γ-BUTYROBETAINE AS A SPECIFIC ANTAGONIST FOR CARNITINE IN THE DEVELOPMENT OF THE EARLY CHICK EMBRYO*

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ABSTRACT

The effect of γ-butyrobetaine alone and with the addition of carnitine on the development of the early excised chick embryo has been studied. γ-Butyrobetaine in appropriate amounts exerts an inhibitory effect which can be relieved or annulled by the inclusion of appropriate amounts of carnitine. This has been interpreted as a metabolite-antimetabolite relationship, in which the normal metabolite, carnitine, is antagonized by the structurally closely related γ-butyrobetaine, and is regarded as evidence of an important role of carnitine in the metabolism of the developing chick embryo.

Since the discovery of carnitine as a necessary growth factor for the mealworm, *Tenebrio molitor*, and several related insect species (Carter *et al.*, 1952) evidence has been accumulating about the universal occurrence of carnitine in material of biological origin (Fraenkel, 1953, 1954). It was hence concluded that carnitine had an important physiological function in all organisms, that the majority of organisms obtained it by biosynthesis, and that only those few incapable of synthesis had to have it supplied as a "vitamin" in the food. This conclusion was based on the fact that many insects and higher animals did not seem to require a dietary source of carnitine, when grown on "synthetic" diets, and still contained carnitine in normal amounts (Fraenkel, 1953; Fraenkel and Friedman, 1957). It was also shown that sizable quantities of carnitine were synthesized during the development of the chick embryo (Fraenkel, 1953). Subsequently it was discovered that γ-butyrobetaine (desoxycarnitine) acted as a specific inhibitor for carnitine in the development of *Tenebrio molitor* (Bhattacharyya *et al.*, 1955). This suggested the use of γ-butyrobetaine as a means of demonstrating the physiological importance of carnitine in a system which normally synthesized it. The system chosen was the chick embryo which had previously been employed for demonstrating the biosynthesis of carnitine. Attempts were first made to inhibit normal development by in-

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jecting γ-butyrobetaine into the egg, and subsequently studying the effect of added carnitine. Preliminary experiments did not give clear-cut evidence of an antagonistic relationship between carnitine and γ-butyrobetaine in the quantities used, and the technique of the experiment was subsequently changed to deal with the excised embryo on a synthetic medium. A brief report of this study has been given elsewhere (Ito and Fraenkel, 1956).

Material and Methods

White Leghorn and Rhode Island red eggs used in this study were obtained from the Poultry Farm of the University of Illinois. Eggs were incubated at 38°C.

Sterile solutions of γ-butyrobetaine and carnitine in Rock's solution were injected through sterilized needles into the yolk of the egg through a small hole at the blunt end. The hole was subsequently sealed with paraffin. The eggs had previously been incubated for 20 hours at 38°.

Cultivation of chick blastoderms was performed on synthetic media, following the technique developed by Spratt (1947, 1948, 1949). Spratt (1949) had shown that early chick embryos explanted on an agar medium containing glucose and minerals continued to develop normally for 24 to 48 hours. In our experiments the media contained 1.25×10^{-3} M glucose which is slightly higher than the minimum level of glucose stated by Spratt as necessary for development *in vitro*. In compounding the medium, views by Howard (1953) on the effect of NaCl concentration on the early chick blastoderms were also taken into consideration. The composition of the medium was as follows:—

Chick-Ringer solution, modified (NaCl, 0.7 per cent; KCl, 0.042 per cent; $CaCl_2$, 0.024 per cent)	35 ml.
0.1 M phosphate buffer, pH 7.2	2 ml.
1.1 per cent $NaHCO_3$	2 ml.
0.02 per cent phenol red (as an indicator)	1 ml.

Agar was added to a concentration of 0.6 per cent.

The eggs were emptied into a modified Rock's solution (NaCl, 0.7 per cent, KCl, 0.042 per cent, $CaCl_2$, 0.024 per cent, $NaHCO_3$, 0.06 per cent) and the blastoderm removed. The developmental stage of the blastoderm was carefully examined. The morphological stages of the embryos were described according to the terminology of Hamburger and Hamilton (1951). Early blastoderms of definite streak or head-process stage were used in some experiments, and slightly older ones, representing one-somite through four-somite stage in others (Fig. 1). The blastoderms were cut into halves and the anterior half was placed on the medium in a watch glass, which in turn was contained in a Petri dish with moistened cotton at the bottom. This dish was covered and kept at 38°C.

The compounds tested for their effects on the development of the blastoderm have the following structures:

$(CH_3)_3\overset{+}{N}CH_2CH(OH)CH_2COO^-$	carnitine
$(CH_3)_3\overset{+}{N}CH_2CH_2CH_2COO^-$	γ-butyrobetaine (desoxycarnitine)

These compounds were used as the hydrochloride salts. L-Carnitine was isolated from meat extract (Carter *et al.*, 1952) and γ-butyrobetaine was synthesized (Bhattacharyya *et al.*, 1955). DL-Carnitine was obtained from the International Minerals and Chemical Corporation, Skokie, Illinois. The culture media after the addition of carnitine and/or γ-butyrobetaine were neutralized before and after autoclaving and the pH of the media adjusted to 7.2.

Observations were made after 20 and 40, and occasionally after 60 hours. Drawings of the explants were made with a camera lucida.

TABLE I

The Effect of Injection into the Egg of γ-Butyrobetaine and Carnitine on the Development of the Chick Embryo

Developmental stages checked after 7 days and numbered according to the system of Hamburger and Hamilton (1951).

γ-Butyrobetaine	L-Carnitine	No. of embryos treated	Undeveloped embryos	Developed embryos with or without defects																		Total
				No. of developmental stage																		
				7	13	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	
mg./egg	*mg./egg*																					
20	None	15	13	—	—	—	1*	—	1	—	—	—	—	—	—	—	—	—	—	—	—	2
10	None	15	7	1	—	—	—	—	—	—	1*	—	—	—	—	1*	1	2	2	—	—	8
1	None	14	4	—	—	—	—	—	—	1*	1	—	—	3‡	2	2	1	—	—	—	—	10
None	10	16	6	—	—	—	—	—	—	—	1*	—	1*	1	—	—	1	3	2	1	—	10
10	10	14	11	—	—	—	1	1§	—	—	—	—	—	—	1*	—	—	—	—	—	—	3
10	1	14	10	—	—	—	—	1*	—	1	—	—	—	—	—	—	—	2	—	—	—	4
1	1	10	1	—	—	1§	1§	—	—	—	—	—	2‖	2	—	—	—	—	1	1	1	9
None	1	11	2	—	1*	—	1*	—	—	—	—	—	1	1	1	—	2	1	1	—	—	9
None¶	None	10	0	—	—	—	—	—	—	—	—	—	2	1	2	3	2	—	—	—	—	10

* Circulatory system little developed.

‡ Eyes defective in 1 embryo.

§ Eyes little developed.

‖ Circulatory system defective in 1 embryo.

¶ Injection of saline only.

RESULTS

(*A*) *Injection into Eggs* (*Table I*).—Solutions of γ-butyrobetaine and carnitine in water were injected in amounts of 0.1 ml. per egg. The pH of the solutions of 100 mg./ml. was approximately 2.1 with either substance. Injection of 0.1 ml. of 0.11 N HCl in Rock's solution (pH 1.35) had no effect on the development of the embryo.

Injection of 10 or 20 mg./egg of γ-butyrobetaine inhibited the development of most embryos and a similar result ensued from the injection of 10 mg/egg. of carnitine. With an adverse effect of carnitine alone in this concentration no beneficial effect of this compound in combination with γ-butyrobetaine could be expected. However, 1 mg. carnitine/egg had no beneficial effect. It is pos-

sible that a careful adjustment of doses of the two substances would have shown a reversal by carnitine of the inhibition due to γ-butyrobetaine. However, this method was subsequently abandoned in favor of a method with explanted embryos, which was considered simpler and more sensitive.

(*B*) *Experiments with Explanted Blastoderms.* In preliminary experiments a wide range of variation from normal development to degeneration was recognized in explants on media containing γ-butyrobetaine. Consequently, the

TABLE II

The Effect of γ-Butyrobetaine and Carnitine on the Development of Excised Early Chick Blastoderms of Definite Streak or Head-Process Stage

The culture medium contained 1.25×10^{-3} M glucose.

γ-Butyrobetaine	L-Carnitine	Developmental class*				Total
		I	II	III	IV	
M	M					
5×10^{-2}	—	0	2	25	8	35
" " "	5×10^{-3}	0	0	5	8	13
" " "	2×10^{-3}	0	8	22	3	33
3×10^{-2}	—	0	5	17	3	25
" " "	2×10^{-3}	1	14	7	0	22
2×10^{-2}	—	5	14	10	1	30
" " "	2×10^{-3}	9	0	0	0	9
" " "	1×10^{-3}	26	10	0	0	36
1×10^{-2}	—	5	27	3	1	36
5×10^{-3}	—	13	10	0	0	23
—	—	26	13	0	0	39
No glucose		0	0	0	12	12

* See text.

results were expressed by the morphological state after 40 hours of cultivation, according to the following classification:

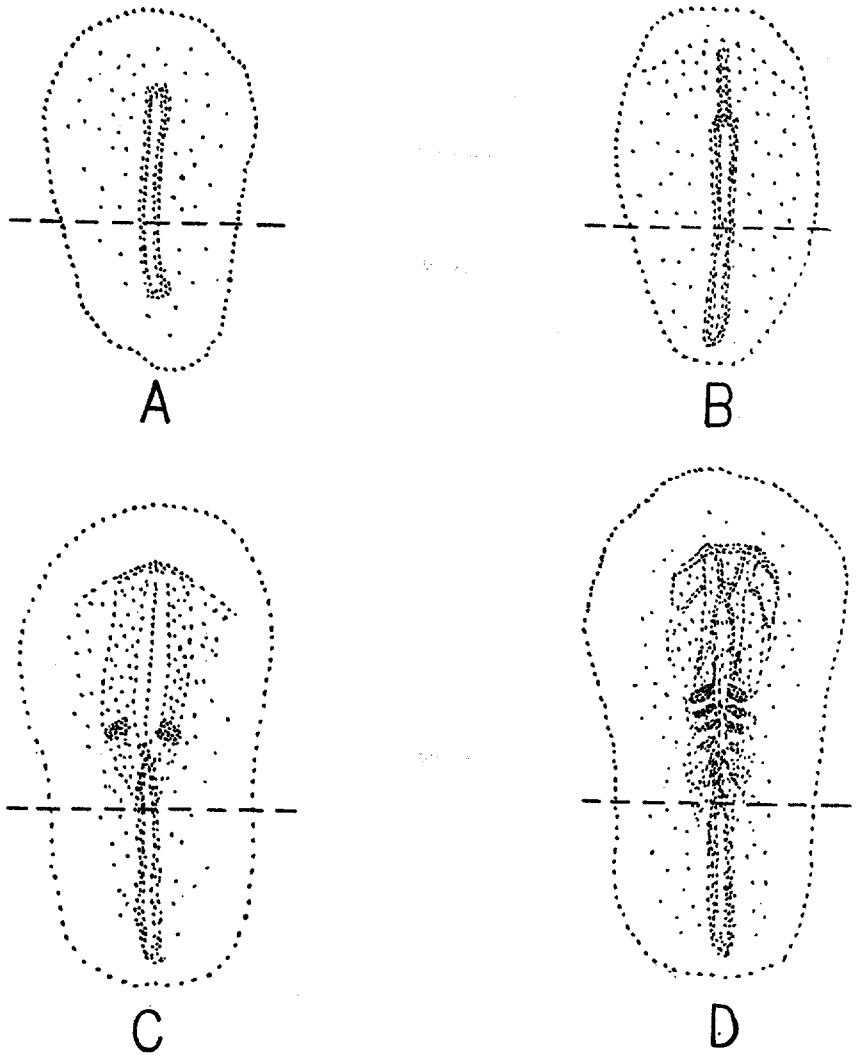
I. Normal development. Formation of the brain, spinal cord, optic vesicles, somites, and heart is normal. Pulsation of the heart is observed.

II. Subnormal development. Almost the same as above, except that no heart beat is observed.

III. Delayed degeneration. The blastoderm develops for the first 20 hours and subsequently degenerates.

IV. Degeneration. Almost no development is observed and the blastoderm disperses completely or becomes a mass of cells showing no differentiation.

1. The Effect of γ-Butyrobetaine and Carnitine on Early Blastoderms (Table II).—Blastoderms of definite streak or head-process stage (Fig. 1 A and B)



FIGS. 1 A to D. Diagrams of early blastoderms.
FIG. 1 A. Definite streak stage.
FIG. 1 B. Head-process stage.
FIG. 1 C. One-somite stage.
FIG. 1 D. Four-somite stage.
The broken line indicates the place where the blastoderm was cut into halves.

explanted on the culture medium without glucose cannot develop and finally degenerate, as indicated in Table II, and Fig. 2 D, while development takes place in the presence of glucose. Here the formation of a brain, spinal cord, optical vesicle, somites, and heart is observed, and in most cases also the pul-

sation of the heart (Fig. 2 A and B). These observations agree with those of Spratt (1949).

With the media containing γ-butyrobetaine a retardation of development or degeneration occurs in the explants. At a relatively higher concentration of

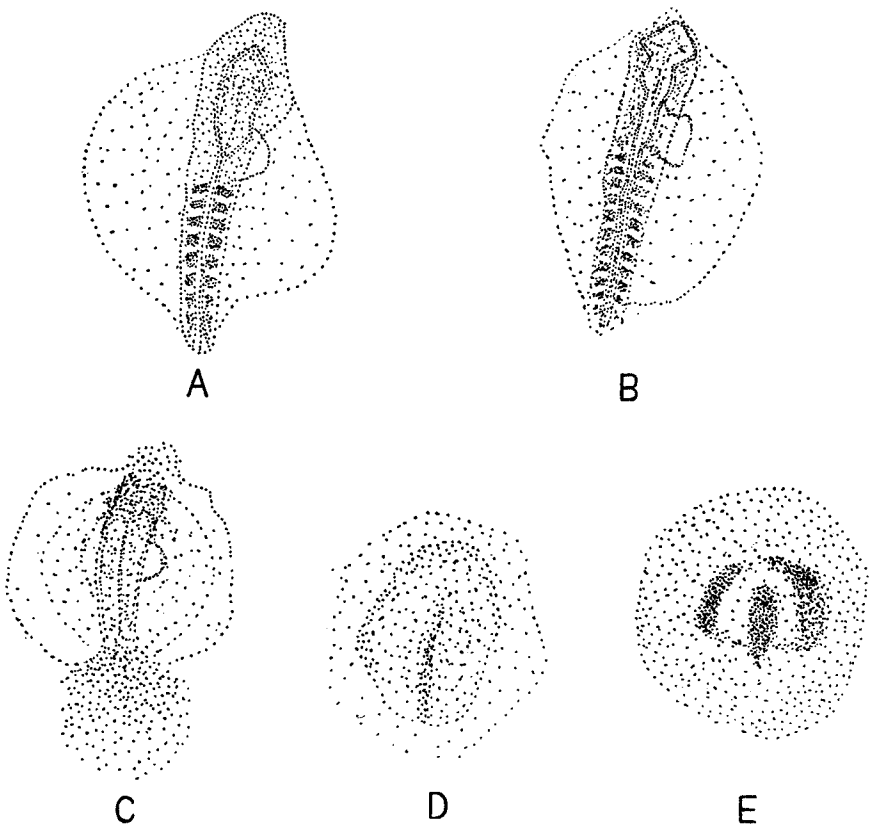


FIG. 2 A to E. Diagrams of developed and degenerated explants on various media.

FIG. 2 A. Normal development, after 20 hours.

FIG. 2 B. Normal development, after 40 hours.

FIG. 2 C. Delayed degeneration, class III, after 20 hours.

FIG. 2 D. Degeneration on glucose-free medium, after 20 hours.

FIG. 2 E. Degeneration in the presence of γ-butyrobetaine and glucose, class IV, after 40 hours.

5×10^{-2}M or 3×10^{-2}M almost all the explants, after a slight initial development during the first 20 hours, degenerate during the next 20 hours. However, a few develop up to stage II and others degenerate shortly after explantation without showing any signs of development. The degenerated explants on media without glucose are usually a thin layer of dispersed cells (Fig. 2 D). However, on a medium containing both glucose and γ-butyrobetaine they usually become

a thick mass of cells (Fig. 2 E). These differences become more evident after 60 hours, because in the former case the explant disperses almost completely, while in the latter it becomes dense without dispersing. In the explants which eventually degenerate following slight development during the first 20 hours, the formation of the somites is usually seen but that of the head-fold and especially of the heart occurs only rarely. As shown in Table II, on media containing 2×10^{-2}M butyrobetaine more than one-half undergoes development and differentiation (stages I and II) and the remainder undergoes initial development

TABLE III

Effect of γ-Butyrobetaine and Carnitine on the Development of Excised Chick Embryos of One-Somite to Four-Somite Stage

The culture medium contained 1.25×10^{-3} M glucose.

γ-Butyrobetaine	L-Carnitine	Developmental class*				Total
		I	II	III	IV	
M	M					
5×10^{-2}	—	0	10	6	0	16
" " "	2×10^{-3}	1	11	3	0	15
3×10^{-2}	—	0	9	0	0	9
" " "	2×10^{-3}	3	12	0	0	15
2×10^{-2}	—	7	3	0	0	10
" " "	1×10^{-3}	10	1	0	0	11
1×10^{-2}	—	9	4	0	0	13
5×10^{-3}	—	6	1	0	0	7
—	—	14	0	0	0	14

* See text.

(stage III), while with 1×10^{-2}M practically all develop to stage II. 5×10^{-3}M had no adverse effect.

2. The Effect of γ-Butyrobetaine on Older Blastoderms (Table III).—Most explants of one-somite through four-somite stage (Fig. 1 C and D) developed at a concentration as high as 5×10^{-2}M and none of them degenerated without some sign of development. Even degenerated explants formed hearts. However, none of the explants showed heart beat. A concentration of 2×10^{-2}M γ-butyrobetaine or lower had no effect. Almost all the explants included in class III showed not only formation of additional somites, but also of headfold and heart.

3. The Antagonistic Effect of Carnitine on the Inhibition of Development by γ-Butyrobetaine in the Early Blastoderms (Table II).—Since γ-butyrobetaine inhibited the development and differentiation of the early blastoderm, experiments were designed to find out whether this inhibition could be reversed by the addition of carnitine to the media. The results are summarized in Table

II. With 5×10^{-2}M butyrobetaine in the medium, the addition of 5×10^{-3}M L-carnitine had a slightly detrimental effect, but that of 2×10^{-3}M L-carnitine somewhat improved development. With 3×10^{-2} or 2×10^{-2}M γ-butyrobetaine the addition of 2×10^{-3} or 1×10^{-3}M L-carnitine very clearly counteracted the inhibition of development caused by γ-butyrobetaine. Eleven out of thirty explants degenerated with the addition to the medium of 2×10^{-2}M γ-butyrobetaine, and none, out of forty-five, after further addition of 2×10^{-3} or

TABLE IV

The Frequencies of the Heart Beat in Chick Embyros Cultured in the Presence of γ-Butyrobetaine and in the Presence or Absence of Carnitine

The explants were held at 26° for 30 minutes prior to counting.

γ-Butyrobetaine	L-Carnitine	No. of explants	Beats/min.
M	M		
2×10^{-3}	—	4	28.5 ± 0.5
2×10^{-2}	2×10^{-3}	9	30.1 ± 3.5

TABLE V

Inhibition of Embryonal Development by Carnitine

The culture medium contained 1.25×10^{-3} M glucose. Explants of definite streak or head-process stage were used.

DL-Carnitine	Developmental class*				Total
	I	II	III	IV	
M					
1×10^{-1}	0	0	0	8	8
4×10^{-2}	0	0	8	2	10
1×10^{-2}	4	4	5	0	13
2×10^{-3}	9	1	0	0	10

* See text.

1×10^{-3}M L-carnitine. With still lower concentrations of γ-butyrobetaine, the addition of carnitine had no significant effect.

The frequency of the heart beat was measured only in the tests with 2×10^{-2}M γ-butyrobetaine and was virtually the same in the presence or absence of carnitine (Table IV).

4. The Antagonistic Effect of Carnitine on the Inhibition of Development by γ-Butyrobetaine in Older Blastoderms (Table III).—The addition of carnitine in amounts of 2×10^{-3} or 1×10^{-3}M to the medium had some favorable effect on the development of blastoderms placed on media which contained 2×10^{-2}, 3×10^{-2}, or 5×10^{-2}M γ-butyrobetaine. Since the inhibitory effect of γ-butyrobetaine in older blastoderms is not as critical as in the earlier stages, the

antagonistic action of carnitine is not as striking. Nevertheless, in some of the explants the heart beat was resumed as an effect of added carnitine.

5. Inhibitory Effects of Higher Concentrations of Carnitine.—In the tests with early blastoderms (Table II) the application of the highest dose of carnitine employed, 5×10^{-3}M, gave a result which suggested an inhibitory effect of carnitine. Subsequently the effect of higher concentrations of carnitine alone, in the absence of γ-butyrobetaine, was investigated. DL-Carnitine was used in these tests. Table V shows that DL-carnitine in amounts of 1×10^{-2}, 4×10^{-2}, and 1×10^{-1} had an increasing inhibitory or toxic effect on the early blastoderms. At the highest concentration the explants did not develop at all and degenerated. The symptoms of degeneration were the same as in the presence of similar quantities of γ-butyrobetaine. It is not believed that the inhibitory effect was due to the use of DL-carnitine instead of L-carnitine. In feeding experiments with *Tenebrio* DL-carnitine had one-half the activity of L-carnitine, and the D-component, while physiologically inert, proved entirely harmless (Friedman *et al.*, 1957).

DISCUSSION

The effect on embryonal development of the injection into the egg of physiologically active compounds has been studied by several authors (Landauer, 1954). Spratt (1950) and Rothfels (1954) have studied the effect of antimetabolites on excised embryos, using the method developed by Spratt (1947, 1948, 1949).

In our experiments with explanted chick blastoderms of definite streak or head-process stage, the addition of γ-butyrobetaine to the media caused an inhibition of development and degeneration. This inhibition was relieved by the addition of appropriate amounts of carnitine. For demonstrating this γ-butyrobetaine–carnitine antagonism, the concentration of these substances in the culture medium was highly critical. There is a relatively narrow margin between too high a dosage of γ-butyrobetaine, the effect of which cannot be fully reversed by carnitine, and too small a dosage which has no significant action. Similarly it appeared that the dosages of carnitine, to be effective, must be below a concentration which is toxic in itself. Furthermore, a striking effect of carnitine was demonstrated only with the very early stages of chick development.

The significant outcome of this experiment is the demonstration of a physiological role of carnitine in a system which normally functions in the absence of added carnitine. It has been shown earlier that carnitine is synthesized in the developing chick embryo (Fraenkel, 1953). This fact, together with the recognition of the ubiquitous occurrence of carnitine in all living matter (Fraenkel, 1954), and the role of carnitine as a vitamin in certain insects, led to the view of an important metabolic role for carnitine in *all* organisms. The specific anti-

carnitine effect of γ-butyrobetaine was first shown in *Tenebrio molitor* which requires carnitine as a vitamin (Bhattacharyya *et al.*, 1955). γ-Butyrobetaine differs from carnitine only by the absence of the OH-group in the beta-position. The interference of γ-butyrobetaine with carnitine metabolism is best explained by the close structural relationship of these compounds, whereby the inhibitor usurps the place of the normal metabolite in a particular reaction. Since a similar antagonistic relationship has now been demonstrated in a different system in which normally carnitine is synthesized, it is concluded that here too carnitine has an important function to fulfill. This is the first instance in which a function of carnitine has been demonstrated in an organism which does not normally require it as a vitamin.

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