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NUCLEIC ACID METABOLISM OF THE DEVELOPING CHICK EMBRYO

II. DESOXYRIBONUCLEIC ACID AND CELL DIVISION

D. H. MARRIAN, A. F. PHILLIPS AND S. M. WERBA
Department of Radiotherapeutics, University of Cambridge (England)

INTRODUCTION

For some time, it was generally believed that the desoxyribonucleic acid (DNA) of a mammalian cell was not retained during cell division, so that two units of DNA had to be newly synthesised[1–5]. More recent work, with arguments derived from cell counts and uptake of labelled phosphate, has not confirmed this view[6–9], thereby bringing mammalian division into line with bacterial division, where the original DNA is retained[8, 10]. The present paper shows that the purine fragments of the DNA of the chick embryo are also retained during development.

METHODS AND MATERIALS

Injection of labelled precursors. Injections of sodium ^{14}C-formate and 2-^{14}C-glycine into fertile eggs were carried out as described previously[11].

Counting techniques

A change in spreading technique has led more certainly to "infinitely thin" conditions. After drying off the solution of the purines in dilute formic acid (pH 2), glass-distilled water (enough to cover the bottom of the planchette) is added, dried off, and the cycle repeated. Counts per aliquot of the original solution are much more consistent and this modification, together with a slight change in planchette size, has led to an increase in the counting rate by a factor of 1.19

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± 0.014 compared with those previously reported. Therefore, when some of the results from our previous paper have been quoted here, they have been multiplied by 1.19 to make the comparison valid. The experiments in this paper have been carried out over a period of about 18 months, during which time the counter efficiency varied slightly. A standard planchette was counted every day and all counting rates have been normalised to a standard count of 1000 (range of standard 1043–937).

Separation of the constitutents from the embryo

The embryos were dissected out and homogenised in ice-cold 0.6 *N* perchloric acid. Acid-soluble adenine (and occasionally guanine) was isolated as before[11], and the tissue residue washed twice with water, twice with alcohol, once with ether, and refluxed 1 hour with about 10 volumes of methanol/chloroform (1/1). The solid was spun down, washed with alcohol, then ether, and dried off *in vacuo*. After the initial homogenising, all operations were carried out in the same tube to eliminate losses.

The following separation of ribonucleic acid (RNA) constituents and DNA purines combines features of the methods of SCHMIDT AND THANNHAUSER[12], and of OGUR AND ROSEN[13]. It leads to the quantitative extraction and estimation of mixed DNA purines in a solution which, after evaporation, was suitable for paper chromatography, elution and radioactivity determination. 25–60 mg dry, defatted tissue were weighed into a stout centrifuge tube and shaken occasionally during 24 hours with 1 ml *N* KOH. The solution was spun (2500 r.p.m./4 min) and the small residue washed with 1 ml *N* KOH and spun again. The combined supernatants were cooled in ice, and treated with 0.66 ml 6 *N* HCl and 0.4 ml 25% trichloracetic acid. The precipitate of DNA was spun down in the cold, washed once with 1 ml of water containing 2 drops of 25% trichloracetic acid, and barium ribonucleotides isolated from the combined supernatants[14]. RNA purines and pyrimidine nucleotides were separated as before. The washed DNA precipitate was stirred occasionally at 80° for 40 min with 2 ml 0.5 *N* perchloric acid, a little Hyflo supercell added, and the whole cooled in ice. The solution was quantitatively filtered into a 10 ml graduated cylinder and the precipitate and paper washed with at least 4 × 1 ml of 0.5 *N* perchloric acid. The filtrate, of known volume, was read against the solvent in a Unicam U.V. Spectrophotometer. The volume of the extract multiplied by the optical density at 270 mμ gave a quantitative estimate of the amount of DNA purines originally present in the tissue sample.

Evaporation of the above solution *in vacuo* to small bulk gave a concentrate from which the DNA purines were separated on Whatman No. 3 paper, eluted, estimated by U.V. spectroscopy and counted as before. In making up the *iso*propanol/HCl solvent described by WYATT[15], it was found necessary to fractionate the *iso*propanol before use, rejecting that portion of the distillate which had an optical density of more than 0.050 at 260 mμ, using water as blank. This purification avoided large variations in the paper blanks (usually about 0.050 read against formic acid pH 2).

EXPERIMENTAL AND RESULTS

The results are given in terms of relative specific activity (R.S.A.), defined as the ratio of the specific activity of the isolated material to that of the administered precursor.

Expt. 1(a). 24 eggs were injected with a solution of sodium ^{14}C-formate (10 mg/ml, $1.78 \cdot 10^5$ c.p.m./μmole) as follows: before incubation, 0.05 ml, then 0.1, 0.2, 0.3, and 0.4 ml at 24-hour intervals. 11 embryos which survived were harvested on the 5th day and were at stage 25 of development[16].

1(b). 18 eggs were incubated for 4 days and then injected with 0.4 ml of the same formate solution. 7 viable embryos were harvested 24 hours later (stage 25). The relative specific activities were as follows:

		1(a)	*1(b)*	
Acid-sol. adenine		1.07	0.56	
RNA	guanine	1.06	0.46	
RNA	adenine	1.52	0.43	
DNA	guanine	1.01	0.24	Calc. 0.67 (see later)
DNA	adenine	1.11	0.40	Calc. 0.67

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Expt. 2(a). 4 eggs were incubated for 4 days and then injected with 0.5 ml of the above formate solution for 4 successive days and the surviving embryo harvested on the 8th day (stage 34).

2(b). 4 eggs were incubated for 7 days and injected with 0.5 ml of the above formate solution. Two viable embryos were harvested 24 hours later (stage 34).

		2(a)	*2(b)*	
Acid-sol. adenine		0.32	—	
RNA	guanine	1.12	0.24	
	adenine	1.12	0.22	
DNA	guanine	1.02	0.16	Calc. 0.32 (see later)
	adenine	0.89	0.16	Calc. 0.32

Expt. 3(a). 20 eggs were injected with sodium ^{14}C-formate solution ($1.78 \cdot 10^4$ c.p.m./μmoles) at the same times as in Expt. 1(a) but using *one half* the amounts. 2 embryos were harvested on the 5th day (stage 24).

3(b). 15 eggs were injected with formate solution ($1.78 \cdot 10^4$ c.p.m./μmoles) as in Expt. 1(b) but using *one half* the amount. 8 embryos were harvested on the 5th day (stage 24).

		3(a)	*3(b)*	
Acid-sol. adenine		—	0.30	
RNA	guanine	—	0.25	
	adenine	—	0.26	
DNA	guanine	0.99	0.20	Calc. 0.67 (see later)
	adenine	0.97	0.20	Calc. 0.67

Expt. 10. 24 eggs were incubated for 7 days and then injected with 0.1 ml formate solution (50 mg/ml, $1.58 \cdot 10^5$ c.p.m./μmole) and 3 embryos harvested after 2, 4, 6, 8, 10, 12 and 24 hours.

		Time in hours						
		2	*4*	*6*	*8*	*10*	*12*	*24*
Acid-sol.	guanine			0.31			0.36	
	adenine	0.097	0.21	0.24		0.51	0.41	0.36
RNA	guanine	0.001		0.064	0.048	0.16	0.15	0.29
	adenine	0.005		0.075	0.044	0.16	0.15	0.29
DNA	guanine	0.004	0.015	0.031	0.052	0.089	0.083	0.15
	adenine	0.004	0.007	0.022	0.036	0.060	0.070	0.14

The incorporation into DNA is shown in Fig. 1.

Expt. 4. 12 eggs were incubated for 4 days and then injected 8 times with 0.1 ml

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2-^{14}C-glycine solution (50 mg/ml, $8.65 \cdot 10^3$ c.p.m./μmole) at 12 hour intervals. 9 embryos were harvested on the 8th day.

Acid-sol.	guanine	0.94
	adenine	1.03
RNA	guanine	0.94
	adenine	1.29
DNA	guanine	1.02
	adenine	1.00

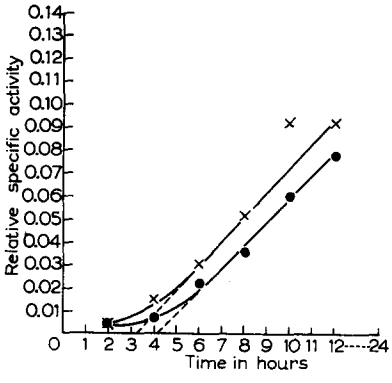

Fig. 1. Incorporation of labelled formate into DNA purines in 7-day embryos. The formate was injected into the air sac at time zero. × – Guanine; ● – Adenine.

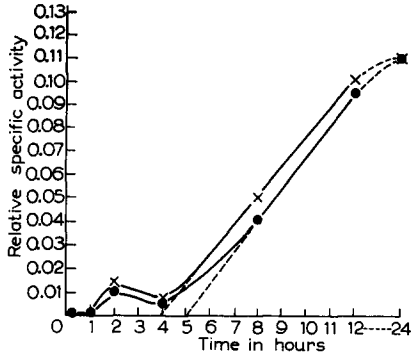

Fig. 2. Incorporation of labelled glycine into DNA purines in 7-day embryos. The glycine was injected into the air sac at time zero. × – Guanine; ● – Adenine.

Expt. 11. A batch of 5 ½-day eggs were injected with 0.2 ml 2-^{14}C-glycine solution (50 mg/ml, $2.71 \cdot 10^4$ c.p.m./μmole) and similarly for 6 ½- and 7 ½-day eggs. All eggs were harvested 12 hours after injection and were at stages 28, 30, and 33, respectively.

		Isotope available		
		5½–6 days	*7½–8 days*	*7½–8 days*
Acid-sol.	guanine	0.31	0.43	0.36
	adenine	0.65	0.88	0.54
RNA	guanine	0.15	0.20	0.11
	adenine	0.16	0.28	0.20
DNA	guanine	0.125	0.15	0.082
	adenine	0.095	0.13	0.089

Expt. 7. 7-day eggs were injected with 0.1 ml 2-^{14}C-glycine solution (50 mg/ml, $2.61 \cdot 10^5$ c.p.m./μmole) and harvested after the time-intervals tabulated (3 embryos at 1, 4 and 8 hours, and 2 at the other times).

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	Time in hours							
	¼	½	1	2	4	8	12	24
Acid-sol. adenine	0.14	0.069		0.34	0.31	0.23	0.37	0.43
RNA guanine	<0.001		0.005	0.016	0.013	0.081	0.16	0.14
RNA adenine	0.004		0.007	0.021	0.016	0.13	0.22	0.21
DNA guanine	<0.002		<0.002	0.012	0.006	0.041	0.081	0.090
DNA adenine	<0.002		<0.002	0.008	0.005	0.033	0.076	0.090

The incorporation into DNA is shown in Fig. 2.

Growth curves of the embryos

A number of embryos at various ages were dissected out and weighed fresh. The total amount of DNA present was determined on the bulked, dried, and defatted tissue for each age, by measurement of optical density of the DNA purines at 270 mμ at pH < 2 (0.5 *N* $HClO_4$). Determinations were made at least in duplicate in each case. The wet weights in grams, and the amounts of DNA in optical density units, are shown in Fig. 3, and the number of embryos of each age is indicated. For the 6-day point, the relationship between the amount of DNA phosphorus and the amount of DNA purines given in the original method of OGUR AND ROSEN[13] was confirmed by our modified method.

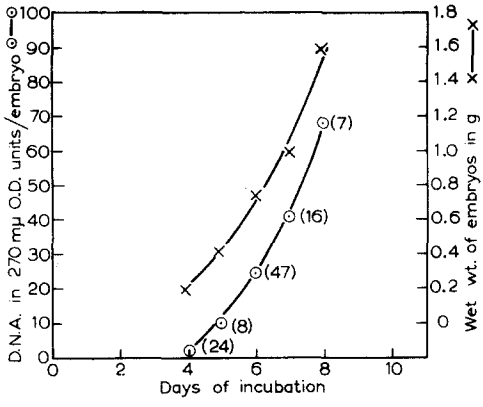

Fig. 3. Growth curves for chick embryos. × – Wet weight (right-hand scale); ◉ – Total DNA (left-hand scale). The number of embryos for each point is shown in parentheses.

Degradation of DNA adenine

Combined DNA adenine fractions from various experiments (2.65·10⁴ c.p.m./μmole) were evaporated to dryness and degraded to 4-amino-5-imidazolecarboxamidine hydrochloride by the method of CAVALIERI *et al.*[17]. The hydrolysate was freed from hydrochloric acid by evaporation and separated by paper chromatography in *iso*propanol/HCl. The product was located, eluted and counted as usual and had a specific activity of 1.34·10⁴ c.p.m./μmole. (The reported extinction co-efficient for this material is 10,710 at 280 mμ (pH 7)[17]; in dilute formic acid (pH 2) the E_m is 9,830 at 280 mμ.)

DISCUSSION

For calculation of the amount of DNA synthesised in a given period, by measurement of the dilution of newly synthesised labelled DNA, both the total amount present and the net increase in the period must be known. These were read off the growth curve of Fig. 3. The determination was checked for the 6-day point by the amount of DNA phosphorus present, which confirms the validity of the modified method

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suggested here. The DNA points are also in excellent agreement with calculations from the NOVIKOFF AND POTTER figures as modified in our first paper[11]. To a close approximation, the increase in DNA units can be taken as linear over any 24-hour period, thereby simplifying later calculations.

It has been our aim, in several of these experiments, to allow an embryo to develop from a stage at which it contains essentially no DNA in the presence of a large excess of labelled purine precursor. Ideally, this would entail injecting a fertile egg before incubation, and continuing injections at frequent intervals during development, as was done in Expts. 1 and 3. As the embryo is sensitive to such treatment in the very early stages, many fail to develop, but those which do appear to be normal in every way. Expts. 2(a) and 4 represent a convenient compromise in that the eggs were incubated for 3 or 4 days before injections were begun. The amount of DNA present at 4 days is so small compared to the amount at 7 or 8 days that only a small error is introduced by the presence of a small amount of unlabelled DNA.

Expt. 4 shows, as expected, that the DNA purines synthesised in the presence of excess labelled glycine attain the same specific activity as the precursor. This means that any molecule of DNA which is synthesised under these conditions must contain purines of the specific activity of the precursor. Therefore, in a different system, such as an embryo which is only allowed labelled precursor for a 12-hour period, the amount by which the newly formed DNA is diluted by that already present will show whether only the net increase in DNA is radioactive, or whether *twice* this increase is radioactive (*i.e.* whether, when a cell divides, the old DNA is retained, or whether two new units of DNA are synthesised).

Experiment 11 combined with Fig. 3 would be expected to give this answer, assuming that injections of labelled glycine are available for DNA synthesis immediately after injection into the air sac. However, Expt. 7 (Fig. 2) shows that there is a lag of about 4 hours before radioactivity is incorporated into DNA under these conditions.

Considering an embryo age x days which is offered excess radioactive precursor and then sacrificed 12 hours later:

Let amount of DNA at $(x + \frac{1}{2})$ days $= D_{x+\frac{1}{2}}$

Let amount of DNA at x days $= D_x$

Then net increase is $D_{x+\frac{1}{2}} - D_x = \delta D$.

Dilution to be expected if only the net increase is radioactive is (1) $\delta D/D_{x+\frac{1}{2}} \times 8/12$ since there is a 4-hour lag.

The dilution to be expected if *twice* the net increase is radioactive is (2) $2\,\delta D/D_{x+\frac{1}{2}} \times 8/12$.

Applying these calculations to the results of Expt. 11, we obtain the following:

Time during which precursor was available	*Expt. 11 Dilution found*		*Dilution calc. according to (1) above*	*Dilution calc. according to (2) above*
	Gu	*Ad*		
5½–6 days	0.13	0.10	0.195	0.39
6½–7 days	0.15	0.13	0.15	0.30
7½–8 days	0.08	0.09	0.15	0.30

It is obvious that the found values are much closer to those calculated for one unit

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synthesised per division, than for two. The divergence, disappointingly great in some cases, is towards less than one unit synthesised, and is never between one and two.

Expts. 1(a), 2(a) and 3(a) showed that the specific activity of newly formed DNA purines was the same as that of injected formate if the latter were present in large excess, the R.S.A. figures for DNA purines being close to 1.0 in each experiment. Thus, as in the case of the excess glycine experiment (Expt. 4) all the DNA finally present must have been labelled completely and to the same extent.

Expt. 10 (Fig. 1) shows that there is about a 4½-hour lag before formate precursor begins to arrive in DNA. The corresponding dilution calculations follow, the "found" column being calculated from DNA incorporation studies in our first paper.

Time during which precursor was available	*Dilution found*		*Dilution calc. according to (1) above*	*Dilution calc. according to (2) above*
	Gu	*Ad*		
4–4½ days	0.21	0.20	0.31	0.62
5–5½ days	0.17	0.15	0.23	0.46
6–6½ days	0.13	0.13	0.15	0.30
7–7½ days	0.10	0.09	0.13	0.26
8–8½ days	0.06	0.05	0.11	0.22

Here again, the results make it highly improbable that 2 units of DNA are synthesised during the mitotic cycle.

It was surprising that the R.S.A. of the purines in experiments 1(a), 2(a) and 3(a) did not approach 2.0, since it is well known that 2 formate carbons are built into the purine molecule[18]. Our first thought was that there was not enough formate to keep an excess always present, but Expt. 3(a), where only one half of the amounts of Expts. 1(a) and 2(a) was offered, still gave the same R.S.A. of 1.0 for DNA purines. The reason for this discrepancy is not clear and will not be pursued further at this time except to say that both the 2 and 8 carbon atoms of the adenine molecule appeared to contain the same amount of radioactivity, as shown by the degradation product lacking carbon atom 2 having almost exactly one half of the specific activity of the starting adenine. Thus it appears that both the 2 and 8 carbons must have a specific activity one half of the injected formate, in which case each atom appears to have been diluted by an equal amount of a non-radioactive one-carbon source.

The point is not relevant to the present paper, which is mainly concerned with the dilution which occurs when the amount of DNA in the system increases under conditions of a large excess of precursor. The calculations depend only upon the observed specific activities of the purines labelled under these conditions.

A further point of uncertainty derives from the results of Expts. 1(b), 2(b) and 3(b), where a 24-hour incorporation into DNA was studied. In 1(b) and 2(b), the R.S.A. observed was about one half of that calculated for one new unit synthesised, and in 3(b) it was even lower, which may have been due to insufficient excess of labelled precursor.

Another small point may be noted: in the timing experiments 10, 11 and 7, and in the results published in the previous paper[11], it always appeared that the specific activity of DNA guanine was fractionally higher than that of DNA adenine, while the reverse order held true in the case of the RNA purines. Similar findings in the

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uptake of glycine by the liver and bone marrow RNA of the hen have been reported by HAMMARSTEN[19].

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SUMMARY

A series of experiments has been performed to find out whether, in developing chick embryos, the purine fragments of desoxyribonucleic acid (DNA) are retained during cell division, or whether the original DNA is broken down and all the DNA of the daughter cells is synthesised afresh.

First, embryos were allowed to develop from an early stage in the continuous presence of excess of a ^{14}C-labelled precursor, either formate or glycine, and the tissue purines were found to reach the same specific activity as the precursor. Next, measurements were made of the total amount of DNA purines in embryos at different stages of development, and thus the average increase in any given period was determined. Finally, embryos were given an excess of the labelled precursor for a limited period only, so that the specific activity of the tissue purines at the end of that time could be used to calculate the actual amount of DNA synthesised during the period so defined. Allowance had to be made for a time lag of about 4 hours between injection of the labelled precursor into the air-sac and the appearance of radioactivity in the DNA.

The amount of DNA synthesised was found to be approximately equal to, and never more than, the expected net increase during the period. The observations therefore show that the original DNA is retained.

Complete labelling of the tissue DNA by labelled glycine when the latter was continuously present in excess, was confirmed by the finding that the specific activity of the DNA purines was equal to that of the precursor. In the case of formate, the same specific activity was found, although the DNA purines would be expected to reach twice the specific activity of the precursor. This finding requires further investigation, but it does not affect the main conclusion.

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