HORMONAL INTERVENTION IN THE UPTAKE OF AMINO ACIDS BY STAPHYLOCOCCI

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PREVIOUS work (Yotis and Stanke, 1966; Yotis, 1967; Yotis and Fitzgerald, 1968; Yotis and Waner, 1968; Yotis and Baman, 1969) has shown that steroids exert an antimicrobial effect *in vitro* and *in vivo* on *Staphylococcus aureus*, and may play a role in protection against staphylococcal infections. Progesterone and certain important androgens, employed in the pharmacological range of 15–40 μg per ml, reduced the growth of Gram-positive organisms in nutrient broth. In addition, staphylococci grew more luxuriantly in serum from control rabbits than in serum from hormone-treated animals. The steroids also effectively retarded the progress of induced furunculosis in rabbits. The present experiments were designed to determine optimum parameters for the hormonal inhibition of the uptake of alanine and glutamic acid by staphylococci, and to correlate this inhibition with the previously observed hormonal intervention in staphylococcal growth.

MATERIALS AND METHODS

Strains. *Staph. aureus* serotypes IX–XIII (ATCC 12606–12610) were maintained by periodic transfer on nutrient agar slants at 4°C. The bacteria were tested for glucose and mannitol utilisation under anaerobic conditions according to the method of Hugh and Leifson (1953) and for gelatin liquefaction, pigmentation, haemolysis, and production of both free and bound coagulase by accepted microbiological techniques (Sonnenwirth, 1963). Strains of *Shigella flexneri* and *Salmonella paratyphi* were obtained from stock culture collections maintained at the Strych School of Medicine and were originally from the American Type Culture Collection.

Chemicals. Crystalline progesterone, testosterone, and oestradiol-17 β were obtained from Sigma Chemical Company and crystalline diethylstilboestrol from Calbiochem. The purity of the compounds was tested by observation of melting points and maximum absorbancies. A specific amount of hormone was dissolved in 95 per cent. ethanol and diluted to a final concentration of 1 or 2 per cent. ethanol. Control cells were exposed to the 1 or 2 per cent. ethanol. L-Alanine-¹⁴C (uniformly labelled, u.l.) and L-glutamic acid-¹⁴C (u.l.) were obtained from Amersham/Searle Corporation, Des Plaines, Illinois, with specific activities of 156 and 249 mCi per m.mole respectively.

Amino acid uptake studies. Bacteria were grown to mid-log phase in tryptic soy broth (Difco) on a rotary shaker at 37°C, harvested by centrifugation, and prepared in smooth suspension as previously described (Yotis and Fitzgerald). All uptake experiments were performed in phosphate buffers (Gomori, 1955) at the indicated pH values. A measured volume of cells was added to 0.1 mCi of the appropriate amino acid in the presence and absence of the hormone(s). Equal volumes of the suspension were filtered at specified times thereafter through Millipore filters (Millipore Corporation, New Bedford, Mass., 0.45 μm

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pore size) pre-saturated with the appropriate unlabelled amino acid at 10^{-3} M. The filter-membranes were immediately washed with five volumes of cold buffer, air-dried, and placed in scintillation fluid (Davidson and Feigelson, 1957). The radioactivity of filters was assayed according to the method of Mans and Novelli (1961) in a Packard Tri-carb liquid scintillation spectrometer model 3320.

Statistical analysis. All data were subjected to statistical analysis (Batson, 1956). A "t" test value greater than 2.00 indicates a probability factor of less than 5 per cent. and is statistically significant.

RESULTS

The effect of bacterial concentration on the hormonal inhibition of amino acid uptake

The initial experiment was designed to determine the bacterial concentration required for optimal hormonal inhibition of amino acid uptake. To this end, staphylococci were suspended in buffer, pH 7.0, at various cellular concen-

TABLE I
Effect of cell concentration on hormonal inhibition of alanine uptake

Bacterial concentration (μ g dry wt per ml)	μ M 14 C-alanine taken up by		Difference (per cent.)	"t"*** for difference
	control cells	progesterone- treated cells		
75	1.20	1.06	11.7	2.09
90	1.80	1.48	17.8	2.23
125	2.25	1.95	13.4	2.11
180	2.95	2.30	22.1	2.96
260	4.30	3.15	26.8	2.89

* Values greater than 2.00 are statistically significant.

trations and each suspension was added to a solution of 14 C-labelled alanine containing progesterone 40 μ g per ml in 1 per cent. ethanol. Similar suspensions were added to control tubes that contained 14 C-alanine in 1 per cent. ethanol without the hormone. Incubation at 24°C was terminated by Millipore filtration 8 min. after addition of the label. The data reveal that progesterone significantly reduces the total uptake of the 14 C-alanine at all bacterial concentrations (table I). Inhibition ranged from 12 to 27 per cent. with maximal effects at the greatest bacterial concentration. All further experiments were performed at a final cellular density equivalent to 260 μ g dry weight per ml after addition of the 14 C-label and the ethanol or ethanol-hormone mixture.

Hormonal effects on various strains of Staph. aureus

Five strains of staphylococci were suspended in buffer, pH 7.0, at 24°C. The uptake of 14 C-alanine was examined in the presence and absence of progesterone at a concentration of 40 μ g per ml. Uptake was terminated 8 min. after addition of the label. Progesterone exerted a significant inhibitory action on all serotypes. A definite variation in the responses of the strains

to the hormone was observed. Serotype X was the most susceptible whereas serotypes XII and XIII were least susceptible (table II).

TABLE II
Differences in susceptibility to hormonal inhibition of alanine uptake exhibited by various serotypes of Staph. aureus

Serotype	μM ^{14}C -alanine taken up by		Difference (per cent.)
	control cells	progesterone-treated cells	
IX	4.99	4.21	15.7
X	2.73	2.15	21.3
XI	3.67	3.08	16.1
XII	4.01	3.52	12.3
XIII	4.63	4.00	13.7

The time-course of the influence of progesterone on the uptake of alanine and glutamic acid

Bacteria were exposed to ^{14}C -alanine with and without progesterone at 40 μg per ml. Cells were removed from the suspensions at the indicated times and analysed for radioactivity (table III). The uptake of alanine by staphylococci was significantly retarded by the hormone. The inhibition was evident

TABLE III
Time-course study of alanine uptake by staphylococci and its inhibition by progesterone

Time (min.)	μM ^{14}C -alanine taken up by		Difference (per cent.)
	control cells	progesterone-treated cells	
5	1.96	1.56	20.4
9	2.83	2.31	18.4
45	6.79	5.76	15.2
120	10.78	9.57	11.2
180	13.63	11.80	13.4
240	16.63	14.49	12.9

during the initial 5 min. and remained relatively constant throughout the incubation period of 240 min. The hormonal inhibition is sustained, even when saturation levels of ^{14}C -alanine are approached after 240 min.

Similar results were obtained in a related series of experiments with glutamic acid in the presence of progesterone 40 μg per ml. However, the inhibition was observed only during the initial $\frac{1}{2}$ –7 min. after the addition of the label; thereafter, both control and hormone-treated cells contained equal amounts of ^{14}C -activity.

At this point it was of interest to determine whether the hormonal effects were exerted on the actual transport of the amino acid into the cell, or on the adsorption of the amino acid to the bacterial cell prior to its assimilation. Identical experiments were undertaken with heat-killed organisms (65°C for 30 min.) and progesterone at 40 μ g per ml. The data indicated that the cells adsorbed insignificant amounts of radioactivity, slightly above the background level.

TABLE IV
Results of comparative studies of the action of progesterone on alanine uptake by bacteria

Species tested and details of observations made	Results obtained at the stated time after addition of ^{14}C -alanine		
	5 min.	9 min.	16 min.
<i>Staphylococcus aureus</i> Index of alanine uptake* by: control cells progesterone-treated cells Difference per cent. "t" for difference	32,180 26,060 19.1 2.15	48,260 38,500 20.3 2.73	74,900 55,240 26.3 3.29
<i>Shigella flexneri</i> Index of alanine uptake* by: control cells progesterone-treated cells Difference per cent. "t" for difference	13,330 12,960 2.8 0.84	17,330 17,940 3.5 0.56	25,720 25,750 0.1 0.01
<i>Salmonella paratyphi</i> Index of alanine uptake* by: control cells progesterone-treated cells Difference per cent. "t" for difference	33,390 34,870 4.4 0.01	48,830 50,860 4.1 1.03	67,210 69,720 3.7 1.02

* Uptake expressed as counts per minute per 30 ml cells.

Action of progesterone on other micro-organisms

Previous work (Yotis and Stanke) showed that steroids inhibited the in-vitro growth of Gram-positive micro-organisms such as *Staph. aureus* and *Bacillus subtilis*, but did not affect the growth of Gram-negative organisms. Thus, it was important to undertake a related series of experiments with Gram-negative organisms. *Shigella flexneri* and *Salmonella paratyphi* were exposed to ^{14}C -alanine under identical conditions of pH, temperature, cell concentration, and progesterone at 40 μ g per ml. The bacteria were removed and assayed for radioactivity at 5, 9 and 16 min. after addition of the label. The results given in table IV show that after 5 min. progesterone inhibited staphylococcal alanine uptake 19 per cent.; after 9 min., 20 per cent.; and after 16 min.,

26 per cent. In direct contrast, *Sh. flexneri* and *S. paratyphi*, in the presence of hormone, showed no significant variation in the amount of ^{14}C -activity taken up compared with that assimilated by corresponding controls.

The effect of hormone concentration

The extent of the inhibition produced by various progesterone concentrations on alanine uptake is indicated in table V. Cells were exposed to the ^{14}C -amino acid in the presence of the hormone at concentrations of 30, 20, 10, 1 and 0 μg per ml. Bacteria were isolated by membrane filtration 8 min. after addition of the label, and assayed for radioactivity. A progressive increase in inhibition was observed with the increasing concentrations of progesterone.

TABLE V

The degree of inhibition of alanine uptake observed at various progesterone concentrations in tests with Staph. aureus

Progesterone concentration (μg per ml)	Index of ^{14}C -alanine uptake*	Difference (per cent.)	"t" for difference
0	43,400	...	...
1	40,709	6.3	1.31
10	37,910	12.7	1.87
20	33,250	23.4	2.35
30	30,920	28.8	2.24

* Uptake expressed as counts per minute per 30 ml cells.

These results were correlated with those of previous work (Yotis and Stanke; Yotis and Fitzgerald) in which staphylococcal growth was retarded by steroids in the pharmacological range of 15–40 μg per ml. It is noteworthy that similar dosages also effectively inhibit the uptake of alanine.

Effect of pH

Because amino acid uptake is mediated by enzyme-like reactions involving permease systems (Cohen and Monod, 1957; Kepes, 1963), it was of interest to observe hormonal effects at various hydrogen ion concentrations. Phosphate buffers were prepared at pH 5.7, 6.5, 7.5 and 8.0. Cells were exposed to ^{14}C -alanine for 8 min. and assayed for radioactivity (table VI). Maximum inhibition occurred at pH values of less than 7.0. At pH 5.7, 140 μmoles of alanine were taken up by hormonally treated bacteria whereas 275 μmoles were taken up by the controls (49 per cent. inhibition); at pH 6.5, 130 μmoles compared with 295 μmoles (56 per cent. inhibition). At pH values above 6.5, there was a rapid decline in alanine uptake and correspondingly less marked inhibitory effects.

Effect of other hormones

The observation that progesterone inhibited the uptake of alanine and glutamic acid prompted a comparative study of the effects of other hormones.

Bacterial uptake of ^{14}C -alanine was observed separately in the presence of progesterone, testosterone and oestradiol- 17β at $40\text{ }\mu\text{g}$ per ml, and diethylstilboestrol at $20\text{ }\mu\text{g}$ per ml. The solubility of the latter compound obliged us

TABLE VI

Hormonal inhibition of alanine uptake by Staph. aureus at various pH values

pH	μM ^{14}C -alanine taken up by		Difference (per cent.)	“ t ” for difference
	control cells	progesterone-treated cells		
5.7	2.75	1.40	49.1	4.29
6.5	2.95	1.30	56.0	4.43
7.5	1.30	1.17	10.0	1.90
8.0	0.76	0.69	9.3	1.66

to use 2 per cent. ethanol as the solvent for all four compounds and in the control studies. Cells were removed at 2, 5 and 9 min. after addition of the label and assayed for radioactivity. Diethylstilboestrol produced maximum

TABLE VII

Effect of various hormones on the uptake of alanine by Staph. aureus

Hormone used for treatment of test bacterial suspension	Test result obtained at the given time after addition of ^{14}C -alanine					
	2 min.		5 min.		9 min.	
	μM ^{14}C -alanine taken up	“ t ” for difference from control	μM ^{14}C -alanine taken up	“ t ” for difference from control	μM ^{14}C -alanine taken up	“ t ” for difference from control
Nil (control)	1.74	...	3.16	...	4.40	...
Testosterone, $40\text{ }\mu\text{g}$ per ml	1.56	2.00	2.85	1.83	3.87	2.11
Progesterone, $40\text{ }\mu\text{g}$ per ml	1.49	2.26	2.62	2.14	3.74	2.31
Oestradiol- 17β , $40\text{ }\mu\text{g}$ per ml	1.52	2.14	2.67	2.22	3.78	2.09
Diethylstilboestrol, $20\text{ }\mu\text{g}$ per ml	0.51	4.47	0.50	3.98	0.55	4.20

inhibition, retarding uptake by 70–80 per cent. Testosterone, oestradiol- 17β and progesterone exerted relatively less marked inhibitory effects (table VII).

In related experiments, 17α -hydroxyprogesterone, which did not retard the growth of *Staph. aureus* in broth (Yotis and Stanke), produced no inhibitory effects on the uptake of alanine and glutamic acid by staphylococci.

DISCUSSION

The data presented indicate that hormones inhibit the uptake of amino acids by *Staphylococcus aureus*. The following results observed in our present study are correlated with the previously observed retardation of staphylococcal growth in broth (Yotis and Stanke, 1966; Yotis, 1967; Yotis and Fitzgerald, 1968; Yotis and Waner, 1968; Yotis and Baman, 1969): (1) progesterone in amounts exceeding 15 μg per ml inhibited uptake of amino acids; (2) maximum reduction of uptake was obtained with diethylstilboestrol; (3) 17 α -hydroxyprogesterone did not significantly alter uptake; and (4) uptake by Gram-negative micro-organisms was not affected by the steroids or by diethylstilboestrol.

The last mentioned observation invites interesting speculation. The steroids retard the growth of staphylococci without penetrating the bacterial cells in significant quantities (Yotis and Fitzgerald), and in some way probably influence extracellular factors in the cell wall or cell membrane. In so doing, direct action may be exerted on specific uptake systems located in the cell membrane, and this in turn retards the growth of the bacteria. A major difference between Gram-negative and Gram-positive organisms is the biochemical structure of the cell wall. A possible explanation of the resistance of Gram-negative organisms to the effect may involve the inability of the organism to bind hormones and thus avoid the postulated subsequent effects on uptake systems.

The pH dependence of hormonal inhibition may reflect steroidal interaction with the enzyme-like permease systems (Cohen and Monod, 1957; Kepes, 1963) for amino acid transport. The data implicate permease systems, in that maximum uptake inhibition (56 per cent.) occurred at that pH value (6.5) associated with maximum uptake of alanine. A further explanation for increase in hormonal inhibition as pH is lowered is the availability of greater numbers of charged groups in the cell surface, resulting in greater attraction and binding of the hormones. The observation that changes in hormonal inhibition rates correspond to pH changes of the suspending medium implicate an extracellular mode of action perhaps at the cell wall or cell membrane.

The results of the present work are correlated with those of previous work and indicate parameters that may be of use in elucidating the mechanism of the retardation of the growth of *Staph. aureus* by these hormones.

SUMMARY

Hormonal effects on the uptake of amino acids by *Staphylococcus aureus* have been studied in relation to the reported antibacterial action of the hormones. Progesterone at 40 μg per ml retarded the uptake of ^{14}C -alanine and ^{14}C -glutamic acid. Diethylstilboestrol, testosterone and oestradiol-17 β exerted similar inhibitory effects, whereas 17 α -hydroxyprogesterone did not. Maximum inhibition of alanine uptake occurred at a cellular density corresponding to 260 μg dry weight per ml, at a pH value between 5.7 and 7.0, and with diethylstilboestrol at a concentration of 20 μg per ml. The assimilation of alanine by *Shigella flexneri* and *Salmonella paratyphi* was not inhibited by progesterone. The data are correlated with inhibitory phenomena previously observed and

indicate parameters for further research toward elucidation of the hormonal mechanism of inhibition of *Staph. aureus*.

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