COMPETITIVE INHIBITORY EFFECTS OF SOME UNNATURAL CYTOSINE NUCLEOSIDES ON THE *IN VITRO* DEVELOPMENT OF THE EARLY CHICK EMBRYO

by

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INTRODUCTION

It has been widely held that embryonic development of the chick is accompanied by a marked acceleration of nucleic acid biosynthesis and accumulation[1]. However, the nature of the mechanism by which the nucleic acids act is still unknown. This study was undertaken to explore the use of some unnatural synthetic cytosine nucleosides as specific structural antagonists in an effort to elucidate the role of the nucleic acids in the developing chick embryo.

That the nucleic acids and their derivatives possess biological activity with respect to embryonic development has been demonstrated in various species. BRACHET[2] has found that the nucleic acids behave as potent embryonic evocators, and has suggested that potency as evocators may be related to nucleic acid content. HAMMETT and co-workers[3,4], in a series of studies on the effects of nucleic acid derivatives on embryonic development, reported that cytosine and thymine accelerate differentiation in *Obelia geniculata*. BIEBER, NIGRELLI AND HITCHINGS[5] have recently reported that a series of purine and pyrimidine analogues inhibit embryonic development of *Rana pipiens* at various stages characteristic for each compound.

As part of a series of studies on the relationship between chemical structure and biological activity in the nucleic acid series, several new nucleosides of uracil and thymine[6,7] and of cytosine and 5-methylcytosine[8] were synthesized and some were tested for biological activity[9]. These pyrimidine-N-glycosides differ from the natural nucleosides with respect to the sugar moiety and the lactol ring structure attached to the pyrimidine base—the natural compounds having 1-N-ribofuranoside or -2-desoxy-ribofuranoside structures whereas these synthetic products contain other sugars in the 1-N-glycopyranoside form[10]. The pyrimidine bases contained in the synthetic nucleosides used in this study are identical with the corresponding natural substances, and the

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glycosidic centers—with the exception of the arabinose nucleosides*—are of the *beta* configuration as in the natural nucleosides[11].

The cytosine and 5-methylcytosine nucleosides used in this study are of particular interest in view of the report by HAMMARSTEN and co-workers[12] that cytidine may be utilized as a precursor in the biosynthesis of pyrimidines in pentose and desoxypentose nucleic acids in the rat. The isolation of 5-methylcytosine from DNA by HOTCHKISS[13] and WYATT[14], its nucleoside[15], and corresponding nucleotide[16], suggests that nucleoside derivatives of this pyrimidine might be involved in similar biosyntheses. Suitable structural antagonists of these nucleic acid components should be extremely useful tools in the problem of nucleic acid metabolism and function.

Following the technique of SPRATT[17], chick embryos, cultured *in vitro* on a synthetic medium, were used as test organisms for the study of these synthetic unnatural nucleosides. This material is particularly appropriate in view of the fact that the chick embryo undergoes morphogenesis and differentiation *in vitro* from the definitive primitive streak or head fold to the 10 to 15 somite stage upon incubation for 20 hours in an exceedingly simple medium containing buffered chick-Ringer solution and glucose. Thus the effects of addition of other metabolites or anti-metabolites may be readily identified. In this way, SPRATT[18] studied the ability of the chick embryo to utilize various sugars and demonstrated that an exogenous source of carbohydrate is required by the embryo for morphogenesis and differentiation. Of 17 sugars tested, SPRATT found that only the naturally-occurring hexoses, D-glucose, D-mannose, and D-fructose, and one disaccharide, D-maltose, were utilized by the embryo. Among sugars not utilized were D-arabinose, D-xylose and D-ribose. D-Galactose supports morphogenesis only in high concentrations.

MATERIALS AND METHODS

Explantation of chick blastoderms. After 20 hours of incubation, the eggs were opened into a dish containing about 100 ml of sterile saline solution. The blastoderm was removed, trimmed of yolk, transected about 0.2 to 0.6 mm posterior to the node at right angles to the head-tail axis, and explanted under sterile conditions to the surface of a semi-solid (0.38 g agar per 100 ml medium) basal medium (buffered Ringer solution) containing the compounds to be tested. Experiments were set up with 5 to 10 embryos per group, and were run repeatedly to confirm results. In each experiment, a group of controls was included in which the embryos were explanted to media containing only buffered Ringer solution with glucose. Embryos were explanted at stages from the primitive streak to 3 pairs of somites. Using SPRATT's[19] criteria for adequacy of the medium, it was possible to determine the effects of the nucleic acid derivatives and their analogues.

Solutions and culture media. Chick Ringer solution, phosphate buffer, bicarbonate buffer, and phenol red indicator solutions were prepared according to SPRATT[18]. Glucose was added either in solid form or in solution to a concentration of 20 mg per 100 ml

* Arabinose nucleosides are synthesized from the condensation product of triacetyl-β-arabinopyranosyl bromide or chloride with 2,4-dialkoxypyrimidine. The mechanism assumes a WALDEN inversion of the anomeric carbon atom to give, in this case, an *alpha* nucleoside. It is to be noted, however, that all the synthetic nucleosides discussed herein possess a *trans* relationship between the aglycon and the 2-hydroxyl group of the sugar as is also the case with their corresponding natural analogues.

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of medium, the level reported by Spratt as the minimal glucose concentration which supports normal morphogenesis and differentiation.

The synthetic nucleosides were added either in solid form or in solution to concentrations of 10^{-3} to 10^{-6} molar. The following compounds were tested in these experiments:

1-D-glucopyranosylcytosine hydrochloride
1-D-galactopyranosylcytosine hydrochloride
1-D-arabinopyranosylcytosine
1-L-arabinopyranosylcytosine
1-D-xylopyranosylcytosine hydrochloride
1-D-xylopyranosyl-5-methylcytosine
1-D-arabinopyranosyl-5-methylcytosine

The effects of natural cytidine were studied in the presence and absence of the unnatural nucleosides in concentrations varying from 10^{-3} to 10^{-6} molar.

All media were prepared as in the following sample case:

Chick Ringer solution	32.0 ml
Phenol red solution	2.0 ml
Glucose stock solution	1.0 ml
Glucopyranosylcytosine · HCl solution	1.0 ml
Powdered agar (solid)	0.15 g

This medium is neutralized with bicarbonate, autoclaved, and 2.0 ml each of phosphate and bicarbonate buffer are added. The pH of the final medium is between 7.5 and 8.5. It is then pipetted in 2.0 ml portions into watchglasses which are placed in sterile Petri dishes containing pads of moist adsorbent cotton to ensure that the atmosphere surrounding the embryo is saturated during the experiment. The embryos are then incubated for 20 hours at 38° C.

Examination of embryos. In representative cases, low power magnification microphotographs were taken and histological sections were made of the embryos before and after *in vitro* incubation to record the gross and microscopic changes in morphogenesis and differentiation.

RESULTS

Normal chick embryos. Normal embryos at the definitive primitive streak or head fold stage were transected about 0.2 to 0.6 mm posterior to the node and cultured on buffered Ringer solution containing 20 mg of glucose per 100 ml of medium for 20 hours at 38° C regenerate tails, develop somites, and show all of the criteria for adequacy of the medium as described by SPRATT[19]. Histological sections showed normal, well-developed neural grooves, mesoderm and endoderm with many mitotic figures, characteristic of healthy, rapidly-developing embryos.

Importance of glucose. When an embryo is cultured on the buffered chick Ringer solution for 20 hours in the absence of glucose, degeneration takes place characterized by a complete loss of structure and a dispersal of cells. Unless glucose is present, this same degenerative picture appears, no matter which natural or unnatural nucleosides are added to the medium.

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Inhibitory effects of unnatural cytosine nucleosides. When glucopyranosylcytosine·HCl was added to the culture medium (containing buffered chick Ringer solution with glucose), marked inhibition of morphogenesis and differentiation was noted. This inhibition was characterized in almost all cases by a failure in tail regeneration and by retarded differentiation of the mesoderm and neural plate. Thus while the controls developed from 10 to 15 pairs of somites, those embryos treated with this synthetic nucleoside generally developed from 0 to 4 somite pairs. The minimum effective concentration of synthetic nucleoside was found to be 10^{-3} molar with respect to the total volume of the medium in which the embryo was cultured. This effect was independent of the glucose concentration, that is, raising the concentration of glucose in the medium several fold did not prevent the inhibition pattern.

Histological sections of the treated embryos showed a decreased number of mitotic figures and a state of extensive cytolysis. Similar, but less marked, inhibitory results were obtained with the compounds: Galactopyranosylcytosine·HCl, D-arabinopyranosylcytosine, Xylopyranosylcytosine·HCl, and D-arabinopyranosyl-5-methylcytosine. Nucleosides without such inhibitory action were: L-arabinopyranosylcytosine, and xylopyranosyl-5-methylcytosine.

Reversal of inhibition by cytidine. Embryos cultured in a medium containing buffered Ringer solution, glucose, glucopyranosylcytosine·HCl (10^{-3} molar) and the natural nucleoside, cytidine (10^{-4} molar), showed completely normal morphogenesis and development indicating that the inhibitory effects of this synthetic nucleoside are negated by the natural analogue at about one-tenth the molar concentration of the unnatural inhibitor. The gross and microscopic examinations of these embryos showed excellent development in almost all cases. Histological examination showed active mitosis with healthy and rapidly developing cells. Cytidine also caused the reversal of the inhibitory effects of galactopyranosylcytosine·HCl. The addition of cytidine alone to a buffered Ringer solution with glucose results in perfectly normal development.

When glucose is omitted from the medium, degeneration with cell dispersion occurs whether or not the natural or unnatural nucleosides are present indicating that the sugar portion of the nucleoside is not utilized by the chick embryo as a carbohydrate source.

Seasonal factor. These experiments were first begun in November, 1950, and the results reported herein were generally consistent until the following March when anomalies began to appear. Experiments in which glucopyranosylcytosine had behaved as an inhibitor were again repeated and in over 50% of the cases no inhibition was noted. Finally, in June of 1951, these experiments were temporarily postponed until the following November on the theory that the effects noted were subject to seasonal variations. This was, indeed, found to be the case. Repetition of these experiments in November unequivocally confirmed the inhibitory action of glucopyranosylcytosine and the ability of the natural product, cytidine, to reverse this inhibition. Thus it has been shown that the action of this synthetic nucleoside as a growth inhibitor diminishes in the Spring and almost disappears by June, but reappears in November and persists until the Spring.

DISCUSSION AND CONCLUSIONS

These results, on over 500 chick embryos, have demonstrated that the use of

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synthetic analogues of the nucleic acid derivatives offers a potential tool for the investigation of mechanisms of synthesis and the role of nucleic acids in living systems.

One can offer as a possible interpretation of these results that in the rapid synthesis of nucleic acids, assumed to take place in the developing embryo, the presence of unnatural structural analogues of certain nucleic acid derivatives enter into the metabolic cycle in a manner incompatible with normal development. In the presence of a true metabolite, however, the organism preferentially selects the natural nucleoside over the unnatural compound. If this mechanism approximates the actual picture, then one would conclude that cytidine, and perhaps other nucleosides, are intermediates in the synthesis of nucleic acids in the chick embryo. Preliminary studies indicate that natural uridine may play a similar role in causing reversal of glucopyranosylcytosine hydrochloride.

The seasonal variations reported above suggest a possible relationship between the reproductive cycle, egg fertility, and the level of nucleic acid metabolism.

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SUMMARY

A study of the effects of a series of unnatural synthetic cytosine nucleosides on the developing chick embryo cultured *in vitro* according to the method of SPRATT[17] showed that the compound glucopyranosylcytosine hydrochloride, when added to a simple medium containing only buffered Ringer solution and glucose, produced marked inhibition of morphogenesis and differentiation.

Similar but less marked inhibition resulted from the 1-D-glycopyranosylcytosine nucleosides of galactose, arabinose, and xylose, and from 1-D-arabinopyranosyl-5-methylcytosine.

The natural nucleoside, cytidine, when added to the medium containing the inhibitor, prevented this inhibition.

The inhibitory effects observed were subject to seasonal variation. Optimum inhibition appeared during the Winter months and diminished in the Spring and Summer.

This study indicates that cytidine may be utilized in the developing chick embryo and that unnatural nucleosides inhibit morphogenesis by a competitive metabolic mechanism.

RÉSUMÉ

Une étude des effets d'une série de nucléosides synthétiques et non-naturels de la cytosine sur les embryons de poulet en développement cultivés *in vitro* suivant la méthode de SPRATT[17], a montré que, ajouté au milieu simple ne contenant que de la solution de Ringer tamponnée et du glucose, le chlorhydrate de glucopyranosylcytosine provoque une inhibition marquée de la morphogénèse et de la différentiation.

Une inhibition semblable mais moins marquée est obtenue par les 1-D-glycopyranosylcytosine nucléosides du galactose, de l'arabinose, et du xylose, ainsi que par la 1-D-arabinopyranosyl-5-méthylcytosine.

Le nucléoside naturel, la cytidine, ajouté au milieu contenant l'inhibiteur, empêche l'inhibition.

Les effets d'inhibition observés sont sujet à des variations saisonnières. L'inhibition optimale apparaît pendant les mois d'hiver et diminue au prentemps et en été.

Cette étude montre que la cytidine peut être utilisée par l'embryon de poulet au cours de son développement, tandis que les nucléosides non naturels inhibent la morphogénèse par un mécanisme métabolique de compétition.

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ZUSAMMENFASSUNG

Eine Untersuchung über die Effekte einer Reihe synthetischer unnatürlicher Cytosin Nukleoside auf die Entwicklung *in vitro* kultivierter Hühnerembryonen (nach der SPRATT Methode[17]) zeigte, dass Glucopyranosylcytosin-Chlorhydrat, dem einfachen nur Ringerlösung und Glucose enthaltenden Medium zugefügt, eine ausgesprochene Inhibition der Morphogenese und Differentiation bewirkt.

Eine ähnliche aber weniger ausgeprägte Inhibition wird von den D-Glycopyranosylcytosin Nukleosiden der Galactose, Arabinose und Xylose bewirkt, wie auch von der D-Arabinopyranosyl-5-methylcytosin.

Das natürliche Nukleosid, Cytidin, hebt, bei Zufügung zu dem den Inhibitor enthaltenden Medium, die Inhibition auf.

Die beobachteten Inhibitionseffekte sind jahreszeitlichen Variationen unterworfen. Die optimale Inhibition erscheint während der Wintermonate und nimmt im Frühling und im Sommer ab.

Diese Untersuchung zeigt, dass Cytidin durch das sich entwickelnde Hühnerembryo benützt wird, während dessen unnatürliche Nukleoside die Morphogenese durch ein competitives Stoffwechselmechanismus hemmen.

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