In steroid SC-5233, $R$ is $CH_3$; in steroid SC-8109, $R$ is H. Results demonstrating aldosterone-blocking activity with SC-5233 are summarized in Fig. 1. Aldosterone in a solution of ethanol in 0.86-percent sodium chloride (20/80 by volume) was subcutaneously injected into adrenalectomized rats, either alone or with oil solutions of SC-5233. Four-hour samples of urine were collected from individual animals for sodium and potassium analyses. Aldosterone alone caused a reduction in the value of the Na/K ratio in the urine, but in the presence of 1.2 and 4.8 mg of SC-5233 per rat this reduction was significantly reversed.

The 19-nor analog, SC-8109, similarly blocked aldosterone activity. For example, administration of 0.98 μg of aldosterone reduced the Na/K ratio to 0.63 ± 0.07 (mean ± standard error). A dose of 1.3 mg of SC-8109 significantly counteracted this effect, giving a ratio of 1.12 ± 0.12. We attach strong importance to these findings because SC-5233 and SC-8109 appear to represent the first known examples of aldosterone blockers.

Several studies were undertaken to define the mechanism of blocking with deoxycorticosterone acetate (DOCA). We used this compound because of its availability, structure, electrolytic effects, and possible similarity in mechanism of action to aldosterone. The results obtained with DOCA and SC-5233 are summarized in the succeeding paragraphs; those obtained with SC-8109 are incomplete (*3, 4*).

A progressive blocking of the action of 12 μg of DOCA on the Na/K ratio was obtained with six different doses of SC-5233, ranging from 0.15 to 4.8 mg. These data showed that approximately 0.24 mg of SC-5233 was required for a 50-percent block of DOCA (*5*). Progesterone, which was recently described as a DOCA-blocker in man (*6*), showed similar activity with a dose of 1.8 mg. Compound SC-8109 was effective at 0.067 mg. By comparison of doses, progesterone, SC-5233, and SC-8109 showed relative activities of 1, 7.5, and 26.8, respectively.

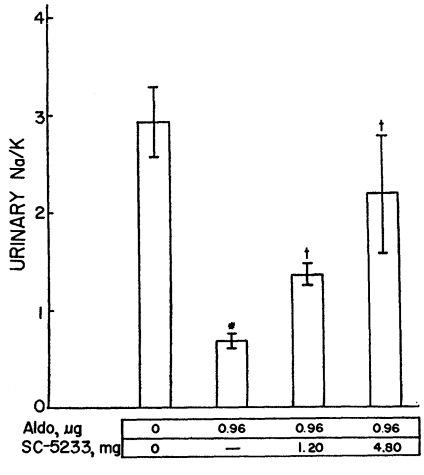


Fig. 1. Effects of SC-5233 in blocking urinary Na/K action of aldosterone in adrenalectomized rats (12 animals per treatment). Standard error is shown by vertical lines. * $P < 0.05$, compared with no treatment; † $P < 0.05$, compared with treatment with aldosterone alone.

Table 1. Effects of various doses of SC-5233 in blocking the action of deoxycorticosterone acetate (DOCA) in adrenalectomized rats.

| Treatment (dose per rat) | | No. of rats | Urinary Na/K (mean ± S.E.*) |
|---|---|---|---|
| SC-5233 (mg) | DOCA (μg) | | |
| | 24 | 20 | 0.47 ± 0.04 |
| | 48 | 19 | 0.54 ± 0.05 |
| 0.6 | 24 | 9 | 0.80 ± 0.13 |
| 1.2 | 48 | 9 | 0.81 ± 0.04 |
| 2.4 | 24 | 9 | 1.35 ± 0.36 |
| 4.8 | 48 | 9 | 1.23 ± 0.15 |
| 9.6 | 24 | 9 | 1.54 ± 0.40 |
| 19.2 | 48 | 8 | 1.62 ± 0.19 |

* S.E. = standard error.

Table 2. Effects of large amounts of deoxycorticosterone acetate (DOCA) in overcoming blocking action of SC-5233 in adrenalectomized rats.

| Treatment (dose per rat) | | Urinary Na/K (mean ± S.E.*) |
|---|---|---|
| SC-5233 (mg) | DOCA (μg) | |
| 2.4 | 48 | 1.09 ± 0.13 |
| 2.4 | 240 | 0.75 ± 0.13 |
| 2.4 | 1200 | 0.69 ± 0.16 |
| | 48 | 0.54 ± 0.10 |

* S.E. = standard error; 15 rats per treatment; 20 untreated controls showed a Na/K response of 1.90.

The effects of SC-5233 in counteracting reduction of the Na/K ratio produced by 24 and 48 μg of DOCA were investigated at two dosage levels and at various ratios (Table 1). Doubling the dose at a fixed ratio of SC-5233/DOCA did not cause significant changes in Na/K values. Increasing ratios of SC-5233/DOCA, however, progressively blocked the effects of 24 and 48 μg of DOCA. These observations suggest that (i) equal blocking results with similar ratios and (ii) blocking increases with larger ratios of SC-5233/DOCA. It would appear that SC-5233 acts as a blocker according to the law of mass action.

The opposite question of reversing the action of SC-5233 with an excess of DOCA was studied (Table 2). The results indicate that the effects of 2.4 mg of SC-5233 were reversed with 240 and 1200 μg of DOCA. We feel that these results demonstrate reversible competition.

In order to rule out the possibility that SC-5233 had a direct effect of increasing the urinary Na/K ratio, instead of specifically blocking DOCA, we performed the following experiment. Doses of 0.0, 1.2, 9.6, and 19.2 mg of SC-5233 alone were given; these doses produced Na/K ratios of 2.15, 2.38, 2.67, and 2.32, respectively (nine rats per treatment). None of the responses with SC-5233 treatment significantly exceeded the control response of 2.15. Increases in the Na/K ratio greater than 0.66 above the control value would occur by chance once in ten trials, whereas in our results the largest increase was 0.52. Thus, SC-5233 does not in itself greatly affect the urinary Na/K ratio.

The results of these studies strongly suggest that SC-5233 and, possibly, SC-8109 exert their effects on electrolytes by competition with DOCA and aldosterone (*7*).

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3. A more detailed report on these investigations and other pharmacological effects of SC-5233 and SC-8109 is in preparation.
4. When given orally, 4.8 mg of SC-8109 significantly reversed the Na/K effect of 12 μg of DOCA from 0.59 ± 0.05 (mean ± standard error) to 0.95 ± 0.14. At the same oral dose, SC-5233 was inactive.
5. The term *percent block* refers to values obtained by the following formula: (net blocking effect of test compound) × 100/(effect of DOCA).
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7. We gratefully acknowledge the valuable technical assistance of Marjorie A. Thomas and Elizabeth D. Griffin. Thanks are due to David W. Calhoun for the statistical work.

30 July 1957

## Sodium Diuresis Induced by Steroidal Antagonists of Aldosterone

For several years it has been known that the adrenal cortex secretes a number of steroidal hormones which increase the tendency of the renal tubules to reabsorb sodium. The adrenal steroid of most importance in the physiological regulation of electrolyte metabolism is aldosterone (*1*). Aldosterone has been shown to play a crucial role in normal physiology in promoting the conservation

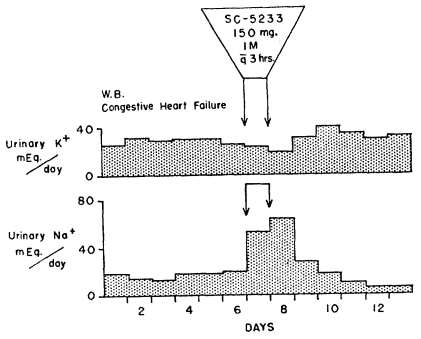


Fig. 1. Effect of SC-5233 on potassium and sodium excretion in a patient with congestive heart failure. Diet was constant from day to day.

of sodium in the face of sodium deprivation. It has also been implicated in the pathogenesis of the abnormal sodium and water accumulation which occurs in the "edematous states," such as congestive heart failure. There has been considerable interest in the possibility that the adrenal cortex may also elaborate a "sodium-losing" hormone (*2*). Identification of such a hormone has not yet been accomplished.

The present report (*3*) submits evidence that certain *synthetic* steroids, 3-(3-oxo-17β-hydroxy-4-androsten-17α-yl)-propionic acid γ-lactone (SC-5233) and its 19-nor analog (SC-8109) can act as natriuretic agents. The evidence strongly suggests that the mechanism of action of the new sodium-losing steroids is that of antagonism of the sodium-retaining action of aldosterone. Because of its earlier availability, SC-5233 was employed in the majority of these experiments. Compound SC-8109 has activity qualitatively like that of SC-5233, and it appears to be effective in smaller doses than SC-5233.

Preliminary studies with adrenalectomized dogs indicated that SC-5233, when administered alone, had no appreciable effect on electrolyte excretion. As previously reported (*4*), deoxycorticosterone (DOC), like aldosterone, regularly induces sodium retention and potassium excretion when it is administered to adrenalectomized dogs. The administration of CS-5233 together with deoxycorticosterone diminished the effectiveness of the latter with respect to both sodium conservation and potassium loss. The degree of attenuation of the effectiveness of DOC was a direct function of the dose of SC-5233 and an inverse function of the dose of DOC employed. These laboratory results confirm those obtained by Kagawa *et al.* with adrenalectomized rats (*5*). It is important to observe that the inhibitory action of SC-5233 is not limited to the sodium-retaining action of DOC but affects also the potassium-losing action of DOC. Numerous compounds (including cortisone) will cause an acute rise in sodium excretion in adrenalectomized dogs and rats. Of the compounds studied thus far, only SC-5233 and SC-8109 have been found to cause a simultaneous *inhibition* of potassium excretion. This change in the qualitative pattern of the electrolytic composition of the urine suggests that the site of action of SC-5233 is the renal tubule.

Metabolic studies in man clearly indicated that SC-5233 is an effective antagonist of aldosterone. In the absence of sodium-retaining steroids, SC-5233 had little or no effect. However, in the presence of sodium-retaining steroids, SC-5233 exhibited its characteristic natriuretic (without kaliuretic) action. This was demonstrated in three situations.

1) In seven patients with varying degrees of edema (due to congestive heart failure or nephrosis) the administration of SC-5233 consistently induced natriuresis (Fig. 1).

2) A patient with negligible adrenal function due to Addison's disease was given SC-5233 while being maintained on a high sodium diet without steroids. SC-5233 failed to induce a convincing change in electrolyte excretion (Fig. 2). When the same patient was maintained on a high sodium diet plus DOCA, the addition of SC-5233 induced a rise in sodium excretion. Potassium excretion not only failed to rise but actually showed a slight fall.

3) Normal subjects receiving a high sodium intake were treated with SC-5233 with no significant effect. (It has been shown repeatedly in the past and was found again in the course of this study that normal subjects on high sodium intake have minimal levels of aldosterone.) The same normal subjects, while receiving a low sodium intake, were again treated with SC-5233; under these circumstances SC-5233 induced an increase in urinary sodium. (As in previous studies, aldosterone was found in the urine in relatively large amounts during the course of the low sodium diet.)

The conclusion appears secure that SC-5233 is effective as a natriuretic agent only in the presence of sodium-retaining

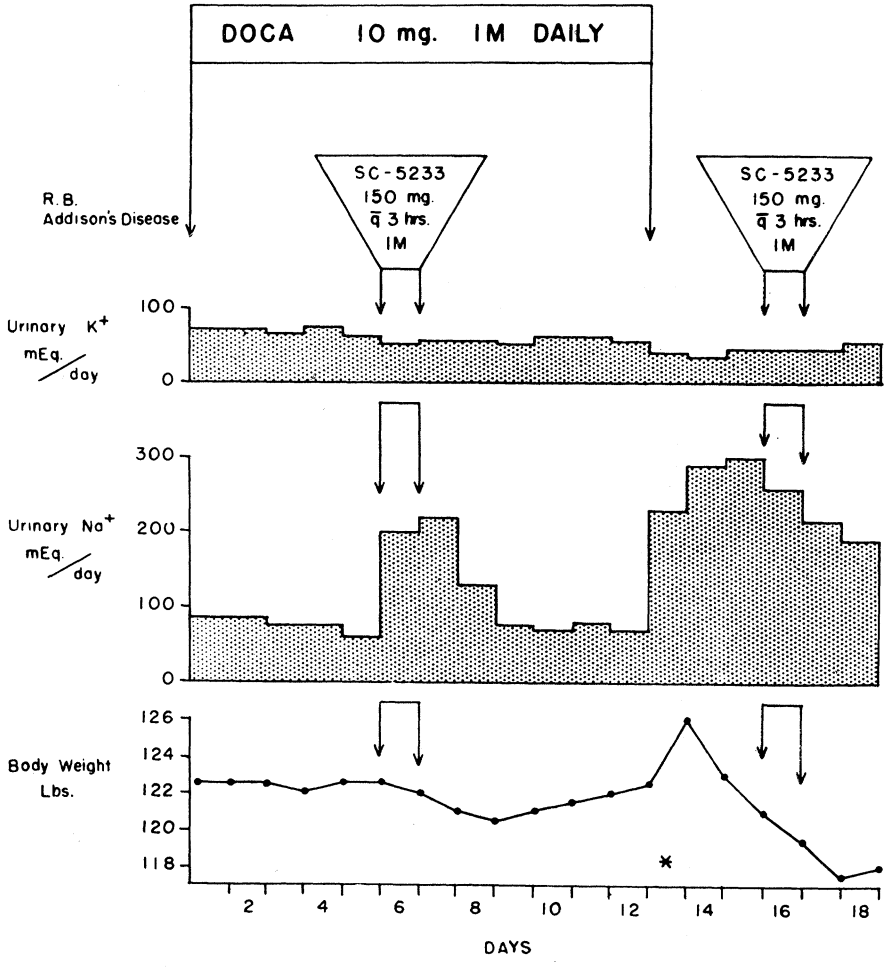


Fig. 2. Effect of SC-5233 on potassium and sodium excretion in a patient with Addison's disease. Diet was constant from day to day. Deoxycorticosterone acetate in sesame oil was injected intramuscularly in doses of 5 mg every 12 hours during the first 12 days. On day 13 (marked by an asterisk) the sodium deficit resulting from the initial treatment with SC-5233 was repaired by ingestion of 18 g of NaCl. SC-5233 was administered intramuscularly in doses of 150 mg (in 3 ml of sesame oil) every 3 hours for 8 doses on day 6 and day 16.

steroids, endogenous or exogenous. The most reasonable interpretation of these findings is that SC-5233 and, presumably, SC-8109 act as antagonists to aldosterone and other sodium-retaining steroids. It is suggested that the mechanism through which these sodium-losing steroids act is that of competition with aldosteronelike steroids for a crucial locus of action within the renal tubular cells. A similar mechanism has been postulated previously to explain the sodium loss which has occasionally been seen during treatment of patients with supraphysiologic amounts of cortisone (*6*) and progesterone (*7*).

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3. This study was supported in part by grants-in-aid from the National Cancer Institute (CY-3107) and from the John A. Hartford Foundation. SC-5233 and SC-8109 were generously provided by G. D. Searle and Company. Donald Island rendered invaluable technical assistance.
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19 August 1957

## Effect of Citrovorum Factor and Peptones on Mouse Leukemia Cells L-5178 in Tissue Culture

A medium has been devised (*1*) which permits the continuous reproduction of mouse leukemia cells (L-5178, a lymphocytic neoplasm of DBA/2 mice, *2*) in culture in the complete absence of nonleukemic cells (*3*). After at least 150 successive generations *in vitro*, the cells have continued to grow in suspension (rather than on the glass surface), and have retained their round-cell character, as well as their capacity to induce fatal leukemia in DBA/2 mice. In addition to serum and other ingredients usually encountered in culture media, certain peptones are required for the continued multiplication of these cells. Data presented in 1928 by Baker and Carrel and extended by Willmer and Kendal (*4*) indicated that various peptones can stimulate the multiplication of mammalian and avian cells in tissue culture. More recently, Waymouth has reported that peptone, in the presence of albumin, replaces serum for the multiplication of strain L mouse cells (*5*). However, the factors in peptone which support cell multiplication have not been identified. Previous studies of the growth responses of *Lactobacillus leichmannii* (ATCC 7830) and *Pedicoccus cerevisiae* [*Leuconostoc citrovorum* (ATCC 8081)] to peptonelike materials and the relationship of the responses to vitamin $B_{12}$, citrovorum factor, and thymidine have been described (*6, 7*).

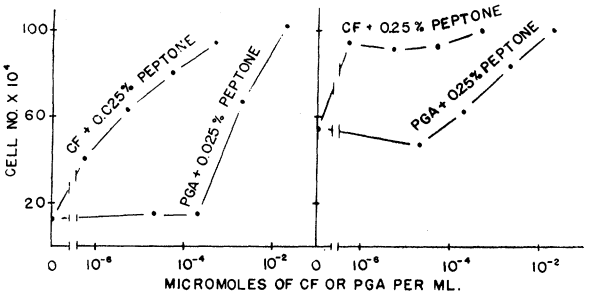


Fig. 1. Reduction of the effect of peptone by citrovorum factor (CF) and the relative activity of pteroylglutamic acid (PGA) and CF in support of cell multiplication. The cells ($2.0 \times 10^5$) were incubated in 1 ml of medium (*1*) for 48 hours; the extent of cell reproduction was determined by hemocytometer counts.

Experiments (*8*) directed toward the isolation of the active factors in the peptone used (*9*) have demonstrated that synthetic 5-formyl-5,6,7,8-tetrahydropteroylglutamic acid (citrovorum factor; CF; folinic acid; leucovorin) partially replaces peptone in the nutrition of these leukemic cells and that the capacity of the cells to obtain functional derivatives from folic acid (pteroylglutamic acid; PGA) is very much less than their capacity to obtain them from citrovorum factor.

The partial replacement of peptone by leucovorin in the nutrition of the neoplastic cells is shown in Fig. 1. The data indicate that, in the presence of 0.025 percent peptone, a stimulatory effect of leucovorin was clearly demonstrable, while at a ten-fold higher level of peptone, very much less leucovorin was required to attain maximal growth. In this 48-hour experiment, at the lower level of peptone, $3.7 \times 10^{-6}$ μmole of the active form of leucovorin per milliliter (*10*) permitted half-maximal growth, while a 400-fold higher concentration of pteroylglutamic acid ($1.5 \times 10^{-3}$ μmole/ml) was required to obtain a similar effect. When cultures were carried for a minimum of ten progressive generations, in triplicate, on limiting levels of the two coenzyme-precursors, the doubling-time was less than maximal and remained constant, and, in addition, equivalent rates of multiplication were obtained with active leucovorin in a concentration 1/5000 that of pteroylglutamic acid. The fact that the doubling-time remained constant indicates that, in the presence of either pteridine derivative, certain components of the peptone are no longer required for the nutrition of the cells. During continuous culture in media containing a much higher level of peptone, 0.5 percent, but only $2.2 \times 10^{-5}$ μmole of pteroylglutamic acid per milliliter, a generation time of less than 22 hours (average, 24 hours) was rarely obtained. In contrast, generation times of 14 to 22 hours (average, 18 hours) were obtained with optimal amounts of active leucovorin ($2 \times 10^{-5}$ μmole/ml) or with very high levels of pteroylglutamic acid ($2 \times 10^{-2}$ μmole/ml) in the presence of low levels of peptone (0.06 percent).

The high levels of folic acid, as compared with the levels of its tetrahydro derivative, which are required to support cell multiplication indicate a very limited capacity of the leukemic cells under these conditions to synthesize coenzymes from pteroylglutamic acid (*11*). The absolute requirement of certain other cell lines for this vitamin is very much less than it is for L-5178, for example, HeLa and L-strain (*12*), a finding which suggests that these cells efficiently convert folic acid to coenzyme forms. Such an interpretation is supported by the fact that citrovorum factor is only 20 to 30 times as effective as pteroylglutamic acid for L-strain cells (*13*). Although the requirement for folic acid of sarcoma 180 cells in tissue culture resembles that of both L-strain and HeLa cells, our colleague, Richard Schindler, has found that this requirement is met (in Eagle's medium, *12*) by active leucovorin in an amount approximately 1/200 that of pteroylglutamic acid.

It is well known that various neoplastic cell lines, *in vivo*, exhibit widely variable sensitivity to the chemotherapeutic action of A-methopterin, an agent which inhibits the enzymic conversion of folic acid to tetrahydro derivatives. It is suggested that the effectiveness of A-methopterin as an inhibitor of the reproduction of various types of cells is markedly influenced not only by the extracellular supply of tetrahydro derivatives of folic acid, but also by the enzymic capacity of such cells to convert folic acid-like compounds to coenzymically active, tetrahydro forms (*14*).

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