Identity of the Site of Action of 3′,5′-Adenosine Monophosphate and Adrenocortocotropic Hormone in Corticosteroidogenesis in Rat Adrenal and Beef Adrenal Cortex Slices*

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ABSTRACT: 3′,5′-Adenosine monophosphate (3′,5′-AMP) has been found to stimulate the transformation of [1-^{14}C]acetate and [7α-^{3}H]cholesterol to corticosterone in rat adrenal slices and to cortisol in beef adrenal cortex slices. When [1-^{14}C]acetate is the corticosteroid precursor, the stimulation of corticosteroid synthesis by 3′,5′-AMP is accompanied by a drop in the radioactivity of the cholesterol arising from the [^{14}C]acetate. The same results were obtained when stimulation of corticosteroidogenesis by adrenocortocotropic hormone (ACTH) was investigated. In addition, neither 3′,5′-AMP nor ACTH stimulated the transformation of [7α-^{3}H]pregnenolone (3β-hydroxy-5-pregnen-3-one) or [7-^{3}H]progesterone (4-pregnene-3,20-dione) to corticosteroids. The results indicate that both 3′,5′-AMP and ACTH stimulate at the same site in the biosynthetic pathway for corticosteroids, i.e., between cholesterol and pregnenolone, and suggest that 3′,5′-AMP is an obligatory intermediate in the action of ACTH.

It has been shown by Haynes (1958) that incubation of beef adrenal slices with ACTH[1] results in an increase in the level of 3′,5′-AMP in this tissue, and in a subsequent study 3′,5′-AMP was found to stimulate corticoid synthesis in rat adrenal slices (Haynes *et al.*, 1959). These results suggested that the stimulation of corticosteroidogenesis by ACTH is mediated via 3′,5′-AMP. Further support for this suggestion was provided by the observation that the addition of 3′,5′-AMP to rat adrenal slices maximally stimulated by ACTH results in essentially no increase in corticoid production over that seen with ACTH alone (Koritz, 1962). If ACTH action on the adrenal cortex is mediated via 3′,5′-AMP, as the foregoing results indicate, then the stimulation of corticoid synthesis by 3′,5′-AMP should take place at the same site as that for ACTH in the biosynthetic reaction sequence leading to corticoid formation. To help answer this question, the site of stimulation of corticoid synthesis by ACTH and 3′,5′-AMP in the adrenal was investigated with the aid of various radioactive precursors of the adrenocorticosteroids. In his pioneering studies, Hechter (1951) concluded, from considerations of the rates with which precursors were converted to corticosteroids, that ACTH probably acts between cholesterol and pregnenolone. In later direct studies, using radioactive precursors, Stone and Hechter (1954) presented evidence that ACTH acts between cholesterol and pregnenolone; these results were consistent with the previous suggestion although pregnenolone was not examined in this study. In some recent reports Savard and Mason (1963) and Savard and Casey (1964) have suggested that LH and ACTH stimulate hormone production in their respective target organs by acting at some site between acetate and cholesterol.

The data presented in this paper indicate that ACTH stimulates hormone production in adrenals from the rat and from cattle at some site between cholesterol and pregnenolone, in confirmation of the conclusions of Hechter (1951) and Stone and Hechter (1954), and that 3′,5′-AMP also stimulates hormone production at a site between cholesterol and pregnenolone. These results, in conjunction with those of Haynes (1958), Haynes *et al.* (1959), and Koritz (1962), indicate that 3′,5′-AMP is probably an obligatory intermediate in ACTH action. The data do not indicate a simulation by ACTH or 3′,5′-AMP at a site between acetate and cholesterol.

Experimental Procedures

Incubations. In experiments with rat adrenals the glands from Sprague-Dawley rats were sectioned and preincubated for 1 hour in Krebs-Ringer-bicarbonate-glucose (200 mg %) according to the procedure of Saffran and Schally (1955). Final incu-

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[1] Abbreviations used in this paper: ACTH, adrenocorticotropic hormone; 3′,5′-AMP, adenosine 3′,5′-cyclic-phosphate; LH, luteinizing hormone.

bation was for 3 hours under 95% O_2–5% CO_2 at 37°. When radioactive cholesterol, pregnenolone, or progesterone were used as substrates, the final incubation medium consisted of 1.8 ml of Krebs-Ringer-bicarbonate-glucose, radioactive substrate, and 0.154 M NaCl or other additions to a final volume of 2.0 ml. When present, ACTH was added at 0.5 unit per beaker and 3′,5′-AMP at 20 μmoles per beaker. Control and experimental conditions (the presence of ACTH or 3′,5′-AMP) consisted of eight incubation beakers each with about 40 mg tissue per beaker. When radioactive acetate was used as the substrate, the final incubation medium consisted of 2.5 ml of Krebs-Ringer-bicarbonate-glucose and 0.154 M NaCl or other additions to a final volume of 3.0 ml. In these experiments control and experimental conditions consisted of one incubation beaker each, with about 500 mg tissue per beaker. When present, ACTH was added at 1.0 unit per beaker and 3′,5′-AMP at 80 μmoles per beaker. [7-^{3}H]Progesterone and [7α-^{3}H]Δ^5-pregnenolone (14 μc and 12 μc, respectively) were added to the dry beakers in benzene, and the benzene was removed under nitrogen so that the steroid was deposited as a dried film on the bottom of the beaker. Sodium [1-^{14}C]acetate was added at the level of 134 μc per incubation beaker. Five μc of [7α-^{3}H]cholesterol was added per incubation beaker in 0.1 ml of an emulsion in Tween-80 (Atlas Powder Co.) prepared essentially according to Constantopoulos and Tchen (1961). The [7α-^{3}H]-cholesterol (100 μc) was taken to dryness under nitrogen, the residue was dissolved in 1 ml of acetone, and 0.6 ml of a Tween-80 solution in acetone (1 mg/ml) was added. After mixing, the acetone was removed under nitrogen and the cholesterol-Tween-80 was dissolved in 2.0 ml of water. Addition of greater than 5μc of [7α-^{3}H]cholesterol in these incubations resulted in no greater counts in the products isolated.

In experiments with beef adrenal cortex, slices of the tissue were prepared with a Stadie-Riggs microtome and 500 mg of tissue per beaker were preincubated in 3.0 ml of Krebs-Ringer-bicarbonate-glucose at 37° under 95% O_2:5% CO_2 for 1 hour. The final incubation was for 3 hours under 95% O_2:5% CO_2 at 37° in a medium consisting of 2.5 ml of Krebs-Ringer-bicarbonate-glucose and 0.154 M NaCl or other additions to a final volume of 3.0 ml. When present, ACTH was added at 1.0 unit per beaker and 3′,5′-AMP at 80 μmoles per beaker. [7-^{3}H]Progesterone and [7α-^{3}H]Δ^5-pregnenolone, both at 20 μc per beaker, were added to the beakers as described above. [7α-^{3}H]Cholesterol (25 μc per beaker) was added in a Tween-80 emulsion (see above), and sodium [1-^{14}C] acetate was added at the level of 93 μc per incubation beaker.

Extraction and Purification of Products. In experiments with rat adrenals, following the incubations with radioactive cholesterol, pregnenolone, or progesterone, the incubation medium was removed, the tissue was rinsed with 0.154 M KCl, and the combined medium and rinse were extracted 3 times with dichloromethane. In all experiments the contents of each beaker were extracted separately and then the extracts from the beakers for a given experimental procedure pooled. With incubations with [1-^{14}C]acetate, the tissue was homogenized in its incubation medium and extracted three times with dichloromethane, and the pooled dichloromethane extract was washed once with 1% sodium acetate and twice with water. The dichloromethane extracts were now dried with sodium sulfate, the solvent was removed under nitrogen, and the residue was chromatographed on paper for 46 hours using the system ligroin–propylene glycol (Savard, 1954). The dried paper strips were surveyed for radioactivity with a chromatogram scanner (Atomic Accessories, Inc.). The radioactive area at or near the origin, which contained the corticosterone, was eluted and rechromatographed on paper for 17 hours using the system toluene–propylene glycol (Burton *et al.*, 1951). The dried chromatograms were scanned for radioactivity, the area corresponding to corticosterone was eluted, and the radioactivity was determined by counting an aliquot dissolved in 10 ml of scintillation fluid (0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-2[5-phenyloxazolyl]benzene in toluene) in a Packard Tri-Carb liquid scintillation spectrometer. Carrier corticosterone (30 mg) was added to the eluate and the material was recrystallized from acetone-ligroin (1:9) to constant specific activity. The material from the last recrystallization was acetylated by the method of Zaffaroni and Burton (1951), the acetate was recrystallized from ethyl acetate–ligroin (5:9.5), and its specific activity was determined.

In experiments with beef adrenal cortex slices, following all incubations, the tissue was homogenized in its incubation medium and extracted three times with dichloromethane. The dichloromethane extracts were treated as described above for the incubation with rat adrenals, but were chromatographed directly on the toluene–propylene glycol system for 46 hours. The radioactive area corresponding to cortisol was eluted and its radioactivity was determined by liquid scintillation counting. Carrier cortisol (30 mg) was added to the eluate and the material was recrystallized from ethyl acetate–ligroin (1:1) to constant specific activity. The material from the last crystallization was acetylated, the acetate was recrystallized from ethyl acetate–ligroin, and its specific activity was determined.

When [1-^{14}C]acetate was used as the substrate in incubations with both rat and beef adrenal tissue, the [^{14}C]cholesterol formed in the absence and presence of ACTH or 3′,5′-AMP was isolated. To this end, the runoffs from the ligroin–propylene glycol chromatograms in the case of incubations with rat adrenals and from the toluene–propylene glycol chromatograms in the case of incubations with beef adrenal cortex were collected and the solvent was taken off under nitrogen. The residue material was saponified and, to ensure adequate digitonide formation, 100 μg of cold cholesterol was added. The saponified

TABLE I: Radiochemical Homogeneity of [^{14}C]Corticosterone and [^{14}C]Cortisol Biosynthesized from [1-^{14}C]Acetate by Rat Adrenal and Beef Adrenal Cortex Slices under Various Conditions.

Tissue	Stage of Purification	Radioactivity (cpm)	Radioactivity (cpm/mmole)	+ ACTH Radioactivity (cpm)	+ ACTH Radioactivity (cpm/mmole)	+ 3′,5′-AMP Radioactivity (cpm)	+ 3′,5′-AMP Radioactivity (cpm/mmole)
Rat adrenal	After carrier corticosterone added	4,100	34,600	4,620	40,000	3,820	28,400
	1st recrystallization	1,340	12,500	2,420	23,000	1,980	17,400
	2nd recrystallization	820	9,400	1,860	20,400	1,500	17,200
	3rd recrystallization	580	8,300	1,720	20,700	1,400	16,600
	4th recrystallization	560	8,600	1,280	20,800	1,040	15,600
	5th recrystallization	460	8,300	930	19,000	960	17,600
	5th mother liquor	72	8,700	190	21,000	160	16,800
	Corticosterone acetate	340	8,200	670	20,200	710	16,900
	Corrected cpm[a]	1,000		2,400		2,300	
Beef adrenal	After carrier cortisol added	25,500	310,000	101,000	1,180,000	120,000	1,450,000
	1st recrystallization	8,300	163,000	52,000	860,000	63,000	1,200,000
	2nd recrystallization	4,700	118,000	34,600	725,000	43,000	1,040,000
	3rd recrystallization	3,500	116,000	24,500	720,000	28,000	1,020,000
	3rd mother liquor	1,000	120,000	8,600	715,000	13,600	1,010,000
	Cortisol acetate	2,500	117,000	17,100	725,000	19,700	1,000,000
	Corrected cpm[a]	9,600		62,000		83,000	

[a] Calculated as follows: Initial cpm × (constant specific activity/initial specific activity). This gives a measure of the radioactivity in the original sample due to corticosterone or cortisol.

TABLE II: Radiochemical Homogeneity of [^{14}C]Corticosterone and [^{14}C]Cortisol Biosynthesized from [7α-^{3}H]Cholesterol by Rat Adrenal and Beef Adrenal Cortex Slices under Various Conditions.

Tissue	Stage of Purification	Radioactivity (cpm)	Radioactivity (cpm/mmole)	+ ACTH Radioactivity (cpm)	+ ACTH Radioactivity (cpm/mmole)	Radioactivity (cpm)	Radioactivity cpm/mmole)	+ 3′,5′-AMP Radioactivity (cpm)	+ 3′,5′-AMP Radioactivity (cpm/mmole)
Rat adrenal	After carrier corticosterone added	12,600	176,000	14,100	192,000	9,740	164,000	12,800	222,000
	1st recrystallization	2,050	35,000	2,700	41,500	1,200	25,000	2,180	51,000
	2nd recrystallization	780	15,600	1,400	28,000	630	17,000	1,370	39,000
	3rd recrystallization	510	11,800	1,000	26,700	400	12,000	1,080	35,000
	4th recrystallization	440	12,000	810	25,500	210	11,500	740	34,600
	4th mother liquor	60	11,800	160	25,000	150	11,900	280	35,100
	Corticosterone acetate	190	11,700	260	25,200	120	11,600	420	34,800
	Corrected cpm[a]	840		1,900		690		2,030	
Beef adrenal	After carrier cortisol added	14,300	168,000	46,700	535,000			58,500	652,000
	1st recrystallization	2,800	62,000	17,000	332,000			21,700	350,000
	2nd recrystallization	1,600	42,000	10,600	370,000			14,200	355,000
	3rd recrystallization	1,100	40,000	7,100	330,000			10,000	350,000
	3rd mother liquor	400	42,000	2,900	333,000			3,900	360,000
	Cortisol acetate	600	40,000	4,900	336,000			6,900	355,000
	Corrected cpm[a]	3,500		29,200				31,700	

[a] See Table I.

TABLE III: The Effect of ACTH and 3′,5′-AMP on the Transformation of Corticoid Precursors to Corticoids by Rat Adrenal and Beef Adrenal Cortex Slices.

Tissue	Precursor	Product Isolated	Corrected cpm[a] in Product			Ratios	
			Control	+ ACTH	+ 3′,5′-AMP	ACTH/ Control	3′,5′-AMP/ Control
Rat adrenal	[1-^{14}C]Acetate	Corticosterone	1,000	2,400	2,300	2.4	2.3
	[7α-^{3}H]Cholesterol	Corticosterone	1,330		3,580		2.7
	[7α-^{3}H]Cholesterol	Corticosterone	840	1,880		2.3	
	[7α-^{3}H]Δ^{5}-Pregnenolone	Corticosterone	2,420,000	2,650,000	3,380,000	1.1	1.4
	[7-^{3}H]Progesterone	Corticosterone	3,900,000	3,900,000	4,380,000	1.0	1.1
Beef adrenal	[1-^{14}C]Acetate	Cortisol	9,600	62,000	83,000	6.5	8.7
	[7α-^{3}H]Cholesterol	Cortisol	3,500	29,200	31,700	8.4	9.0
	[7α-^{3}H]Δ^{5}-Pregnenolone	Cortisol	770,000	760,000	850,000	1.0	1.1
	[7-^{3}H]Progesterone	Cortisol	890,000	870,000	810,000	1.0	0.9

[a] See Table I.

material was now submitted to digitonin precipitation followed by cleavage of the collected digitonides in pyridine according to the method of Bergmann (1940). Carrier cholesterol (10 mg) was added to the sample. The mixture was then converted to the dibromide and free cholesterol was regenerated as described by Schwenck and Werthessen (1952). The process of bromination and regeneration was repeated until constant specific activity of the cholesterol was obtained. Aliquots of the cholesterol were examined by liquid scintillation counting and by the Liebermann-Burchard reaction.

Chemicals. Sodium [1-^{14}C]acetate (45 mc/mmole), [7α-^{3}H]cholesterol (4.7 c/mmole), [7α-^{3}H]Δ^{5}-pregnenolone (1.3 c/mmole), and [7-^{3}H]progesterone (9.5 c/mmole) were obtained from New England Nuclear Corp. These steroids showed no impurities on the chromatographic systems used in this study. 3′,5′-AMP was obtained from Sigma Chemical Co. or Schwarz BioResearch, Inc. Nonradioactive steroids were obtained from Sigma. Dichloromethane (Spectrograde) was obtained from the Fisher Scientific Co. The ligroin used in recrystallizations had a boiling range of 66–75°. The ACTH used was a product from Parke, Davis and Co.

Results

Tables I and II show in detail the data obtained from typical experiments where rat adrenal slices or beef adrenal cortex slices were incubated with [1-^{14}C]acetate or [7α-^{3}H]cholesterol in the absence and presence of ACTH or 3′,5′-AMP. The radioactive corticosterone or cortisol from similar incubations with [7α-^{3}H]Δ^{5}-pregnenolone or [7-^{3}H]progesterone were subjected to the same work-up and the results reported for these substrates are also based on constant specific activity of both free and acetylated corticosterone and cortisol. These data serve not only to demonstrate the transformation of the radioactive substrates to corticosterone or cortisol in incubations with rat adrenal slices or beef adrenal cortex slices, respectively, but also to give a measure of the extent of transformation of the radioactive precursors into these substances under the indicated experimental conditions.

In Table III are presented representative data on the effects of ACTH and 3′,5′-AMP on the transformation of the indicated radioactive corticosteroid precursors into corticosterone in rat adrenal slices and into cortisol in slices of beef adrenal cortex. It is apparent that there was no effect of either ACTH or 3′,5′-AMP on the transformation of progesterone to corticosteroids in adrenals from the two species. These results are typical of six experiments run. It is also seen that neither ACTH nor 3′,5′-AMP had an effect on the transformation of pregnenolone to cortisol in beef adrenal slices and that ACTH had no effect on the conversion of pregnenolone to corticosterone in rat adrenal slices (six experiments). However, some effect by 3′,5′-AMP on the conversion of pregnenolone to corticosterone in rat adrenal slices appears to be present. A total of five experiments was run with pregnenolone in the absence and presence of 3′,5′-AMP and gave an average ratio of 3′,5′-AMP/control of 1.30 with a standard deviation of $\pm$0.23. There is some doubt if this stimulation by 3′,5′-AMP is significant, especially in view of the results obtained with the beef adrenal. When either [^{14}C]-acetate or [^{3}H]cholesterol is the substrate, it is seen that both ACTH and 3′,5′-AMP stimulate their transformation to corticosterone in rat adrenal slices and to cortisol in beef adrenal cortex slices (eight experiments with [^{3}H]cholesterol and four experiments with [^{14}C]acetate). It should be noted that not only do both ACTH and 3′,5′-AMP stimulate to about

TABLE IV: Radiochemical Homogeneity of [^{14}C]Cholesterol Biosynthesized from [1-^{14}C]Acetate by Beef Adrenal Cortex Slices under Various Conditions.

Stage of Purification	Control Radioactivity (cpm)	Control Radioactivity (cpm/mmole)	+ ACTH Radioactivity (cpm)	+ ACTH Radioactivity (cpm/mmole)	+ 3′,5′-AMP Radioactivity (cpm)	+ 3′,5′-AMP Radioactivity (cpm/mmole)
After carrier cholesterol added	555×10^3	215×10^5	368×10^3	142×10^5	280×10^3	108×10^5
After first bromination	140×10^3	101×10^5	32×10^3	24.4×10^5	27×10^3	24.4×10^5
After second bromination	30×10^3	103×10^5	11×10^3	24.6×10^5	9×10^3	24.0×10^5
Corrected cpm[a]	260×10^3		63×10^3		62×10^3	

[a] See Table I.

TABLE V: The Effect of ACTH and 3′,5′-AMP on the Formation of [^{14}C]Cholesterol from [1-^{14}C]Acetate by Rat Adrenal and Beef Adrenal Cortex Slices.

Tissue	Experiment	Corrected cpm[a] in Cholesterol: Control	+ ACTH	+ 3′,5′-AMP
Rat adrenal	1	2.78×10^3	1.26×10^3	0.79×10^3
Beef adrenal	2	260×10^3	63×10^3	62×10^3
	3	51×10^3	22×10^3	15×10^3

[a] See Table I.

the same extent, but that approximately the same stimulation of the formation of radioactive corticosteroids is obtained whether [1-^{14}C]acetate or [7α-^{3}H]-cholesterol is the substrate. This can be seen especially with beef adrenal cortex slices where there is a day-to-day variation in the response to ACTH and 3′,5′-AMP, due, presumably, to differences in the metabolic state of the glands obtained from the slaughter house on different days. Thus, in three experiments the ACTH/control ratios of corrected cpm present in cortisol were 3.2, 8.4, and 5.3 with [7α-^{3}H]cholesterol as the substrate, and 2.0, 6.5, and 8.1 with [1-^{14}C]acetate as the substrate. In the same three experiments the 3′,5′-AMP/control ratios were 3.6, 9.0, and 4.9 with [7α-^{3}H]cholesterol as the substrate, and 2.1, 8.7, and 5.0 with [1-^{14}C]acetate as the substrate.

Parenthetically, it should be noted that, in initial incubations with rat adrenal slices when [7α-^{3}H]-cholesterol was present as a dried film on the bottom of the beaker, we were able to detect only a slight transformation to [^{3}H]corticosterone. It was only with the use of the [^{3}H]cholesterol–Tween 80 preparation that adequate transformation to [^{3}H]corticosterone was found. However, before using Tween 80 with the radioactive substrate, it was determined that this detergent, at the concentrations used, had no effect on ACTH stimulation of corticoid synthesis from endogenous precursors in rat adrenal slices, using the assay procedure of Saffran and Schally (1955).

The data of Tables IV and V show that, following incubations with [1-^{14}C]acetate, the cholesterol in the adrenal slices becomes labeled and that the extent of labeling is decreased under conditions where the transformation of [1-^{14}C]acetate to corticosteroids is increased, i.e., in the presence of ACTH or 3′,5′-AMP. It is to be noted that both ACTH and 3′,5′-AMP decrease the radioactivity of cholesterol to approximately the same extent, just as these substances increased the transformation of [1-^{14}C]acetate to corticosteroids to about the same extent (Table III).

Discussion

The data presented show that the location of the stimulation of corticosteroidogenesis by ACTH and by 3′,5′-AMP are identical. Both substances stimulate the synthesis of corticosterone in rat adrenal slices and of cortisol in beef adrenal cortex slices from [1-^{14}C]-acetate and from [7α-^{3}H]cholesterol. In incubations where [1-^{14}C]acetate is the corticosteroid precursor, the stimulation of corticosteroid synthesis by both substances is accompanied by a drop in the radioactivity of the cholesterol arising from the [1-^{14}C]acetate, and the extent of stimulation by both substances

is approximately equal. In addition, neither substance stimulates corticosteroid synthesis from [7α-^{3}H]pregnenolone or [7-^{3}H]progesterone. These results indicate that the stimulatory effect of both ACTH and 3′,5′-AMP resides at the same site in the biosynthetic pathway for corticosteroids and that this site is located between cholesterol and pregnenolone (see following discussion). At least two compounds, 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol, intervene between cholesterol and pregnenolone (Solomon *et al.*, 1956; Shimizu *et al.*, 1961, 1962; Constantopoulos *et al.*, 1962), and ACTH and 3′,5′-AMP may stimulate at different steps in the transformation of cholesterol to pregnenolone. However, this does not seem likely in view of the observation that 3′,5′-AMP does not further stimulate corticoid synthesis in rat adrenal slices maximally stimulated by ACTH (Koritz, 1962). The present results, in conjunction with those of Haynes (1958), Haynes *et al.* (1959), and Koritz (1962) indicate that 3′,5′-AMP is probably an obligatory intermediate in the stimulation by ACTH of corticoid synthesis in the adrenal.

The stimulation by ACTH (and 3′,5′-AMP) of corticosteroid synthesis from acetate and from cholesterol but not from pregnenolone or progesterone is most easily interpreted as a stimulation between cholesterol and pregnenolone. This is also indicated by the results from incubations with [1-^{14}C]acetate, where it is seen that the stimulation of corticosteroid synthesis by ACTH is accompanied by a drop in the radioactivity of the cholesterol arising from the [1-^{14}C]acetate. Thus, with the slow step in corticosteroidogenesis residing between cholesterol and pregnenolone, stimulation at this point by ACTH would result in a decrease in the accumulated radioactive cholesterol synthesized from [1-^{14}C]acetate. The observation that ACTH stimulates the transformation of both [^{14}C]-acetate and [^{3}H]cholesterol to corticosteroids to approximately the same extent also supports this interpretation.

The foregoing conclusions assume that acetate is converted to corticosteroids via cholesterol. Stone and Hechter (1954), on the basis of data which showed that corticosteroid synthesis from cholesterol is stimulated by ACTH to a much greater extent (>10-fold) than from acetate, concluded that either ACTH must inhibit the synthesis of cholesterol from acetate or that acetate may be transformed to corticosteroids by an ACTH-insensitive pathway which does not involve cholesterol as an obligatory intermediate. The data presented in this study appear to eliminate both these possibilities. However, they do not eliminate the possibility of an acetate-to-corticosteroids pathway which does not pass through cholesterol and which is ACTH sensitive. In this view, the ACTH- or 3′,5′-AMP-mediated decrease in the radioactivity of cholesterol originating from [^{14}C]acetate could be the result of an increased cholesterol-independent transformation of acetate to corticosteroids and a proportionate decrease in the transformation of acetate to cholesterol. Such an interpretation appears unlikely since it would mean that ACTH must now act at two sites, viz., between cholesterol and pregnenolone and between acetate and corticosteroids, and, in addition, it makes it difficult to account for the observation that ACTH stimulates the transformation of both acetate and cholesterol to corticosteroids to approximately the same extent. Of greater consequence is the direct evidence on the obligatory role of cholesterol provided by the experiments of Werbin and Chaikoff (1961) and Krum *et al.* (1964). Werbin and Chaikoff found, after feeding [4-^{14}C]cholesterol to guinea pigs, that the urinary cortisol had the same specific activity as the adrenal cholesterol and that the specific activity of the cortisol did not change after the administration of ACTH in the face of an increase in the total amount of urinary cortisol. Similarly, Krum *et al.*, after feedings dogs [4-^{14}C]cholesterol, found that the specific activities of the adrenal steroids and the adrenal-free cholesterol were identical and that ACTH administration did not change these specific activities. These results indicate that cholesterol is an obligatory intermediate in the biosynthetic pathway of adrenocortical steroids, and it may be concluded that the stimulation by ACTH of acetate to corticosteroids is by virtue of the prior transformation of acetate to cholesterol.

The foregoing data and considerations make unlikely the suggestion by Savard and Mason (1963) and Savard and Casey (1964) that ACTH may stimulate corticosteroidogenesis at some point anterior to cholesterol. With such a locus for ACTH stimulation, it is difficult to explain the effect of ACTH on corticosteroid synthesis from cholesterol and on the decrease in radioactivity of cholesterol synthesized from [^{14}C]acetate, as well as the results of Werben and Chaikoff (1961) and of Krum *et al.* (1964) where a stimulation by ACTH of acetate to cholesterol or to any other corticosteroid precursor should result in a drop in the specific activity of the isolated corticosteroids. The drop in adrenal cholesterol seen after the administration of ACTH (Long, 1947; Rosenfeld, 1955) is also at variance with a locus of ACTH action anterior to cholesterol.

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Structure of the Cell Wall of *Staphylococcus aureus*, Strain Copenhagen. III. Further Studies of the Disaccharides*

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ABSTRACT: Two disaccharides have previously been obtained in high yield from the cell wall of *Staphylococcus aureus* by the use of hydrolytic enzymes. Disaccharide 1 is *N*-acetylglucosaminyl-*N*-acetylmuramic acid and disaccharide 2 is *N*-acetylglucosaminyl-*N*,*O*-diacetylmuramic acid. After hydrolysis of disaccharide 2 with β-acetylglucosaminidase, *N*,*O*-diacetylmuramic acid was prepared. Data obtained from periodate oxidation of these compounds coupled with their susceptibility to β-acetylglucosaminidase indicate that both disaccharides are β-1,4- linked and that the *O*-acetyl group of disaccharide 2 is on the 6- position of *N*-acetylmuramic acid.

The high reducing power and positive Morgan-Elson reaction of the disaccharides are due to their unusual susceptibility to hydrolysis at alkaline *p*H. These and other anomalous properties of the compounds are discussed in relation to data previously obtained (J.-M. Ghuysen and J. L. Strominger (1963), *Biochemistry 2*, 1119).

By means of enzymatic degradation two disaccharides were isolated in high yield from the cell wall of *Staphylococcus aureus* (Ghuysen and Strominger, 1963b). These compounds contained *N*-acetylglucosamine and either *N*-acetylmuramic acid or *N*,*O*-diacetylmuramic acid with the muramic acid residue at the reducing end. The data obtained suggested that the disaccharides were β-1,6- linked. A similar linkage had been proposed for a disaccharide obtained in lower yield from the cell wall of *Micrococcus lysodeikticus* (Salton and Ghuysen, 1959, 1960; Perkins, 1960). Jeanloz *et al.* (1963) have recently synthesized β-1,6-*N*-acetylglucosaminyl-*N*-acetylmuramic acid. Its properties were different from those of the disaccharide from *M. lysodeikticus* and they suggested that the natural disaccharide was β-1,4- linked. As a consequence, the structures of the two disaccharides from *S. aureus* have been reinvestigated, and the data obtained are presented in this paper.

Materials and Methods

Cell Walls and Enzymes. Cell walls of *S. aureus* were prepared as described previously except that after isolation by differential centrifugation (Ghuysen and Strominger, 1963a) they were further purified by treatment with trypsin and ribonuclease in 0.05 M potassium phosphate buffer, *p*H 7.0. *Chalaropsis* B enzyme (Hash, 1963) was kindly donated by Dr. J. C. Hash of Lederle Laboratories, and *Streptomyces* acetylmuramyl-L-alanine amidase was prepared as previously described (Ghuysen *et al.*, 1962). D-Glucosamine-6-phosphate *N*-acetylase was prepared from yeast according to the method of Brown (1962). β-Acetylglucosaminidase was prepared from pig epidi-

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