5. Details of the biological phase of the research are in preparation.
6. The generous supply of the tetracycline antibiotics provided by Lederle Laboratories Division of the American Cyanamid Co., and Chas. Pfizer and Co., is hereby gratefully acknowledged.
7. Model SC-5041, Hanovia ultraviolet light, Hanovia Chemical and Manufacturing Co., Newark, N.J. (366 mμ).
8. Visking Corp., Chicago, Ill.
9. The completion of dialysis at this time was shown by the fact that further dialysis yielded no more precipitate of the fluorophore when the dialyzate was made alkaline.
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Prevention of Toxicity of Amethopterin for Sarcoma-180 Cells in Tissue Culture

The present paper is a preliminary report on the finding that sarcoma-180 (S-180) cells grow normally when the function of folic acid is prevented by amethopterin (Methotrexate), if the medium is supplemented with products of the biosynthetic reactions dependent on folic acid cofactors. It is known that certain microorganisms which require folic acid for growth—for example, *Streptococcus faecalis* 8043—can grow in the absence of this vitamin in a medium containing thymine and adenine or guanine (*1*). That folic acid is one of the nutritional requirements for the growth of mammalian cells in tissue culture was shown by Eagle (*2*). The inhibition of the growth of sarcoma-180 cells in such a medium by amethopterin has been demonstrated (*3*).

The techniques of Eagle (*2, 4*) were used. The medium contained 5 percent thoroughly dialyzed horse serum. The cells were grown in the experimental media for 7 days. The growth of the cells in the presence of amethopterin in a medium containing hypoxanthine, thymidine, and glycine is shown in Table 1. Disintegration of the cells occurred if hypoxanthine or thymidine was omitted from the mixture. In the absence of glycine, some growth was observed, indicating either a small amount derived from the dialyzed horse serum or some formation of glycine, possibly from the exogenous L-threonine (*5*). When glycine was added to this medium, the growth was comparable to that of the control. The complete mixture fully supported the growth of sarcoma-180 cells even in the presence of amethopterin at concentrations 10,000 times that ordinarily required for complete inhibition (Table 1). In body fluids, the concentrations of such compounds could be critical to the effectiveness of amethopterin on neoplastic cells *in vivo*. Similar compounds in the crude medium (chicken plasma clot) might also explain the failure of amethopterin to inhibit sarcoma-180 cells, as reported by Biesele (*6*).

When the function of folic acid was prevented by amethopterin, it was found that sarcoma-180 cells were able to utilize adenine, adenosine, deoxyadenosine, hypoxanthine, and inosine equally well; guanosine supported slower growth of sarcoma-180 cells under these conditions; that is, there was only a threefold increase in 7 days, whereas xanthine and xanthosine were inactive. These results indicate that some "adenine" was derived from guanosine, but the extent to which "guanine" or "adenine," or both, were derived from xanthine and xanthosine was insignificant.

In the presence of amethopterin, thymidine, and glycine, the cells disintegrate if purines are not supplied in the medium (Table 1). Under such conditions it is unlikely that purine synthesis *de novo* occurs. Thus, the single purine in the medium must serve as the sole source, not only of adenine and guanine of nucleic acids, but also of all the coenzymes containing purines as structural constituents. Accordingly, this technique appears to be useful for the study of the pathways of purine metabolism. The present work demonstrates for the first time that mammalian cells (sarcoma-180) are fully capable of using exogenous purines for growth and multiplication.

Further work demonstrated that thymidine could be replaced by thymidylic acid for the growth of sarcoma-180 cells in the presence of amethopterin, glycine, and a purine, but thymine and thymine-riboside had no activity in this respect. A mixture of thymine and deoxyadenosine did not replace a mixture of thymidine and adenine, indicating that this type of transdeoxyribosidation did not occur.

It is seen that the presence of amethopterin creates new requirements for the growth of the tumor cells *in vitro*. When these requirements are met by preformed purines, thymidine, and glycine, inhibition of growth might still be achieved if the utilization of even one of these compounds were prevented. Logical combinations for chemotherapy are thus suggested. The new requirements created by amethopterin probably differ in different species and tissues. It is already known that differences exist in the abilities of various tissues and species to utilize preformed purines *in vivo* (*7*). In addition, rabbit fibroblasts, unlike sarcoma-180 cells, require exogenous L-serine for growth in tissue culture (*8*).

Table 1. Growth of sarcoma-180 cells in tissue culture in the presence of amethopterin. Folic acid (pteroylglutamic acid) was present at a concentration of $2 \times 10^{-7} M$.

Varied supplements in the medium				Degree of cellular multiplication†
Amethopterin* (M)	Hypoxanthine $3 \times 10^{-5} M$	Thymidine $3 \times 10^{-5} M$	Glycine $1 \times 10^{-4} M$	
0				5.7‡
3×10^{-8}				0.70
3×10^{-7}				0.34
3×10^{-7}	+	+	+	5.7§
3×10^{-4}	+	+	+	6.1
3×10^{-7}		+	+	0.60
3×10^{-7}	+		+	0.43
3×10^{-7}	+	+		1.8

* 4-Amino-10-methyl-pteroylglutamic acid.
† Referred to inoculum as 1; determined by the method of Oyama and Eagle (*11*); correlation of the protein determinations with cell counts is discussed by Oyama and Eagle (*11*).
‡ Control.
§ The culture has been carried for 3 weeks under these conditions and is being maintained.

Thymine or thymidine increased the rate of the development of amethopterin resistance in *Streptococcus faecalis* 8043 (*9*). The effect of similar factors on the development of amethopterin resistance in mammalian cells is under study (*10*).

MAIRE T. HAKALA
Department of Experimental Therapeutics, Roswell Park Memorial Institute, Buffalo, New York

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Gastric Secretagogue Effect of Lysine Monohydrochloride

There have been recent references in medical literature to the use of lysine monohydrochloride as a nutritional supplement (*1*). Certain clinical responses, such as increase of appetite, weight gain, and rapid restoration of hemoglobin values, have been reported. Such effects might be produced by either or both of these mechanisms: (i) correction of pre-

existing lysine deficiency and (ii) pharmacodynamic properties of lysine. In an attempt to evaluate what influence the ingestion of lysine has on human physiology, we have undertaken a number of studies. The one reported here concerns the influence on gastric secretion.

Twenty-one adult volunteers were given two test meals each, the first consisting of bread and water and the second of an equal amount of bread and water with 5 g of lysine monohydrochloride added. The two meals were separated by an interval of 1 week. Fractional gastric analysis followed each test meal, and the amounts of free hydrochloric acid and pepsin were determined in samples taken every 15 minutes for 1 hour. The methods used for these analyses are those described by Hawk, Oser, and Summerson (*2*).

The significance of all figures obtained by analysis was determined by t and P values obtained from the formula

$$t = \frac{M_1 - M_2}{\sqrt{(\text{S.E.}_1)^2 + (\text{S.E.}_2)^2}}$$

Values of P are charted from Fischer's table. Values are considered significant when the P value is less than .01.

Eighteen of the 21 subjects showed significant differences of gastric secretions after the two test meals. Whenever the addition of lysine was accompanied by an alteration, the change was an increase. The mean values of all data are given in Table 1. The incidence of effects on pepsin and hydrochloric acid secretion together or separately is as follows: increased pepsin and increased HCl, 12 subjects; increased pepsin but no increase of HCl, three subjects; no increase of pepsin but increased HCl, three subjects; no increase of pepsin or HCl, three subjects.

The addition of 5 g of lysine monohydrochloride to a bread and water test meal significantly increased gastric secretion of hydrochloric acid and pepsin, or both, in 18 of 21 subjects studied. Further work is being done to explain and localize this action. It may be of some significance that previous work has developed evidence that amino acids, either by action in the intestinal tract (*3*) or when given intravenously by systemic action (*4*), promote gastric secretion. The present study may indicate that part of the effect of lysine given as a nutritional supplement results from the stimulation of gastric secretion. This, of course, does not preclude or compete with any importance which lysine may have as a participant in anabolic processes (*5*).

Table 1. Effect of addition of lysine monohydrochloride to diet.

Time (min)	Mean values (units)		Difference	
	No lysine	With lysine	Mean	S.E.*
	Hydrochloric acid			
15	3.7	2.5	−1.1	1.12
30	13.4	24.2	10.8	5.95
60	13.7	33.7	20	2.07
	Pepsin			
15	−48	5	53	2.31
30	1	102	101	5.35
60	30	169	139	8.51

* The standard error was determined from the formula $\sqrt{\frac{(\text{SD}_1)^2}{N} + \frac{(\text{SD}_2)^2}{N}}$

ARTHUR M. SACKLER
LAWRENCE H. SOPHIAN
van Ophuijsen Institute, New York, New York

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Delayed Pain as a Peripheral Sensory Pathway

The existence of "second pain" as a "genuine sensory phenomenon" was recently questioned (*1*). There was also the more interesting implication that a delayed type of "C-fiber pain" does not exist at all as a separate pain sensation. "Double pain" can be aroused by an appropriate noxious stimulus if it is applied to the skin far enough from the central nervous system (*2, 3*). The "second" or slower pain is felt at a distinct time interval after the first or fast component, and it is usually qualitatively different from the first, being more a diffuse ache than a sharp prick, more unpleasant, longer lasting, and like the pain that is aroused from deeper structures below the skin (*4*). Landau and Bishop (*4*) point out that this delayed type of pain has a greater threshold for most types of stimuli than does sharp pricking pain, although its threshold is lower for certain stimuli. They also present evidence (evidently missed by Jones) that the delayed pain sensation can be masked by the faster sharp pain; this makes it difficult to detect double pains under certain conditions of stimulation.

I suggest, therefore, that what the experimental work reported by Jones (*1*) amounts to is a careful description of some conditions of stimulation in which second pain is not demonstrable. For example, threshold electric stimuli will never elicit second pain, even in "480 separate determinations," if those receptors and nerve fibers subserving fast pain are electrically more excitable than those subserving slow pain sensations. The more slowly conducting, amyelinated C fibers, as well as their receptor endings (*4*), have a higher electric threshold than any other nerve-fiber group.

Jones concluded that second pain, when it does appear, is an experimental "artifact" rather than a "genuine sensory phenomenon." These distinctions have little meaning without careful definition for this context. Jones' main contention would appear to be that if a noxious stimulus were delivered at the same instant to any of the receptors that subserve pain, only a fast single, and not a double, pain could be produced—that is, that there is only one type of pain-sensory system; but her negative results do not prove this. If it is difficult [although not impossible (*4*)] to find a stimulus which excites the receptors that subserve slow pain without also exciting other types of receptors, this difficulty cannot be used as proof that slow pain is not a "genuine" and distinct sensory modality. More definitive evidence on this point must be sought from other experimental procedures. It is important, therefore, to analyze some of the criticisms (*1, 5*) of these other, more positive lines of evidence.

Although the total reaction time for perception of pain is obviously not a measure of conduction time, the *increase* in reaction time found when the stimulus is moved to a more distal point (*2*) can be a measure of conduction velocity in the sensory fibers. Jones dismisses such evidence by citing a more recent report (*6*) to show that the increase in reaction time with a more distal stimulus is small and relatively insignificant. But this negative finding concerned touch sensations, not pain (*7*). If the impulses that are responsible for touch sensation are transmitted in group A fibers of even the small variety, for example, at 10 m/sec, then the increase in reaction time that could be expected when the stimulus is moved 50 cm distally would be only 0.05 second, and difficult to detect. On the other hand, if delayed pain is mediated by C fibers, at 1 m/sec, the difference in reaction time would be 0.5 second. This interval, which is easily detectable, was found to be the actual difference in reaction time to slow pain for the conditions stated (*2*).

Although all cutaneous senses show an increasing delay in perception during