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ON THE EFFECT OF SOME ADENINE DERIVATIVES ON THE INCORPORATION *IN VITRO* OF ISOTOPICALLY LABELLED COMPOUNDS INTO THE NUCLEIC ACIDS OF EHRLICH ASCITES TUMOR CELLS

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(Received December 11th, 1958)

SUMMARY

1. The incorporation of [^{14}C]formate into DNA thymidylic acid of Ehrlich ascites tumor cells *in vitro* is slightly stimulated by addition of adenine or adenosine. Deoxyadenosine has, however, a pronounced inhibitory effect on this process. All 3 adenine compounds reduce the incorporation of [^{14}C]formate into DNA purine deoxyribotides to very low levels.

2. The specific radioactivity of pooled acid-soluble thymine compounds is not reduced by the addition of either adenine or deoxyadenosine to cell suspensions. The total radioactivity of acid-soluble thymine compounds is reduced in the presence of adenine, but in the presence of deoxyadenosine this activity is doubled. Addition of adenine, adenosine or deoxyadenosine reduces the total radioactivity of the purines of the acid-soluble fraction.

3. The incorporation *in vitro* of [^{32}P]orthophosphate into DNA of Ehrlich ascites tumor cells is slightly activated by adenine or adenosine but is markedly reduced by deoxyadenosine. A 50 % inhibition of the rate is obtained at about 1 μmole of deoxyadenosine/ml cell suspension. The effect of deoxyadenosine is unchanged after 3 recrystallizations and is abolished by weak acid hydrolysis. At concns. up to about 2 μmoles/ml the 3 adenine compounds have little effect on [^{32}P]labelling of RNA, but all inhibit it at high concns. (6.6 μmoles/ml).

4. It is postulated that deoxyadenosine inhibits the DNA synthesis in Ehrlich ascites tumor cells *in vitro*.

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INTRODUCTION

TOTTER has shown that the carbon of formate serves as a precursor for the methyl group of DNA* thymine both *in vivo*[1] and *in vitro*[2]. PRUSOFF has recently found[3] that both cytidine and deoxycytidine and to a lesser extent uridine and deoxyuridine stimulate the incorporation of [^{14}C]formate into DNA thymine of both rabbit bone marrow and Ehrlich ascites tumor cells *in vitro*.

In the present paper, the effect is reported of some adenine derivatives on the incorporation of [^{14}C]formate into DNA bases and in acid-soluble purine and thymine derivatives of Ehrlich ascites carcinoma cells *in vitro*. Under the same exptl. conditions the effect of these adenine derivatives on the incorporation of [^{32}P]orthophosphate into RNA and DNA has been investigated. A preliminary note has been published[4].

MATERIALS

Deoxyadenosine, deoxyinosine and deoxycytidine-HCl were obtained from the California Foundation for Biochemical Research. Sodium[^{14}C]formate with a specific activity of 2 mC/mmole and [^{32}P]orthophosphate in sterilized isotonic solution were obtained from the Radiochemical Centre, Amersham, England. Deoxyribonuclease was a product of GEA, Copenhagen, and phosphodiesterase was prepared from snake venom according to the procedure of BUTLER[5]. Deoxyribose was prepared from deoxyinosine by treatment with Dowex-50, H^+. A solution (500 μl containing 25 μmoles) of deoxyinosine was shaken with Dowex-50, H^+ (100 mg) for 2 min at 100°. The resulting solution contained less than 1 % of the original light absorbency at 260 mμ, and 80 % of the original amount of deoxyribose.

The Ehrlich mouse ascites carcinoma cells were kindly furnished by Dr. G. KLEIN, Karolinska Institutet, Stockholm, and have been maintained by transfers made by interperitoneal injection of ascites fluid into mice of strain, St/EH. The cells used for expts. were obtained 4 to 6 days after transplantation of the tumor cells. After decapitation of the animals the ascites fluid was drained through an abdominal cut into centrifuge tubes containing a small amount of heparin. The tumor cells which usually were contaminated with erythrocytes were collected by centrifugation, and the wet wt. of the cells was determined. A cell suspension was prepared by mixing each gram of packed cells with 2 ml of TYRODE's solution.

METHODS

The DNA deoxyribonucleotides of cells incubated with [^{14}C]formate were isolated essentially as described by DOWNING AND SCHWEIGERT[6]. The residue resulting from washing and fat extraction of the acid-insoluble fraction was dissolved in 1 ml of 0.5 *N* KOH and digested at 37° overnight. The digest was cooled to 0°, and 0.45 ml

* The abbreviations used in the text are as follows: DNA, deoxyribonucleic acid; RNA, ribonucleic acid; dAMP, deoxyadenosine monophosphate; dGMP, deoxyguanosine monophosphate; dCMP, deoxycytidine monophosphate; TMP, thymidine monophosphate, TCA, trichloro acetic acid.

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of 60 % $HClO_4$ were added. The resulting precipitate was suspended in water, and K_2CO_3 (0.5 *M*) was added until neutrality was obtained. The resulting solution was transferred to a dialyzing bag and was dialyzed against 10 l of running distilled water. After addition of about 2 μmoles of $MgCl_2$ to the dialyzed solution the DNA present was hydrolyzed by treatment with 0.1 mg deoxyribonuclease for 5 h at 37° at pH 7, followed by phosphodiesterase (0.5 mg at pH 9.3 for 2 h at 37°). After addition of one vol. of 5 % $HClO_4$ the 4 deoxyribonucleotides present in the supernatant fraction were separated and isolated by either anion exchange chromatography (Expt. 1, Table I) or paper chromatography (Expts. 2 and 3, Table I). When the former method was used the $HClO_4$ filtrate was adjusted to about pH 8 with KOH; the $KClO_4$ formed was allowed to settle at 0°, and the supernatant soln. was passed through a column of an anion exchanger (Dowex-1 formate, 18 cm × 0.2 cm^2). The deoxyribonucleotides were eluted with a linear concn. gradient (from 0 *M* to 1 *M*) of ammonium formate at pH 4.5. They appeared as 4 discrete peaks in the following order, dCMP, TMP, dAMP and dGMP. The individual peaks appeared at ammonium formate concns. of about 0.09, 0.24, 0.41 and 0.58 *M*, respectively. This procedure is similar to that recently described by CANELLAKIS AND MANTSAVINOS[7]. For further purification each of the deoxyribonucleotide fractions was treated with Norit and chromatographed on filter paper in ethanol–ammonium acetate solvent[8], pH 7.6. When only paper chromatography was used for isolation, the $HClO_4$ filtrate of the enzyme digest was directly treated with Norit. The Norit eluate was evaporated to a small vol., and the individual deoxyribonucleotides were isolated by paper chromatography using a borate containing solvent[9]. For further purification each single spot was eluted and rechromatographed in ethanol–ammonium acetate solvent[8]. The specific radioactivity of each of the eluted deoxyribonucleotides was determined. For the estimation of the purine deoxyribotides the diphenylamine test for deoxyribose as described by BURTON[10] was used, and for the pyrimidine deoxyribotides the tryptophane test for deoxyribose as described by COHEN[11] was used.

The purine and pyrimidine derivatives present in the acid-soluble fraction of the cells were isolated by treatment with Norit. The recovery ranged from 80–90 %. The free bases were obtained by hydrolysis with formic acid[12], and were separated by paper chromatography in WYATT's solvent[13]. Each compound was eluted for determination of radioactivity. The thymine fractions were rechromatographed on filter paper several times; the solvents were water and water–saturated *n*-butanol[14]. The thymine concns. of the eluates were determined by spectral differences between neutral and alkaline solutions. A factor of $4.3 \cdot 10^3$ was used for the molar increase in extinction coefficient at 300 mμ, when the soln. was brought from neutrality to alkalinity (*i.e.*, 1 *N* with respect to NH_4OH).

The specific radioactivities of the nucleic acid phosphate of cells incubated with [^{32}P]orthophosphate were determined in the following way. The RNA fractions and the DNA fractions were obtained by the procedure described by KIT *et al.*[15]. Aliquots of the incubation mixture were treated with $HClO_4$, and the resulting precipitates were washed with lipid solvents. The RNA was extracted with 1 ml of 1.2 *N* $HClO_4$ at 0–2° overnight. The residue was digested with KOH at 37°, and after neutralization and addition of TCA, the precipitate was washed with 4 % TCA; the DNA was extracted at 90° for 15 min with 1 ml of the same soln. The phosphate of the 2 nucleic acid fractions was obtained as orthophosphate by ashing in a H_2SO_4–HNO_3 mixture.

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The specific radioactivity of [^{32}P]orthophosphate was determined according to ERNSTER *et al.*[16].

[^{14}C]radioactivity was determined by counting the samples in extremely thin layers in a windowless gas flow counter. [^{32}P]radioactivity was determined with an end-window Geiger counter.

All determinations were performed in duplicate.

RESULTS

Experiments with [^{14}C]formate

The influence of the addition of adenine, adenosine or deoxyadenosine on the incorporation of [^{14}C]formate both into DNA deoxyribonucleotides and into acid-soluble purine and thymine compounds of suspensions of Ehrlich ascites tumor cells *in vitro* has been investigated.

In Table I is given the specific radioactivity of the two DNA purine deoxyribotides and of DNA thymidylic acid from cells incubated under different exptl. conditions. No significant specific radioactivity of DNA deoxycytidylic acid was found in any case, which suggests that there was no significant incorporation of ^{14}C into either the pyrimidine ring or the deoxyribose of DNA. It appears from the table that in the control expts. the specific radioactivity of DNA thymidylic acid is much larger (5- to 15-fold) than that of the two DNA purine deoxynucleotides. Of these two, that of DNA deoxyguanylic acid is 1.2 to 1.5 times higher than that of the DNA deoxyadenylic acid. Similar results have been reported by SMELLIE *et al.*[17]. While the addition of adenine and of adenosine to the cell suspension has some stimulating effect on the incorporation of formate into DNA-TMP, the addition of deoxyadenosine has a pronounced inhibitory effect on this process. The specific activity of DNA-TMP

TABLE I

EFFECT OF ADENINE, ADENOSINE AND DEOXYADENOSINE ON THE INCORPORATION OF [^{14}C]FORMATE INTO DNA DEOXYRIBONUCLEOTIDES

Each vessel contained about 430 mg packed cells (wet wt.), 5.0 μmoles of deoxycytidine-HCl, 56 μmoles of glucose, 37 μmoles of sodium succinate, 0.5 μmoles and $2 \cdot 10^6$ counts/min of sodium [^{14}C]formate and 1.3 ml of TYRODE's solution. The final vol. was 2.2 ml. Incubation was performed in 30-ml Erlenmeyer flasks under air at 37° with shaking in a Dubnoff metabolic incubator. The figures in parentheses are derived from counts less than 50% above the background.

Compound added	*μmoles/ml*	*Expt. No.*	*Incubation time min*	*Specific activity (counts/min/μmole) of DNA deoxyribonucleotides isolated*		
				dGMP	*dAMP*	*TMP*
None		1	135	2,400	1,390	21,300
		2	210	3,750	2,750	19,350
		3	180	3,940	3,290	20,800
Adenine	2.2	1	135	(119)	(0)	23,300
	2.2	2	210	(0)	(0)	21,200
Adenosine	2.2	1	135	(203)	(61)	32,200
	2.2	2	210	(0)	(0)	21,000
Deoxyadenosine	2.2	1	135	(45)	(12)	1,340
	2.2	2	210	(270)	(0)	1,820
	3.2	3	180	(0)	(0)	1,060

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is in this case reduced by a factor ranging from 10 to 20. The specific radioactivities of the 2 DNA purine deoxyribotides are reduced to very low values in the presence of any of the three adenine compounds tested.

With regard to the decrease in specific activity of DNA-TMP caused by deoxyadenosine, at least 2 possible explanations exist. (1) The biosynthesis of DNA proceeds with normal rate, but deoxyadenosine reduces, by an isotope dilution effect, the specific radioactivity of the precursors of DNA-TMP resulting in a similar reduction of the specific activity of the TMP in DNA. (2) The specific radioactivity of the precursors of DNA-TMP is not decreased by the presence of deoxyadenosine, in which case the rate of DNA synthesis would be reduced.

Thymidine and thymidine phosphates may serve as precursors of DNA thymine[18,19]. The thymine part of these compounds plus free thymine have been isolated from the acid-soluble fractions of cells incubated with [^{14}C]formate as described under Table I. In Table II are given the analyses of these thymine fractions obtained after repeated paper chromatographic purification. The specific radioactivity of this compound is in no case lowered by the presence of adenine or deoxyadenosine in the incubation mixture, and in fact is always higher under these conditions than in the case of the control. This finding suggests, that the specific radioactivity of the precursors of DNA-TMP is not decreased in the presence of deoxyadenosine, and would thus exclude the possibility mentioned under (1), favouring the idea that deoxyadenosine inhibits DNA synthesis under the conditions used. The figures for the total radioactivity recovered in acid-soluble thymine compounds show some reduction in the presence of adenine; in the presence of deoxyadenosine these figures are, however,

TABLE II

RADIOACTIVITY OF ACID-SOLUBLE THYMINE COMPOUNDS

Exptl. conditions as described for Table I. Concn. of compounds added: 2.3 μmoles/ml cell suspension.

Compound added	*Expt. No.*	*Total activity (counts/min)*	*Specific activity (counts/min/μmoles)*	*Relative specific activity**
None	4	73,500	813,000	20.0
	5	63,000	890,000	19.7
	6	—	1050,000	23.3
Adenine	4	55,600	888,000	21.6
	5	41,000	961,000	21.7
	6	41,900	1440,000	31.9
Deoxyadenosine	4	146,600	905,000	22.2
	5	148,000	1450,000	32.0
	6	108,000	1380,000	31.4

* $\frac{\text{Specific radioactivity of thymine}}{\text{Specific radioactivity of formate}} \times 100$.

about twice as high as those in the control expts. This shows that the supposed decreased rate of DNA synthesis in the presence of deoxyadenosine is not due to an inhibition of the reactions responsible for incorporation of formate into the methyl group of thymine. It rather suggests that some of the [^{14}C]labelled thymine com-

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pounds, which otherwise would have been incorporated into DNA, under these conditions accumulate as acid-soluble thymine compounds.

The radioactivity of the recovered pool of purine derivatives obtained from the acid-soluble fractions is shown in Table III. The total radioactivity of these compounds is markedly lowered when adenine or adenosine is added to the cell suspension. The addition of deoxyadenosine has a less pronounced inhibitory effect on the labelling of the purines of the acid-soluble fraction.

TABLE III

TOTAL RADIOACTIVITY OF THE PURINES OF THE ACID-SOLUBLE FRACTION

Exptl. conditions as for Table II.

Compound added	*None*	*Adenine*	*Adenosine*	*Deoxyadenosine*
Total radioactivity	48,900*	6,900*	12,580**	23,600*

* Average of 3 expts. ** Average of 2 expts.

Experiments with ^{32}P

The effect of added adenine, adenosine, deoxyadenosine or of adenosine plus guanosine on the incorporation of [^{32}P]labelled orthophosphate into the RNA fractions and DNA fractions of suspensions of Ehrlich ascites tumor cells *in vitro* has been studied. The results of expts. performed under conditions similar to those described in Table I appear in Fig. 1. Under all the exptl. conditions used, the specific radioactivity of both DNA phosphate and of RNA phosphate increased linearly with time at least up to 3 h. In the case of DNA, addition of adenine, adenosine or adenine plus guanosine has some stimulating effect on the rate of incorporation of ^{32}P (the slopes correspond to rates of 123, 141 and 147 %, respectively, of the control). Deoxyadenosine, however, inhibited the rate of incorporation of ^{32}P strongly (the rate is 15 % of that of the control). In a duplicate expt. (not shown) performed with another batch of cells, similar results were obtained (the rates of incorporation of ^{32}P corresponded to 106, 118, 129 and 8.1 % of the control in presence of adenine, adenosine, adenosine plus guanosine, and deoxyadenosine, respectively). These data support the interpretation of the expts. with [^{14}C]formate, *i.e.*, that deoxyadenosine has, under the conditions used, a pronounced inhibitory effect on DNA synthesis *in vitro* in Ehrlich ascites tumor cells. Adenine, adenosine, and adenosine plus guanosine seem, however, to have some stimulating effect on this process.

For the RNA fraction from the same expt. (Fig. 1), none of the added compounds has much effect on the incorporation of ^{32}P. Adenine and deoxyadenosine have a slight inhibitory effect (the rates are 94 and 89 % of the control, respectively). Adenosine has a slight activating effect (the rate is 114 % of the control), whereas adenosine plus guanosine has no significant effect. In a duplicate expt. (not shown) with another batch of cells, the rates of incorporation of ^{32}P corresponded to 86, 78, 107 and 93 % of that of the control expt. in the presence of adenine, deoxyadenosine, adenosine and adenosine plus guanosine, respectively. Thus, the strong inhibitory effect of deoxyadenosine in the concn. used on incorporation of ^{32}P is shown to be almost specific for DNA.

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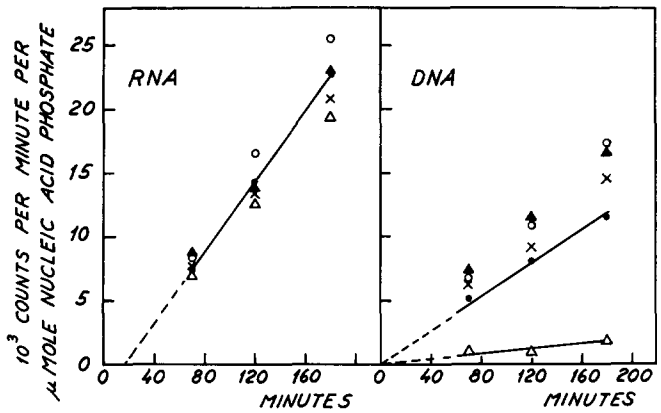

Fig. 1. The effect of adenine derivatives on incorporation of $^{32}PO_4^{\equiv}$. The exptl. conditions were the same as described for Table I except that 16 μC of $^{32}PO_4^{\equiv}$ were added to each vessel instead of [^{14}C]formate. ● control; × plus adenine; ○ plus adenosine; △ plus deoxyadenosine; ▲ plus adenosine plus guanosine. Concn. of each of the compounds added to the exptl. vessels: 2.2 μmoles/ml cell suspension.

When this type of expt. is performed with the added compounds in thrice the above concn. (*i.e.* 6.6 μmoles/ml cell suspension), most of them appear to be inhibitory towards incorporation of ^{32}P in the nucleic acids. As seen in Fig. 2, the increase in specific radioactivity of the nucleic acid phosphate for both RNA and DNA is linear at least up to 3 h, except in the presence of adenosine, where the rate of incorporation into DNA decreases after 1 h; after 3 h it is 57 % of that of the control. In the presence of adenine and deoxyadenosine the rate is 56 and 9.5 %, respectively. Adenosine plus guanosine have a slight activating effect (the rate is 114 % of the control). Under these conditions, the presence of any of the added compounds causes varying degrees of inhibition of the rate of incorporation into RNA. Deoxyadenosine is the most inhibitory (the rate is 43 % of the control); adenine, adenosine, and adenosine plus

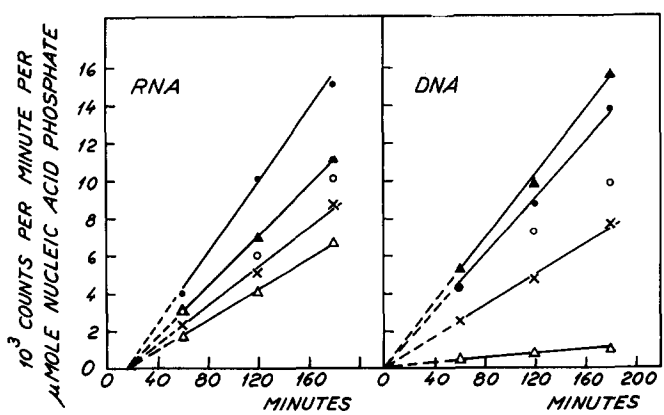

Fig. 2. The effect of high concns. of adenine derivatives on incorporation of $^{32}PO_4^{\equiv}$. The exptl. conditions were as described for Fig. 1 except that 20 μC of $^{32}PO_4^{\equiv}$ were added to each vessel, and the purine derivatives added were present in three times as high concns. (*i.e.* 6.6 μmoles/ml cell suspension). ● control; × plus adenine; ○ plus adenosine; △ plus deoxyadenosine; ▲ plus adenosine plus guanosine.

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guanosine have progressively less effect (the rates are 53, 55 and 74 %, respectively, of the control). In a duplicate expt. (not shown) with another batch of cells, the following rates of incorporation as % of the control were obtained in the presence of added deoxyadenosine, adenine, adenosine and adenosine plus guanosine, respectively: for RNA 51, 58, 82 and 84 %; for DNA 12, 53, 80 and 110 %.

The effect of different concns. of deoxyadenosine on the rate of incorporation of ^{32}P into RNA and DNA has also been investigated. A 50 % inhibition is obtained at about 1 μmole/ml cell suspension (Fig. 3). A low and almost constant rate (corresponding to about 85 % inhibition) is obtained with concns. above 2.5 μmoles/ml. The rate of incorporation into RNA is, however, not significantly influenced by concns. The rate of incorporation RNA is, however, not significantly influenced by concns. of deoxyadenosine up to about 2 μmoles/ml cell suspension. At higher concns. deoxyadenosine inhibits the rate, and 50 % inhibition is obtained at about 7 μmoles/ml. This inhibitory effect may, however, be of an unspecific type, since all compounds tested appear to inhibit incorporation of ^{32}P into RNA at this concn. (see Fig. 2).

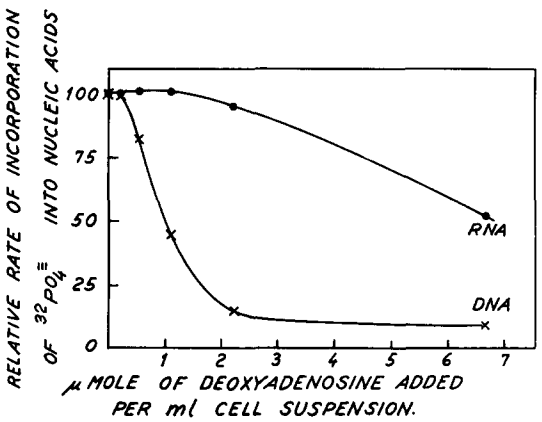

Fig. 3. Variation of rate of incorporation of $^{32}PO_4^{\equiv}$ with deoxyadenosine. The exptl. conditions were as described for Fig. 1. The rate of incorporation of ^{32}P into the nucleic acids of the cells in the control vessel is arbitrarily given as 100. The curves shown are the average of 2 expts.

Eight different control expts. have shown that the average rate of increase of specific [^{32}P]radioactivity is 1.7 times higher for RNA than for DNA. It can be seen from Figs. 1, 2, 4 and 5 that there is often a time lag of 20 to 30 min for the incorporation of ^{32}P into RNA, whereas this did not usually occur with DNA.

Since the inhibitory effect of deoxyadenosine might have been due to an impurity present in the preparations used rather than to the compound itself, the effect of mild acid hydrolysis was tested. A solution of deoxyadenosine was made 0.06 *N* with regard to HCl, treated at 100° for 10 min, cooled, and neutralized with NaOH. Paper chromatography suggested complete hydrolysis to adenine and deoxyribose. This treatment resulted in complete abolition of the inhibitory effect on incorporation of [^{32}P]orthophosphate into DNA (Fig. 4). The hydrolysate had in fact a slight stimulating effect similar to that of the same concn. of a mixture of equimolar amounts of adenine and deoxyribose. Neither hydrolyzed deoxyadenosine nor deoxyribose plus adenine had any great effect on the incorporation of ^{32}P into RNA. Furthermore, the

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inhibitory effect of deoxyadenosine was unchanged after the commercial product had been thrice recrystallized from water. This appears from Fig. 5 which also shows that deoxyribose alone had no effect on the rate of incorporation of [^{32}P]orthophosphate into either DNA or RNA.

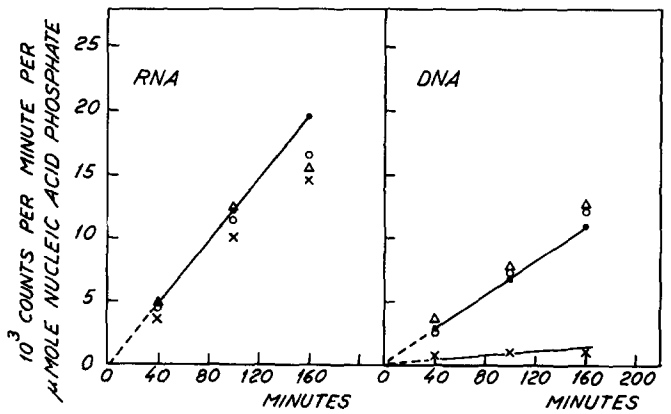

Fig. 4. The effect of acid hydrolyzed deoxyadenosine on incorporation of $^{32}PO_4^{\equiv}$. The exptl. conditions were as described for Fig. 1. Each vessel contained 20 μC of $^{32}PO_4^{\equiv}$. ● control; × plus deoxyadenosine; ○ plus hydrolyzed deoxyadenosine; △ plus adenine plus deoxyribose. Concns. of each of the compounds added to the exptl. vessels: 2.2 μmoles/ml cell suspension.

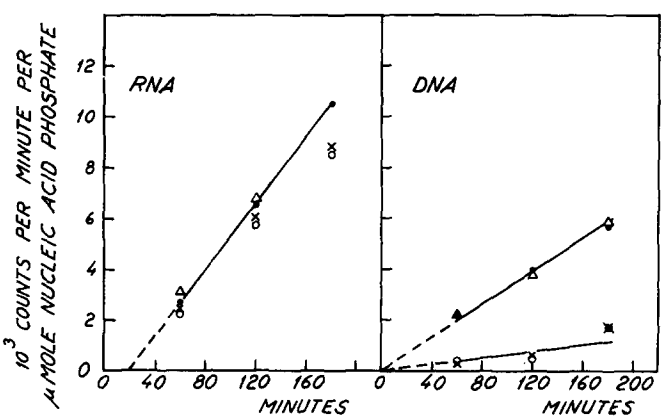

Fig. 5. Effect of recrystallized deoxyadenosine and of deoxyribose on incorporation of $^{32}PO_4^{\equiv}$. Exptl. conditions as described for Fig. 1. Each vessel contained 10 μC of $^{32}PO_4^{\equiv}$. ● control; × plus deoxyadenosine, commercial product; ○ plus deoxyadenosine, three times recrystallized; △ plus deoxyribose. Concn. of the compounds added to the exptl. vessels: 2.2 μmoles/ml cell suspension.

DISCUSSION

The above data show that when Ehrlich ascites cells are incubated with [^{14}C]formate, the specific radioactivity of the pooled acid-soluble thymine compounds is not reduced in the presence of added deoxyadenosine. This agrees with the possibility that the inhibitory effect of deoxyadenosine on incorporation of [^{14}C]formate into DNA-TMP is due to a decreased rate of DNA synthesis. This concept is supported by the similar

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inhibitory effect of deoxyadenosine on the incorporation of [^{32}P]orthophosphate into DNA. The latter effect is abolished by weak acid hydrolysis but is unchanged after 3 recrystallizations. It is postulated, therefore, that deoxyadenosine directly or indirectly inhibits DNA synthesis in Ehrlich ascites tumor cells *in vitro* under the exptl. conditions used. The inhibitory effect seems to be specific for DNA, since incorporation of ^{32}P into RNA is not appreciably affected by concns. up to 2 μmoles/ml cell suspension. From these observations it may be expected that deoxyadenosine has an inhibitory effect on the mitosis of certain cell types *in vitro*.

The depressing effect of adenine, adenosine and, to a smaller extent, of deoxyadenosine on the total radioactivity of the pooled purines of the acid-soluble fraction obtained in expts. with [^{14}C]formate, suggests that some reaction(s) leading to incorporation of ^{14}C into the purine ring and, therefore, possibly also the *de novo* synthesis of the purine ring, is inhibited to varying extents in the presence of these 3 compounds. A similar sparing action of adenine on the need for *de novo* synthesis from formate in rats is suggested by observations of GOLDTHWAIT AND BENDICH[20]. The marked decrease in the specific activity of DNA purine deoxyribotides found in the presence of added adenine and adenosine is, therefore, probably a result of both an isotope dilution of precursors of the DNA purines and of a decreased rate of incorporation of [^{14}C]formate into the purine ring.

ACKNOWLEDGEMENTS

Excellent assistance was given by Miss HANNE TROEDSSON in the later part of the work. The investigation was supported by grants from "Augustinusfonden", Fabrikant Einar Willumsens mindelegat and The Lilly Research Laboratories; all of these are gratefully acknowledged.

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