellate somatic cells and 16 large gonidia (asexual reproductive cells). The female spheroid resembles an asexual one in its size and number of somatic cells, but it is distinctive in having 45 or more small dense eggs rather than the 14–16 large gonidia. The male spheroid is said to be dwarf because it has a maximum of only 512 cells, of which there is a 1:1 ratio of somatic to spermatogenous cells.

New individuals are formed by the gonidia of the asexual spheroid undergoing a series of cleavages whose patterns to form the asexual, the male, and the female embryos are distinctive. The pattern of cleavage, i.e., sexual or asexual, depends on the presence or absence of the sexual hormone produced by the males. In the formation of an asexual embryo (in both male and female strains), the gonidia are morphologically differentiated by unequal cleavages of the cells in the anterior half of the embryo at the division of the 32-celled stage; in the female embryo, the morphological differentiation of eggs occurs by unequal cleavages in the anterior two-thirds of the embryo at the division of the 64-celled stage; finally, in the formation of the male embryo, the spermatogenous cells are formed by unequal cleavages of all the cells at the last

divisions in the embryo, thus producing the typical 1:1 ratio of somatic cells to spermatogenous cells.

Culture methods

Both male and female strains were grown in Volvox medium, a very dilute medium designed by Provasoli and Pintner (10), but the medium was adjusted to pH 8 rather than pH 7 as formerly used. Sodium acetate was added to the medium to a concentration of 0.05% at pH 7.5 when males were grown for production of the sexual hormone. Illumination of 12,000 lux intensity on a 16-hr light/8-hr dark cycle and a temperature of 28–30° during the light period (20° during the dark) resulted in a generation time of 48 hr, and thereby a population increase by a factor of 15, since there are 14–16 asexual reproductive cells in each spheroid. Large populations were grown in half-filled 500-ml or 2000-ml Erlenmeyer flasks through which sterile air was bubbled.

Sexual spheroids appear spontaneously in the male strain at an approximate frequency of 1 in every 20,000 embryos, due in part at least to spontaneous gene mutations (11). A spontaneous male in an asexual population of the male strain will secrete enough hormone to initiate the production of male spheroids in the next generation. In order to grow large populations of the male strain for production of the hormone, it was necessary to grow the inoculum first in small volumes (1 parental spheroid/50 ml of Volvox medium/250 ml prescription bottle) which after 7 days' growth could be examined and those cultures discarded which showed premature male production. Usually two bottles (each containing approximately 45,000 young asexual spheroids) were added to each 2000-ml flask containing 1000 ml of Volvox medium with 0.05% sodium acetate. Thus each flask would have 90,000–100,000 small asexual spheroids whose gonidia would form male spheroids in the next generation. To insure that these spheroids would be males, 1 ml of a sterile solution containing the hormone was added to each large flask. Males were formed within 2–3 days, and sperm packets could be observed in these males 24 hr later. The flasks were kept on the lighted shelves with constant aeration until 48 hr after the sperm had been released, by which time the sperm had disintegrated. The contents of the flasks were put into plastic bags and frozen at −20°. Fluids formed under optimum conditions could be expected to stimulate 100% formation of sexual embryos in the bioassay at dilutions of 10^7 or less, becoming limiting at the 10^8 dilution with 50% or less of the embryos being sexual. However, such conditions were not always achieved, inasmuch as lack of synchrony in the inoculum would result in inhibition of later developing males by the dissolution of early males which would make the medium less conducive to the best growth.

The bioassay

The bioassay of the hormone in fluids from sexual populations of the male strain uses the HK10 female strain as the detector. Serial 1/10 dilutions are made in Volvox medium and then inoculated with 75+ young asexual spheroids of the female strain. Within 48 hr the asexual reproductive cells of the inoculum will have formed 1000+ embryos. In those dilutions where the hormone is not in limiting concentration, all, or nearly all, of the offspring will be female rather than asexual. In only one tube of a dilution series will be found a mixture of female and asexual offspring, an indication that in that dilution the hormone was in limiting concentration. All dilutions greater than this will contain no females among the offspring. Thus, one need score only the offspring in a single tube of any dilution series. Details of the assay method have been published earlier (5).

Concentration and purification procedures

The frozen fluid from male cultures was thawed in 2-liter batches, filtered through glass wool to remove large debris, and centrifuged at 10,000 × *g*. After adjusting it to pH 5, the fluid was passed rapidly (500–600 ml/hr) through a column of carboxymethyl cellulose (2.6 × 20 cm; Bio-Rad Laboratories) which had been equilibrated with 0.001 M citrate–phosphate buffer (pH 5). The very dilute nature of the medium in which the male *Volvox* had been grown made it unnecessary to dialyze the fluid prior to its passage through the column. All (99%) of the activity remained on the column.

After washing with 0.001 M citrate phosphate buffer, 0.1 M NaCl in 0.05 M citrate–phosphate buffer (pH 5) was applied to the column and the active substance was eluted in 200 ml (after discarding a 35-ml void volume). The pH of this eluant was adjusted to pH 6.5 with 1 N NaOH and the resultant salt buffer was frozen for storage.

Salt buffer from three 2-liter runs was thawed, combined, and then flash-evaporated at 40° to a volume of approximately 45 ml. This concentration resulted in a heavy white precipitate, but the activity remained in the supernatant. The concentrate was centrifuged for 10 min in a clinical centrifuge at 1500 × *g*. The supernatant was then put on a column (Pharmacia 26/100) of Sephadex G-75 with 0.1 M ammonium acetate (pH 7) as the eluant. This preparative column was run at 50 ml/hr, and the eluant collected in 10-ml fractions monitored with an ultraviolet absorptiometer (280 nm). Peaks of absorption were seen in fractions 15–20 (molecular weight >70,000), 21–30, and 35–46 (low-molecular-weight substances), but the bulk of the activity was only in fractions 21–30, reaching a maximum usually in 25 and 26. The peak of activity was slightly before the peak seen when α-chymotrypsin (molecular weight 25,000) was run on the same column as a marker protein. Fractions 21–30 of several runs were then combined, flash-evaporated to 1/10 volume and rechromatographed on the Sephadex G-75 column. The active fractions were then combined and lyophilized.

Further purification was achieved by chromatography on a Sephadex G-50 column (0.8 × 80 cm) with 0.05 M NH_4HCO_3 (pH 8.5) as equilibration buffer and eluant (2 ml/hr). The elution profile, as recorded by ultraviolet absorption (280 nm), showed one small and one large band of high-molecular-weight and one of low-molecular-weight. The active principle was in the second band of high-molecular-weight. The ultraviolet absorption coincides exactly with the activity. The active fractions were combined and lyophilized.

Characterization

Qualitatively, the white fluffy material gives strong protein reactions, sugar reaction, and a faint, but definite, phosphate reaction. On a weight basis, it contains 62% protein (standard: bovine-serum albumin) and 40.5% sugar (standard: mannose). The spectrum of the dialyzed factor at pH 8 (0.1 M Tris·HCl) shows a minimum at 252 nm ($A^{1\%}_{1\,cm} = 2.4$), a maximum at 273 nm ($A^{1\%}_{1\,cm} = 3.3$), and a shoulder at 283 nm. The spectrum has a somewhat asymmetric shape and, for a simple protein, there is an unusually high absorption in the 255- to 265-nm range ($A_{280/260} = 1.2$). At pH 13 (0.1 M NaOH) the spectral maxi-

TABLE 1. *Amino acid analysis of Volvox carteri hormone (from 55 μg of substance)*

Amino-acid residue	nmol	Residues (His = 2)
Asp:	27.6	13
Thr:	25.5	12
Ser:	34.9	16
Glu:	23.5	11
Pro:	19.5	9
Gly:	30.0	13
Ala:	28.6	13
Cys:	6.2	3
Val:	20.1	9
Met:	4.8	2
Ile:	15.8	7
Leu:	22.7	10
Tyr:	9.2	4
Phe:	7.8	4
Lys:	11.8	6
His:	4.6	2
Arg:	18.9	8–9
Trp:		1 (?)
	sum:	143–144

mum shifts to 283 nm ($A^{1\%}_{1\,cm}$ = 2.7). However, it was not possible to calculate the tryptophan content from the spectral changes (12).

The highly purified factor gives a reaction of 100% in the bioassay at a concentration of 10^{-10} g/liter and 14.4% reaction at 10^{-11} g/liter. On heating a 2 mg/ml solution of the active material in 0.05 M NH_4HCO_3 for 30 min at 60°, it retains full activity. The spectrum of the heated solution has a maximum at 260 nm ($A^{1\%}_{1\,cm}$ = 2.6) and a shoulder at 275 nm ($A^{1\%}_{1\,cm}$ = 2.2). The sex hormone is heat stable up to 80° (15 min); at 90° (15 min) 90% of the activity was lost. The biological activity is also retained after treating the material with 6 M guanidine·HCl at room temperature for 24 hr. The chaotrope even seems to stabilize the substance against heat inactivation. A low-molecular-weight cofactor could not be dissociated or split off the molecule. The active principle is not dialyzable. It is salted out by 80% ammonium sulfate saturation but not adsorbed on charcoal, DEAE cellulose, or QAE Sephadex. 10% acetic acid does not precipitate the material, but it is coagulated by 10% trichloroacetic acid. Since a glycoprotein was indicated by survey analyses, several chemical data were collected and the molecular behavior of the conjugated protein was studied.

The active substance chromatographs as a single band (R_F 0.61) on cellulose thin layers (prewashed with butanol–acetic acid–water, 4:1:5) in 0.05 M NH_4HCO_3 as solvent. The activity coincides with the protein localization. The molecular weight, by comparison with standard proteins on Sephadex G-25 (13), is 25,500. On electrophoresis on cellulose thin layers at pH 6.5 it migrates as a single zone slightly towards the anode. Disc electrophoresis (staining in and destaining with 10% trichloroacetic acid) shows a diffuse weakly staining zone close to the origin and another unsharp band corresponding to a molecular weight of about 25,000 (14). The activity is spread as a zone between the two. Sodium dodecyl sulfate gel electrophoresis (15) in a 7.5% gel gave a well-defined single band at 28,400 ± 700 daltons (standards: ribonuclease, trypsin, triosephosphate dehydrogenase, bovine-serum albumin). Thus, the protein is a single chain, not composed of subunits. The relatively high value in comparison with the other data on molecular weight is probably due to the high carbohydrate content of the glycoprotein (16). This also is indicated by a relative shift of the mobility in a 10% gel. Sucrose gradient centrifugation (17) gives a molecular weight of 26,000, assuming a partial specific volume of 0.700. On sedimentation velocity analysis, the highly purified preparations (concentration = 0.65 to 2.7 mg/ml in 0.05 M NH_4HCO_3) form a well-defined symmetrical slow band ($s^\circ_{20,w}$ = 1.57 ± 0.07) and another fairly sharp and symmetrical faster moving band ($s^\circ_{20,w}$ = 2.7). Both of them contain the activity. The diffusion coefficient for the slow band was determined in 0.05 M NH_4HCO_3 as $D^\circ_{20,solv.}$ = 4.16 ± 0.02 × 10^{-7}. From these hydrodynamic data a molecular weight of 30,600 ± 5% is calculated and a dissymmetry constant of f/f_0 = 1.66 is derived, representing a rather elongated, asymmetric molecule. It readily forms dimers and also apparently polymers at higher concentrations. It seems that the active proteide is in concentration-dependent, reversible equilibrium with multiple aggregates, whereas on column chromatography, when the concentration was about 0.2 mg/ml, only the monomer is eluted.

The amino-acid analysis shows no unusual amino acids (Table 1). Based on histidine as unit, the minimal molecular weight is 7750. There are 6 lysine and 8–9 arginine residues; tryptic digestion after performic acid oxidation (18) shows a minimum of 12 and a maximum of 15 tryptic peptides. There are three strongly anionic peptides, derived from cysteine stretches. Thus, the molecular weight of the protein moiety is around 15,000 (2 × 7750), corresponding to an overall molecular weight of 28,200 under the assumption of a 55:45 protein to sugar ratio. The amount of amino sugars, as revealed in the amino-acid analyzer, corresponds to about 5% of the total sugars. No N-terminal amino acid could be detected; the amino terminal is either closed by a small substituent or by the glycosidic moiety. The peptide was iodinated by ICl (iodine monochloride) in glycine buffer (pH 9.5) (19), almost without loss of biological activity. Gas chromatographic sugar analysis after methanolysis, acetylation, and trimethylsilylation (20) of the material gave (internal standard: pertrimethylsilylmannitol) arabinose (6.6%), xylose (25.5%), mannose (15.6%), galactose (4.6%), glucose (32.5%), unknown peak (*N*-acetylgalactosamine?) (11.3%), and *N*-acetylglucosamine (3.9%).

These data taken together demonstrate that the sexual hormone produced by the male population of *Volvox carteri* f. *nagariensis* is a glycoprotein of 27,500–30,000 daltons, composed of only one polypeptide chain which has a typical composition, except for a very low value for tryptophan and a strikingly high carbohydrate content. The main components of the nonprotein material are xylose and glucose. The ratio: pentoses to hexoses to aminohexoses is 2:3.5:1. The hormone tends to aggregate reversibly at higher concentrations in 0.05 M NH_4HCO_3.

Discussion

Numerous instances of apparent chemical control of the sexual processes in plants have been reported, but few have been analyzed in any detail (21). In some plants the substances serve to bring together gametes of the compatible mating types or sexes, e.g., *Allomyces* (22), *Ectocarpus* (23), and *Chlamydomonas* (24). In others, the differentiation of the sexual struc-

tures which produce the gametes depends on the secretion of active compounds by one or both compatible individuals. Thus, in *Mucor* (25) and *Achlya* (26) the hormones initiate the differentiation of special sex organs on siphonaceous plant bodies, while in certain ferns (27) the hormones effect a special pattern of cell cleavage and differentiation resulting in the formation of antheridia, the multicellular male reproductive organs. The hormone secreted by the male *Volvox* is similar in effect to that of ferns, but the hormones are unrelated chemically. In the ferns the antheridiogens are thought to be related to the gibberellins, and, indeed, in some species the response to both antheridiogens and various gibberellins is the same. The antheridiogens from different ferns do not show the extreme species-specificity seen in the sexual hormones from the various species of *Volvox* (27, 28).

The morphogenetic hormone of *Volvox carteri* f. *nagariensis* is a medium sized glycoprotein of high (45%) carbohydrate content whose overall composition bears similarities to certain other plant glycoproteins (29) in its lack of neuraminic and uronic acids. No fucose and other deoxy sugars were detected; however, an unknown sugar (tentatively identified as the —gas chromatographically similar—*N*-acetylgalactosamine), was found present in appreciable quantities. The amount of amino sugars, as determined with the amino-acid analyzer, accounts only for the amount of *N*-acetylglucosamine estimated independently by gas chromatography. If the unknown sugar were *N*-acetylgalactosamine, the relative amounts of the two amino hexoses would be inversely proportional to those of the corresponding hexoses. The amino-acid analysis showed a relatively low content in sulfur amino acids. Whereas the protein moiety consists of but one polypeptide chain, it is not clear whether the carbohydrate moiety is also a single polymer. Although the binding amino acids are not known, the high content of acidic amino acids and of serine is conspicuous. There is no terminal amino group. It might be one site of carbohydrate binding or blocked by acylation. The absorbance of the hormone at 280 nm corresponds well with the amount of aromatic amino acids found, assuming at the most only one tryptophan, which, however, has so far not been detected independently. The absorbance of the material in the 250- to 270-nm range is higher than would be expected for a simple glycoprotein. Changes of the spectrum with pH may be explained by the presence of a very tightly bound heterocyclic (nucleotidic) base. However, it was not possible to liberate and identify such a compound. Other striking features are the relatively low sedimentation constant and the similarly low diffusion constant, which compensate to a molecular weight comparable to that found by independent means. The frictional ratio (f/f_0) points to a rather elongate shape such as that of other glycoproteins of biological activity. The sites of dimerization and further aggregation are probably charged parts of the molecule.

Nothing is known about the biochemistry of the response initiated by the sexual hormone in *Volvox*. It would appear to function only as an initiator of the pathway of development leading to a sexual embryo, for it is equally effective in both the male and the female strains; and it has been clearly demonstrated that the particular events of development in the embryogeny are under the control of various genetic loci. The identification of two genetic loci, one linked to sex, which result in the formation of a sexual embryo without the addition of the sexual hormone, has been used to postulate a scheme of biochemical action in which the hormone serves as a co-repressor (11). With the present demonstration of the chemical nature of the hormone and the possibility of iodination without loss of biological activity, it may now be possible to test this hypothesis using labelled hormone, immunological procedures, etc.

In the bioassay of the highly purified hormone a 14.4% reaction was obtained at a concentration of 10^{-11} g/liter or 3×10^{-16} M, assuming a molecular weight of 30,000. Approximately 100 gonidia per ml are involved in the bioassay, and thus it figures that 1800 molecules per gonidium resulted in the 14.4% reaction. The actual number of molecules necessary to effect the reaction in a gonidium is probably much lower. Earlier experiments have shown that increasing the inoculum per ml 20-fold did not change the level of the reaction; larger numbers of gonidia have not been tried inasmuch as such numbers result in problems of limiting CO_2, illumination, and media composition. Pall (30) has published data from studies of the fraction of sexual induction as a function of inducer concentration and he concluded that only 2 molecules of the hormone are actually required to make the gonidium form a sexual embryo rather than an asexual one.

Volvox offers possibilities for investigations of the control of development and differentiation in a multicellular organism using biochemical approaches generally more applicable to populations of microorganisms. Furthermore, the ease with which *Volvox* can be cultivated and its sexual phases evoked allows one to investigate genetic phenomena in this haploid autotrophic organism. The development of the many approaches to problems of cellular differentiation will, of course, depend on investigators with different interests and competencies; therefore, the male and female strains of *Volvox carteri* f. *nagariensis* have been deposited in the Culture Collection of Algae at Indiana University, Bloomington, for unrestricted distribution (31).

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1. Dobell, C. (1932) *Antony van Leeuwenhoek and His Little Animals* (Staples Press, London), pp. 256–262.
2. Powers, J. H. (1907) "New forms of *Volvox*," *Trans. Amer. Microsc. Soc.* **27**, 123–149; Powers, J. H. (1908) "Further studies in *Volvox*, with descriptions of three new species," *Trans. Amer. Microsc. Soc.* **28**, 141–175.
3. Darden, W. H., Jr. (1966) "Sexual differentiation in *Volvox aureus*," *J. Protozool.* **13**, 239–255.
4. Starr, R. C. (1968) "Cellular differentiation in *Volvox*," *Proc. Nat. Acad. Sci. USA* **59**, 1082–1088.
5. Karn, R. W. (1972) Differentiation of reproductive cells in *Volvox obversus*. Ph.D. thesis, Indiana University, Bloomington; Kochert, G. (1968) "Differentiation of reproductive cells in *Volvox carteri*," *J. Protozool.* **15**, 438–452; McCracken, M. D. & Starr, R. C. (1970) "Induction and development of reproductive cells in the K-32 strains of *Volvox rousseletii*," *Arch. Protistenk.* **112**, 262–282; Starr, R. C. (1969) "Structure, reproduction, and differentiation in *Volvox carteri* f.

nagariensis Iyengar, strains HK 9 & 10," *Arch. Protistenk.* **111**, 204–222; Starr, R. C. (1972) "Sexual reproduction in *Volvox dissipatrix*," *Abstr. Br. Phycol. J.* **7**, 284; Vande Berg, W. J. & Starr, R. C. (1971) "Structure, reproduction, and differentiation in *Volvox gigas* and *Volvox powersii*," *Arch. Protistenk.* **113**, 195–219.

6. Starr, R. C. (1970) "Control of differentiation in *Volvox*," in *Changing Syntheses in Development*, ed. Runner, M. N., *Develop. Biol. Suppl.* **4**, 59–100.
7. Starr, R. C. (1972) "Sexual reproduction in *Volvox dissipatrix*," *Abstr. Br. Phycol. J.* **7**, 284.
8. Darden, W. H., Jr. (1970) "Hormonal control of sexuality in the genus *Volvox*," in *Phylogenesis and Morphogenesis in the Algae*, ed. Frederick J. F. and Klein, R. M., *Ann. N.Y. Acad. Sci.* **175**, 757–763.
9. Ely, T. H. & Darden. W. H. (1972) "Concentration and purification of the male-inducing substance from *Volvox aureus* M5," *Microbios* **5**, 51–56.
10. Provasoli, L. & Pintner, I. J. (1959) "Artificial media for freshwater algae; problems and suggestions," in *The Ecology of Algae*, eds. Tryon, C. A. & Hartman, R. T. (Spec. Publ. No. 2, Pymatuning Lab. of Field Biology, University of Pittsburgh), pp. 84–96.
11. Starr, R. C. (1972) "A working model for the control of differentiation during development of the embryo of *Volvox carteri* f. *nagariensis*," *Soc. Bot. Fr., Memoires* 1972, pp. 175–182.
12. Bencze, W. L. & Schmid, K. (1957) "Determination of tyrosine and tryptophane in protein," *Anal. Chem.* **25**, 1193–1196.
13. Andrews, P. (1965) "The gel-filtration behavior of proteins relative to their molecular weight over a wide range," *Biochem. J.* **96**, 595–606.
14. Hedrick, J. C. & Smith, A. J. (1968) "Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis," *Arch. Biochem. Biophys.* **126**, 155–164.
15. Weber, K. & Osborn, M. (1969) "The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis," *J. Biol. Chem.* **244**, 4406–4412.
16. Bretscher, M. A. (1971) "Major human erythrocyte glycoprotein spans the cell membrane," *Nature New Biol.* **231**, 229–230.
17. Martin, R. G. & Ames, B. N. (1961) "A method for determining the sedimentation behavior of enzymes: Application to protein mixtures," *J. Biol. Chem.* **236**, 1372–1379.
18. Rao, D. R., Beyreuther, K. & Jaenicke, L. (1973) "A comparative study of pig and sheep brain glutamine synthetase: Tryptic peptides and thiol groups," *Eur. J. Biochem.* **35**, 582–592.
19. Roholt, O. A. & Pressman, D. (1972) "Iodination—Isolation of peptides from the active site," in *Methods in Enzymology*, eds. Hirs, C. W. H. & Timasheff, S. N. (Academic Press, New York), Vol. 25, Part B, pp. 438–449.
20. Donike, M. (1969) "*N*-Methyl-*N*-trimethylsilyl-trifluoracetamid, ein neues Silylierungsmittel aus der Reihe der silylierten Amide," *J. Chromatogr.* **42**, 103–104.
21. Machlis, L. (1972) "The coming of age of sex hormones in plants," *Mycologia* **64**, 235–247.
22. Machlis, L., Nutting, W. H., Williams, M. W. & Rapoport, H. (1966) "Production, isolation, and characterization of sirenin," *Biochemistry* **5**, 2147–2152.
23. Müller, D. G., Jaenicke, L., Donike, M. & Akintobi, T. (1971) "Sex attractant in a brown alga; chemical structure," *Science* **171**, 815–817.
24. Wiese, L. (1969) "Algae," in *Fertilization*, eds. Metz, C. B. & Monroy A. (Academic Press, New York), Vol. 2, pp. 135–188.
25. van den Ende, H. & Stegwee, D. (1971) "Physiology of sex in Mucorales," *Bot. Rev.* **37**, 22–36.
26. Barksdale, A. W. (1969) "Sexual hormones of *Achlya* and other fungi," *Science* **166**, 831–837.
27. Voeller, B. R. & Weinberg, E. S. (1969) "Evolutionary and physiological aspects of antheridium induction in ferns," in *Current Topics in Plant Science*, ed. Gunckel, J. E. (Academic Press, New York), pp. 79–93.
28. Endo, M., Nakanishi, K., Näf, U., McKeon, W. & Walker, R. (1972) "Isolation of the antheridiogen of *Anemia phyllitides*," *Physiol. Plant.* **26**, 183–185.
29. Gottschalk, A. & Buddecke, E. (1972) "Occurrence and distribution of glycoproteins," in *Glycoproteins*, ed. Gottschalk, A. (Elsevier Publishing Co., Amsterdam), 2nd ed., pp. 529–564.
30. Pall, M. (1973) "Sexual induction in *Volvox carteri:* a quantitative study," *J. Cell Biol.* **59**, 238–241.
31. Starr, R. C. (1971) "The culture collection of algae at Indiana University—additions to the collection July 1966–July 1971," *J. Phycol.* **7**, 350–362.