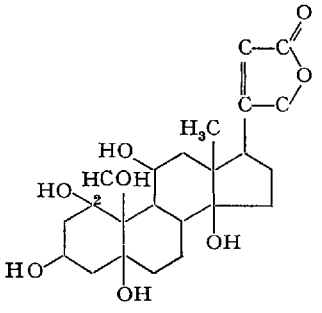
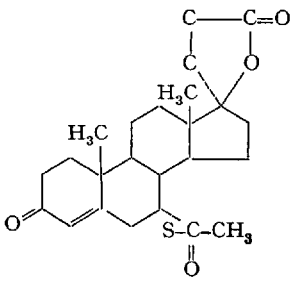
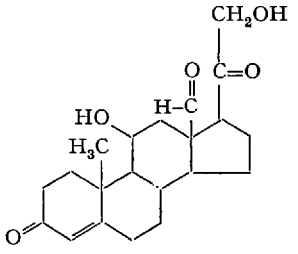


Ouabagenin



SC 9420 (Aldactone)



Aldosterone (Aldehyde form)

centration (3×10^{-3} *M* in 0.1 ml of ethanol) similar to that used for the steroids with lactone rings, which KAHN and ACHESON found to be active on cation transport in their experiments in cold-stored erythrocytes[10]. The same amount of pure ethanol was added to the control aliquots. Ouabain was added at the concentration of 2.5×10^{-5} g in 0.1 cm^3 of distilled water; the same amount of pure water was added to the control samples.

The quantities of potassium removed from the incubation medium and the quantities of sodium set free in the same medium during incubation are shown in the Table. SC 9420 had no measurable effect on these changes under the conditions of the experiment. On the contrary, Ouabain blocked potassium uptake by, and sodium output from, incubated cold-stored human erythrocytes.

Our experiments do not support the assumption that steroidal antagonists of aldosterone compete with aldosterone-like hormones for the enzymatic mechanism which controls cation transport not only within the renal tubular cells, but also within the blood red cells and possibly within the body cells in general.

Riassunto. Nonostante l'affinità strutturale con l'uabaina, lo spirolattone antialdosteronico SC 9420 non esercita la stessa azione del glicoside cardiocinetico sugli scambi attivi che si manifestano *in vitro* attraverso la membrana di eritrociti umani incubati a 37°C dopo 6 giorni di conservazione a 4°C. Non trova quindi conferma sperimentale l'ipotesi che entrambe queste sostanze competano con l'aldosterone in qualche fase del processo enzimatico di trasporto degli elettroliti a livello di tutte le membrane cellulari dell'organismo.

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On the Mechanism of Action of Some 4-Aminoanalogues of Folic Acid

The biochemical mechanism of action of the antileukemic active 4-amino-analogues of folic acid, aminopterin and amethopterin has been found to be in a strong inhibition of the folic acid hydrogenases[1–6]. In this way, the formation of the coenzymatically active tetrahydrofolic acid may be prevented and therefore the biosynthesis of thymidylate and purines inhibited. In order to prepare less toxic antimetabolites of folic acid with another mechanism of action, some 4-amino-analogues of coenzymatically active folic acid derivates have been prepared in our laboratory[7]. This paper presents the inhibiting action of these substances on some enzyme systems converting folic acid into coenzymatically active single carbon carriers. Besides the folic acid hydrogenases, the serine-aldolase[8] and the hydroxymethyltetrahydrofolic acid dehydrogenase[9,10] from pigeon liver were studied as the hydroxymethylation coenzyme generating systems. From the enzymes participating in the formation of formylation coenzymes, the ATP-activated formylase of tetrahydrofolate[11], using formate as the formyl source, the formiminotransferase and deaminase, described recently by TABOR and WYNGARDEN[12], and the aerobic formylase[13] were examined. The enzyme system leucovorin-cyclodehydrase[14] converting leucovorin into its coenzymatically active imidazoline-derivate was included in the enzymes studied. Table I shows the inhibitory properties of several 4-amino-analogues of folic acid on the above-mentioned enzyme systems achieved from acetone powders of mammalian and avian liver in a partially purified form.

The inhibiting power of the 4-amino-analogues mentioned could be observed neither on the aerobic formylase of folic acid nor on the tetrahydrofolic acid formylase using formate as the formyl donor with the inhibitor concentrations equimolecular to the substrate concentration. The most strongly inhibited enzyme systems are the folic acid hydrogenases which are inhibited by 8.6×10^{-9} *M* aminopterin, or 6.2×10^{-9} *M* amethopterin. The substitution of the nitrogen atom N^{10} by the formyl or hydroxymethyl group, and the hydrogenation of the pyrazine ring, diminish the inhibition activity more than 10–100 times.

Of the enzymes formylating the tetrahydrofolate, the system formiminotransferase + cyclodeaminase only was inhibited by all 4-amino-analogues tested, but only concentrations 10^{-4} *M* caused the 50% inhibition of the enzymes. As tested by the Lineweaver-Burk test, the

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Tab. I. The inhibition of some enzyme systems converting folic acid into coenzymatically active derivates by 4-aminoanalogues of folic acid metabolites

Enzyme	Leucovorin cyclodehydrase			Folic acid reductase + dihydrofolic acid reductase		
Method of activity determination	ΔE at 342 mμ in the incubation mixture (an imidazoline derivate of tetrahydrofolate)			Determination of diazotable amine after decomposition of tetrahydrofolate formed with acid		
Antimetabolite	1[a]	2	3	1	2	3
Aminopterin	no inhibition at $0.5 \cdot 10^{-3}$ *M*			$8.6 \cdot 10^{-9}$	0.0038	0.000016
N^{10}-Formylaminopterin	no inhibition at $0.5 \cdot 10^{-3}$ *M*			$3.5 \cdot 10^{-8}$	0.0167	0.0000724
N^{10}-Formyltetrahydroaminopterin				$4.3 \cdot 10^{-7}$	0.19	0.00082
N^5-Formyltetrahydroaminopterin	$4.3 \cdot 10^{-4}$	202.0	0.76	$1.2 \cdot 10^{-5}$	2.3	0.01
Tetrahydroaminopterin	$1.43 \cdot 10^{-3}$	634.0	2.38	$1.0 \cdot 10^{-6}$	0.46	0.002
N^{5-10}-Methylentetrahydroaminopterin	$1.0 \cdot 10^{-3}$	477.0	1.78	$4.5 \cdot 10^{-7}$	0.202	0.00088
N^{10}-Hydroxymethylaminopterin				$1.19 \cdot 10^{-6}$	0.526	0.0023
N^{10}-Hydroxymethyltetrahydroaminopterin				$7.6 \cdot 10^{-8}$	0.338	0.00146
Trihydroxymethyltetrahydroaminopterin	no inhibition at $0.5 \cdot 10^{-3}$ *M*			$2.0 \cdot 10^{-6}$	0.885	0.00325
N^{10}-Methylaminopterin	$4.6 \cdot 10^{-4}$	266.0	0.83	$6.2 \cdot 10^{-9}$	0.00275	0.0000119
N^{10}-Methyltetrahydroaminopterin	no inhibition at $0.5 \cdot 10^{-3}$ *M*					

[a] 1. Molar concentration of the inhibitor causing the 50% inhibition. 2. Absolute concentration of the inhibitor (μg/ml) causing the 50% inhibition. 3. Molar inhibitor — substrate relation.

Tab. II

Enzyme	Serine aldolase + hydroxymethyltetrahydrofolic acid dehydrogenase			Hydroxymethyltetrahydrofolic acid dehydrogenase			Formiminotransferase + cyclodeaminase		
Method of activity	ΔE at 355 mμ (anhydroleucovorin) after $HClO_4$ deproteinization								
Antimetabolite	1[a]	2	3	1	2	3	1	2	3
Aminopterin	$1.8 \cdot 10^{-4}$	79.0	0.518	$1.9 \cdot 10^{-4}$	84.2	0.549	$1.5 \cdot 10^{-4}$	67.0	0.43
N^{10}-Formylaminopterin	$3.2 \cdot 10^{-4}$	142.0	0.93	$2.3 \cdot 10^{-4}$	103.0	0.67	$2.7 \cdot 10^{-4}$	122.0	0.78
N^{10}-Formyltetrahydroaminopterin							$2.3 \cdot 10^{-4}$	104.0	0.67
N^5-Formyltetrahydroaminopterin	$6.9 \cdot 10^{-5}$	30.6	0.20	$1.7 \cdot 10^{-4}$	73.7	0.48	no inhibition at $0.4 \cdot 10^{-3}$ *M*		
Tetrahydroaminopterin	$3.7 \cdot 10^{-5}$	16.5	0.109	$2.2 \cdot 10^{-5}$	9.7	0.0634	$3.9 \cdot 10^{-5}$	173.0	1.106
N^{5-10}-Methylentetrahydroaminopterin	$7.3 \cdot 10^{-6}$	3.2	0.0205	$7.4 \cdot 10^{-6}$	3.3	0.0209	$8.2 \cdot 10^{-4}$	365.0	2.34
N^{10}-Hydroxymethylaminopterin							$1.86 \cdot 10^{-5}$	82.6	0.53
N^{10}-Hydroxymethyltetrahydroaminopterin	$3.14 \cdot 10^{-4}$	139.5	0.911	$8.5 \cdot 10^{-5}$	38.1	0.247	$3.62 \cdot 10^{-4}$	160.0	1.02
Trihydroxymethyltetrahydroaminopterin	$3.25 \cdot 10^{-5}$	14.1	0.094	$3.28 \cdot 10^{-5}$	14.6	0.214	$2.5 \cdot 10^{-3}$	1130.0	16 1
N^{10}-Methylaminopterin							$7.0 \cdot 10^{-4}$	312.0	2.0
N^{10}-Methyltetrahydroaminopterin							$5.85 \cdot 10^{-3}$	2600.0	16.7

[a] 1. Molar concentration of the inhibitor causing the 50% inhibition. 2. Absolute concentration of the inhibitor (μg/ml) causing the 50% inhibition. 3. Molar inhibitor – substrate relation.

inhibition may not be of competitive nature. The enzyme leucovorin-cyclodehydrase was mostly inhibited by the structural analogue of leucovorin, but the inhibition was of the same order as in the case of the formimino-system.

As for the hydroxymethylating systems, the manometric method for the serine-aldolase determination described by BLAKLEY[8], appeared to be unsuitable for the inhibition studies. Thus, the activity of the hydroxymethyltetrahydrofolic acid dehydrogenase was determined with serine and tetrahydrofolate as substrates. The activity of both the serine-aldolase and the dehydrogenase has been determined in these experiments. For the determination of the dehydrogenase activity only, N^{5-10}-methylentetrahydrofolic acid was used as the substrate. From the comparison of the inhibition of both enzymes tested, it can be concluded that the serine-aldolase may not be inhibited by lower concentrations of the inhibitors than the dehydrogenase.

In two enzyme systems tested, it was found that the structural 4-amino-analogue of the substrate was attacked by the enzyme. The enzymic formylation of aminopterin into N^{10}-formylaminopterin[15] was described earlier. In the course of the studies on the serine-aldolase, the conversion

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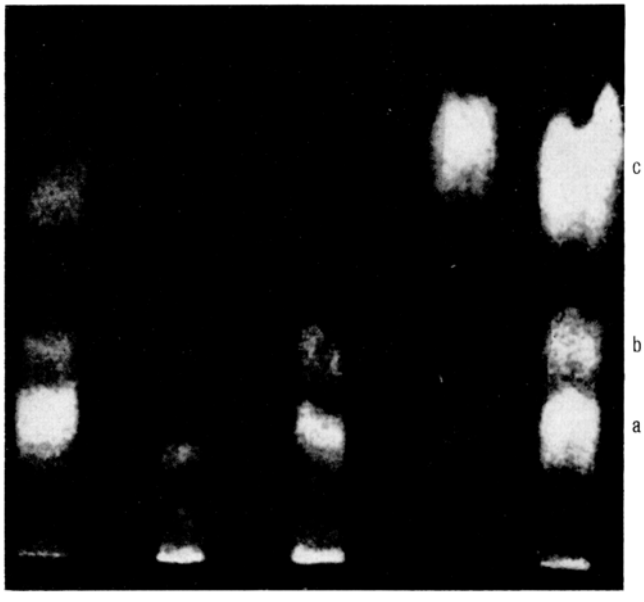

The chromatogram of the incubation mixture of serine aldolase from pigeon liver with tetrahydroaminopterin (0.5% sodium carbonate, ascending technique).
Samples: 1. serine aldolase + tetrahydroaminopterin + serine. 2. sample without enzyme. 3. sample without serine. 4. synthetic N^{5-10}-methylentetrahydroaminopterin. 5. the mixture of sample 1. with the synthetic N^{5-10}-methylentetrahydroaminopterin.
Zones: a) light-green fluorescent decomposition product of tetrahydroaminopterin, probably 2,4,6-triaminopteridine. b) tetrahydroaminopterin. c) N^{5-10}-methylentetrahydroaminopterin.

of tetrahydroaminopterin into N^{5-10}-methylentetrahydroaminopterin was detected by means of paper chromatography of the reaction mixtures. The product showed identical behaviour with that prepared by the non-enzymic hydroxymethylation of tetrahydroaminopterin by low concentrations of formaldehyde. However, the dehydrogenation of the substance mentioned to the N^{5-10}-methylen-tetrahydroaminopterin could not be observed. From the results presented here, it can be supposed that from the enzyme systems of folic acid turnover the hydrogenases are most strongly inhibited by the 4-amino-analogues of folic acid derivates. Even the hydrogenated and N^5 or N^{10} substituted 4-amino-analogues, which are much less active than aminopterin or amethopterin themselves, show the strongest activity on the folic acid hydrogenases. However, in the case of the last-mentioned substances, the inhibition of the hydroxymethyltetrahydrofolic acid dehydrogenase should be considered.

The results of the toxicity determination of the above-mentioned 4-amino-analogues for mice[16] do not agree with the enzyme inhibition observed. Thus another mechanism of action might be supposed; i.e., the interference with the natural folic acid coenzymes in the single carbon transfer reactions. The inhibition of the thymidylate synthetase and the glycinaminoribotide and aminoimidazolcarboxamide ribotide transformylases will be reported later.

Zusammenfassung. Der Einfluss einiger 4-Aminoanalogen der coenzymatisch aktiven Folsäurederivate auf die Enzyme der Folsäureumwandlungen wurde verfolgt. Die stärkste Hemmung zeigen alle 4-Aminoanaloge auf die Hydrogenase der Folsäure, wobei die Hydrierung des Pyrazinringes dieselbe deutlich vermindert. Eine mässige, jedoch deutlich schwächere Hemmung von nonkompetitiver Natur wurde bei der Hydroxymethyltetrahydrofolsäuredehydrogenase, Formiminotransferase und Leukovorincyclodehydrase festgestellt. Weiter wurde die enzymatische Hydroxymethylierung von Tetrahydroaminopterin durch Serinaldolase aus der Taubenleber beobachtet.

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Institute of Hematology and Laboratory of Protein Metabolism, Charles' University (Prague), 8. November 1960.

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Establishment of an Epithelial Cell Strain from Calf Liver in Continuous Culture

Calf liver cells were isolated in September 1958 for various researches in the following way. Fetal calf liver was minced and the fragments were explanted on glass without plasma in a culture medium containing 25% calf serum and 0.5% lactalbumine hydrolysate (N.B.C.) in Hanks balanced salt solution[1]. The cultures were incubated at 37°C. Media were changed every 2–3 days. Subcultures were made after about a month by trypsinizing (0.5% trypsin in a salt solution deficient in Ca^{++} and Mg^{++}). In the beginning growth of the cells was slow, but after the 5th subculture (March 1959) the growth was more regular, quicker and clearly epithelial as a close packed pavement epithelium. Cells from various explants were kept in culture. In the early stages most explants often formed epithelial cells, but after prolonged culture the cells were fibroblastlike. One of the subcultures however grows as a sheet of cells on the glass wall. After establishment of these cells in continuous culture the serum percentage of the medium was reduced gradually to 5%. The doubling time for this strain is two to three days and the mitotic index 2–3%. The shape of the cells from this strain (KaLe) is polygonal (see Fig.). The mean nuclear size, measured in a culture fixed with formol-alcohol-aceticacid and stained with iron hematoxylin, is 16 μ. (The mean nuclear size of liver tissue fixed and stained on the same manner is 7 μ.) There are some 'giants', 6–7%, whose nuclear size varies from 23–42 μ. The number of nucleoli per nucleus varies from 1–7. There is no relation between number of nucleoli and size of the nucleus. The number of chromosomes is 70 ± 2 (normal fetal liver cells 60). The cells on the photograph were fixed and stained with 2% $AgNO_3$[2]. Between the cells a 'cement substance' is clearly visible, which is stained by the silver. (Cultures of cartilage cells, stained with $AgNO_3$, never gave staining of such 'cement substance' although some aneuploid strains grow as epithelial sheets as well.) The mitochondria of the liver cells are mostly spherical, some are filamentous. The plasma of the liver cells fixed with alcohol 70% and stained with the PAS reaction is clearly PAS positive. Physiologically they are thus liver cells. Up to now it was impossible to cultivate this KaLe strain as separate cells in suspension. We tried it with shake and stir cultures, but always groups of cells were found. It is probable that the cells are held together by the cement substance. Biochemical research on this strain will be published elsewhere.

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