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Neurotransmitter Synthesis by Neuroblastoma Clones

(neuroblast differentiation/cell culture/choline acetyltransferase/acetylcholinesterase/tyrosine hydroxylase/axons–dendrites)

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ABSTRACT **Neuroblastoma clones were examined for choline acetyltransferase (EC 2.3.1.6), tyrosine hydroxylase (EC 1.14.3.a), acetylcholinesterase (EC 3.1.1.7), and also for neurite formation. One clone does not form axons or dendrites. Three types of clones were found with respect to neurotransmitter synthesis: cholinergic, adrenergic, and clones that do not synthesize acetylcholine or catechols. All clones contain acetylcholinesterase. These results show that genes determining neurotransmitter species can be expressed in dividing cells, that the parental programs of gene expression are inherited, and that dividing cells can be programmed with respect to their ability to communicate with other cells.**

Elegant biological studies have yielded much information pertaining to the problem of how neural circuits form as the nervous system is assembled. However, virtually nothing is known about the molecular mechanisms for synapse formation. The problem ultimately must be defined in terms of the genetic program for generating different cell types and the steps that determine the specificity of neurons in forming functional synapses.

The neuroblastoma system established by Augusti-Tocco and Sato (1) provides an unusual opportunity to explore steps in neuron differentiation and function. The cells multiply rapidly *in vitro*, yet exhibit many properties characteristic of differentiated neurons (2–7). In this report, the properties of additional clones derived from the mouse neuroblastoma are described. Three cell types, cholinergic cells, adrenergic cells, and cells that do not synthesize acetylcholine or catecholamines, were detected.

METHODS AND MATERIALS

Cells. Mouse neuroblastoma C-1300 cells were grown as described (7). Some clones were obtained in two stages: first, a well-isolated colony of cells in agar was picked and then cloned by isolation of a single cell with a stainless-steel cylinder. In other cases, cells were added to petri dishes containing broken coverslips; each glass shard with a single cell was then transferred to a separate dish.

Chromosomes were analyzed by incubation of cells in logarithmic growth for 6–12 hr with 15–300 μM colcemide (*N*-desacetyl-*N*-methyl-colchicine) obtained from Ciba; chromosomes were spread by the method of Merchant, Kahn, and Murphy (8).

Choline Acetyltransferase (EC 2.3.1.6) Assay. Cell monolayers were washed 3 times with an isotonic salt solution; then cells and protein were harvested by scraping and washing with 10 mM potassium phosphate buffer (pH 6.8)–1 mM EDTA (potassium salt). The recovered suspension was sonicated for 5 min at 3°C, divided into small portions, and stored in a vapor-phase liquid-nitrogen freezer.

Choline acetyltransferase activity was assayed by a method modified (manuscript in preparation) from that of Schrier and Shuster (9). Each reaction contained the following components in a final volume of 0.05 ml, except where noted: 50 mM potassium phosphate buffer (pH 6.8), 200 mM NaCl, 1 mM EDTA (potassium salt), 2.5 mM choline iodide, 0.5% Triton X-100 (Packard), 2.2 mM [^{14}C]acetyl CoA (10 Ci/mol), 0.1 mM neostigmine methylsulfate, and 0–0.5 mg of homogenate protein. Each reaction was incubated at 37°C for 10 min; then 0.5 ml of H_2O at 3°C was added and the diluted reaction and 2 subsequent 1.0-ml washes were passed through a 0.5 × 5 cm column of Bio-Rad AG 1-X8 resin (Cl^- form, 100–200 mesh). Each eluate was collected in a scintillation vial; 10 ml of scintillation solution [1000 g Triton X-100–2 liters of toluene–165 ml Liquifluor (New England Nuclear Co.)] was added and radioactivity was determined. The counting efficiency for ^{14}C was 80–90%.

Duplicate or triplicate homogenates were prepared and each was assayed for choline acetyltransferase activity at 4 concentrations of protein. The rate of reaction was proportional to enzyme concentration within the range 5–350 pmol of [^{14}C]acetylcholine formed per 10 min. Assay reproducibility with replicate homogenates was ±15%. Each value reported is the average of values obtained with 2–3 homogenates.

^{14}C-Labeled reaction products in column eluates were characterized by paper chromatography or electrophoresis. Reactions were modified so that the specific activity of the [^{14}C]acetyl CoA was 40–50 Ci/mol, and choline chloride rather than choline iodide was used. Solutions containing ^{14}C-labeled products (25–30 μl of a column eluate); 0.2 μmol of unlabeled acetylcholine, and 0.2 μmol of unlabeled acetylcarnitine were subjected to ascending paper chromatography for 16–24 hr with 1-propanol–0.1 N acetic acid 3:1. Chromatograms were dried and sprayed with the Dragendorf reagent (18) to visualize acetylcholine or acetylcarnitine. The chromatogram was cut into 1.0 × 0.5 cm segments, and the radioactivity of each was determined with a scintillation counter.

Acetylcholinesterase (EC 3.1.1.7) Assay. The enzyme was assayed as described by Blume *et al.* (7).

Tyrosine Hydroxylase (EC 1.14.3.a) Assay. Cell monolayers were washed, harvested, and sonicated as described above,

except that cells and protein were harvested in 0.1 M potassium phosphate buffer, pH 6.2.

Tyrosine hydroxylase activity was assayed by a modification of the methods described by Nagatsu, Levitt, and Udenfriend (10), and by Shiman, Akino, and Kaufman (11).

Each reaction contained the following components in a final volume of 0.05 ml: 0.1 M potassium phosphate buffer (pH 6.2), 0.5 mM L-[3,5-di ^{3}H]tyrosine (12 Ci/mol, from Amersham-Searle), 0.3 mM 6,7-dimethyl-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine (Calbiochem), 0.25 mM NADPH (sodium salt), about 27 μg of sheep-liver dihydropteridine reductase protein [purified through the second ammonium sulfate-precipitation step of Kaufman (12)], and 0–0.5 mg of homogenate protein. Reactions were incubated at 34°C for 10 min, and were stopped by the addition of 0.5 ml of 0.17 N acetic acid at 3°C and assayed as described by Nagatsu *et al.* (10).

93% of the $^3H^+$ released from L-[3,5-^{3}H]tyrosine was recovered in the column eluate; appropriate corrections were applied to reported values. An internal standard of ^{3}H OH then was added to each sample and radioactivity again was determined. The counting efficiency for ^{3}H was about 30%.

The rate of reaction was proportional to the concentration of homogenate protein for values reported. Duplicate homogenates were prepared and each was assayed for tyrosine hydroxylase at four protein concentrations; reproducibility was ±25%. Average values are reported. Protein was assayed by a modification of the method of Lowry (13).

Characterization of the ^{3}H-labeled product of the Tyrosine Hydroxylase Reaction. The tyrosine hydroxylase reaction contained 0.1 mM *p*-bromo-*m*-hydroxybenzyloxyamine, an inhibitor of aromatic L-amino acid decarboxylase, in addition to the components described above. ^{3}H-Labeled products formed during incubation were adsorbed to alumina and separated from [^{3}H]tyrosine as described by Nagatsu *et al.* (14), except that ^{3}H-labeled products were eluted with 0.2 N HCl. Recovery of 3,4-dihydroxyphenylalanine was 78%. An appropriate correction was applied to values reported. The [^{3}H]catecholamines then were characterized by paper and thin-layer chromatography with the following solvents: 1-butanol-glacial acetic acid-H_2O 12:3:5; methylethylketone-formic acid-H_2O 24:1:6; ethylacetate-glacial acetic acid-H_2O 15:15:10; and 1-butanol-1 N acetic acid-ethanol 35:10:10. Thin-layer chromatography was performed with Eastman Chromogram Sheet 6065 (20 × 20 cm). Spots were located after development by spraying with ethylenediamine ferricyanide solution (15) to locate catechols, or with ninhydrin to locate tyrosine.

RESULTS

Cell types

The specific activities of tyrosine hydroxylase, choline acetyltransferase, and acetylcholinesterase found with homogenates of the neuroblastoma tumor grown *in vivo* and different clonal cell lines derived from this tumor are shown in Table 1. Values obtained with mouse L-cells, a fibroblastic cell line, and mouse brain are also given for comparative purposes. The specific activity of choline acetyltransferase from tumor was about 2% that of mouse brain. Acetylcholine synthesis was detected with most cell extracts, including L-cells, and other established cell lines not shown here; however, the rate of acetylcholine synthesis was <2% that of mouse brain (1–10 pmol of acetylcholine formed per min per mg of protein). The neuroblastoma-tumor and mouse-brain specific activities of tyrosine hydroxylase were <3% that of adrenal medulla.

Of the 21 clones derived from neuroblastoma C-1300 that were assayed for tyrosine hydroxylase and choline acetyltransferase, 12 clones were inactive with respect to both enzymes; 6 clones were cholinergic with high choline acetyltransferase activity; and 1 clone was adrenergic, with tyrosine hydroxylase specific activity about 200-fold higher than that of brain. Two cell lines were found with low activities of *both* tyrosine hydroxylase and choline acetyltransferase, but further evidence is needed to distinguish between a cholinergic-adrenergic cell type and a mixture of adrenergic cells and cholinergic cells. No cells were found with *high* activities of both tyrosine hydroxylase and choline acetyltransferase. These results show that three, possibly four, classes of neuroblastoma cells with respect to neurohormone synthesis can be derived from neuroblastoma C-1300.

The specific activity of acetylcholinesterase was high with all neuroblastoma clones tested. In addition, electrically excitable cells were found with each cholinergic, adrenergic, and inactive neuroblastoma clone tested.

Homogenates were prepared from stationary-phase cells rather than from logarithmically growing cells because neuroblastoma C-1300 tyrosine hydroxylase, choline acetyltransferase and acetylcholinesterase activities respond to regulatory mechanisms, and are 30-, 4-, and 25-fold higher, respectively, in nondividing than in logarithmically multiplying cells (7, 16, and unpublished data).

Genetic heterogeneity was examined by determination of the modal number of chromosomes per cell. The mouse neuroblastoma arose spontaneously in 1940; a modal value of 66–70 chromosomes was found by Levan in 1956 (17). Modal values of 59 and 118 were found with cholinergic clones, 104 and 192 with adrenergic clones, and 100–120 with inactive clones. It is clear that neuroblastoma cells contain more chromosomes than does a diploid mouse cell, and that clones differ from one another in their chromosome content.

Neuroblastoma clones without tyrosine hydroxylase or choline acetyltransferase may synthesize other transmitter-like compounds. Histamine was detected in uncloned neuroblastoma cells that had been subcultured frequently at a concentration of 17 pmol/mg protein (unpublished data), a value similar to that of rat brain. However, glutamic acid decarboxylase was not detected with neuroblastoma extracts (unpublished data).

Subclones

A cholinergic neuroblastoma clone and two inactive clones were recloned, and the properties of the sublines then were studied (Table 2). 23 of the 26 subclones derived from cholinergic clone NS-20 contained active choline acetyltransferase, and closely resembled the parent-cell type; however, 3 clones were found with relatively low activities of choline acetyltransferase.

Three sublines derived from inactive clone N-4, and 5 sublines derived from inactive clone N-18, were similar to the parent cells. However, 2 subclones of N-18 contained higher activities of choline acetyltransferase than the parent clone. These results show that most, but not all, subclones resemble the parental type.

Clone N-1, with both tyrosine hydroxylase and choline

TABLE 1. *Types of neuroblastoma C-1300 clones*

Source of homogenate	Tyrosine hydroxylase	Choline acetyl-transferase	Acetyl-cholin-esterase	Modal number of chromosomes
	pmol product formed per min per mg of protein			
Mouse L cells, clone A9		2	440	
Mouse brain	5	550	69,000	
Neuroblastoma tumor, *in vivo*	0	10*	130,000	66–70†
Cholinergic clones				
NS-20Y		920	47,000	
NS-20	0	490	48,000	59
NS-26	1	437	47,000	116
NS-25	1	125	55,000	59
NS-18	1	76	80,000	59
NS-21	0	52	36,000	61
NS-16	0	43	67,000	60
Adrenergic clones				
N1-106	60	5*	179,000	104
N1E-115	980	0.1	256,000	192
Inactive clones				
N-1A-103	0	2	19,000	101
N-3	9	4	174,000	
N-4	4	0.1	46,000	105
N-7	6	0	99,000	
N-8	2	2	42,000	
N-9	11	1	53,000	
N-10	1	3	55,000	
N-11	0	5*	404,000	
N-12	0	4	346,000	
N-13	0	6	130,000	
N-18	2	2	105,000	110
13 other clones		5*		
Uncertain (clonal homogeneity not established)				
N-1	70	22	23,000	106
N-5	25	13	151,000	109

* Estimate based on determination of ^{14}C-labeled products in column eluates.
† Data of Levan, cited by Hauschka (17).

acetyltransferase, was recloned, and the enzyme activities of the sublines were determined (Table 3). Unlike the other neuroblastoma clones, N-1 cells do not extend axons or dendrites. The cell population was homogeneous initially, consisting of cells that were attached well to the surface of a petri dish an

TABLE 2. *Subclones of cholinergic and inactive clones*

Cell line	Choline acetyl-transferase	Tyrosine hydroxylase
	pmol of product formed per min per mg of protein	
Cholinergic clone NS-20	100–750*	
23 Subclones	100–930*	
3 Subclones	25–55*	
Inactive clone N-4	0.1	5
3 Subclones	1	0
Inactive clone N-18	2	2
5 Subclones	5	2
2 Subclones	16	1

* Estimate based on determination of ^{14}C- labeled products in column eluates.

usually formed short spikes (<30 μm), but were entirely devoid of neurites 100–2000 μm in length. However, after the 10–20th subculture, some relatively large cells were observed with long, branched neurites.

N-1 then was recloned and 19 subclones and 10 colonies were examined. Two N-1 subclones were found that were without neurites, tyrosine hydroxylase, or choline acetyltransferase; however, acetylcholinesterase was present and cells with electrically active membranes were found when one clone was examined. Fifteen adrenergic clones with neurites were found. Each clone studied contained tyrosine hydroxylase and acetylcholinesterase activities, but cells were almost devoid of choline acetyltransferase activity. One clone (N1-106) with neurites contained 104 chromosomes per cell; other cell lines contained about 200 chromosomes per cell. Several nonadrenergic clones with neurites were found; clone N1E-113 contained 205 chromosomes per cell. These results show that the N-1 cell population is heterogeneous and contains adrenergic and nonadrenergic cells. No cholinergic or adrenergic-cholinergic cell type was found.

Clonal morphology

Four types of neuroblastoma clones, incubated in the absence of serum to stimulate neurite extension, are shown in Fig. 1:

TABLE 3. *Axon-minus and adrenergic subclones of neuroblastoma clone N-1*

Subclones	Tyrosine hydroxylase	Choline acetyl-transferase	Acetyl-cholin-esterase	Modal number of chromosomes
	pmol of product formed per min per mg of protein			
Parent clone N-1†	70	22	23,000	106
Axon-dendrite minus subclones				
N1A-103†	0	2	19,000	101
N1A-104†	1	5		
Adrenergic subclones				
N1-106	60	5*	179,000	104
N1E-115	980	0.1	256,000	192
N1E-124	350	0.9	175,000	207
N1E-125	330	1.3	98,000	
N1E-116	225	0.5	109,000	202
N1E-122	215	0.4	40,000	
N1E-110	150			
N1E-126	122	0.3	73,000	202
N1E-114	93	0.1	74,000	
N1E-112	63			
N1E-128	60			
N1E-127	60			
N1E-123	40			
N1E-111	32			
N1E-118	17			
Minus tyrosine hydroxylase subclones				
N1E-113	6			205
N1E-117	1			

* Estimate based on determination of ^{14}C-labeled products in column eluates.
† All subclones were positive for neurites, except those marked †.

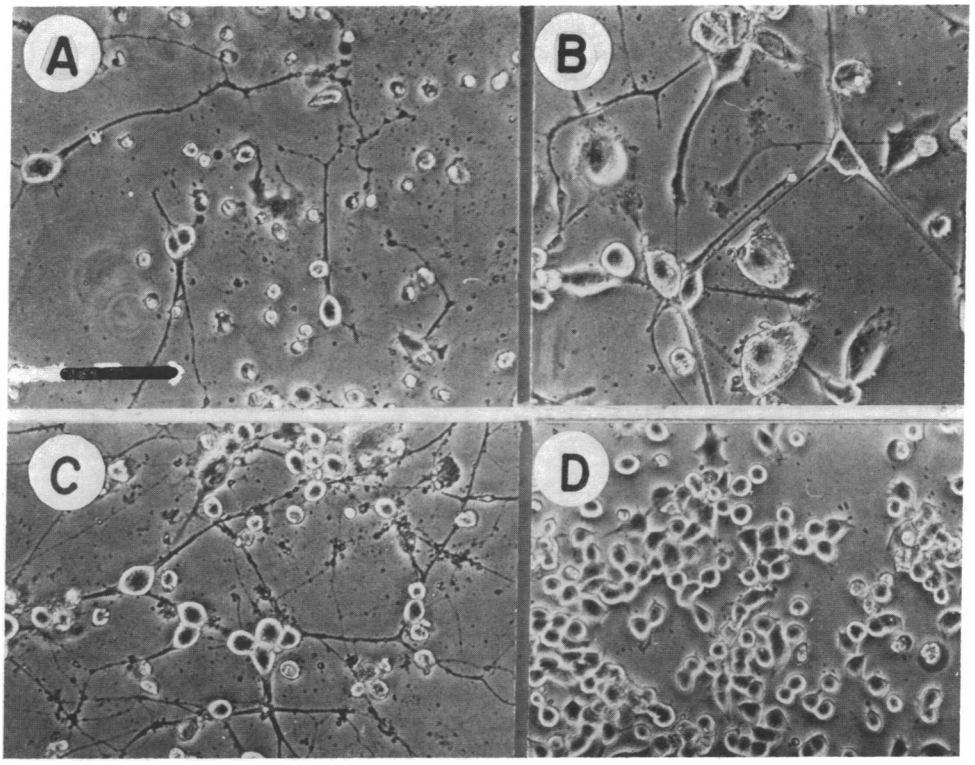

FIG. 1. Axon–dendrite formation by (*A*) cholinergic clone NS–20; (*B*) adrenergic clone N1E; (*C*) inactive clone N–18; and (*D*) inactive clone N1A–103, which does not form axons or dendrites.

Cells were incubated in growth medium without serum for five days to stimulate neurite formation. The scale shown in *A* applies to all panels, and corresponds to 10 μm.

TABLE 4. *Neuroblastoma C-1300 clones*

Cell type	Acetylcholinesterase and excitable membranes	Neurites	Choline acetyltransferase	Tyrosine hydroxylase	Modal number of chromosomes
Axon-minus	+	−	−	−	101
Inactive	+	+	−	−	110
Cholinergic	+	+	+	−	59, 116
Adrenergic	+	+	−	+	104, 200

cholinergic, adrenergic, inactive with neurites, and the clone lacking neurites. Cells from each clone adhered well to the surface of the petri dish, but only the axon-minus line (N1A-103) was devoid of long neurites. Axon-minus cells and N-18 cells that form long neurites were mixed and cultivated in the same flasks for more than a week; however, no influence of one cell type upon the other was detected.

Product identification

Neuroblastoma N-1E tyrosine hydroxylase activity was dependent upon a pteridine cofactor (6,7-dimethyl-2-amino-4-hydroxy-5,6,7,8-tetrahydropteridine). Tritium release from L-[3,5-^{3}H]tyrosine agreed well (within 5%) with [^{3}H]dihydroxyphenylalanine formation. The labeled product of the tyrosine hydroxylase reaction formed in the presence of an inhibitor of aromatic amino-acid decarboxylase was characterized by paper and thin-layer chromatography with four solvents. Between 86 and 99% of the applied tritiated product was identical in chromatographic mobility with authentic dihydroxyphenylalanine. Clone N-1 cells, in the absence of an inhibitor of aromatic amino-acid decarboxylase, synthesize 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylethylamine, norepinephrine, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxyphenylethylglycol, 3-methoxytyrosine, 3-methoxytyramine, and 3-methoxy-4-hydroxyphenylacetic acid (unpublished data). Catechols formed by clone C-1300 have been characterized also by Schubert, Humphreys, Baroni, and Cohn (2), and by Anagnoste, Goldstein, and Broome (4).

The ^{14}C-labeled products of the choline acetyltransferase reaction were eluted from the column and routinely characterized by paper chromatography. 84–99% of the labeled product formed with homogenates of cholinergic cells was identical in chromatographic mobility to authentic acetylcholine. However, only 2–25% of the labeled material formed with adrenergic or inactive homogenates was acetylcholine. The major contaminant was identified as [^{14}C]acetyl carnitine (unpublished data).

DISCUSSION

Clones of neuroblastoma C-1300 were examined for two enzymes required for neurotransmitter synthesis, choline acetyltransferase and tyrosine hydroxylase, catalyzing acetylcholine formation and the first step in norepinephrine synthesis, respectively. A summary of data is shown in Table 4. Three types of clones were found: (*a*) clones that form little or no acetylcholine or catechols; (*b*) cholinergic clones; and (*c*) adrenergic clones. One additional clone was found with relatively low cholinergic and adrenergic activities, but we do not know whether this is a fourth cell type or a mixture of adrenergic and cholinergic cells. However, no cells were found that *actively* synthesize both acetylcholine and catechols.

Tumors of cholinergic neurons have not been reported before. They probably afflict man, but may not have been recognized for lack of a diagnostic test. Since few mammalian cells other than neurons have high activities of choline acetyltransferase, the enzyme and acetylcholine can be used as specific diagnostic markers for cholinergic neuroblastomas. Since acetylcholinesterase was found with all neuroblastoma cell types, the enzyme apparently is not a specific marker of cholinergic neurons.

At least six types of neurons or corresponding tumors that arise from the neural crest can be distinguished on the basis of transmitter synthesis: those synthesizing acetylcholine; 3,4-dihydroxyphenylalanine, 3,4-dihydroxyphenylethylamine, norepinephrine, epinephrine, and sensory neurons that do not synthesize these compounds. The three types of neuroblastoma thus exhibit properties expected of neural-crest neurons. Although neuroblastoma stem cells capable of giving rise to cholinergic, adrenergic, and inactive cells were not found, it seems likely that such cells normally exist.

The available information suggests that there are relatively few kinds of universal neurotransmitters in the nervous system of both vertebrates and invertebrates. Most neurons probably are greatly restricted with respect to the number of kinds of neurotransmitters that can be synthesized. We find cholinergic, adrenergic, and inactive neuroblastoma cell types, but have not detected cells capable of synthesizing *both* acetylcholine and catechols at rapid rates. It seems likely that the expression of a gene required for the synthesis of one neurotransmitter may restrict the expression of genes for alternate neurotransmitters. For example, a product derived from the choline acetyltransferase gene directly or indirectly might restrict the expression of genes for tyrosine hydroxylase, and vice versa. Simultaneous expression of acetyltransferase and tyrosine hydroxylase genes might inactivate both genes, or might result in a balanced state of mutual inhibition; i.e., a cell with low cholinergic and adrenergic activities. Whether a product of one neuron affects the expression of genes for transmitter synthesis of neighboring neurons is a problem for future study.

Analysis of sublines derived from clonal cells demonstrates that most, but not all, resemble the parent type. The mechanisms underlying altered gene expression are not known. Gene expression may be reversible or mutations may affect structural or regulatory genes. In any event, the loss of a step that commits a cell to one developmental pattern may enable the cell or its descendants to differentiate along an alternate pathway.

Thousands of cell generations have elapsed since tumor C-1300 originated, and extensive genetic heterogeneity in the

cell population is expected. By a relatively simple experimental approach, it may be possible to obtain lines that express genes that are characteristic of many kinds of neurons from a single neural tumor cell; i.e., with two sequential selective procedures: first, selection for dedifferentiated cells might yield cell populations enriched in stem cells; then selection for differentiated cells might yield cells that follow alternate programs of differentiation.

We conclude that genes determining the species of neurotransmitter synthesized can be expressed in dividing cells, and that the parental programs of gene expression are inherited and perpetuated for hundreds of cell generations. Similarly, normal neuroblasts may be destined with respect to transmitter synthesis and may generate different types of clonal cell populations that are programmed with regard to their ability to establish functional communication with other cell types before synapses are formed.

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1. Augusti-Tocco, G. & Sato, G. (1969) *Proc. Nat. Acad. Sci. USA*, **64**, 311–315.
2. Schubert, D., Humphreys, S., Baroni, C. & Cohn, M. (1969) *Proc. Nat. Acad. Sci. USA*, **64**, 316–323.
3. Olmsted, J. B., Carlson, K., Klebe, R., Ruddle, F. & Rosenbaum, J. (1970) *Proc. Nat. Acad. Sci. USA*, **65**, 129–136.
4. Anagnoste, B. F., Goldstein, M. & Broome, J. (1970) *Pharmacologist*, **12**, 269.
5. Nelson, P., Ruffner, W. & Nirenberg, M. (1969) *Proc. Nat. Acad. Sci. USA*, **64**, 1004–1010.
6. Seeds, N. W., Gilman, A. G., Amano, T. & Nirenberg, M. W. (1970) *Proc. Nat. Acad. Sci. USA*, **66**, 160–167.
7. Blume, A., Gilbert, F., Wilson, S., Farber, J., Rosenberg, R. & Nirenberg, M. (1970) *Proc. Nat. Acad. Sci. USA*, **67**, 786–792.
8. Merchant, D. J., Kahn, R. H. & Murphy, W. H., (1960) *Handbook of Cell and Organ Culture* (Burgess Publishing Co., Minneapolis, Minn.), pp. 198–200.
9. Schrier, B. K. and Shuster, L. (1967) *J. Neurochem.* **14**, 977–985.
10. Nagatsu, T., Levitt, M. & Udenfriend, S. (1964) *Anal. Biochem.* **9**, 122–126.
11. Shiman, R., Akino, M. & Kaufman, S. (1971) *J. Biol. Chem.*, **246**, 1330–1340.
12. Kaufman, S. (1962) in *Methods in Enzymology, Vol. V*, ed. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York) p. 802.
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, **193**, 265–275.
14. Nagatsu, T., Levitt, M. & Udenfriend, S. (1964) *J. Biol. Chem.*, **239**, 2910–2917.
15. Schneider, F. H. & Gillis, C. N. (1965) *Biochem. Pharm.* **14**, 623–626.
16. Amano, T., Richelson, E. & Nirenberg, M. (1971) *Fed. Proc.* **30**, 1085.
17. Hauschka, T. S., Kvedar, B. J., Grinnell, S. T. & Amos, D. B. (1956) *Ann. N.Y. Acad. Sci.* **63**, 683–705.
18. Bregoff, H. M., Roberts, E. & Delwiche, C. C. (1953) *J. Biol. Chem.* **205**, 565–574.