Correction. In the article "Interactions between molecules (subfactors) released by different T cell sets that yield a complete factor with biological (suppressive) activity" by Wlodzimierz Ptak, R. W. Rosenstein, and Richard K. Gershon, which appeared in number 7, April 1982, of Proc. Natl. Acad. Sci. USA (79, 2375–2378), the authors request the following corrections.

Maria Ptak should be added as the second author. Her address is Copernicus University, Cracow, Poland. In the footnotes of Table 1 on p. 2376, the second sentence should begin, "The supernatant of these cells was mixed . . ." The following acknowledgment should have appeared on p. 2378.

This work was supported in part by Grants CA 29606 and CA 14216 from the National Institutes of Health and by funds from the Maria Sklodowska-Curie Fund (Polish-American Agreement) and the Polish Academy of Sciences (10.5).

Correction. In the article "Corticotropin and β-endorphin-like materials are native to unicellular organisms" by D. LeRoith, A. S. Liotta, J. Roth, J. Shiloach, M. E. Lewis, C. B. Pert, and D. T. Krieger, which appeared in number 6, March 1982, of Proc. Natl. Acad. Sci. USA (79, 2086–2090), the authors request that the following error be corrected. In the legend to Fig. 5 (p. 2089), line 11 should read:

column B profile and is absent from the column A profile. The high
**Corticotropin and β-endorphin-like materials are native to unicellular organisms**

*(Tetrahymena pyriformis)* **[high-pressure liquid chromatography/gel filtration/radioreceptor assay/bioassay]**

**D. LeRoith*, A. S. Liotta†, J. Roth*, J. Shiloach‡, M. E. Lewis§, C. B. Pert§, and D. T. Krieger‡

*Diabetes Branch and *Pilot Plant, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205; Biological Psychiatry Branch, National Institute of Mental Health, Bethesda, Maryland 20205; and *Department of Medicine, Mt. Sinai School of Medicine, New York, New York 10029

Communicated by Floyd E. Bloom, December 3, 1981

**ABSTRACT** Multiple molecular forms of immunoreactive corticotropin (ACTH) and β-endorphin were present in extracts of a unicellular eukaryote (*Tetrahymena pyriformis*). One form of immunoreactive ACTH reacted similarly with two different ACTH antisera (one specific for the 11-24 sequence and the other with determinants within sequences 1-14 and 17-39) and migrated with synthetic hACTH-(1-39) in a gel filtration system. This form also exhibited ACTH bioactivity in a dispersed rat adrenal cell bioassay with, a mean immunoassay/bioassay ratio of 1.5. Gel filtration revealed multiple size classes of immunoreactive β-endorphin; a major peak of radioreceptor activity was detected which exhibited a Kd, similar to that of authentic β-endorphin. A major portion of immunoreactive β-endorphin-sized material exhibited retention times similar to those of synthetic human and camel β-endorphin upon reverse-phase high-pressure liquid chromatography. These distinctive properties and specificities would seem to exclude the presence of limited homologies with sequences present in other proteins. High molecular weight material containing both ACTH and β-endorphin antigenic determinants was also demonstrated, suggesting, but not proving, the presence of a common precursor molecule.

In recent years, classic neuropeptides have been discovered in extraneural vertebrate tissues (1), and both gut and pituitary hormones have been found in the brain (2). We have reported that a material similar to insulin is present in unicellular eukaryote organisms—a protozoan, *Tetrahymena pyriformis*, and two fungi, *Neurospora crassa* and *Aspergillus fumigatus*—as well as in a prokaryote, *Escherichia coli* (3). In order to extend these findings, we looked for the presence of other peptide hormones in *T. pyriformis*.

The present study presents evidence of the presence, in *Tetrahymena*, of materials similar to corticotropin [ACTH-(1-39); adrenocorticotropic hormone] β-endorphin, and high molecular weight material containing both ACTH and β-endorphin antigenic determinants. Opiate radioreceptor activity was also found. Thus, in addition to insulin, unicellular organisms also contain peptides similar to ACTH and β-endorphin, suggesting that the origins of these peptides are much earlier evolutionarily than was previously thought.

**MATERIALS AND METHODS**

**Culture and Extraction of Tetrahymena.** The ciliated protozoan *T. pyriformis* was grown in large-volume fermenters at 30°C under conditions of controlled aeration in defined medium, without added macromolecules or serum (3). At the end of the logarithmic growth period (36–48 hr), they were harvested, separated from the medium by continuous centrifugation, weighed, and then homogenized in a Manton Gaulin homogenizer. Tissue was extracted in 10 vol of 0.1 M HCl/0.22 M formic acid. The homogenate [batches equivalent to 80 g (wet weight) were processed separately] was defatted 10 times with ethyl acetate/ether and then lyophilized to approximately 1/10 the original volume and the supernatant was shaken with 3 vol of dichloromethane. This step resulted in precipitation of the bulk of the large proteins without significant loss of immunoreactive ACTH or β-endorphin-like material. The aqueous phase was then concentrated on Sep-Pak (Waters Associates) C18 reverse-phase cartridges (25 ml per cartridge). The cartridges were washed with 25 ml of 0.01 M trifluoroacetic acid (F3CCOOH) and then eluted with 5 ml of 0.01 M F3CCOOH/60% acetyl nitrile (CH2CN). Eluates were combined and evaporated to dryness under reduced pressure.

The samples [equivalent to 240 g (wet weight)] were reconstituted in 2.5 ml of 0.1 M HCl/0.22 M formic acid and subjected to gel filtration on a 1.5 × 100 cm column of Sephadex G-50 (fine) with 0.22 M formic acid as eluent. Culture medium was also carried through the entire tissue culture and extraction process (in the absence of *Tetrahymena* cells) in order to determine if the ACTH and endorphin-like material detected were due to the presence of components other than the *Tetrahymena* cells (i.e., 50 liters of medium was processed in this manner). No immunoreactive ACTH or β-endorphin-like material was detected in a concentrate of the cell-free medium; an equal concentrate of *Tetrahymena* extract contained 1 pmol of immunoreactive β-endorphin-like material. This would exclude possible contamination by media or sources within the fermentation vessels or within the assay procedures utilized for detection of ACTH and endorphin-like material.

**Assay Methods.** ACTH radioimmunoassay (4). Two antisera were used. Antiserum 7C, raised in a rabbit, contains antibody molecules directed toward determinants in the NH2-terminal region of the ACTH molecule. ACTH-(1-13) and human ACTH-(1-39) (hACTH) react on an equimolar basis; α-melanotropin (melanocyte-stimulating hormone) reacts in a parallel fashion at approximately 58% molar crossreactivity. Rat "pro-opiocortin" (the ACTH-lipotropin precursor molecule) purified from pituitary intermediate lobe by gel filtration and ion exchange chromatography also reacts with the antiserum on an approximately 70% molar basis, whereas α-, β-, and γ-endorphins, and human β-lipotropin do not crossreact. Four microliters of this antiserum is sufficient to completely immunoprecipitate 2.7 pmol of hACTH-(1-39). The West antiserum (National Institutes of Health, Bethesda, Maryland) was used.

**Abbreviations:** F3CCOOH, trifluoroacetic acid; CH2CN, acetyl nitrile; ACTH, corticotropin (adrenocorticotropic hormone); hACTH, human ACTH.

† To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.
Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases) reacts strongly with ACTH-(11–24) but with decreased affinity in a non-parallel fashion with rat pro-opiocortin and not at all with ACTH-(1–10) or ACTH-(17–39).

To exclude the possibility that apparent reactivity of the gel-filtered extract in the immunoassay was due to interference—e.g., degradation of the radiolabeled ACTH, or inhibition of immunoprecipitation—two additional experiments were performed. In experiment 1, $^{125}$I-labeled ACTH was incubated under conditions of the assay but without first antibody, in the presence and absence of gel-filtered extract that was rich in apparent ACTH immunoreactivity. After 2 days, 4 µl (an excess) of ACTH antibody was added and precipitated in the usual way with second antibody; 85–92% of the $^{125}$I-labeled ACTH was precipitated irrespective of whether or not extract had been present. In the second experiment, a sample of the extract prior to gel filtration was incubated with labeled ACTH for 2 days. This was then chromatographed on an SF-Sephadex cation exchange column. Labeled ACTH eluted in the same position as radiolabeled ACTH that had been incubated with authentic unlabeled ACTH.

**Immunosorption with Sepharose-immobilized affinity-purified ACTH antiserum.** This was performed as described (5, 6). Immunodepletion (ACTH bioassay) studies were performed with affinity-purified ACTH, β-endorphin, and bovine serum albumin antisera prepared as described (5, 6). The anti-β-endorphin immunoglobulin was prepared from the same antisera used for radioimmunoassay. The anti-ACTH immunoglobulin was prepared from an antisera containing binding sites directed towards both the NH$_2$ and COOH termini of hACTH-(1–39).

**ACTH bioassay.** This was performed as described (7). In preliminary experiments (data not shown), immunoreactive ACTH-sized material was serially diluted and incubated in the dispersed rat adrenal gland cell ACTH bioassay. The extract caused a dose-dependent increase in steroidogenesis. Parallelism with the synthetic hACTH standard was demonstrated over a 10-fold range, by analysis of variance, thus permitting quantification of the bioassay. Labeled ACTH eluted from the column in the same manner as authentic hACTH-(1–39).

**β-Endorphin radioimmunoassay** (9). Two β-endorphin antisera were used. The antigenic determinants reside within residues 19–27 of the COOH-terminal sequence of the β-endorphin molecule. Camel and human β-endorphin and human β-lipotropin react on an equimolar basis with both antisera. Rat pro-opiocortin also reacts on a nearly equimolar basis, whereas ACTH-(1–39), α-, γ-endorphin, and α-melanotropin do not react at all with these antisera. To exclude nonspecific effects by the extract in the immunoassay, two additional experiments were performed as described for the ACTH immunoassay.

**Opiate radioreceptor assay.** The 10,000 x g pellet of homogenized rat cerebrum was used as the source of receptor and β-endorphin was used as the standard unlabeled peptide; the assay was carried out as described (10, 11) with the use of both $^3$H-labeled [d-Ala$^2$,D-Leu$^5$]enkephalin and $^3$H]naloxone as ligands.

**High-pressure liquid chromatography.** This was performed with instrumentation as described (12).

**RESULTS**

**Presence of ACTH-like Material.** Immunoreactive ACTH content of the extract was 0.164 pmol/g (wet weight) when assayed with the NH$_2$-terminal-directed antisera 7C. The extract exhibited parallelism with the standard curve over a 1:10 dilution. Sephadex G-50 gel filtration revealed multiple partially resolved peaks of immunoreactive ACTH. A prominent peak eluted with the K$_m$ of hACTH-(1–39) standard, indicating that this material was the approximate size of the standard (Fig. 1). Material in this peak ( aliquots of fractions 102–104) was pooled, concentrated, and rerun on a similar column (1.5 x 90 cm); >78% of the immunoreactive ACTH again eluted at the expected position of authentic hACTH-(1–39) (data not shown). Fractions comprising this peak were pooled and concentrated on a C$_8$ Sep-Pak, and the extract was incubated for 24 hr at 37°C in the presence of 10 mM dithiothreitol. Dilutions (1:20) of the extract demonstrated parallelism (analysis of variance) with the synthetic hACTH-(1–39) standard in the ACTH bioassay (Fig. 2). The mean immunoreactive (midportion ACTH antibody)-to-bioreactive ACTH ratio of the extract was 1.5 (range, 1.2–1.7). Synthetic hACTH-(1–39) similarly processed yielded an average immunoreactive-to-bioreactive ratio of 1.1 (range 1–1.2). The similarity of these ratios, however, in no way ensured that material reactive in the ACTH radioimmunoassay was responsible for the ACTH bioreactivity of the extract. Therefore, a series of immunodepletion studies were performed to investigate this possibility.

In one experiment (Fig. 2), incubation of extract with affinity-purified anti-ACTH immunoglobulin prior to bioassay resulted in a marked decrease in bioreactivity of the Tetrahymena extract (>80% loss of bioreactivity compared to concentrations obtained by assay of extracts preincubated with bioassay medium). In contrast, when dilutions of the extract were preincubated with either anti-bovine serum albumin immunoglobulin or antihuman β-endorphin immunoglobulin, no loss of bioreactivity was noted (data not shown).

In another experiment, Tetrahymena extract was incubated with anti-ACTH immunoglobulin and then percolated through a Sepharose-immobilized protein A column; the column effluent was bioassayed. Under the conditions used, virtually all
of the anti-ACTH material was retained on the column; if the Tetrahymena bioactive ACTH-like material were specifically bound by the ACTH immunoglobulin, no bioreactivity should be associated with the effluent. Bioassay of multiple dilutions of the effluent revealed that >90% of such activity was removed (Fig. 2). When the column technique was used with either anti-β-endorphin or anti-serum albumin immunoglobulin, <10% of bioassayable material was removed from the extract (data not shown).

Presence of β-Endorphin-like Material. Immunoreactive β-endorphin concentration of the extract was 0.294 pmol/g (wet weight) when assayed with the COOH-terminal β-endorphin antiserum. The fractions obtained from the first gel filtration experiment were also analyzed for the presence of β-endorphin-like material with this antiserum. Multiple peaks of β-endorphin immunoreactivity were detected. The majority of the material eluted with an apparent molecular weight similar to that of authentic β-endorphin. The remainder of the activity was distributed in peaks eluting before and after the authentic β-endorphin marker (Fig. 3 Upper). Radioreceptor assay of alternate gel filtration fractions, utilizing 3H-labeled [Dala, DLeu]enkephalin, revealed a broad peak of opiate-like activity; the summit of the peak eluted with the approximate apparent size of authentic β-endorphin (Fig. 3 Lower). When these same fractions were tested in the radioreceptor assay utilizing [3H]naloxone, no opiate-like activity was detected. Receptor activity was approximately 50-fold greater than immunoreactivity. Similar discrepancies have been described for rat brain extracts when immunoassayable [Met]- and [Leu]enkephalin content was compared to radioreceptor activity by using displacement of 3H-labeled[Leu]enkephalin (13).

In order to investigate whether any of the material reactive in the radioreceptor assay was also reactive in the β-endorphin immunooassay, an aliquot of pooled fractions (fractions 100–120 of Fig. 3 Lower) containing 2.5 pmol of immunoreactive β-endorphin-sized material (150 pmol of radioreceptor activity) was immunoprecipitated with anti-β-endorphin immunoglobulin, and the supernatant was assayed by both radioreceptor assay and immunooassay. The immunooassay value was decreased to 0.43 pmol; the radioreceptor value was decreased to 22 pmol.

When aliquots of fractions 100–122 (K clot, region for authentic human β-endorphin) were pooled, concentrated, and rerun on a similar column, most of the immunoreactive β-endorphin material eluted in the same region (data not shown). Aliquots of fractions 103–118 of the rerun column were pooled and passed through a C18 Sep-Pak cartridge, and the eluent was taken to dryness and oxidized with H2O2 in 0.1 M CH3COOH (to convert methionine-containing peptides to the sulfoxide forms). Reverse-phase high-pressure liquid chromatography of this sample was then performed as described in Fig. 4. All fractions were assayed for immunoreactive β-endorphin content. The major portion of the immunoreactive β-endorphin appeared to be distributed in several poorly resolved molecular species exhibiting retention times similar to those of camel and human β-endorphins and related peptides. When additional Tetrahymena extract is available, further analysis will be necessary to resolve this material into homogeneous peaks. Material eluting near the positions of [Met]- and [Leu]enkephalin are not related to those peptides because the antiserum used does not crossreact with them.

High Molecular Weight Material Contains the Dual Antigenic Determinants of ACTH and β-Endorphin. The gel filtration patterns of Tetrahymena extract revealed both high molecular weight immunoreactive ACTH and β-endorphin-like
material (see Figs. 1 and 3 Upper). It is possible that both antigenic determinants were present within the same molecule(s), as detected in the two radioimmunoassays, as is known to occur with pituitary high molecular weight material. Initially, to test this hypothesis, an aliquot of *Tetrahymena* extract [equivalent to approximately 180 g (wet weight)] was passed through a microcolumn containing affinity-purified ACTH antiserum covalently bound to Sepharose 4B (column A). Twenty-five micrograms of synthetic hACTH-(1–39) was added to a similar aliquot of extract and similarly percolated through another anti-ACTH column to control for specific binding (column B). Both column effluents were subjected to NaDodSO₄/polyacrylamide gel electrophoresis. All fractions obtained were assayed in the β-endorphin radioimmunoassay.

Consistent with the Sephadex gel filtration study, high molecular weight immunoreactive β-endorphin exhibiting an apparent molecular weight >25,000 was present in the NaDodSO₄/polyacrylamide gel electrophoresis profile of the column B effluent (Fig. 5). In contrast, such high molecular weight material was not detected in the profile of the column A effluent. This result is consistent with the idea that such high molecular weight material contains both ACTH and β-endorphin antigenic determinants. Twenty-five micrograms of hACTH-(1–39) (in 200 µl) was then applied to column A, which was presumed to have specifically bound the high molecular weight β-endorphin by recognition of ACTH determinants; the column was stopped on both ends and maintained at room temperature for 16 hr. Then 2 ml of radioimmunoassay buffer was passed through the column, and the effluent was concentrated, and subjected to NaDodSO₄/polyacrylamide gel electrophoresis. Gel slices were assayed for immunoreactive β-endorphin. Almost all of the immunoreactive β-endorphin detected exhibited a molecular weight >25,000. The distribution of immunoreactive β-endorphin was qualitatively similar to that of the multiple high molecular weight forms seen in the column
subjects, from 1.36 to 1.4 (19)]. In considering structure–function relationships with regard to such biological activity, ACTH analogues smaller than ACTH-(1–18) have markedly decreased potencies on a molar basis. It is generally agreed that amino acids 5–10 are required for binding of ACTH to the ACTH-specific receptor and 11–18, for ACTH activation of corticosterone production (20, 21).

These data indicate that the 4500-dalton ACTH-like material in the protozoa is indeed similar to ACTH-(1–39), and the distinctive specificities noted, which correspond to three overlapping regions that span ACTH-(1–39), would seem to exclude the presence of limited homologies with sequences present in other proteins (22). Unequivocal confirmation of the present tentative identification of ACTH-like material must await sequence determination (either of the peptides themselves or by using cDNA technology), a Herculean task at the moment given the low concentrations of this material in the protozoa.

Interestingly, we also find high molecular weight material that has both endorphin-like and ACTH-like immunoreactivities. This suggests that the protozoan contains a gene(s) that codes for a common precursor similar to the gene(s) that code(s) for the authentic pituitary common precursor in vertebrates, although we obviously have not demonstrated a precursor–product relationship in this preliminary investigation.

The β-endorphin-like material demonstrated in the present study was characterized by Sephadex gel filtration, high-pressure liquid chromatography, polyacrylamide gel electrophoresis, and radioreceptor activity. The presence of greater amounts of radioreceptor-active material than of immunoreactive β-endorphin-like material prompted us to look for other ligands which would bind to the receptor. The presence of immunoreactive dynorphin-like material was in fact detected (unpublished data). This finding could indicate the presence of a product from yet a different gene(s) in addition to the presence of insulin (3), ACTH-endorphin-like material, and a variety of gastrointestinal and other anterior pituitary and posterior pituitary-like peptides that we have found (unpublished data) in this organism. Alternatively, many of these peptides could be within a very large "pre-vertebrate" precursor molecule.

The presence of immunologically active ACTH-like material has been reported only in vertebrate species, and likewise the pituitary and adrenal are organs that arose early in vertebrate evolution, or about 400 million years ago. If the ACTH-like material we detected in Tetrahymena is the ACTH progenitor, this extends its origin to about 1 billion years ago (23).

We do not know the function of these peptides in the protozoa. However, our finding in protozoa of material that interacts with vertebrate receptors strengthens the possibility that this material may also serve a physiological function in the protozoa (24). This function could be exercised through the extracellular fluid medium from one organism to another or the messenger function could exist entirely within a single organism.

The present findings are in accord with our finding of insulin-like and other peptides (i.e., somatostatin) (unpublished data) in protozoa that closely resemble the peptide hormones and neuropeptides of the vertebrates. They suggest that the messenger peptides that have been thought to be unique to vertebrates, or possibly to include very complex invertebrates, and to be neuronal in origin are actually evolutionarily much older and go back at least as far as the simplest eukaryotes, and, in the case of insulin (25) and human chorionic gonadotropin (15), to bacteria as well.

This work was supported in part by the Lita Annenberg Hazen Charitable Trust.