PRIMARY CULTURES OF DISSOCIATED SYMPATHETIC NEURONS

III. Changes in Metabolism with Age in Culture

RICHARD E. MAINS and PAUL H. PATTERSON

From the Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115. Dr. Mains' present address is the Department of Biology, University of Oregon, Eugene, Oregon 97403.

ABSTRACT

Several biochemical parameters of dissociated sympathetic neurons from superior cervical ganglia of the newborn rat were monitored as a function of age in culture. The neurons, which were grown in the virtual absence of non-neural cells, displayed a striking increase in their ability to synthesize and accumulate catecholamines. This capacity increased 50-fold during a 3-wk period in vitro, after which it appeared to reach a steady level. The major change took place during the second week. The time course of this change was not affected by plating the neurons at a higher cell density. The change in the catecholamine metabolism was far greater in magnitude and quite different in time course from the overall growth of the cells which was monitored by the incorporation of radioactive tyrosine into protein, lipid synthesis from radioactive choline, and incorporation of radioactive uridine into acid-precipitable material. Of the total tyrosine used by the cultures, the proportion devoted to catecholamine synthesis increased to 25% (a 10-fold rise) during the 3-wk period. This changing pattern of metabolism in the cultures suggested a process of maturation which may be similar to neuronal development in vivo.

INTRODUCTION

In the two preceding papers we described methods for making reproducible cultures of sympathetic neurons and for studying aspects of their neurotransmitter metabolism (Mains and Patterson, 1973 a,b). One aim of that work was to establish a system for studying neuronal development and interaction in vitro. In the experiments described here we have used this system to examine the change in the ability of the neurons to synthesize and accumulate radioactive catecholamines as they grew during a period of several weeks in culture. In order to assess the selectivity of this change in neurotransmitter metabolism, it was then compared to the alterations in protein, lipid, and RNA metabolism of the cells during the same period in culture.

MATERIALS AND METHODS

All experiments were made on low density cultures of sympathetic neurons isolated from the superior cervical ganglia of newborn rats. Except where noted, the preparation and maintenance of the cultures, the radioactive compounds used, and the methods of incubation were the same as those described in the two preceding papers (Mains and Patterson, 1973 a,b).
**Growth in Radioactive Choline**

The Leibovitz's medium (L-15-air growth medium) lacked unlabeled choline and was supplemented with 0.8 µCi/ml [methyl-\(^{14}\)C]choline (41 mCi/mmol; New England Nuclear, Boston, Mass.). The concentration of choline was thus about one-third of that in L-15-air, but nearly three times that in commercial L-15. To determine radioactive lipids the cultures were washed for 10–15 min in plating medium and scraped off the dish into 6 ml of chloroform:methanol (2:1). The organic phase was washed with saline (Patterson and Lennarz, 1971), taken to dryness under a stream of air, and counted in 10 ml of Aquasol (New England Nuclear).

**Growth in Radioactive Uridine**

L-15-air growth medium in this set of experiments included 0.1 µCi/ml [2-\(^{14}\)C]uridine (53 mCi/mmol; New England Nuclear). To determine acid-precipitable radioactivity the cultures were washed for 10–15 min in plating medium and scraped off the dish into 4 ml of 10% trichloroacetic acid. After adding 0.05 ml of 60 mg/ml bovine serum albumin, the sample was centrifuged 5 min at 1000 g. The pellet was resuspended in another 4 ml of trichloroacetic acid and centrifuged again. The pellet was taken up in 1 ml of 0.4 M sodium borate, 1% sodium dodecyl sulfate, pH 9.0, and counted in 10 ml Aquasol.

**RESULTS**

**Changes in Catecholamine Metabolism**

As previously described (Mains and Patterson, 1973 a) our standard assay for the ability of the neurons to synthesize and accumulate catecholamines involved an 8-h incubation in radioactive tyrosine followed by electrophoretic and, occasionally, chromatographic separation of labeled products. To examine systematically how this synthetic capacity varies with time, the assay was made with cultures of many different ages. In all cases the cultures were initiated from ganglia of newborn to 1-day old rats. The results were found to be consistent for any particular age among different cell platings (see also Mains and Patterson, 1973 b) so that the values for all experiments at a given age (i.e., within 1 day) were pooled. The results for 167 cultures, showing radioactive catecholamine production in an 8-h period as a function of culture age, are given in Fig. 1. It can be seen that during the first 3 wk in culture the ability of the neurons to synthesize and accumulate radioactive catecholamines from labeled tyrosine increased about 50-fold. This capacity rose exponentially during the second week in vitro and leveled off thereafter. The "over 20" point includes cultures as old as 45 days.

It was clearly of interest to ask whether the 50-fold exponential increase in catecholamine production was simply a reflection of a large exponential phase of growth during the second week in culture or did it represent a specific differentiation? To examine this question we monitored three parameters of growth in the cultures as a function of age: protein (tyrosine), lipid (choline), and RNA (uridine) metabolism.

**Changes in Tyrosine Metabolism**

Tyrosine is taken up by the neuronal cultures and incorporated into protein, catecholamines, and other metabolites. It was previously found that the free tyrosine pool is quite small relative to the total uptake and that the vast majority of the tyrosine is incorporated into protein (Mains and Patterson, 1973 b). Thus, most of the radioactivity accumulated by the neurons during an 8-h incubation is found in protein, and may be taken as an approximation of the rate of protein synthesis. Data for the total radioactivity were obtained during the experiments shown in Fig. 1. The relationship between the 8-h rate of accumulation of radioactivity and the age of the cultures is shown in Fig. 2. It can be seen that the accumulation rate rose gradually with age and at 3-wk was about five times that of the initial 1–2 day figure. Note the
FIGURE 9. Cultures of various ages were incubated for 8 h with [3H]tyrosine and the total radioactivity accumulated was determined.

absence of a lag during the first week seen for catecholamines. Thus the magnitude and time course of changes in tyrosine uptake and accumulation were quite different from the increase in catecholamine production with age. It should be emphasized that non-neural cells do not make a significant contribution to the metabolism of the L-15-air cultures utilized in these experiments (Mains and Patterson, 1973a).

Changes in Lipid Metabolism

Another parameter of growth which was investigated was the incorporation of radioactive choline into lipid. The procedure was to grow the cells in the presence of labeled choline (from the time of plating) and periodically remove some of the cultures and extract the lipids. Although this experimental procedure is different from the one used for examination of tyrosine incorporation into protein and catecholamines, the accumulation of radioactive lipid should provide another parameter of cellular growth (this also applies to the uridine experiments presented below). Some of these cultures were also tested for their ability to produce catecholamines from [3H]tyrosine during the usual 8-h incubation. The data from an experiment of this type are shown in Fig. 3. The dashed line is the result for catecholamine production from Fig. 1 and the open circles show the catecholamine production by the neurons of two ages grown in the presence of radioactive uridine. The cells were unaffected by the radioactive uridine as judged by catecholamine production. From day 6 to 16, radioactive catecholamine synthesis and accumulation in an 8-h period increased 22-fold, while in the same period uridine incorporation into acid-precipitable material doubled. Thus the changes described for catecholamine production are not matched in size or time by the increases in protein, lipid, or RNA accumulation.
Relationship of Tyrosine Metabolism and Catecholamine Metabolism

As previously pointed out, in the initial 3-wk period catecholamine synthesis and accumulation increased about 50-fold, while in the same period the total incorporation of radioactive tyrosine into all cellular products increased fivefold. Thus the proportion of tyrosine taken up by the cells which is converted to catecholamine rises as the neurons grow in culture. This rise is illustrated in Fig. 5. The data indicate that up to 8 days in culture, 2-3% of the tyrosine taken up by the neurons was used for production of radioactive catecholamines, while in cultures older than 2.5 wk 25 ± 4% of the radioactive tyrosine was converted into labeled catecholamines. These figures indicate the striking degree to which the neuronal tyrosine metabolism centers around neurotransmitter synthesis.

Cell Density and Catecholamine Metabolism

A factor which might influence catecholamine production in culture is the density at which the neurons are plated. In an earlier series of experiments a strong density dependence was, in fact, found: the lower the density, the smaller the net synthesis of catecholamine per plated cell (unpublished observations). In these earlier observations the highest cell density was below the standard density used in the experiments reported in this and the preceding two papers (Mains and Patterson, 1973 a,b).

Of particular interest in the present experiments was the question whether the catecholamine changes could be accelerated by plating more neurons per dish. Possibly the dramatic increase in catecholamine synthesis during the second week in culture was due to an increase in the mass of cellular material or to the number of cell contacts. An experiment to address this question involved plating the neurons at about three times their usual cell density and measuring the catecholamine production at various ages. The higher cell density was achieved by dissociating the neurons from the usual number of rats and then plating them in one-third the usual number of dishes. The rates of catecholamine production as a function of the ages of the cultures are shown in Fig. 6. The dashed line is the data from cells at the usual density (Fig. 1) multiplied by 2.85 (the factor which gives the best fit to the data). The fact that the factor is so close to the approximate threefold increase in cell density indicates that catecholamine production on a per cell basis was not affected by increased cell density. With the correction for the increase in cell density...
were plated into one-third the usual number of dishes giving an approximately threefold higher cell density. Catecholamine production during 8-h incubations was then determined for cultures of various ages (closed circles). The dashed line is the data from Fig. 1 (obtained at the usual cell density) multiplied by 4.85 (see text) and replotted for comparison.

number, the data from the higher cell density experiment fit the standard curve quite well. The initial lag period of about 1 wk was unchanged. Above a certain cell density then, the maturation of catecholamine metabolism was not dependent on nerve cell density.

**DISCUSSION**

The cultures of dissociated sympathetic neurons underwent a 50-fold increase in their rate of synthesis and accumulation of catecholamines during several weeks in culture, the major changes occurring between days 6 and 18. During that period the rate of chase of labeled catecholamines also increased threefold (Mains and Patterson, 1973 b). Thus, the change with age shown in Fig. 1 is a real increase in the rate of synthesis and accumulation, rather than a decrease in the rate of breakdown or loss. There are many possible mechanisms which could account for the observed changes with age, including higher levels of tyrosine hydroxylase, improved reuptake of transmitter, increased storage capacity in vesicles, etc. It should be noted, however, that the changes in catecholamine metabolism are not due to changes in the rate at which the intracellular tyrosine pool becomes labeled, since previous data established that the tyrosine pool labels to a steady-state value rapidly in both young and old cultures (Mains and Patterson, 1973 b).

Since the incorporation of tyrosine into protein, the production of lipid from choline, and the synthesis of acid-precipitable material from uridine all changed more gradually with age in the cultures (Figs. 2, 3, and 4), the changes in catecholamine production were not simply a function of growth or enlargement of the neurons. It appears instead that the neurotransmitter metabolism of the cells underwent a significant differential change during the second week in culture. The fact that tyrosine, choline, and uridine incorporation all increased linearly from the beginning or middle of the first week, suggests that if the lag in catecholamine production during the first week were due simply to a process of recovery from the isolation procedure, then catecholamine metabolism is more sensitive than the other parameters.

The increased emphasis on catecholamine production in older cultures, in which 25% of the total radioactivity accumulated during 8 h is present as dopamine or norepinephrine, shows a similarity in time-course to the change in vivo in the levels of the synthetic enzyme activities, which increase 4–8-fold in the second and third weeks after birth in the rat superior cervical ganglion (Thoenen et al., 1972 a). Other aspects of the morphological and biochemical maturation of rat sympathetic neurons take about 3 wk, with major changes occurring in the second and third postnatal weeks (Eranko, 1972 a,b). The nerve endings in peripheral targets develop on the same time scale as the cell bodies in sympathetic ganglia (e.g. Machado et al., 1971; de Champlain et al., 1970; Iversen et al., 1967) which makes comparisons with the cultures (which contain both cell bodies and processes) easier. However, much more remains to be done before rigorous quantitative comparisons can be made between the development seen in vitro and in vivo. Specifically, it will be necessary to measure in the cultures parameters which have been investigated in vivo, such as the catecholamine enzyme activities and total catecholamine levels.

The data show that, above a certain cell density, increases in the number of neurons per dish did not affect the time course of the changes in catecholamine production. It seems probable that plating the cells at a higher density shortened the time period before the neurons came into contact as well...
as increasing the number of interneuronal contacts. Thus it is possible that the sympathetic neurons are not influencing each other's development of catecholamine production at the normal plating density used in these cultures. Also relevant in this context is the observation that the presence of the non-neural cells of the ganglion did not affect catecholamine production by the neurons (Mains and Patterson, 1973 a). Other influences which are likely to affect the development of catecholamine production are the preganglionic (spinal cord) neurons (Black et al., 1972), cells from sympathetic target tissues (iris, heart, etc). and the level of nerve growth factor (Thoenen et al., 1972 b). Experiments are currently in progress on the effects of these influences on the timing and magnitude of the changes in catecholamine metabolism reported here.

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REFERENCES

Black, I. B., I. A. Hendry, and L. L. Iversen. 1972. Effects of surgical decentralization and nerve growth factor on the maturation of adrenergic neurons in a mouse sympathetic ganglion. J. Neurochem. 19:367.

deChamplain, J., T. Malmfors, L. Olson, and C. Sachs. 1970. Ontogenesis of peripheral adrenergic neurons in the rat: Pre- and postnatal observations. Acta Physiol. Scand. 80:276.

Eranko, L. 1972 a. Ultrastructure of the developing sympathetic nerve cell and the storage of catecholamines. Brain Res. 46:159.

Eranko, L. 1972 b. Postnatal development of histochemically demonstrable catecholamines in the superior cervical ganglion of the rat. Histochem. J. 4:223.

Iversen, L. L., J. deChamplain, J. Glowinski, and J. Axelrod. 1967. Uptake, storage, and metabolism of norepinephrine in tissues of the developing rat. J. Pharmacol. Exp. Ther. 157:509.

Mains, R. E., and P. H. Patterson. 1973 a. Primary cultures of dissociated sympathetic neurons. I. Establishment of long term growth in culture and studies of differentiated properties. J. Cell Biol. 59:329.

Mains, R. E., and P. H. Patterson. 1973 b. Primary cultures of dissociated sympathetic neurons. II. Initial studies on catecholamine metabolism. J. Cell Biol. 59:346.

Machado, A. B. M. 1971. Electron microscopy of developing sympathetic fibers in the rat pineal body: the formation of granular vesicles. Prog. Brain Res. 34:171.

Patterson, P. H., and W. J. Lennarz. 1971. Studies on the membranes of bacilli, I. Phospholipid biosynthesis. J. Biol. Chem. 246:1062.

Thoenen, H., R. Kettler, and A. Saner. 1972 a. Time course of the development of enzymes involved in the synthesis of norepinephrine in the superior cervical ganglion of the rat from birth to adult life. Brain Res. 40:459.

Thoenen, H., A. Saner, R. Kettler, and P. U. Angeletti. 1972 b. Nerve growth factor and preganglionic cholinergic nerves; their relative importance to the development of the terminal adrenergic neuron. Brain Res. 44:593.