IN VITRO STUDIES

ON

THE BIOSYNTHESIS OF OXYTOCIN

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By

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A B S T R A C T

In vivo and *in vitro* studies on the biosynthesis of vasopressin in the supraoptic nuclei of the dog and guinea pig[1] using ^{35}S-cysteine and ^{3}H-tyrosine have suggested that vasopressin could be synthesized by way of a precursor, which is modified to release active hormone. *In vivo* injection of ^{3}H-tyrosine into the cerebrospinal fluid of rats[2] had resulted in incorporation of the label into both oxytocin and vasopressin.

In this work, an attempt was made to develop an *in vitro* system for the biosynthesis of oxytocin. Incubations of either ^{3}H-isoleucine or ^{14}C-leucine with rat hypothalamic neuronal perikaya, ribosomes and cell sap, cell sap, fractions of cell sap and homogenate, and incubations of ^{3}H-isoleucine and ^{14}C-leucine with rat hypothalamic tissue fragments were analyzed for the incorporation of label into purified hormone. Gel filtration, partition chromatography, high voltage electrophoresis, and thin layer chromatography were applied, followed by measurement of radio-activity and biological activity.

It is concluded that in no reproducible case was either radioactive isotope incorporated into material with the chromatographic and biological properties of oxytocin. Other radioactive products of incubation were detected in hypothalamic cell sap, ribosomes and cell sap, and homogenate. In hypothalamic homogenate incubations, considerable degradation of both oxytocin and other material absorbing

at 280 nm was observed.

It is suggested that future investigations should attempt to first develop isolation procedures for the labelled hormone produced *in vivo*, and then reduce the complexity of the system in small stages, through the cultured hypothalamic-neurohypophyseal system of Sachs[3] to simpler *in vitro* systems.

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[1] For review, see Sachs, H., P. Fawcett, Y. Takabatake, and R. Portanova (1969). Biosynthesis and release of vasopressin and neunophysin. Rec. Prog. Hormone Res. 25, 447

[2] Pickering, B., and C. Jones (1971). Isolation of radioactive oxytocin and vasopressin from the posterior pituitary gland of the rat after the injection of labelled tyrosine into the cerebro-spinal fluid. Endocr., 49, 93.

[3] Sachs, H., R. Goodman, J. Osinchak, and J. McKelvy (1971). Supraoptic neunosecretory neurons of the guinea pig in organ culture. Biosynthesis of vasopressin and neurophysin. Proc. Nat. Acad. Sci. USA, 68, 2782.

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INTRODUCTION

The control and feedback mechanisms in homeostasis have been a tempting puzzle for biochemists and physiologists. Knowledge of the control of enzyme function, metabolic pathways, eclllular activity, and tissue organization have each contributed to the understanding of life. The understanding or regulation of hormone production and secretion has been one of the most instructive, as the remote control of the endocrine system lends it its usefulness. However, before one can study regulation, the basic pathway of synthesis must be known.

The studies of Bargmann and Scharrer, and Scharrer and Scharrer (1,36) revealed that the mammalian neurohypophyseal hormones, oxytoxin and vasopressin, are synthesized in the para-ventricular and supraoptic nuclei of the hypothalamus, respectively, and transported in neurosecretory granules the length of the axons to the posterior pituitary gland, or neurohypophysis, where they are stored in large quantities to be released into the blood stream upon the appropriate stimulus.

Oxytocin, which causes both uterine contraction during labor, and milk ejection during lactation, seems to be released at the appropriate times by nervous stimuli. Vasopressin, which causes vascular constriction, is more noted for its antidiuretic effect. Its secretion is elicited by hyperosmolality, dehydration, or hemorrhage. Studies by Daniel and Lederis and by Nordmann et al (5, 23), on the

intact hypothalamoneurohypophyseal system (HNS) *in vitro* have shown that acetylcholine stimulates the release of both oxytocin and vasopressin, and Dyball and Koizumi (8) have shown that this releast is concurrent with excitation of the supraoptic and paraventricular nuclei.

Nevertheless, while there is some evidence on the short term effects on hormonal release, the long term control of hormone biosynthesis is poorly understood. Jones and Pickering (15) have shown that up to 5 days water deprivation or sodium chloride inhibition results in a decreased neurohypophyseal content of oxytocin and vasopressin in rats, while Takabatake and Sachs (42) have shown that 4 days of water deprivation results in an increased biosynthesis of vasopressin in guinea pig hypothalamic slices incubated *in vitro*. In addition, in the hereditary diabetes insipidus (DI) of the Brattleboro strain of rats studied by Valtin and co-workers (43), vasopressin synthesis is impaired. It has been suggested that in this animal oxytocin is synthesized and released at a normal or high rate, perhaps in compensation for the lack of vasopressin. Sachs and his co-workers have studied the biosynthesis of vasopressin in some detail. Using unanaesthetized dogs, he infused ^{35}S-cysteine into the third ventricle for up to 36 hours and studied the incorporation of radioactivity into material with vasopressin properties and into its carrier protein, neurophysin (30, 31).

In later *in vitro* studies in guinea pig hypothalamic median eminence slices, Takabatake and Sachs (35) found a time lag in the incorporation of ^{35}S-cysteine into vasopressin-like material as compared to incorporation into the total protein. Furthermore, Sachs and

Takabatake (35) found that radioactive incorporation into vasopressin, which was considerable after six hours of incubation, was negligible after incubation with puromycin, but that incubation with puromycin after one and a half hours of incubation with ^{35}S-cysteine did not prevent the appearance of labelled vasopressin at six hours.

Sachs proposed the following model: In the cell bodies of the supraoptic nucleus, a larger polypeptide precursor of vasopressin is synthesized in the endoplasmic reticulum, by a ribosome dependent system. This precursor molecule would be transported from the endoplasmic reticulum to the Golgi apparatus, where neurosecretory material is packaged into neurosecretory granules (NSG) which then would move down the axons to the nerve endings in the neurohypophysis. At some point the precursor would be modified to release vasopressin, which would be bound to neurophysin, present in the NSG. The NSG then would release both vasopressin and neurophysin, upon stimulation, into the capillaries of the posterior pituitary vascular plexus.

The concept of a polypeptide precursor is not unique to the neurohypophyseal hormones. The most notable cases include the biosynthesis of insulin in the beta cells of the pancreas, a process wherein two disulphide bridged chains are formed by cleavage of a single larger polypeptide (Steiner et al 1969). Another example lies in the release of angiotensin from the alpha-globulin angiotensinogen, which is split by the enzyme renin. Evidence for precursors both to other hormones (e.g. glucagon, parathyroid hormone, gastrin) has recently been forthcoming (22, 4).

The structures of some of the hypothalamic releasing factors have been recently elucidated. Few studies relating to their biosynthesis have been done. Mitnick and Reichlin (20) reported incorporation of ^{14}C-glutamic acid, ^{14}C-histidine and ^{3}H-proline into the thyrotropic releasing hormone, a tripeptide with the following sequence: L-pyroglutamyl - L-histidyl - L-prolinamide. These authors claimed that the synthesis of the compound occurs in a ribosome-free system. McKelvy and Schaer (18), in cultured whole HNS, reported in vitro incorporation of ^{14}C-histidine and ^{3}H-proline into material which showed the chromatographic behaviour of TRH.

The structures of oxytocin and vasopressin are similar and their places of synthesis are closely related, so that it was plausible to suggest that their mode of synthesis was also related.

However, their different characteristics are worthy of note. While vasopressin is synthesized in the supraoptic nucleus, oxytocin is synthesized primarily in the paraventricular nucleus, although Sokol (39), by destroying portions of the hypothalamic nuclei in DI rats, has shown some oxytocin to be produced outside of the paraventricular nucleus. Also, the distribution of the hormones in the HNS varies considerably. In the rat the vasopressin: oxytocin (V:O) ratio is 2.3:1 in the hypothalamus and 1.6:1 in the posterior pituitary, while in the dog, the hypothalmic V:o ratio is 15.5:1, and that of the posterior pituitary, 1.2:1 (11). This would suggest that the release of oxytocin from a precursor could be spatially different, or that the rate of transport of oxytocin from the paraventricular nucleus could be faster than that of vasopressin from the supraoptic nucleus. There is evidence that bovine

NSG containing vasopressin and neurophysin II equilibrate at higher density than those containing oxytocin and neurophysin I (6, 7). The differential release of oxytocin and vasopressin to different stimuli has been noted (12).

This study was undertaken in order to establish a system for the in vitro biosynthesis of oxytocin, so that the intracellular or extracellular control mechanisms for hormone production could be studied in a simplified system. It was desired to examine the similarities and differences between the biosynthesis of oxytocin and vasopressin.

METHODS

I EXCISION OF HYPOTHALAMIC TISSUE AND PITUITARY GLAND

Rats were stunned with a blow on the back of the head, then decapitated. The heads were placed in ice water and dissected as soon as possible. The skull was exposed by cutting the skin sagittally from the back of the nect to the nose. The back of the skull and surrounding tissue were removed and the skull split lengthwise with one cut, and opened on each side. The brain was exposed, then carefully lifted from the front, cutting the optic nerves and brain stem. A 0.5-0.6 mm cube bounded anteriorly by the front of the optic chiasma and posteriorly by the pons was excised, and split horizontally to remove cortical material. The remaining oblong was presumed to contain the hypothalamic areas.

The pituitary was found in the sella turcica at the base of the brain, covered by a thin dura membrane, the sella turcica tenterium, which was carefully pulled away with a needle. Once exposed, the pituitary was gently removed with a narrow spatula.

II PREPARATION OF HYPOTHALAMIC MATERIAL AND INCUBATION

A. Cell Bodies

1. Preparation

Neuronal cell bodies were prepared by the method of Sellinger et al. (37). Hypothalamic tissue was minced with a scalpel on a glass slide into two volumes of suspending medium: 7.5% polyvinylpyrrolidone (w/v), 1% bovine albumin (w/v), in 10 mM $CaCl_2$. The mince was passed through a 333μ nylon bolting cloth three times using a truncated plastic syringe, through 110μ and 333μ mesh thrice, then thrice through 73μ and

333μ mesh. The suspension was layered on 1.75 M sucrose in a centrifuge tube and centrifuged at 20,000 rpm in an SB-283 rotor for 30 minutes at 4°C. (33,000 g-hr). The suspension layer and sucrose were removed with a Pasteur pipette, leaving a pellet of purified neuronal perikarya, which was resuspended in Eagle's minimal essential medium (MEM) without glucose, and centrifuged as before. The pellet was resuspended in 0.2 ml of MEM. A smear of the pellet was viewed under phase contrast microscopy at 1000 X magnification.

2. Incubation

Neuronal perikarya and stock solutions were combinet to make a suspension containing glucose, 0.6%, freshly prepared glutamine, 1mM, 7 non-essential amino acids, 1mM, and either 2 μCi/ml ^{3}H-isoleucine or 1 μCi/ml ^{14}C-leucine, all made up to 1 ml with MEM. The mixture was incubated for 4 hours at 37°C under a humidified 95% air/5% CO_2 mixture.

The incubation was stopped by the addition of an equivalent volume of 0.25% acetic acid followed by 10 strokes of a Potter-Elvehjem (teflon on glass) homogenizer, and placed on a boiling water bath for 15 minutes. All samples were stored frozen when not applied immediately to gel filtration.

B. Hypothalamic Homogenate, Cell Sap, and Ribosomes.

1. Preparation

Hypothalamic tissue was minced in two volumes of Medium A (0.05M Tris-HCl, pH 7.8, 0.02 M KCL, 0.005 M $MgSO_4$, in 0.25 M sucrose) with scissors, then homogenized with ten strokes of a teflon homogenizer. The supernatant from a 15 minute centrifugation at 10,000 rpm in an A

321 rotor (2300 g-hr) at 4°C was used for hypothalamic homogenate incubations. Otherwise, the suspension was centrifuged at 12,000 rpm in an A 211 rotor for 15 min. (3500 g-hr) and the residue discarded. "Cell sap" was the supernatant obtained from a one hour centrifugation at 35,000 rpm in an A 321 rotor at 4°C (100,000 g-hr).

Ribosomes were prepared by a method similar to that of Ragnotti et al (27) as advised by Dr. G. R. Lawford. The 12,000 rpm supernatant was homogenized with 10 strokes with a one-tenth volume of 14.3 sodium deoxycholate (DOC). The homogenate was then layered over 1 M sucrose, and centrifuged two hours at 56,000 rpm in an A 321 rotor (500,000 g-hr). The supernatant was discarded and the pellet either frozen or resuspended in a small volume of Medium A.

2. Incubation

Incubation was performed in a protein synthesis system similar to that of Nandi Majumdar and Trachewsky (21). Materials were in the following concentration in a total volume of 1 ml: 100 mM KCL, 40 mM NaCl, 50 mM Tris-HCI pH7.8, 5 mM $MgSO_4$, 10 mM beta-mercaptoethanol, 5mM PEP, 2 mM ATP, 1 mM GTP, 50 μg/ml pyruvate kinase, 0.2 mM of 19 cold amino acids, at least 1 mg/ml of ribosomes in suspension, of protein in cell sap or of homogenate, and 1 μCi/ml of ^{14}C-leucine. Incubations were made in duplicate, and pooled before analysis.

Incubations were terminated by the addition of an equal volume of 0.5% acetic acid, followed by 15 min. incubation on a boiling water bath. Occasionally the boiling was continued for a shorter period.

C. Hypothalamic Tissue Fragments

1. a. Preparation

Hypothalamic tissue was bisected sagitally, one half being used for control, the other for experimental incubation. Each half was sliced into 1 mm fragments with a razor blade. The pooled slices from six halves were incubated in a Petri dish in 10 ml of 90% Eagle's MEM containing 2 mM glutamine, 0.5% glucose, 1 mg/ml streptomycin, 1000 μU/ml sodium penicillin G, and 10% fetal calf serum, which was brought to 0.028 M in HEPES and 0.01 M in Tricine, both pre-adjusted to pH 7.2, according to Sachs et al (34).

1. b. Preparation

Eagle's MEM, lacking the amino-acids that corresponde to the added radioactive amino acids was used. During the incubation, 2 μCi of ^{14}C-leucine and 20 μCi of ^{3}H-isoleucine were added. The preparation and incubation was otherwise identical to Preparation 1.a.

2. Incubation

Radioactive amino acids (1 μCi ^{14}C-leucine and 8 μCi ^{3}H-isoleucine) were added, and the tissue was incubated at 37°C under a humidified atmosphere of 95% air and 5% CO_2. At the end of the incubation, the tissue was centrifuged at 2000 rpm for 5 min, (57 g-hr), the medium decanted, the tissue washed with 2 ml of fresh medium, recentrifuged, and the media pooled.

The tissue was transferred to a homogenizer with an equal volume of 0.5% acetic acid, homogenized with 10 strokes of the teflon pestle at speed 0.5 and boiled for two minutes, then centrifuged at 2000 rpm for 5 minutes (57 g-hr).

III ANALYTICAL METHODS

A Gel Filtration

Sephadex® G-25 Superfine and G-15 and G-10 Fine cross-linked dextran beads were prepared according to the manufacturers description (24). The glass parts of the Pharmacia K 15/90 columns were siliconized with Siliclad®. The gel was swollen 3 hours, de-aerated under vacuum, and poured in 0.2 N acetic acid at room temperature. Care was taken to keep a constant, low, flow rate (about 10 ml/hr) when the gel was packing. The column was equilibrated with 0.1N formic acid. The void volume (V_o) was determined by the application of a 0.1% solution of Blue Dextran 2000® to the column, eluted with 0.1N formic acid. Optical density (O.D.) was measured at 620 nm. The V_o was approximately 50 ml.

Samples of 2 - 4 ml were applied to the gel bed and 2 ml fractions were collected at a rate of 10 - 20 ml/hr, using a pressure head of 80-120 cm of water.

The Sephadex C-100 Superfine K 15/90 column was prepared and eluted with 0.05 M Tris-HCl, pH 7.8, 5 mM beta - mercaeptoethanol, in the cold room in the same fashion as described above.

B Partition Chromatography in Sephadex

Partition chromatography was performed as described by Yamashiro (45). Columns were either 0.9 x 150 cm with sintered glass bases, a minimized dead volume, and ground glass adaptors, or were Pharmacia K 15/90 columns. All tubing was made of teflon. Sephadex G-25 Fine gel was swollen in 0.2 N acetic acid for three hours, and deaerated. The gel was carefully poured in one batch into a column one-

third prefilled with 0.2 N acetic acid, and allowed to settle without flow. Flow was then started at about 10 ml/hr, and the column equilibrated overnight at a pressure head of 80-100 cm of water.

Using deaerated solvents, a two phase n-butanol:benzene:pyridine: glacial acetic acid: water (600:200:100:0.9:900) solvent system was equilibrated in a separatory funnel. The column was equilibrated with 1.3 column volumes (V_c) of the lower phase of the solvent system. The V_o was measured as the volume of lower phase displaced from the entry of the uppper phase into the gel bed until its emergence. The column was equilibrated with 0.25 V_c of the upper phase, then the sample was applied, dissolved in upper phase. The pressure head was increased to 200 cm of water following this stage. Fractions were collected every 2 ml. The column was regenerated by washing it with pyridine: 0.2 N acetic acid (3:5), then re-equilibrated in 0.2 N acetic acid.

C Column Ion Exchange Chromatography

Ion exchange was performed using the weakly acidic cation exchanger BioRex 70®. The resin was soaked in 4 N HCl for one day in the cold room, then washed 4 times with water. The pH was adjusted to 5.6 using 2 N NaOH, the supernatant decanted, and the remaining slurry was suspended in 0.25 N deaerated acetic acid. The Pharmacia K 9/15 column was partly filled with 0.25 N acetic acid, and the resin packed under a slow flow rate.

1. A sample of 0.1 μCi ^{14}C-leucine and 0.025 mg of synthetic oxytocin* (10 U) was applied, and the column was eluted with 200 ml of 0.25 N

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* Synthesized and assayed in this laboratory (Appendix I)

acetic acid, 200 ml water, 100 ml 0.25 N ammonium acetate pH 5.3, 50 ml water, and 50 ml of 30% pyridine: 4% acetic acid (42) successively. The first and last fractions were evaporated to dryness and redissolved in 10 ml of Tyrode's physiological solution.

D Thin Layer Chromatography (TLC)

Samples dissolved in aqueous or organic solvent were spotted on 5 x 20 cm silica gel-aluminum backed plates. Samples were dried at room temperature. The chromatography tank was equilibrated overnight in the upper phase of butanol:acetic acid:water (4:1:5) (44). Plates were developed for about 7 hours, dried thoroughly in a 70°C oven overnight, and peptide visualized by I_2 treatment in a closed chamber.

E High-Voltage Electrophoresis (HVE) at pH 6.5

HVE was performed similarly to Ryle et al on a Savant flat plate apparatus (29), using the dye-markers of Stevenson (41). Samples were applied on a 95 x 27 cm strip of Whatman 3MM paper. Small samples (5 1) of 1% methyl green (MG), crystal violet (CV) and xylene cyanol FF (XCFF), were used as markers. Each electrode compartment was filled with about 500 ml of pH 6.5 buffer (water:pyridine:glacial acetic acid, 879:100:3). The ionogram was developed at 3-4 Kv, with a current of about 100 mA. The power was disconnected when methyl green reached a line drawn 30 cm towards the cathode, and the sheet hung to dry.

F Protein Determination

Optical density (O.D.) of fractions from gel filtration were measured at 280 nm, and plotted without further conversion.

An estimation of protein concentration was done using the formula: $1.55\ O.D._{280nm} - 0.76\ O.D._{260nm}$ = mg/ml protein (16).

When synthetic polypeptide was isolated from partition chromatography, 0.2 ml aliquots were taken to dryness and redissolved in 0.2 ml water, and protein estimated by the method of Lowry et al (17). O.D. was read at 700 nm.

G Ribosomal concentration was estimated using the formula:

$1\ O.D._{260nm} = 74.6\ \mu g/ml$ (38).

Ribosomal purity was confirmed by the ratio $O.D._{260nm} / O.D._{280\ nm}$. Only preparations with a value greater than 1.75 were used.

H Determination of Radioactivity

1. Water Soluble Samples

Aliquots of 0.1 ml from gel filtration or ion exchange chromatography were counted for ^{3}H or ^{14}C at 4°C in 10 ml of Bray's solution, or in 10 ml of the scintillation fluid recommended by Beckman (5 g PPO, 100 g naphthalene, in 1, 4-dioxane up to one liter).

2. Organic Solvent Soluble Samples

Aliquots of 0.5 ml were counted for ^{3}H or ^{14}C either in Bray's solution, in Beckman's recommended sciltillation fluid, or in toluene-PPO (5 g PPO, 0.3 g POPOP, in toluene up to one liter).

3. Silica Gel Samples

TLC plates with ^{3}H or ^{14}C were divided horizontally in 0.5 cm strips, and each strip was scraped into a scintillation vial and counted in 10 ml of toluene-PPO.

4. Samples on Paper

Three cm wide strips from HVE containing ^{14}C were scanned on a strip counter, or were cut into 1 x 3 cm strips, each strip cut into two

and the pieces counted in 10 ml of Toluene-PPO.

J. Bioassay

Mouse mammary gland strip contraction activity was determined by the method of Roca et al (28), following the basic procedure of Mendez-Bauer et al for the rabbit (19). A female mouse between 10 and 15 days of lactation was killed and the lower mammary gland tissue was excised into Tyrode's solution. A strip about 3 mm wide x 15 mm long was cut out and freed of connective tissue, and suspended from a Grass FT03C force displacement transducer in a 2.5 ml bath of Tyrode, at room temperature. The tension was adjusted to between 300 and 500 mg, and the tissue left to stabilize for 30 minutes. Standardization of isometric contraction was performed using posterior pituitary working standard solution or prestandardized synthetic oxytocin in Tyrode's solution. After the peak of the contraction was reached (about 2 minutes), the strip was rinsed four times with Tyrode's solution. Samples were injected every 7 - 8 minutes. Three-point or four-point assays were performed. Activity was expressed as units of milk ejecting-like activity.

RESULTS

Since the neurons themselves, and not the accompanying glial cells were presumed to be the sites of biosynthesis (36), a preparation which produced purified neuronal cell bodies was used in the initial experiments. The first attempt at incorporation into hypothalamic cell bodies from rats was inconclusive as the potential areas of biological activity obtained from partition chromatography were not counted for radioactivity prior to bioassay.

A second such incubation was performed with tissue from ten rats, but the visually estimated yield was lower than in the first experiment, presumably because the suspension which was layered on sucrose was so thick as to prevent the cell bodies from sedimenting. After Sephadex G-25 gel filtration and partition chromatography, the radioactivity was high in the areas R_f 0.0-0.92, 0.32-0.28, and 0.28-0.24. However, bioassay of these peaks after evaporation and solution in Tyrode's solution showed no detectable activity.

It was also of interest to look into a cell free system which might provide a more direct route to finding the possible components of the biosynthetic system. To examine this possibility, an incubation of hypothalamic ribosomes and hypothalamic cell sap with ^{14}C-leucine was performed. Partition chromatography (Fig 1) revealed radioactive materials having R_f 1.0, 0.76, 0.64, 0.54, 0.25, and 0.084. Bioassay of evaporated, pooled peaks of R_f 0.25 and 0.084 revealed no biological activity.

At this point, experiments being performed in the same laboratory by Dr. A. Nandi Majumdar indicated that a ribosome-free cell sap system could be capable of incorporating ^{14}C-leucine into material behaving like oxytocin. Partition chromatography of the material corresponding to both radioactivity and biological activity on gel filtration gave material including a peak of R_f .22($R_{f\,oxytocin}$=0.24), which could represent biosynthesized oxytocin (Fig 2). Similar partition chromatography of synthetic oxytocin mixed with ^{14}C-leucine had previously shown not to give a radioactive peak in the area R_f 1.0 - 0.21 (Fig 3).

Therefore, an incubation of hypothalamic cell sap for one hour with ^{14}C-leucine was performed and analyzed. Partition chromatography revealed material at three peaks of R_f 0.65, 0.56, 0.27, and at two peaks between R_f 0.21 and 0.12, with a total biological activity of 2.0, 1.8, o.87, 0.54 and 0.54 mU respectively. Some material corresponding to R_f 0.20 - 0.14 was lost in this experiment.

Although the bioassay results seemed inconsistent with the idea of only one oxytocic principle, the possibility of a system of biologically active precursors was presented, and an attempt to isolate the factors involved in their synthesis was initiated by a purification of cell sap by G-100 gel filtration <u>prior</u> to incubation with ^{14}C-leucine. Incubation of peaks (I + II + III), I, II, and III from Sephadex G-100 gel filtration (Fig 4) with 1.0 μCi of ^{14}C-leucine, and subsequent analysis of each incubation revealed that except where chromatography was interrupted, each incubation showed material at peaks of R_f about 1.0-0.85, 0.50, 0.40-0.34, 0.29-0.22, and 0.21-0.18 on partition chromatography (Table I).

Moreover, all the material at consistent peaks which was bioassayed appeared to be biologically active to some degree or other, bringing into question both the purity of the radioactive ^{14}C-leucine and the selectivity of the bioassay.

Partition chromatography of a second sample of non-incubated ^{14}C-leucine (Fig 5A) showed radioactive material at R_f 0.91, 0.55, 0.43, 0.21, 0.17 and 0.041, essentially the same pattern of radioactive peaks which had been detected after incubation (Fig 5B). It was apparent that impurities present in the ^{14}C-leucine had appeared in the chromatographic run.

There existed the possibility that material from a sample of synthetic oxytocin purified on the same column was releasing small quantities of biologically active material, and that the peaks at R_f about 0.096-0.080 and 0.070 were the only new ones resulting from incubation. Evaporation and lyophilization of the ^{14}C-leucine peak of Fig 5A, and its reapplication to partition chromatography, showed that this procedure alone could be sufficient for the formation of the impurities, as has been shown by A. Nandi-Majumdar, and therefore other methods of separating oxytocin from radioactive contaminants were investigated.

In addition, slow contractions of the mouse mammary gland strip induced by very small amounts of organic solvent were affecting the slectivity of the bioassay, which is otherwise remarkably unresponsive to potentially biologically active contaminating materials beside oxytocin (9).

A similar incubation of hypothalamic cell sap with ^{14}C-leucine was analyzed on gel filtration, followed by high voltage electrophoresis (HVE) at pH 6.5 of the radioactive peak for both a control (0 hr) and experimental (3 hr) incubation. Test runs of both synthetic oxytocin and ^{14}C-leucine (Fig 6A) on HVE had shown there to be no significant radioactivity in the area corresponding to oxytocin. The control and experimental incubations revealed that although no radioactivity appeared in the oxytocin area (Fig 6B, C) there was a considerable production of material in the 3 hr incubation alone which travelled towards the anode.

In an experiment performed in this laboratory by A. Nandi-Majumdar, the pooling of material of R_f 0.24 on partition chromatography from several different experiments produced, on TLC in the upper phase of butanol:acetic acid:water (4:1:5) a peak of R_f 0.34, which corresponds to that of oxytocin. Furthermore, in two other solvent systems radioactive material corresponding in R_f to oxytocin was detected. It was calculated from the quantities of radioactivity contributed from each experiment that the incubation of female rat hypothalamic cell sap with ^{14}C-leucine for 2 hr was the experiment which produced this material. Consequently, this experiment was repeated. In my hands the gel filtration and partition chromatography showed no radioactivity at R_f 0.24 (Figs 7, 8), while the R_f 0.21 and 0.18 areas from partition chromatography for the 0 hr and 2 hr incubations gave essentially identical patterns on TLC in the uppper phase of butanol:acetic acid: water (4:1:5), and revealed no significant radioactivity in the area R_f 0.34 (Fig 9). However, comparison of partition chromatography from 0 and 2 hr incubations revealed a large peak of R_f 0.11 in the 2 hr

incubation only.

Since radioactive oxytocin was not being produced in detectable quantities, it was presumed that either some components or required subcellular organization were missing, or else that degradative enzymes were destroying any product. To test both these possibilities, an incubation of hypothalamic homogenate with ^{14}C-leucine was performed. Sephadex G-25 gel filtration of the control (0 hr) and experimental (3 hr) incubations, followed by measurement of the O.D. at 280 nm and the radioactivity, revealed that a notable shift of 280 nm absorbing material to areas of later elution, and hence lower molecular weight, had occurred. Partition chromatography indicated a small amount of radioactive material of R_f 0.23, which HVE revealed to be neutral material, and a much larger amount of radioactive material which could not be accounted for by impurities in the ^{14}C-leucine, at R_f 0.14. This experiment was repeated in the presence of synthetic oxytocin (0.2 mg/ml), added as a carrier, and analyzed on Sephadex G-25 gel filtration, which showed that following incubation, in addition to the shift of 280 nm absorbing material, only 5% of the control biological activity was present (Figs 10, 11). There was, however, no change in the pattern of radioactivity.

In an experiment designed to retain cellular organization, hypothalamic tissue fragments from female rats were incubated either 0 or 6.5 hr with 1 μCi of ^{14}C-leucine in the culture medium used by Sachs (34). The homogenized, 0.25% acetic acid extracted tissue was subjected to Sephadex G-25 gel filtration, and revealed significant radioactive uptake over the course of 6.5 hr.

The experiment was repeated with the addition of both ^{3}H-isoleucine (8 μCi) and ^{14}C-leucine (1 μCi), both of which were taken up in significant amounts at 6.5 hr (Figs 12, 13). A second, later eluting peak of ^{3}H on Sephadex G-25 gel filtration was detected in the 6.5 hr incubation. Partition chromatography of material in the fractions on gel filtration containing both ^{3}H and ^{14}C demonstrated that no ^{14}C was present in the area where oxytocin might be expected, yet a small but significant increase in ^{3}H from the 6.5 hr incubation had occurred in the R_f 0.24 area (Fig 14). This material was collected, evaporated and lyophilized, and applied to TLC. There was no significant radioactivity in the R_f 0.34 area (Fig 15).

Presuming that if incorporation into biosynthesized oxytocin was occurring it could be limited by competition with unlabelled amino acids corresponding to the labelled tracers added, this procedure was repeated in a medium lacking unlabelled leucine and isoleucine. Incubation was continued for either 0 or 8 hr. No increase in the R_f 0.24 peak from partition chromatography was observed, and it was concluded that this small amount of material was not the product of a process which was dependent on the concentration of cold amino acid.

DISCUSSION

Table I shows that in none of the incubations I have performed is there both radioactivity and biological activity attributable to oxytocin. Possible synthesis of oxytocin in the cell body preparation cannot be excluded, as the first incubation revealed considerable biological activity, but radioactivity was not measured, and the second incubation was made with estimated low quantities of cells, which produced no detectable biological activity.

In those cases where radioactive impurities could have been masking the presence of labelled oxytocin on partition chromatography, as in early incubations of cell sap, later experiments with the same system but different modes of separation, such as HVE or TLC showed there to be no radioactivity associated with the area in which oxytocin might be expected.

During incubations of cell sap, cell sap with ribosomes, and hypothalamic homogenate, with ^{14}C-leucine, several products are formed with R_fs varying between 0.070 and 0.096 (Fig 5B), 0.11 (Fig 2), 0.084 (Fig 1) and 0.14 respectively on partition chromatography. Material lyophilized from Sephadex G-25 gel filtration of a 0 hr and a 3 hr hypothalamic cell sap incubation indicated the production of a net anion at 3 hr when analyzed on HVE at pH 6.5 (Fig 6C).

It is evident that considerable degradation of oxytocin and 280 nm absorbing material occurs during a three hr incubation of hypothalamic homogenate (Figs 10, 11). This is in accord with Hooper's

(13, 14) reports that oxytocinases are present in dog hypothalamic mitochondrial fractions.

There is an uptake of ^{14}C-leucine by hypothalamic tissue fragments in comparison to 0 hr controls during 6.5 and 8 hr incubation (Figs 12, 13), and there appears to be little degradation of 280 nm absorbing material in this system.

These data may contribute to the design of future in vitro systems for the synthesis of oxytocin, but, until it can be shown that oxytocin biosynthesis can be followed in vivo, it appears fruitless to pursue in vitro systems.

Success may depend on using radioactive tracers of high specific activity, and on long term infusion, perhaps into the intracisternal space, as done in rats by Pickering and Jones (25), or into the third ventricle of the dog as described by Sachs (32). It may require using larger, or greater numbers of animals to obtain sufficient material for analysis, and also upon the choice of the species and condition of the animal whose biosynthetic system might be expected to be active, such as DI rats, females which are approaching parturition or are lactating, or animals which are given high salt drinking water.

If this were successful, the in vitro systems which offer most promise include the cultured whole HNS of Sachs (34), which would preserve not only cellular but some physiological organization, or, if such a case were fortuitously found, a tumour from a patient with a syndrome analogous to the syndrome of inappropriate antidiuretic hormone (SIADH) used by George et all (10) for in vitro vasopressin biosythesis.

It might be worthwhile indeed to attempt to reproduce the experiments of Sachs for vasopressin biosynthesis before extending such a system to oxytocin.

Isolation procedures for oxytocin could include, in addition to those already used (Table II), the use of bovine albumin rather than oxytocin as a carrier (25), milder extraction procedures (overnight extraction at 4°C in 0.2 N acetic acid (15), and subcellular fractionation before extraction. Analytical methods could include CM- or DEAE-cellulose, polyacrylamide gel electrophoresis, or affinity chromatography on neurophysin-cellulose (26).

Should a system producing both labelled oxytocin and neurophysin be found, a comparison of their specific activities might indicate any biosynthetic relationship between the two or any other biosynthesized materials.

Other lines of approach to the problem could consist of attempts to mimic the mechanism of release of a precursor which could precede active oxytocin. The addition of 9-deamido-oxytocin to a hypothalamic system might demonstrate whether amidation was part of the sequence of activation of oxytocin.

It is known that N-leucyl-oxytocin when incubated with dialyzed liver cell sap or pregnancy serum releases leucine and the active hormone (2). This kind of potential enzymatic activity might be worth investigating in a hypothalamic system.

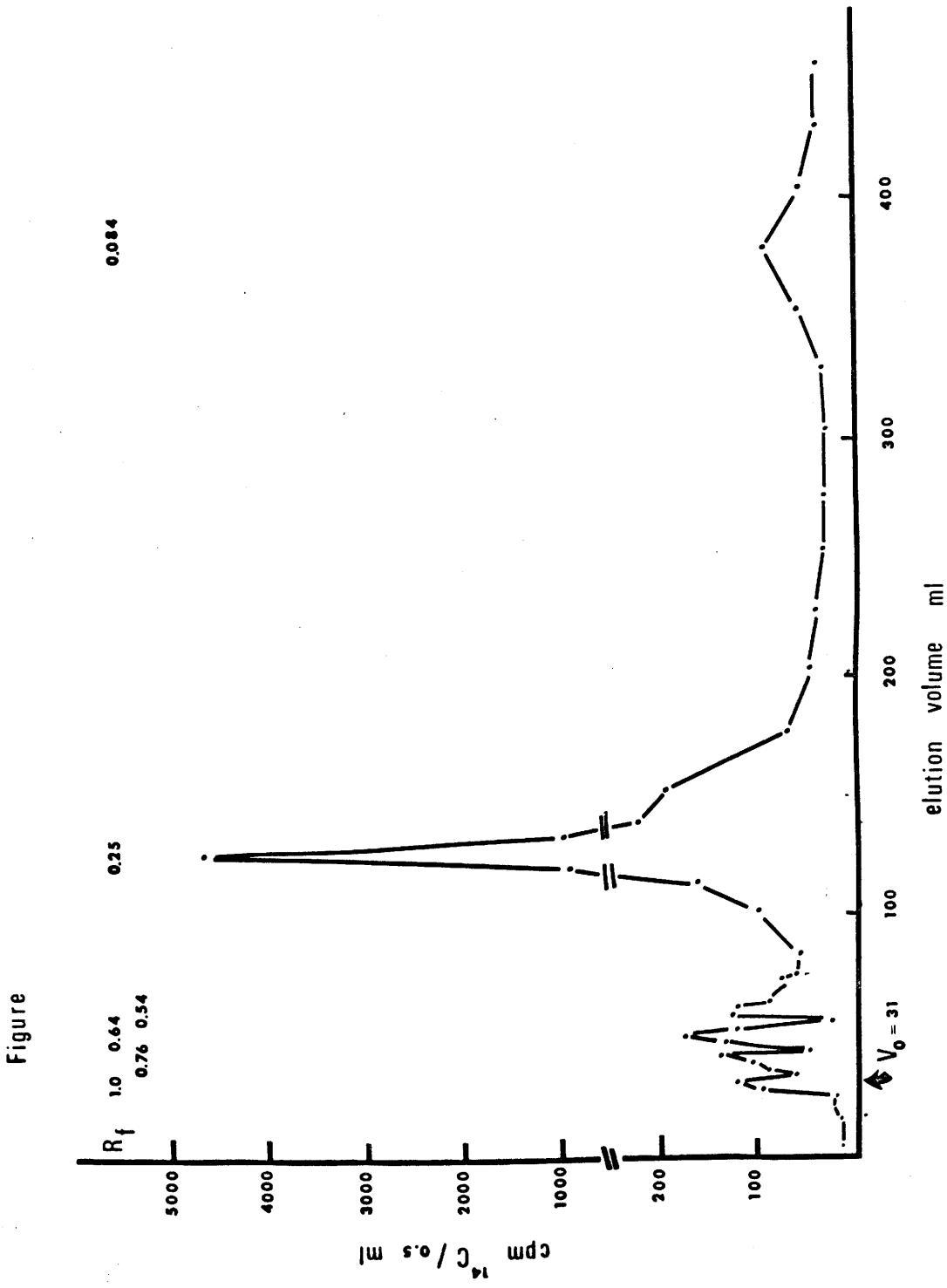

Figure 1: Partition chromatography of lyophilized radioactive material from Sephadex G-25 gel filtration of an incubation of rat hypothalamic ribosomes and cell sap with ^{14}C- leucine.

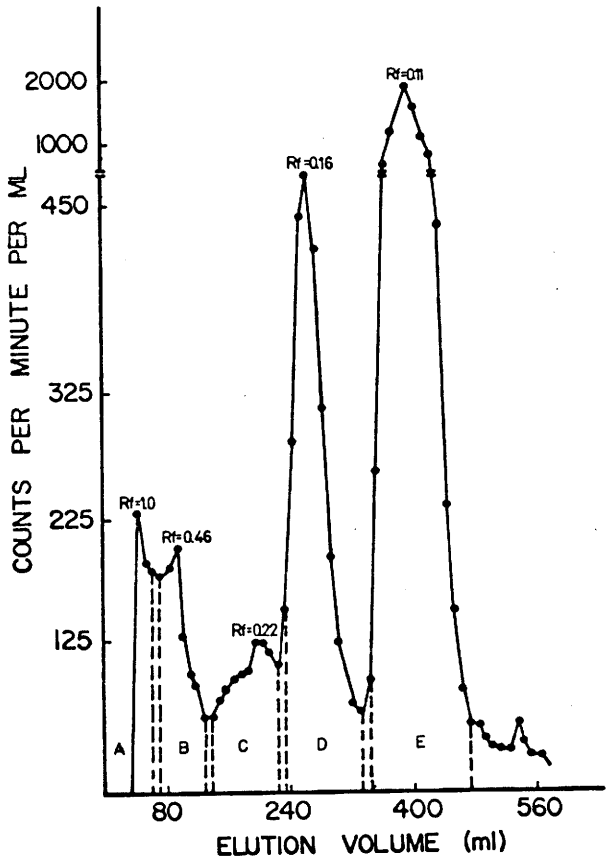

Figure 2: Partition chromatography of lyophilized radioactive material from Sephadex G-25 gel filtration of an incubation of rat hypothalamic cell sap. (Courtesy of Dr. A. Nandi Majumdar.)

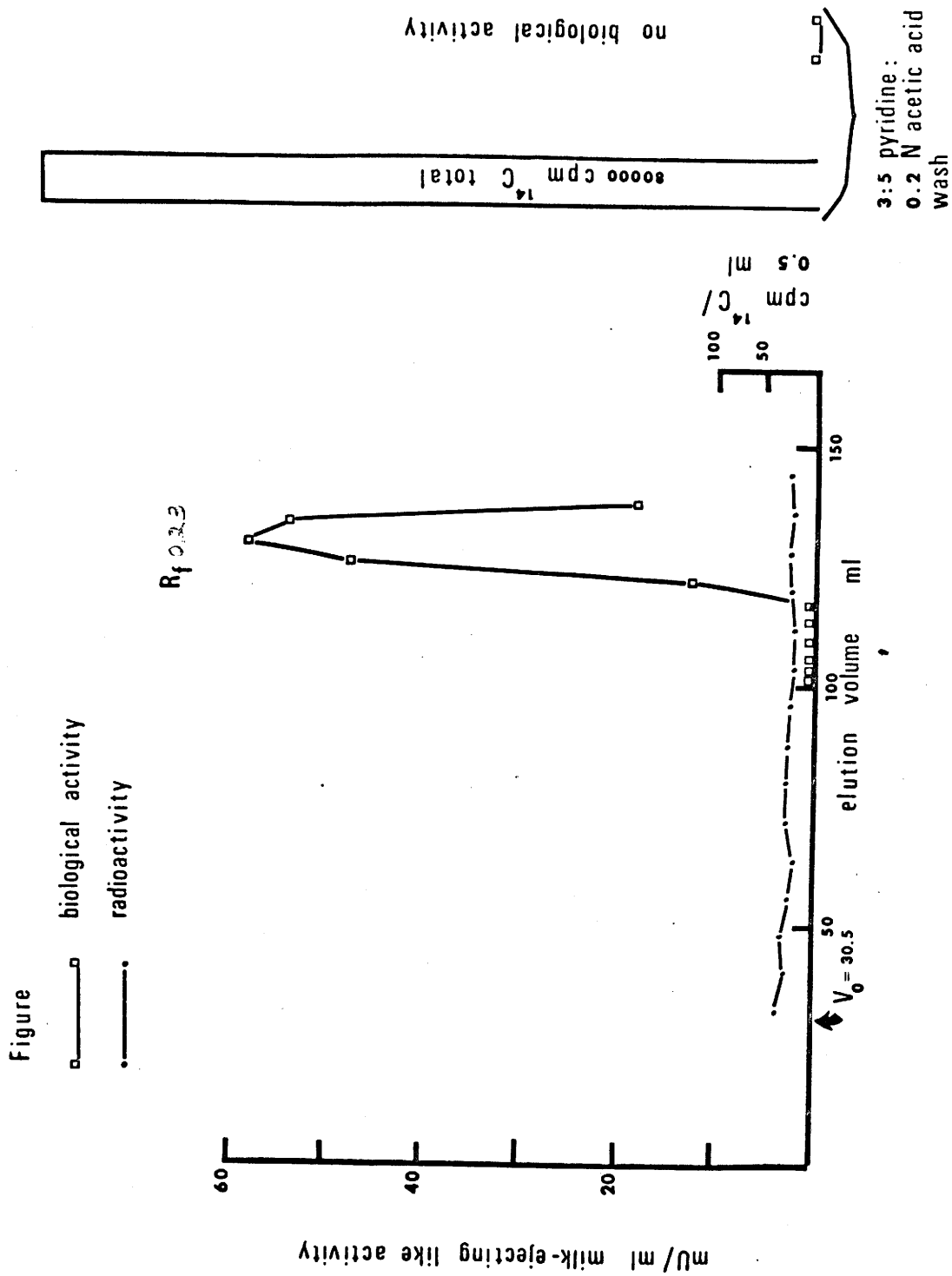

Figure 3: Partition chromatography of a sample of synthetic oxytocin (196 U.) and ^{14}C-leucine (0.1 μCi.)

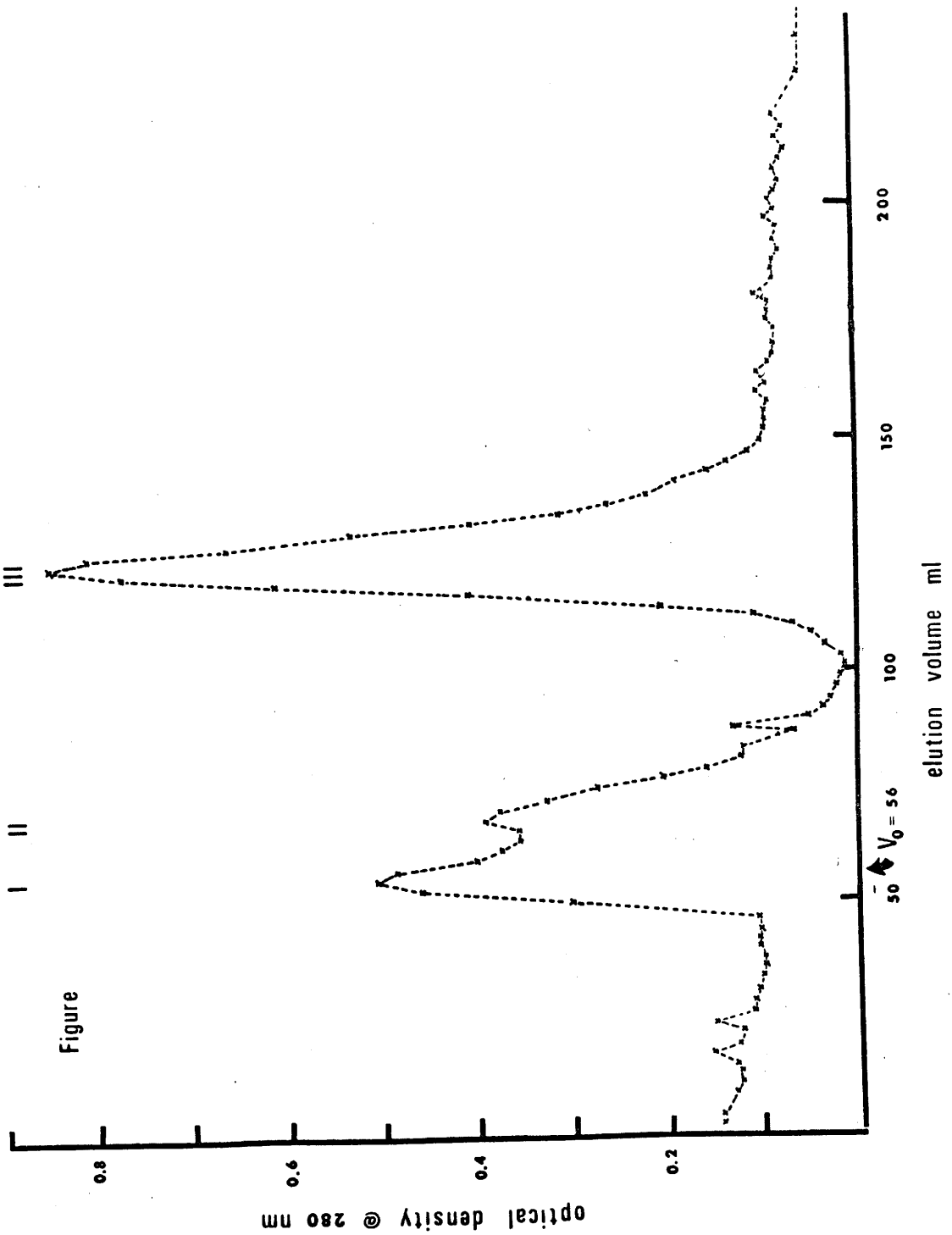

Figure 4: Sephadex G-100 gel filtration of hypothalamic cell sap. Material from peaks I, II, and III were incubated singly and in combination with ^{14}C-leucine.

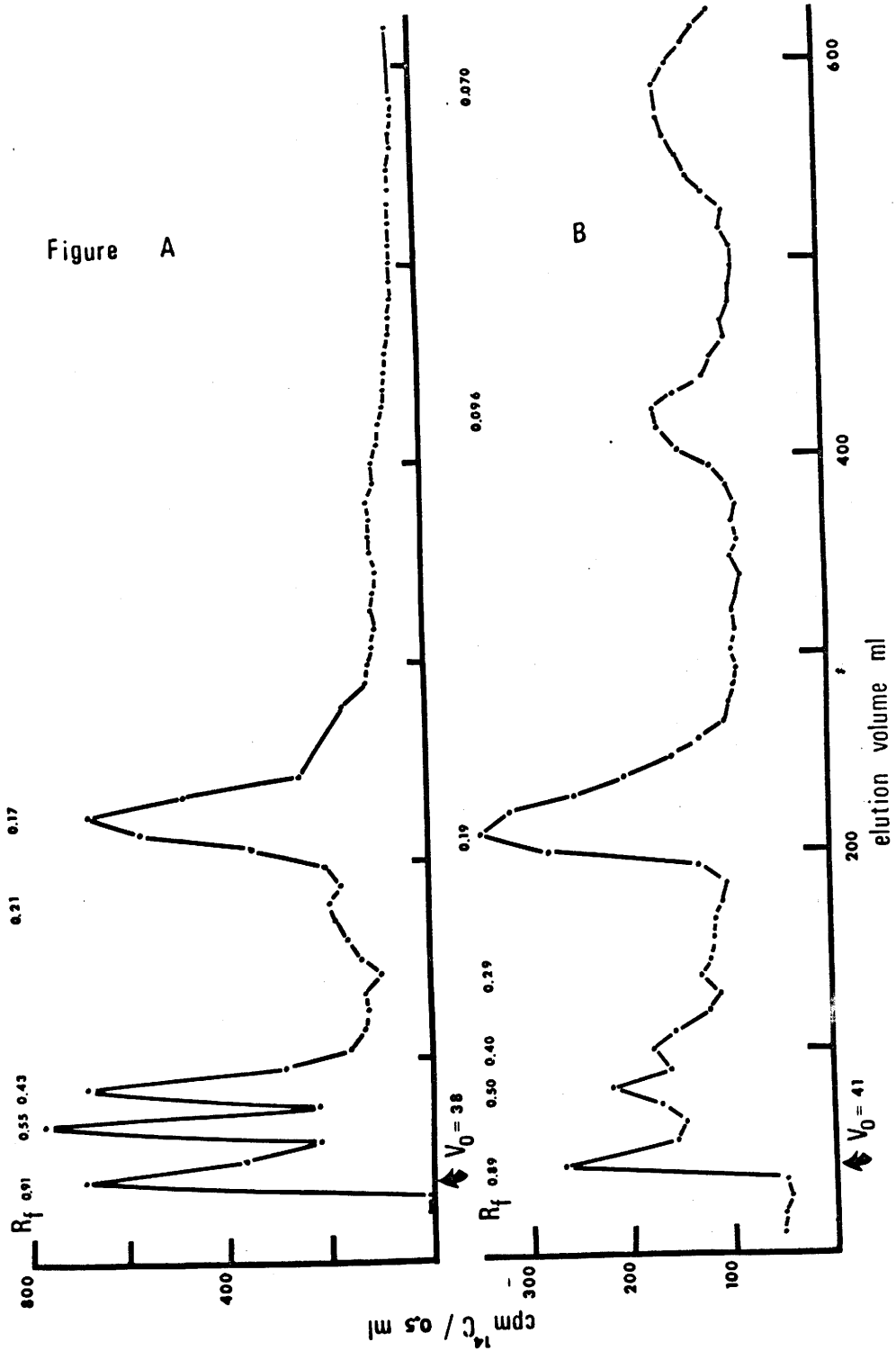

Figure 5A: Partition chromatography of ^{14}C-leucine (4 μCi).

Figure 6A: Partition chromatography of lyophilized radioactive material from Sephadex G-25 gel filtration of peak III (Fig. 4) with ^{14}C-leucine.

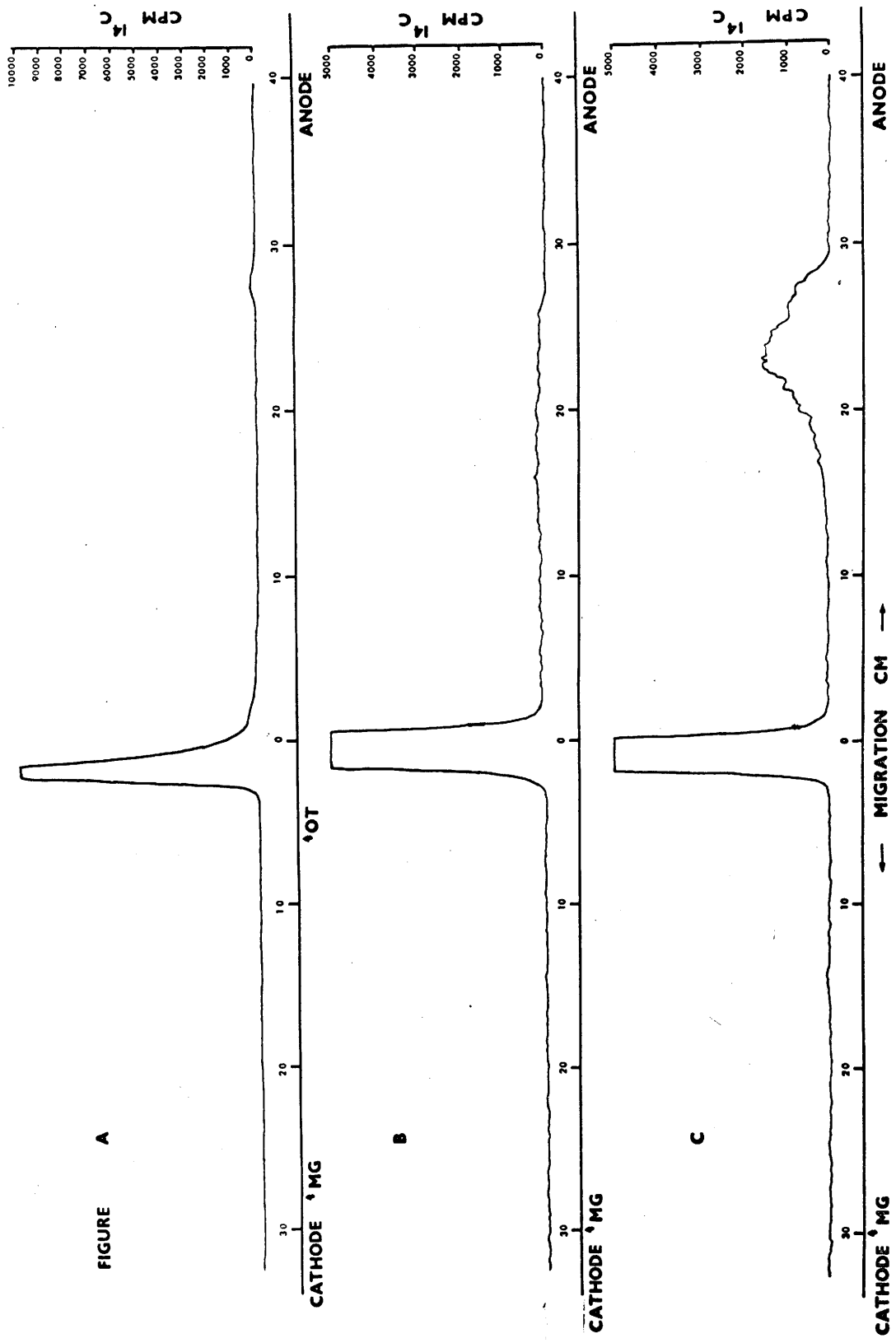

Figure 6A: High voltage electrophoresis of ^{14}C-leucine (1 μCi)
OT = oxytocin MG = methyl green

Figure 6B: High voltage electrophoresis of a 0 hr incubation of hypothalamic cell sap with ^{14}C-leucine.

Figure 6C: High voltage electrophoresis of a 3 hr incubation of hypothalamic cell sap with ^{14}C-leucine.

Figure

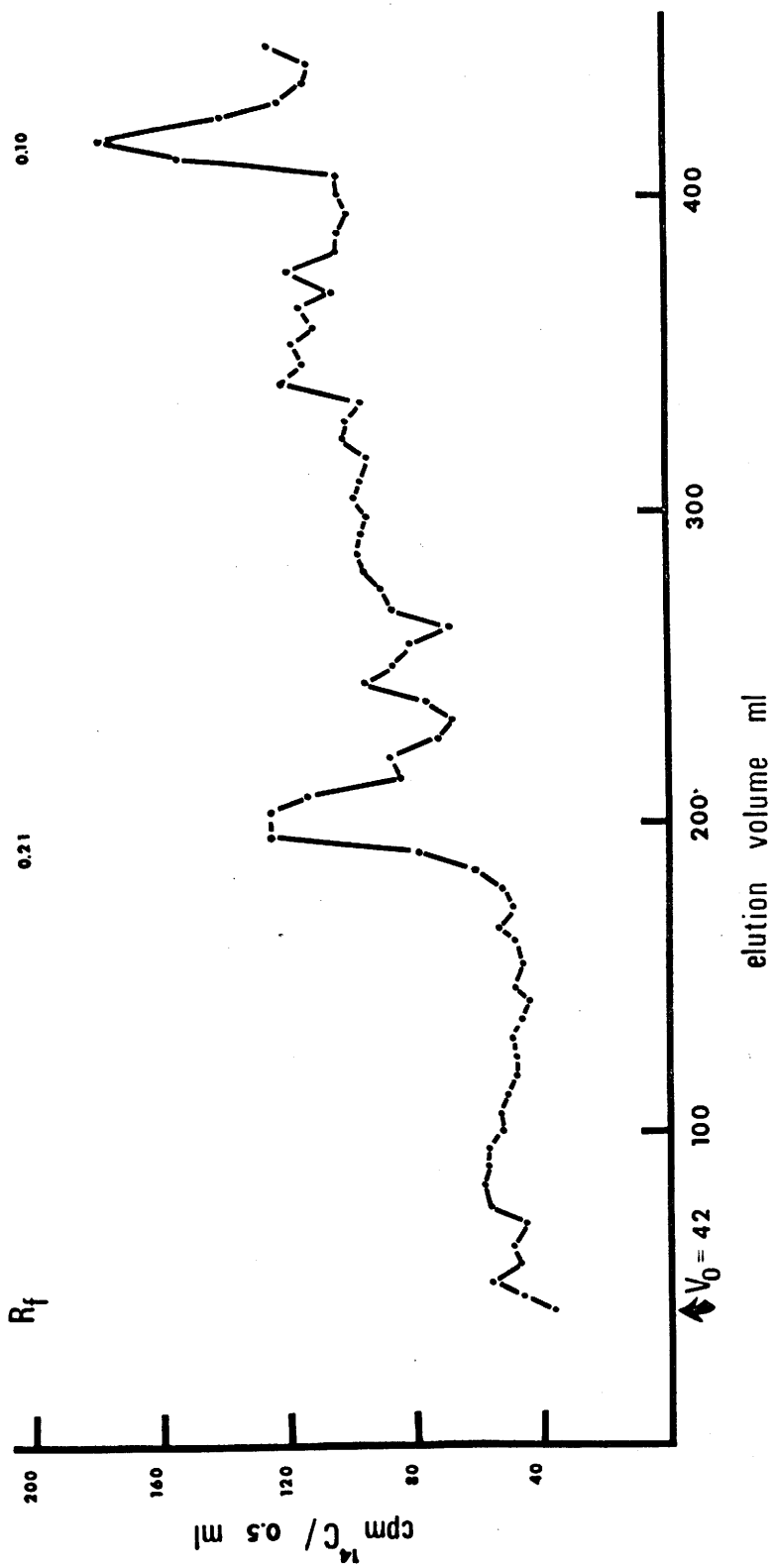

Figure 7: Partition chromatography of radioactive material lyophilizedfrom a Sephadex G-25 gel filtration af a 0 hr incubation of female rat hypothalamic cell sap with ^{14}C-leucine.

Figure

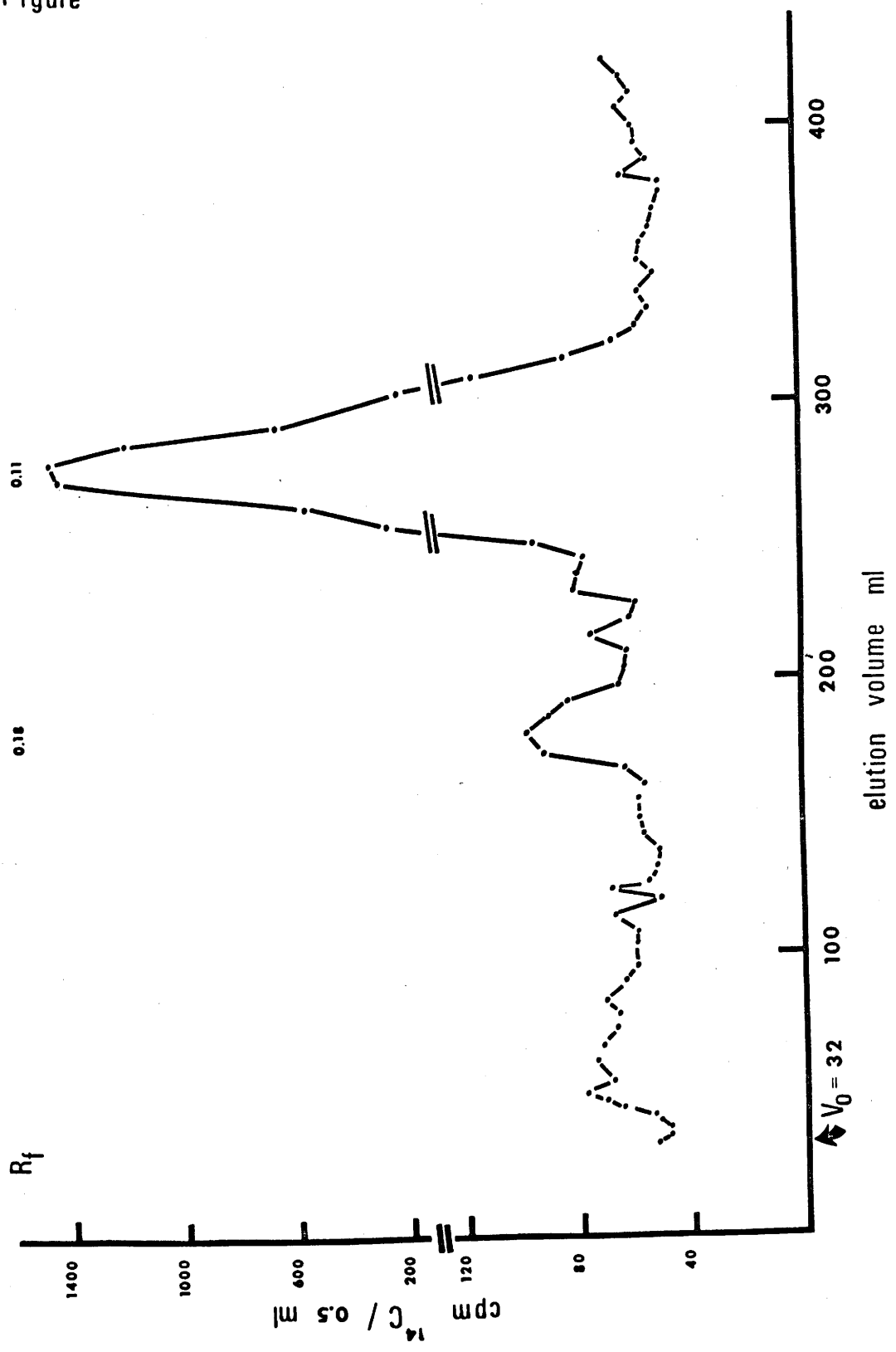

Figure 8: Partition chromatography of radioactive material lyophilized from a Sephadex G-25 gel filtration af a 2 hr incubation af female rat hypothalamic cell sap with ^{14}C-leucine.

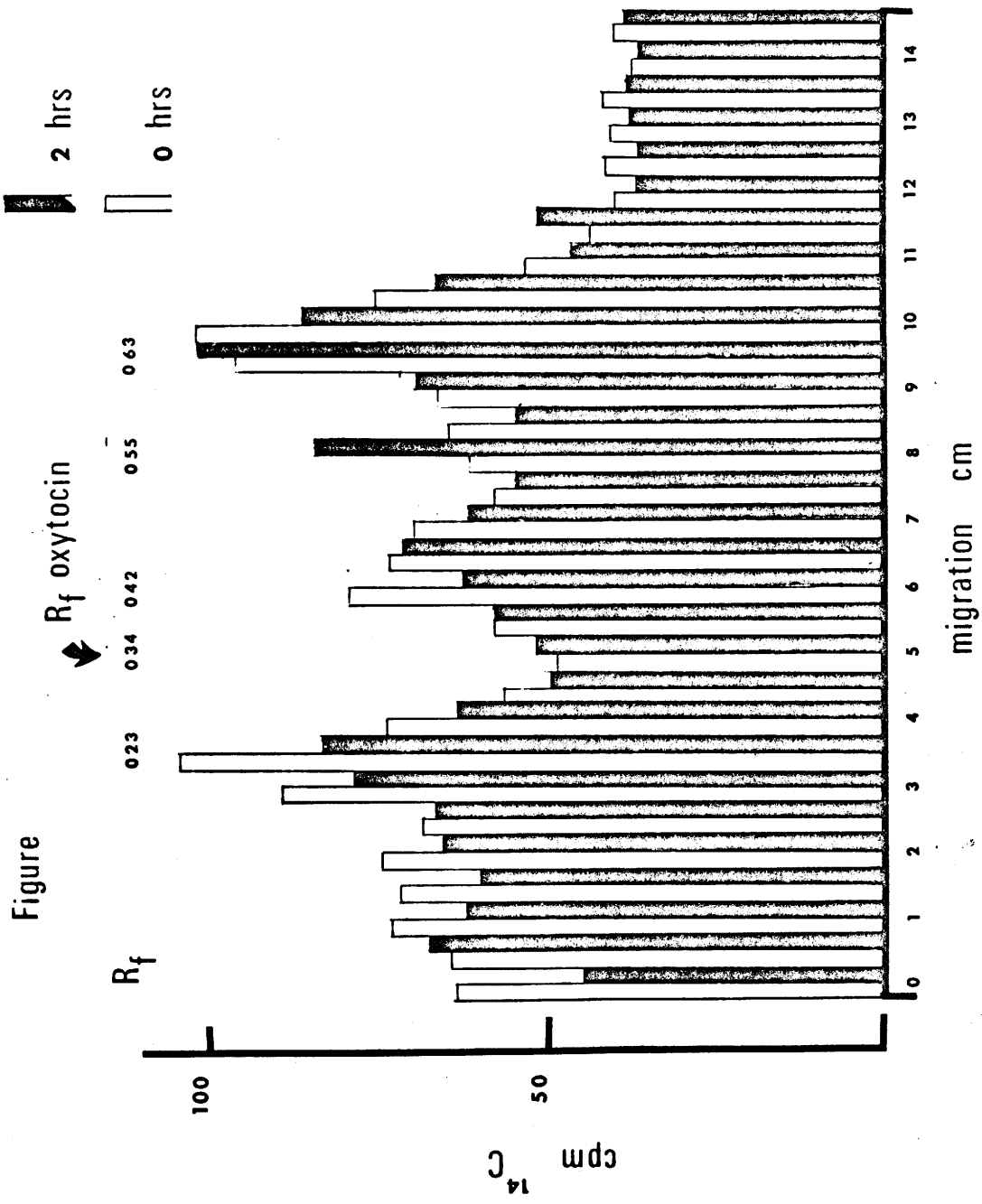

Figure 9: Thin layer chromatography of radioactive material of R_f 0.21 and 0.18 on partition chromatography (Figs 7,8) of a 0 hr and a 2 hr incubation of female rat hypothalamic cell sap with ^{14}C-leucine.

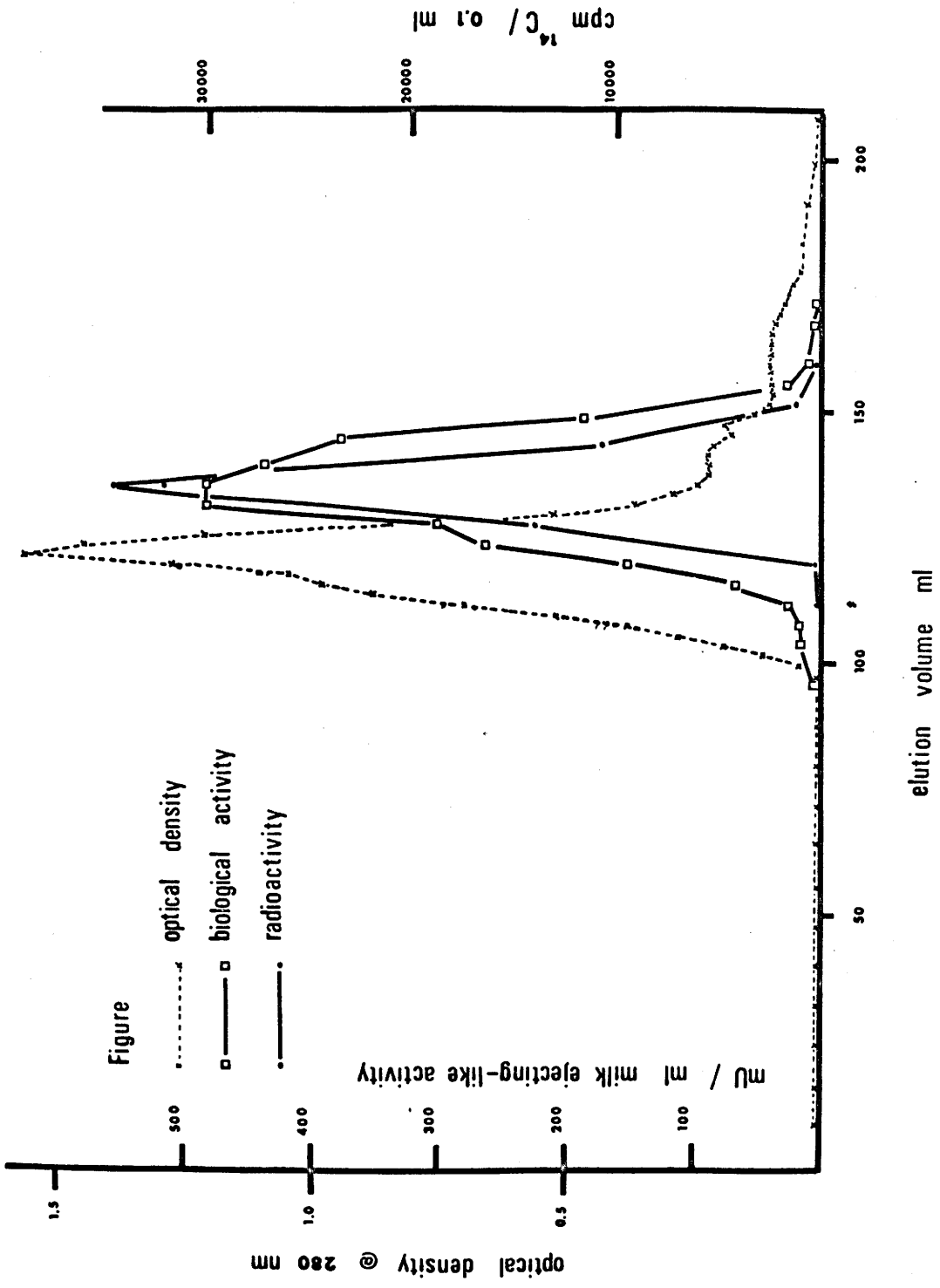

Figure 10: Sephadex G-25 gel fitration of a 0 hr incubation of female rat hypothalamic homogenate with ^{14}C-leucine and synthetic oxytocin.

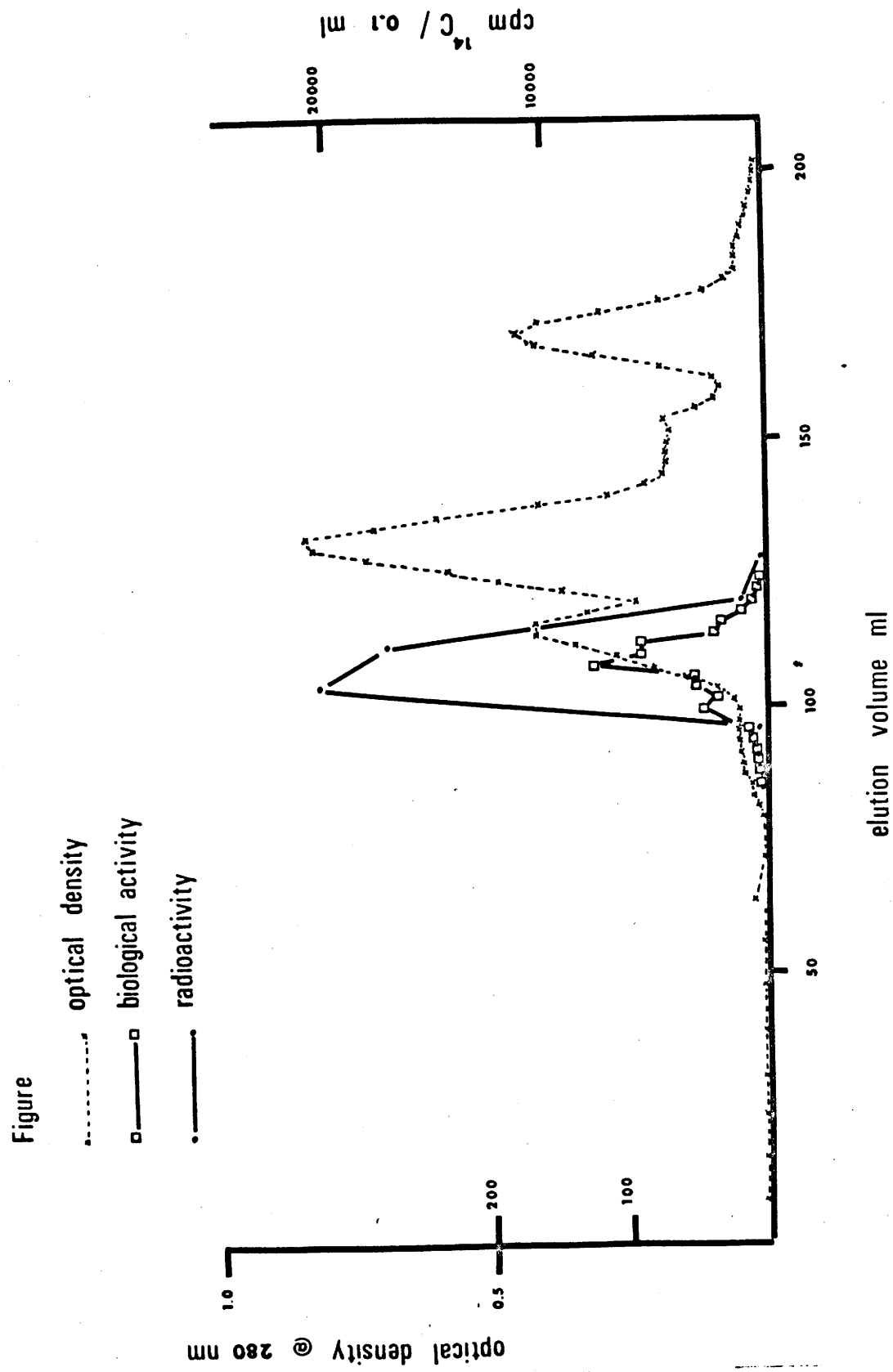

Figure 11: Sephadex G-25 gel filtration of a 3 hr incubation of female rat hypothalamic homogenate with ^{14}C-leucine and synthetic oxytocin.

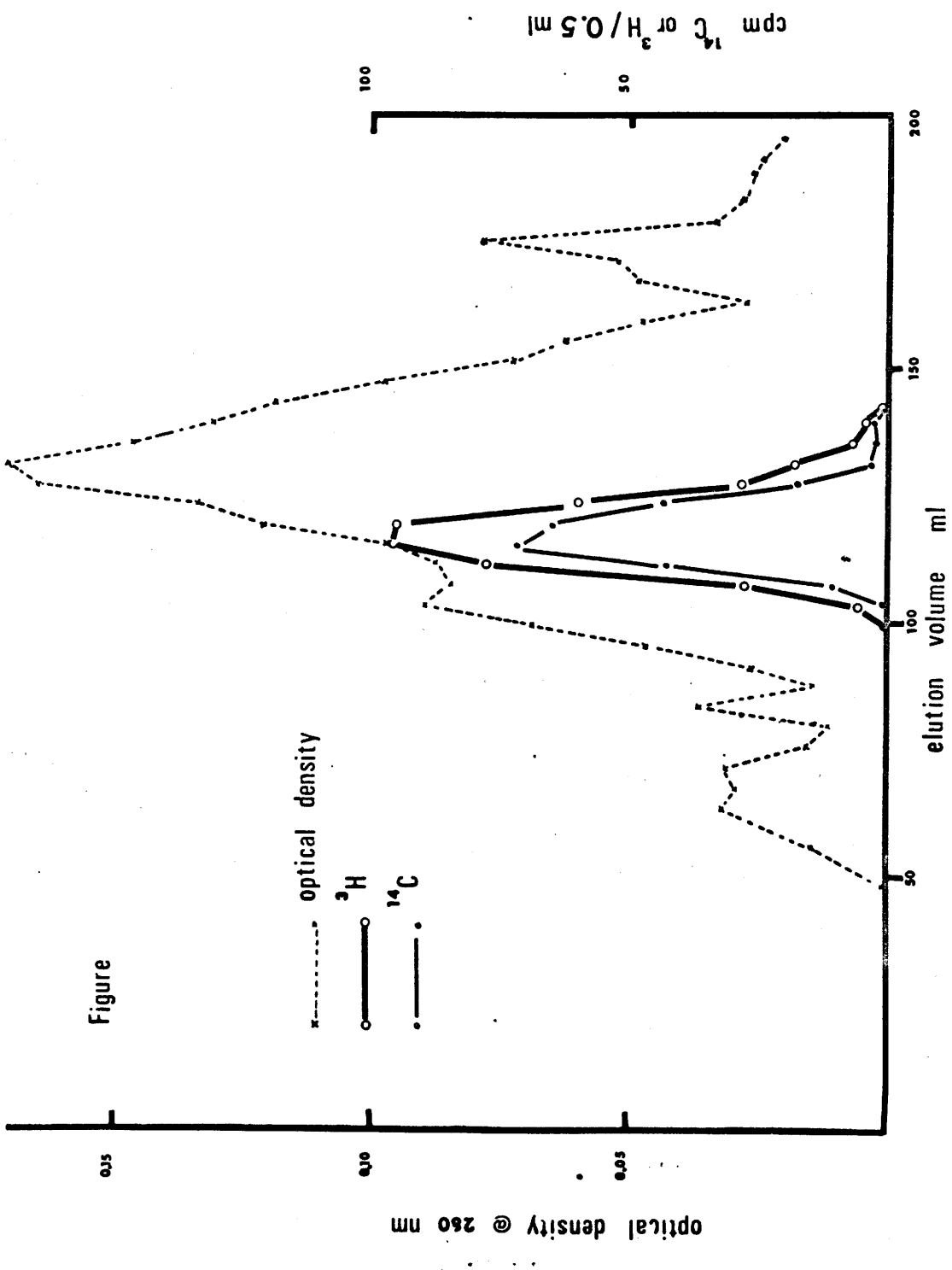

Figure 12: Sephadex G-25 gel filtration of a 0hr incubation of female rat hypothalamic tissue fragments with ^{3}H-isoleucine and ^{14}C-leucine.

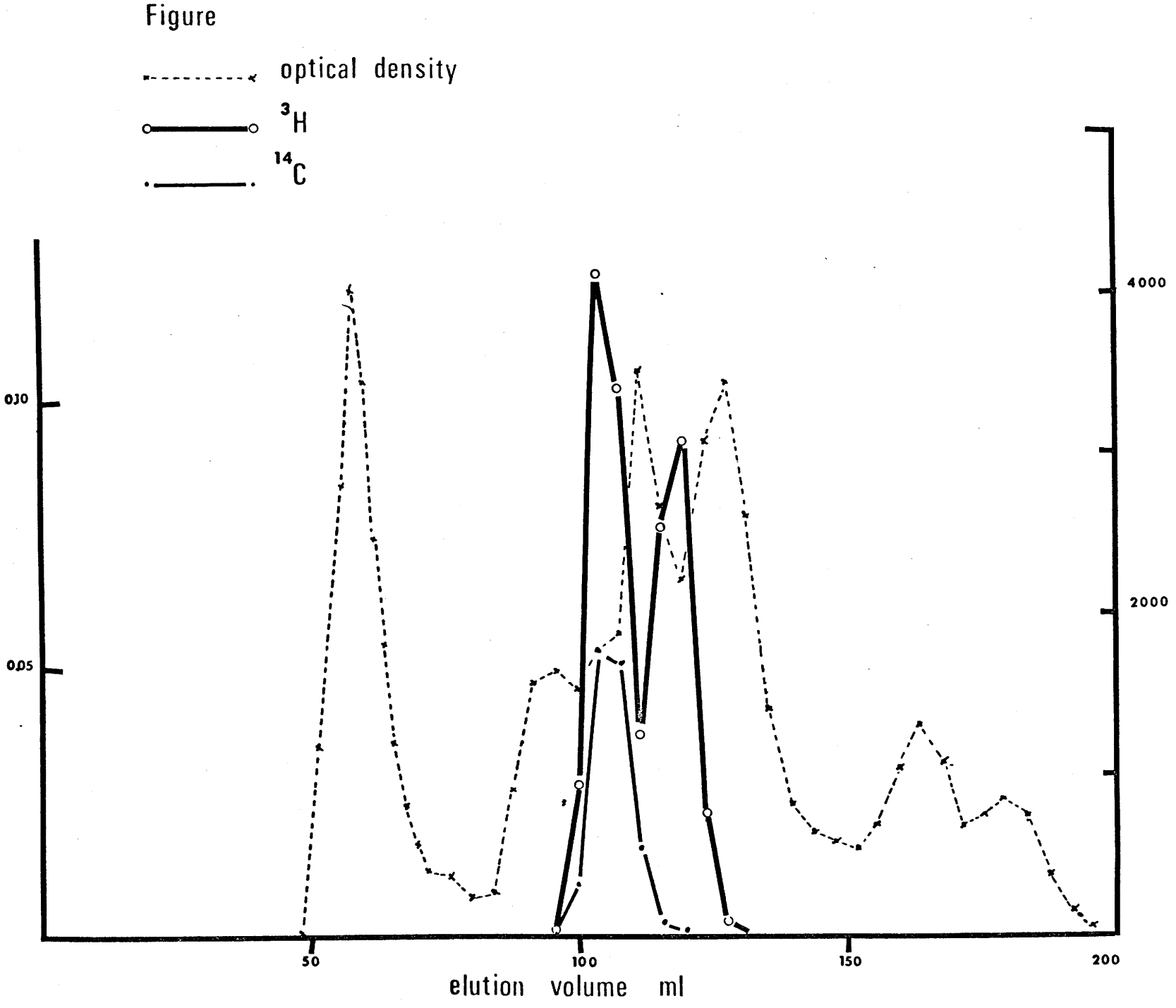

Figure 13: Sephadex G-25 gel filtration of a 6.5 hr incubation of female rat hypothalamic tissue fragments with ^{3}H-isoleucine and ^{14}C-leucine.

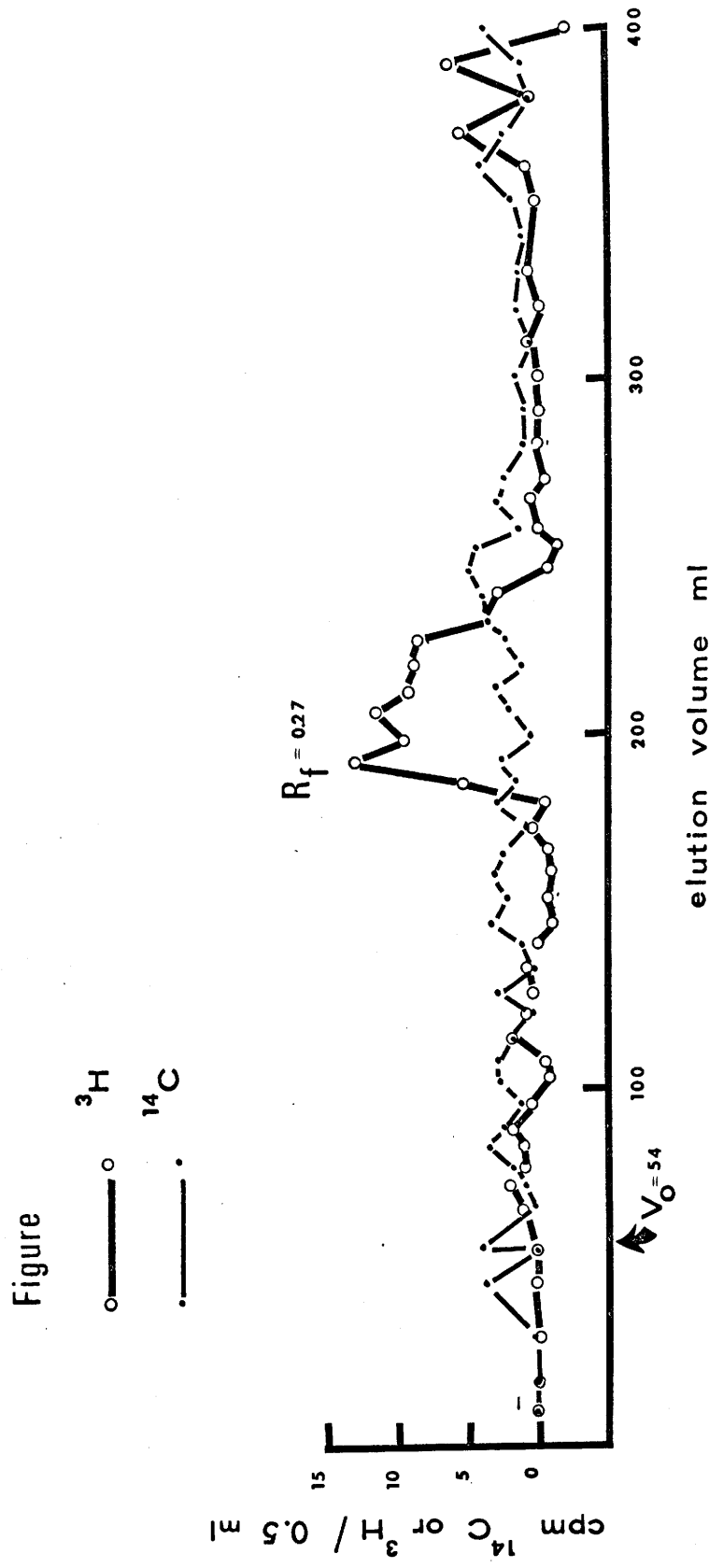

Figure 14: Partition chromatography of the fractions containing both ^{3}H and ^{14}C on gel filtration (Fig 13) of a 6.5 hr incubation of female rat hypothalamic tissue fragments with ^{3}H-isoleucine and ^{14}C-leucine.

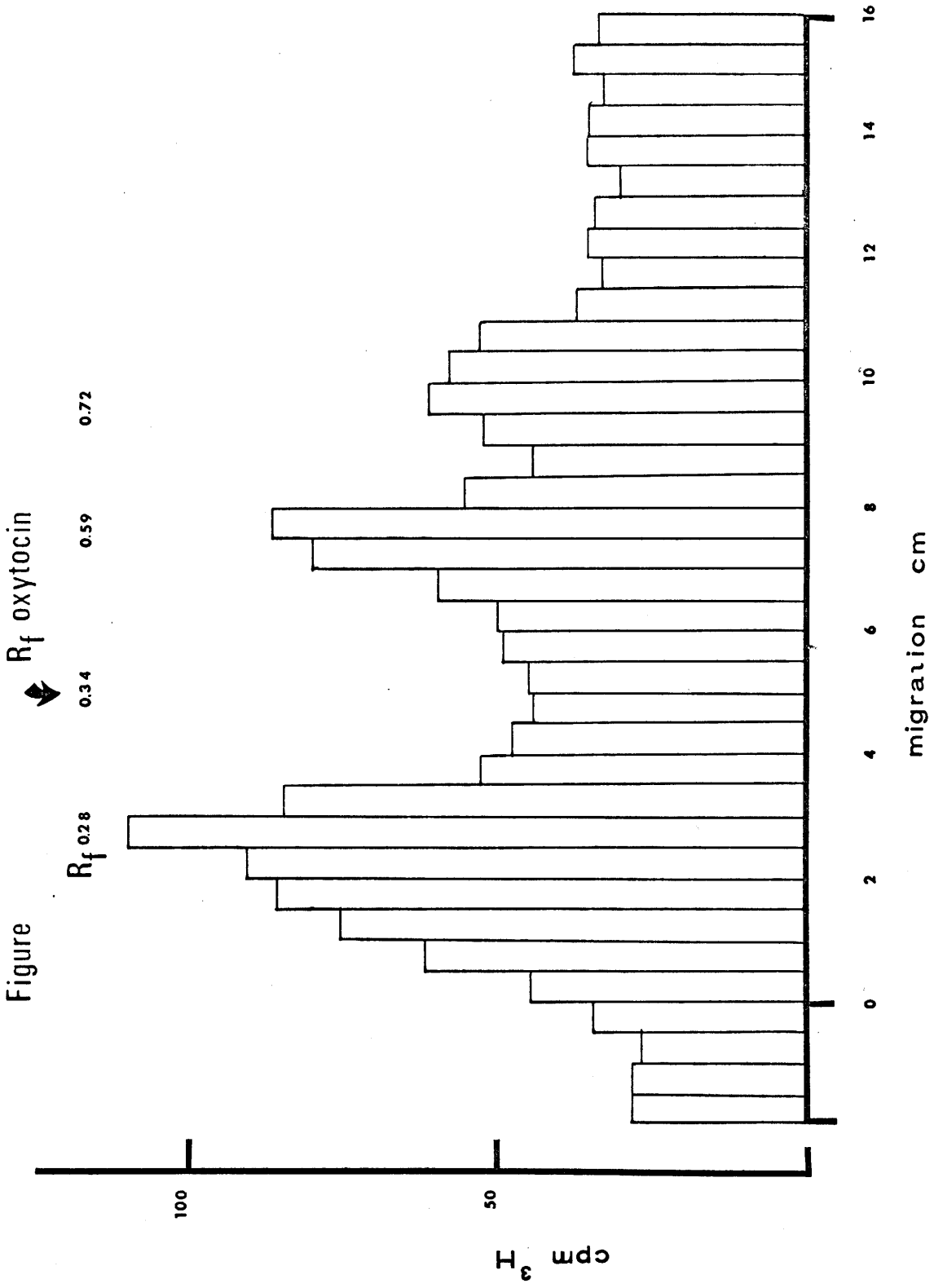

Figure 15: Thin layer chromatography of R_f 0.27 area from partition chromatography described in Fig 14.

TABLE I

Analysis of Incubations

Type of incubation	Radioactive Tracer	Treatment*	Fraction (R_f)	Radio-activity (cpm)**	Biol-ogical Activity#
Neuronal cell bodies in 1 ml, 4 hr.	^{3}H-isoleucine 1 μCi/ml	-			6.25
		GF	0.46	8,012	-
		GF, PC	0.3-0.2	?	0.625
Neuronal cell bodies 2 x 1 ml, 4 hr.	^{14}C-leucine 1 μCi/ml	GF	0.48	55,137	
		GF, PC	1.0 -0.92	116	-
		" "	0.62-0.53	44	int##
		" "	0.47-0.42	30	-
		" "	0.32-0.24	118	-
		" "	0.17	47	int
		" "	0.093	58	int
		" "	0.079	58	int
		" "	0.070	42	int
Ribosomes with cell sap, 2 x 1 ml, 1 hr.	^{14}C-leucine 1 μCi/ml	GF	0.48	32,931	
		GF, PC	1.0	120	int
		" "	0.76	138	
		" "	0.64	175	
		" "	0.54	120	
		" "	0.25	4,650	-
		" "	0.084	85	-
Cell sap, 2 x 1 ml, 1 hr. (Courtesy of Dr. A. Nandi Majumdar)	^{14}C-leucine 1.5 μCi/ml	GF	0.47	34,043	
		GF, PC	1.0	540	0.96
		" "	0.46	480	0.93
		" "	0.22	297	2.2
		" "	0.16	410	0.75
		" "	0.11	1,563	2.2
Cell sap, 2 x 1 ml, 1 hr.	^{14}C-leucine 1 μCi/ml	GF	0.48	47,766	
		GF, PC	1.0-0.65	100	2.0
		" "	0.50	115	1.8
		" "	0.27	120	0.27
		" "	$\approx$ 0.21	2480	0.54
		" "	$\approx$ 0.12	2460	0.54

** On GF, radioactivity is expressed as the maximum cpm/ 0.1 ml.
On PC, radioactivity is expressed as the maximum cpm/ 0.5 ml.
On HVE, radioactivity is expressed as the maximum cpm/ 1 x 3 cm strip.
On TLC, radioactivity is expressed as the maximum cpm/ 0.5 x 5 cm strip.

* GF = gel filtration, PC = partition chromatography, HVE = high voltage electrophoresis, TLC = thin layer chromatography.

mU milk ejecting-like activity

Interference by organic solvent?

Analysis of Incubations (Table I cont'd)

Type of incubation	Radioactive Tracer	Treatment	Fraction (R_f)	Radio activity (cpm)	Biological Activity
Purified cell sap (I + II + III) 2 x 1 ml, 1 hr.	^{14}C-leucine 1 Ci/ml	GF	0.45	39,500	
		GF, PC	> 1.0	-	1.9, 3.1
		" "	1.0	63	8.0, 8.5
		" "	0.5	97	8.25
		" "	0.36	69	
		" "	0.22	75	2.9 (ave)
		" "	0.18	169	12.8 (ave)
		" "	0.13	113	3.5
		" "	0.10	(83)	4.5
		" "	0.081	(61)	1.8
Purified cell sap I. 2 x 1 ml, 1 hr.	^{14}C-leucine 1 Ci/ml	GF	0.50	41,000	
		GF,PC	0.85	112	0.36
		" "	0.51	130	0.16
		" "	0.34	60	
		" "	0.25	70	
		" "	0.21	271	0.13
		" "	0.16	86	
		" "	0.08	68	0.30
Purified cell sap II. 2 x 1 ml, 1 hr.	^{14}C-leucine 1 Ci/ml	GF	0.49	60,000	
		GF, PC	> 1.0	[illegible]	0.23
		" "	1.0	103	0.16
		" "	⩽ 0.28	316	0.25
		" "	⩽ 0.25	67	0.18
		" "	0.09	103	
Purified cell sap III, 2 x 1 ml, 1 hr.	^{14}C-leucine 1 Ci/ml	GF	0.46	43,000	
		GF, PC	> 1.0	-	
		" "	0.89	267	
		" "	0.50	220	
		" "	0.40	175	
		" "	0.29	125	
		" ",TLC	0.091-0.12	19	
		" " "	0.22-0.25	18	
		" " "	0.35-0.38	18	
		" " "	0.48-0.52	37	
		" "	0.19	343	
		" "	0.096	123	
		" "	0.070	155	
Cell sap, 2 x 1 ml, 0 hr.	^{14}C-leucine 1 μCi/ml	GF	0.42	14,500	
		GF, HVE	0.0 *		

* μ_{MG}, or the mobility with respect to methyl green.

Analysis of Incubations (Table I cont'd)

Type of incubation	Radioactive Tracer	Treatment	Fraction (R_f)	Radio-activity 9 cpm)	Biol-ogical Activity
Cell sap, 2 x 1 ml, 3 hr.	^{14}C-leucine 1 μCi/ml	GF	0.48	31,000	
		GF, HVE	0.0		
		" "	- 0.85		
Cell sap, 2 x 1 ml, 0 hr.	^{14}C-leucine 1 μCi/ml	GF	0.42	29,300	
Cell sap, 2 x 1 ml, 3 hr.	^{14}C-leucine 1 μCi/ml	GF	0.42	23,300	
		GF, PC	0.23	235	
		" ", HVE	0.0	250	
Cell sap, 2 x 1 ml, 0 hr.	^{14}C-leucine 1 μCi/ml	GF	0.50	50,000	
		" , PC	0.21	124	
		" "	0.10	178	
		" ",TLC of PC	R_f 0.21		
		" " "	0.23	108	
		" " "	0.42	78	
		" " "	0.55	-	
		" " "	0.63	102	
Cell sap, 2 x 1 ml, 2 hr.	^{14}C-leucine 1 μCi/ml	GF	0.48	57,000	
		" , PC	0.18	96	
		" "	0.11	1460	
		" ", TLC of PC	R_f 0.18		
		" " "	0.23	83	
		" " "	0.42	71	
		" " "	0.55	84	
		" " "	0.63	102	

TABLE II

Comparison of Treatments of Synthetic Oxytocin and ^{14}C-Leucine

Sample	Treatment	Fraction (R_f)	Radio-activity recovered (cpm)	Radio-activity recovered (%)	Biological activity recovered (mU milk ejecting-like activity)	Biological activity recovered (%)
1.0 U oxytocin	Control				9.8, 8.6	92
	5 min boiling				6.7, 7.5	71
^{14}C-leucine 0.1 μCi, and 1.96 U oxytocin	G- 10 gel filtration	0.67-0.63	78,856	41	1.0	51
^{14}C-leucine, 0.1 μCi, 197 U oxytocin	G-15 gel filtration	0.53	132,146	59	≫18250	≫13
^{14}C-leucine 0.1 μCi, and 197 U oxytocin	G-25 gel filtration	0.42	49,080	25	84,308	43
^{14}C-leucine 0.1 μCi, 1.96 U oxytocin	Partition chromatography	0.23 pyridine acetic acid wash	80,000	41	760	39
^{14}C-leucine 4 μCi	"	0.91	22,368	0.28		
	"	0.55	17,959	0.22		
	"	0.43	20,461	0.25		
	"	0.21	13,529	0.16		
	"	0.17	45,154	0.55		
	"	0.041	7,146,000	87		
^{14}C-leucine 0.1 μCi, 9.8 oxytocin U	ion exchange on Bio-Rex 70					
	0.25N HOAc	200 ml	133,000	60	140	1.4
	water	200 ml	8,600	3.8	-	-
	0.25N AmmOAc pH 5.3	100 ml	4,600	2.0	5,000	51
	water	50 ml	2000	0.8	620	63
	30 % Pyridine : 4 % HOAc	50 ml	660	0.2	320	3.2
^{14}C-leucine 1.0 μCi	HVE @ pH 6.5	Neutral	422,684	22		

APPENDIX I: MATERIALS

Chemicals

Formic acid, 97% was from Aldrich Chemical Company Ltd. Cadmium acetate, 1, 4-dioxane reagent A.C.S. and magnesium sulphate were from Allied Chemicals. Ethylene glycol and naphthalene analyzed reagent were from Baker Chemical Co. Polyvinylpyrrolidone (PVP) average M.W. 40,000 and xylene cyanol FF were from B.D.H. Acrylic cation exchange resin Bio-Rex® 70 (100-200 mesh) was from Biorad.

Ammonium acetate A.C.S., calcium chloride. $2H_2O$ A.C.S., crystal violet, cupric sulphate .5 H_2O A.C.S., Folin-Ciocalteau phenol reagent 2N, formic acid 90%, glucose A.C.S., hydrogen peroxide 30% (stored at 5° C), magnesium chloride A.C.S., methyl green, ninhydrin, potassium chloride A.C.S., pyridine A.C.S., sodium bicarbonate A.C.S., sodium chloride A.C.S., sodium hydroxide A.C.S., sodium phosphate monobasic A.C.S., sodium potassium tartrate A.C.S., and tris (hydroxymethyl) amino methane (Tris, THAM®) were from Fisher.

POPOP and PPO scintillation grade were from Fraser Medical Supplies Ltd., Lyphogel® was from Gelman Instrument Co. Eagles's minimal essential medium, fetal calf serum, 100X penicillin G (10,000 U/ml) streptomycin (10,000 µg/ml) solution, and 100X Eagle's vitamin solution were from GIBCO.

Toluene reagent and n-butanol reagent were from MacArthur Chemical Co. Acetone A.R., benzene A.R., iodine U.S.P. resublimed crystals, and methanol (absolute) A.R., were from Mallinckrodt. L-Alanine, L-aspartic acid, L-histidine .HCl.H_2O, L-lysine. HCl, L-methionine, L-serine, L-threonine, L-tryptophan and ammonium sulphate special enzyme grade were from Mann.

Carbon dioxide gas was from Matheson. Silica-gel TLC sheets F254 0.25 mm thickness aluminum support were from Merck. Puromycin. 2HCl was from Nutritional Biochemicals . Bovine albumin was from Pentex. Blue Dextran 2000 and Sephadex® G-10, G-15, and G-25 Fine, and G-25 and G-100 Superfine epichlorohydrin cross-linked dextran beads, were from Pharmacia Fine Chemicals. Sodium deoxycholate and sucrose special density gradient grade were from Schwartz-Mann. L-Arginine, L-asparagine.H_2O, L-glutamine, L-glutamic acid, glycine, L-isoleucine, L-leucine, L-phenylalanine, L-proline, L-tyrosine, L-valine, and 5'-adenosine triphosphate (disodium salt) (ATP), 5'-guanosine triphosphate (sodium salt) (GTP) and phosphoenolpyruvate (trisodium salt hydrate) (PEP), all three stored at -20°C, HEPES, tricine and pyruvate kinase crystalline suspension in ammonium sulphate, stored at 5°C, were from Sigma. Nylon bolting cloth of 333 μ, 110 μ, and 73 μ pore width was obtained from B. and S.H. Thompson and Co. Ltd. Posterior Pituitary Reference Standard, stored at 5°C, was obtained from the U.S. Pharmacopeia. L-Cysteine was prepared in this laboratory by Dr. A. Nandi Majumdar from cysteine tosylate. Mr. A. Wong and Mrs. L.Calhoffer prepared the synthetic oxytocin (LS-1-48B, GL-1-28; 1S-1-48A) in this laboratory from a protected nonapeptide which was already prepared by the stepwise nitrophenyl ester method of Bodanszky and du Vigueaud (3). Dr. D. Yamashiro also kindly supplied synthetic oxytocin (DY-XIII-48) prepared by the same procedure. Water was either double distilled from glass or filtered and deionized by Continental ion exchangers.

Radiochemicals

Generally labelled ^{3}H-isoleucine (1.25 Ci/mM) and uniformly labelled ^{14}C-leucine (250 mCi/mM) were obtained from New England Nuclear.

Animals

Charles River, Sprague-Dawley, Wistar and Hooded rats and lactating mice were obtained from the Animal Quarters, Division of Health Sciences, McMaster University.

Equipment

A Minor Clinical Centrifuge from O.H. Johns Scientific was used for low speed centrifugation. High speed preparations were done with the I.E.C. B-20 centrifuge and the B-60 ultracentrifuge, equipped with A321, A211, and SB-282 rotors. Cell body preparations were inspected with a Nikon Model MS Inverted Microscope, and incubated in an incubator from O.H. Johns Scientific. Homogenization was performed with a Coframo RZR1-64 motorized homogenizer. Small samples were transferred using 50μl, 20μl, and 10μl Eppendorf micropipets, and materials were weighed on either a Mettler P1200 top loader or on a Mettler H54 semi-micro balance. A Radiometer Copenhagen pH-meter 26 was used for pH adjustments. Gel filtration, ion exchange chromatography and partition chromatography were performed in Pharmacia K 9/15, K 15/90 and K 25/100 columns. Fractions were collected with an LKB Ultrorac 7000 lyophilizer in a Virtis 10-100 lyophilizer evacuated with a General Electric oil vacuum pump or in a Thermovac Industries Corp. lyophilizer.

Optical density was measured using either a Gilford Model 2400 UV Spectrophotometer, or a Beckman DB-GT UV Spectrophotometer. TLC plates were scanned in a Chromato-Vue UV scanner from Ultra-Violet Products Ltd. High voltage electrophoresis was performed on a Savant Instruments Inc., flat plate apparatus. Radioactivity was determined with a Nuclear Chicago Unilux III Scintillation System, or an Actigraph III strip counter. Bioassays were performed with a Grass Model 7 Polygraph equipped with a Grass FT03C force displacement transducer.

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