Inhibition of Incorporation of Thymidine into Deoxyribonucleic Acid by Amino Acid Antagonists in Vivo*

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Radioactive precursors are not incorporated into deoxyribonucleic acid in vivo until 18 hours after partial hepatectomy (1, 2). This lag period may represent the time required for the synthesis of some specific protein which enables DNA synthesis to occur (3). It is known that inhibitors of protein synthesis will inhibit the synthesis of bacteriophage DNA if they are added before DNA synthesis begins (4–8). It is therefore of interest to determine whether inhibitors of protein synthesis injected into rats during the first 18 hours after partial hepatectomy will inhibit DNA synthesis.

Use of thymidine as a specific precursor of DNA is well established (3, 9–17). It is a particularly good precursor since, aside from phosphorylated derivatives and degradation products, it is incorporated specifically into DNA with negligible diversion of the radioactivity into RNA (9, 10, 12). This high degree of specificity has been extremely useful in studies of the organization and duplication of chromosomes (14) where tritium-labeled thymidine is incorporated only into the new DNA formed during chromosome division and not into DNA already present. In addition, in regenerating rat liver homogenates, tritium-labeled thymidine is incorporated into deoxypolynucleotide in diester linkages which are indistinguishable from the linkages of nonradioactive thymidine in natural DNA (15). All these findings suggest that thymidine incorporation into regenerating rat liver DNA in vivo may be used to measure the rate of DNA synthesis.

It is shown in this paper that ethionine and p-fluorophenylalanine and β-2-thienylalanine hydrochloride strongly inhibit the incorporation of thymidine into DNA in vivo. Under certain conditions, chloramphenicol also inhibits incorporation.

EXPERIMENTAL

Methods

Male rats were supplied by Tierzuchterei Brunger (Halle, Westfalen, Germany). They were allowed to feed ad libitum on barley and a synthetic mixture of ground whole wheat, powdered skim milk, yeast, cod liver oil, olive oil, sugar, and calcium and phosphate salts. In addition, they were supplied daily with fresh leafy vegetables. After at least 1 week on this diet three lobes of the liver were removed by partial hepatectomy (18). After the operation, the rats were given only water until the time of sacrifice. The time schedule for injection of inhibitors and tritium-labeled thymidine is given in the legend for Table I.

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RESULTS

Table I shows the specific activity of regenerating rat liver DNA after the injection of thymidine into rats which had been injected with 0.9% sodium chloride solution or analogues of amino acids. Three normal nonhepatectomized rats injected with NaCl solution and tritium-labeled thymidine under identical conditions, had specific activities of 96, 63, and 90 c.p.m. per mg of DNA, 4 hours after the thymidine injection.

The large variation from animal to animal, both in control and experimental groups of Table I, is probably a function of the success of the partial hepatectomy as well as the unavoidable necessity of working with rats of different ages and weights. In addition, errors in the intraperitoneal injection of thymidine or the test compound may account for the very low value observed in one animal injected with NaCl solution and in one animal in-
Male rats (185 to 336 g) were injected intraperitoneally with 0 to 14 ml of 0.9% NaCl solution or with the compound to be tested 9.5 to 10.5 hours after partial hepatectomy. At 21.5 to 22.5 hours after the operation they were injected intraperitoneally with 0.5 ml of water containing 10 PC of tritium-labeled thymidine (obtained from the Schwarz Laboratories, Mount Vernon, New York). Exactly 2 hours after the thymidine injection (24 hours after operation) each rat was killed and the specific activity of DNA in the regenerating liver was determined as outlined in "Experimental."

Ethionine "hydrochloride" (1/4 of the inhibitor present as the hydrochloride) and methionine hydrochloride were prepared by dissolving 3 g of DL-ethionine (Sigma Chemical Company) or DL-methionine (Eastman Organic Chemical Dept.) in 54 ml of NaCl solution and 6 ml of 1 N HCl, and heating. After cooling to less than 40°, this solution or dilutions of it were injected intraperitoneally at the dosage (mg per g body weight) indicated. Other injected solutions contained the following: (a) 1 g of DL-ethionine or DL-methionine (Eastman Organic Chemicals) homogenized in 20 ml of NaCl solution, (b) 50 mg of methionine sulfone or methionine sulfoxide (Nutritional Biochemicals Corporation) per ml of NaCl solution; (c) 423 mg of p-fluorophenylalanine (Sigma Chemical Company, and California Foundation for Biochemical Research) suspended with homogenization in 17 ml of NaCl solution. A 1:5 dilution of this suspension was used for dosages of 0.1 mg per g body weight or less; (d) 300 mg of 8-2-thienylalanine (N.m.eq) and 0.6 meq HCl dissolved (with heating) in 12 ml of NaCl solution.

The possibility that the inhibition observed with ethionine hydrochloride might be due to the use of HCl to dissolve the ethionine was tested by injecting a solution of methionine hydrochloride which had been prepared in the same way as the solutions of ethionine hydrochloride. It may be seen in Table I that two rats injected with 0.5 mg of methionine hydrochloride per g body weight had liver DNA with specific activities of 2170 and 2220, while of the inhibitor present as the hydrochloride. Errors in DNA isolation are probably not responsible for any of the variation, since the results of triplicate independent analyses of each homogenate usually agreed within ±7% of the average.

Because of the insolubility of ethionine in saline, sufficient HCl was added to convert 1/4 of the ethionine to ethionine hydrochloride. Injecting 1 mg of ethionine hydrochloride per g body weight in a 250-g rat would thus involve the injection of approximately 0.5 meq of HCl buffered by 1.5 meq of ethionine.

It may be seen that all dosages of ethionine hydrochloride above 0.033 mg per g body weight produced a significant inhibition. The minimal inhibition at levels of ethionine hydrochloride above 0.25 mg per g was more than 90% when compared with saline-injected controls. It might also be mentioned that a minimal inhibition of at least 90% was obtained when 0.75 mg of ethionine hydrochloride per g body weight of rat was injected at any time ranging from 3 to 22 hours after partial hepatectomy. The 11 rats used in this experiment were injected with thymidine 22 hours after the operation and killed exactly 2 hours later as usual.

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All inhibitors injected as the hydrochlorides in these experiments were converted to the "hydrochloride" form by the addition of 1/4 equivalent of HCl per equivalent of inhibitor in a similar fashion.
When methionine hydrochloride was injected at a level of 1 mg per g body weight, marked inhibition of thymidine incorporation was observed. This inhibition may be related to the inhibitory effect of methionine on rat growth when fed in large amounts (20-22). However, neutral suspensions of methionine at a level of 1 mg per g body weight produced no significant inhibition. It remains to be determined whether this difference between neutral suspensions and solutions of methionine hydrochloride is due to differences in the rate at which methionine is absorbed or to the effect of the higher amount of hydrochloric acid injected.

Neutral suspensions of ethionine gave marked but variable inhibition at all levels above 0.066 mg per g body weight. The extent of the inhibition appeared to be about the same as when solutions of ethionine in dilute HCl were injected. For example, the minimal inhibition at levels of 0.1 mg per g body weight was 66% for the ethionine hydrochloride and 78% for two rats injected with the ethionine suspension. Similarly, at a level of 0.33 mg per g body weight the minimal inhibition was above 94% in both cases.

Two analogues of methionine other than ethionine were also tested. It may be seen that the specific activity of DNA after injection of either methionine sulfoxide or methionine sulfone was well above the minimal value observed for saline-injected controls. Although methionine sulfoxide is known to be an antagonist of glutamic acid in bacteria (23-25) it has not been shown to inhibit protein synthesis in mammalian tissue. When injected into rats it may be reduced to methionine and used as such (26, 27). Little is known about the metabolism and activity of methionine sulfoxide in mammalian tissues except that it is rapidly excreted in the urine after intraperitoneal injection (28). This rapid excretion may account for the failure of the sulfoxide to inhibit thymidine incorporation 12 to 14 hours after the methionine sulfoxide was injected. The sulfoxide is not used by bacteria (29-31) and it inhibits the utilization of methionine by Lactobacillus arabinosus (32).

The results obtained when rats were injected with saline suspensions of p-fluorophenylalanine and p-2-thienylalanine are also presented in Table I. It may be seen that p-fluorophenylalanine is a powerful inhibitor of the incorporation of thymidine into DNA. On the other hand, a neutral suspension of p-2-thienylalanine failed to give any inhibition except in one rat (possibly an error in the intraperitoneal injection). When \( \frac{1}{4} \) of a solution of p-2-thienylalanine was partially converted to the hydrochloride and injected into two rats at a level of 0.5 mg per g body weight, a 98% inhibition was observed. As noted earlier, injection of methionine which had been partially converted to the hydrochloride in the same manner resulted in only slight inhibition of thymidine incorporation at the same dosage level. These facts indicate that failure to absorb undissolved p-2-thienylalanine may be responsible for the failure to observe inhibition when neutral suspensions are injected.

Allyl glycine at dosages of 0.5 mg per g body weight caused convulsions and death within 1.5 hours after injection in two rats. At a dosage of 0.2 mg per g rat, one rat had mild convulsions and died within 2 hours. The other died within 10 hours. This compound appeared to be so toxic to the animal as a whole that examination of lower dosages for a specific effect on the incorporation of thymidine was not undertaken. No previous reports of the activity of allyl glycine in producing convulsions could be found after an extensive search of the literature. Allyl glycine does inhibit the growth of Escherichia coli and Saccharomyces cerevisiae (33).

The effect of chloramphenicol on thymidine incorporation has also been tested. Four rats (273 to 300 g) injected with unwarmed suspensions of chloramphenicol (0.3 to 0.5 mg per g body weight) incorporated thymidine into DNA at the same rate as control rats injected with NaCl solution. However, when the solution was heated in an attempt to dissolve the slightly soluble chloramphenicol (and cooled to less than 40°C before injection) a minimal inhibition ranging from 25 to 90% (3 rats) and from 90 to 96% (3 rats) was obtained. Failure to observe inhibition in the first four rats may have been due to the lack of absorption of the undissolved compound. The extent of inhibition observed when heated solutions were injected may be related to the amount of chloramphenicol dissolved before injection. From these preliminary experiments, it can be stated that chloramphenicol that has been partly dissolved in saline by warming does inhibit the incorporation of thymidine into DNA in vivo. However, a closer correlation of inhibition with dosage and with treatment of the solution before injection cannot be made at the present time.

**DISCUSSION**

Ethionine is known to be a potent inhibitor of the synthesis of at least 7 enzymes (34 and references cited). In addition, p-fluorophenylalanine and p-2-thienylalanine inhibit the synthesis of proteins and are antagonists of phenylalanine in different systems (35-40). Chloramphenicol also inhibits protein synthesis (41-48), although it does not appear to be a simple antagonist of any naturally occurring amino acid (49-50).

The inhibition of thymidine incorporation by amino acid analogues suggests that these compounds may be inhibiting the synthesis of some specific protein required for DNA synthesis. However, the results presented here in no way rule out other possible explanations. For example, in the case of ethionine, the inhibition might be due directly to antagonistic effects of S-adenosylmethionine (and derived S-adenosyl compounds) synthesized from ATP and ethionine (51). If a large part of the injected ethionine did react with ATP in this way, the resulting decrease in ATP might contribute to inhibition of thymidine incorporation. A similar reaction between methionine and ATP with the formation of S-adenosylmethionine (52 and references cited) might explain why methionine also produced inhibition in some cases.

If the amino acid analogues used in these experiments are inhibiting the synthesis of enzymes required for the incorporation of thymidine into DNA, then the nature of the enzymes involved must be examined. The fact that specific activities of less than 100 c.p.m. per mg of DNA are obtained with normal, nonhepa-
tectomized rat livers as compared with specific activities of 2700 to 9620 for 24-hour regenerating livers, proves that the enzymes responsible for the incorporation observed increase at least 27-fold in activity and/or amount during regeneration. It is unlikely that some unknown exchange mechanism permits thymidine to be incorporated into DNA without concomitant synthesis of DNA in regenerating liver but not in normal liver. Instead, the enzymes that incorporate thymidine in the present experiments in vivo are probably identical with the enzymes already known to be involved in the incorporation of thymidine in vitro (15 and references cited). The fact that the activity and/or amount of enzymes for phosphorylation of thymidine and for polymerization of deoxynucleotides increase during liver regeneration (3) strengthens this view.

It must be emphasized that no evidence is provided by the data in this paper either as to the nature of the inhibition or the type of enzymes involved. The mechanism whereby amino acid analogues inhibit the incorporation of thymidine into DNA must be clarified by further experiments.

SUMMARY

Ethionine and p-fluorophenylalanine, when injected intraperitoneally at a level of 0.3 to 0.5 mg per g body weight, inhibit the uptake of tritium-labeled thymidine into deoxyribonucleic acid of regenerating rat liver to a minimal extent of 92%. Inhibition has also been observed with L-2-thienylalanine hydrochloride, chloramphenicol, ethionine hydrochloride and high concentrations of enzymes involved. The mechanism whereby amino acid analogues inhibit the incorporation of thymidine into DNA must be clarified by further experiments.

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