Inhibition of the Steroid-Induced Synthesis of Δ^5-3-Ketosteroid Isomerase in *Pseudomonas testosteroni* by a New Purine Deoxyribonucleoside Analog: 6-Chloro-8-aza-9-cyclopentylpurine

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SUMMARY

Analogs of 2′-deoxyadenosine (2′-AdR) were used to determine the structural requirements for inhibition of the steroid-induced synthesis of Δ^5-3-ketosteroid isomerase in *Pseudomonas testosteroni*. Compounds more active than 2′-AdR included 4-aminopyrazolo[3,4-*d*]pyrimidine-2′-deoxyribonucleoside and 6-chloro-9-cyclopentylpurine. The data obtained with many compounds indicate that: (a) substitution of the amino group of adenine with chlorine enhances activity, (b) replacement of C with N in position 8 of the purine ring increases activity, (c) replacement of deoxyribose in position 9 of 2′-AdR with substituents containing a 2′-hydroxyl (as in adenosine, 3′-AdR, and 9-(2′-hydroxycyclopentyl)adenine) causes a loss of activity, while nonhydroxyl-containing substituents (as in 2′,3′,5′-trideoxyadenosine and 9-cyclopentyladenine) retain activity (thus, direct phosphorylation of the analogs is not prerequisite to inhibitory activity), and (d) unnatural derivatives of 2′-AdR containing either L-deoxyribose in β-configuration or D-deoxyribose in α-configuration are inactive. These findings were used to guide the design and synthesis of 6-chloro-8-aza-9-cyclopentylpurine (689). This compound, which cannot be phosphorylated directly, markedly inhibited the synthesis of induced enzymes in *P. testosteroni* at a concentration (0.3 mM) that was significantly less inhibitory to the synthesis of total protein and to the incorporation into protein of L-leucine-1-^{14}C. The inhibition of Δ^5-3-ketosteroid isomerase activity was not attributable either to prevention of uptake of the inducer or to direct inhibition of enzyme activity. These data suggest that 689 inhibits relatively selectively a process critically involved in the inductive synthesis in *P. testosteroni* of Δ^5-3-ketosteroid isomerase.

INTRODUCTION

The microorganism *Pseudomonas testosteroni* is capable of utilizing certain C_{19} and C_{21} steroids as its sole source of carbon (1). Concerned with the degradation of these steroids are the following known enzymes that are induced by the presence of the steroidal substrate; 3β,17β-hydroxysteroid dehydrogenase, 3α-hydroxysteroid dehydrogenase, and Δ^5-3-ketosteroid isomerase (2, 3). The induced synthesis of these steroid-metabolizing enzymes is sensitive to dactinomycin and puromycin, inhibitors of the DNA-directed synthesis of RNA and of the synthesis of protein, respectively (4).

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During an investigation of factors involved in the control of the formation of induced enzymes in *Pseudomonas testosteroni*, it was found that 2′-deoxyadenosine (2′-AdR), a normal component of DNA, prevents the induced formation of enzymes under conditions that permit microbial growth (5). 2′-AdR, after its conversion to 2′-deoxyadenosine-5′-triphosphate (dATP), inhibits the synthesis of DNA in both mammalian (6–10) and bacterial (11, 12) systems; accordingly, the possibility that such an anabolite was responsible for the inhibition of the formation of enzyme activity had to be entertained. In a preliminary communication (13), it was reported, however, that several analogs of 2′-AdR, some of which contained substituents in position 9 that cannot be phosphorylated directly, also interfered with the androgen-induced appearance of Δ^5-3-ketosteroid isomerase (subsequently referred to as *isomerase*). In this paper, studies are described of the structural requirements for inhibition of steroid-induced enzyme synthesis by compounds that are related to 2′-AdR; of the agents tested, 6-chloro-8-aza-9-cyclopentylpurine (hereinafter referred to as 689) was the most potent. Furthermore, in *Pseudomonas testosteroni* this purine deoxyribonucleoside analog caused significantly more inhibition of the induced synthesis of isomerase than of the formation of total protein.

MATERIALS AND METHODS

Compounds. L-Leucine-1-^{14}C (34.2 μC/μmole) and androst-4-ene-3,17-dione-4-^{14}C (45.3 μC/μmole) were obtained from New England Nuclear Corporation, Boston, Massachusetts. 6-Chloro-8-aza-9-cyclopentylpurine (689) was prepared by the dropwise addition at 0° with stirring (20 min) of an aqueous solution of sodium nitrite (4.5%) to a suspension of 5-amino-6-chloro-4-cyclopentylaminopyrimidine (1.13 g) (14) in 2 N acetic acid (12.5 ml) (15). The reaction mixture was stirred for an additional 30 min, during which time it warmed to room temperature; the solid that separated was collected by filtration and washed with cold water (1.12 g, 93% yield). An analytical sample was recrystallized from hot water, m.p. 66–69°, $\lambda_{max}^{H_2O}$ 263.5 mμ (ϵ 9150). The compound was stored in a desiccator at −20° and was dissolved in dimethylsulfoxide (DMSO) just prior to its addition to the bacterial suspension.

Analysis

Cal'd for $C_9H_{10}ClN_5$:	C, 48.33; H, 4.51; Cl, 15.85; N, 31.31
Found:	C, 48.09; H, 4.41; Cl, 15.66; N, 31.13

Inhibition of the steroid-induced synthesis of Δ^5-3-ketosteroid isomerase. The methods employed in the study of the inhibition of enzyme induction by 2′-AdR and various related compounds were similar to those described previously (5). An inoculum of the organism was taken from a petri dish and grown overnight at 30° in a liquid medium which contained lactate, Casamino acids, and yeast extract, to which no steroid was added. Of this culture, 4.3 ml was added to 100 ml of growth medium at the beginning of each experiment. Incubation of 5 ml of the inoculated medium was carried out in conical fermentation flasks (125 ml) at 30° in a gyrorotatory shaker. To study the process of enzyme induction, 1.25 mg of Δ^4-androstene-3,17-dione, the inducer, dissolved in 0.05 ml of DMSO, was added to 5 ml of inoculated medium 2 hr after the initiation of incubation. Unless stated otherwise, the compounds tested for the inhibition of enzyme induction were dissolved in DMSO and added to the culture 3 hr after the addition of the inducer, to give a final concentration of 6 mM. Because of the limited supply of many of the compounds, some were added as solids and an appropriate amount of DMSO was added separately (the final concentration of DMSO did not exceed 2%, which was nontoxic to *P. testosteroni*). After a total period of 7 hr (involving exposure during only the final 2-hr period to potential inhibitors of both growth and enzyme induction), the turbidity of dilutions of the cultures was measured with a Zeiss spectrophotometer (660 mμ). The bacterial suspension was centrifuged in Corex tubes for 10 min at 5°

(25,000 *g*). After discarding of the supernatant medium, the cells were resuspended in sodium-potassium phosphate buffer (pH 7, 0.1 M) and centrifuged. Subsequent to decantation of the supernatant fluid, 10 ml of acetone (−50° to −70°) was added to the centrifuge tubes; after standing at room temperature for a few minutes, the frozen material was broken up with a spatula, stirred and recentrifuged. The acetone was discarded, and the air-dried residues were incubated with phosphate buffer for 1 hr at 30° in the same tubes (closed with silicone stoppers) placed horizontally in the gyrorotatory shaker. A final centrifugation for 10 min gave a clear supernatant fluid that was assayed for isomerase activity. The assay involves direct measurement of the rate of increasing absorption at 248 mμ as Δ^4-androstene-3,17-dione is formed from the corresponding Δ^5-compound (16).

Determination of the synthesis of protein. Protein synthesis was measured by determining the rate of incorporation of L-leucine-1-^{14}C into residual protein by fractionation of a sample of bacterial suspension followed by collection of the desired fraction on Millipore membranes by filtration (17). The Millipore filters were immersed in vials containing a mixture of PPO: POPOP:ethanol:toluene,[4] and radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer.

Determination of the total cellular content of nucleic acid and proteins. Samples of bacterial suspension were mixed with ice-cold 0.4 N perchloric acid for 15 min, after which the suspension was centrifuged. The resulting precipitate was washed with ice-cold 0.4 N perchloric acid, and after centrifugation and decantation of the supernatant material, 0.4 N perchloric acid again was added to the precipitate and the mixture was heated at 95° for 30 min. A final centrifugation gave a clear supernatant solution; this was analyzed for DNA by the method of Burton (18), and for RNA by the procedure of Mejbaum (19). The remaining precipitate was digested at room temperature with 0.6 N NaOH for 30 min and then analyzed for its protein content by the method of Lowry *et al.* (20). The reference standards for DNA, RNA and protein were salmon sperm sodium deoxyribonucleate, yeast ribonucleic acid and bovine serum albumin, respectively.

[4] 100 mg of *p-bis*[2-(5-phenyloxazolyl)]benzene (POPOP), 8 g of 2,5-diphenyloxazole (PPO), 600 ml of absolute alcohol, and 1400 ml of toluene.

RESULTS

The Effect of 2′-Deoxyadenosine (2′-AdR) and Its Analogs upon the Activity of the Steroid-Induced Synthesis of Δ^5-3-Ketosteroid Isomerase

The compounds[5] tested for activity as inhibitors of the synthesis of the isomerase are listed in Table 1. Whereas 2′-AdR produced a marked inhibition of the Δ^4-androstene-3,17-dione-induced increase in the activity of isomerase, such compounds as purine, adenine, adenosine, deoxyinosine, and deoxyguanosine exerted only minor inhibitory effects. Such naturally occurring pyrimidine derivatives as deoxythymidine, deoxycytidine, and uridine were ineffective, as was deoxyribose. Although 4-aminopyrazolo[3,4-*d*]pyrimidine, in which the carbon and nitrogen atoms in positions 7 and 8 of adenine are exchanged, produced no greater inhibition of enzyme activity than was caused by adenine itself, the deoxyribose-containing derivative of this analog corresponding in structure to 2′-AdR, caused 78% inhibition of the synthesis of induced enzyme at a level of only 3 mM, while for 72% inhibition by 2′-AdR, a level of 6 mM was required (5). Although the substitution of nitrogen for carbon in position 8 can increase activity (see below), 8-azahypoxanthine, like hypoxanthine deoxyribonucleoside, was inactive (8-azaadenine was too insoluble to study, and the deoxyribonucleoside was not available). Replacement of the hydrogen atom on carbon 8 of the parent compound with various groups, or

[5] The following compounds were not soluble in either DMSO or in the aqueous culture medium in the amounts employed: 9-(2,3-dihydroxypropyl)-adenine, 4-amino-9-*n*-hexylpyrazolo[3,4-*d*]pyrimidine, 7-methyladenine, 9-methyladenine, 6-hydroxylaminopurine, 2,6-diaminopurine, 8-azaadenine, 3′,5′-diethylthio(threo)-2′,3′,5′-trideoxyadenosine.

TABLE 1
The effect of various compounds upon enzyme induction in Pseudomonas testosteroni[a]

Compound	Isomerase activity[b] (% inhibition)
Purine and pyrimidine nucleosides	
2′-Deoxyadenosine	72
Adenosine	16
Deoxyinosine (hypoxanthine deoxyribonucleoside)	14
Deoxyguanosine	13
Deoxycytidine	0
Deoxythymidine	0
Uridine	0
Purine bases	
Purine	24
Adenine	39
Sugars	
Deoxyribose	2
Modifications of adenine	
4-Aminopyrazolo[3,4-*d*]pyrimidine	35
6-Chloropurine	63
8-Azahypoxanthine	0
Modifications of the adenine moiety of 2′-deoxyadenosine	
8-Iodo-2′-deoxyadenosine	7[c]
8-Bromo-2′-deoxyadenosine	42
8-Methoxy-2′-deoxyadenosine	48
8-Mercapto-2′-deoxyadenosine	0
8-Amino-2′-deoxyadenosine	16
2,6-Diaminopurine-2′-deoxyribonucleoside	13
9-(2′-Deoxy-β-D-ribofuranosyl)purine	6
4-Aminopyrazolo[3,4-*d*]pyrimidine-2′-deoxyribonucleoside 6 mM	83
3 mM	78
2 mM	66
1 mM	52
6-Chloropurine-2′-deoxyribonucleoside	33
Modifications of the sugar moiety of adenosine	
3′-Deoxyadenosine	8
2′,5′-Dideoxyadenosine	71
2′,3′-Dideoxyadenosine	58
2′,3′,5′-Trideoxyadenosine	74
3′,5′-Di-*O*-acetyl-2′-deoxyadenosine	5
3′-*O*-Acetyl-2′-deoxyadenosine	66
2′-*O*-Methyladenosine	23
3′-Ethylthio(threo)-2′,3′-dideoxyadenosine	70
5′-*O*-Pivalyl-5′-deoxyadenosine	0
5′-Ethylthio-2′,5′-dideoxyadenosine	0
2′,3′-Dideoxy-didehydroadenosine, 6 mM	81
3.4 mM	69
5′-Ethylthio-2′,3′,5′-trideoxy-2′,3′-didehydroadenosine	60
9-(Tetrahydro-2-furyl)adenine	38
Adenine arabinoside (9-β-D-arabinofuranosyladenine)	0
9-Ethyladenine	34

TABLE 1 (*Continued*)

Compound	Isomerase activity[b] (% inhibition)
9-*n*-Pentyladenine	41
9-Cyclopentyladenine	71
9-(2-Hydroxycyclopentyl)adenine	28
9-Cyclohexyladenine	30
Modifications of both the adenine moiety and the sugar moiety of 2′-deoxyadenosine	
6-Methylamino-9-(tetrahydro-2-furyl)purine	31
1-Cyclohexyl-4-aminopyrazolo[3,4-*d*]pyrimidine	94
1-Methyl-4-aminopyrazolo[3,4-*d*]pyrimidine	66
6-Chloro-9-(cyclopentyl)purine	94
6-Chloropurine ribonucleoside	0
Tubercidin (7-deazaadenosine)	22[c]
Steric modifications	
2′-Deoxy-β-L-adenosine	3[c]
9-(2′-Deoxy-α-D-ribofuranosyl)adenine	14
6-Chloro-9-(2′-deoxy-α-D-ribofuranosyl)purine	6

[a] As described under Materials and Methods, the bacterial cells were added to growth medium and maintained at 30° in a shaking incubator for 7 hr. The compounds studied, dissolved in DMSO to give a final concentration of 6 mM unless specified otherwise, were added 5 hr after the initiation of incubation. Several compounds investigated were not soluble in DMSO or in the aqueous medium of the bacterial suspension; these are listed in footnote 5.

[b] The percentage of enzyme activity was calculated for each experiment, and the mean of all the experiments is shown.

[c] The compound was added as a solid and the appropriate amount of DMSO was added separately.

the introduction of an amino group in position 2 of the adenine moiety, as well as the removal of the amino function in position 6 [9-(2′-deoxy-β-D-ribofuranosyl)purine], yielded compounds of relatively little or even no inhibitory activity. High activity was retained in compounds in which the hydroxyl groups of the sugar moiety of 2′-AdR were either partially or completely absent; thus, even 2′,3′,5′-trideoxyadenosine was as effective an inhibitor as was the parent compound. These findings suggest that phosphorylation of these compounds is not required for activity as inhibitors of induced enzyme synthesis. Analogous evidence was supplied by data obtained with 9-(tetrahydro-2-furyl)adenine; this hydroxyl-free compound, in which the terminal methyl group of trideoxyadenosine has been replaced by H, was about one-half as active as trideoxyadenosine. Since this synthetic compound is a mixture of α- and β-isomers, and other compounds with the α-configuration were inactive (see below), it can be assumed that the activity of the furyl derivative is attributable only to the β-isomer; this situation could exist also with 6-methylamino-9-(tetrahydro-2-furyl)purine, which caused 31% inhibition.

Compounds in which the 2′-position of 2′-AdR was modified, as in adenosine, 3′-deoxyadenosine, 2′-*O*-methyladenosine, adenine arabinoside (1-β-D-arabinofuranosyladenine), tubercidin (7-deazaadenosine), and 6-chloropurine ribonucleoside, were not active inhibitors. Alteration of the 3′-position of 2′-AdR did not decrease the activity, as indicated by the data obtained using 3′-*O*-acetyl-2′-deoxyadenosine and 3′-ethylthio(threo)-2′,3′-dideoxyadenosine (if it may be assumed that the substituent in position 3′ is not removed by the organism). 2′,3′-Dideoxy-didehydroadenosine also was quite effective in blocking the induction of isomerase activity. Compounds in which the deoxyribose moiety of either 2′-AdR or 4-aminopyrazolo[3,4-*d*]pyrimidine-2′-deoxyribonucleoside was replaced with

straight-chain alkyl groups (methyl, ethyl, *n*-pentyl) or cyclized alkyl groups (cyclopentyl, 2-hydroxycyclopentyl, cyclohexyl) also were investigated: the compound showing the highest activity was 9-cyclopentyladenine, which bears the closest structural similarity to 2′-AdR. Again, activity was reduced by insertion of a hydroxyl group in position 2′, as in 9-(2′-hydroxycyclopentyl)adenine, a situation analogous to the relationship between 2′-AdR and adenosine. Except for 5′-ethylthio-2′,3′,5′-trideoxy-2′,3′-didehydroadenosine, substitution on position 5′ (as in 3′,5′-di-*O*-acetyl-2′-deoxyadenosine, 5′-*O*-pivalyl-2′-deoxyadenosine, and 5′-ethylthio-2′,5′-dideoxyadenosine) led to a loss of inhibitory activity.

Substitution of the 6-amino group with chlorine had quite variable effects, dependent upon other alterations in structure; thus, 6-chloropurine was significantly more active than adenine itself, while as might be predicted from findings already cited with adenosine, the ribonucleoside was inactive. The 2′-deoxyribonucleoside of 6-chloropurine appeared to be less active than 2′-AdR, but it is doubtful that a 6 mM concentration was maintained. With the 9-(cyclopentyl)purines, however, the 6-chloro derivative was significantly more active than the 6-amino compound, a difference even more marked in the 8-aza series (see below).

Modifications that somewhat enhanced the inhibitory activity of 2′-AdR are to be found in 1-cyclohexyl-4-aminopyrazolo[3,4-*d*]pyrimidine and 6-chloro-9-(cyclopentyl)-purine. With changes in stereospecificity, as seen in 2′-deoxy-β-L-adenosine, 9-(2′-deoxy-α-D-ribofuranosyl)adenine, and 6-chloro-9-(2′-deoxy-α-D-ribofuranosyl)purine, as compared to the corresponding compounds of natural configuration, the capacity to inhibit enzyme induction was lost.

Since the inhibitory activity was enhanced when (a) a chlorine atom was introduced into position 6 of 9-cyclopentylpurine, (b) a nitrogen atom replaced carbon in position 8 of adenine, and (c) the sugar moiety was replaced by a five-membered ring without hydroxyl groups, the design and synthesis of 6-chloro-8-aza-9-cyclopentylpurine (compound 689) was carried out. This compound, as shown in Table 2, was among the most effective of the agents tested in blocking the synthesis of induced enzyme (75% inhibition at 0.25 mM). As shown in Table 3, replacement of the chlorine atom in position 6 of 689 by either hydroxyl, amino, or hydrazino groups led to a loss of activity.

TABLE 2

The effect of various concentrations of 6-chloro-8-aza-9-cyclopentylpurine (689) upon the induction of Δ⁵-3-ketosteroid isomerase activity in Pseudomonas testosteroni.

Two hours after inoculation, the inducer, Δ⁴-androstene-3,17-dione, was added; 689 was added 2 hr later. After a total period of 7 hr the cells were analyzed for total enzyme activity. The conditions of the 30° incubation are described under Materials and Methods.

Concentration of 689 (mM)	Isomerase activity (% inhibition)
1.0	96
0.5	95
0.3	93
0.25	75
0.15	17
0.10	0

TABLE 3

The effect of structural modifications in position 6 of 6-chloro-8-aza-9-cyclopentylpurine (689) upon the inhibition of induction of Δ⁵-3-ketosteroid isomerase activity in Pseudomonas testosteroni.

At 2 hr after inoculation, Δ⁴-androstene-3,17-dione was added; 2 hr subsequently the compound under test, dissolved in DMSO, was added to give a final concentration of 0.3 mM. After a total incubation period of 7 hr at 30° the cells were analyzed for total enzyme activity (see Materials and Methods).

Compound	Isomerase activity (% inhibition)
6-Chloro-8-aza-9-cyclopentylpurine	93
6-Hydroxy-8-aza-9-cyclopentylpurine	18
6-Amino-8-aza-9-cyclopentylpurine	19
6-Hydrazino-8-aza-9-cyclopentylpurine	0

TABLE 4

The effect of 6-chloro-8-aza-9-cyclopentylpurine (689) upon enzyme induction, growth, and nucleic acid and protein synthesis in Pseudomonas testosteroni

At 2 hr after inoculation of cells into a growth medium at 30°, the inducer, Δ^4-androstene-3,17-dione, was added; 2 hr later 689 was added to the culture; 3 hr later samples were removed and analyzed for turbidity, total content of DNA, RNA, protein, and Δ^5-3-ketosteroid isomerase activity, as described under Materials and Methods. L-Leucine-1-^{14}C was added 1 hr after 689 in experiment A, and simultaneously with 689 in experiment B (see Materials and Methods).

		Percent of control					
			Total content/ml			L-Leucine-1-^{14}C	Total
Expt.	Conc. 689 (mM)	Turbidity	DNA	RNA	Protein	into residual protein	Δ^5-3-ketosteroid isomerase activity
A	0.3		59	80	87	64	13
	0.2		74	89	86	71	—
	0.1		80	77	92	86	109
B	0.3	89	51	103	79	52	2
	0.2	97	79	88	91	81	9
	0.1	103	93	94	96	82	62

TABLE 5

The effect of 6-chloro-8-aza-9-cyclopentylpurine (689) upon the uptake of the inducer, Δ^4-androstene-3,17-dione, by Pseudomonas testosteroni

Cells were added to growth medium at 30°, as described under Materials and Methods. At 2 hr after inoculation, Δ^4-androstene-3,17-dione-4-^{14}C (250 μg/ml, 6×10^6 cpm) was added, and at the desired interval 0.1-ml samples of bacterial suspension were removed and added to phosphate buffer (pH 7.0, 0.1 M). The suspension was filtered and washed with buffer on Millipore membranes and then the radioactivity was determined. Samples of bacterial suspension to which the inducer was added simultaneously with 689 (0.3 mM, 4 hr after inoculation) were treated similarly.

Time (hr) after addition	Cpm $\times 10^{-3}$	
Addition of inducer in absence of 689 (beginning 2 hr after inoculation)		
0.25	52.7	
1.0	63.9	
2.0	66.0	
Addition of inducer and 689 simultaneously (beginning 4 hr after inoculation)		
	Inducer only	Inducer + 689
0.5	58.3	77.2
2.0	39.8	66.6

As shown in Table 4, compound 689, at a level of 0.3 mM, markedly inhibited the synthesis of DNA while the formation of RNA and protein was decreased to a much lesser extent. The inhibition of DNA synthesis was accompanied by an even greater inhibition of the formation of induced enzyme. The biosynthesis of another of the steroid-induced enzymes, 3α-hydroxysteroid dehydrogenase (2), was inhibited to the same extent by 689 as was the isomerase. The formation of total protein, as measured by the fixation of leucine-1-^{14}C into residual protein was considerably less sensitive to inhibition by 689 than was the steroid-induced formation of the isomerase.

The data in Table 5 indicate that the inhibition of enzyme activity is not attributable to prevention of the uptake of the inducer, Δ^4-androstene-3,17-dione, by the organism; thus, cells appear to contain maximum amounts of the inducer prior to exposure to 689, and this uptake was not prevented when the steroid was added subsequent to 689. The decrease in radioactivity of Δ^4-androstene-3,17-dione at 6 hr after inoculation in control cells probably reflects a loss of inducer due to catabolism.

To eliminate the possibility that inhibition of the formation of induced enzyme by 689 was the result of direct inhibition of enzyme activity, the compound (0.3 mM

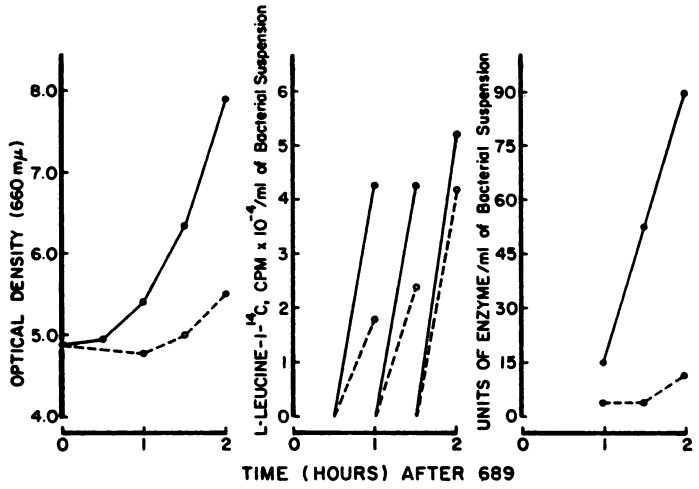

FIG. 1. *The duration of inhibition of the synthesis of induced enzyme and protein by 689, 0.3* mM

Two hours after inoculation of cells into growth medium at 30° (see Methods), the inducer, Δ^4-androstene-3,17-dione, was added. DMSO or 689 in DMSO was added to 5-ml samples of bacterial suspension 2 hr later and after either 0.5, 1, or 1.5 hr L-leucine-1-^{14}C (2 μC; 8 μg) was added. After allowing 30 min for incorporation of the labeled precursor, the flasks were removed and analyzed for turbidity, Δ^5-3-ketosteroid isomerase activity, and incorporation of radioactivity into protein as outlined in Methods. One unit of enzyme produces a change in absorbancy of 1 optical density unit per minute. Control, ●——●; 689-treated ○- - - -○.

and 1 mM) was added directly to the enzyme-containing buffered extract of the acetone-powdered cells (see Materials and Methods) and incubated for 30 min at 30°; significant loss of enzyme activity did not occur.

Since some of the inhibitory effects of 689 at 0.3 mM diminished more rapidly than others, it was possible to separate the metabolic alterations induced by this agent (Fig. 1); thus, the inhibition of the synthesis of total protein was waning within 1.5 hours after the addition of 689, while the blockade of the formation of induced enzyme was of longer duration. Two hours after the addition of 689, 0.3 mM, the synthesis of total protein in the treated cells was inhibited only 23% when the activity of the isomerase was reduced by about 87%.

DISCUSSION

It has been shown that 2′-AdR after phosphorylation can inhibit DNA synthesis by blocking the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates (6–12); hence, such a metabolite possibly could account for the inhibition of the steroid-induced synthesis of enzyme. The data obtained with 2′,3′,5′-trideoxyadenosine and 689 (compounds that cannot be directly phosphorylated) indicate, however, that either the inhibition of induced-enzyme synthesis or the inhibition of the formation of DNA by 689 is not dependent upon the participation of a phosphorylated derivative. 4-Aminopyrazolo-[3,4-*d*]pyrimidine, a purine analog in which the nitrogen atom of position 7 and the carbon atom of position 8 are exchanged, inhibits the *de novo* synthesis of purines (21), and 6-chloropurine inhibits the synthesis of guanine nucleotides (22). Since both of these modifications, a chlorine atom in (purine) position 6 and an 8-aza substitution, are found in 689, it is possible that the compound exerts an inhibitory effect upon the purine nucleotide synthetic pathways; however, the fact that the formation of DNA is inhibited to a much greater extent than is that of RNA, indicates that the total activity of the compound involves other inhibitory activities. The effect of 689 upon the synthesis of nucleic acids and protein is similar to that observed in "thymine-

less death" (23) (i.e., an accumulation of RNA and protein relative to DNA occurs in treated cells). The findings presently available suggest that 689 may inhibit the synthesis of thymidylate, an effect that cannot be circumvented by the addition of thymidine, which cannot be utilized by *P. testosteroni* for DNA synthesis (unpublished observations).

During the course of these experiments, it was reported (24) that 2′,3′-dideoxyadenosine, an analog of AdR that we have shown (13) to be capable of inhibiting enzyme induction, produces an effect in *Escherichia coli* that is similar in some respects to "thymineless death." The authors hypothesized that this analog is incorporated into DNA, with resultant interruption in the biosynthesis of DNA because of the absence of the 3′-hydroxyl group necessary for the formation of 3′,5′-phosphodiester linkages; the synthesis of RNA and protein, however, were affected to a much smaller extent. It is possible that 2′,3′-dideoxyadenosine exerts its effects in this manner, but the compound also could act in a manner analogous to 689, by inhibiting the formation of thymine-containing deoxyribonucleotides. Since 2′,3′-dideoxyadenosine is capable of being phosphorylated, an additional possibility is that it exerts an effect upon the enzyme ribonucleoside diphosphate reductase, in a manner similar to that of dATP.

It appears that 689 has some selectivity of action with respect to the inhibition of synthesis of induced enzymes, as compared to that of total protein. This property is not the result of a direct effect of 689 upon the entrance of the inducer into the cells or on the activity of the induced enzyme, but further work will be necessary to account for the relatively greater susceptibility of the synthesis of induced enzymes, as compared to the formation of total protein.

It has been observed (unpublished observations) that if the inducer is added at a time when the cells are in the logarithmic phase of growth, no lag in the appearance of induced enzyme occurs. Therefore, as shown in Fig. 1, if the effect of 689 upon enzyme induction were attributable to an interference with protein synthesis, the recovery of this process that is observed 2 hr after exposure of cells to 689 should have been accompanied by an immediate rise in the activity of the induced enzyme. The fact that the induction of the synthesis of new molecules of the isomerase did not recover from inhibition at a similar rate suggests that a relatively specific inhibition occurs.

The chlorine atom in position 6 of 689 could make this compound a potent "alkylating agent"; thus, alkylation of various macromolecules could account for some or all of the observed phenomena. Since the halogen atom in position 6 of 689 is relatively more reactive than 6-chloropurine-2′-deoxyribonucleoside and 6-chloro-9-cyclopentylpurine, this phenomenon may account for the greater inhibitory activity of 689. The finding that substitution of the chlorine atom with either hydroxyl, amino, or hydrazino groups results in marked loss of inhibitory activity (Table 3) supports the concept of alkylation. On the other hand, the even greater lability of fluorine, as compared to chlorine, in compounds of this type makes mandatory a study of 6-fluoro-9-cyclopentylpurine, its 8-aza derivative, and related compounds. It would appear that the rigid conformational requirements necessary for the inhibition of induced enzyme synthesis which have been described, may be related to the need for a precise fit of 2′-AdR, 689, and intermediate compounds on a "receptor site," while a halogen in position 6 may afford an opportunity for irreversible inactivation of the receptor.

The precise site and mechanism of the inhibitory action of 689 and its congeners on the synthesis of nucleic acids, and on proteins in general, as well as on that of induced enzymes in particular, are under investigation.

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