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STUDIES ON THE INCORPORATION OF THYMIDINE INTO DNA BY RAT-LIVER HOMOGENATES *IN VITRO*

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SUMMARY

[^{3}H]thymidine is incorporated into DNA by homogenates of normal and 24 h regenerating liver. Regenerating liver homogenates are somewhat more active than normal liver homogenates. The incorporation of thymidine is not inhibited by ethionine, *p*-fluorophenylalanine, or β-2-thienylalanine when these compounds are added to incubation flasks in solid form. However, chloramphenicol inhibits incorporation by 19 to 91 % at concentrations ranging from 0.38 m*M* to 6.21 m*M*.

The ability of both normal and regenerating liver homogenates to incorporate thymidine into DNA is markedly inhibited by the injection of ethionine 8–14 h before the liver is excised. This suggests that the injected ethionine prevents the synthesis of an enzyme necessary for thymidine incorporation, or inhibits its activity.

INTRODUCTION

It has been demonstrated that the incorporation of [^{3}H]thymidine into regenerating rat liver DNA *in vivo* can be strongly inhibited by the intraperitoneal injection of

Abbreviation: DNA, deoxyribonucleic acid.

ethionine, ethionine hydrochloride, *p*-fluorophenylalanine, and *β*-2-thienylalanine hydrochloride[1]. Chloramphenicol also inhibits incorporation under certain conditions. It was therefore desirable to examine the action of these compounds in more detail by using an *in vitro* system.

METHODS

The conditions used for incubation are given in the legend of Table I. Methods used for isolation of the DNA and the sources of the inhibitors tested have been given previously[1]. Rats used in these experiments were either obtained from Germany (Tierzuchterei Brunger, Halle, Westfalen, Germany) or from a local rat colony originating from the German source. All regenerating livers in these experiments were excised 24 h after partial hepatectomy.

TABLE I

THE EFFECT OF CHLORAMPHENICOL ON THE INCORPORATION OF THYMIDINE INTO DNA

The incubation medium contained the following components at the final concentrations indicated: Glutamate, 10 m*M*; succinate, 3 m*M*; pyruvate, 10 m*M*; magnesium chloride, 3 m*M*; ATP, 0.4 m*M*; fructose, 6 m*M*; phosphate buffer, pH 7.4, 10 m*M* and sufficient sucrose to make the medium 0.25 *M* sucrose before the addition of the homogenate. The final concentration of the pooled regenerating liver homogenate (24 h after partial hepatectomy) from two male rats (Beirut) in 0.25 *M* sucrose was 10%. Each 25-ml Erlenmeyer incubation flask also contained 4 *μ*C of [^{3}H]thymidine (Schwartz Laboratories; specific activity 360 mC/mmole) in a final volume of 4 ml. Chloramphenicol was weighed out into dry flasks before other additions, and concentrations given are those which would be present if all the chloramphenicol had dissolved. Incubation was for 45 min at 37°. The specific activities of DNA from three flasks incubated without chloramphenicol were 3010, 2830, and 2790, or an average of 2880.

Chloramphenicol ($M \times 10^{-3}$)	*Counts/min/mg DNA*	*% Inhibition*
0.00	2880	0
0.38	2340	19
0.76	1820	37
1.25	1900	34
1.63	1620	44
1.97	1560	46
2.27	1380	52
2.73	621	78
3.17	490	83
3.86	397	86
4.71	319	89
6.21	247	91

RESULTS AND DISCUSSION

When rat-liver homogenates were incubated with a mixture of cofactors, substrates, and [^{3}H]thymidine, the thymidine was incorporated into DNA. Incorporation followed a definite time course. In one experiment, for example, the specific activity of the DNA after 0, 15, 30, and 60 min of incubation was 5, 340, 950, and 1235 counts/min/mg DNA respectively. No significant radioactivity was observed in DNA isolated

from flasks that were identical to the others except that they were not incubated. This result indicates that the isolation method completely removes all trace of acid-soluble compounds such as the radioactive thymidine and its phosphorylated derivatives.

When analogs of amino acids were added directly to flasks containing fortified homogenates of 24 h regenerating rat liver and incubated for 45 min, it was shown that chloramphenicol produced an inhibition of approximately 75 %. Ethionine, *p*-fluoro-phenylalanine and *β*-2-thienylalanine did not show any significant inhibition. In each case, 4 mg of the analog was added in solid form to flasks prepared as described in Table I. The failure to observe inhibition with analogs other than chloramphenicol may be due to the fact that these compounds are not very soluble in water. Another possible explanation is that these compounds have no direct inhibitory effect on the enzymes concerned with thymidine incorporation, but that inhibition observed *in vivo*[1] is due to some other action, such as inhibition of enzyme synthesis.

The inhibition of thymidine incorporation by chloramphenicol was examined in more detail by correlating the concentration of chloramphenicol with the per cent inhibition as shown in Table I. It may be seen that inhibition ranging from 19 to 91 % can be obtained with chloramphenicol at concentrations of 0.38 to 6.21 m*M*. The only definitely established primary effect of chloramphenicol at a metabolic level is the inhibition of protein synthesis although the effect on other reactions has been rather extensively investigated[2,3]. It is known that in some cases chloramphenicol inhibits the uptake of precursors into nucleic acids[4–8] while in other cases nucleic acid synthesis is stimulated or not affected[9–13]. It has not been established that the effect on nucleic acid synthesis (when it is observed) is due only to the inhibition of protein synthesis.

The specific activities of DNA obtained after incubation of [^{3}H]thymidine with normal and regenerating rat-liver homogenates are presented in Table II as the "control" values. It may be seen that there is a wide variation in the ability of homogenates to incorporate thymidine into DNA, but that the regenerating liver homogenates appear to be approximately twice as active as the homogenates of normal liver, if mean values are compared.

The reason for variation from rat to rat and from experiment to experiment has not yet been determined. The values presented in Table II are the average of results from duplicate or triplicate flasks containing aliquots of the same homogenate. Values for these duplicate analyses rarely varied more than $\pm$ 8 % from the average, indicating that the variation observed is not due to errors of incubation, isolation of DNA or determination of specific activity. The extent of homogenization within wide limits does not have a significant effect on the incorporation. In addition, aging the homogenate for 1 h in an ice bath produces only a very slight increase in incorporation. A more significant stimulation (60 %) is obtained by aging the homogenate for 4 h.

The incubation medium used in these experiments is one that was found to be satisfactory for the incorporation of orotic acid into RNA[14]. BOLLUM AND POTTER[15] have also published a note on the incorporation of thymidine into normal and regenerating liver homogenates using a sucrose system that differs in several respects from the one used in this work. In their experiment, BOLLUM AND POTTER observed

that a regenerating rat-liver homogenate showed 44 times as much activity as a normal rat-liver homogenate. It is not yet known why slight differences in the incubation conditions produce this difference in results.

TABLE II

THE EFFECT OF ETHIONINE INJECTION ON THE INCORPORATION OF THYMIDINE INTO DNA OF NORMAL AND REGENERATING RAT-LIVER HOMOGENATES

Rats in the "control" columns received no injection. Rats in the "ethionine" columns received one intraperitoneal injection of ethionine (0.5 mg/g body weight) at times ranging from 8–14 h before the time of sacrifice. One rat with a regenerating liver (specific activity of 155) received an injection of 1 mg of ethionine hydrochloride/g body weight. The conditions of incubation are given in Table I. Each value represents the results obtained with a homogenate from one rat, except for one value (specific activity of 258) where a pooled homogenate was used. In most cases, the value is the average specific activity of DNA obtained from duplicate or triplicate flasks containing the same homogenate. These data represent the results of 9 experiments in which both males and female rats over a wide weight were used. Although the females tended to give DNA with a lower specific activity, this trend was not clear cut, and the data were not strictly comparable since male and female rats were never tested in the same experiment under exactly identical conditions. Incubation was for 45 min at 37° for most experiments, but in several cases, 60 min incubation times (indicated by parantheses in the Table) were used.

Counts/min/mg DNA			
Normal liver		*Regenerating liver*	
Control	*+ Ethionine*	*Control*	*+ Ethionine*
296	33	(295)	75
345	41	(470)	83
378	48	673	131
392	55	695	155
425	73	895	212
(480)		1010	258
(548)		1020	286
635		(1520)	*
(785)		1580	
911		1660	
(1060)		(2530)	
(1240)		2880	
(1790)		3870	
548**	48**	1020**	155**

* One value of 408 has been omitted since the colorimetric determination of DNA in duplicate samples gave values which were unaccountably low, indicating a procedural error.

** Mean values for the column.

It is known that the intraperitoneal injection of ethionine at a level of 0.5 mg/g body weight inhibits the incorporation of [^{3}H]thymidine into DNA by 97 %[1]. This inhibition was examined in more detail by incubating [^{3}H]thymidine with liver homogenates obtained from rats injected with ethionine (see Table II). If the mean values of injected and uninjected rats are compared, it may be seen that the ethionine injection has inhibited the thymidine incorporation in normal and regenerating liver homogenates by 91 and 84 %, respectively.

This marked inhibition of the ability of both normal and regenerating liver homogenates to incorporate [^{3}H]thymidine into DNA indicates that ethionine has prevented the synthesis or inhibited the activity of some enzyme required for

thymidine incorporation. It should be of interest to determine whether it is the enzymes that phosphorylate thymidine, or the enzyme that polymerizes the deoxynucleotides to DNA, (or both) which are less active or lacking in homogenates as a result of the ethionine injection.

Deoxynucleotides other than thymidine are required as co-substrates for thymidine incorporation if a true DNA molecule is being synthesized. The possibility that ethionine might inhibit thymidine incorporation by lowering the level of these co-substrates was tested by adding deoxynucleotides of cytosine, adenine and guanine to flasks simultaneously at levels of 0.4 mg/flask in one set of four flasks and at levels of 4 mg/flask in a second series of four flasks. These flasks contained the usual incubation medium as well as normal liver homogenates from rats that had been injected with saline or with ethionine. In no case was significant stimulation of incorporation observed. Instead, in three out of four flasks at the 4 mg level, the specific activity of DNA was lower than control values by 52, 69, and 81 %. These results indicate that the injection of ethionine has not lowered the tissue levels of deoxynucleotides. Thus, the reason why injection of ethionine inhibits the incorporation of tritium-labeled thymidine into DNA *in vitro* still remains to be elucidated.

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