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.

L. LAMY[8] and C. G. MELTON[9]

Department of Biology, Bishops University, Lennoxville (Québec, Canada), and Department of Biochemistry, University of Western Australia, Perth 6009 (Australia), 9 April 1975.

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[8] Present address: Champlain College, Lennoxville, Québec, Canada.
[9] To whom reprint requests should be sent, at the Perth address. Research Fellow of the Raine Medical Research Foundation.

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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Nuclear and syncytial counts on embryos reared in saline (control) or in 18 mM chloramphenicol (CAP) from 0.7 to 7 h post-fertilization, at which time the inhibitor medium was replaced by fresh saline

Age (h)	Control		CAP	
	Nuclei	Syncytia	Nuclei	Syncytia
2.5	2, 2, 2	2, 2, 2	2, 2, 2	2, 2, 2
3.5	4, 4, 4	4, 4, 4	4, 4, 4	4, 4, 4
4.5	4, 6, 8	4, 4, 8	4, 4, 8	4, 4, 8
6	8, 16, 16	8, 12, 16	4, 8, 8	4, 8, 8
6.5	16, 16, 31	15, 16, 16	12, 16, 16	8, 16, 16
7	–	–	CAP removed	
7.5	32, 32, 32	16, 32, 32	8, 16, 16	2, 7, 8
8	–	–	8, 16, 16	4, 6, 10
9	66, 67, 72	32, 64, 68	16, 16, 16	8, 12, 12
10	65, 74, 88	64, 66, 72	12, 16, 38	12, 14, 27
11	76, 112, 127	74, 108, 116	33, 64, 66	32, 47, 59

At a given time point, nuclear and syncytial counts are from the same 3 complete, serially reconstructed embryos.

any dose produce blastomere fusion[1]. (Another complete inhibitor, azide, produced cleavage arrest and was not reported to produce any other cleavage anomaly[9]). Since partial but not complete respiratory inhibitors produced refusion, the effects of another partial respiratory inhibitor were assessed to determine whether blastomere refusion is somehow due to partial respiratory inhibition per se. Thenoyltrifluoroacetone [4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione] blocks mitochondrial electron transport by specifically inhibiting succinic dehydrogenase[10,11] but permits electron flow through the NADH branch.

Inhibitor results. Thenoyltrifluoroacetone (Baker) did not produce blastomere refusion at any dose tested (90% normal cleavage at 1 μM). It did produce ordinary cleavage arrest followed by cytolysis at higher doses (100% arrest at doses above 10 μM). The stage of arrest was progressively earlier the higher the dose[12]. Thus, this partial inhibitor resembles the complete inhibitors cyanide and dinitrophenol in its developmental effects, rather than rotenone and chloramphenicol, suggesting that it is the NADH branch specifically that is involved in the refusion effect, rather than 'partial respiratory inhibition' in some general sense.

Another inhibitor relevant to elucidating the mechanism of blastomere refusion has been thoroughly tested for cleavage effects. Cycloheximide is a well-known inhibitor of non-mitochondrial protein synthesis[4] We have consistently failed to repeat an earlier observation of very erratic blastomere refusion following cycloheximide treatment[2] and conclude that the earlier results were invalid, or at least not reproducible enough to affect our conclusions about the fusion mechanism. Numerous more recent tests on thousands of embryos and two separate lots of cycloheximide (Sigma and Calbiochem) have failed to produce a single case of blastomere fusion. Instead, cycloheximide very consistently produced the same spectrum of results as KCN, dinitrophenol, and thenoyltrifluoroacetone. Uniform cleavage arrest at progressively earlier stages with increasing dose was produced in all embryos beginning at a dose of 14 μM cycloheximide, with an abrupt disappearance of potency at 11 and 7 μM, each of which doses allowed 98% normal cleavage.

Histological analyses. These were based primarily on the technique of serial reconstruction of whole embryos[1] with major attention being paid to the number of completely separated syncytia per embryo, i.e., the number of continuous networks of membrane-enclosed cytoplasm regardless ofthe number of nuclei and blastomeric lobes making up each syncytium. A small subjective error inherent in these numbers arose from semantic ambiguities over occasional blastomeres separated by what may have been either unpigmented (regressing?) membranes or no membranes. These were usually scored as non-membranes. (The possibility that all 'reunited' blastomeres recorded below were still separated by light microscope-invisible membranes is highly unlikely because such invisible membranes would have to have been tortuously following the interdigitating contours of the densely packed yolk platelets of the egg, a behaviour which to our knowledge is never observed of other plasma membrane systems). Histological and embryological methods employed and sources of frogs and inhibitors were as previously described[1], except that all embryos were chemically dejellied[13] before inhibitor incubation, and some histological sections were stained with carmine and aniline blue rather than hematoxylin and eosin to differentiate cell membranes. For photographic illustrations of the internal progression of blastomere refusion see LAMY and MELTON[1] and MOUNTS[12].

The Table shows that chloramphenicol-treated embryos were less cellular than their controls from 6 h onward. More important is the demonstration that the number of syncytia in treated embryos decreased during the course of the effect. This internal blastomere fusion and inferred membrane breakdown paralleled external observations of the reunion of blastomeres into irregular mounds, both processes being most severe in 7- to 8-h-old embryos. Nuclear division was also arrested or retarded by 6 h post-fertilization.

The Table also traces the course of the reversal of the refusion effect following chloramphenicol removal at seven hours post-fertilization. Note the time lag before recleavage occurs: the most severe blastomere fusion is not reached until after drug removal in this experiment. There was an approximately similar lag before the renewal of nuclear division. At 16 h post-fertilization (not shown) the ex-treated embryos were very highly cellular, but still somewhat less so than their controls (for histological illustration see MOUNTS[12]). These embryos continued to develop apparently normally and hatched into swimming tadpoles at approximately the same time as their controls about 5 days later.

In addition to the demonstration of true blastomere refusion and its reversal in chloramphenicol-treated embryos, we have completed the similar histometric demonstration of blastomere refusion in rotenone treated embryos reported in part earlier[1,2]. To summarize these results[1,2] for the crucial period of maximum refusion, the decrease in the average number of syncytia in 0.5 μM rotenone treated embryos during less than 1 h was dramatic – from over 10 to under 3 syncytia. At 7.2 h post-fertilization the numbers of syncytia in 3 treated, externally typical, serially reconstructed embryos were 10, 10, and 11; at 7.5 h, 5 typical embryos from the same dish had 3, 6, and 5 syncytia; at 8.0 h, 4 such embryos

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had 2, 3, 4, and 1 syncytia. Controls during the same period (3 embryos per time point) averaged 21.3, 32, and 30.3 syncytia, respectively.

This report effectively establishes that true refusion of blastomeres occurs in chloramphenicol- and rotenone-treated embryos, presumably accompanied by membrane disassembly, and that it is reversible if the inhibitor is not an irreversible mitochondrial binder. This system is now attractive for exploring the metabolic linkage between respiration and membrane biogenesis and perhaps for differentiating between membrane synthesis and maintenance.

Summary. Rotenone and high doses of chloramphenicol, both of which specifically inhibit electron transport between NADH and flavoprotein in the respiratory chain, caused fully separated *Rana pipiens* blastomeres to refuse, as shown by syncytium counts on embryos reconstructed from serial sections. With chloramphenicol, the effect was completely reversible: re-cleavage and normal development followed drug removal. The blastomere fusion effect was not produced by the succinic dehydrogenase-specific respiratory inhibitor, thenoyltrifluoroacetone, nor by a non-mitochondrial protein synthesis inhibitor, cycloheximide, both of which instead produced simple arrest of cleavage.

P. Mounts[14] and C. G. Melton[15]

Department of Biology, University of Pittsburgh, Pittsburgh (Pennsylvania 15260, USA), and Department of Biochemistry, University of Western Australia, Perth 6009 (Australia), 9 April 1975.

[14] Present address: MRC Mammalian Genome Unit, Department of Zoology, University of Edinburgh, Edinburgh, U.K. This work is part of a Masters Thesis submitted to the Faculty of Arts and Sciences of the University of Pittsburgh.

[15] To whom reprint requests should be sent, at the Perth address. Research Fellow of the Raine Medical Research Foundation.

Tubuloreticular Structures in Hepatic Endothelial Cells in a Case of Malignant Melanoma Liver Metastasis

Tubuloreticular structures have been described in several human diseases, namely viral, neoplastic and connective tissue diseases[1–3]. They have been identified in the hepatic sinusoidal endothelium by Kovacs et al.[4] in a case of systemic lupus erythematosus. To our knowledge there are no other references concerning their presence in human liver endothelial cells. The unknown significance of these structures and the controversy regarding their relationship to diseases make them an interesting biological finding. This report describes the presence of tubuloreticular structures in the hepatic endothelial cells in a case of malignant melanoma liver metastasis.

Methods. A percutaneous liver biopsy was performed by Menghini needle in a 48-year-old white man with liver metastasis of a malignant melanoma. The patient had no clinical or laboratory evidence of any other disease, such as collagen disease. The fragments were fixed in cacodylate-buffered 3% glutaraldheyde pH 7.3 and postfixed sequentially in veronal acetate-buffered 1% osmium tetroxide pH 7.3 and veronal acetate-buffered 0.5 uranyl acetate pH 5.8[5]. Following dehydration in ethanol, they were embedded in Epon 812[6]. The ultrathin sections were stained with lead citrate and examined on a Phillips EM 300 electron microscope.

Results and discussion. Tubuloreticular structures were frequently identified within the hepatic sinusoidal endothelium. They presented 20–22 nm diameter branched tubules associated with dilated cisternae of the endoplasmic reticulum. The tubular arrays appeared identical in dimensions and location to structures described by others in tumors, collagen and viral diseases[2, 7, 8]. No tubuloreticular structures were found, either in the tumor cells or in the hepatocytes.

Despite the accumulated literature concerning these structures, their nature and pathological significance, remains unsolved. Their ubiquitous presence in a variety of cell types and diseases with unrelated aethiology

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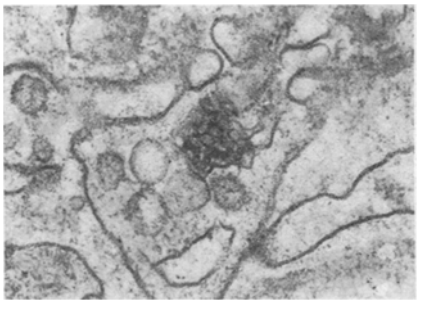

Fig. 1. Tubuloreticular structure within a sinusoidal endothelial cell.

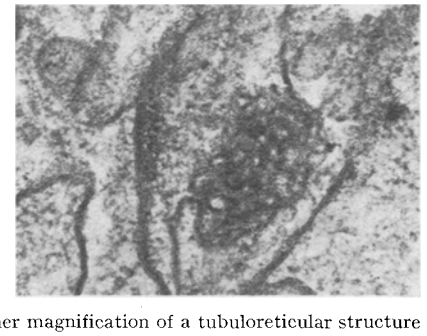

Fig. 3. Higher magnification of a tubuloreticular structure shown in Figure 2.