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Characterization of Proteins in Flagellates and Growing Amebae of *Naegleria fowleri*

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Polypeptides of whole-cell extracts of *Naegleria fowleri* flagellates and growing amebae were resolved by two-dimensional polyacrylamide gel electrophoresis. Autoradiograms of the [^{35}S]methionine-labeled polypeptides of amebae and flagellates were analyzed by two-dimensional densitometry to determine whether there were correlations between intracellular concentration of a protein and subunit size or charge. The majority of the polypeptides of amebae and flagellates had molecular sizes in the range of 20 to 60 kilodaltons. The radioactivity per polypeptide species in the size range of 20 to 60 kilodaltons was greater in amebae than in flagellates. The greatest number of polypeptides detected in amebae and flagellates was in the isoelectric focusing range of pH 6 to 7. The radioactivity per polypeptide species in the isoelectric focusing gradient below 6.3 was greater in amebae than in flagellates. Polypeptides in the size range of 20 to 60 kilodaltons had a median isoelectric point below pI 6.3, whereas those larger than 60 kilodaltons had a median pI value above 6.3. These data indicated that molecular size and charge were not entirely independent variables and that the size and charge of a polypeptide might have an important influence in determining its intracellular concentration in both amebae and flagellates. Autoradiograms were also compared so that changes in intracellular protein complement and concentrations occurring during differentiation could be recognized. The relative amounts of a limited number of polypeptides increased markedly, and others decreased markedly, during enflagellation.

Amebae of *Naegleria fowleri* can be induced to transform into flagellates by subculture from growth medium to non-nutrient buffer (22). The enflagellation process requires about 180 min, during which time several structures associated with motility appear de novo (21). The synthesis de novo of flagellar tubulin during differentiation in another ameboflagellate, *N. gruberi*, has been studied in some detail (5, 8, 9). To synthesize new proteins in the absence of exogenous nutrients, stored materials and degradation products from macromolecules must provide the needed precursors. Selective protein synthesis, presumably controlled at the level of transcription, and differential protein degradation proceed simultaneously during enflagellation. Accordingly, the enflagellation process in *N. fowleri* is a useful model for studying the regulation of the intracellular protein content in a eucaryotic protist.

Those proteins in eucaryotic cells that degrade most rapidly seem to have synthetic rates that change dramatically, whereas the proteins with slow degradative rates appear to have relatively constant rates of synthesis (10). Accordingly, the steady-state levels in eucaryotic cells of proteins that are synthesized or degraded rapidly change rapidly in response to new conditions (2, 20). Correlations have been observed which indicate that there are relationships among large molecular size, acidic isoelectric point, and rapid turnover rate (7, 10). To test the proposition that inherent qualities of proteins are important in the regulation of their intracellular concentration, the proteins in whole-cell extracts of amebae and flagellates of *N. fowleri*, separated by two-dimensional polyacrylamide gel electrophoresis (PAGE) (16–18), were characterized and compared with respect to molecular size, charge, number of species, and amount. It is anticipated that the concentrations of particular polypeptide species will change markedly during enflagellation, whereas the relative concentrations of other polypeptide species will remain rather constant (8). The analysis of the data provides an overall view of the changes in intracellular protein content occurring in *N. fowleri* during enflagellation.

The complements of polypeptide species in amebae and flagellates of *N. fowleri* were generally the same with respect to molecular size and charge, although the relative amounts of a limited number of polypeptides increased markedly, and others decreased markedly, during enflagellation. Polypeptides having a molecular size of

20 to 60 kilodaltons and a median pI value below 6.3 were more abundant in amebae than in flagellates, whereas polypeptides in the size range of 60 to 100 kilodaltons and with a median pI value above 6.3 were more abundant in flagellates. There was a distinct correlation between molecular size and charge of polypeptide in amebae and flagellates.

MATERIALS AND METHODS

An enflagellating strain of *N. fowleri* (nN68) and a non-enflagellating variant (nF69) were used (21). *N. fowleri* amebae were grown axenically in 10 ml of Nelson medium (11, 15, 24) in unagitated 75-cm^2 tissue culture flasks (Falcon Plastics, Oxnard, Calif.) at 37°C. The proteins of growing *N. fowleri* were radiolabeled by addition of 50 μCi of [^{35}S]methionine (1087.1 Ci/mmol) per ml at the mid-logarithmic phase of growth. Cells were incubated with radiolabeled amino acid for 24 h (approximately 3.5 generations); during this period, the culture grew to a late-logarithmic population density of 1.6 to 2.5 million amebae/ml. Radiolabeled amebae were harvested, washed free of medium, and prepared for electrophoresis or subcultured to Page saline (19) and shaken at 42°C to induce enflagellation (21). The flagellates, formed during a 3.5-h incubation of *N. fowleri* at 42°C in a gyratory water bath, were separated from untransformed amebae. The remaining amebae attached to the culture vessel within a few minutes once agitation was stopped, leaving only flagellates in the supernatant fluid. In one experimental series, 200 μCi of [^{35}S]methionine per ml was added to previously unlabeled amebae after subculture to Page saline to induce enflagellation. The flagellates radiolabeled during differentiation were harvested as described above.

Radiolabeled flagellates or growing amebae were washed thoroughly with Page saline (19) warmed to 37°C. The washed cells were sedimented by centrifugation and suspended in 0.5 ml of ice-cold disruption buffer (10 mM Tris 50 μg of DNase I per ml, 50 μg of RNase A per ml, and 5 mM $MgCl_2$, pH 7.4). Amebae or flagellates were ruptured by three cycles of freezing and thawing; the resulting extracts were lyophilized. The lyophilized material was suspended in lysis buffer I at a concentration of 10^7 cell-equivalents per 250 μl. Lysis buffer I contained 0.5% sodium dodecyl sulfate (SDS), 9.5 M urea, 0.2% ampholytes (pH 3 to 10), and 5% 2-mercaptoethanol, supplemented with 10 mM lysine (12, 17). The two known proteolytic activities of *N. fowleri* (21) were inactivated by lysis buffer I. After 5 min at 25°C, an equal volume of lysis buffer II (9.5 M urea, 2% Nonidet P-40, 1.6% ampholytes [pH 5 to 7], 0.4% ampholytes [pH 3 to 10], and 5% 2-mercaptoethanol, supplemented with 10 mM lysine [16, 17]) was added. The samples were held at −20°C until subjected to electrophoresis on the same day.

Isoelectric focusing (IEF) was achieved with mixed ampholytes (1.6% pH 5 to 7 and 0.4% pH 3 to 10) at 400 V for 17 h, followed by 800 V for 1 h. SDS-PAGE was carried out with uniform 12% acrylamide gels, but otherwise as described previously (16). Sample volumes were adjusted so that approximately equal numbers of cell-equivalents were loaded for each preparation. After electrophoresis, gels were fixed and stained in 10% acetic acid, 25% isopropanol, and 0.04% Coomassie blue, followed by destaining in several changes of 10% acetic acid–20% methanol. The gels were dried; Kodak X-Omat R film was exposed to the dried gels for several time periods. Comparisons of polypeptide patterns of amebae and flagellates were made on autoradiograms of gels exposed for time periods such that the product of radioactivity loaded and exposure time was equivalent to 820,000 cpm for 21 days. The data presented here are based on three independent experiments and two-dimensional PAGE separations.

Autoradiograms were scanned systematically, using a digital drum-scanning microdensitometer (model C4100; Optronics International, Chelmsford, Mass.). Each datum was corrected for film background density, and the corrected density readings were contoured manually (14). The resulting contour patterns corresponded to the patterns of polypeptides observed on the autoradiograms. The data were apportioned into intervals along both the IEF and SDS-PAGE dimensions for enumeration and computer quantitation of the contoured peaks. The IEF dimension was divided arbitrarily into 6-mm sections corresponding to 30 record columns. The pH corresponding to each section was measured to relate the arbitrary divisions to the pH gradient in the IEF gel. The SDS-PAGE dimension was organized into intervals of 20 kilodaltons by a computer program that calibrated the vertical coordinate against size standards (14.3 to 200 kilodaltons) that were run in the SDS-PAGE dimension with each sample (26).

Ampholytes, SDS, acrylamide, *N,N'*-methylenebisacrylamide, and molecular size standards were obtained from Bio-Rad Laboratories, Richmond, Calif. Urea (ultrapure grade) was obtained from Schwarz/Mann, Orangeburg, N.Y. Tris, DNase I, RNase A, and Coomassie blue R-250 were from Sigma Chemical Co., St. Louis, Mo. Nonidet P-40 was obtained from Bethesda Research Laboratories, Rockville, Md., and [^{35}S]methionine (translation grade) was purchased from New England Nuclear Corp., Boston, Mass.

RESULTS

Several hundred [^{35}S]methionine-labeled polypeptides of *N. fowleri* were resolved by two-dimensional electrophoresis and detected by autoradiography (Fig. 1). The proteins were prelabeled during growth of the amebae as described in Materials and Methods. The distribution of polypeptides revealed in autoradiograms was similar to that revealed by staining with Coomassie blue (Fig. 2) with respect to both positions within the gel and relative intensities of the spots. The small number of densitometric data points (pixels) with values at the upper limit (0.7% relative to the total number of data points having values above background) confirmed that the exposure chosen (product of radioactivity in becquerels and time in seconds = 2.5×10^{10} disintegrations) did not unduly exceed the film capacity. Comparisons of spot intensities within the limits of film capacity, therefore, could be used to gauge the relative amounts of radiolabeled polypeptides. The faintest detectable poly-

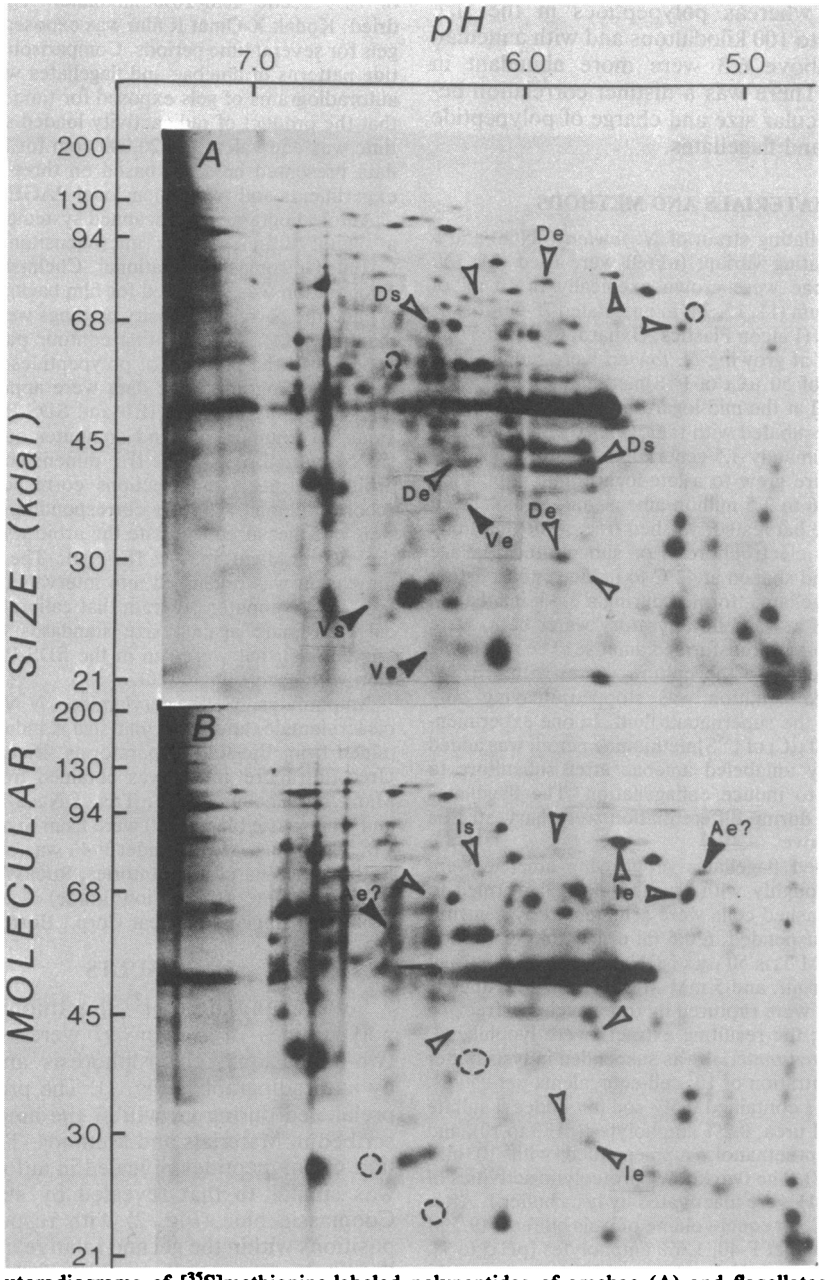

FIG. 1. Autoradiograms of [^{35}S]methionine-labeled polypeptides of amebae (A) and flagellates (B) of *N. fowleri*. The proteins were prelabeled in amebae and, after various manipulations, resolved by IEF and SDS-PAGE as described in Materials and Methods. Positions of size standards run in the same gels are shown. Qualitative changes are indicated by solid arrows (spot present) and dashed outlines (spot not detected); quantitative changes are indicated by open arrows. Abbreviations: Is, increase related to starvation; Ie, increase related to enflagellation; Ds, decrease related to starvation; De, decrease related to enflagellation; Vs, polypeptides that vanished only during enflagellation; Ae?, polypeptides appearing in flagellates but also present in amebae of the non-enflagellating strain before and after incubation in non-nutrient buffer; Kdal, kilodaltons. Indicated changes are examples of those enumerated in Table 1.

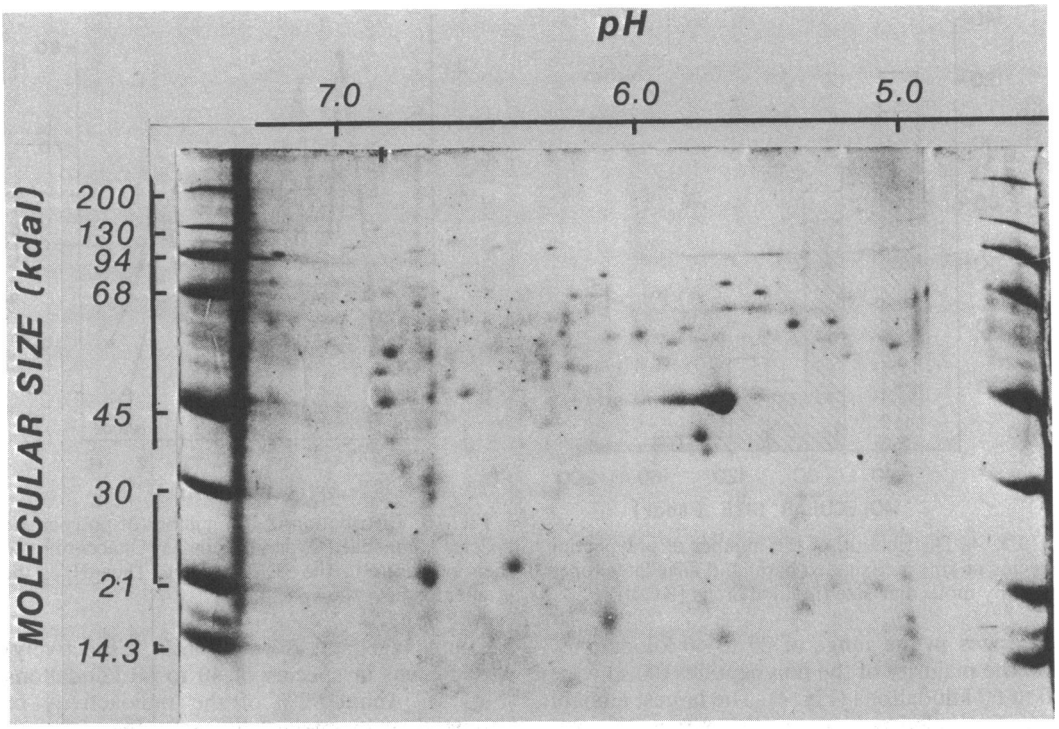

FIG. 2. Coomassie blue-stained polypeptides of amebae of *N. fowleri* resolved by IEF and SDS-PAGE. Kdal, kilodaltons.

peptide spot had a sum of 9 digital units (26); the most abundant polypeptide in Fig. 3 had a sum of 183,000 digital units. The amount of the most abundant radiolabeled polypeptide on this gel, therefore, was 20,000-fold greater than the amount of a polypeptide at the lower limit of detection (Fig. 3). By using several different exposure times to allow quantitation of more dense and less dense spots, the range in polypeptide amount was extended to 10^5-fold.

The greatest number of radiolabeled polypeptides in ameba and flagellate extracts had molec-

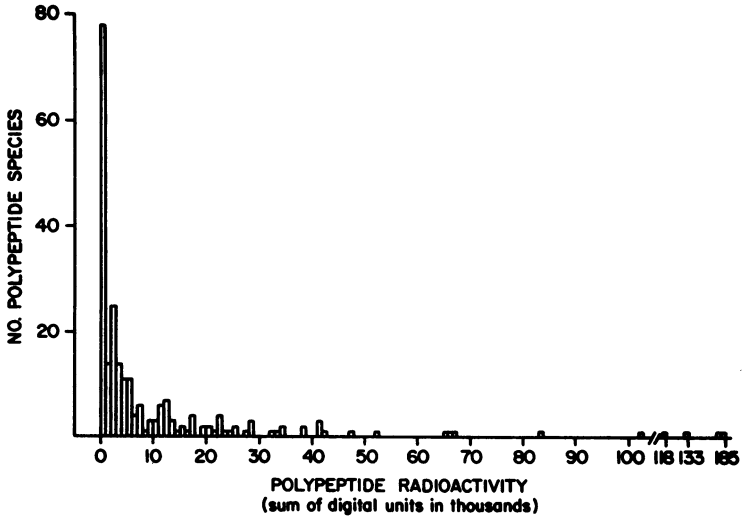

FIG. 3. Frequency distribution of the amount of polypeptide for individual species. The most abundant polypeptide is present at an estimated 10^8 molecules/ameba (9, 24).

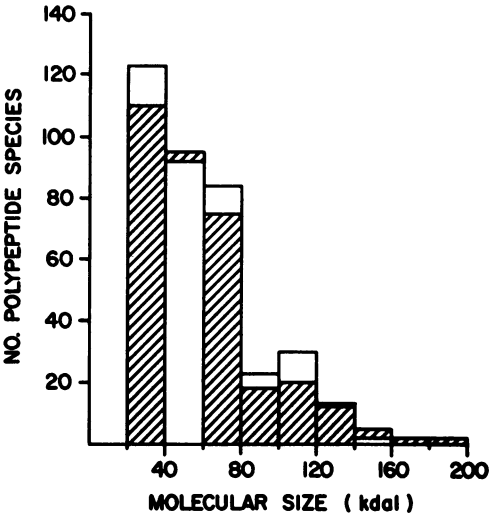

FIG. 4. Distribution of the number of polypeptide species of amebae (shaded bars) and flagellates (open bars) by molecular size (in kilodaltons [Kdal]).

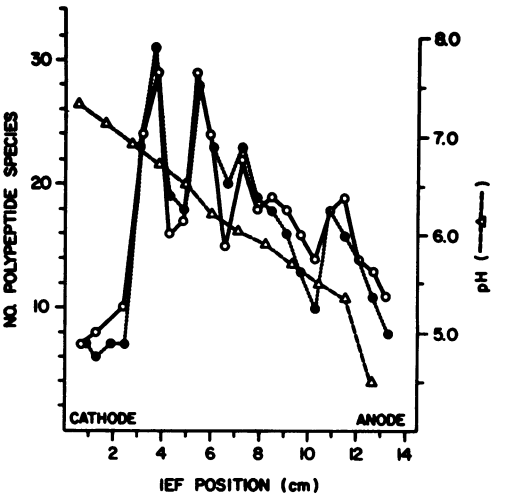

FIG. 6. Distribution of the number of polypeptide species of amebae (●) and flagellates (○) according to their positions in the IEF gradient. The pH of the gradient is also shown (△).

ular sizes in the range of 20 to 40 kilodaltons, and the majority of the polypeptides (60%) were 20 to 60 kilodaltons (Fig. 4). The largest amount of radiolabeled polypeptide in extracts of amebae and flagellates (63 and 46%, respectively) was present in species of 40 to 60 kilodaltons (Fig. 5). About 92% of the radioactivity of polypeptides in amebae, but only 69% of that of flagellates, was in the size range of 20 to 60 kilodaltons. Only 6% of the polypeptide radioactivity of amebae was in the size range of 60 to 100 kilodaltons, whereas 28% of that of flagellates was in this size range (Fig. 5). The mean radioactivity per polypeptide species in the molecular size range of 20 to 40 kilodaltons was less than the mean amount in the range of 40 to 60 kilodaltons for both amebae and flagellates. From 40 to 200 kilodaltons, the mean amount of polypeptide tended to decrease with increasing molecular size for both amebae and flagellates. There was a greater amount of polypeptide per spot for amebae than for flagellates in the size range of 20 to 60 kilodaltons, whereas the converse was true in the range of 60 to 100 kilodaltons.

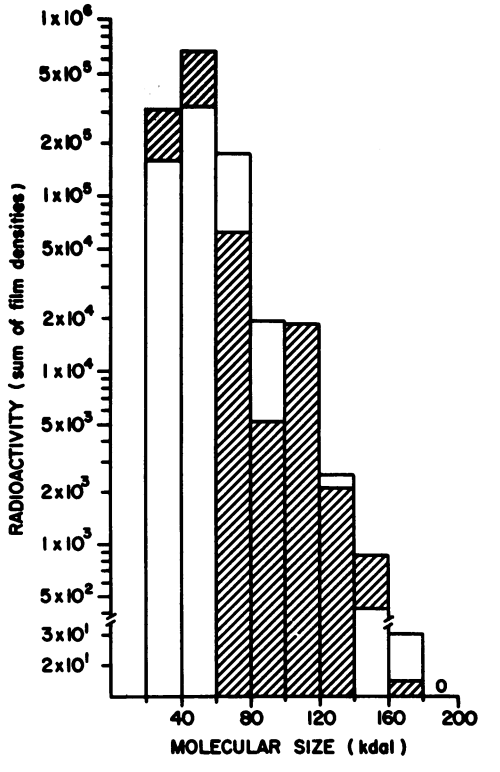

FIG. 5. Distribution of the amounts of polypeptides of amebae (shaded bars) and flagellates (open bars) by molecular size (in kilodaltons [Kdal]).

The distribution of polypeptide species along the IEF dimension was similar for amebae and flagellates. A greater number of radiolabeled polypeptides in ameba and flagellate extracts had isoelectric positions between pH 6 and 7 than in more acid portions of the gels (Fig. 6). The number of polypeptides in extracts of both amebae and flagellates decreased as the pH of the IEF gradient decreased. The amount of polypeptide in extracts of amebae or flagellates was not markedly different at various positions in the pH gradient. There was less polypeptide in the gradient below pH 6.3 for flagellates than for amebae (Fig. 7). Similarly, there was less polypeptide per spot for flagellates than for amebae at pH values below 6.3.

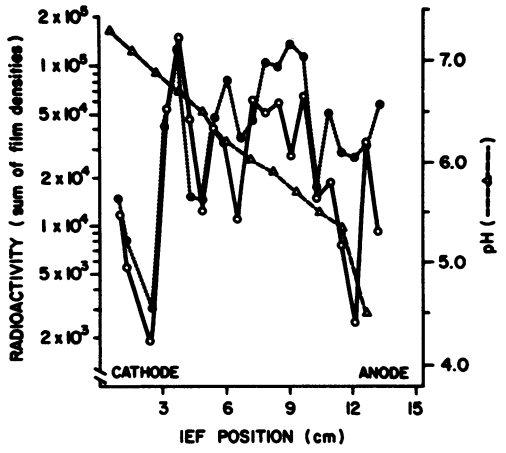

FIG. 7. Distribution of the amounts of polypeptide species of amebae (●) and flagellates (○) according to their positions in the IEF gradient. The pH of the gradient is also shown (△).

TABLE 1. Changes in polypeptide concentration during starvation or enflagellation[a]

Nature of change	No. of polypeptide changes related to:	
	Starvation	Enflagellation
Appeared	3	None detected
Increased	2	14
Decreased	7	6
Vanished	3	5

[a] A change was related to starvation if it occurred in both the enflagellating strain and the non-enflagellating variant after subculture to non-nutrient buffer. A change was related to enflagellation if it occurred in the enflagellating strain but not in the non-enflagellating variant after subculture to non-nutrient buffer. More than 300 polypeptide spots were compared.

The overall correlation for both amebae and flagellates between subunit charge and size, using the median polypeptide as the indicator of central tendency, was that the isoelectric point increased as molecular size increased (Fig. 8A). Similarly, for both amebae and flagellates, the median amount of polypeptide in smaller species had lower pI values than did the median amount in species of larger size (Fig. 8B).

During the process of enflagellation, the complement of the polypeptides detected on autoradiograms was not markedly altered, but a small number of qualitative and quantitative differences were noted. Some of these changes occurred in both the enflagellating strain and the non-enflagellating variant after subculture to non-nutrient buffer, whereas others were observed only when amebae and flagellate preparations were compared (Table 1; Fig. 1). The largest number of polypeptide changes observed were enflagellation-related increases; however, few novel polypeptides were detected. The relative concentration of some polypeptide species decreased in both the enflagellating and the non-enflagellating strain after subculture to non-nutrient buffer; a similar number of decreases were specific to the enflagellating cells. Eight polypeptides displayed unexpected changes; they were present in growing amebae of the non-enflagellating strain and in both strains after subculture to non-nutrient buffer but absent in growing amebae of the enflagellating strain.

There was continued protein synthesis during differentiation. Previously unlabeled amebae subcultured to non-nutrient buffer supplemented

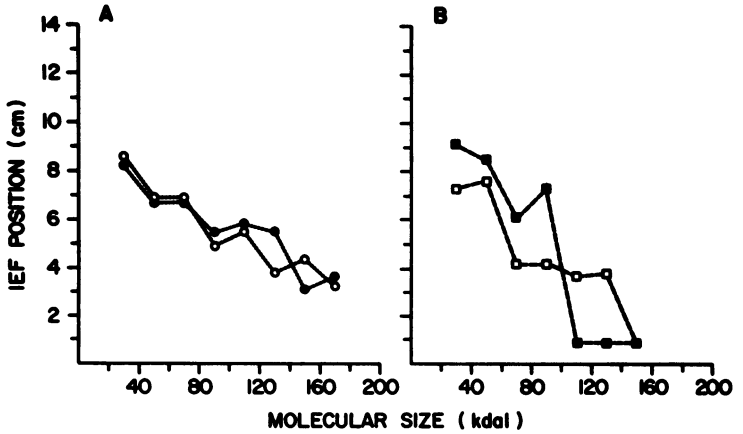

FIG. 8. Relationship between molecular size (in kilodaltons [Kdal]) and charge of polypeptides of amebae (closed symbols) and flagellates (open symbols). Each point represents the position in the IEF gradient at which the median species (A) or median amount of labeled polypeptide (B) is located within each of the molecular size ranges. The relationship between IEF position and the pH of the gradient is similar to those shown in Fig. 6 and 7.

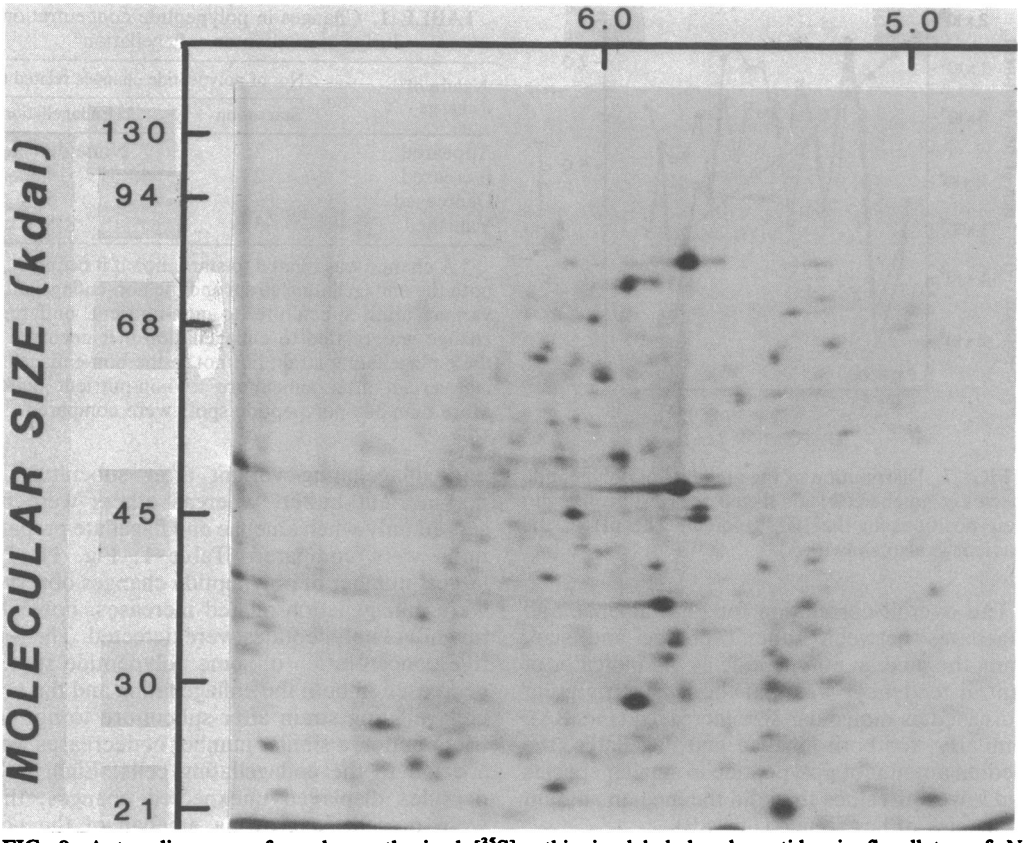

FIG. 9. Autoradiograms of newly synthesized [^{35}S]methionine-labeled polypeptides in flagellates of *N. fowleri*. The proteins were labeled with [^{35}S]methionine during differentiation in non-nutrient buffer. Kdal, kilodaltons.

with [^{35}S]methionine incorporated the label, although to a lesser extent than did growing amebae. Autoradiograms of flagellates radiolabeled during differentiation demonstrated widespread distribution of [^{35}S]methionine among the several hundred polypeptides resolved by two-dimensional electrophoresis (Fig. 9).

DISCUSSION

The polypeptide profiles in two-dimensional gels are similar, whether visualized by Coomassie blue staining or by autoradiograms of [^{35}S]methionine-labeled polypeptides. These results indicate that the polypeptides of *Naegleria* have similar methionine contents and that estimates of amount of polypeptide based on radiolabeled methionine are useful. Several polypeptide species are abundant in the cell, being present at an estimated concentration of 10^8 molecules/amebae. Cellular tubulin, for example, makes up about 12% of the total cell protein of *Naegleria* (9), actomyosin constitutes 0.7% (13), and flagellar tubulin constitutes 0.15% (9). The abundant polypeptides, therefore, are probably structural in nature and might be expected to be relatively stable. For example, the cytoskeletal ameboid motility system remains present in *N. gruberi* flagellates inasmuch as reversion to ameboid form and motility may occur almost instantly under a variety of conditions (8). The majority of the polypeptides detected in our survey are present at an estimated 10^3 to 10^4 molecules/ameba.

Amebae of *N. fowleri* reproducibly transform into flagellates after subculture to non-nutrient buffer (21). A wide spectrum of proteins are synthesized during enflagellation, indicating that the nutritional deprivation used to initiate this differentiation process does not involve a general shutdown of protein synthesis. Moreover, essentially a full complement of cellular proteins is being synthesized during enflagellation. The transformation process is inhibited by cycloheximide (Woodworth, John, and Bradley, unpublished data), indicating that the new protein synthesis is required. The precursors for these new macromolecules must come from stored material and by degradation of preexisting proteins (5). Most of the polypeptides, previously radiolabeled with [^{35}S]methionine, are not per-

ceptibly changed with respect to subunit size, charge, and relative amount. These results indicate that most of the *Naegleria* proteins examined are relatively stable during enflagellation and that they are not covalently modified extensively during differentiation. Particular polypeptides, however, may be modified in both amebae and flagellates by phosphorylation, amidation, dephosphorylation, deamidation, etc. (6). The steady-state level of a polypeptide is generally lower for proteins whose degradation is relatively rapid, and it is these polypeptides which are most responsive to environmental changes (1). Many enzymes and regulatory proteins, particularly those critical to differentiation, are likely to be present at concentrations of less than 10^3 molecules/ameba and therefore may not have been detected in this study.

A few polypeptides do decrease in amount, or vanish, during enflagellation. Presumably these proteins are no longer needed by cells in a nonnutritive medium and can be degraded to supply the precursors and energy required for new macromolecular syntheses. A few polypeptides appear in enflagellating and non-enflagellating strains, presumably as a consequence of nutritional deprivation. These new polypeptides may represent modification of preexisting proteins or newly synthesized polypeptides. No new flagellation-specific polypeptide has been detected, indicating that most changes associated with differentiation in *Naegleria*, as in *Saccharomyces* (23), may involve regulatory processes other than extensive synthesis of unique proteins, such as quantitative shifts, redistribution within the cell or altered supramolecular interactions, or some combination of these possibilities. It is also conceivable that the non-enflagellating variant is capable of producing most of the polypeptides necessary for differentiation but is blocked at some critical step in subunit interaction. The presence of some polypeptides in amebae of the non-enflagellating variant and in flagellates, but absent in amebae of the enflagellating strain, may indicate that the differentiation program is perturbed in the variant.

Quantitative analysis of autoradiograms of samples from amebae and flagellates discloses correlations among charge, size, and amount with polypeptides that are not evident from visual examination of the autoradiograms. Polypeptides having molecular sizes between 60 to 100 kilodaltons and pI values above 6.3 become more abundant during differentiation than those 20 to 60 kilodaltons in size and with pI values below 6.3. Possible explanations for the observed results include (i) an increase in the rate of synthesis of larger, more neutral polypeptides relative to that of smaller more acidic polypeptides, (ii) a decrease in the rate of degradation of larger, more neutral polypeptides relative to that of smaller, more acidic polypeptides, and (iii) an enrichment of larger, more neutral polypeptides as a result of selective export of smaller, more acidic polypeptides during enflagellation. In addition, the amount of polypeptide per spot in the size range of 40 to 60 kilodaltons is greater in both amebae and flagellates than other molecular size classes. It is not known at this time whether the higher levels achieved by particular polypeptide classes reflect faster rates of synthesis, slower rates of degradation, or both. Nevertheless, the subunit size and charge of polypeptides appear to play a significant role in determining their intracellular concentration (7). Our data indicate that size and charge are not entirely independent variables as proposed by some workers (3, 4, 10). A correlation between molecular size and charge has also been reported previously by other investigators using several different eucaryotic models (7, 18). The direction of the correlation observed for whole-cell *Naegleria* polypeptides is the opposite of that observed for soluble proteins in mammalian cells. In *Naegleria*, large polypeptides tend to have neutral pI values, and small polypeptides tend to have acidic pI values.

ACKNOWLEDGMENT

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