Polyamines and Nucleic Acid Metabolism in Chick Embryo

INCORPORATION OF LABELLED PRECURSORS INTO NUCLEIC ACIDS OF SUBCELLULAR FRACTIONS AND POLYRIBOSOMAL PATTERNS

By G. MORUZZI, B. BARBIROLI AND C. M. CALDARERA

Istituto di Chimica Biologica dell'Università di Bologna, Bologna, Italy

(Received 31 October 1967)

1. An increase in polyamine concentration, caused by inhibiting the amine oxidase activities with iproniazid, increased the incorporation of [^3H]orotic acid into chick-embryo RNA and DNA. On the other hand, a decrease in polyamine concentration, obtained by causing an increase in amine oxidase activities, decreased [^3H]orotic acid incorporation into nucleic acids. This was particularly evident for nuclear DNA and ribosomal RNA. 2. Polyribosomal patterns obtained by sucrose-density-gradient centrifugation showed highest radioactivity in the regions of 259s and 280s aggregates in those embryos in which the polyamine contents were enhanced, whereas a decrease in the radioactivity was observed when the polyamine concentrations were decreased. 3. The activity of DNA-dependent RNA polymerase, assayed in the same experimental conditions, also varied in the same fashion with changes in polyamine concentration.

Spermine and spermidine have been found in large amounts in organs and tissues such as pancreas (Rosenthal & Tabor, 1956), thymus, spleen and liver (Jänne, Raina & Siimes, 1964). Polyamines are also present in several micro-organisms (Herbst & Snell, 1949). Although the biological role of these amines is still unknown, these compounds are essential growth factors for some micro-organisms (Herbst & Snell, 1948; Sneath, 1955), and putrescine, spermine and spermidine are growth factors for a mammalian cell line (Ham, 1964).

In addition, polyamines are present in appreciable quantities in the embryos of several cereals (Moruzzi & Caldarera, 1964), and spermine is a growth-promoting factor for explants of Jerusalem artichoke *in vitro* (Bertossi, Bagni, Moruzzi & Caldarera, 1965).

Caldarera, Barbiroli & Moruzzi (1965) showed, during chick-embryo development, similar changes in content of polyamines and nucleic acids under conditions in which the polyamine content was experimentally modified.

These observations, the relationship between spermine, thymidine and folinic acid metabolism (Turner, Lansford, Ravel & Shive, 1963), the increase of spermidine in regenerating liver (Dykstra & Herbst, 1965) and the effect of polyamines on RNA synthesis in a polyauxotrophic strain of *Escherichia coli* (Raina & Cohen, 1966), imply a relationship between polyamines and nucleic acids.

The present paper describes the possible relation-

ship between polyamines and nucleic acid metabolism. We have studied the incorporation of [^3H]orotic acid into the nucleic acids of chick-embryo subcellular fractions, the polyribosomal profiles and DNA-dependent RNA polymerase activity in conditions in which polyamine contents were experimentally modified.

MATERIALS AND METHODS

Embryos and injection technique. Embryos were obtained from White Leghorn $\times$ New Hampshire fertilized eggs obtained from a commercial source and incubated at 39° in a relative humidity of 65% with forced air circulation.

On the tenth day of incubation, the egg shell was swabbed with 70% (v/v) ethanol. Spermine and spermidine in sterile solutions were injected into the yolk sac in a volume of 300–400 μl .; the punctures were then sealed with a mixture of paraffin wax and beeswax. The injected embryos were not turned thereafter.

[^3H]Orotic acid was injected in sterile solution into the air space and the embryos were used after 6 hr. Sodium [^3H]formate was injected in the same way and embryos were used 2 hr. later for sucrose-density-gradient analysis.

Iproniazid. Iproniazid (16mm) was injected in sterile solution (0.5 ml.) into the yolk sac of embryos as an amine oxidase inhibitor.

Materials. Spermine and spermidine were obtained from Fluka A.-G., Buchs SG, Switzerland; iproniazid was from F. Hoffmann–La Roche and Co. Ltd., Basle, Switzerland; RNA and DNA were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; nylon bolting cloth (110 mesh) was from John Stainer Ltd., Manchester; Dowex 1 resin (X8; 200–400 mesh) was from Pierrel

Associates, Milan, Italy; 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were from Nuclear-Chicago Corp., Des Plaines, Ill., U.S.A. [^3H]Orotic acid (5c/m-mole), sodium [^3H]formate (227 mc/m-mole) and [^{14}C]ATP (27.1 mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks.

Preparation of subcellular fractions. All procedures were performed at 0–4°. Embryos were freed from the extra-embryonic membranes and yolk sac, and immediately homogenized with 4 vol. of ice-cold 0.25 M-sucrose containing MgCl_2 (3 mM) in a glass Potter homogenizer fitted with a Teflon pestle (A. H. Thomas Co., Philadelphia 5, Pa., U.S.A.); homogenization was complete after 25–30 slow up-and-down movements, with a pestle speed of 1100 rev./min. The homogenates were filtered through two layers of nylon bolting cloth to remove clumps and connective tissue attached to unbroken cells. Nuclei were sedimented by centrifuging the filtered homogenates at 800g for 10 min. This pellet was washed twice by resuspending it in several volumes of the homogenizing medium and resedimenting as described above. The supernatant fluid was centrifuged at 8000g for 15 min. to sediment mitochondria. The mitochondrial pellet was discarded. The post-mitochondrial supernatant fluid was carefully removed and centrifuged at 105 000g for 60 min. in the Spinco model L centrifuge to sediment microsomes. Ice-cold 70% (w/v) HClO_4 was added dropwise to the post-microsomal supernatant, to a final concentration of 10% (w/v), to obtain the acid-soluble free nucleotides.

Incorporation of [^3H]orotic acid into nucleic acids of subcellular fractions. [^3H]Orotic acid (100 μC) in sterile solution was injected into the air space of each of the embryos, which were used 6 hr. after the injection. Each subcellular fraction (nuclei, microsomes and soluble supernatant), obtained as described above, was resuspended in water. Proteins and nucleic acids were precipitated with 2 vol. of ice-cold 10% (w/v) HClO_4 , centrifuged down and washed with 1 vol. of 5% HClO_4 , and then lipids were extracted with successive washings with ethanol, acetone and ethanol-diethyl ether (3:1, v/v). The precipitate was suspended in 0.3 N-KOH and incubated for 60 min. at 37° (Fleck & Munro, 1962). DNA and proteins were precipitated by the addition of 5% (w/v) HClO_4 , after neutralization with 6 N-HCl, and removed by centrifugation from the supernatant RNA digest. DNA was separated from proteins by extraction with 5% HClO_4 for 15 min. at 90° with occasional stirring. RNA and DNA digests were neutralized with cold 5 N-KOH and kept in the refrigerator overnight, and the KClO_4 was centrifuged down. The E_{260} value was measured.

^3H -Labelled acid-soluble nucleotides. The post-microsomal supernatant, treated with HClO_4 as described above, was kept at 4° for 30 min. and then centrifuged at 1400g for 10 min. The acid supernatant was decanted, and the residue was resuspended in a volume of ice-cold 5% (w/v) HClO_4 equal to that of the original suspension and centrifuged as before. The extracts and the washings were combined and neutralized with ice-cold 5 N-KOH and kept in the refrigerator overnight; the KClO_4 was removed by centrifugation. The extracts containing acid-soluble nucleotides were put on an ion-exchange resin column (12 cm. $\times$ 1.2 cm. diam.) of Dowex 1 (X8; formate form; 200–400 mesh) (Hurlbert, Schmitz, Brumm & Potter, 1954). This was followed by 50 ml. of water, a volume that would

displace any non-exchangeable material. Elution of total free nucleotides with 10 N-formic acid was then carried out till no extinction at 260 m μ was found in the eluted fluid (about 50 ml.).

Distribution of polyribosomes. [^3H]Formate (50 μC) was injected into the air space and the embryos were used 2 hr. later.

Ribosomes were obtained from the mitochondria-free supernatant treated with sodium deoxycholate (final concn. 1.3%, w/v) from a whole-embryo homogenate to which bentonite had been added (0.1 mg./g. of fresh tissue) (Petermann, 1964), and which had then been centrifuged at 105 000g for 60 min. Ribosomes suspended in a medium containing KCl (50 mM), MgCl_2 (1 mM) and tris-HCl buffer, pH 7.6 (20 mM) (Tata & Widnell, 1966) (about 25 000 E_{260} units), were layered over 26 ml. of a linear 15–30% (w/v) sucrose density gradient. After centrifugation at 24 000 rev./min. in a Spinco SW 25.1 rotor for 180 min., 0.5 ml. fractions were collected by puncturing the bottom of the tube and their E_{260} values were measured. Radioactivity was determined as described below. Sedimentation coefficients were calculated as described by Martin & Ames (1961) and Wettstein, Staehelin & Noll (1963).

Measurement of radioactivity. The radioactivity of ^3H -labelled RNA, DNA and acid-soluble free nucleotides of subcellular fractions (containing about 1.5–2 mg. of nucleic acids) and of sucrose-density-gradient fractions was measured in a Nuclear-Chicago model 725 automatic scintillation spectrometer. The samples were dried completely in a vacuum oven at 70°, and the dried samples were redissolved at room temperature in 0.5 ml. of water. After 1 hr., 10 ml. of a liquid scintillator (Bray, 1960) was added and the radioactivity was measured. Under these conditions all ^3H -labelled samples were counted with an efficiency of 20%.

Assay of RNA polymerase. The two DNA-dependent RNA polymerase activities were determined in purified chick-embryo nuclei by using the conditions described by Tata & Widnell (1966). Nuclei were purified as described by Widnell & Tata (1964). The incubation medium for the Mg^{2+} -activated reaction, carried out in the absence of $(\text{NH}_4)_2\text{SO}_4$, contained in 0.5 ml.: 50 μmoles of tris-HCl buffer, pH 8.5; 2.5 μmoles of MgCl_2 ; 10 μmoles of cysteine; 3 μmoles of NaF; 0.3 μmole of each of GTP, CTP and UTP; 0.015 μmole of non-radioactive ATP; 0.005 μmole of [^{14}C]ATP; 0.1 ml. of nuclear suspension containing about 0.15 mg. of DNA. Incubation was for 15 min. in air at 37°, after which time the reaction was stopped by the addition of 5 ml. of ice-cold 0.5 N- HClO_4 and 400 μg . of carrier RNA.

The incubation medium for the Mn^{2+} -activated RNA polymerase reaction, carried out in the presence of $(\text{NH}_4)_2\text{SO}_4$, contained in 0.5 ml.: 50 μmoles of tris-HCl buffer, pH 7.5; 2 μmoles of MnCl_2 ; 0.05 ml. of a solution of $(\text{NH}_4)_2\text{SO}_4$, saturated at room temperature and adjusted to pH 7.5 with aq. NH_3 soln.; 0.3 μmole each of GTP, CTP and UTP; 0.015 μmole of non-radioactive ATP; 0.1 ml. of nuclear suspension. After preincubation for 15 min. at 37° the reaction rate was followed by the addition of 0.005 μmole of [^{14}C]ATP, and the reaction was stopped, after a further 45 min. of incubation, by the addition of 5 ml. of ice-cold 0.5 N- HClO_4 and 400 μg . of carrier RNA.

The precipitates were washed twice with 5 ml. of ice-cold 0.5 N- HClO_4 and twice with 5 ml. of ethanol. The residues

Table 1. *Effect of spermine, spermidine and iproniazid on the incorporation of [³H]orotic acid into the nucleic acids of subcellular fractions in chick embryos*

Group no.	Embryos injected with	10 ⁻³ × Sp. radioactivity (counts/min./mg.)			
		Nuclear DNA	Nuclear RNA	Ribosomal RNA	Total free nucleotides
1	0.9% NaCl soln. (control)	4.6 ± 0.31	36.3 ± 1.90	13.5 ± 0.97	288 ± 14
2	Spermine	3.2 ± 0.26 (-30%; P < 0.01)	23.5 ± 1.90 (-35%; P < 0.001)	10.2 ± 0.55 (-24%; P < 0.01)	184 ± 12 (-19%; P < 0.05)
3	Spermidine	2.2 ± 0.18 (-52%; P < 0.001)	17.7 ± 0.98 (-51%; P < 0.001)	5.6 ± 0.31 (-58%; P < 0.001)	167 ± 10 (-27%; P < 0.01)
4	Iproniazid	6.5 ± 0.51 (+41%; P < 0.01)	43.7 ± 2.70 (+20%; P < 0.05)	20.4 ± 1.61 (+51%; P < 0.01)	275 ± 15 (+21%; P < 0.05)
5	Iproniazid + spermine	7.7 ± 0.59 (+67%; P < 0.001)	46.4 ± 3.24 (+28%; P < 0.02)	21.9 ± 1.53 (+62%; P < 0.001)	300 ± 17 (+31%; P < 0.01)
6	Iproniazid + spermidine	7.4 ± 0.51 (+61%; P < 0.001)	47.3 ± 3.31 (+30%; P < 0.02)	21.5 ± 1.47 (+59%; P < 0.001)	308 ± 17 (+35%; P < 0.01)

Sodium chloride (0.9%) (control), spermine (5 μmoles), spermidine (2.5 μmoles) and iproniazid (8 μmoles) (as indicated) were injected into the yolk sac of each of a group of embryos on the tenth day of incubation. When iproniazid was given, alone or in combination with the polyamines, a second injection was given 36 hr. after the first. The [³H]orotic acid was administered 90 hr. after the first injection, and all determinations were carried out 6 hr. later. Specific radioactivities are given as the means ± s.e.m. of eight groups of two embryos each. For groups 2-6, the percentage changes from the corresponding values in group 1 and the *P* values of the differences are given in parentheses.

were dissolved in 0.5 ml. of formic acid; 5 ml. of ethylene glycol monomethyl ether was added, followed by 10 ml. of scintillator fluid (Bray, 1960). Under these conditions the counting efficiency for all samples was about 50%.

DNA was determined by the method of Fleck & Munro (1962).

RESULTS

Table 1 shows the incorporation of [³H]orotic acid into the nucleic acids of subcellular fractions. Groups 2 and 3, injected with spermine and spermidine respectively, show a decreased specific radioactivity of nuclear DNA, nuclear RNA and ribosomal RNA. On the other hand, large increases are found in the specific radioactivity of nuclear DNA, nuclear RNA and ribosomal RNA in group 4 (embryos injected with iproniazid). A further increase in specific radioactivity is found in groups 5 and 6 (embryos injected with iproniazid plus spermine and iproniazid plus spermidine respectively). The specific radioactivities of acid-soluble free nucleotides change in a similar fashion.

The sucrose-density-gradient analysis of ribosomes obtained from the deoxycholate-treated post-mitochondrial supernatant (Fig. 1) shows a shift of radioactivity in polyribosomes towards the heavier aggregates in groups 4, 5 and 6 (embryos injected with iproniazid, iproniazid plus spermine and iproniazid plus spermidine respectively); there is also a concomitant increase in the radioactivity in the regions of 206s, 259s and 280s (peaks *d*, *e* and *f* respectively). On the other hand, the groups injected with spermine or spermidine (groups 2 and 3 respectively) show a decreased radioactivity in the region of the heaviest polyribosomal aggregates.

The activity of nuclear DNA-dependent RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) is shown in Table 2. The results show that both the Mg²⁺-activated and Mn²⁺-ammonium sulphate-activated RNA polymerase reactions are modified, in agreement with the other results. This behaviour is particularly evident for the Mn²⁺-activated reaction: this enzymic activity is depressed in the groups of embryos injected with spermine and spermidine (groups 2 and 3 respectively), whereas the embryos treated with iproniazid show an activity increased by 43% (group 4). A further increase in this enzymic activity is obtained in those embryos that are injected with iproniazid plus spermine or plus spermidine (groups 5 and 6 respectively).

DISCUSSION

The observation that there is an increase in the incorporation of [³H]orotic acid into DNA under conditions in which there is an increase in spermine or spermidine content can be correlated with the

Fig. 1. Effect of spermine, spermidine and iproniazid on polyribosomal sedimentation profiles in chick embryos. The experiment was carried out as described in Table 1 and in the text. Embryos were injected with: (1) 0.9% NaCl soln.; (2) spermine; (3) spermidine; (4) iproniazid; (5) spermine + iproniazid; (6) spermidine + iproniazid. Peak $a=73$ s; $b=113$ s; $c=147$ s; $d=206$ s; $e=259$ s; $f=280$ s. —, E_{260} ; ----, radioactivity. All the embryos were injected (into the air space) with $50\mu\text{C}$ of sodium $[^3\text{H}]$ formate, and polysomes were extracted 2 hr. later.

Table 2. Effect of spermine, spermidine and iproniazid on DNA-dependent RNA polymerase activity in chick embryos

Experimental details are given in Table 1 and in the text. Results are the averages of at least three determinations of pooled embryos (four embryos per determination). Duplicate experiments agreed within $\pm 10\%$. The value for Mg^{2+} -activated RNA polymerase activity in the control was $141\mu\text{moles}$ of $[^{14}\text{C}]\text{AMP}$ incorporated/15 min./mg. of DNA, and that for Mn^{2+} - $(\text{NH}_4)_2\text{SO}_4$ -activated RNA polymerase activity in the control was $375\mu\text{moles}$ of $[^{14}\text{C}]\text{AMP}$ incorporated/45 min./mg. of DNA.

Group no.	Embryos injected with	RNA polymerase activity (% of control)	
		Mg^{2+} -activated	Mn^{2+} - $(\text{NH}_4)_2\text{SO}_4$ -activated
1	0.9% NaCl soln. (control)	100	100
2	Spermine	57	30
3	Spermidine	41	52
4	Iproniazid	125	143
5	Iproniazid + spermine	133	162
6	Iproniazid + spermidine	137	168

quantitative modifications of spermine or spermidine concentrations in the embryo. In fact, in embryos injected with iproniazid, and in which the increase in spermine content is 47% and that of spermidine 49%, as shown previously (Caldarera *et al.* 1965), the rate of incorporation of orotic acid into DNA is increased by 41%. In embryos injected with spermine together with iproniazid, in

which the spermine content is increased by 70%, the incorporation rate is increased by 67%, and in that injected with spermidine plus iproniazid the incorporation rate is increased by 61%.

The specific radioactivity of RNA varies in the same way as that of DNA; it is possible to observe a significant increase in the rate of incorporation of orotic acid into both nuclear and cytoplasmic RNA.

This effect is particularly evident for ribosomal RNA. On the other hand, under conditions in which the polyamine content decreases (Table 1, groups 2 and 3) there is a general fall in the incorporation rate of [³H]orotic acid into all nucleic acid species studied. However, one might point out that the increased biosynthetic activity could be partly correlated with the increased acid-soluble radioactivity.

These results, particularly the increased incorporation rate of [³H]orotic acid into ribosomal RNA, led us to study the profiles of ribosomal aggregates by sucrose-density-gradient centrifugation. The methods of polyribosomal analysis in sucrose-density-gradient centrifugation allow us to ascertain the cytoplasmic content of rapidly labelled RNA and the ability of ribosomes to aggregate on messenger RNA strands.

Under our experimental conditions there is a shift in the distribution of radioactivity in polyribosomes towards the heavier aggregates and a marked increase in the labelling of RNA in the polysomal regions of 206s, 259s and 280s particles in the groups of embryos in which there are higher polyamine contents, whereas the size of labelled polyribosomal aggregates fell in those embryos in which polyamine content was low.

These results may help us to understand the positive effect of spermine and spermidine on the amino acid incorporation rate at the ribosomal level observed by Martin & Ames (1962). On the other hand it has been observed that polyamines exert a marked protection against the effect of levorphanol, which inhibits selectively the synthesis of ribosomal RNA in *E. coli* (Simon, Cohen & Raina, 1966).

To investigate these effects more thoroughly, we assayed the DNA-dependent RNA polymerase activity under the same experimental conditions. In particular we studied the two different reactions described by Widnell & Tata (1966) for isolated whole nuclei: the first in the presence of Mg²⁺ and in the absence of ammonium sulphate, the other in the presence of Mn²⁺ and ammonium sulphate. The product, according to Widnell & Tata (1966), of the Mg²⁺-activated RNA polymerase reaction catalysed by a preparation of rat liver nuclei, in terms of base composition and nearest-neighbour frequency, is a ribosomal type of RNA, whereas that of the Mn²⁺-ammonium sulphate-activated RNA polymerase reaction is a more DNA-like RNA.

Both reactions show appreciable increases in all

groups of embryos in which the polyamine contents are enhanced, and this is particularly evident for the Mn²⁺-activated reaction. The increased RNA polymerase activity could explain the higher [³H]orotic acid incorporation rate into the ribosomal RNA and the higher radioactivity found in the heaviest polyribosomal aggregates.

The variations of polyamine concentrations, resulting from different physiological needs of the cells, indicate that spermine and spermidine may have a role in the control of protein synthesis.

This work was supported by a grant from the Consiglio Nazionale delle Ricerche, Rome, Italy.

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