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Inhibitory Effect of Bicarbonate on ATP Levels and Cholesterol Biosynthesis in Liver Homogenates.* (26118)

LEMUEL D. WRIGHT, ESTHER ENGLERT, LAN-FUN LI AND ROBERTA TRAGER
Graduate School of Nutrition, Cornell University, Ithaca, N. Y.

Rat liver homogenate preincubated with protamine loses the capacity to convert mevalonic acid (MVA) to non-saponifiable material (NSF, largely cholesterol)(1). This loss of synthetic capacity is associated with the absence of ATP in these homogenates. ATP is present and conversion of MVA to NSF occurs if the homogenates are supplemented prior to incubation with RNA, DNA or polyethylene sulfonate. Thus it would appear that there is a requirement for a relatively non-specific polyanion that is essential for maintenance of ATP levels in liver homogenates. During studies on the specificity of protamine as an inhibitor of respiration or of oxidative phosphorylation it was observed in a number of experiments that protamine that had been trypsinized in sodium bicarbonate solution was no longer deleterious but the sodium bicarbonate solution in which protamine had been trypsinized was quite inhibitory. More extended study has now shown that bicarbonate is a potent inhibitor of the aerobic system concerned with maintenance of ATP levels in homogenates and that trypsinized protamine, amino acids of the urea cycle, or canavanine, for reason to be discussed later, reverse, at least over a narrow range, this inhibition. In this connection the studies of Miller and Evans who showed that bicarbonate is an inhibitor of cytochrome oxidase are pertinent(2).

Methods and materials. The biosynthetic experiments involved preincubation of 5 ml aliquots of a 200 xg supernatant fraction of rat liver homogenate prepared as previously described(3,4,5) with 6 ml amounts of solution containing 1 mg ATP, 10 mg DPN, 100 mg sodium succinate, various amounts of sodium bicarbonate, and other supplements to be studied. Each flask was aerated with a stream of oxygen and preincubated with agitation for 30 min at 37°. Following preincubation each flask was opened and 1 ml MVA-2-C^{14} solution added, the contents aerated and the flasks then reincubated for an additional 3 hr. After final incubation, homogenates were saponified, extracted with petroleum ether, the ether extracts dried with sodium sulfate, filtered, evaporated, taken up in scintillation mixture, and counted. In experiments involving a determination of ATP content of homogenates at time of MVA addition following preincubation the contents of paired flasks were treated with an equal volume of cold 4% perchloric acid. The precipitate that formed was removed by centrifugation and the supernatant solutions were freed of excess perchloric acid by careful neutralization with cold 10% potassium hydroxide.

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TABLE I. Biosynthesis of NSF by Liver Homogenate as Influenced by Preincubation with Various Sodium and Potassium Salts.

Exp.	Level of material tested, mg/flask	NSF, cpm
1		7242
	10 $NaHCO_3$	7716
	20 "	8922
	30 "	6128
	50 "	21
	10 NaCl	7919
	20 "	7522
	30 "	6873
	50 "	6740
2		5825
	10 $NaHCO_3$	7123
	20 "	7731
	50 "	28
	10 $KHCO_3$	6359
	20 "	8240
	50 "	20
	10 NaCl	5955
	20 "	6094
	50 "	5631

The solutions were centrifuged to remove potassium perchlorate and the supernatant solutions were subjected to anion exchange chromatography and gradient elution on Dowex-1 as described by Allfrey and Mirsky (6). ATP was determined strectrophotometrically in the eluates. Protamine sulfate was obtained from the Mann Research Laboratories. The amino acids of the urea cycle and canavanine were all of the L form and were obtained from Nutritional Biochemical Corp. or California Corp. for Biochemical Research.

Results. The results summarized in Fig. 1 show that biosynthesis of NSF from MVA by liver homogenates is completely inhibited by preincubation with about 0.05 M sodium bicarbonate. Note in particular the steep slope of the curves at levels of sodium bicarbonate just below those showing complete inhibition. Levels of sodium bicarbonate of about 0.02 M are stimulatory. Separate studies have established that the inhibitory effect of sodium bicarbonate in the amounts studied is not attributable to alterations in the pH of the homogenates (no detectable change in pH with up to 50 mg of sodium bicarbonate per flask).

Fig. 2 shows that a control homogenate of liver preincubated without bicarbonate maintained an adequate level of ATP as determined by anion exchange chromatography on Dowex-1 formate. Biosynthesis of NSF by homogenate in a paired flask supplemented with MVA following the preincubation period was excellent (7505 cpm). On the other hand, homogenate of liver preincubated with 0.05 M bicarbonate did not maintian a level of ATP and biosynthesis of NSF in a paired flask supplemented with MVA following the preincubation period was negligible (20 cpm).

Biosynthesis of NSF from MVA is not affected by preincubation of homogenates with sodium chloride (Table I). Preincubation with potassium bicarbonate is accompanied by increased activity of the system at intermediate levels and by complete inhibition at high levels as shown previously with sodium bicarbonate.

The data of Table II demonstrate that protamine digested with trypsin is active in reversing the inhibitory effect of sodium bicarbonate. Protamine freshly dissolved in buffer or protamine digested in bicarbonate without trypsin is inactive. Separate studies showed that the effect of trypsinized protamine is not due to the trypsin. It should be pointed out that the results summarized in Table II were obtained only when the level of sodium bicarbonate used was just maximally inhibitory. At higher levels of sodium bicarbonate no reversal of inhibition was ob-

TABLE II. Biosynthesis of NSF by Liver Homogenate as Influenced by Preincubation with Sodium Bicarbonate and Various Protamine Preparations.

$NaHCO_3$, mg/flask	Additional supplement, mg/flask	NSF, cpm
		6950
40		25
"	4 Protamine	21
"	8 "	15
"	12 "	27
"	20 "	19
"	4 Protamine, incubated	28
"	8 *Idem*	29
"	12 "	21
"	20 "	18
"	4 Protamine, incubated with trypsin	23
"	8 *Idem*	66
"	12 "	222
"	20 "	6312

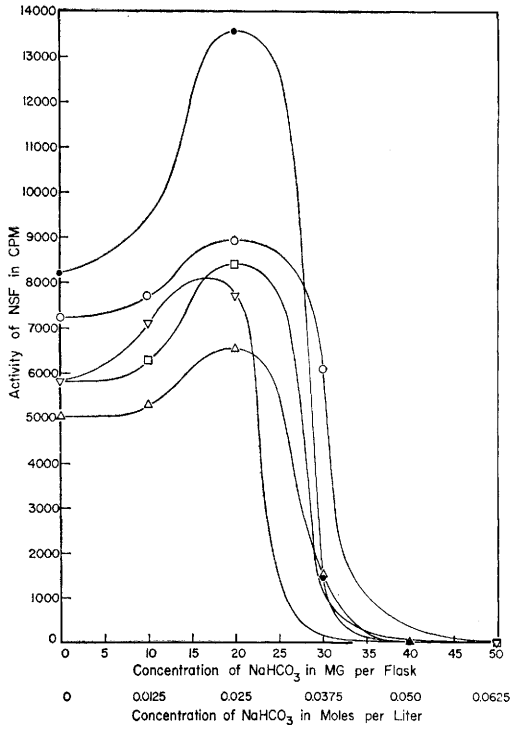

FIG. 1. Biosynthesis of NSF by liver homogenate as influenced by preincubation with various levels of sodium bicarbonate.

served with trypsinized protamine. For this reason, to avoid negative experiments from which no conclusions could be drawn, subsequent experiments have involved either the use of graded levels or an excess of materials to be evaluated against several levels of bicarbonate.

The activity of trypsinized protamine but not undigested protamine against bicarbonate inhibition of the liver system suggested that this activity may be due to arginine which is present in protamine to the extent of 85-90%. The data of Table III, Exp. # 1, show that arginine does indeed reverse bicarbonate inhibition. Some explanation of the figures obtained is in order. As noted earlier, intermediate levels of bicarbonate stimulate the system. Thus if a compound reverses the effect of bicarbonate at intermediate levels of bicarbonate this stimulation should be abolished by the reversing agent. The results obtained with 20 mg of sodium bicarbonate per flask show that this is the case. With no added bicarbonate or arginine (control flask)

TABLE III. Biosynthesis of NSF by Liver Homogenate as Influenced by Preincubation with Sodium Bicarbonate and Amino Acids of the Urea Cycle

Exp.	$NaHCO_3$, mg/flask	Additional supplement, mg/flask	NSF, cpm
1			5012
	10		5308
	"	4 Arginine	4653
	"	8 "	5158
	"	20 "	4725
	20		6541
	"	4 "	4915
	"	8 "	5345
	"	20 "	5058
	30		1508
	"	4 "	1840
	"	8 "	1816
	"	20 "	6399
	40		35
	"	4 "	22
	"	8 "	14
	"	20 "	106
2			6120
	20		10343
	"	20 Ornithine	7406
	"	" Citrulline	7695
	"	" Arginine	7150
	"	" Canavanine	6853
	30		906
	"	20 Ornithine	8005
	"	" Citrulline	6505
	"	" Arginine	8706
	"	" Canavanine	9885
	40		44
	"	20 Ornithine	291
	"	" Citrulline	29
	"	" Arginine	310
	"	" Canavanine	755

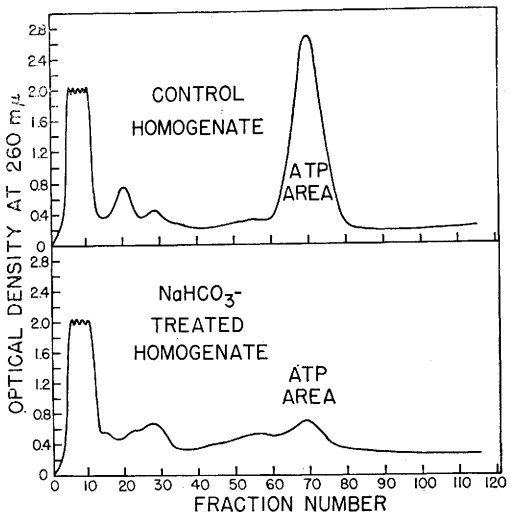

FIG. 2. ATP content of rat liver homogenates preincubated with and without added sodium bicarbonate. Level of sodium bicarbonates employed was 0.05 M.

5012 cpm in NSF were observed. With 20 mg of sodium bicarbonate per flask 6541 cpm in NSF were observed. However, with 20 mg of sodium bicarbonate per flask plus 20 mg of arginine per flask 5058 cpm in NSF were observed. On the other hand, with 30 mg of sodium bicarbonate per flask 1508 cpm in NSF were observed (70% inhibition rather than stimulation). However, with 30 mg of sodium bicarbonate per flask plus 20 mg of arginine per flask 6399 cpm in NSF were observed (essentially complete reversal of bicarbonate inhibition).

The results obtained with arginine suggested that this amino acid reverses bicarbonate inhibition of aerobic activity in essentially whole homogenates of liver by stimulating the Krebs-Henseleit urea cycle. Accordingly a number of amino acids of the cycle as well as canavanine were compared for activity. The data of Table III, Exp. #2 show that all the compounds are active. It is apparent from a study of the results that the order of increasing activity is: citrulline, ornithine, arginine, and canavanine. Thus with no added bicarbonate or amino acid (control flask) 6120 cpm in NSF were observed. With 20 mg of sodium bicarbonate per flask 10343 cpm in NSF were observed. However, with 20 mg of sodium bicarbonate per flask this stimulation was reduced to 7695 cpm with citrulline, to 7406 cpm with ornithine, to 7150 cpm with arginine and to 6853 cpm with canavanine. Again at a level of 30 mg of sodium bicarbonate per flask where inhibition of the conversion of MVA to NSF is 85% complete (6120 cpm in control *vs.* 906 cpm in the sodium bicarbonate flask) 6505 cpm were found in the citrulline flask, 8005 cpm in the ornithine flask, 8706 cpm in the arginine flask and 9885 cpm in the canavanine flask. The results obtained with 40 mg of sodium bicarbonate per flask are compatible with the same order of activity.

Discussion. The results presented demonstrate that bicarbonate is a potent inhibitor of a complete system in aerobic homogenates that is essential for conversion of MVA to NSF. It seems established that bicarbonate is an inhibitor of respiration or of oxidative phosphorylation that is essential for maintenance of ATP levels required for phosphorylation of MVA, phosphorylation of MVA-5-P and decarboxylation and dehydration of MVA-5-PP. These studies do not establish the locus of inhibition but Miller and Evans have shown both with plant and animal preparations that bicarbonate is an inhibitor of cytochrome oxidase(2). Inhibition of cytochrome oxidase by bicarbonate could account for the results presented in this paper. It is perhaps significant that in the two studies bicarbonate showed complete inhibition at 0.05 M. The mechanism by which bicarbonate inhibits cytochrome oxidase has not been established.

It seems clear that the amino acids of the urea cycle reverse bicarbonate inhibition by stimulating the cycle to convert bicarbonate to urea. Reversal of high levels of bicarbonate is apparently not achieved because of limited activity of the cycle. It would appear that citrulline is less active than the other amino acids studied because conversion of citrulline to arginine requires the expenditure of 1 mole of ATP in an ATP limited system without the uptake of bicarbonate. On the other hand, ornithine or arginine (after conversion to ornithine by urease) reduces the level of bicarbonate by 1 mole for every mole of ATP expended. No completely satisfactory mechanism can be offered to explain the fact that canavanine is more active than arginine in reversal of bicarbonate inhibition.

Summary. Bicarbonate is a potent inhibitor of a system essential for maintenance of ATP levels that are required for conversion of mevalonic acid to non-saponifiable material (largely cholesterol) by liver homogenates. This bicarbonate inhibition, over a narrow range of concentration, is reversed by trypsinized protamine or by amino acids of the urea cycle or by canavanine.

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Influence of Bacterial Pyrogen on Transplantable Tumors. (26119)

GEORGE T. MANILLA, PAUL S. NICHOLES AND CLARKE G. MCCARTHY
(Introduced by S. Marcus)
Department of Bacteriology, University of Utah College of Medicine, Salt Lake City

Crude filtrates from microbial cultures have been shown to be effective oncolytic agents and numerous investigators have observed spontaneous regression of tumors following acute infections. The historical significance affixed to microbes and microbial products in malignancy has been reviewed by other authors(1-4).

During studies in this laboratory on potentiation of the immune response by bacterial pyrogens(5), spontaneously occurring tumors of mice were noted to regress after administration of pyrogen. The work reported here was undertaken to determine the effect of these pyrogens on mouse transplantable tumors. Of the 3 tumors studied, only the subcutaneous transplants of the sarcoma S-37 tumor responded to treatment, as evidenced by tumor regression and a decreased mortality rate.

Materials. The mouse tumors utilized in these experiments were a sarcoma strain S-37, an adenoma strain EO771, and a leukemia strain 1498. The sarcoma S-37 was maintained in this laboratory by serial transplantation in the ABC strain of mice. Likewise the adenoma strain EO771 and the leukemia strain 1498 were both maintained by periodic transplantation in C57BL mice. The ABC and C57BL strains of mice used in the subcutaneous and intraocular homologous transplant experiments were purchased from Roscoe B. Jackson Memorial Cancer Inst., Bar Harbor, Maine. The Swiss albino mice employed in the intracerebral heterologous transplant experiment were obtained from a local source. All animals were allowed free access to their food supplies of Purina Laboratory Chow and water.

The tumor-bearing animals were treated with various doses of the bacterial pyrogenic substances PC-3 and/or PC-6 which were fractions derived from the parent pyrogen PC-1 (Piromen).*

Methods and results. Preliminary work suggested that it was advisable to determine the therapeutic value of pyrogen on tumors at different implant sites as well as on different tumor strains. The effect of these pyrogens on both subcutaneous and intracerebral transplants of the S-37 tumor was ascertained.

The other tumors selected for either subcutaneous or intraocular transplant sites were first removed from a carrier animal and minced with scissors in a small quantity of sterile 0.85% sodium chloride solution. Small fragments of tumor tissue were introduced into the animals by the semi-automatic trocar method of Fuson and Eichwald(6). Subcutaneous tumor transplant of the sarcoma and leukemia strains were implanted into the left axillary region of the recipient mice. Intraocular adenoma transplants were implanted into the anterior chamber of the animal's right eye. For intracerebral transplantation, the trocar method proved inadequate; therefore, the S-37 tumors were removed from the carrier mice and the tumors briefly homogenized in sterile saline with a Waring blendor until the large tissue particles were noticeably disrupted. The Swiss albino mice

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