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14. Additional sets of RI lines derived from crosses of inbred strains AKR/J by C57L/J, DBA/2J by C57BL/6J, SWR/J by C57L/J and C3H/HeJ by C57BL/6J are being developed (B. A. Taylor, personal communication).
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Biosynthesis of a Vasotocin-Like Peptide in Cell Cultures from Pineal Glands of Human Fetuses

Abstract. *Cultured cells from pineal glands of human fetuses release into their media a substance that has antidiuretic and hydroosmotic activities. The ratio of these activities as well as their susceptibility to tryptic digestion, specific oxidative inactivation by tyrosinase, and reductive inactivation by sodium thioglycollate, indicates the presence of a basic peptide, presumably identical with arginine vasotocin. The total amount of this peptide released into the culture media during 38 days of incubation is about ten times greater than the amount contained in nonincubated pineal glands from fetuses of the same age, strongly suggesting that fetal ependymal cells from the pineal gland can synthesize in vitro a peptide similar to arginine vasotocin.*

Katsoyannis and du Vigneaud's first synthesis (*1*) of arginine vasotocin (AVT) in 1958 (an example of the synthesis of a hormone before it was identified as a natural product) led to the discovery that this is a widespread octapeptide hormone, present in all species so far investigated, from the most primitive living vertebrates (*2*) to man (*3*). In mammals, we first identified AVT in the pineal gland (*4, 5*), and this conclusion has been directly confirmed by the chemical analysis of pineal AVT (*6*). The extraordinary evolutionary stability of AVT suggests that this ancestral molecule may be elaborated by some primitive structure common to all vertebrates. Pavel (*7*) has suggested that pineal AVT may be elaborated by secretory ependymal cells, the most primitive secretory cells of the brain (*8*). If this postulate is true, then ependymal secretory cells from the pineal gland, when cultured in vitro, should have the ability to synthesize AVT. Because, in the fetal pineal gland of mammals (*9*) including man (*10*), the only secretory activity so far detected is located in ependymal cells, we therefore studied human fetuses. Male fetuses, aged 95 to 115 days after ovulation, were obtained aseptically within 15 to 30 minutes of therapeutic abortion. The pineal glands of six fetuses were extracted in 0.25 percent acetic acid as described (*7*) and served as controls for comparison prior to incubation. The pineal glands from four other fetuses were used for cell cultures. Brain tissue (from the region of the frontal lobe) of the same fetuses was used as control tissue in cell cultures. The pineals and neural tissue used for cell culture were minced to provide a crude dissociated cell suspension. The cells and cell clumps corresponding to each gland were suspended in 1 ml of Hanks medium supplemented with 10 percent calf serum and 2.5 percent N16 medium. The culture tubes contained 1 ml of medium and were maintained in a nearly horizontal position at 37°C and *p*H 7, without rocking, for 6 to 8 days. Cell attachment to the bottom of the tubes was complete within 24 hours. On day 8, when the cell cultures were established, the media were first changed, and the tubes were inserted in a rocker incubator system. The culture media were changed every 5 days thereafter. The assay methods used have been described (*4*), and include rat antidiuretic, rat uterus, and frog bladder (*Rana temporaria*) assays. The U.S.P. Posterior Pituitary Reference Standard was used as standard in antidiuretic assays. Synthetic oxytocin (Syntocinon, Sandoz) restandardized against the U.S.P. Standard, was used as standard in hydroosmotic assays. Synthetic AVT (provided by M. Bodanszky, Case Western Reserve University, Cleveland) was used for the calculation of the hydroosmotic rat antidiuretic ratio. Highly purified crystalline trypsin (twice crystallized and chymotrypsin free, salt free, lyophilized; Serva laboratories), tyrosinase (salt free, lyophilized; Worthington Biochemicals), and sodium thioglycollate (British Drug House) were used for tryptic digestion, specific oxidative inactivation, and reductive inactivation, respectively. Every 5 days, the culture media from the pineal cultures from the four fetuses were pooled, adjusted to 4 ml, and assayed for antidiuretic or hydroosmotic activities. No rat uterus activity could be detected in the culture media (that is, less than 25 μunit/ml). Hydroosmotic and antidiuretic activities of the culture media are shown in Table 1. The culture media from day 18 were pooled, and hydroosmotic activity was determined after it was incubated with trypsin and tyrosinase. One milliliter of culture media was incubated with 20 μg of trypsin at *p*H 8 and 38°C for 3 hours. The other half was incubated at the same temperature and *p*H without trypsin. For specific oxidative inactivation, 1 ml of culture media was incubated for 1 hour at *p*H 7.5 and 37°C with tyrosinase (25 μg/ml). The other half was incubated at the same temperature and *p*H without tyrosinase. The hydroosmotic activity was nearly completely destroyed after trypsin and tyrosinase incubation; that is, when the control water flux was 3 μl per 20 minutes, the fluid movement in response to untreated medium was 34 μl per 20 minutes, whereas the response to the medium treated with trypsin or tyrosinase was 6 μl and 5 μl per 20 minutes, respectively. Both the controls and

Table 1. Hydroosmotic and antidiuretic activities of cell culture media from pineal glands of human fetuses expressed in U.S.P. microunits per milliliter ± the standard error.

Day	Activity	
	Hydroosmotic	Antidiuretic
8	3750 ± 634	
13		19 ± 3
23	3100 ± 428	
28		18*
33		20*
38	3480 ± 458	
Mean	3440	19

* The antidiuretic value of day 28 and day 33 represents the equivalent of a single assay used as control in inactivation studies.

the samples incubated with the trypsin or tyrosinase were assayed on the hemibladders from the same frogs. The culture media from day 28 were pooled, and antidiuretic activity was determined after incubation with trypsin and tyrosinase as described above. The antidiuretic activity was reduced by trypsin and tyrosinase incubation from 18 μunit/ml to less than 5 μunit/ml. The culture media from day 33 were also pooled, and antidiuretic activity was determined after reductive inactivation with sodium thioglycollate. Thioglycollate solution in Sorensen's phosphate buffer (0.1 ml) was added to 0.9 ml of culture media. Controls (0.9 ml) were incubated with 0.1 ml of phosphate buffer at the same temperature and *p*H without sodium thioglycollate. The antidiuretic activity of culture media incubated with 0.01*M* and 0.05*M* sodium thioglycollate for 3 hours at *p*H 7.4 and 37°C was reduced from 20 μunit/ml to 8 μunit/ml and less than 5 μunit/ml, respectively. Since sodium thioglycollate affects bladder permeability (*11*) no hydroosmotic reductive inactivation experiments were performed. Neither in the culture media from brain tissue of the same fetuses, nor in the control media, could antidiuretic or hydroosmotic activities be detected (hydroosmotic activity of < 500 μunit/ml and antidiuretic < 5 μunit/ml). Antidiuretic activity was quantified in three samples (days 13, 28, and 33); but hydroosmotic activity was not estimated on the same samples. If we assume that the hydroosmotic activity of the days 13, 28, and 33 was about that measured in the three samples shown in Table 1, we estimate the ratio of hydroosmotic to antidiuretic activity to be 181. This does not differ significantly from the activity ratio of 195 that we found for synthetic AVT by the same assays. The total amount of the AVT-like peptide released into the culture media during 38 days (as deduced from the biological activities assayed every 5 days) is about ten times greater than the amount contained in the nonincubated pineal glands of the same age [the hydroosmotic activity from the six nonincubated glands being equivalent to 2600 ± 825 (standard error) U.S.P. μunit per gland]. From day 40 onward, both antidiuretic and hydroosmotic activities of culture media progressively decreased. The antidiuretic and hydroosmotic activities of the culture media from pineal glands from human fetuses, the ratio of these activities as well as their susceptibility to tryptic digestion, to specific oxidative inactivation by tyrosinase, and to reductive inactivation by sodium thioglycollate indicate the presence of a peptide containing at least one basic amino acid (*12*), a tyrosine residue (*13*), and a disulfide bond (*14*). These characteristics are consistent with the specific pharmacological profile of AVT (*15*). The total amount of the AVT-like peptide released into the culture media during 38 days of incubation is about ten times greater than the amount contained in the nonincubated glands of the same age, which suggests that pineal ependymal cells can synthesize in vitro a basic peptide presumably identical with AVT. On the other hand, as deduced from the ratio of biological activities in conjunction with the enzymatic degradation results, there is no evidence for the presence of vasopressin or oxytocin in the culture media, suggesting that ependymal cells are not able to synthesize the natural analogs of AVT. If chemical analysis substantiates our results, our study demonstrates for the first time the biosynthesis of AVT by secretory specialized ependymal cells. If ependymal cell secretion represents the first and the most primitive secretory activity of the brain (*8*) and ontogenetically precedes neurosecretion (*16*), then the synthesis of AVT in a primitive ependymal cell would represent the first step in the ontogenetic molecular evolution of the brain octapeptides.

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Thyroid Hormone Action: A Cell-Culture System Responsive to Physiological Concentrations of Thyroid Hormones

Abstract. *Cells from a rat pituitary tumor cell line will respond in vitro to physiological concentrations of* L-*thyroxine and* L-*triiodothyronine. The cells are grown in a culture medium that contains serum from a hypothyroid calf. Dose-response relationships of a variety of thyronine derivatives indicate that this system has a specificity of response which is similar to that observed in vitro.*

Thyroid hormones affect the growth, development, and metabolism of virtually all tissues of higher organisms. In spite of extensive studies in vivo, the mechanism of action of the thyroid hormones remains to be defined. Although studies in vivo are necessary in order to evaluate the effects of the hormones on the entire organism, an in vitro system offers significant advantages for the study of the action of the thyroid hormones at the molecular level.

Investigations in vitro of the mechanisms of action of L-thyroxine (T4) and L-triiodothyronine (T3) at physiological concentrations (*1*) have not been successful. The hormone concentrations required to induce biological effects in subcellular components (such as mitochondria) range from 10^4 to 10^6 times greater than physiological free hormone concentrations (*2*). Although other investigators have used cell-culture systems to study the action of the thyroid hormones, the cells responded only minimally, even at high hormone concentrations (*3*). In addi-