HEME AND REGULATION OF EMBRYONIC HEMOGLOBIN SYNTHESIS

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Chick blastoderms were cultured on media containing δ amino levulinic acid (DAL; a precursor of heme) in order to evaluate the biological role of DAL in the regulation of the commencement of hemoglobin (Hb) synthesis. The data show that DAL is probably not the biologically relevant control factor for initiation of Hb synthesis.

The possibility that heme regulates the initiation of Hb synthesis is based on the independence of early Hb synthesis from nucleic acid synthesis (Wilt, 1965; 1967) and the stimulation of early Hb synthesis by DAL, in vitro (Levere and Granick, 1967; Wainwright and Wainwright, 1966; Wilt, 1966). A detailed model of the regulation of the rate of early Hb synthesis has been proposed in which the rate of heme production, controlled by the activity of DAL synthetase, is the central regulator of the rate and time of onset of Hb production (Levere and Granick, 1967). This model may be distinguished from one in which heme is necessary for Hb production, but is not the essential regulatory element. The primary purpose of this communication is to show that DAL is probably not the biologically relevant regulatory agent for the initial phases of Hb synthesis in the chick embryo. This conclusion was reached from the results of experiments in which the effect of DAL was examined in the presence of a rich medium or a minimal glucose-salts medium. DAL only increased Hb formation on the minimal medium, a condition in which control Hb formation is reduced.

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Chick embryos at the 3-6 somite stage were cultured on agar media by the watch glass technique (Spratt and Haas, 1960). The embryos were dissected in buffered chick saline; the area opaca was removed to the border of the sinus terminalis, and the embryos were separated into right and left halves by cutting through the primitive streak (and its imaginary extension). The half embryos were placed dorsal side up on the medium and cultured for 18-30 hours in a moist incubator (gassed with 5% CO_2 in air in the case of Earle's saline medium). At the termination of the culture period embryos were floated off the medium, inspected, and rinsed in saline. Embryos were collected by centrifugation, excess saline removed, and the tissue homogenized in 1ml of 1.0% digitonin (Levere and Granick, 1967). Hb content was determined using the colorimetric method of Hell (1964). DNA determinations were carried out on portions of the homogenate extracted with 0.5M perchloric acid at 80° for 15 minutes by the method of Cerrioti (1952). Protein content was determined by the method of Lowry (1951) using bovine pancreatic ribonuclease as a standard.

RESULTS AND DISCUSSION

The effect of the composition of the culture medium on the commencement of Hb synthesis was examined. The data presented in Table I show the results of a typical experiment. Half embryos were cultured for 24 hours in the presence or absence of DAL, and the response to DAL on the very rich (whole egg) (Spratt and Haas, 1960) or minimal (Earle's solution) medium was compared. The accumulation of Hb on the two types of media is very different. On rich medium about 4µg of Hb per half embryo is synthesized in the cultures, and there is no significant difference between embryos cultured with or without DAL. On a glucose salts medium, the synthesis of Hb is very much less, about 5% of that on rich medium.

The stimulation of Hb formation by DAL on minimal medium may be very dramatic, resulting in a 2 to 3 fold increase in Hb content. Some half

Table I

Hb Synthesis, In vitro

µgm/half blastoderm

	Whole egg media						Earle's saline media					
	-DAL			+DAL			-DAL			+DAL		
	Hb	Prot.	DNA	Hb	Prot.	DNA	Hb	Prot.	DNA	Hb	Prot.	DNA
	6.75	49.2	4.1	6.4	62.4	5.0	0.52	30.8	0.88	0.66	40.0	1.35
	5.32	33.6	4.1	2.6	50.8	6.8	0.12	34.4	1.15	0.62	24.0	1.6
	3.0	70.4	5.5	1.64	73.6	7.3	0.27	33.6	2.5	0.44	35.2	2.05
	3.4	40.0	3.6	4.0	64.0	5.9	0.11	21.2	1.2	0.43	32.0	1.8
	1.48	60.8	4.1	2.88	40.0	4.5	0.04	35.2	2.5	0.66	20.8	1.55
	5.04	48.0	3.2	4.72	57.6	4.1	0.02	40.8	1.35	0.20	32.0	2.05
Av:	4.17	50.2	4.1	3.71	58.1	5.6	0.18	29.3	1.60	0.50	31.0	1.73
Hb/DNA		1.06			0.73			0.154			0.31	

Hemoglobin, total protein, and total DNA content were determined on individual dual chick blastoderm halves after 24 hours in culture. Control and experimental halves on a given medium were prepared from a single embryo. +DAL cultures contained $1x10^{-3}$M DAL, and controls contained 10^{-3}M succinate and 10^{-3}M glycine. Whole egg medium is identical to the rich medium of Spratt and Haas (1960) and contains 2 parts of egg extract and 1 part of 1.2% agar. Earle's saline medium contains commercial Earle's saline (Gibco) and 1% agar. All media contained 130 units/ml of penicillin and 130 units/ml of streptomycin sulfate.

blastoderms respond poorly, however, just as found by Levere and Granick (1967), and this results in large standard deviations. The quantitative aspects of the response to DAL in Earle's saline is similar to the data of Levere and Granick (1967), who used different analytical procedures. Table II summarizes results from a larger number of cases which show that the response to DAL on Earle's saline is, indeed, significant ($p<0.01$). The content of Hb/µg of DNA is much greater in embryos cultured on rich medium than in embryos cultured on poor medium. There is little or no difference in the protein and DNA content of half blastoderms cultured with or without DAL, but half embryos cultured on rich medium have more DNA and protein at

the end of the culture period than do those cultured on Earle's solution. Apparently embryos on rich medium have fared much better in culture.

Blastoderms cultured on agar media containing only egg albumen (Wilt, 1965), Medium 199, or whole egg medium in which the egg extract concentration was reduced from 67% to 5% behaved just as they did when cultured on Earle's saline. The response to DAL on these less rich media was not detectable when the concentration of DAL was lowered from 10^{-3}M to $5x10^{-5}$, nor was an increase of DAL concentration to 10^{-2}M effective in bringing about detectable stimulation on the rich whole egg medium. Other more specific and sensitive methods of determining Hb content of the blastoderms have been developed and applied to this experimental situation; these results will form the basis of a subsequent communication, but it is worth while to mention here that they confirm the above results in every respect.

During the past 3 years we have cultured many hundreds of blastoderms on DAL. Some blastoderms will develop a diffuse orange color not seen in controls, even on rich media. However, these blastoderms do not have an elevated Hb content, nor more extensive blood islands, nor obviously greater numbers of red cells. They do show high concentrations of porphyrins when examined by the fluorescence technique of Granick and Levere (1965).

Table II

Summary of Hb Synthesis, In vitro

	Whole Egg		Earle's Saline	
	-DAL	+DAL	-DAL	+DAL
Hb/DNA	1.12±0.314	0.82±0.372	0.151±0.129	0.325±0.112

The amount of Hb (µgm) formed in half embryos of a given DNA content (in µgm) is presented from results on 41 half blastoderms in each category. Media and other conditions are the same as for Table I except that 10^{-3}M glutamic acid was used in the controls instead of succinate and glycine.

The whole egg extract was examined for the presence of heme precursors. Egg extract was deproteinized with 10% trichloroacetic acid, and the acid soluble portion examined for porphobolinogen, DAL and other amino ketones by a modification (Marver et al., 1966) of the method of Urata and Granick (1963). No porphobolinogen, DAL nor amino ketone were detected, even though $5x10^{-5}$M DAL would have been detectable. In other experiments radioactive DAL* was added to egg extract before analysis, and 85% of the isotope was recovered, the greatest part of the loss occurring during deproteinization. The specific activity of the recovered DAL was unchanged.

The results confirm those presented by Levere and Granick (1967), Wainwright and Wainwright (1966) and Wilt (1966). DAL may result in accumulation of porphyrins in the young chick blastoderm. On some media it also results in pronounced stimulation of the early embryonic Hb synthesis, and DAL may -- indeed -- become rate limiting for Hb synthesis. On rich media approximating conditions in vivo, however, DAL is apparently not the biologically relevant regulatory factor for the initial rate of Hb synthesis. Heme is no doubt necessary for Hb synthesis. It is apparently not sufficient. The data show the powerful stimulus of whole egg extract for Hb synthesis and general health of the cultures is not due to any DAL it may contain.

This interpretation is consistent with previous studies. Spratt (1947) showed that the chick blastoderm will continue morphogenesis and differentiation on glucose-salts medium, but that the health and performance of blastoderms was much improved as the medium was enriched. Studies showing an involvement of heme in Hb biosynthesis (London et al., 1968) all show heme is necessary for Hb synthesis and that it may become rate limiting; I know of no evidence that supply of heme is sufficient for the regulation of Hb synthesis. The results do not rule out the hypothesis that extra genomic

* C^{14} DAL (15.5mC/mM) was purchased from the French Atomic Energy Commision; H^3 DAL (300mC/mM) was purchased from New England Nuclear.

factors are involved in initiating Hb synthesis in the chick embryo. The results do rule out the view that synthesis of DAL (presumably resulting in increased levels of heme) is the relevant and unique controlling factor in the initiation of Hb synthesis.

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