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Embryonic Development and Mitochondrial Function

1. Effects of Chloramphenicol Infusion on the Synthesis of Cytochrome Oxidase and DNA in Rat Embryos during Late Organogenesis

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Summary. Cytochrome oxidase, which is partially synthesized by the mitochondrion, was used as a measure for the development of mitochondrial function in rat embryos during the late stage of organogenesis. For this purpose the specific inhibitor of mitochondrial protein synthesis, chloramphenicol (CAP), served as a tool. Due to the rapid elimination rate of CAP from rats, a method for continuous infusion which would not cause immobilization to the animals was devised.

1. Pharmacokinetic studies proved that CAP reaches the embryo before placentation. Concentrations of CAP in the embryo are as high as they are in the maternal serum (about 20 μg/ml serum or g embryo) and thus are sufficiently in supply for the inhibition of mitochondrial protein synthesis, if 1000 mg/kg CAP are infused intravenously per 24 hrs. CAP is partially excluded from the embryonic compartment after the placental barrier has fully developed: whereas CAP concentration in the maternal serum remains at about 20 μg/ml, the concentration in the embryonic compartment drops to about 10 μg/g embryonic tissue during day 13 of gestation.

2. The average cytochrome oxidase activity per cell is very low (about 1 nmole O_2/min × μg DNA^{-1}) in embryonic tissue as it is in many other rapidly proliferating tissues. It is 15—60 times higher in slowly proliferating tissues, as, for example, the adult rat liver or brain (> 14 nmoles O_2/min × μg DNA^{-1}).

3. When the infusion technique is applied on day 12 of gestation, a sufficiently high concentration of CAP in embryonic tissue can be obtained to inhibit the synthesis of cytochrome oxidase. In contrast to tissues of an adult organism—as in the case of liver after partial hepatectomy—in embryonic tissues this limitation in the availability of cytochrome oxidase apparently results in a critical reduction of energy production, which subsequently affects DNA synthesis and embryonic growth.

4. The possible relevance and applicability of these experimental findings to man is discussed.

Key words: Embryonic Development — Mitochondrial Protein Synthesis — Chloramphenicol — Continuous Intravenous Infusion — Cytochrome Oxidase.

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Since mammalian embryos during the phase of organogenesis develop at a very rapid rate, a sufficient supply of energy must be guaranteed. Little data are available on biogenesis and function of mitochondria—the cell organelle of highly efficient ATP production—and even less is known about their possible role during embryonic growth and differentiation processes (cf. Neubert *et al.*, 1975). Several changes in the metabolic behavior of mammalian embryos have been reported to take place at the stage of placentation. These changes suggest a shift from a partially anaerobic metabolism towards an energy supply provided by the mitochondrial machinery (Neubert *et al.*, 1971; Cox and Gunberg, 1972a and b).

We have, therefore, been concerned with the question whether there is a connection between mitochondrial function and growth and differentiation processes occurring at the time of placentation. For this purpose chloramphenicol (CAP) served as a tool. This antibiotic is known to inhibit not only protein synthesis at the 70 s ribosomal level of bacteria, but also amino acid incorporation in mammalian mitochondria. It does not severely affect protein synthesis of 80 s ribosomes of the mammalian cells (cf.: Roodyn and Wilkie, 1968). Cytochrome oxidase is a typical membrane-bond mitochondrial enzyme. The de novo synthesis of this last and essential component of the respiratory chain is CAP-sensitive.

Another purpose of this paper is to discuss the question as to what extent data on embryotoxicity from animal experiments may be further used as a reference model for man.

Materials and Methods

Animal Breeding and Drug Treatment

Female Wistar spf rats of the strain SW 72 (Winkelmann, Kirchborchen, Germany), weighing 150—170 g, were kept under a reverse day/night cycle. The light period lasted from noon to 2 a.m. The animals were mated with males of the same strain between 6 a.m. and 9 a.m. The 24 hrs period following the detection of sperms in the vaginal smears was called day 0 of pregnancy. Altromin-1324 and water were fed ad libitum. Rat embryos were used at the late stage of organogenesis (day 11—14 of gestation). The rats were decapitated, the embryos quickly removed and homogenized in 0.32 M sucrose, 2 mM tris-(hydroxy-methyl)-methyl-2-aminoethane-sulfonic acid (TES-) buffer, pH 7.4, 3 mM $MgCl_2$.

Continuous intravenous infusion was used since it was necessary to maintain sufficiently high CAP serum levels in order to induce a depression of mitochondrial protein synthesis during the experimental period (Oerter and Bass, 1972). A method developed by Löbbecke *et al.* (1969) was modified in such a way as to allow up to 15 rats to be infused simultaneously over a period of several days. A plastic catheter (outer diameter 0.3 mm) was placed into one of the tail veins. The catheter was connected to a metal swivel. This device was made to rotate freely, so as not to hinder the animals in their mobility. Biting of the tubings was prevented by a plexiglass housing which covered all of the tail, as can be seen in Fig. 1.

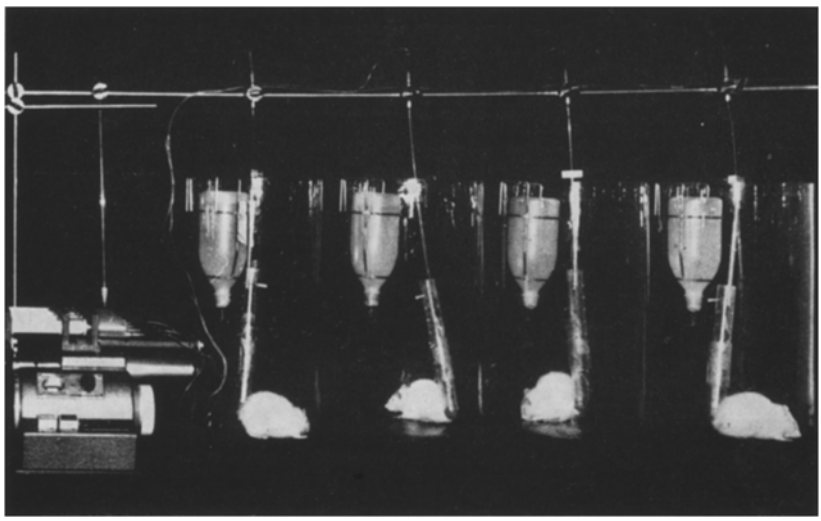

Fig. 1. Apparatus used for continuous intravenous infusion of chloramphenicol. For easier observation the rats were placed in glass jars with a diameter of 20 cm. Within this area the rats could move and turn freely, hindered only by their tail caged in plexiglass in order to prevent biting of the infusion tubing. CAP was infused with a pump from Braun, Melsungen (type Unita) which delivered 10 ml per 24 hrs and rat

Determination of Chloramphenicol (CAP)

CAP was determined colorimetrically using a method developed by Glazko *et al.* (1949) and modified later by Vömel and Spingler (1959). This modification implies that only microbiologically active molecules containing the nitro group were to be extracted by ethyl acetate. Metabolites like glucuronides, however, were to remain in the aequous phase. The nitro group is then reduced by $TiCl_3$, the solution reacidified with 4 N HCl and diazotized by $NaNO_2$. After the elimination of the excess nitrite by the use of ammonium sulfamate, the diazonium salt is then coupled with N-napthyl (1) ethylene-diammoniumdichloride and the colored product determined at 546 nm in a Gilford 2400 spectrophotometer.

Measurement of Cytochrome Oxidase (cytox) Activity

Cytox (cytochrome a, a_3, E.C. 1.9.3.1.) activity was measured polarographically according to Schnaitman *et al.* (1967). A 2 ml reaction chamber with a Clark oxygen electrode type YSI 4004 (Yellow Spring Instrument Co., Yellow Springs, Ohio, U.S.A.) linked to a 10 mV recorder was used. The temperature was kept at 25°C. Composition of the medium: 100 mM potassium phosphate, pH 7.4, 0.2 mM N,N,N′,N′-tetramethyl-p-phenylendiamine dihydrochloride (TMPD), 11.6 mM sodium ascorbate, 0.05 mM cytochrome c, 5—100 µl homogenate corresponding to 1—300 µg DNA, depending on the source of the tissue. The reaction was started by the addition of cytochrome c after incubating the homogenate with the nonionic detergent Lubrol WX (0.95% final concentration) for 15 min at 0°C.

Determination of DNA

DNA was determined by using the method as developed by Burton (1956) and its modification as described by Neubert *et al.* (1968). After several washings with cold 2% perchloric acid DNA was extracted with 5% perchloric acid at 75°C (15 min).

Reagents Used

TMPD: Fluka, Buchs, Switzerland; cytochrome c: type III, Sigma Co.; Lubrol WX: ICI-Chemicals. All other reagents were of analytical grade.

Results

Conditions for the Measurement of Cytochrome Oxidase Activity

The use of isolated mitochondrial fractions in the polarographic estimation of cytochrome oxidase has been described (Schnaitman *et al.*, 1967). However, it must be emphasized that pure mitochondrial fractions from embryonic tissue cannot be isolated easily. Total cell DNA, therefore, serves as a better reference than mitochondrial protein. It enables us to measure the amount of this mitochondrial component per cell or even per whole embryo. For these reasons cytox was measured in homogenates. It first had to be established whether this method could be equally well applied to homogenates.

Only after the addition of detergents can reproducible results for mitochondrial fractions be obtained (Smith and Camerino, 1963). We have found that the same holds true for homogenates. Cytochrome c, which is added in excesss amounts, is kept at a reduced state by TMPD and ascorbate. However, ascorbic acid solutions may consume oxygen without enzyme present (Barron *et al.*, 1936); this autoxidation was described when preparations of cytochrome c were used (Borei, 1945). Lehninger *et al.* (1954) found such an autoxidation in 2 out of 3 commercially available cytochrome c preparations. We investigated the influence of cytochrome c concentration on the rate of oxygen consumption with and without the presence of enzyme. Fig. 2a shows the oxygen consumption in relation to cytochrome c concentration without enzyme added (autoxidation). In Fig. 2b activity was determined as a function of substrate concentration (cytochrome c) at constant enzyme concentration and plotted with (II) and without (I) substraction of the blank. The reaction rate I represents the sum of two independent functions. The initial rate resembles a normal metabolic substrate binding curve. Oxygen consumption with higher concentrations of cytochrome c might be interpreted as resulting from high blank values due to cytochrome induced autoxidation. Regular enzyme kinetics result after substraction of the blank, the K_m for cytochrome c being 1.25×10^{-5} M. This agrees well with the value reported for beef heart mitochondria:

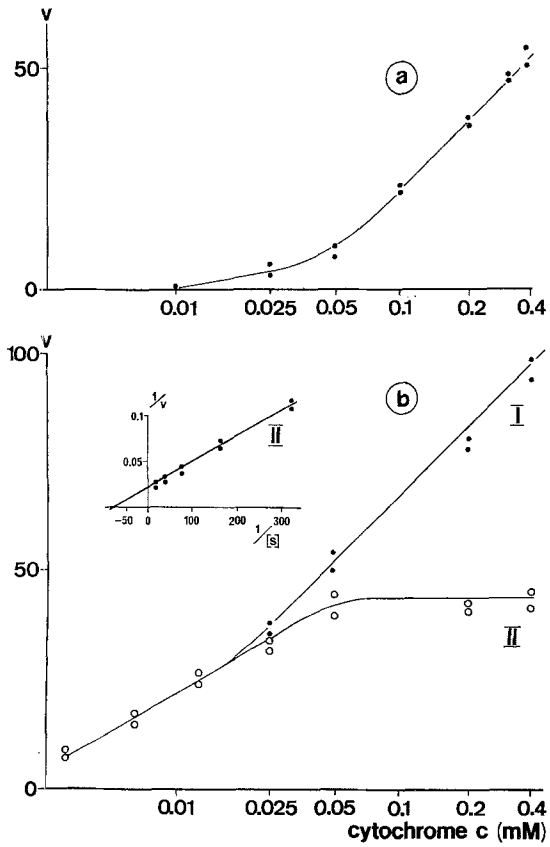

Fig. 2 a and b. Dependence of oxygen consumption on substrate concentration (cytochrome c). (a) No enzyme added, (b) at constant enzyme concentration, results are plotted with (II, open circles) and without (I, closed circles) substraction of the blank values from Fig. 2a. In the Lineweaver-Burk-plot the corrected values are shown, V = nmoles oxygen consumed per minute. 2 ml system, 25°C. For composition of the medium see "Methods"

1.2×10^{-5} M (Wharton and Tzagoloff, 1967). The linear relationship between enzyme concentration and activity measured is given in Fig. 3 a—d for homogenates from rat embryos, liver, kidney, and brain. The concentration of cytochrome c used in these and the following experiments was 0.05 mM.

Cytochrome Oxidase Activity in Tissues with Different Proliferation Rates

Using several rapidly proliferating and slowly proliferating tissues as comparisons, data for cytochrome oxidase activities per DNA were

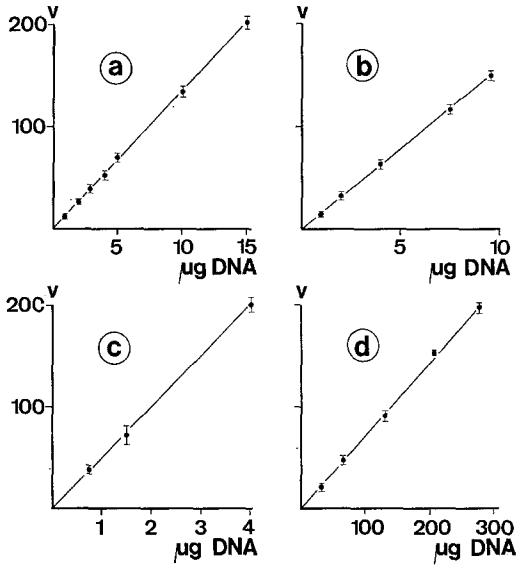

Fig. 3a–d. Linearity between oxygen consumption mediated by cytochrome oxidase and enzyme concentration. Homogenates were preincubated at 0°C with Lubrol WX (0.95% final concentration) for 15 min. The amount of enzyme added is expressed as µg DNA. (a) adult rat liver, (b) adult rat kidney, (c) adult rat brain, (d) rat embryo (day 14 of gestation), V = nmoles oxygen consumed per minute. 2 ml system, 25°C. Concentration of cytochrome c: 0.05 mM. For composition of the medium see "methods"

Table 1. Activity of cytochrome oxidase in tissues with different proliferation rates

	Bone marrow (guinea pig)	Embryo (rat) day 14 of gestation	Testis (rat)	Kidney (rat)	Liver (rat)	Liver (72 hrs after partial hepatectomy)	Brain (rat)
cytochrome oxidase activity (nmoles oxygen consumed × min^{-1} per µg DNA	0.2 ± 0.03	0.94 ± 0.1	3.0 ± 0.2	17 ± 3	15.43 ± 2.13	14.07[a] ± 1.26 7.55[b] ± 0.9	49 ± 9

[a] Without CAP treatment; [b] 1000 mg CAP/kg infused intravenously per 24 hrs.
M ± S.D. of at least 6 estimations.
Cytox and DNA were measured as described under "Methods".

compiled in Table 1. Cytox activity is high in adult-almost non-proliferating—tissues like the liver, brain, and kidney, but much lower in rapidly proliferating tissues like bone marrow, embryonic material, and

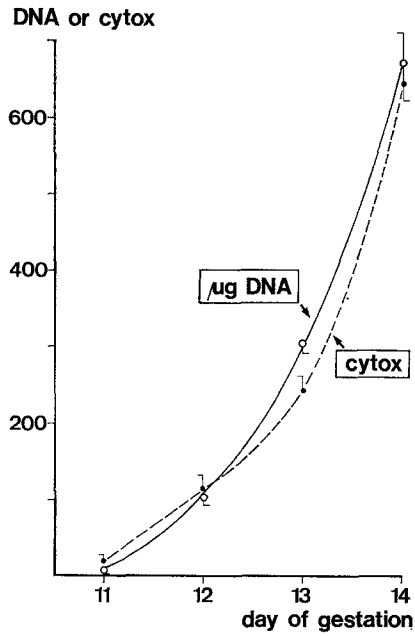

Fig. 4. DNA content and cytochrome oxidase activity during embryonic development (day 11—14 of gestation), whole rat embryos (homogenate). Abscissa: day of gestation, Ordinate: µg DNA per embryo (open circles), nmoles oxygen consumed (cytox) per minute, per embryo (closed circles). For experimental details see "Methods"

testes. Rat embryonic tissue is about 15—20 fold less active than adult rat liver and about 40—60 fold less than adult rat brain when compared on a DNA basis.

Cytochrome Oxidase Activity during Embryonic Development

In Fig. 4 data are given for the increase of cytox and DNA per embryo during the late stage of organogenesis. Cytox activity increases from day 11—14 of gestation by a factor of about 45, the increase in DNA content is even higher. This results in a slight decrease in the cytox/DNA ratio from 1.2 on day 11 to 0.9 on day 14. The increase in DNA content per embryo indicates an average of almost 6 replications over the 72 hrs period studied.

Cytochrome Oxidase Activity in Embryonic Tissue after Chloramphenicol Treatment

Pregnant rats received a continuous intravenous infusion of CAP over a 48 hrs period from day 12 to 14 of gestation in order to study the

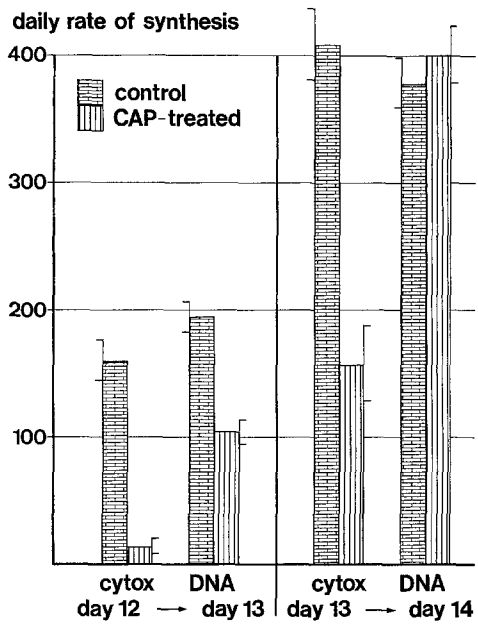

Fig. 5. Influence of CAP infusion on the net increase of DNA and cytochrome oxidase during embryonic development (day 12—13; day 13—14 of gestation). DNA: Net increase in 24 hrs in μg per embryo. Cytox: Net increase in 24 hrs in nmoles consumed per minute per embryo. 1000 mg CAP/kg infused intravenously per 24 hrs. The values are the means ± S.D. from 11—14 experimental animals (equal to 7—9 estimations) for each day and group

possible correlation between mitochondrial function and embryonic growth and differentiation during the late stage of organogenesis. Initial experiments performed in our laboratory had shown that—due to the very short half-life (25 min) of the antibiotic in rats (Alvin and Dixit, 1974; Ferrari and Della Bella, 1974)—doses of 1000 mg CAP infused per kg and day were needed to reach serum levels of about 20 μg/ml in the mother animals. This same dose was used for the following experiments. Cytox and DNA were used as parameters for mitochondrial and overall embryonic development, respectively. The total amounts of DNA and cytox per embryo on day 12, 13 and 14 of gestation were measured. These data were then used to calculate the daily net increase of the two cell components between day 12 and 13, day 13 and 14 in controls and CAP treated animals (Fig. 5). During the first 24 hrs period (day 12—13 of gestation) the CAP infusion almost completely blocked the synthesis of cytox within the embryo. The normal increase in the DNA content per embryo is also found to be reduced by about 50%.

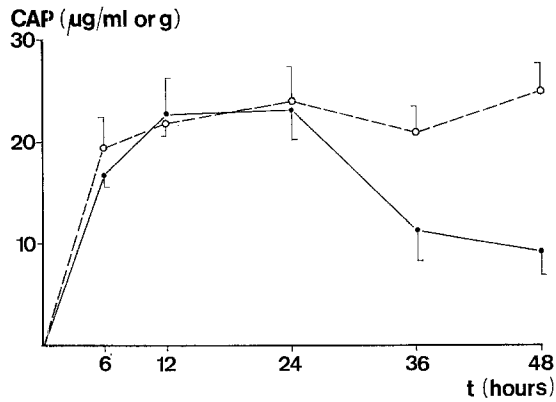

Fig. 6. Concentration of active (non-metabolized) CAP in maternal serum and embryo. Ordinate: o-----o µg CAP per ml serum, •——• µg CAP per g (wet weight) embryo. Abscissa: Duration of CAP infusion, 1000 mg CAP/kg infused intravenously per 24 hrs. 0—24 hrs of infusion = day 12 of gestation, 24—48 hrs of infusion = day 13 of gestation. The values are the means ± S.D. from 12—15 estimations in each category

When CAP infusion is continued for a second 24 hrs period (day 13—14 of gestation) cytox synthesis has partially resumed and DNA is synthesized at a rate equal to controls, so that a growth retardation can no longer be observed.

Chloramphenicol Concentration in Maternal Serum and Embryonic Tissue during Infusion

Since CAP at the dose used was no longer effecting embryonic growth on day 13 of gestation, we measured the concentration of active CAP in maternal serum and in embryonic tissue on day 12 and 13 of pregnancy. Whereas serum levels have reached their maximum after 6—12 hrs of infusion and remain constant over a 48 hrs period, CAP concentration in the embryo decreases significantly during the second 24 hrs period of infusion (Fig. 6).

Discussion

Cytochrome Oxidase Activity and Its Influence on Embryonic Development

In our studies cytochrome oxidase was used as an example for a mitochondrial component which is partially synthesized by the mitochondrion itself (Weiss *et al.*, 1971; Rubin and Tzagoloff, 1973). After having established conditions to reliably measure cytox activity in homogenates, cytox activities per cell were compared in tissues with different growth rates. Interestingly enough, tissues with a compara-

tively low growth rate exhibit a rather high ratio of cytox/DNA, whereas several rapidly proliferating tissues like the embryo, bone marrow and testes show considerably lower values. Using older fetuses Jacovic *et al.* (1971) also had found lower cytox activity in embryonic liver as compared to adult tissue. The low activity of cytox per DNA found in rat embryo is still sufficient for a very rapid embryonic development.

The antibiotic CAP has been used extensively for studies on mitochondrial protein synthesis *in vivo* and *in vitro*. It was shown that CAP *in vitro* does not influence protein synthesis taking place at the 80 s ribosomes of the mammalian cell (Kroon, 1965; Lamb *et al.*, 1968; cf. Roodyn and Wilkie, 1968). Experiments conducted in our laboratory have confirmed these findings with embryonic tissue as well.

It was seen in our studies that a 24 hrs infusion of CAP from day 12—13 of gestation leads to a cytox/DNA reduction of about 50%. CAP infusion for 72 hrs following partial hepatectomy under similar experimental conditions also reduces cytox per DNA by about 50% (Table 1). Such a comparison of the percentages of inhibition, however, can be misleading. The amounts of cytox and DNA synthesized during the experimental period have to be evaluated separately for a better correlation of the effects of CAP on regenerating rat liver and on rat embryos. When such calculations are performed it becomes evident that for both experimental setups *cytox synthesis* is blocked considerably by CAP (embryo: Fig. 5; partial hepatectomy: Kroon and De Vries, 1969). Drastic differences become obvious when the influence of CAP on *liver regeneration* and on *embryonic growth* are compared: Whereas the doubling of the DNA content normally taking place within 72 hrs after partial hepatectomy occurs equally well in the presence of CAP (Firkin and Linnane, 1969; Kroon and De Vries, 1969), overall embryonic development is severely impaired if the synthesis of cytox is drastically inhibited by CAP. The threefold increase in DNA content per embryo normally occurring between day 12 and 13 of gestation is reduced by 50%.

We explain this difference of the pharmacological effect of CAP on these two proliferating tissues in the following way: there is apparently an excess of cytox activity in liver tissue so that its "dilution" per cell by cell division during regeneration under CAP is still compatible with a normal regeneration process. Chance and Schindler (1965) had suggested from the evaluation of other data that cytox in adult tissues is not the rate limiting step in oxidative phosphorylation. In a tissue with a low average amount of cytox per cell and high frequency of cell division, for example embryonic tissue, CAP treatment and the subsequent "dilution" of cytox per cell does lead to cytox becoming the rate limiting step in oxidative phosphorylation. Growth is apparently stopped when oxidative ATP synthesis is reduced below a critical level.

When CAP infusion is continued for a second 24 hrs period (day 13—14) its effect on embryonic growth levels off and this is paralleled by the finding that cytox synthesis can be detected again. This indicates that there is a minimal threshold level of cytochrome oxidase activity needed for normal proliferation rates. Once this minimum of cytox can be synthesized, proliferation may proceed. Under these experimental conditions this results in CAP becoming less effective.

Pharmacokinetics of Infused Chloramphenicol

CAP, given to rats, is excreted very rapidly as glucuronide in the bile (Uesugi *et al.*, 1974). It can be calculated that up to 70—80 mg of CAP can be eliminated per hour and kg. The resulting half life of CAP in rat serum is approximately 25 min (Alvin and Dixit, 1974; Ferrari and Della Bella, 1974). No pharmacokinetic data on CAP in the embryonic tissue have so far been available. Therefore, we measured the concentration of active CAP over the full 48 hrs period described above. Our findings show (Fig. 6):

1. that CAP in the serum of the mother animals reaches steady state conditions after 6—12 hrs of infusion,

2. that CAP reaches the rat embryo before and after placentation,

3. that—assuming one μg CAP/g wet weight (embryo) roughly corresponds to one μg CAP/ml (serum)—CAP concentrations in the serum of the mother and in the embryo are very similar during the first 24 hrs period of infusion,

4. that, however, CAP concentration in the embryo during the second 24 hrs period is significantly lower when compared with the maternal serum.

There are two possible explanations for this: the embryo becomes capable of inactivating CAP between day 12 and 13. This explanation is, however, unlikely, as studies on drug metabolizing enzymes have shown that these reactions—including the capability to glucuronidate—become active in experimental animals only after birth (Fouts and Adamson, 1959; Dutton, 1963).

We favor a second possibility: the placental barrier on day 12 of gestation is different from that on day 13, so that CAP is partially excluded from the embryonic compartment. During the second day of CAP infusion cytox synthesis is still on the average reduced by about 50%, and CAP does not inhibit the steep increase in DNA synthesis which normally occurs. Our evidence suggests that for rat embryos an inhibition of cytox synthesis by more than 50% is necessary before effects on embryonic growth at this stage of development can be detected. From our results it can be further concluded that 20 μg CAP/g embryonic

wet weight leads to a drastic block in the cytox synthesis as well as to a considerable growth retardation, whereas 10 μg/g wet weight still inhibits cytox synthesis but does not affect DNA synthesis. This very well corroborates the *in vitro* experiments performed in our laboratory. These experiments indicated that 10 μg/ml CAP inhibit about 50% of the embryonic mitochondrial protein synthesis, whereas 20 μg/ml CAP inhibits 80—90% (Jäger and Bass, 1975).

Possible Significance of These Findings for Conclusions Relevant to Man

A comparison of the amounts of CAP given to humans for therapeutic reasons (30 mg/kg × day) with the amounts needed to produce embryotoxic effects in rats (1000 mg/kg × day) shows that the latter seems to be a very high dose. But in order to establish a valid comparison the serum concentrations reached in these two species have to be evaluated: whereas the doses applied differ by a factor of 33, the resulting serum levels differ only by a factor of about 4. Under these conditions the CAP dose mentioned yields serum levels of 4—6 μg/ml in man (Walter and Heilmeyer, 1969), and our dose given to rats results in serum levels of 20 μg/ml (Fig. 6). The apparent discrepancy between the two species is explained by the ability of the rat liver to detoxify CAP much faster than the human organism, whereby a much longer half life results in humans: about 200 min (Bartman, 1974) as compared to about 25 min in rats.

From our data it can be concluded:

a) Significance of Pharmacokinetic Studies in Teratology. Pharmacokinetic data must be available on both man and animal species if conclusions regarding teratogenic hazards possibly existing in man are to be drawn from teratological animal experiments. We feel that up until now this point has not been often adequately acknowledged in teratological studies.

b) Likelihood of Embryotoxic Effects in Man Induced by Chloramphenicol. A number of conclusions may be drawn regarding the likelihood of embryotoxic actions induced in man by administering therapeutic doses of about 30 mg chloramphenicol per kg and day, provided that two premises are fulfilled:

α) first premise: CAP is not concentrated within the human embryo or fetus in any higher concentration than that occurring in the maternal serum—as has been shown in our studies with experimental animals. Up until now data on CAP concentration in human fetuses have been published only for the perinatal period (Scott and Warner, 1950; Ross *et al.*, 1950). Here similar concentrations for both maternal and cord blood are reported.

β) second premise: CAP dose dependently leads to a similar degree of inhibition in human embryonic tissue as compared to that found in

our animal experiments. All studies performed to date with a variety of animal tissues have provided no clue for possible differences in the concentration of CAP required to inhibit mitochondrial protein synthesis. However, in order to complete information on this, data on the susceptibility of human fetal mitochondrial protein systhesis to CAP are desirable.

Data available do not justify the assumption that levels above 5 μg CAP/g tissue will be reached in the human embryo or fetus after therapeutic dosage. It can be assumed, on the contrary, that for prolonged periods CAP concentrations will be even well below that level. Since concentrations of 5—10 μg/ml according to our *in vitro* studies block mitochondrial protein synthesis by only 30—50%, such concentrations may not be expected to lead to embryotoxic effects in man. However, we wish to stress the point that we have only little information on the teratogenic effects of drug combinations. It, therefore, cannot be ruled out that an inhibition of embryonic cytochrome oxidase by CAP to about 30—50% does potentiate the effects which other embryotoxic agents may have. Systematic studies on such drug combinations are in progress in our laboratory. Clear-cut dose response relationships will always be needed in further investigations on embryonic development in experimental animals under conditions which impair mitochondrial protein synthesis *in vivo*. Some such experiments can be performed more easily with the use of the analogue thiamphenicol which principally acts in the same way as CAP but has a half-life of about 50 min in rats (Ferrari and Della Bella., 1974). Results of studies performed with this chemotherapeutic agent will be reported later.

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