Polyamines and Nucleic Acids during Development of the Chick Embryo

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1. A higher concentration of polyamines (spermine, spermidine, putrescine and cadaverine) during development of the chick embryo was observed between the fifth and tenth day of incubation; the concentrations of nucleic acids showed a parallel increase. 2. When spermine (5 μ moles) was injected into the yolk sac of embryos at the tenth day of incubation, a high amine-oxidase activity was noted and the spermine and spermidine concentrations were decreased; also, there was a remarkable decrease in RNA and DNA concentrations and a parallel increase in that of total free nucleotides. 3. On the other hand, when iproniazid (16 μ moles) was injected there was an inhibition of amine-oxidase activity and a similar increase in the concentrations of spermine and spermidine and of nucleic acids, whereas that of total free nucleotides decreased. 4. Another group of embryos injected with spermine and iproniazid together showed a remarkable increase in spermine and spermidine concentrations and a parallel increase in those of RNA and DNA, and a decrease in that of total free nucleotides.

The presence and distribution of the polyamines spermine, spermidine, cadaverine and putrescine in various living organisms have been studied by several authors (Fischer & Bohn, 1957; Hirsch & Dubos, 1952; Tabor, Tabor & Rosenthal, 1961). Spermine and spermidine are of particular interest because of their localization in certain organs such as pancreas, liver, prostate and seminal fluid. These compounds are also present in a number of micro-organisms, and for some of them they are an essential growth factor (Herbst & Snell, 1949).

Moruzzi & Caldarera (1964) showed the presence of polyamines in appreciable quantities in the embryo of several cereals (wheat, oats, barley etc.), and Bertossi, Bagni, Moruzzi & Caldarera (1965) pointed out that spermine is a growth-promoting factor for explants of Jerusalem artichoke *in vitro*. However, Keister (1958) showed that polyamines show affinity for nucleic acids, and Mahler, Mehrotra & Shays (1961) and Tabor (1962) demonstrated a stabilizing effect on DNA. Spermine, spermidine and putrescine stimulate the synthesis of RNA catalysed by RNA polymerase when the reaction is primed by native DNA (Krakow, 1963; Weiss & Fox, 1964). Relationships between spermine and folic acid have been demonstrated; in particular, spermine is involved directly or indirectly in some way in the metabolism of C₁ units (Turner, Lansford, Ravel & Shive, 1963).

These observations led us to study the inter-

relations of nucleic acid and polyamines in the chick embryo under conditions of nucleic acid synthesis.

MATERIALS AND METHODS

Embryos. 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. The eggs were placed on racks tilted at 30° to the horizontal, and turned twice daily.

Materials. Putrescine, cadaverine, spermidine and spermine were obtained from Fluka A.-G., Buchs, Switzerland; RNA and DNA were from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; Amberlite XE-64 (CG-50) resin was obtained from British Drug Houses Ltd., Poole, Dorset; Dowex 1 resin (X8; 200–400 mesh) was from Pierrel Associate, Milan, Italy; Nessler's reagent and 1-fluoro-2,4-dinitrobenzene were from E. Merck, Darmstadt, Germany.

Extraction of polyamines and acid-soluble nucleotides. Embryos were freed from the extra-embryonic membranes and yolk sac, and immediately frozen, weighed and homogenized with ice-cold 1 N-HClO₄ in a glass Potter homogenizer. The homogenates were kept at 4° for 30 min. and then centrifuged at 1400g for 15 min. The acid supernatants were decanted and the residues suspended in a volume of cold 0.5 N-HClO₄ acid equal to that of original homogenate and centrifuged as before. The extracts and the washing were combined and neutralized with ice-cold 5 N-KOH and kept in the refrigerator overnight; the KClO₄ was removed by centrifugation and the extracts were put on a column for ion-exchange chromatography.

Acid-soluble nucleotides were extracted with the same method by using respectively 0.6N- and 0.2N-HClO₄.

Ion-exchange chromatography. The extract containing polyamines was passed through a column (22 cm. $\times$ 1.2 cm.) of Amberlite XE-64 (CG-50) resin (K⁺ form; 100–200 mesh) at a flow rate of 0.7 ml./min. This was followed by 50 ml. of water, a volume that would displace any non-exchangeable material. Gradient elution (Tabor, Rosenthal & Tabor, 1958) was carried out with 1.67M-Na₂SO₄ in 0.1M-phosphate buffer, pH 7.1, as eluting fluid; at the start the mixing flask contained 200 ml. of water. Fractions of volume 3 ml. were collected on a Sargent fraction collector. The flow rate of the column was 0.8 ml./min. Acid-soluble free nucleotides extracted as described above were put on an ion-exchange resin column (20 cm. $\times$ 1.2 cm. diam.) of Dowex 1 (X8; formate form; 200–400 mesh) (Hurlbert, Schmitz, Brumm & Potter, 1954). Gradient elution with formic acid-ammonium formate was then carried out (Caldarera, Budini, Barbiroli & Rabbi, 1962). The mixing flask contained 500 ml. of water at the start, and the following solutions were fed into it from a reservoir: 0.5M-formic acid (500 ml.); 1M-formic acid (500 ml.); 2M-formic acid (500 ml.); 4M-formic acid (500 ml.); 0.2M-ammonium formate in 4M-formic acid (900 ml.); 0.4M-ammonium formate in 4M-formic acid (500 ml.); 0.8M-ammonium formate in 4M-formic acid (250 ml.); the flow rate through the column was adjusted to be as close to 0.7 ml./min. as possible.

Colorimetric estimation of polyamines. The method of Rosenthal & Tabor (1956) was used. A 2 ml. portion of each fraction was mixed with 0.5 ml. of saturated Na₂B₄O₇ in a small test tube and 0.25 ml. of 1-fluoro-2,4-dinitrobenzene solution was added (this solution was prepared by pipetting 0.65 ml. of 1-fluoro-2,4-dinitrobenzene into 50 ml. of reagent-grade acetone). The tube was shaken and placed in a water bath at 60° for 10 min. On removal 0.5 ml. of 5N-HCl was added; after cooling to room temperature, the colour was extracted with cyclohexanone (for putrescine and cadaverine) and with ethyl acetate (for spermidine and spermine), and read at 420 m μ .

Identification of polyamines. Identification of the eluted compounds was carried out by paper chromatography with ethylene glycol monomethyl ether-propionic acid-water (14:3:3, by vol.) saturated with HCl as the solvent; the *R_F*

values were compared with those of the authentic compounds. Besides these, electrophoretic separations with 65 mM-sulphosalicylic acid, pH 3.5, were used (Raina, 1963). Dinitrophenyl derivatives of polyamines were prepared and the spectra examined according to the method of Dubin & Rosenthal (1960).

Identification and estimation of free nucleotides. The effluent from the column was monitored at 260 m μ with an automatic ultraviolet analyser (Vanguard model 1056). Then it was collected on a Sargent fraction collector in 5 ml. fractions. Each fraction's extinction was measured at 260 m μ in a Beckman model DU spectrophotometer. The identity of the nucleotides was established by determining the extinction at 260 and 275 m μ (the readings at 275 m μ assist in the interpretation of the chromatograms, since the approximate *E*₂₇₅/*E*₂₆₀ ratios are characteristic of the various nucleotides). This was followed by verification with paper-partition chromatography with known controls (Magasanik, Vischer, Doniger, Elson & Chargaff, 1950). The molar extinction values of known pure nucleotides were used for the calculation of the concentrations of the peaks.

Determination of RNA and DNA. The Schneider (1946) method, with HClO₄ instead of trichloroacetic acid as the extractant, was followed and the amounts of RNA and DNA were calculated by measuring the extinction at 260 m μ in a Beckman model DU spectrophotometer and comparing the readings with pure RNA standards hydrolysed with 0.5N-KOH for 20 hr. at 37°, and DNA hydrolysed with 5% HClO₄ for 15 min. at 90°.

Enzyme assay. Spermine-oxidase and spermidine-oxidase activities were estimated by the measurement of NH₃ liberated by the amine-oxidase reaction. The diffusion of NH₃ was carried out in Conway (1957) units and the NH₃ collected in the central compartment of a Conway dish in 3 ml. of 0.01N-H₂SO₄. The reaction mixture (final volume 3 ml.) contained: sodium phosphate buffer, pH 7.4 (0.1M); spermine, 75 μ moles (or spermidine, 100 μ moles); whole embryo homogenate (15%; about 3–4 mg. of protein) centrifuged at 1000g for 15 min. at 0°. The reaction mixture was incubated at 37° for 6 hr. The NH₃ was measured photometrically by means of Nessler's reaction. A standard curve was used to measure the liberated NH₃. Protein N was determined by the usual micro-Kjeldahl technique.

Table 1. Concentrations of polyamines and nucleic acids in the developing chick embryo

Polyamines were determined by ion-exchange chromatography on Amberlite XE-64 resin (K⁺ form); gradient elution was carried out with 1.5M-Na₂SO₄ in 0.1M-phosphate buffer, pH 7.1. Polyamines are expressed as μ moles/g. dry wt. RNA and DNA were separated according to the Schneider method and estimated at 260 m μ , and are expressed as mg./g. dry wt. Values represent the means $\pm$ S.E.M. of eight groups of embryos.

No. of embryos in each group	Unincubated eggs	Incubated for:						4 days after hatching
		2 days	5 days	7 days	10 days	14 days	21 days	
	... 10	100	50	15	6	6	6	6
Spermine	None	2.49 $\pm$ 0.18	3.54 $\pm$ 0.17	3.25 $\pm$ 0.30	3.70 $\pm$ 0.21	2.44 $\pm$ 0.17	0.60 $\pm$ 0.03	1.78 $\pm$ 0.14
Spermidine	None	1.85 $\pm$ 0.12	5.73 $\pm$ 0.40	6.73 $\pm$ 0.49	5.49 $\pm$ 0.38	4.03 $\pm$ 0.33	1.27 $\pm$ 0.66	2.08 $\pm$ 0.15
Cadaverine	None	2.77 $\pm$ 0.20	5.68 $\pm$ 0.44	3.76 $\pm$ 0.34	3.02 $\pm$ 0.20	1.00 $\pm$ 0.07	0.60 $\pm$ 0.04	Traces
Putrescine	None	2.40 $\pm$ 0.19	5.00 $\pm$ 0.34	3.29 $\pm$ 0.33	2.63 $\pm$ 0.23	0.86 $\pm$ 0.06	0.52 $\pm$ 0.03	Traces
RNA	—	32.41 $\pm$ 1.16	37.77 $\pm$ 0.89	58.68 $\pm$ 1.45	58.21 $\pm$ 1.72	51.63 $\pm$ 1.07	20.08 $\pm$ 0.35	24.19 $\pm$ 0.39
DNA	—	14.12 $\pm$ 0.96	17.94 $\pm$ 0.99	22.45 $\pm$ 0.44	20.84 $\pm$ 0.31	16.42 $\pm$ 0.32	7.54 $\pm$ 0.23	9.11 $\pm$ 0.20

Injection technique. The egg shell was swabbed with 70% (v/v) ethanol. Spermine and iproniazid in sterile solutions were injected into the yolk sac in a volume of 300–400 μ l.; the punctures were then sealed with a mixture of paraffin wax and beeswax. The injected eggs were not turned thereafter.

RESULTS

The first observation concerns the polyamine and nucleic acid concentrations during the successive stages of embryonic development. In a preliminary investigation on unincubated eggs, we could not detect polyamines (Table 1), whereas polyamines, especially spermine (2.5 μ moles/g. dry wt.), were present in appreciable quantities in blastoderms of fertilized eggs after 48 hr. of incubation. The concentrations of these compounds progressively increased and reached maximum values between the fifth and tenth day of incubation and then successively decreased; on the fourth day after hatching, a new increase in spermine and spermidine concentrations was observed, whereas cadaverine and putrescine concentrations continued to decrease. RNA and DNA concentrations, determined under the same experimental conditions, showed the same behaviour as those of the polyamines; RNA underwent greater variations. Spermine-oxidase and spermidine-oxidase activities (Fig. 1) varied.

The results reported in Table 2 show the effect of injection of spermine and iproniazid in chick embryos at the tenth day of incubation.

The embryos injected with spermine (5 μ moles) showed, after 96 hr., an amine-oxidase activity increased by 97% and at the same time a decrease in spermine and spermidine concentrations. Also, there was a remarkable decrease in the concentrations of RNA and DNA and a parallel increase in that of total free nucleotides.

Another group of embryos, at the tenth day of incubation, was injected with iproniazid (8 μ moles)

Fig. 1. Spermine-oxidase (○) and spermidine-oxidase (●) activities during development of the chick embryo. Details of the assay method are given in the Materials and Methods section.

Table 2. Effect of spermine and iproniazid on spermine-oxidase activity and the concentrations of polyamines, nucleic acids and free nucleotides in the chick embryo

	Group 1	Group 2	Change from that with 0.9% NaCl (%)	P	Group 3	Change from that with 0.9% NaCl (%)	P	Group 4	Change from that with 0.9% NaCl (%)	P	Group 1-4 (group 1-4)
Spermine-oxidase activity	Embryos injected with 0.9% NaCl	Embryos injected with spermine			Embryos injected with iproniazid			Embryos injected with spermine and iproniazid			
Spermine	0.59 ± 0.04	1.16 ± 0.07	+97	<0.001	0.48 ± 0.03	2.47 ± 0.19	+47	0.43 ± 0.03	2.87 ± 0.22	+70	<0.001
Spermidine	1.68 ± 0.12	1.13 ± 0.08	-32	<0.01	3.75 ± 0.32	3.75 ± 0.32	+49	4.13 ± 0.34	4.13 ± 0.34	+64	<0.01
RNA	2.52 ± 0.20	1.83 ± 0.14	-27	<0.01	58 ± 0.62	58 ± 0.62	+18	64 ± 0.68	64 ± 0.68	+30	<0.001
DNA	49 ± 0.61	39 ± 0.50	-20	<0.001	19 ± 0.14	19 ± 0.14	+27	20 ± 0.18	20 ± 0.18	+33	<0.001
Free nucleotides (total)	15 ± 0.14	13 ± 0.11	-13	<0.001	1051 ± 115	1051 ± 115	+44	1668 ± 116	1668 ± 116	+34	<0.01
	2510 ± 200	3627 ± 326	+44	<0.01							

Spermine (5 μ moles) was injected into the yolk sac of each of a group of embryos at the tenth day of incubation. Iproniazid (8 μ moles) was injected into the yolk sac of each of another group of embryos at the same time of incubation and again 36 hr. later. The final group of embryos was injected with spermine (2.5 μ moles) and iproniazid (8 μ moles) together at the tenth day of incubation and the injection repeated after 36 hr. All determinations were carried out 96 hr. after the first injection. Spermine-oxidase activity is expressed as μ moles of NH_3 produced in 6 hr./mg. of N. Polyamines are expressed as μ moles/g. dry wt. Nucleic acids are expressed as mg./g. dry wt. Free nucleotides are expressed as μ moles/100 mg. dry wt. Values represent the means $\pm$ s.e.m. of eight groups of six embryos each.

and 36 hr. later reinjected with the same dose. The second injection was done to maintain the inhibition of amine-oxidase activity longer than 36 hr.; in fact, with one injection the inhibition reaches maximum values between 24 and 36 hr. (Fig. 2) and quickly falls. The inhibition of amine-oxidase activity obtained with iproniazid resulted in an increase in spermine and spermidine concentrations. This change was followed by an increase in RNA and DNA concentrations, whereas that of total free nucleotides were decreased by 58%.

Fig. 2. Inhibition of spermine-oxidase activity by iproniazid in the chick embryo. Iproniazid ($8\mu\text{moles}$) was injected into the yolk sac of the embryos at the tenth day of incubation; the second injection ($8\mu\text{moles}$) (arrow) was done to maintain the inhibition longer than 36 hr. (broken line). Experimental details are given in the text.

Finally, another group of embryos were injected with spermine ($2.5\mu\text{moles}$) and iproniazid ($8\mu\text{moles}$) together at the tenth day of incubation and the injections repeated after 36 hr. These embryos showed a large increase in spermine and spermidine concentrations and a parallel increase in those of RNA and DNA, whereas that of total free nucleotides were decreased.

The variations in the concentrations of the individual free nucleotides in the same experiments are shown in Table 3.

Embryos injected with spermine showed a 60% increase in the concentration of purine nucleotides and a higher increase in those of triphosphate nucleotides: ATP increased by 100%, CTP by 98%, GTP by 50% and UTP by 170%. There was a decrease in the concentration of pyrimidine nucleotides in embryos injected with iproniazid and this was due mainly to the uridine nucleotides (80% decrease); the concentration of adenine and cytidine nucleotides was decreased by 60%. The fall in nucleoside triphosphate concentration is considerable. In those embryos injected with spermine and iproniazid together, the concentration of pyrimidine nucleotides decreased by 76%. No significant variations in the concentrations of total adenine nucleotides were noted, whereas that of ATP was increased by 100%.

Table 3. *Effect of spermine and iproniazid on the concentrations of free nucleotides in the chick embryo*

Experimental details are given in the text and Table 2. Values are given as means $\pm$ S.E.M. of eight groups of six embryos each. Free nucleotides are expressed as $\mu\text{moles}/100\text{mg. dry wt.}$

	Embryos injected with 0.9% NaCl	Embryos injected with spermine	Embryos injected with iproniazid	Embryos injected with spermine and iproniazid
AMP	317 ± 20.8	368 ± 31.2	206 ± 21.3	84 ± 7.9
ADP	365 ± 24.3	378 ± 35.6	Traces	263 ± 23.2
ATP	404 ± 29.5	820 ± 55.8	192 ± 13.5	816 ± 65.4
Adenine nucleotides	1086 ± 72	1566 ± 110	398 ± 29	1163 ± 89
GMP	40 ± 5.3	60 ± 4.9	57 ± 4.3	52 ± 4.3
GDP	Traces	Traces	Traces	Traces
GTP	120 ± 10.9	180 ± 15.2	Traces	Traces
Guanine nucleotides	160 ± 15	240 ± 20	57 ± 4	52 ± 4
IMP	Traces	190 ± 18.2	274 ± 26.1	125 ± 11.1
Total purine nucleotides	1246	1996	726	1340
UMP	111 ± 11.9	46 ± 3.9	Traces	Traces
UDP	173 ± 19.3	180 ± 17.2	Traces	Traces
UTP	146 ± 12.3	398 ± 31.6	Traces	Traces
UDP-acetylglucosamine	258 ± 24.3	252 ± 20.8	90 ± 7.7	145 ± 15.1
UDP-glucose	165 ± 14.2	168 ± 15.8	80 ± 7.3	170 ± 14.6
Uridine nucleotides	853 ± 73	1044 ± 91	170 ± 14	315 ± 28
CMP	308 ± 29.4	383 ± 25.3	152 ± 11.7	13 ± 1.4
CTP	103 ± 11.4	204 ± 22.3	Traces	Traces
Cytidine nucleotides	411 ± 40	587 ± 52	152 ± 12	13 ± 1
Total pyrimidine nucleotides	1264	1631	322	328

DISCUSSION

These experiments show a possible relationship between polyamines and nucleic acids. During development of the chick embryo the polyamines are biosynthesized in considerable quantities from embryonic cells from the earliest stages of development; their concentrations reach a maximum between the fifth and tenth day of incubation. These results agree with those of Raina (1963). In each stage of the embryonic development the concentrations of RNA and DNA also show a parallel behaviour (Table 1). On the other hand, it is in this period of development that one observes the most evident morphological modifications and most active protein synthesis in the embryo. Further investigations, in which the content of embryonic polyamines was experimentally modified, confirmed these preceding observations.

Injection of spermine into the yolk sac (Table 2) results in an increase in amine-oxidase activity, and, consequently, an increased oxidation of polyamines. Spermine, by causing an increase of this enzymic activity, leads to a decrease of the polyamine concentration in the embryo. Correspondingly, the concentrations of nucleic acids also decrease. Under these conditions, the concentration of total free acid-soluble nucleotides increases; this increase is due mainly to the triphosphates, precursors of the nucleic acids. This phenomenon may be due partly to the catabolic products of the polyamines, such as the amino aldehydes, which are toxic (Tabor, Tabor, McEwen & Kellogg, 1964).

However, an increase in the concentrations of spermine and spermidine was obtained with iproniazid, an inhibitor of the amine oxidases, with a simultaneous increase in the concentrations of RNA (18%) and DNA (27%). The concentration of total free nucleotides in this case decreases, and this is particularly evident for the triphosphates.

To confirm this behaviour, we injected into the yolk sac one of the main polyamines, spermine, together with iproniazid; we obtained an increase in the concentration of spermine, accompanied by a further increase in the concentrations of both RNA (30%) and DNA (33%), whereas that of total free nucleotides decreased. However, among the triphosphates only the concentration of ATP increased.

Therefore, not only in physiological conditions, but also in two extreme experimental conditions, when the polyamine concentrations increase and decrease, the concentrations of nucleic acids show in each case a corresponding behaviour. The concentrations of total free nucleotides, especially the triphosphates, always agree with these changes. This can be attributed, respectively, to their higher or lower utilization.

All these observations show the existence of a

relationship between polyamines and nucleic acids. The observations by Krakow (1963) and Weiss & Fox (1964) that polyamines stimulate polynucleotide-phosphorylase activity *in vitro* may help to explain this relationship. Polyamines could also have such an action *in vivo* by stimulating the biosynthesis of nucleic acids, especially DNA, during polymerization controlled by RNA polymerase and DNA polymerase.

The present results, and especially those on the free nucleotides, seem in accordance with this hypothesis.

Such a view could also explain the significance of the distribution of the polyamines in Nature, and particularly their presence in organs and tissues in which there is rapid cellular proliferation (Raina, 1963; Herbst & Dykstra, 1964; Bertossi *et al.* 1965).

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