Changes in the Nuclear Polyamine Content of Chick Erythrocytes during Embryonic Development

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The polyamine content of the circulating erythrocyte population in the embryonic chick was studied during its development. Total cellular polyamine content fell dramatically between 5 and 7 days of development, paralleling the decrease in metabolic activity exhibited by these cells. Nuclei were isolated from the erythrocytes by a non-aqueous technique, which not only eliminated the polyamine loss that occurred with aqueous isolation, but also prevented redistribution of the polyamines from the cytoplasm. Nuclear spermidine and spermine contents decreased markedly between 5 and 6 days of development from 31 to 10 pmol/ μ g of DNA and from 33 to 18 pmol/ μ g of DNA respectively. Thereafter the spermine content remained constant, but the spermidine content continued to decline. Good correlations between spermidine and RNA contents were observed in both cells and nuclei, and similarly between spermine and RNA contents in cells, but no such correlation was observed between spermine and RNA in nuclei.

There is considerable evidence that polyamines are involved in the regulation of cell growth (for reviews see Bachrach, 1973; Cohen, 1971), and they are recognized as growth factors for bacteria (Herbst & Snell, 1948; Martin *et al.*, 1952) and mammalian cells in culture (Ham, 1964; Pohjanpelto & Raina, 1972). Changes in the rate of cell proliferation are always paralleled by changes in polyamine content, as has been observed, for example, in regenerating rat kidney (Russell & Lombardini, 1971), developing embryonic rat brain (Pearce & Schanberg, 1969) and early sea-urchin development (Kusunoki & Yasumasu, 1976). These changes have been further analysed by comparing increases in cell number with polyamine biosynthesis and content in rat brain tumour cells (Heby *et al.*, 1975).

The circulating erythrocyte population of the chick embryo undergoes marked changes during development. Up to day 5 the cells are immature and are mainly polychromatic erythrocytes. At later stages of development the population of erythrocytes is increasingly composed of different forms of inactive mature erythrocyte (Lucas & Jamroy, 1961). Therefore it is of interest to study the polyamine content of these cells during development. The distribution of polyamines between the nucleus and cytoplasm is of particular interest because of the possibility of the interaction of these molecules with both RNA and DNA (Bachrach, 1973; Cohen, 1971).

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Polyamines will readily redistribute during aqueous cell fractionation, as has been observed with isolated ribosomes (Tabor & Kellogg, 1967), tRNA (Cohen, 1971) and on bacterial cell walls and protoplasts (Bachrach & Cohen, 1961). In the present paper the polyamine content of erythrocyte nuclei has been measured after non-aqueous isolation of the nuclei, a technique that effectively prevents redistribution.

Materials and Methods

Isolation of erythrocytes

Blood from 5–18-day embryonic chicks was collected from White Leghorn hen's eggs. The cells were washed twice in ice-cold phosphate-buffered saline (Dulbecco & Vogt, 1954) before being taken up into heparinized haematocrit tubes and centrifuged at 50g for 5 min to separate debris and leucocytes from the erythrocytes. Cell number was estimated by means of a Coulter counter (Coulter Electronics Inc.).

Biochemical assays

A suspension of erythrocytes in 1M-NaCl was acidified with concentrated HClO_4 to produce a 0.2M- HClO_4 solution. Alternatively, the glycerinated nuclear pellet was taken up in 0.2M- HClO_4 . The cell or nuclear suspension was sonicated with an MSE 100W ultrasonic disintegrator for one 10s burst, cooled in ice for 30 min and centrifuged at 1000g for 10 min. The resultant supernatant was used for the polyamine assay and the pellet for the macromolecular assays. The polyamines were quantified by the

dansylation method (Seiler & Wiechmann, 1967). DNA was assayed by the diphenylamine method (Burton, 1956) with calf thymus DNA as standard, and RNA was assayed by the orcinol method (Ceriotti, 1955) with yeast RNA as standard.

Nuclear isolation

Non-aqueous method (Kirsch *et al.*, 1970). At least 10^8 cells were taken up in 0.5 ml of phosphate-buffered saline and placed in a 30 mm-diam. plastic Petri dish. The dish was floated on liquid N_2 in an atmosphere of Freon-12 (ISC Chemicals) to freeze the cells. These were then freeze-dried under vacuum of 1.3 mPa at -40°C for 3 days. The dried cells were taken up into 10 ml of dried glycerol, cooled in ice for 10 min and homogenized in a Polytron sonicator/homogenizer (Kinematica G.m.b.h., Lucerne, Switzerland). It was usually necessary to follow an initial 45 s burst at speed setting 5 by three or four 15 s bursts (with 5–10 min cooling on ice between bursts). This procedure routinely liberated more than 95% of the nuclei, as estimated by phase-contrast microscope examination. The glycerol homogenate was layered on to a 3-chloropropane-1,2-diol (Koch-Light)/glycerol mixture (3:17, v/v) and centrifuged at 170 000g in a Beckman SW50L rotor for 45 min. The supernatant was discarded, leaving a pellet consisting of packed erythrocyte nuclei.

Aqueous method A (Schildkraut & Maio, 1968). Erythrocytes were taken up in 6 ml of solution I (1.5 mM- CaCl_2 /10 mM-Tris/HCl, pH 7.5) and homogenized in a close-fitting Dounce homogenizer: 100 strokes of the pestle were used, the mixture being allowed to cool in ice after every tenth stroke. The homogenate was layered on top of solution II (1.5 mM- CaCl_2 /10 mM-Tris/HCl/2.2 M-sucrose, pH 7.5) and centrifuged at 55 000g in a Beckman SW25 rotor for 45 min. The supernatant was discarded, leaving a pellet of nuclei, which by scanning electron microscopy was found to be pure.

Aqueous method B (Elgin & Bonner, 1970; Purkayastha & Neelin, 1966). Erythrocytes were taken

up in approx. 20 vol. of 0.1 M-sodium phosphate buffer, pH 7.0. A solution of saponin (Fisons) was added to produce a final concentration of 0.15% (w/v), and the suspension was stirred at 4°C for 20 min. The resultant mixture was carefully layered over solution III (3 mM- CaCl_2 /10 mM-Tris/HCl/0.3 M-sucrose, pH 7.0) and centrifuged at 1000g for 15 min. The pellet was resuspended in solution III, layered over solution IV (3 mM- CaCl_2 /10 mM-Tris/HCl/2.2 M-sucrose, pH 7.0) and centrifuged at 55 000g in a Beckman SW25 rotor for 45 min. The supernatant was discarded, leaving the nuclear pellet, which by scanning electron microscopy was found to be pure.

Aqueous method C (Berkowitz *et al.*, 1969). Erythrocytes were washed in 50 ml of solution V (0.2 mM- MgCl_2 /1 mM- K_2HPO_4 /0.32 M-sucrose, pH 6.8) and centrifuged at 1000g for 6 min. The pellet was then resuspended in 50 ml of solution VI (10 mM-NaCl/1 mM- K_2HPO_4 , pH 6.8) for 15 min at 0°C before the cells were centrifuged at 800g for 10 min. The pellet was resuspended in 25 ml of solution VII (0.3% Triton N-101 in 0.1 mM- MgCl_2 /0.1 mM- K_2HPO_4 /0.32 M-sucrose, pH 6.3) and the cells were homogenized at 0°C by ten strokes in a loose-fitting Dounce homogenizer and then five passes in a tight-fitting homogenizer. The homogenate was centrifuged at 800g for 10 min. The pellet was again taken up in solution VII, homogenized and recentrifuged. The supernatant was discarded, leaving the nuclear pellet, which was found to be pure by scanning electron microscopy.

Chemicals

All chemicals were obtained from either Sigma or BDH Chemicals, unless otherwise stated. The radiolabelled precursors were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Results

Comparison of nuclear isolation methods

Nuclei were isolated from erythrocytes of 6-day chick embryos by various methods. As viewed by scanning electron microscopy the nuclei isolated

Table 1. Comparison of polyamine and DNA contents of erythrocyte nuclei isolated by different methods. Erythrocytes were obtained from 6-day chick embryos and their nuclei isolated by the techniques detailed in the Materials and Methods section. The polyamine and DNA contents of the nuclei were assayed as described in the Materials and Methods section. Each value represents the mean $\pm$ S.E.M. of three to eight determinations.

Nuclear isolation technique	Polyamine content (pmol/ μg of DNA)			DNA content (pg/nucleus)
	Spermine	Spermidine	Putrescine	
Non-aqueous method	18.0 ± 1.6	10.4 ± 0.6	10.7 ± 0.6	2.3 ± 0.6
Aqueous method A	12.8 ± 1.9	2.1 ± 0.1	2.9 ± 0.1	1.9 ± 0.8
Aqueous method B	7.9 ± 0.8	1.0 ± 0.1	1.4 ± 0.1	1.8 ± 0.6
Aqueous method C	10.4 ± 1.5	4.6 ± 0.6	1.1 ± 0.1	2.4 ± 0.6

non-aqueously were intact and, with the exception of a few small strands of cytoplasm, devoid of contamination. The nuclei derived by aqueous method A were not as clean, and pieces of cytoplasm were observed attached to the nuclei. Nuclei obtained by aqueous method B were very contaminated with cytoplasm, but nuclei isolated by aqueous method C were clean and less contaminated with cytoplasmic strands than nuclei produced by the other two aqueous methods.

Nuclei isolated non-aqueously and by aqueous method A were reddish-brown, but nuclei isolated by the other aqueous methods were white. The difference in nuclear colour is probably accounted for by the loss of haemoglobin from those nuclei isolated by procedures involving the use of detergent. It has been established that 10% of cellular haemoglobin is found in the erythrocyte nucleus (Small & Davis, 1970), and its loss during isolation indicates considerable disruption of nuclear content.

The polyamines from the nuclei of 6-day embryonic chick erythrocytes were assayed after isolation by the various techniques described. The results are summarized in Table 1 and clearly demonstrate that the non-aqueously isolated nuclei contain significantly more of all polyamines than do the nuclei isolated by any of the aqueous methods. Furthermore, although

all polyamines are depleted in aqueous nuclear preparations, spermine is consistently the least affected. The spermine content in these nuclei varies between 40 and 65% of the value for non-aqueous preparations, whereas spermidine (20–50%) and putrescine (20–30%) values are significantly lower. This suggests that spermine may be more tightly bound than the other polyamines in the nucleus.

Since the polyamine values are expressed as a function of the nuclear DNA content, it is possible that the higher polyamine values are due to loss of DNA during non-aqueous isolation. However, there does not appear to be any significant loss of DNA from these nuclei as compared with nuclei isolated by the aqueous methods (Table 1). It is also possible that the high content of polyamine found in the non-aqueous nuclear pellet is due to contamination by cytoplasmic polyamine. To assess this possibility, erythrocytes were 'contaminated' with either [^{14}C]spermidine or [^{14}C]spermine before the nuclei were isolated in the usual manner. The amount of radioactive polyamine added to each sample was 22.7 nmol, a value greater than the amount of the same polyamine initially present in the sample. In this situation polyamine redistribution during the isolation procedure would result in radioactive contamination of the nuclear polyamine. The results of this experiment are

Table 2. *Percentage of total nuclear polyamine labelled after addition of [^{14}C]polyamine to the erythrocytes before non-aqueous isolation*

Erythrocytes were taken up in 0.5 ml of phosphate-buffered saline containing either 2.5 μCi of spermidine (122 mCi/mmol) or 2.5 μCi of spermine (115 mCi/mmol), and immediately frozen in Freon-12 over liquid N_2 . Nuclei were isolated by the non-aqueous method, and the amount of polyamine present was measured as described in the Materials and Methods section. The dansylated polyamine spots (which represent a known proportion of the original sample) were cut from the chromatography plates, immersed in 10 ml of scintillant [4 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene] and counted for radioactivity in a Packard Tri-Carb liquid-scintillation counter. N.D., Not determined.

Age of embryo (days)	^[14C] Polyamine added	Polyamine present in spot on chromatography plate (nmol)	^[14C] Polyamine present in spot on chromatography plate	
			(d.p.m.)	(% of total polyamine present)
6	Spermine	Background (0)	22.4	—
		Spermine (1.24)	334.9	0.11
		Spermidine (N.D.)	22.1	—
		Putrescine (N.D.)	22.5	—
14	Spermine	Background (0)	21.6	—
		Spermine (0.48)	87.0	0.07
		Spermidine (N.D.)	21.9	—
		Putrescine (N.D.)	22.1	—
5	Spermidine	Background (0)	22.5	—
		Spermine (N.D.)	22.5	—
		Spermidine (0.63)	631.6	0.39
		Putrescine (N.D.)	22.2	—
14	Spermidine	Background (0)	22.1	—
		Spermine (N.D.)	21.6	—
		Spermidine (0.15)	105.4	0.27
		Putrescine (N.D.)	21.9	—

Fig. 1. Changes in the polyamine content of the circulating erythrocytes during development of the chick embryo. Erythrocytes were isolated from chick embryos from 5 to 18 days of age and the polyamine content was determined by the dansylation method as described in the Materials and Methods section. Each value is the mean $\pm$ S.E.M. for three to ten determinations. (a) Putrescine; (b) spermidine; (c) spermine.

Fig. 2. Changes in the polyamine content of nuclei isolated from erythrocytes at different times during the development of the chick embryo.

Nuclei were isolated from erythrocytes by the non-aqueous method and the polyamine and DNA contents of each sample were assayed as described in the Materials and Methods section. Each value is the mean $\pm$ S.E.M. for three to eight determinations. (a) Spermine; (b) spermidine.

summarized in Table 2. The amount of labelled polyamine in the nuclei was determined and compared with the total polyamine present. Only a very small proportion of the polyamine was found to be labelled, 0.07–0.11% for spermine and 0.27–0.39% for spermidine. It is possible that this radioactivity is located in the residual glycerol of the nuclear pellet.

Polyamine content of erythrocytes

The two main features of the changes in polyamine content are that the content falls considerably between days 5 and 9 and that the amounts of different polyamines fall by different degrees. The decline in putrescine content (Fig. 1a) does not begin until after day 6 and eventually falls to 15 pmol/ 10^6 cells. Spermidine also decreases (Fig. 1b) to 10% of its value on day 5, and, except for the 6-day value, shows a similar time course to putrescine. However, spermine content (Fig. 1c), although showing a marked decrease between days 5 and 9, only falls to 30% of the day-5 value. As a result of these changes the ratio of spermidine to spermine alters significantly as embryonic development proceeds, and falls from 1.20:1 at day 5 to 0.48:1 at day 9.

There is considerable evidence from other systems that indicates that the polyamines, especially spermidine, are associated with RNA. In this system both RNA and polyamine decrease in a similar manner during development. There is a linear relationship between spermidine and RNA but the relationship between the decrease in spermine and RNA is more complex (Fig. 3a).

Polyamine content of erythrocyte nuclei

The changes in nuclear polyamine content during maturation of the erythrocyte nuclei are summarized in Fig. 2. The spermine content (Fig. 2a) falls from 33 pmol/ μ g of DNA in nuclei of the proliferating day-5 cells to 18 pmol/ μ g of DNA in nuclei of day-6 cells, which are mainly quiescent and do not undergo further proliferation. From 6 to 18 days the spermine

Fig. 3. Correlation between polyamine content and RNA content of the erythrocytes and their nuclei

Erythrocytes were isolated from chick embryos from 5 to 18 days of age and the polyamine and RNA contents of the cells determined as described in the Materials and Methods section. Nuclei were isolated from erythrocytes by the non-aqueous method and their polyamine and RNA contents likewise determined. ○, Spermidine; ●, spermine. (a) Erythrocytes; (b) nuclei.

content remains relatively constant. The spermidine content (Fig. 2b) likewise falls between days 5 and 6, from 31 to 10 pmol/μg of DNA. Thereafter it continues to decline, falling to less than 5 pmol/μg of DNA by day 18. When the nuclear values are expressed as a function of total cell polyamine the trends are quite different. Nuclear spermidine remains fairly constant at 40% of total cell spermidine throughout maturation and irrespective of the activity of the cell. However, spermine increases markedly from 40 to 80% of total cell spermine, paralleling the increased inactivity of the erythrocyte population.

There is a good correlation between nuclear RNA and spermidine content. However, spermine content

shows little relationship to the amount of RNA present (Fig. 3b).

Discussion

Many studies have been made of polyamine content in systems that undergo changes in the rate of cell proliferation. From these investigations two general points emerge about polyamine content. In any tissue that undergoes a marked change in its growth rate, changes in the polyamine concentrations also occur, so that the polyamine content is higher in rapidly growing cells than in slowly growing or non-proliferating cells. Secondly, there is a significant change in the relative proportions of the polyamines, which gives rise to a high spermidine/spermine ratio in rapidly growing systems (due mainly to an increase in spermidine content) and a low ratio in slowly growing ones. The data presented in this paper likewise demonstrate that polyamine content of erythrocytes falls as they become mature non-proliferating cells (Fig. 1). Furthermore, the proliferating cells of the 5-day embryo exhibit the highest spermidine/spermine ratio, but thereafter, as more inactive cells enter the circulation, this ratio falls considerably.

The roles that polyamines play in the cell are not fully understood at present, but we now have fairly good indications as to their most probable sites of action. Polyamines have a high affinity for nucleic acids (Cohen, 1971; Bachrach, 1973) and in many situations polyamine content parallels RNA content. This is true in the embryonic chick erythrocytes (Fig. 3), which exhibit very high correlation coefficients between spermidine and RNA and between spermine and RNA.

Only a few studies have been made of nuclear polyamine content. An indirect study was carried out by comparing the polyamine content of the blood from species that differ in having nucleated and enucleated erythrocytes (Shimizu *et al.*, 1965). It was found that the nucleated erythrocytes have a much higher spermine content, suggesting that spermine is selectively concentrated in the nucleus. Nuclei have been isolated from calf thymus and rat liver cells (Stevens, 1966) by using the non-aqueous Behrens (1932) technique. The results obtained by this method indicate that the nuclear and cytoplasmic concentrations of spermidine and spermine do not differ significantly. It is not known, however, whether redistribution of polyamine occurred during the isolation of the nuclei by this procedure. However, studies of karyoplasts derived by cytochalasin-B enucleation of mouse L cells have indicated that both spermidine and spermine are concentrated in the nucleus (McCormick, 1977). Finally, the distribution of polyamine was examined in salivary-gland cells of *Drosophila* larvae by using a low-temperature radioautographic method. The results from this

experiment suggest that spermidine is preferentially associated with nuclei in cells at the distal end of the gland, but not elsewhere (Dion & Herbst, 1967).

The data in the present paper demonstrate that nuclear spermine, unlike spermidine, increases relative to the cytoplasmic content as the erythrocyte population becomes more inactive (Fig. 2). The simplest interpretation of the data is that spermidine is evenly distributed throughout the cell, irrespective of nuclear activity, and its distribution parallels that of RNA (Fig. 3). There is a large literature supporting the view that spermidine is bound to ribosomes and possibly tRNA in the cell (Cohen, 1971; Bachrach, 1973). In contrast, spermine is conserved in the inactive nucleus as the rest of the cell becomes depleted. Spermine showed little correlation with RNA content in the nucleus, which suggests that it has a role other than stabilizing RNA. It is known that, at physiological concentrations, spermine will condense chromatin (Anderson & Norris, 1960; Busch *et al.*, 1967; Rao & Johnson, 1971) and will prevent chromosomes from decondensing (Davidson & Anderson, 1960), and this suggests that the spermine that is selectively conserved in the inactive erythrocyte nucleus may be involved in the condensation of chromatin which occurs during erythrocyte maturation. The situation in the mature erythrocyte nucleus is complicated, as the non-proliferating state is the result of terminal differentiation. This involves, among other things, loss of protein and histone F1 from the nucleus and appearance of histone F2c (Murray *et al.*, 1968). It is therefore possible that the conservation of spermine is more related to this differentiation state than to a general function of condensing chromatin which is common to other cells. However, the finding that a non-aqueously isolated HeLa-cell chromosome preparation also contains substantial quantities of spermine (Goyns, 1979) supports the concept of spermine enrichment in condensed chromatin.

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