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BIOSYNTHESIS OF DEOXYRIBONUCLEIC ACID, RIBONUCLEIC ACID AND PROTEIN *IN VIVO* BY NEONATAL MICE AFTER A TOXIC DOSE OF CYCLOPHOSPHAMIDE*

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Abstract—Swiss–Webster mice received 80 mg/kg of cyclophosphamide 1 day after birth and were allowed to develop or were given 100 μCi/kg of [^{14}C]labeled thymidine, uridine or leucine at various times afterwards. Growth retardation and increased mortality were noted during the postnatal period. DNA synthesis, as measured by [^{14}C]thymidine incorporation, was inhibited in the liver, brain and carcass for at least 3 days after cyclophosphamide treatment. DNA synthesis was reduced 1 day after treatment to 8·4, 26 and 25 per cent of control in the liver, brain and carcass respectively. RNA synthesis, as measured by [^{14}C]uridine incorporation, was reduced only in the liver and brain. Liver RNA synthesis was reduced to 36 and 64 per cent of control at 1 and 5 days after cyclophosphamide treatment respectively. Brain RNA synthesis was reduced to 78 and 64 per cent of control 1 and 4 days after treatment. [^{14}C]leucine incorporation, taken as a measure of protein synthesis, was not affected in a manner which would indicate that drug treatment altered this parameter of differentiation. The data suggested that cyclophosphamide neonatal toxicity may be related to a prolonged inhibition of DNA and RNA synthesis during the first 5 days of life. This observation was correlated with the slow rate of cyclophosphamide activation and excretion in neonatal mice.

CYCLOPHOSPHAMIDE, a clinically useful alkylating antineoplastic agent, has potent toxic properties in developing systems. Cyclophosphamide was teratogenic in many mammalian species[1–4] but the spectrum of effects differed from other alkylating agents.[5] Growth retardation and increased mortality, in addition, were produced in day-old mice[6,7] by doses of cyclophosphamide which produced only a mild degree of leucopenia and no mortality in adults.[8] Treated mice at maturity were morphologically abnormal with small legs, ears and snouts. These effects on development were dose related and were not induced by nor-nitrogen mustard an alkylating agent with inherent alkylating activity.[7] Ifosfamide, a cyclophosphamide analog, produced similar teratogenicity and neonatal toxicity.[9] Cortisone treatment[10] and nutritional deprivation[11] of neonates reduced animal growth but failed to produce the morphologic abnormalities characteristic of cyclophosphamide and ifosfamide.

Perinatal mice metabolically activated cyclophosphamide to alkylating metabolites at a slow rate but reached adult activity between 2 and 3 weeks of age.[12] The elimination kinetics of [^{14}C]cyclophosphamide suggested that neonatal toxicity was pro-

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duced by the prolonged release of alkylating metabolites by the immature cyclophosphamide-activating system of the newborn.[13] A correlation, in addition, was established between toxicity and alkylating metabolites of cyclophosphamide by drug pre-treatment which increased or decreased the cyclophosphamide-activating ability of the newborn.

The present study was undertaken to investigate these unique toxic properties of cyclophosphamide by correlating biochemical lesions with drug levels and abnormal development. [^{14}C]thymidine incorporation into DNA was measured because this process was sensitive to alkylating agents.[14] [^{14}C]leucine incorporation into protein was examined because protein synthesis was an important aspect of differentiation.[15] The biosynthesis of RNA was studied to determine if drug treatment affected this parameter in the utilization of genetic information. Organ and body weights were measured to correlate biochemical effects with abnormal development.

METHODS

Neonatal Swiss–Webster mice were obtained from timed pregnancies started in this laboratory. Litters were born and individually housed in clear plastic shoe box cages with a 12-hr light–dark cycle and free access to food (Purina Lab Chow) and water.

Cyclophosphamide was administered subcutaneously (s.c.) 24–48 hr after birth at a dose of 80 mg/kg in a volume of 20 ml of normal saline/kg. This treatment significantly reduced the growth rate and increased mortality.[7] Control animals received an equal volume of normal saline. Some animals were allowed to develop and body and organ weights were measured at various times up to 35 days of age. [^{14}C]labeled precursors of DNA, RNA or protein were administered s.c. at a dose of 100 μCi/kg in a volume of 10 ml/kg at various intervals after cyclophosphamide, and the animals were sacrificed by decapitation at the indicated times. The liver and brain from one to three mice in a litter were removed and pooled to represent a single experimental unit. The tissue was weighed and homogenized in 5 ml of 0·2 N perchloric acid (PCA). The bodies were homogenized in distilled water with a Polytron homogenizer (Brinkmann). A portion of the homogenate was made 0·3 N in PCA and stored at least 15 min on ice.

[^{14}C]*thymidine incorporation.* Thymidine-2-^{14}C (50 mCi/m-mole ICN Tracer Lab) was administered 1 hr prior to sacrifice. After tissue homogenization the precipitate was washed with successive 5-ml washes of 0·2 N PCA containing 4 mM non-radioactive thymidine, 0·2 N PCA, 95% ethanol saturated with sodium acetate, ethanol–ether (3:1) and ether. The precipitate was heated at 37° for 1 hr in 4 ml of 0·3 N sodium hydroxide, acidified with 3 ml of 30% trichloroacetic acid (TCA), and stored on ice for 10–15 min. The precipitate was washed twice with 5 ml of 5% TCA and then heated twice in 5 ml of 5% TCA for 15 min on a boiling water bath. The supernatants, after heating, were saved for the measurement of radioactivity and DNA.

[^{14}C]*uridine incorporation.* Uridine-2-^{14}C (50·8 mCi/m-mole ICN Tracer Lab) was administered 1 hr prior to sacrifice. After tissue homogenization the precipitate was washed with successive 5-ml portions of 0·2 N PCA, 0·2 N PCA containing 4 mM non-radioactive uridine, two 0·2 N PCA washes, 95% ethanol saturated with sodium acetate, ethanol–ether (3:1) and ether. The precipitate was heated at 37° for 1 hr in

4 ml of 0·3 N sodium hydroxide, acidified with 3 ml of 30% TCA and stored on ice for 10–15 min. The precipitate was washed with 2·5 ml of 10% TCA and the supernatant was combined with the supernatant after alkaline hydrolysis for the measurement of radioactivity and RNA.

[^{14}C]*leucine incorporation.* L-Leucine (UL)^{14}C (197 mCi/m-mole, ICN Tracer Lab) was administered 30 min prior to sacrifice. After tissue homogenization the precipitate was washed with successive 5-ml washes of 0·2 N PCA containing 40 mM leucine and 0·2 N PCA. The precipitate was heated at 70° for 30 min in 0·6 N PCA. The precipitate was washed with successive 5-ml portions of 95% ethanol saturated with sodium acetate, ethanol–ether (3:1) and ether. The precipitate was dissolved in 0·3 N sodium hydroxide overnight at 40° prior to the measurement of protein and radioactivity.

Determination of DNA, RNA and protein. DNA was estimated in the TCA extract by the reaction of deoxyribose with diphenylamine.[16] The DNA was quantified using a standard curve prepared from calf thymus DNA. RNA was estimated by the orcinal procedure for determining ribose.[17] Yeast RNA was used as the standard. Protein was measured by the method of Lowry *et al.*[18] using bovine serum albumin as the standard.

Measurement of radioactivity. Radioactivity incorporated into DNA, RNA and protein was measured in Bray's solution[19] using liquid scintillation counting. [^{14}C]leucine incorporation into protein was measured by dissolving an aliquot of the alkaline solution in Hyamine Hydroxide (Packard) and heating the sample for at least 8 hr at 40° prior to the addition of Bray's solution.

Statistics. Statistical analysis was performed by Student's *t*-test.[20] The level of significance was chosen as $P < 0{\cdot}05$.

RESULTS

Day-old mice treated with 80 mg/kg of cyclophosphamide weighed only 60 per cent of control body weights 35 days after treatment. Livers and brains were significantly smaller at this time (50 and 84 per cent of control, respectively) although the organ to body weight ratios were changed only for the brains where an increase was observed (165 per cent of control). These values, which were used as a measure of biologic development, were not different from control during the first week after treatment.

[^{14}C]precursors of DNA, RNA and protein were incorporated into the respective macromolecules of neonatal mice. There was no indication of developmental changes in these values during the 5-day period after saline treatment.

DNA synthesis, measured by [^{14}C]thymidine incorporation, was significantly inhibited in neonatal liver (Fig. 1), brain (Fig. 2) and carcass (Fig. 3) 24 hr after cyclophosphamide. DNA synthesis, at this time, was 8·4, 26 and 25 per cent of control respectively. DNA synthesis remained reduced in the liver and brain for at least 5 days after drug treatment. [^{14}C]thymidine incorporation into DNA of carcasses from treated mice returned to control values 5 days after treatment.

Hydroxyurea, an inhibitor of DNA synthesis, was used as an internal control to distinguish between real incorporation and non-specific binding of [^{14}C]thymidine to DNA.[21] Hydroxyurea, 500 mg/kg administered s.c. to neonatal mice 1 hr before a [^{14}C]thymidine pulse (1 hr), reduced the specific activity of liver, brain and carcass

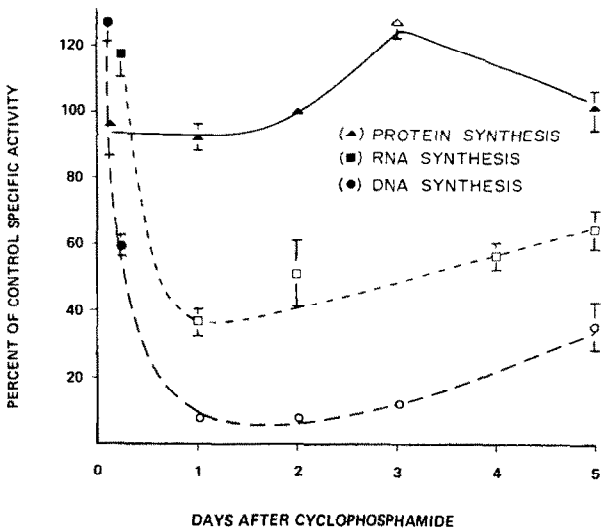

FIG. 1. DNA, RNA and protein synthesis in the liver of cyclophosphamide-treated animals expressed as a per cent of control values. Mice received either 80 mg/kg of cyclophosphamide or normal saline 1 day after birth and 100 μCi/kg of [^{14}C]labeled thymidine, uridine or leucine at various times afterwards. Precursor incorporation into DNA, RNA and protein was measured at various times after treatment. The specific activity of macromolecules from treated animals was expressed as a per cent of the values simultaneously determined in controls. The average control specific activities were: 39,000 dis./min/mg of DNA, 4000 dis./min/mg of RNA and 1000 dis./min/mg of protein. The values represent the mean $\pm$ S.E. for three to six determinations. Open symbols indicate values that are significantly different from control ($P < 0{\cdot}05$).

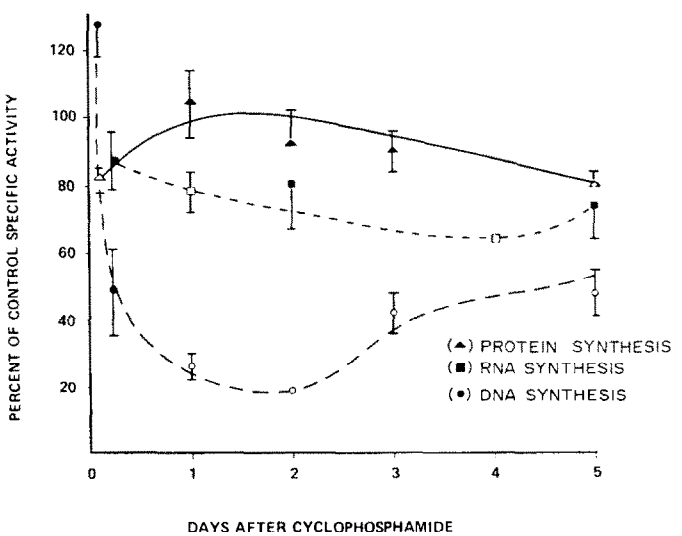

FIG. 2. DNA, RNA and protein synthesis in the brain of cyclophosphamide-treated animals expressed as a per cent of control values. See legend to Fig. 1 for method. The average control specific activities were: 3000 dis./min/mg of DNA, 1000 dis./min/mg of RNA and 500 dis./min/mg of protein.

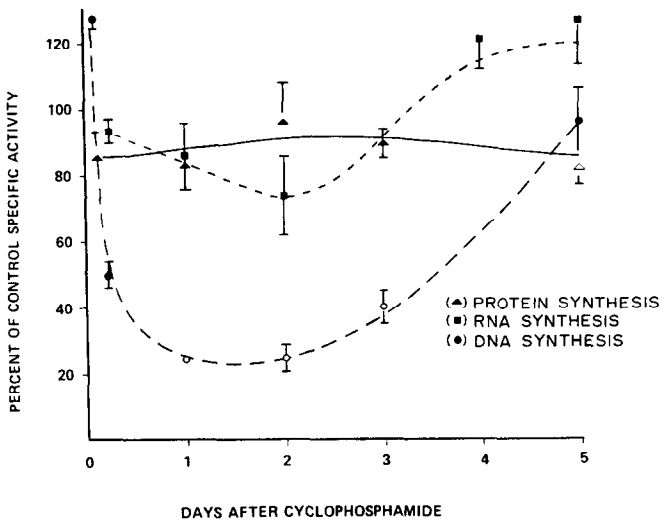

FIG. 3. DNA, RNA and protein synthesis in the carcass of cyclophosphamide-treated animals expressed as a per cent of control values. See legend to Fig. 1 for method. The average control specific activities were: 15,000 dis./min/mg of DNA, 3000 dis./min/mg of RNA and 1000 dis./min/mg of protein.

DNA to less than 1 per cent of the value found in control animals (Table 1). This observation indicated that radioactivity associated with the DNA isolated from neonatal mice was a result of incorporation rather than non-specific binding. Day-old mice treated with 500 mg/kg of hydroxyurea appeared normal and grew at a normal rate for at least 4 weeks despite the dramatic initial inhibition of DNA synthesis.

TABLE 1. HYDROXYUREA INHIBITION OF [^{14}C]THYMIDINE INCORPORATION INTO DNA—RELATIVE DNA SPECIFIC ACTIVITY IN HYDROXYUREA-TREATED NEONATAL MICE*

Liver	Brain	Carcass
0·27 ± 0·03†	0·83 ± 0·27	0·54 ± 0·05

* Hydroxyurea (500 mg/kg) 1 hr before a 1-hr [^{41}C]thymidine pulse in 48-hr-old mice.

† Mean ± S. E. (three observations) of DNA specific activity in hydroxyurea-treated animals expressed as a per cent of control values.

The incorporation of [^{14}C]uridine into total RNA was reduced in neonatal liver between 1 and 5 days after cyclophosphamide (Fig. 1). RNA synthesis in livers from treated mice ranged from 36–64 per cent of control at 1 and 5 days after treatment. RNA synthesis was reduced in brain to 78 and 64 per cent of control on days 1 and 4 after treatment (Fig. 2).

The incorporation of [^{14}C]leucine into total protein did not reveal a consistent pattern which would indicate that drug treatment altered protein synthesis. Protein

synthesis was increased in the liver 3 days after treatment (Fig. 1) and reduced in the brain 3 hr and 5 days after treatment (Fig. 2). Protein synthesis, in addition, was not significantly altered in the liver or brain at 7, 9, 12 or 21 days after drug treatment (Table 2).

TABLE 2. PROTEIN SYNTHESIS IN MICE TREATED WITH CYCLOPHOSPHAMIDE—RELATIVE SPECIFIC ACTIVITY OF PROTEIN*

Days after treatment†	Liver	Brain
7	120 ± 20‡	86 ± 7
9	82 ± 7	74 ± 13
12	106 ± 7	102 ± 3
21	132 ± 25	118 ± 11

* Thirty-min pulse of 100 μCi/kg of [^{14}C]leucine.
† Cyclophosphamide 80 mg/kg 24–48 hr after birth.
‡ Mean ± S.E. (two to three observations) of protein specific activity expressed as a per cent of control.

DISCUSSION

The pharmacokinetic properties of cyclophosphamide differed in neonatal and adult mice.[13] Radioactivity in the plasma of neonatal mice, after [^{14}C]cyclophosphamide, reached peak values 32 min after treatment and was eliminated by apparent first-order kinetics. The half-life of plasma radioactivity was 8·8 hr and the time for elimination of 99 per cent of the plasma radioactivity was calculated to be 2·4 days. The elimination of radioactivity from the plasma of adults, on the other hand, was rapid and biphasic with an initial half-life of 1·9 hr. The parent compound represented 76 and 44 per cent of total radioactivity in plasma from cyclophosphamide-treated neonatal mice at 1 and 8·5 hr respectively. The half-life for the elimination of radioactivity from the liver and brain was 7 and 11 hr respectively.* A dose of nor-nitrogen mustard, which was equimolar with 80 mg/kg of cyclophosphamide, was cleared from the plasma within 1 hr and failed to affect the growth and development of mice.[7]

The pharmacokinetic properties of a teratogenic dose (20 mg/kg) of [^{14}C]cyclophosphamide during embryonic development indicated that the maternal system provided a route for the rapid elimination of cyclophosphamide from the embryo.[22] Radioactivity in maternal plasma and embryos reached peak levels between 16 and 32 min after [^{14}C]cyclophosphamide and attained equilibrium at very low levels of radioactivity by 256 min after treatment. DNA synthesis was inhibited by 12 hr in the maternal liver and embryo but returned to normal at 24 and 48 hr after cyclophosphamide.[23] There was no inhibition of RNA synthesis and protein synthesis was not affected until 72–96 hr after treatment.

The earliest biochemical lesion detected in the present study was an inhibition of [^{14}C-]thymidine incorporation into DNA. This effect occurred 1 day after treatment in all of the tissues studied and lasted for at least 5 days in the liver and brain. This biochemical lesion in neonatal mice was remarkably different from the transient inhibition of DNA synthesis which was correlated with a more rapid elimination of [^{14}C]cyclophosphamide in adult and embryonic mice.[23] The delayed inhibition of DNA synthesis

* Unpublished observation, this laboratory.

noted in neonates was also reported for cyclophosphamide in hamster plasmacytomas and other alkylating agents in both hamster plasmacytomas and Hela cells.[14,24] Cortisol, in addition, inhibited DNA synthesis in rat brain during the period of drug treatment.[25]

The role of reduced DNA synthesis in cyclophosphamide toxicity was complicated by the observation that agents which inhibit DNA synthesis do not necessarily affect development. Hydroxyurea, for example, administered to pregnant rats, in doses that reduced DNA synthesis, did not interfere with embryonic development.[26] Similar results were obtained in the present study because 500 mg/kg of hydroxyurea dramatically inhibited DNA synthesis in neonatal mice but did not affect their growth and development. Since the reduction in DNA synthesis induced by hydroxyurea lasted only 24 hr in rat embryos, the difference between the effects of hydroxyurea and cyclophosphamide may be due to prolonged inhibition of DNA synthesis by cyclophosphamide. Cyclophosphamide alternatively may produce toxicity by a mechanism unrelated to inhibition of DNA synthesis.

Cyclophosphamide inhibited RNA synthesis in the liver between 1 and 5 days after treatment and in the brain at 1 and 4 days after treatment. This inhibition was prolonged relative to the effects previously reported in embryos and adults.[23] This data was consistent with the observation that cyclophosphamide inhibited the synthesis of RNA *in vivo* by hamster plasmacytomas at 24 and 48 hr after treatment.[24] The more prolonged inhibition of RNA synthesis observed in the present study may be due to a reduced DNA template surface for the transcription of RNA molecules. The ability of cyclophosphamide to depress RNA synthesis, in addition, may depend on the amount of the genome being actively transcribed at the time of treatment.

The lack of a consistent change in [^{14}C]leucine incorporation into protein in the present study could be explained on the basis that drug treatment altered protein synthesis qualitatively rather than quantitatively. Drug toxicity, therefore, may be associated with altered tissue specific protein patterns rather than a general depletion of proteins. Developmental changes in the free amino acid pool alternatively may have masked changes in protein synthesis associated with drug toxicity.

In the present study, organ weights after treatment indicated that cyclophosphamide interfered with both liver and brain growth. The liver to body weight ratios demonstrated that drug treatment did not produce a selective reduction in liver growth since body weights were reduced to the same extent as liver weights. Brains, however, were not proportionally affected. Increased brain to body weight ratios was also a consequence of malnourishment during development.[11] Brain growth, relative to body growth in both cyclophosphamide-treated and malnourished neonates, as a result, was spared. Cortisol treatment in rats during the first 4 days after birth produced similar changes.[25]

The biochemical lesions observed in the present study occurred several days before a reduction in growth of the animals was detected. The delayed inhibition of growth suggested that the animals have either a reserve capacity to maintain growth (e.g. long lived or masked messenger RNA) or that it takes a period of time for toxicity to be manifested (e.g. failure of a system to mature). A reduction in DNA synthesis inhibits growth by hyperplasia while a reduction in RNA synthesis prevents the cells from increasing in size and differentiating. The tissue, as a result, may be unable to mature or conduct biochemical operations compatible with life.

The observations made in the present study suggested that cyclphosphamide neonatal toxicity may be produced in affected cells by an alteration in the utilization of genetic information at the transcription level. The study, in addition, demonstrated that the pharmacological properties of a drug, which were different in neonatal and adult animals, predisposed an immature animal to increased toxicity.

REFERENCES

1. P. GERLINGER, J. V. RUCH and J. CLAVERT, *C. r. Seanc. Soc. Biol.* **157,** 1973 (1963).
2. P. GERLINGER, *C. r. Seanc. Soc. Biol.* **158,** 2154 (1964).
3. T. VON KREYBIG, *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **252,** 173 (1965).
4. J. E. GIBSON and B. A. BECKER, *Cancer Res.* **28,** 475 (1968).
5. J. E. GIBSON and B. A. BECKER, *Teratology* **4,** 141 (1971).
6. H. NORDLINDER, *Experientia* **25,** 1296 (1969).
7. R. D. SHORT and J. E. GIBSON, *Experientia* **27,** 805 (1971).
8. F. D. HAYES, R. D. SHORT and J. E. GIBSON, *Proc. Soc. exp. Biol. Med.* **139,** 417 (1972).
9. J. S. BUS and J. E. GIBSON, *Proc. Soc. exp. Biol. Med.* **143,** 965 (1973).
10. S. SCHAPIRO, *Proc. Soc. exp. Biol. Med.* **124,** 771 (1965).
11. J. DOBBING, in *Developmental Neurobiology* (Ed., W. A. HIMWICH), pp. 241–61. Thomas, Springfield, Illinois (1970).
12. R. D. SHORT and J. E. GIBSON, *Toxic. appl. Pharmac.* **19,** 103 (1971).
13. J. S. BUS, R. D. SHORT and J. E. GIBSON, *J. Pharmac. exp. Ther.* **184,** 749 (1973).
14. J. J. ROBERTS, T. P. BRENT and A. R. CRATHORN, in *The Interaction of Drugs and Subcellular Components in Animals Cells* (Ed. P. N. CAMPBELL), p. 5. Churchill, London (1968).
15. E. H. DAVIDSON, *Gene Activity in Early Development*, pp. 38–41. Academic Press, New York (1968).
16. K. BURTON, *Biochem. J.* **62,** 315 (1956).
17. J. CERIOTTI, *J. biol. Chem.* **214,** 59 (1955).
18. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
19. G. A. BRAY, *Analyt. Biochem.* **1,** 279 (1960).
20. R. G. D. STEEL and J. H. TORRIE, *Principles and Procedures of Statistics*, p. 43. McGraw-Hill, New York (1960).
21. C. G. MORLEY and H. S. KINGDON, *Analyt. Biochem.* **45,** 298 (1972).
22. J. E. GIBSON and B. A. BECKER, *J. Pharmac. exp. Ther.* **177,** 256 (1971).
23. R. D. SHORT and J. E. GIBSON, *Teratology* **6,** 129 (1972).
24. G. P. WHEELER and J. A. ALEXANDER, *Cancer Res.* **29,** 98 (1969).
25. M. COTTERRELL, R. BALÁZS and A. L. JOHNSON, *J. Neurochem.* **19,** 2151 (1972).
26. W. J. SCOTT, E. J. RITTER and J. G. WILSON, *Devl. Biol.* **26,** 306 (1971).