Naunyn-Schmiedeberg's Arch. Pharmacol. 288, 7—16 (1975)

Inhibition of RNA Synthesis, a Possible Mode of the Embryotoxic Action of Hydroxyurea

R. Krowke and G. Bochert

Pharmakologisches Institut der Freien Universität Berlin, Abt. "Embryonal-Pharmakologie"

Received May 21, 1974/Accepted January 17, 1975

Summary. ^{14}C-u-glucose and ^{32}P-phosphate were given intravenously to pregnant rats, treated and untreated with hydroxyurea (HU). The incorporation of radioactivity into a variety of cell components of the embryo was measured. With this double-labelling technique it is possible to survey the effects of embryotoxic drugs on various pathways in mammalian embryos *in vivo*. Onset, extent and duration of the metabolic changes were measured after application of "non-teratogenic" and teratogenic doses of hydroxyurea (HU). 3 hrs after application of hydroxyurea only DNA synthesis is affected, whereas after 5 hrs RNA synthesis is also inhibited.

Key words: Hydroxyurea — Mammalian Embryo — Nucleic Acid — *in vivo*.

Studying drug-induced abnormal mammalian development many investigators still apply macroscopic-morphological criteria exclusively. In order to obtain a deeper insight into mammalian embryonic metabolism and drug-induced abnormalities biochemical data are required.

A double-labelling technique has been developed, using ^{14}C-glucose and ^{32}P-phosphate, to follow metabolic changes produced by embryotoxic drugs *in vivo* (Krowke, 1970; Krowke *et al.*, 1971 a—c; Bochert, 1973).

In this paper we report the results of studies on the teratogenic action of hydroxyurea (HU). This cytostatic agent is believed to selectively interfere with DNA synthesis. Chaube and Murphy (1966) were the first to observe malformations following a treatment of pregnant rats with this drug. In our studies the extent and time course of alterations in some metabolic pathways of the embryo are measured and correlated with the result of the abnormal development.

Experimental Conditions

Experimental Animals. Wistar rats (strain SW 72, purchased from Winkelmann, Paderborn, Federal Republic of Germany) of 180—200 g, were kept at a reversed day-night cycle (light from noon to 2.00 a.m). The animals were fed Altro-

Send offprint requests to: R. Krowke, Pharmakologisches Institut der Freien Universität Berlin, Abteilung Embryonal-Pharmakologie, D-1000 Berlin 33, Thielallee 69/73.

This work was supported by grants from the Deutsche Forschungsgemeinschaft given to the Sonderforschungsbereich 29, "Embryonale Entwicklung und Differenzierung Embryonal-Pharmakologie".

min-R and water ad libitum. They were mated for 2 hrs (8.00—10.00 a.m.). The 24 hrs period following mating was called day 0 of pregnancy if sperms were detected in vaginal smears.

Reagents. All chemicals used were p.a. substances purchased from Merck, Darmstadt, Federal Republic of Germany; the enzymes were bought from Boehringer, Mannheim, Federal Republic of Germany. ^{14}C-u-glucose (spec. act. 320 μCi/μmol) and ^{32}P-phosphate (spec. act. 50—140 Ci/mgP) were supplied by the Radiochemical Center Amersham Buchler, Braunschweig, Federal Republic of Germany.

Administration of Drugs and Labelled Precursors. Pregnant rats were used on day 12 of pregnancy. 5 hrs before sacrifice groups of at least 4 rats received an injection of 250 mg, 500 mg, or 750 mg HU/kg. 2 hrs later an injection of a mixture of ^{14}C-u-glucose and ^{32}P-inorganic-phosphate was given intravenously.

In the second experimental series the drug (250 mg/kg) was administered together with the isotopes intravenously. The incorporation of label into the different cell fractions was studied 45 min, 90 min, or 180 min after the injection.

Fractionation of Embryonic Cell Components. A fractionation procedure was used (Krowke *et al.*, 1970) based on the procedures of Schmidt-Tannhauser (1945) and Ogur-Rosen (1950). The method and results regarding the purity of the fractions obtained have been published before (Krowke *et al.*, 1971 a—c). The embryos of one rat were homogenized in 0.2 N perchloric acid (PCA) and aliquots equivalent to about 4 embryos were used for the fractionation of the cell components: After "washing" with PCA, *lipids* were extracted from the precipitate with alcohol/ether (3+1). *RNA* was hydrolized with pancreatic RNase 100 μg/ml, pH 7.5— breaking of cell components was achieved by freezing the samples in liquid nitrogen and thawing in ice water several times. This step was followed by an alkaline hydrolysis (0.3 N NaOH, 37°C for 60 min) which liberated mostly sulphated glycosaminoglycans. This fraction was evaluated for ^{14}C incorporation only. Other carbohydrates were removed by treatment with α-amylase. Subsequently, *DNA* was hydrolysed in 0.5 N PCA for 15 min at 75°C. The acid-insobuble residue of the fractionation was solubilized in 20% NaOH and contains predominantly *protein.*

The hydrolysed DNA fraction is known to be contaminated to some extent with ^{14}C-labelled by-products so that the specific activity can be about 10% too high compared with DNA obtained from isolated and highly purified nuclei by phenol extraction (Krowke, unpublished).

The radioactivity of aliquots of all fractions was counted in a liquid scintillation counter (Packard Tricarb 3380 or Nuclear Chicago Isocap 300). Data were corrected for the spill-over in the low energy channel and counting efficiency was calculated from external standardization. DNA was measured according to Burton (1956).

Results

A survey on the labelling kinetics with the two precursors in non-treated animals is given in Table 1. A closer analysis of the different kinetics for the two precursors has been published before (Krowke *et al.*, 1971 b). For easier documentation the data for the drug-treated animals are given as a percentage (M ± S.D.) of the controls.

Two kinds of experimental series have been performed: 1. Time dependence of an interference of HU with metabolic pathways of the embryo. These experiments were executed with a "non-teratogenic" dose of 250 mg/kg. 2. A dose dependence as evaluated from data obtained 5 hrs after the injection of the drug.

Table 1. Incorporation of ^{14}C-glucose and ^{32}P-phosphate into acid soluble and insoluble material of rat embryos (day 12). M ± S.D. All the data are obtained after i.v. injection of 1 mCi/kg each ^{14}C-glucose and ^{32}P-phosphate. n = number of experiments

Min	n	Acid soluble	Lipid	RNA	NaOH	Carbohydr.	DNA	Protein
^{14}C-dpm/μg DNA								
45	5	450 ± 150	53 ± 10.3	35 ± 6.2	11 ± 1.7	6 ± 1.2	10 ± 1.0	35 ± 5.1
90	4	220 ± 60	78 ± 20	54 ± 16	16 ± 4.1	9.5 ± 3.1	25 ± 7.2	77 ± 20
180	8	170 ± 43	195 ± 20	62 ± 9.1	20 ± 3.2	9.2 ± 3	38.3 ± 4.3	90 ± 10
^{32}P-dmp/μg DNA								
45	5	185 ± 50	1.3 ± 0.4	3.4 ± 1.0			1.1 ± 0.2	
90	4	305 ± 20	4.9 ± 0.5	19.7 ± 1.3			6.5 ± 0.8	
180	8	461 ± 52	12.3 ± 1.7	58.8 ± 7.1			26.1 ± 3.5	

A. Localization and Duration of the Interference with Embryonic Metabolism by HU

The incorporation of radioactivity into DNA was found to be reduced as early as 45 min after i.v. injection of 250 mg/kg HU (Fig. 1). According to Chaube and Murphy (1966) and our own results this is a "non-teratogenic" dose if gross malformations are evaluated. Since the inhibition of incorporation is already 50% (^{14}C) or even more (^{32}P) 45 min after application of the drug, the onset of the effect of HU may be expected to occur quite early after the injection. Due to experimental difficulties it is hard to study the incorporation of radioactive label—especially that of ^{32}P—earlier than 30 min after the injection of the isotopes. The inhibitor is effective for several hours—even with the rather small dose used in these studies—since the inhibition is not released until 3 hrs after application.

From the data of Fig. 1 it is evident that the incorporation of ^{32}P-radioactivity into DNA is inhibited more extensively (about 70—80%) than is the incorporation of ^{14}C-glucose fragments (about 50%). For the evaluation of the incorporation data the radioactivity found in the acid-soluble fraction has to be taken into account. While the radioactivity accumulated in this fraction was about the same as in the controls 45 min after injection of the inhibitor, a considerably higher amount of radioactivity—especially of ^{14}C—was present after 90 min. However, 180 min after the injection the radioactivity had returned to control values again.

Since ^{14}C-radioactivity of the maternal serum of the drug-treated animals was also increased 90 min after the injection, the elevated values in the acid-soluble components of the embryo are apparently the result of an increased availablility of radioactive precursors from the maternal to the embryonic compartment.

^{14}C-radioactivity was, in accordance with these findings, also found increased 90 min after the injection in the lipid, RNA, and protein fractions (Fig. 1). Therefore, there was no evidence of HU affecting the other metabolic pathways of the embryo—such as lipid, RNA, or protein syntheses—under these experimental conditions and the effect was confined to DNA metabolism. It is noteworthy that the incorporation of both precursors into DNA did not exceed 50% with regard to ^{14}C and 80% of ^{32}P and no complete inhibition was achieved during the period of time investigated. However, it has to be taken into account that, due to the increased availability of the ^{14}C-precursor in the acid soluble fraction 90 min after the injection, the incorporation data into DNA at this time do not reflect a true picture and the actual degree of inhibition is higher than the percentage shown in Fig. 1.

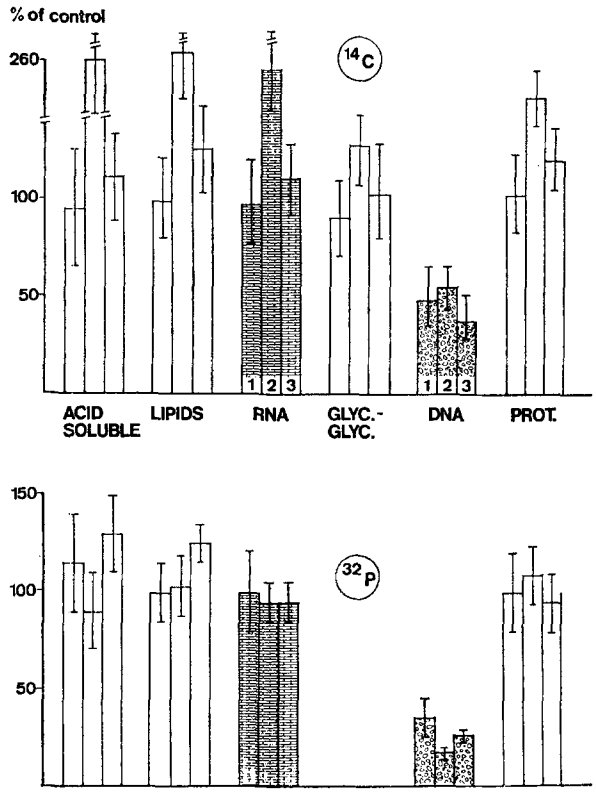

Fig. 1. Effect of 250 mg hydroxyurea/kg on the incorporation of ^{14}C-glucose and ^{32}P-phosphate into different fractions of rat embryos on day 12 of pregnancy. The isotopes were applied i.v.together with the drug. All data are given as $\overline{M} \pm S.D.$ and are expressed as percentage of the control values (cf. Tab. 1) which were measured as dpm/μg DNA. The radioactivity was measured after 45 (1), 90 (2), and 180 (3) min

B. Dose-Response Relationship and Localization of the Metabolic Block

In the second experimental series the effect of three different doses of HU (250, 500, and 750 mg/kg) was studied over a period of 5 hrs (5 hrs drug action, 3 hrs isotope incorporation). The lowest of these doses was just "non-teratogenic" while the two higher doses were able to induce clear-cut gross abnormalities such as deformities of the cerebrum.

As shown in Fig. 2 the data obtained with 250 mg/kg supplement the results of the first experimental series and indicate that the metabolic block in DNA metabolism was largely reversed within the last 2 hrs of the experiment. However, with the higher doses the incorporation of labelled precursors into DNA was still inhibited by more than 50%

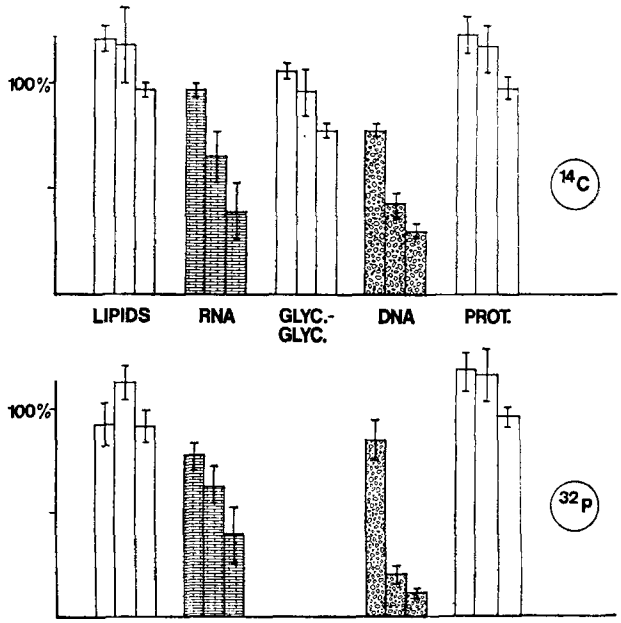

Fig. 2. Dose-response relationship of the inhibition of embryonic metabolism by HU. $\bar{M} \pm$ S.D. of at least four different experiments per dose. Different doses of hydroxyurea were given to pregnant rats on day 12 of pregnancy (columns from left to right 250 mg, 500 mg, 750 mg per kg). 2 hrs later the animals received an i.v. injection of ^{14}C-glucose and ^{32}P-phosphate 1 mCi/kg of each isotope. After another 3 hrs (i.e. 5 hrs drug action, 3 hrs isotope incorporation) the animals were killed and the embryos fractionated as described. Data are expressed as per cent of the control values. Control data are given in Table 1

when the experimental period was extended to 5 hrs. Again the degree of inhibition was higher for the incorporation of ^{32}P than that of ^{14}C-glucose fragments. To some extent the interpretation of the data is again rendered difficult by the finding that the ^{14}C-radioactivity found in the acid-soluble fraction is increased over that of the controls in the studies using 250 and 500 mg/kg HU.

The interference by HU is not confined to the incorporation of the precursors into the DNA fraction, but, the metabolic pathways leading to the labelling of the RNA fractions are also affected. Apparently the block in RNA metabolism takes a longer period to manifest itself than that affecting DNA synthesis. Using the dose of 250 mg/kg HU an impairment of RNA synthesis—as judged from the incorporation of ^{32}P—was not evident during a 3 hrs interval (Fig. 1) but became obvious when the experimental period was incerased to 5 hrs (Fig. 2). No significant effect on the incorporation of ^{14}C-fragments into RNA was seen

with this dose. But with 500 and 750 mg/kg HU a dose dependent decrease of incorporation of both the precursors into RNA could be demonstrated.

Discussion

Embryonic tissue at the stage of organogenesis is the most rapidly proliferating of mammalian tissues (Köhler *et al.*, 1972) having generation times of 8 hrs or even less. In the early stages of organogenesis its proliferation rate even surpasses very rapidly growing tumor tissues in tissue culture (Burk *et al.*, 1967). At the same time numerous differentiation processes are induced. If nucleic acid synthesis is inhibited this might result in an embryotoxic effect. But in very few studies has the extent and duration of such an inhibition been measured in order to demonstrate a causal relationship between biochemical changes and abnormal development.

The biochemical action of HU has been examined predominantly in cell cultures. But this may not be the action responsible for the teratogenic effect on mammals *in vivo*. Therefore, we have studied the effect of HU on the metabolic pathways in rat embryos and have tried to correlate the findings with the induction of gross morphological abnormalities.

Chaube and Murphy (1966) demonstrated that hydroxyurea causes malformations in rats. The amount of 300 mg/kg administered on day 12 of gestation was reported the lowest teratogenic dose. This finding was completely confirmed in our experiments. The comparatively high dose may be required because of the short half-life of HU which in maternal tissue of rats was reported to be 20 min, and 45 min for rat embryos of day 18 of gestation (Rajewski *et al.*, 1971). The LD_{50} in rats was determined to be 4.7 g/kg (Murphy, 1965).

HU is thought to block DNA synthesis due to an inhibition of the thioredoxin system at the level of ribonucleotide diphosphates. Skoog and Nordenskjöld (1971) showed in mouse cell cultures that the concentrations of deoxyribonucleotide triphosphates were greatly decreased in the presence of hydroxyurea. These authors demonstrated that the smallest pool (dGTP) is the first to be exhausted and thereby an inhibition of DNA synthesis is induced. Scott *et al.* (1971) measured the incorporation of ^{3}H-thymidine into the acid soluble material of 12 day old rat embryos *in vivo* after application of HU to the mother animals in the range of 250 to 1000 mg/kg. These authors found a dose and time dependent reduction of incorporated radioactivity. But as Schneider and Greco (1970), Dobson and Cooper (1971), and Morley and Kingdon (1971) showed, thymidine is catabolized in liver tissue and incorporated into macromolecules other than DNA (mainly lipids and protein), so that the measurement of label derived from thymidine in the acid-insoluble material cannot be taken as a valid measure of DNA synthesis.

We were interested in obtaining information on whether the action of HU in embryonic tissues is confined to the inhibition of replication only or whether this drug also interferes with other metabolic pathways, and on the degree of inhibition of DNA synthesis as well as the period of time over which replication is blocked. For this purpose a double-labelling technique was used which had proved valuable already in several previous studies (Krowke *et al.*, 1971 a—c). The two radioactive precursors—^{14}C-glucose and ^{32}P—were chosen after evaluating a variety of different metabolites for this purpose (Krowke, 1970). The advantages of the use of these precursors are:

a) They do not alter the physiological concentrations in the maternal serum or in the maternal and embryonic cells nor the concentrations of these precursors or of their metabolites;

b) a number of different cellular components become labelled rapidly (Table 1).

The procedure—using glucose and phosphate as precursors for studying embryonic metabolism *in vivo*—can be employed for screening substances for their possible interference with a variety of metabolic pathways in the embryos. Inhibition of the synthesis of main cellular components such as DNA, RNA, or protein can be detected and compared with the occurrence of developmental abnormalities. More specific precursors very often enter the embryonic compartment less readily, give only limited information and are often too expensive for these purposes. By using HU as a model substance we also tried to determine whether our methods (Krowke, 1970; Bochert *et al.*, 1973) can localize effects in various metabolic pathways of mammalian embryos after *in vivo* application. Measurement of the flow rates in metabolic pathways of the embryo under drug-induced pathological conditions is faced with enormous difficulties because of a very complex situation. The rate is the result of several variables including enzyme reactions, pool sizes, and permeability in the maternal and embryonic compartments. Therefore, our data do not represent real incorporation rates, but they certainly give a hint of a drug-induced interference with a metabolic pathway. These problems will be dicussed elsewhere in more detail (Krowke and Neubert, 1975). Our studies show that the metabolic block produced by HU certainly is not only confined to the DNA synthesis but also includes RNA metabolism. To our knowledge such an interference of HU with RNA metabolism has not been described for any other experimental system before. It will be of interest to test whether such an effect also can be observed in tumor tissues and other systems known to be sensitive to the action of HU.

Light and electron microscopic examinations showed that extensive cell necroses occurred in the mid zone of the wall of brain vesicles and in the blastemas of the limb buds 3 hrs after injection of HU at a dose

of 250 mg/kg or more (Merker, personal communication). Similar necroses have been described by Scott *et al.* (1971), and Ritter *et al.* (1971, 1973) to occur in those tissues where malformations can be detected at later stages of development. Thus areas of cell necroses were found especially in limb buds. As parts of the developing brain are also affected by HU, disturbances of central nervous function after birth may result (Butcher *et al.*, 1973).

It seems unlikely that inhibitors of DNA synthesis should lead to rapid and extensive cell death within such a short time. Other antimetabolites as cytosinarabinoside (ara-C) or fluorodeoxycytosine, which also inhibit DNA synthesis in mammalian tissue *in vivo* within 1—2 hrs (Krowke *et al.*, 1971 a—c; Bochert and Krowke, 1972) do not cause the appearance of necroses until much later. As shown by our experiments with the teratogenic dosages of 500 and 750 mg/kg, not only DNA but also RNA synthesis is affected (Fig. 1). It is possible that the effect of HU on RNA synthesis is the cause of rapid cell death. We suggest that this is the main reason for cell death as early as 3 hrs after application of the drug. Since such a pronounced effect on the total RNA synthesis was observed after 5 hrs with a teratogenic dose of HU and since it is well known that the major portion of the RNA synthesized during this time interval is ribosomal RNA, it seems fair to conclude that the inhibition of synthesis of specific RNA species made in relatively small amounts (e.g. mRNAs) could not be detected by our methods but may occur much earlier and be responsible for cell death.

Most of the experiments with hydroxyurea and similar compounds were exclusively focussed on DNA synthesis (e.g. Scott *et al.*, 1971). Effects occurring on other pathways in mammalian embryos, for instance RNA synthesis, are frequently not detected as the method chosen is not flexible enough and allows examination of one fraction only, e.g. DNA fraction by measuring thymidine incorporation.

References

Bochert, G., Krowke, R.: Effect of 5-fluoro-deoxycytidine (FCdr) on DNA metabolism of mouse embryos (CF1). Teratology **8**, 216 (1973)

Bochert, G., Mewes, P., Krowke, R.: Labelling of RNA and DNA moieties of mammalian embryos *in-vivo* by ^{14}C-u-glucose and ^{32}P-orthophosphate. Naunyn-Schmiedeberg's Arch. Pharmacol. **277**, 413—428 (1973)

Burk, K. Y., Woods, M., Hunter, Y.: On the significance of glycolysis for cancer growth, with special reference to Morris rat hepatomas. J. nat. Cancer Inst. **38**, 839—863 (1967)

Burton, K.: A study of conditions and mechanism of the reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. **62**, 315 (1956)

Butcher, R. E., Scott, W. J., Kazmaier, K., Ritter, E. J.: Postnatal effects in rats of prenetal treatment with hydroxyurea. Teratology **7**, 161—166 (1973)

Chaube, S., Murphy, M. L.: The effects of hydroxyurea and related compounds on the rat fetus. Cancer Res. **20**, 1448—1457 (1966)

Chaube, S., Murphy, M. L.: Protective effect of deoxycytidilic acid (CdMP) on hydroxyurea—induced malformation in rats. Teratology **7**, 79—88 (1973)

Dobson, R. L., Cooper, M. F.: Incorporation of radioactivity from thymidine into mammalian glucose and glycogen. Biochim. biophys. Acta (Amst.) **254**,393—401 (1971)

Köhler, E., Merker, H.-J., Ehmke, W., Wojnorowicz, F.: Growth kinetics of mammalian embryos during the stage of differentiation. Naunyn-Schmiedeberg's Arch. Pharmacol. **272**, 169—181 (1972)

Krowke, R.: Studies on the incorporation of ^{14}C-glucose and ^{32}P-phosphate into nucleic acids and other cell components of rat embryos *in vivo*. In: R. Bass, F. Beck, H.-J. Merker, D. Neubert, and B. Randhahn: Metabolic pathways in mammalian embryos during organogenesis and its modification by drugs. Berlin: Freie Universität 1970

Krowke, R., Pielsticker, K., Siebert, G.: A simple fractionation procedure for studying incorporation of radioactively labelled precursors into mammalian main cell components. Naunyn-Schmiedegs Arch. Pharmak. **271**, 121—124 (1971a)

Krowke, R., Siebert, G., Neubert, D.: Biochemical screening test with ^{14}C-glucose and ^{32}P-phosphate for the evaluation of embryotoxic effects *in vivo*. Naunyn-Schmiedebergs Arch. Pharmak. **271**, 274—288 (1971b)

Krowke, R., Siebert, G., Neubert, D.: Effect of 5-fluoro-deoxycytidine, 5-fluorouracil and cycloheximide on the incorporation of ^{14}C-glucose and ^{32}P into different cell components of rat embryos *in vivo*. Naunyn-Schmiedebergs Arch. Pharmak. **268**, 229—234 (1971c)

Krowke R., Neubert D.: Embryonic intermediary metabolism under normal and pathologial conditions. In: Handbook of Teratology (J. G. Wilson, ed.) Sect. III; 6. New York: Academic Press, 1975 (in press)

Morley, C. G. D., Kingdon, H. S.: Use of ^{3}H-thymidine for measurement of DNA synthesis in rat liver—a warning. Analyt. Biochem. **45**, 298—305 (1971)

Ogur, M., Rosen, G.: The nucleic acids of plant tissue: I. The extraction and estimation of deoxypentose nucleic acid and pentose nucleic acid. Arch. Biochem. **25**, 262 (1950)

Rajewsky, M F., Fabricius, E., Hülser, D. F.: Synchronisation *in vivo*: Temporary inhibition of DNA synthesis in the rat embryo with hydroxyurea. Exp. Cell Res. **66**, 489—492 (1971)

Ritter, E. J., Scott, W. J., Wilson, J. G.: Teratogenesis and inhibition of DNA synthesis induced in rat embryos by cytosine arabinoside. Teratology **4**, 7—14 (1970)

Ritter, E. J., Scott, W. J., Wilson, J. G.: Relationship of temporal patterns of cell death and development of malformation in rat limb. Possible mechanism of teratogenesis with inhibition of DNA synthesis. Teratology **7**, 219—226 (1973)

Schmidt, G., Tannhauser, S. J.: A method for determination of deoxyribonucleic acid, ribonucleic acid and phosphorproteins in animal tissues. J. biol. Chem. **161**, 83—89 (1945)

Schneider, W. C., Greco, A. E.: Incorporation of pyrimidine deoxyribonucleosides into liver lipids and other components. Biochim. biophys. Acta (Amst.) **228**, 610—626 (1970)

Scott, W. J., Ritter, E. J., Wilson, J. G.: DNA synthesis inhibition and cell death associated with hydroxyurea teratogenesis in rat embryos. Develop. Biol. **26**, 306—315 (1971)

Skoog, L., Nordenskjöld, B.: Effects of hydroxyurea and 1-β-D-arabinofuranosylcytosine on deoxyribonucleotide pools in mouse embryo cells. Europ. J. Biochem. **19**, 81—89 (1971)