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STUDIES OF CHICK ADRENAL MEDULLA IN ORGAN CULTURE*

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Abstract—Chick adrenol medullae were maintained in organ culture using Eagle's minimal essential medium (MEM) fortified with 1% glucose and 10% fetal calf serum. This medium was as good as or better than other more highly enriched media tested. After an initial loss of 50 per cent of their catecholamine content and tyrosine hydroxylase activity at 24 hr, the glands survived for up to 8 days without further losses. In contrast to adrenal medullae of other species, the chick adrenals contain only muscarinic receptors. Both fresh and cultured glands secreted catecholamines when acetylcholine, carbamylcholine or urocholine was added to the medium, but no secretion occurred when nicotine was added. The secretory response was blocked by atropine but not by hexamethionium. Aminophylline by itself had a slight but significant stimulatory effect and potentiated the response to carbamylcholine. Cyclic-AMP, dibutyryl cyclic-AMP and cyclic-GMP had no effect on secretion by themselves or in the presence of aminophylline. After 1–3 days in culture, the glands maintained their ability to synthesize epinephrine and norepinephrine from tyrosine at about one-half the rate of fresh glands and were able to incorporate [^{3}H]leucine into protein at a greater rate than fresh glands. However, after stimulation with carbamylcholine, the glands were unable to recover their catecholamine content or induce an increase in tyrosine hydroxylase activity. Additions of nerve growth factor, dibutyryl-cAMP, dexamethasone and ACTH to the culture media did not enable the glands to recover their catecholamine stores or increase their tyrosine hydroxylase activity.

Tissue culture has proven to be a valuable tool for studies of organs removed from the more complex environment of the intact animal. Tissue cultures of neuroblastoma [1, 2] and sympathetic ganglia [3] have demonstrated the feasibility of this method for studying tyrosine hydroxylase induction. Richelson [1, 2] and Waymire *et al.* [4] have shown increases in tyrosine hydroxylase activity in response to cyclic-AMP as well as sodium butyrate in cultured neuroblastoma. Larrabee [5] has demonstrated that sympathetic ganglia can survive in artificial media, and the neurons continue to respond to acetylcholine stimulation for over a week after their isolation *in vitro*. Silberstein *et al.* [6] have more specifically shown that neonatal mouse adrenals in organ culture increased their tyrosine hydroxylase activity 24 hr after exposure to high concentrates of KCl. This report describes biochemical and pharmacological studies of chick adrenal glands maintained in organ culture with reference to the adrenal medullary cells.

EXPERIMENTAL PROCEDURE

Tissue culture. Chick embryos, 18–20 days old, and 1-day-old chicks were of the Vanress–Arbor Acre and Vanress–Whiterock strains. Adrenal glands from these chicks were aseptically removed, cleansed of excess tissue and placed on individual millipore filters (0·2 μm) which were floated on 1 ml of tissue culture media in individual organ culture dishes. Dishes were covered with plastic tops and placed in a 37° incubation chamber which was gassed with 95% O_2–5% CO_2. Media were replaced every 24 hr.

Several media, with and without supplements indicated in Table 1, were tested for their ability to maintain the adrenal medulla in culture. Eagle's minimum essential medium [7] and medium CMRL-1066 [8] were prepared from stock solutions purchased from Grand Island Biologicals. Medium AN-54 was prepared according to the procedure of Ling *et al.* [9]. Maintenance of the medulla in culture is defined as the ability of the glands to maintain their levels of catecholamines and tyrosine hydroxylase activity and to respond to carbamylcholine.

Analytical procedures. Glands were homogenized in glass-to-glass tubes in 1 ml water. A 100-μl aliquot was taken from the whole homogenate for dopamine-β-hydroxylase assay and a 50-μl aliquot was added to 1 ml of 3·5% perchloric acid for catecholamine assay. The remaining homogenate was centrifuged at 26,000 *g* for 20 min. A 100-μl aliquot of this supernatant was used for tyrosine hydroxylase assay by the method of Waymire *et al.* [10]. Dopamine-β-hydroxylase was assayed spectrophotometrically by the method of Nagatsu and Udenfriend [11]. Catecholamines were measured by the ferricyanide–trihydroxyindole method [12].

Secretion. Secretion was determined by measuring the catecholamine levels in the media after a 1-hr incubation in the presence or absence of secretogogues. To determine the sensitivity of the glands to various secretogogues, the glands were first preincubated for three 1-hr periods in secretogogue-free media to establish the basal rate of secretion. The secretogogue was then added to the media and the incubations were continued for another hr, at which time the amounts of catecholamines released were determined.

Biosynthesis of catecholamine. Catecholamine bio-

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synthesis was studied by measuring the conversion of tyrosine to dopamine, norepinephrine and epinephrine. Glands were incubated for 1 hr in tyrosine-free media (1 ml) and then transferred to 0·5 ml of the same media containing 0·5 μCi [U-^{14}C]-tyrosine (sp. act., 500 Ci/mole) and 10^{-4} M iproniazid (phenylisopropyl-hydrazine, monoamine oxidase inhibitor). The incubations (2 hr) were carried out at 37° in small glass vials covered with rubber stoppers. Each vial was irrigated with 95% O_2–5% CO_2 during the incubation. The reaction was stopped by placing the vials in ice. The incubation media were collected in test tubes and the glands were washed with fresh media (0·5 ml); the washes were combined with the incubation media. To the glands and their media, in separate tubes, were added 0·1 ml of catecholamine mixture (epinephrine, 4 g/l.; norepinephrine, 4 g/l.; dopamine, 2 g/l., dissolved in 0·1% EDTA–0·01 M HCl), 0·1 ml ascorbic acid (20 mM) and 1·0 ml perchloric acid (7%) (PCA). The glands were homogenized and the protein was removed from both the media and homogenate by centrifugation at 26,000 *g* for 10 min. The supernatants were neutralized with K_2HPO_4 (2 M) to pH 6·5 and, after chilling in ice, the potassium perchlorate was removed by centrifugation. Norepinephrine and epinephrine were separated from dopamine by ion-exchange chromatography as described by Häggendal [13]. Catecholamines were identified by thin-layer chromatography as described by Johnson and Bookma [14].

Protein synthesis. The procedure used for determining protein synthesis was the incorporation of [^{3}H]-leucine into PCA-precipitate material. Glands were incubated in leucine-free media (1 ml) for 1 hr at 37° to reduce the intracellular level of leucine. The glands were then transferred to 0·2 ml of leucine-free media containing 5 μCi/ml of [^{3}H]-leucine (sp. act., 29·8 Ci/m-mole) and incubated for 1 hr at 37° in the presence of air. At the end of the reaction, the glands were homogenized in 1 ml PCA (0·4 M) and centrifuged at 26,000 *g* for 10 min. The precipitate was washed four times with 2 ml PCA (0·2 M) and dissolved overnight in NaOH (0·5 M). A 0·2-ml aliquot was placed in 15 ml of scintillation fluid and counted. The remaining NaOH–protein was used for protein determination according to the method of Lowry *et al.* [15].

Statistics. Student's *t*-test was used to determine statistical significance [16]. A P value of less than 0·05 was considered significant.

Materials. Isotopes were obtained from New England Nuclear Corp. and crystalline beef liver catalase (DBO assay) from Sigma. Nerve growth factor was a gift from Dr. R. A. Bradshaw and L-aromatic amino acid decarboxylase was prepared according to Waymire *et al.* [10]. All other materials were obtained from readily available sources.

RESULTS

Stability of the adrenal medulla in culture. A survey of different media was made to determine the medium that would provide optimal survival of the adrenal medulla. As a measure of stability, the criteria used were the abilities of the glands to maintain their catecholamine stores, tyrosine hydroxylase and dopamine-β-hydroxylase activities, and their ability to respond to a secretory stimulus.

Comparison of the stability criteria for three media—MEM, AN-54 and CMRL-1066—with and without supplements (Table 1), indicated that MEM containing 10% fetal calf serum and 1% glucose was better than the more enriched media in maintaining the adrenal medulla in culture. In comparison to MEM there was a greater loss of tyrosine hydroxylase activity and no difference in the amounts of catecholamines but a complete loss in the response to carbamylcholine when medium AN-54 was used. When medium CMRL-1066 was used there was a complete loss of tyrosine hydroxylase activity at 48 hr, a complete loss of catecholamines at 24 hr and a loss of the response to carbamylcholine. However, when the amount of glucose in CMRL-1066 was increased from 0·1 to 1·0%, there was greater stability of tyrosine hydroxylase and the response to carbamylcholine was maintained. Addition of trace elements, lipids, vitamins and dexamethasone to MEM had either no effect or decreased the survival parameters of the gland. When the concentration of glutamine was reduced to 10% of the original content in MEM the glands did not survive 24 hr. In the ensuing studies, MEM containing 10% fetal calf serum and 1% glucose was the standard incubation medium.

Stability of catecholamines and their synthesizing enzymes in culture. The stability of the adrenal glands in culture, indicated by their ability to retain their catecholamines, tyrosine hydroxylase and dopamine-β-hydroxylase activities, was examined over a 5-day period. During the first 24 hr, the tyrosine hydroxylase activity and catecholamine content declined about 50 per cent, but thereafter there was little or no change (Table 2). Smaller losses of dopamine-β-hydroxylase occurred and there was a decrease of 25–30 per cent in the first 24 hr in culture but no changes thereafter.

Biosynthesis of catecholamine in organ culture. The formation of dopamine and norepinephrine plus epinephrine from tyrosine by glands maintained in organ culture for periods up to 3 days was determined (Table 3). After 24 hr in culture media, there was a 50 per cent decrease in the formation of norepinephrine plus epinephrine but no further decreases up to 3 days. There was a somewhat larger decrease in the formation of dopamine from tyrosine during the first 24 hr, but no further decreases occurred throughout the experimental period.

Protein synthesis. The ability of the glands to incorporate [^{3}H]-leucine into PCA-precipitable material was used as a measure of protein synthesis. In contrast to losses in catecholamines, enzyme activities and ability to synthesize catecholamines, there was a small but significant increase in leucine incorporation (Table 4).

Secretion—Effects of cholinomimetic drugs on the adrenal medulla. These studies were undertaken to determine the ability of the cultured adrenal medulla to maintain a response to secretogogues and to determine the type of receptors present. Preliminary studies had shown that the acetylcholine- or carbamylcholine-elicited response is Ca^{2+} dependent in cultured chick adrenals [17]. Therefore, Ca^{2+} was present in all of the studies presented in this section.

In most species studied, the adrenal medulla, like

Table 1. Stability of adrenal medulla in various culture media*

Media	Tyrosine hydroxylase (% of initial amount) 24 hr	Tyrosine hydroxylase (% of initial amount) 48 hr	Catecholamine (% of initial amount) 24 hr	Catecholamine (% of initial amount) 48 hr	Carbamylcholine response†
MEM‡	38 ± 9 (5)	37 ± 4 (4)	50 ± 8 (3)	25 ± 6 (4)	+
AN-54‡	41 ± 6 (6)	8 ± 2 (8)	47 ± 5 (6)	33 ± 4 (8)	−
CMRL-1066‡	14 ± 3 (6)	0 (6)	0 (6)	0 (6)	−
CMRL-1066 + 1% glucose‡	36 ± 3 (8)	39 ± 3 (7)			+
MEM + trace elements‡		40 ± 5 (8)		42 ± 7 (8)	+
MEM + lipid concentrate‡		45 ± 11 (4)		25 ± 7 (4)	+
MEM + An-54 vitamins	35 ± 2 (11)	16 ± 3 (4)	40 ± 2 (11)	18 ± 9 (4)	+
MEM, 29·2 mg/l. glutamine	0	0	0	0	−
MEM, 10% serum, 1% glucose§	44 ± 2 (4)	52 ± 9 (3)	50 ± 3 (4)	48 ± 2 (3)	++
MEM, 10% serum, 1% glucose ACTH (0·4 U/l.) + dexamethosone (10^{-4} M)§	45 ± 3 (4)	48 ± 4 (4)			++

* Left and right glands from 20-day embryos and 1-day chicks were removed and cultured. Their stability parameters were measured at 24 and 48 hr. In each case, carbamylcholine (2×10^{-4} M) was given to the right gland at 24 hr. Tyrosine hydroxylase activity and catecholamine levels were measured at 24 and 48 hr in the left gland and are expressed as the percentage of activity of control fresh glands. The results are expressed as mean ± S.E.M. and the number in each group appears in parentheses. Trace elements, lipid concentrate, and vitamins used are in concentrations present in medium AN-54. The contents of fresh glands were: 20-day embryo, tyrosine hydroxylase, 11 ± 0·6 nmoles/hr/gland (N = 6); catecholamines, 3·8 μg/gland (N = 6); 1-day chicks, tyrosine hydroxylase, 18 ± 2 nmoles/hr/gland (N = 5); catecholamines, 6 ± 1 μg/gland (N = 5).

† The response to carbamylcholine was in the range of 3-fold (+) to 6-fold (++) increase in the release of catecholamines over controls.

‡ 20-day embryos.

§ 1-day chicks.

its developmental homologue, autonomic ganglia, contains a mixture of both nicotinic and muscarinic receptors [18–23]. Low doses of nicotine evoke secretion but high doses cause a paralytic effect. Pilocarpine, a muscarinic analogue, also evokes a secretory response which is selectively abolished by a representative muscarinic blocker, atropine. Acetylcholine has both nicotinic and muscarinic activity. Secretion evoked by acetylcholine is partially abolished by the presence of either hexamethonium or atropine and is completely abolished by the presence of both hexamethonium and atropine.

The data in Table 5 show the effects of carbamylcholine, urocholine and cholinergic inhibitors on the chick adrenal medulla in culture. Chick adrenals from either embryos or 1-day-old chicks respond to carbamylcholine with a 3- to 6-fold increase in catecholamine secretion. This response is blocked by atropine but not by hexamethonium, which suggests the presence of only muscarinic receptors. Further evidence to support the presence of muscarinic receptors is given by the 3-fold increase in catecholamine secretion evoked by urocholine (predominantly muscarinic activity), which is blocked by atropine but not by

Table 2. Stability of catecholamine content, tyrosine hydroxylase and dopamine-β-hydroxylase in cultured adrenal glands*

Age of cultured gland (hr)	Tyrosine hydroxide (nmoles/hr/gland) Left	Right	% Remain	Dopamine-β-hydroxylase (nmoles/hr/gland) Left	Right	% Remain	Catecholamines (μg/gland) Left	Right	% Remain
24	46 ± 2 (4)	20 ± 2 (4)	44	82 ± 11 (4)	63 ± 7 (4)	77	7·5 ± 0·5 (4)	4·5 ± 0·7 (4)	50
48	48 ± 12 (2)	25 ± 7 (2)	52	106 ± 11 (2)	79 ± 7 (2)	75	11 ± 0·7 (3)	5·3 ± 0·3 (3)	48
72	62 ± 15 (3)	38 ± 6 (3)	51	112 ± 11 (3)	76 ± 9 (3)	68	12 ± 2 (3)	5 ± 0·9 (3)	36
96	63 ± 3 (3)	31 ± 4 (3)	49	87 ± 3 (3)	62 ± 8 (3)	71	15 ± 2 (3)	6 ± 0·5 (3)	40
120	57 ± 4 (3)	27 ± 2 (3)	47	93 ± 13 (3)	68 ± 9 (3)	73	12·6 ± 2 (3)	4·8 ± 0·4 (3)	38

* Left and right glands from 1-day-old chicks were removed; all left glands were assayed as controls and all right glands were cultured in modified MEM as described in the Experimental section. At 24-hr intervals, a group was removed and assayed as described in Methods. Each gland in culture was compared to its contralateral gland assayed shortly after removal from the chick. The values are expressed as the mean ± S.E. and the number of glands in each group appears in parentheses.

Table 3. Biosynthesis of catecholamines by cultured adrenal glands*

Catecholamines	Culture age (hr)			
	0	24	48	72
Epinephrine + norepinephrine (nmoles/gland/2 hr)	0·60 ± 0·02 (4)	0·30 ± 0·07 (4) P < 0·01	0·26 ± 0·03 (4) P < 0·001	0·26 ± 0·05 (4) P < 0·001
Dopamine (nmoles/gland/2 hr)	0·94 ± 0·03 (4)	0·30 ± 0·03 (4) P < 0·001	0·49 ± 0·05 (4) P < 0·001	0·33 ± 0·04 (4) P < 0·001

* Glands were cultured and catecholamine biosynthesis was measured as described in Methods. Values are the mean ± S.E.M. The numbers in parentheses are the number of glands in each group. The amounts of dopamine and epinephrine plus norepinephrine in the media and tissue were determined separately as described in Methods. The data are the amounts of catecholamines found in the media plus the glands. In uncultured glands, 50 per cent of the amines were found in the media. After 24 hr, the amount found in the media was 40 per cent; and after 48 and 72 hr, the amount was 24 per cent. P values are relative to uncultured glands.

hexamethonium. The glands did not respond to nicotine nor did the presence of nicotine have any effect on the secretion evoked by carbamylcholine (Table 6).

The possibility that nicotinic receptors did not survive in culture or that they do not develop until a later age was investigated using fresh adrenal glands from 1- and 14-day-old-chicks. The data in Table 7 again indicate the absence of nicotinic receptors in the chick adrenal.

Cyclic AMP has been implicated in the release of catecholamines from the adrenal glands [24] and

Table 4. Incorporation of [^{3}H]-leucine into protein by adrenal glands in culture*

	Culture age (hr)					
	0	24	48	72	96	120
[^{3}H]-leucine incorporation (cpm/μg protein)	32 ± 1 (18)	47 ± 2 (4) P < 0·001	64 ± 4 (3) P < 0·001	56 ± 4 (3) P < 0·001	60 ± 7 (3) P < 0·001	62 ± 12 (3) P < 0·03
Total protein (μg)	44 ± 3 (18)	48 ± 3 (5)	55 ± 6 (4)	60 ± 3 (4) P < 0·002	62 ± 4 (4) P < 0·002	66 ± 8 (3) P < 0·02

* Control left glands and cultured right glands were prepared as described in Table 2. At 24-hr intervals, a group of glands were removed and incorporation of [^{3}H]-leucine into protein was measured as described in Methods.

Table 5. Secretory response of cultured glands to cholinomimetics*

Age	Treatment†	Catecholamine secretion (% of control)
16-day embryo	CbCH	489 ± 33 (4)‡
38 ± 2 (18) ng/hr/gland	CbCh + C_6	439 ± 43 (4)‡
	CbCh + Atp	109 ± 5 (4)§
18-day embryo	CbCh	263 ± 42 (3)‖
59 ± 8 (6) ng/hr/gland	CbCh + C_6	243 ± 28 (3)‖
	CbCh + Atp	112 ± 11 (3)§
1-day chicks	CbCh	491 ± 97 (4)‡
44 ± 3 (15) ng/hr/gland	CbCh + C_6	670 ± 115 (4)‡
	Urch	273 ± 30 (5)‖
	Urch + C_6	271 ± 28 (5)‡
	Urch + Atp	105 ± 10 (5)‡

* Secretory studies were done on glands maintained in culture for 24 hr. Preliminary studies showed that the best response was given when the concentration was 2×10^{-3} M for all drugs used. Values are expressed as mean ± S.E. and the number in each group appears in parentheses. The numbers listed under the age of adrenal glands are the basal rates of secretion.

† CbCh, carbamylcholine; C_6, hexamethonium; Urch, urocholine; Atp, atropine.

‡ P < 0·001.

§ NS.

‖ P < 0·02.

Table 6. Effect of nicotine of secretion by cultured adrenal glands*

Treatment	Catecholamine secretion (% of control)
Nicotine (10^{-3} M)	124 ± 5 (4)†
Nicotine (10^{-5} M)	119 ± 4 (4)†
Nicotine (10^{-7} M)	116 ± 5 (4)†
Nicotine (10^{-8} M)	93 ± 1·5 (4)†
Nicotine (10^{-9} M)	112 ± 7 (4)†
CbCh	562 ± 136 (4)‡
CbCh + nicotine (10^{-3} M)	460 ± 67 (4)§
CbCh + nicotine (10^{-5} M)	743 ± 118 (4)‖

* Secretion studies were done on 24-hr cultured glands removed from 1-day-old chicks as described in Table 5. The concentration of carbamylcholine used was 2×10^{-3} M.
† NS.
‡ $P < 0{\cdot}001$.
§ $P < 0{\cdot}005$.
‖ $P < 0{\cdot}05$.

Table 7. Secretory response of fresh glands to cholinomimetics*

Age of chick	Treatment	Catecholamine secretion (% of control)
1 day	Nicotine	99 ± 4 (4)†
49 ± 5 (10) ng/hr/gland	CbCh	341 ± 33 (4)‡
	CbCh + C_6	306 ± 15 (4)‡
	CbCh + Atp	102 ± 3 (4)†
14 days	CbCh	341 ± 74 (3)‡
91 ± 6 (6) ng/hr/gland	Nicotine	99 ± 4 (3)†
	KCl, 50 mM	240 ± 18 (3)‡
	CbCh + C_6	364 ± 61 (3)‡
	CbCh + Atp	112 ± 11 (3)†

* Secretory studies were done as described earlier, but fresh uncultured glands were used. The concentration of nicotine used was 2×10^{-4} M and all other drugs were 2×10^{-3} M. Values are expressed as mean ± S.E. and the number in each group appears in parentheses. The numbers listed under the age of the adrenal glands are the basal rates of secretion.
† NS.
‡ $P < 0{\cdot}05$.

sympathetic nerves [25, 26], but there is contradictory evidence [27]. The studies reported in Table 8 were carried out to determine whether cyclic AMP was involved in secretion by chick adrenal glands in organ culture. Aminophylline causes a moderate secretory response, but neither cAMP nor cGMP stimulates catecholamine secretion, nor do they potentiate the response to aminophylline or carbamylcholine. The nature of the response to aminophylline is not known, but aminophylline is known to mobilize intracellular Ca^{2+} [28] which may contribute to the potentiated response. Aminophylline is also known to inhibit the cAMP-degrading enzyme, phosphodiesterase, which should raise the intracellular level of cAMP, but the failure of either cAMP or dibutyryl-cAMP to stimulate secretion makes this mechanism unlikely.

Studies of induction of tyrosine hydroxylase. Previous studies [29] have shown that neurogenic stimulation of the adrenal medulla in rats results in an increase in tyrosine hydroxylase within 12–24 hr after onset of the stimulus. In denervated glands, no such increase occurs. However, repeated injections of acetylcholine do cause an increase in tyrosine hydroxylase activity in the denervated gland. It has also been shown that denervated glands, after depletion of their catecholamines by reserpine treatment, recover their catecholamine content at a much slower rate than

Table 8. Effect of cyclic nucleotides on secretion by cultured adrenal glands*

Treatment	% Control
Aminophyline	154 ± 19 (4)†
Aminophyline + cAMP	150 ± 9 (4)†
Aminophyline + dBcAMP	138 ± 7 (4)
dBcAMP	108 ± 12 (4)
cGMP	87 ± 5 (5)
CbCh	365 ± 55 (4)†
CbCh + aminophylline	647 ± 197 (4)†
CbCh + dBcAMP	379 ± 67 (4)†
CbCh + cAMP	415 ± 60 (4)†
CbCh + aminophyline + dBcAMP	777 ± 89 (4)†

* These studies were done with 24-hr cultures as described earlier. Cyclic-GMP was used in a concentration of 2×10^{-4} M and the other drugs were 2×10^{-3} M. dBcAMP, dibutyryl-cyclic AMP; cAMP, cyclic AMP; cGMP, cyclic GMP; CbCh, carbamyl choline.
† Statistically significant, $P < 0{\cdot}05$.

Table 9. Effect of stimulation on catecholamine content and tyrosine hydroxylase activity of cultured adrenal glands*

Period after stimulation (hr)	Catecholamines (μg/gland)		
	Left	Right	Right/Left × 100
24	4·2 ± 0·2 (4)	2·3 ± 0·1 (4)	55
48	6·8 ± 0·5 (4)	3·2 ± 0·3 (4)	48
96	7·9 ± 0·4 (3)	3·5 ± 0·2 (3)	48
120	7·8 ± 0·3 (3)	3·1 ± 0·1 (3)	40
	Tyrosine hydroxylase (nmoles/hr/gland)		
	Left	Right	Right/left
24	6·4 ± 0·6 (4)	6·0 ± 0·3 (4)	0·94

* Left and right glands from 1-day chicks were cultured individually in modified media MEM. At 24 hr, the right gland was transferred to media containing acetylcholine (2×10^{-4} M) and physostigmine (2×10^{-4} M). Incubation was continued for another 24 hr, at which time a group of gland pairs was assayed for catecholamines; the media in the others were replaced with fresh drug-free media and incubation was continued. At the intervals stipulated above, a group of gland pairs was removed and assayed for catecholamines and tyrosine hydroxylase. Figures are the average ± S.E.M.

do intact glands. The rate of recovery in the denervated glands can be stimulated by acetylcholine injections to the rate of recovery of the intact control gland. Therefore, studies were carried out to determine whether stimulation of adrenal glands in organ culture could cause an increase in their tyrosine hydroxylase content and whether the glands could recover their catecholamine content.

In the studies reported in Table 9, the catecholamine content and tyrosine hydroxylase activity were measured for various periods of time after treatment with acetylcholine as described in the legend. After 24 hr of exposure of the right gland to carbamylcholine, the catecholamine content had dropped to 50 per cent of the control left gland, but during the subsequent 4 days there was no recovery of catecholamine stores. After 24 hr of stimulatory treatment, there was no difference in tyrosine hydroxylase activity between the treated right gland and the control left.

Variations in preincubation time and time of exposure to secretogogue were used in an attempt to provoke stimulation of tyrosine hydroxylase activity. Glands were preincubated for 24–48 hr prior to exposure to carbamylcholine and were assayed for tyrosine hydroxylase activity after exposure to the drug for 24–48 hr. These variations in treatment had no effect on tyrosine hydroxylase activity (Table 10).

Other factors such as NGF [30], dibutyryl-cyclic AMP [31, 32] and ACTH [33, 34], which have been demonstrated to stimulate tyrosine hydroxylase activity in other tissues, had no stimulatory effect on tyrosine hydroxylase in cultured chick adrenal medullas. The possibility of inhibitors of tyrosine hydroxylase being produced during culture was ruled out by the additivity of enzyme activity in mixing experiments.

DISCUSSION

The studies presented herein describe the development of a system of tissue culture which provides for the functional survival of the chick adrenal medulla. Functional viability is defined as the ability to secrete, synthesize and store catecholamines, which is indicated by the responsiveness of the glands to secretogogues and their maintenance of tyrosine hydroxylase activity and catecholamine content. The usefulness of the system as a model for studying induction of tyrosine hydroxylase was tested.

To determine the level of enrichment required to maintain the adrenal medulla in a functional state,

Table 10. Effect of stimulation on tyrosine hydroxylase activity of cultured adrenal glands*

Preincubation time (hr)	Duration of carbamylcholine treatment (hr)	Tyrosine hydroxylase activity (nmoles/hr/gland)†		Right/left
		Left	Right	
24	24	5·5 ± 1·5 (5)	3·6 ± 0·4 (5)	0·65
48	24	6·2 ± 0·7 (7)	5·5 ± 0·7 (7)	0·89
48	48	6·1 ± 1·0 (7)	5·9 ± 0·5 (7)	0·97

* Left and right adrenal glands from 1-day-old chicks were cultured individually in modified media MEM. After 24 or 48 hr of preincubation, carbamylcholine (2×10^{-4} M) was added to the media of the right gland and incubation was continued for 24 and 48 hr. Tyrosine hydroxylase activity of the right gland was compared to that of the control left gland (right/left).

† Values are means ± S.E.M.

a survey of various media was made. The studies show that medium MEM, modified by the addition of insulin, fetal calf serum and glucose, was better than the more enriched media tested. During the first 24 hr of culture, the glands lost approximately 50 per cent of their catecholamine content and tyrosine hydroxylase activity and about 25 per cent of their dopamine-β-hydroxylase activity. This seems to be a respectable level of survival compared to cultured neuroblastoma cells [32] which lose 75 per cent of their tyrosine hydroxylase activity after 3 days of incubation.

As a further test of survival, it was shown that the glands maintained their secretory response to carbamylcholine. The secretory response of the glands was tested with secretogogues of both specific and mixed receptor activity. The effects of receptor specific antagonists on these responses were also determined. With the use of these probes, the nature of the receptors has been defined. It has been shown that in all experiments the excised chick glands, either fresh or cultured, responded to carbamylcholine and this response was blocked by atropine (muscarinic antagonist) and not by C_6 (nicotinic antagonist). The presence of muscarinic receptors is further indicated by the stimulatory response to urocholine (predominantly muscarinic activity), which is blocked by atropine and not by C_6. The absence of nicotinic receptors was shown by the lack of response to nicotine and by the fact that nicotine neither potentiated nor inhibited the response to carbamylcholine. This finding is surprising because in other species [18–23] the receptors of the adrenal medulla are mixed but predominantly nicotinic, which is consistent with their morphogenetic relationship to sympathetic ganglia.

Experiments were carried out to determine whether the absence of nicotinic receptors was due to their loss during organ culture, or whether nicotinic receptors developed only after birth. The possible lability of nicotinic receptors to culture conditions was ruled out because of the similarity in the pattern of response to the agonist and antagonist of both fresh and cultured glands. Also the similarity in response of 1- and 14-day-old chick glands showed no development of nicotinic receptors. The possibility has not, however, been ruled out that nicotinic receptors are labile to the gland being excised, but this seems unlikely.

Studies of secretion by other tissues have implicated cAMP in its familiar role as second messenger in the stimulus–secretion coupling. Up until now, the cat is the only species in which cAMP has been demonstrated to evoke catecholamine secretion by the adrenal medulla [24]. In the vas deferens–hypogastric nerve preparation, dibutyryl-cAMP or aminophylline did not by itself cause secretion of noradrenaline, but either in the presence or absence of Ca^{2+}, it potentiated the response to nerve stimulation [35]. For this reason, the effects of cAMP, dibutyryl-cAMP and aminophylline on catecholamine secretion in cultured chick glands were examined. The results show that cAMP, dibutyryl-cAMP or cyclic GMP had no effect on secretion either by themselves or in combination with other drugs. However, aminophylline had a small stimulatory effect by itself and potentiated the response to carbamylcholine. Because dibutyryl-cAMP had no effect on secretion, the role of aminophylline in raising the intracellular level of cAMP is unlikely. It is suggested that aminophylline may function to mobilize the intracellular pool of Ca^{2+} due to its reported effect on Ca^{2+} binding by microsomes [28].

Examinations of the synthesis of catecholamines from tyrosine in intact cultured glands showed a loss of catecholamine-synthesizing capacity which paralled the loss of tyrosine hydroxylase activity measured *in vitro*. After 72 hr in culture, there was approximately a 50 per cent decrease in the overall formation of noradrenaline and a 60–65 per cent decrease in the formation of dopamine. The activity of tyrosine hydroxylase in the intact gland was only about 2–3 per cent of the activity measured *in vitro*, but since different substrate concentrations of tyrosine were used (2 μM in experiment with intact glands vs 20 μM in measurement *in vitro*), one cannot make any clear statement regarding the regulation of the enzyme in the intact gland, but it would appear that only a relatively small fraction of the enzymatic capability is utilized. In contrast to losses in the capacity for catecholamine synthesis, there was an increase in the ability of the cultured glands to incorporate [^{3}H]-leucine into protein.

Failure to recover the catecholamine stores was not due to failure either of catecholamine synthesis or protein synthesis. The data in Table 3 show that even with low substrate concentrations of tyrosine (2 μM) the glands were capable of synthesizing 1 μg norepinephrine in approximately 30 hr. At this rate, if all the norepinephrine were retained, recovery should have been complete in about 4 days or, at the very least, significant increases above the 24-hr level in catecholamine control should have occurred. Additionally, it should be pointed out that the recovery studies were carried out in media containing tyrosine at a concentration of 200 μM.

Failure to recover the catecholamine stores may have been due to a number of factors. After treatment with carbamylcholine, the basal level of secretion upon removal of the drug may have increased to the extent that increased levels of synthesis may have been required to maintain a new steady state. The data in Table 5 show that unstimulated glands which have been in culture for 24 hr and have reached a steady state level of catecholamines have a basal release rate of 40–60 ng/hr/gland. If this basal rate were increased after prolonged exposure to carbamylcholine, the gland might not have been able to synthesize catecholamines rapidly enough to replete their released stores. A second factor may have been the failure to increase the levels of tyrosine hydroxylase activity. Other studies [36, 37] have shown that denervated rat adrenal glands which have been depleted of their catecholamine stores by reserpine treatment and which do not increase their tyrosine hydroxylase activity recover their catecholamine stores at a lower rate than do intact adrenals similarly depleted and which increase their tyrosine hydroxylase activities. A third factor which may be responsible for failure to recover the catecholamine stores is that the glands in culture are unable to synthesize new storage vesicles.

Conditions have been developed here for the maintenance of the adrenal medulla, enabling it to survive

a 50 per cent loss in tyrosine hydroxylase activity and catecholamine content and still respond to physiological secretogogues. The gland was, however, unable to recover the catecholamines lost during stimulation and was also unable to induce the synthesis of tyrosine hydroxylase. Some reasons for the lack of recovery may be use of an incorrect method of applying the stimulus, lack of the proper receptor, failure to form a specific inducer, or absence of a nutritional factor in the medium. Numerous examples exist where a specific vitamin, hormone or other supplement is required to maintain normal function of an organ in culture.

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