Inhibition of Respiration and of Respiratory Development by Different Doses of Chloramphenicol in Frog Embryos (*Rana pipiens*)

The respiratory level in frog embryos is low during cleavage and increases strikingly during later embryonic development. The same is true of the sensitivity of development to respiratory inhibitors (see LAMY and MELTON[1]). The question arises as to the role of respiratory development, i.e., mitochondrial biogenesis, in the respiratory increase. We have made a preliminary approach to this question by utilizing the antibiotic chloramphenicol, which is well known as a specific inhibitor of mitochondrial protein synthesis and hence of mitochondrial biogenesis but not of cytoribosomal protein synthesis[2].

We reported previously the discovery that high doses of chloramphenicol (4.5–18 m*M*) produced a dramatic and unusual disturbance of very early development involving re-fusion of blastomeres during cleavage[1,3]. This effect was interpreted as being due to direct respiratory inhibition rather than to inhibition of mitochondrial protein synthesis, because of the high doses required plus the discovery[4,5] that high doses of chloramphenicol directly inhibit respiration at the site 1 NADH dehydrogenation step. Furthermore, two other inhibitors, rotenone and the L-threo isomer of chloramphenicol, share with (D-)chloramphenicol the NADH dehydrogenation effect[4,5] and also the blastomere fusion effect[1], but neither of them inhibits mitochondrial protein synthesis at lower doses as does chloramphenicol[4,5].

We now confirm that cleavage disturbing doses of chloramphenicol do directly inhibit respiration in these embryos, while lower doses do not. Lower doses, however, while not inhibiting respiration directly, do eventually produce a permanent depression of respiration (relative to controls) in continuously treated embryos, concomitant with appreciable developmental retardation and a certain amount of arrest in these later stage (5–9 day) embryos.

Methods. Inhibitor and frog (*Rana pipiens*) sources and embryological methods were as detailed previously[1], except that all embryos were chemically dejellied at the beginning of development[6]. Respiration of embryos was measured at 23.0° with a Clark type polarographic electrode (Yellow Springs Instrument Co. Biological Oxygen Monitor, Model 53) in 3 ml of 10% Holtfreter's solution agitated by a 6 mm plastic coated bar magnet rotating at about 80 rev/min. The voltage signal was recorded until a steady trace was maintained for 10 to 15 min. The number of embryos per determination was 20 on the 1st and 2nd days of development, 10 embryos on the 3rd through 5th days, and 5 embryos thereafter.

Results. Figure 1 shows the respiration of embryos placed in chloramphenicol around 2 h after fertilization (prior to first cleavage) at concentrations which do (4.5 and 9 m*M*) and do not (0.9 and 2.1 m*M*) bring about the cleavage anomaly previously described[1,3]. At the two lower doses respiration was normal, differing from that of controls by no more than 9% (average 1.4%).

Chloramphenicol at 9 m*M* (3 mg/ml) elicited a profound disturbance of respiration. Within 2 h after treatment was begun oxygen consumption declined and reached 83% inhibition in about 3 h. Oxygen uptake then rose sharply over the next 2 h to well above the control level. During this rapid rise the earliest visible signs of blastomere fusion appeared (arrows in Figure 1) and increased in severity as

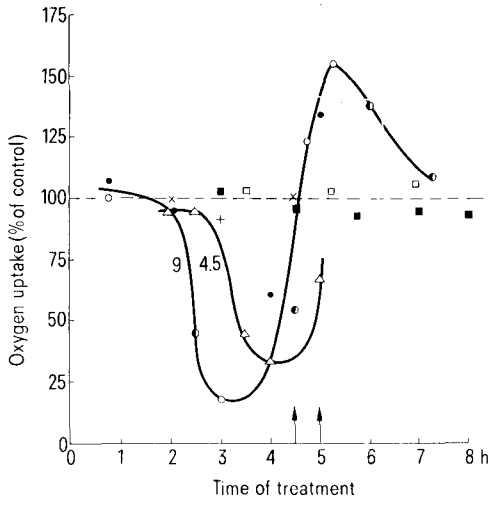

Fig. 1. Oxygen uptake by cleaving embryos incubated in various doses of chloramphenicol, beginning about 2 h post-fertilization. Arrows on abscissa indicate beginning of blastomere refusion in embryos at 9 and 4.5 m*M*, respectively. ○, ●, ◐, 9 m*M* (3 separate experiments and clutches); △, 4.5 m*M*; □, ■, 2.1 m*M*; X, +, 0.9 m*M*.

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Table I. Effect on respiration of late additions of chloramphenicol at doses which do not affect development within 24 h

Dose (m*M*)	Time of addition (h post-fertilization)				
	48	73	123	169	198
0	1.5	3.2	6.6	11.4	9.3
0.9	–	3.2	7.0	11.6	9.4
2.1	–	3.2	6.9	11.8	9.5
3.0	1.6	–	–	–	9.2

Each value (μl O_2/h/embryo 1 day after drug addition) is the average of 3 to 5 determinations; data from 3 different experiments on separate clutches of eggs (48 h, 73–169 h, and 198 h).

Table II. Effects on embryonic development of continuous incubation in doses of chloramphenicol which do not affect respiration directly

Dose (m*M*)	No. of embryos	Percent retarded[a] at day								
		1	2	3	4	5	6	7	8	9
0	167	0	0	1	2	6	3	4	3	3
0.9	228	0	1	2	2	2	2	2	3	3
2.1	105	2	36	69	6	5	47	50	52	52
3.0	185	4	100	62	17	12	38	56	60	61

[a] Including a few totally arrested embryos, especially at 3 m*M* on days 6–9 when about $^1/_{10}$ of all embryos were arrested or dead. Retarded and arrested embryos were not removed but were scored each day.

oxygen consumption peaked and began to decline again. Embryos in 4.5 m*M* seemed to follow a parallel pattern of respiratory disturbance, but with a lag of about 1 h in onset relative to 9 m*M* embryos and a lower maximum inhibition (about 67%). Again the initial indications of blastomere fusion appeared during the steep increase in oxygen consumption following maximal inhibition.

Addition of 9 m*M* chloramphenicol at 2 or 3 days of development also inhibited respiration (40–50%, again followed by a rise above controls). Added at 4 days it resulted in death and cytolysis within 24 h. In contrast, later additions of 3, 2.1, or 0.9 m*M* has no notable effect on development within 24 h and did not inhibit respiration (Table I).

Continuous incubation of embryos in 2.1 and 3 m*M* chloramphenicol from the beginning of development, on the other hand, did result in eventual retardation of development (Table II). There was a transient retardation of development during day 2 or 3, from which the embryos recovered by day 4. By day 6 about half of the embryos had again become retarded and in several cases permanently arrested. These developmental effects were accompanied by a depression of respiration in the non-retarded embryos relative to controls (Figure 2). The respiratory results are typified rather than summarized in Figure 2, since different control series gave uptake curves of differing absolute magnitude: a given experimental series is meaningful only in relation to control results from the same clutch of eggs. Experimental uptake in different series reached 20 to 50% depression relative to the same-clutch controls. This depression sometimes began gradually at day 4 to 6 of development, but became unambiguous by day 6 or 7 in most cases as in Figure 2, concomitant with the developmental retardation. Maximum inhibition was usually reached by day 8 or 9. This depression in general did not result in an actual decrease in respiration relative to earlier stages, merely a curtailed increase relative to controls. The controls by day 9 had increased oxygen uptake by 2–3 fold relative to day 5 controls and 20–25 fold relative to day 1 controls.

Continuous incubation in 0.9 m*M* chloramphenicol affected neither development (Table II) nor respiration. Two series were measured daily from day 1 to day 8 or 10 of development, with daily renewal of 0.9 m*M* chloramphenicol. Respiration of treated embryos differed from that of the controls by an average of only 0.1%. After the noisy first day (9%) it was never more than 5% below the control value on any one day.

Discussion. The first day results accord with our earlier interpretation of the blastomere fusion effect of high doses of chloramphenicol, that it is due to direct interference with respiration rather than to inhibition of mitochondrial protein synthesis[1,3]: only fusion-producing does affected early respiration. Furthermore, only inhibitors of the NADH branch of the respiratory chain (D- and L-chloramphenicol and rotenone) produce blastomere fusion[1], while total respiratory inhibitors (cyanide) and uncouplers (dinitrophenol) do not[1], nor does a succinate branch inhibitor (thenoyltrifluoroacetone)[3]. This specificity is interesting in view of the finding that chloramphenicol did not totally inhibit respiration at fusion doses. Even more interesting was the secondary re-elevation of respiration almost exactly concomitant with the beginning of blastomere fusion at both doses (Figure 1). It is unclear, however, whether this rise is either a cause or effect of the fusion, since it also occurred at later stages where fusion was not produced. Respiratory studies with other inhibitors are in progress to help elucidate this point.

On the other hand, the chronic effects of the lower doses of chloramphenicol (2.1 and 3 m*M*) on both development and respiration at later stages are tentatively interpreted as being mediated by mitochondrial protein synthesis impairment resulting in impaired mitochondrial biogenesis, since these doses did not inhibit respiration directly. In turn we interpret the retardation of development as a consequence of the mitochondrial impairment, since a slightly lower dose (0.9 m*M*) not affecting respiratory development also did not affect embryonic development. This implies a dependence of developmental progress on this hypothesized mitochondrial biogenesis. The extent of respiratory depression (mitochondrial deficiency?) re-

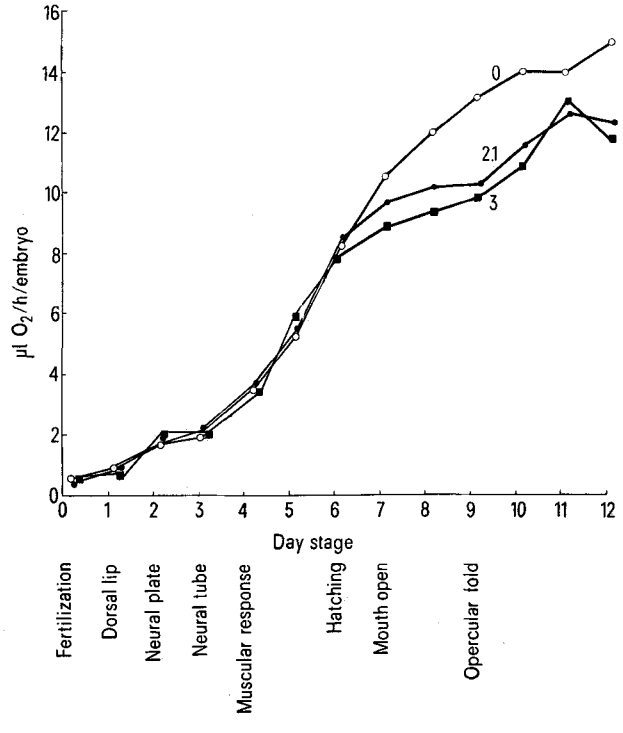

Fig. 2. Oxygen uptake by embryos from a single clutch incubated at 18 °C in the continuous presence of 0, 2.1, or 3 m*M* chloramphenicol, renewed daily. Measurements were made on the least abnormal looking embryos at a given stage. Days 0–2 and 11–12 points, single determinations; days 3–10, average of duplicates; ○, untreated controls; ●, 2.1 m*M*; ■, 3 m*M*.

quired to retard development may be greater than the 20–25% observed in Figure 2 and replicate experiments, since those measurements were made on the non-retarded embryos in the treated dishes. In either case, by the simple criterion of ongoing development the amount of respiration normally found in these embryos would seem to be greater than they actually require.

The observed 25-fold rise in control embryonic oxygen consumption may be due to mitochondrial growth or to enhanced activity of pre-existing mitochondria, or both. The relative contribution of these two mechanisms remains to be clarified by future experiments, including direct measurements of mitochondrial titres. Since there is roughly a 3-fold increase in the embryo's total 'non-yolk' cytoplasmic volume by this time (day 9)[6], an at least comparable 3-fold increase of mitochondrial titre (or size) might be expected. Mitochondrial concentration, or specific mass, may also increase during development. If not, the mitochondria must be about 7- to 8-fold more active at these later stages. The latter result would not be surprizing, however, in view of the respiratory activation-by-homogenization result of SPIEGELMAN and STEINBACH[7] on early embryos of this species, although that observation may be an artifact of increased oxygen access.

Summary, High doses of chloramphenicol (4.5 and 9 m*M*) directly inhibited respiration in conjunction with an unusual cleavage anomaly (blastomere refusion) in embryos of the frog, *Rana pipiens*. Lower doses (2.1–3 m*M*) did not affect respiration or cleavage and, when added later, did not directly affect respiration or development at any tested stage. Continuous incubation in the latter doses, however, did eventually, by day 6 or 7, produce a parallel retardation of embryonic development and of respiratory development relative to controls, suggestive of impaired mitochondrial biogenesis. A lower continuous dose (0.9 m*M*) affected neither respiration nor development at any stage.

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Respiratory Inhibition and Reversible Fusion of Frog Blastomeres

Cleaving embryos of *Rana pipiens* can be induced to undergo blastomere refusion by treatment with chloramphenicol or rotenone[1,2]. As observed externally, blastomeres of 16–32 cell embryos reunite into uncleaved mounds of irregular size and random distribution, usually connected by bridges of various sizes. The bridges are underlain by surface coat lined tunnels, seen in histological sections as pigmented eyelets, suggesting that the bridges arose by external fusion of adjacent blastomeres. The whole network of externally observed uncleaved mounds tends to form a single continuum in severe cases, although chloramphenicol stops short of obliterating all cleavage furrows. With rotenone, membrane breakdown is much more extensive, usually returning the embryo to a one-cell appearance, and the effect is irreversible except occasionally at the lowest doses. With chloramphenicol, on the other hand, drug removal allows the mounds to recleave and the embryos to resume normal-looking development to hatching and beyond.

The contribution of the present investigation is to ascertain by histological analysis that the inhibitor-treated blastomeres do indeed re-fuse, rather than simply fail to fuse in the first place deeper inside the embryo followed by regression or relaxation of incomplete cleavage furrows. We confirm that true refusion – reunion of completely separated blastomeres – does accompany the fully reversible effect of chloramphenicol, as was already indicated preliminarily for the irreversible effect of rotenone[1,2]. We thus verify that the two drugs produce identical effects as regards both internal morphology and the course of their etiology. In addition, we have sought to further the elucidation of the biochemical mechanism of refusion by assessing the developmental effects of two other pertinent inhibitors, thenoyltrifluoracetone and cycloheximide.

The site of action of the inhibitors producing blastomere fusion is suggested by the high dose of chloramphenicol required (above 3 m*M*) and by the simulation of the effect by rotenone and by the L-threo isomer of chloramphenicol[1]. D- but not L-chloramphenicol at low doses (10 μM) inhibits mitochondrial protein synthesis in eukaryotes[3,4]. At over 100-fold higher concentrations, both D- and L-chloramphenicol directly inhibit respiration at the level of step 1 NADH dehydrogenation, with a concomitant inhibition of non-mitochondrial protein synthesis[4–6]. Only these higher doses produce blastomere fusion and only those doses of chloramphenicol (4.5 m*M* and higher) producing blastomere fusion influence respiration during cleavage, while slightly lower doses (3 m*M* and less) do neither[7]. Rotenone also specifically inhibits respiration between NADH and flavoprotein[8], at doses comparable to those producing blastomere fusion[1]. Furthermore, rotenone binds to mitochondria irreversibly[8], which would explain the fact that its cleavage effect is not reversible while that of chloramphenicol is reversible.

When NADH oxidation is inhibited by chloramphenicol or rotenone, the other branch of the respiratory chain (succinate pathway) is presumably still functional. This presumption is reinforced by the finding that the general cleavage retarding/arresting effects of rotenone were partially alleviated by simultaneous addition of sodium succinate[1]. The blastomere fusion effect, however, was not prevented by succinate. This discrepancy agrees with expectation in view of the fact that a complete respiratory inhibitor (cyanide) and an uncoupler (dinitrophenol) produced cleavage retardation or arrest but did not at

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