material used in this study corresponded to the major monosulfate product of synthesis blocked at positions 5 and 6; on the basis of chemical tests this product should have a sulfate at position 3. Reactivity of this compound to agents that oxidize ascorbate and to the ferric chloride test indicated a blocked position 3 on the ascorbate sulfate. Preliminary ^{13}C nuclear magnetic resonance showed the product was not substituted on positions 5 or 6 and indicated probable substitution at position 3. Because structural study is not complete, unambiguous assignment of structure is not possible. On the basis of the experimental evidence given above, the compound is presumed to be ascorbate-3-sulfate.

After addition of synthetic carrier ascorbate-3-sulfate, radioactive material in the S fraction was fractionated by chromatography on a Dowex-1 formate column eluted with a linear gradient system of formate and formic acid as described by Mead and Finamore (*3*). The major radioactive component was doubly labeled with the same ratio of ^{14}C to ^{3}H as the ingested ascorbate. This fraction had a maximum absorbancy at 254 nm in alkaline or neutral solution corresponding to the values given by Mead and Finamore for ascorbate sulfate in brine shrimp cysts. The materials in this formate fraction and the original S fraction were then cochromatographed against chemically synthesized ascorbate-3-sulfate in the systems listed in Table 1. In all cases, correspondence of a positive iodine vapor reaction was observed for this metabolite of ascorbic acid and the synthetic ascorbate-3-sulfate.

Direct column chromatography on DEAE cellulose of both freshly voided urine and the S fraction of urine from normal adult males showed a substance that absorbs ultraviolet light and that is eluted by the sulfuric acid linear gradient at the position corresponding to synthetic ascorbate-3-sulfate. When the S fraction from normal male urine was cochromatographed on DEAE cellulose with ascorbate-3-[^{35}S]sulfate, there was exact coincidence at the position of the ascorbate-3-sulfate between the ^{35}S radioactivity and the urinary material that absorbs ultraviolet light.

Under conditions of high intake of ascorbate in man (100 or more milligrams per day) much ingested ascorbate is excreted unchanged (*2*). The rapid decomposition of free ascorbic acid in urine makes especially difficult the isolation and identification of true urinary metabolites. Only on a highly restricted intake of ascorbate can the essential metabolites of ascorbate be easily observed. Recent experiments with human volunteers indicate that this metabolism of ascorbate is about 3 percent of the body pool per day (*2*). Fractionation of these essential metabolites in human urine by means of the lead acetate precipitation method of carbohydrate chemistry shows that there are a variety of products somewhat comparable to those we have recently observed in ascorbate metabolites excreted by the guinea pig. It seems likely that the metabolites precipitated by lead acetate at *p*H 4 and at *p*H 8 are in part glucuronide derivatives and carboxylic acids formed by oxidative processes. The S fraction corresponds to about 25 percent of the total metabolites, and in it the major radioactive component is ascorbate-3-sulfate.

The demonstration of ascorbate-3-sulfate in human urine and in brine shrimp cysts, as well as our studies which show its existence in guinea pig, rat, and trout urine, suggests that this compound is a fairly ubiquitous metabolite of ascorbate in higher animal systems. The biological role of ascorbate sulfate is of some interest. It could act as a sulfate donor, as has been suggested by Chu and Slaunwhite (*4*) and Mumma (*5*). It could have a function in transport across cellular membranes in as much as ascorbate-3-sulfate is a di-negative ion at physiological *p*H's and appears to interact strongly with metal ions. It could also be part of the ascorbate pool in man and animals. The ascorbate sulfate could also play a part in the transport of ascorbate across the blood-brain barrier and in the concentration of ascorbate in brain tissue. Hammarström and others have demonstrated that free ascorbate does not rapidly cross this barrier (*6*).

This ascorbate sulfate derivative adds one more substance to the growing list of compounds that suggests a greater biochemical role for ascorbate than that of a simple antioxidant. Blaschke and Hertting (*7*) have recently demonstrated 2-methylascorbate in the rat. Earlier, Kiss and Neukom (*8*) have suggested that 2-methylindol ascorbate was the ascorbigen of cabbage. In addition, a variety of 2- and 3-ascorbate derivatives have been prepared by chemical means. The presences of a number of yet unidentified six-carbon metabolites in human and guinea pig urine indicate the complexity of essential ascorbate metabolism.

E. M. BAKER, III
D. C. HAMMER, S. C. MARCH
B. M. TOLBERT, J. E. CANHAM
U.S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado 80240

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25 February 1971; revised 10 May 1971

Leucylglycinamide Released from Oxytocin by Human Uterine Enzyme

Abstract. *Uteri of pregnant and nonpregnant women contain enzymic activities which inactivate oxytocin. A potent enzyme, which has been partially purified from uterine homogenates, cleaves the prolyl–leucyl peptide bond of oxytocin. This finding associates for the first time the release of the dipeptide leucylglycinamide with the degradation of neurohypophyseal hormones.*

Extracts of human uterine tissue inactivate the neurohypophyseal hormone oxytocin (*1*, *2*). To clarify the underlying enzymic reactions, we have identified products of oxytocin digestion. We adapted a fractionation procedure, used previously in studies (*3*) of uterine extract from rats, for the partial purification of uterine extracts from pregnant and nonpregnant women; the former at a time of elective caesarean section. We used strips of the uterine wall of

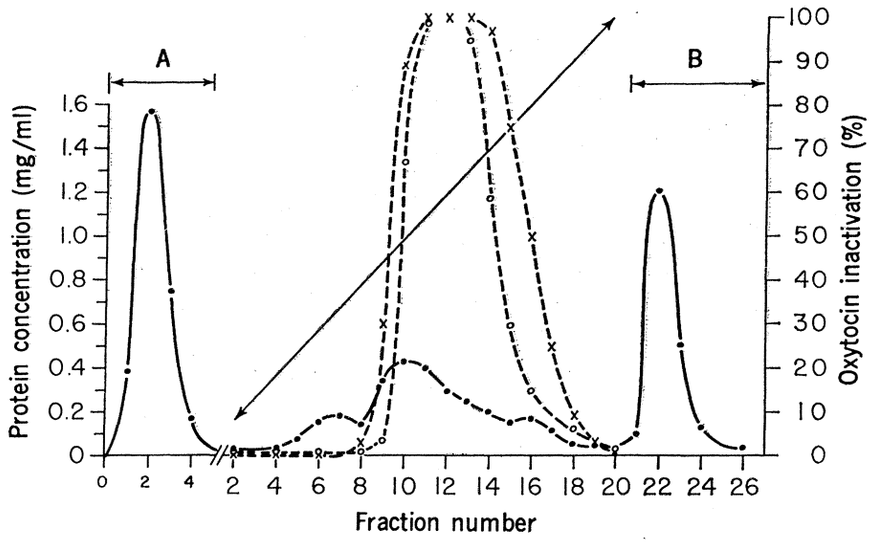

Fig. 1. Inactivation of [9-glycinamide-1-^{14}C]oxytocin ([^{14}C]oxytocin) by a soluble fraction of uterine tissue obtained from a nonpregnant woman. The figure depicts the amount of protein eluted (● —— ●), the percentage of oxytocin inactivation as evaluated by the rat uterotonic assay (x --- x), and the percentage of leucylglycinamide released (○---○) from [^{14}C]oxytocin. *A* and *B* refer, respectively, to 0.01*M* phosphate buffer and 0.75*M* phosphate buffer with 1*M* NaCl. The long arrow indicates the linear phosphate buffer gradient from 0.01 to 0.15 mole/liter.

full thickness from serosa to endometrium. The samples were washed repeatedly with ice-cold saline and then quickly frozen at approximately −70°C. Each gram of frozen tissue was homogenized in 10 ml of 0.01*M* sodium phosphate (*p*H 6.5, referred to as buffer) at 0°C in a Virtis model 45 disintegrator for 1 minute. The homogenate was centrifuged at 105,000*g* for 20 minutes, and the supernatant was applied to a column of diethylaminoethyl cellulose (1.0 g per 2 g of tissue) equilibrated with buffer at 4°C. As the extract was applied to the column and during a subsequent wash of the resin bed with 50 ml of buffer, the column effluent was collected in 9.6-ml fractions. Then the column was eluted with a phosphate buffer having a linear concentration gradient from 0.01 to 0.15 mole/liter; 21 fractions (4.8 ml) were collected. Subsequently the column was stripped with seven 9.6-ml fractions of 1.0*M* NaCl in 0.075*M* phosphate buffer.

The protein elution curve and the pattern of oxytocin inactivation by uterine homogenate from a nonpregnant woman is shown in Fig. 1. Only the gradient eluate was tested; residual oxytocin activity in various fractions was determined by the rat uterotonic bioassay (*3*). Qualitatively the results with uteri from nonpregnant women are identical to our previous findings (*2*) with uteri from pregnant women in that two fractions [eluted with 0.04 to 0.06*M* phosphate buffer (referred to as fraction 1) and eluted with 0.075*M* phosphate buffer containing NaCl (referred to as fraction 2)] are capable of inactivating oxytocin. We conclude from direct identification of the products of oxytocin digestion and of various biologically active oxytocin analogs, which are resistant to aminopeptidase action as well as to redox reactions involving the disulfide bond (*4*), that fraction 2 contains an aminopeptidase. No such conclusion could be drawn in the previous paper (*2*) about reaction products formed during digestion of oxytocin with enzyme fraction 1. This report is specifically concerned with the enzymic activity associated with that fraction.

We prepared radioactively labeled [9-glycinamide-1-^{14}C]oxytocin ([^{14}C]-oxytocin) (*5*) which allows a product identification with partially purified enzyme preparations. Samples (0.5 ml) of fraction 1 were incubated with 0.5 μg of [^{14}C]oxytocin dissolved in 0.05 ml of water at 37°C at *p*H 6.5. The reaction was stopped by heat treatment after 2 hours. The digest was lyophilized and dissolved in 0.1 ml of water. Then it was subjected to high-voltage electrophoresis with two buffers, 10 percent acetic acid–pyridine (*p*H 3.6, 1500 volts, 3.5 ma/cm, 2 hours) (S_1) and 1 percent formic acid–pyridine (*p*H 2.5, 1500 volts, 1.5 ma/cm, 2 hours) (S_2). Oxytocin (which had been incubated with buffer alone as a control), glycine, glycinamide, leucylglycinamide, and prolylleucylglycinamide served as markers during the electrophoretic separation of digest products. Two radioactive peaks were detected and measured with a Baird Atomic Scanogram II on the electrophoretogram (*6*). The compound migrating closer to the origin in both buffer systems was identified by comparison with authentic [^{14}C]oxytocin as the hormone (in S_1, ~ 6.0 cm; in S_2, ~ 5.5 cm). The other peak, which traveled further toward the anode, was leucylglycinamide (in S_1, ~ 13 cm; in S_2, ~ 15 cm). The structure of leucylglycinamide was confirmed in an independent experiment. Oxytocin (2.5 μg of [^{14}C]oxytocin and 22.5 μg of unlabeled oxytocin) was digested with a sample of fraction 1 (tube 12) for 3 hours. The digest was subjected to electrophoresis (*7*) in S_2. The area corresponding to the position of leucylglycinamide was eluted with 10 percent acetic acid, lyophilized, and hydrolyzed for 22 hours at 110°C with 6*N* HCl. When a sample of the hydrolyzate was subjected to electrophoretic separation [^{14}C]glycine was detected. Another sample applied to a modified Beckman amino acid analyzer (model 121C) revealed the presence of leucine and glycine in a molar ratio of 1 : 1.3.

From the above results we conclude that human uterus contains an enzyme capable of inactivating oxytocin by cleaving the prolyl–leucyl peptide bond, thereby releasing the carboxy terminal dipeptide leucylglycinamide. Although earlier studies of oxytocin-responsive tissues other than human uterus have shown that the inactivation of this hormone can occur by the release of the carboxy terminal glycinamide (*2, 3, 7, 8*) and cleavage of the cysteinyl–tyrosyl linkage with (*9*) or without (*10*) prior reduction of the disulfide bond, the release of the dipeptide leucylglycinamide marks a newly discovered locus for the inactivation of oxytocin.

RODERICH WALTER
H. SHLANK
J. D. GLASS
I. L. SCHWARTZ
T. D. KERENYI
Departments of Physiology and
Obstetrics and Gynecology,
Mount Sinai Medical and
Graduate Schools,
City University of New York,
New York 10029

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11. Supported in part by service grants AM-13567 and AM-10080 from the U.S. Public Health Service.

12 April 1971

Human Fetal Cerebellar Cortex: Organization and Maturation of Cells in vitro

Abstract. *Human fetal cerebellar cortex was maintained up to 5 months in vitro. Important features included early migration of granule neurons followed by maturation of Purkinje and granule neurons. Unique areas of organization developed in which a rim of leptomeningeal cells surrounded an explant and its outgrowth zone; these areas subsequently grew as well-defined units.*

In this report we describe cell maturation and the development of a highly organized pattern of growth in organotypic cultures of human fetal cerebellar cortex. Using specimens removed surgically for the purpose of terminating pregnancy (*1*), we prepared explants from ten human fetuses, varying in gestational age from 10 to 19 weeks. Cerebellar cortex, with leptomeninges intact, was undercut and removed, deep cerebellar nuclei and brainstem structures being avoided. The tissue was sectioned into small serial blocks which were suspended in 15 ml of nutrient mixture F-12 (*2*). They were distributed among five to ten plastic petri dishes (Falcon), each dish containing several fragments. Explants were grown directly on the surface of the dish or on sterile glass cover slips previously introduced. Complete feeding medium consisted of 80 parts of nutrient mixture F-12, 20 parts of fetal calf serum, one part of fresh L-glutamine (200 mmole/liter), one part of nonessential amino acids (GIBCO), and one part of 50 percent glucose in water (which raised the total glucose content to 680 mg/100 ml). Collagen and plasma clot were not used, nor were antibiotics or embryo extract.

Observations were made on living cultures and on fixed cultures terminated after varied periods of growth. Phase, dark-field, bright-field, and polarizing optics were used to examine cultures stained with methylene blue, phosphotungstic acid-hematoxylin (PTAH), luxol fast blue, the trichrome method, or Bodian's silver protargol method. For electron microscopy, cultures were fixed in phosphate-buffered glutaraldehyde, treated with osmium tetroxide, dehydrated, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate. They were examined with an RCA EMU-3F electron microscope.

After a few days in culture, cells migrated from the explant in steadily increasing numbers. The nuclei of cells migrating earliest were pale and moderately large. The cytoplasm was broad and sheetlike, eventually giving rise to one or more processes of variable length and thickness. These cells were interpreted as immature astrocytes (*3*). By the end of the first week slender neuritic processes emerged from the explant, followed several days later by migration outward of the cell bodies from which they arose. These cells were small and migrated in groups. Their nuclear size (major diameter 7 to 10 μm), nuclear chromatin pattern, and scanty cytoplasm which gave rise to long, thin, unipolar or bipolar processes which stained strongly after silver reduction were characteristic of immature granule neurons (Fig. 1A). Ultrastructural appearance of a cell from a similar cluster of early migrating small cells is seen in Fig. 1B. At

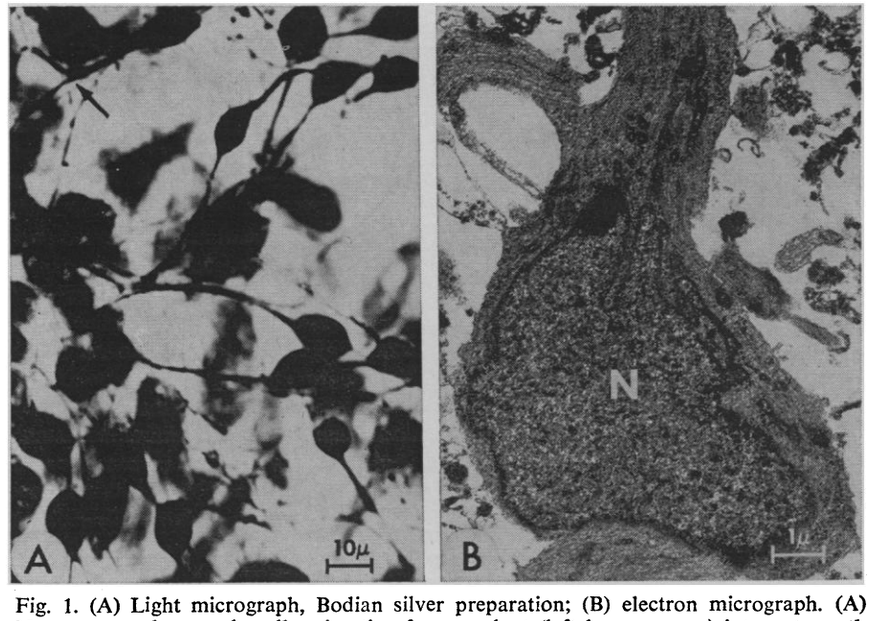

Fig. 1. (A) Light micrograph, Bodian silver preparation; (B) electron micrograph. (A) Numerous early granule cells migrating from explant (left lower corner) into outgrowth zone. Argyrophilic processes are prominent. Arrow (upper left) points to a bifurcating process. Culture of cerebellar cortex from a 16-week-old fetus after 39 days in vitro. (B) Cell from similar group of early migrating small neurons. A thin rim of cytoplasm surrounds the nucleus (*N*) and gives rise to a long process, only the initial part of which is shown. From 16-week-old fetus after 14 days in vitro.