Nucleic Acid Precursor Requirements of
Mycoplasma laidlawii

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SUMMARY
The nucleic acid precursor requirements of Mycoplasma laidlawii strain A were determined by using a partially defined medium. Adenine, guanosine and cytidine were found to be the minimal growth requirements. However, best growth was obtained with undegraded RNA or oligoribonucleotides. Thymidine was not essential for growth when folinic acid was present in the medium; folic acid was completely inactive. M. laidlawii utilized the 2' - and 3'-ribonucleotides most poorly. The dephosphorylation of these mononucleotides to nucleosides rendered them growth promoting.

INTRODUCTION
The effects of ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) on the growth of several Mycoplasma strains in a partially defined medium were described by Razin & Knight (1960b). The saprophytic Mycoplasma laidlawii strain A did not grow in the basal medium alone (Razin & Knight, 1960a) but grew on addition of suitable concentrations of RNA and DNA. Thymidine was found to be the moiety of DNA essential for growth. The fragments of RNA molecule needed for growth were not defined. Chemical or enzymic degradations of RNA to fragments smaller than oligonucleotides abolished its growth-promoting activity. It was therefore suggested that an oligoribonucleotide is required for growth of M. laidlawii strain A. The main purpose of the present work was to define the fragments of RNA molecule essential for growth of M. laidlawii strain A. Improvement of Razin & Knight’s partially defined medium enhanced growth and permitted a more correct analysis of nucleic acid precursor requirements. In the latter medium, RNA could be replaced by adenine + guanosine + cytidine. However, growth of the test organism was best with undegraded RNA or with the oligonucleotides derived from it.

METHODS
Organism. Mycoplasma laidlawii strain A (PG 8) was received through the courtesy of Dr D. G. l’l. Edward (The Wellcome Research Laboratories, Beckenham, Kent).

Media. A modified Edward medium (Razin & Oliver, 1961) was used for keeping stock cultures and for growing the organisms used as inoculum in the nutrition experiments.
The nutrition experiments were carried out in a modified partially defined medium
In their medium, consisting of inorganic salts, Casamino acids, vitamins, nucleic acids and glucose, 10% (v/v) pooled inactivated human serum was added instead of the horse serum used previously. This modification improved growth of the test organism without altering its requirements for nucleic acid precursors. The modified medium without added RNA and DNA will be referred to as basal medium.

Pyrex glassware was used in all experiments. Before use it was cleaned in chromic acid mixture and thoroughly rinsed in distilled water. The basal medium was dispensed in 9 ml. quantities into 6 in. x % in. sterile screw-cap test tubes. The solutions of nucleic acids or their degradation products to be tested were added to give a final volume of 10 ml. Sterilization of nucleic acids and degradation product solutions was carried out by passage through sintered glass filters or by steaming for 20 min. (Merrifield & Dunn, 1950).

Conditions of growth. Five ml. liquid Edward medium were inoculated with 0.1 ml. stock culture and incubated for 24 hr. at 37°C. The organisms were harvested and washed once with 0.01 M-K$_2$HPO$_4$ buffer, pH 7.0 (Butler & Knight, 1960). Each tube of experimental medium received 0.1 ml. of a 1/10 dilution of the washed suspension. Viable counts showed the initial inoculum to contain about $10^5$ to $10^6$ viable particles/ml. medium. Inoculated test tubes were incubated statically in air at 37°C. Growth was usually estimated after incubation for 96 hr.

Assessment of growth. The extent of growth was measured by titration of the acid formed by the organisms during growth (Razin & Knight, 1960a).

Chemicals. Ribonucleic acid (Na salt from yeast; Na-RNA), deoxyribonucleic acid (Na salt from thymus gland; Na-DNA), purines, pyrimidines, nucleosides, nucleosides-2'- and 3'-phosphates, nucleosides-5'-phosphates and ribonuclease (×4 crystallized, bovine pancreas) were the products of L. Light and Co. Ltd. (Colnbrook, Bucks). Folinic acid (Citrovorum factor) and aminopterin (4-amino-pteroylglutamic acid) were obtained from the American Cyanamid Co. (Lederle Laboratories Division, New York, N. Y., U.S.A.). Folic acid was the product of Hoffmann La Roche & Co. Ltd. (Basel, Switzerland). Acid phosphatase (wheat germ) was purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Viper venom (Vipera palestinae) was obtained from the Zoology Department of the Hebrew University, Jerusalem, through Dr D. Nelken. The venom was freeze-dried over P$_2$O$_5$ at 0.3 mm. Hg. (Centrifugal freeze-drier, W. Edwards and Co., London).

Degradation of ribonucleic acid. Acid hydrolysis of RNA to oligonucleotides was done by the method of Merrifield & Woolley (1952) as described by Razin & Knight (1960b). Degradation of RNA to oligonucleotides and pyrimidine mononucleotides by ribonuclease and to nucleosides by crude viper venom was carried out as described previously (Razin & Knight, 1960b). Hydrolysis of RNA to nucleoside-2'- and -3'-phosphates was done by disso ving $300$ mg. Na-RNA in 20 ml. 0.5N-NaOH. The solution was incubated at 25°C for 24 hr., neutralized with HCl and made up to volume for bioassay. The nucleoside-2' and -3'-phosphates obtained by the alkaline hydrolysis of RNA were dephosphorylated to give nucleosides by acid phosphatase. Five mg. of this enzyme were added to 6.5 ml. of the mononucleotides solution, which was brought to pH 5.3 and incubated at 37°C for 22 hr. Inorganic phosphorus determination (Umbreit, Burris & Stauffer, 1957) at the end of the
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incubation period showed the degradation of 90% of the mononucleotides to nucleosides.

Dialysed human serum was prepared by the method described previously (Razin & Knight, 1960a).

**RESULTS**

**Requirement of Mycoplasma laidlawii for thymidine**

Previous work has shown that the requirement of *Mycoplasma laidlawii* for DNA might be completely replaced by thymidine and to a lesser extent by thymine (Razin & Knight, 1960b). Further study has now shown that *M. laidlawii* can grow

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**Fig. 1**

The growth response of *Mycoplasma laidlawii* to folinic acid. Test tubes contained 10 ml. basal medium supplemented with 50 µg. Na-RNA/ml. and various concentrations of folinic acid. Initial inoculum $2 \times 10^5$ viable particles/ml. Acid production determined after incubation at $37^\circ$ for 96 hr.

**Fig. 2**

Growth inhibition of *Mycoplasma laidlawii* by aminopterin and its annulment by folinic acid. Test tubes contained 10 ml. basal medium supplemented with 50 µg. Na-RNA/ml. and various concentrations of aminopterin. One series of test tubes contained 4 m\mu g. folinic acid/ml. (○); the other, 400 m\mu g. folinic acid/ml. (●). Initial inoculum $5 \times 10^4$ viable particles/ml. Acid production determined after incubation at $37^\circ$ for 114 hr.

in the absence of added DNA or thymidine when folinic acid is added to the medium. The growth response of *M. laidlawii* to various concentrations of folinic acid is presented in Fig. 1. A concentration of 4 m\mu g. folinic acid/ml. sufficed for optimal growth and 0.4 m\mu g./ml. enabled about 50% of optimal growth.

Folic acid was completely inactive in replacing thymidine, even at the highest concentration tested (50 µg./ml.).

Aminopterin inhibited growth promoted by folinic acid. This growth inhibition was overcome by raising the concentration of folinic acid or by incorporating thymidine (50 µg./ml.) into the medium (Fig. 2).
Requirement of Mycoplasma laidlawii for RNA components

The medium used for these experiments contained thymidine or folinic acid in amounts sufficient for optimal growth. Degradation of RNA to oligonucleotides by treatment with 6N-HCl for 1–3 min. at 25°C (Merrifield & Woolley, 1952) did not significantly affect its growth-promoting activity. Further hydrolysis of the oligonucleotides by longer treatment with acid rapidly abolished their growth-promoting activity (Fig. 3).

Figure 3. The effect of acid hydrolysis of RNA to oligonucleotides on its growth-promoting activity for Mycoplasma laidlawii. Test tubes contained 10 ml. basal medium supplemented with 50 μg. thymidine/ml. and different concentrations of: undegraded RNA (●); RNA hydrolysed by 6N-HCl at 25°C for 1 min. (○); 3 min. (▲); 7 min. (×). Initial inoculum 10^5 viable particles/ml. Acid production determined after incubation at 37°C for 96 hr.

Fig. 4. The effect of RNA and its degradation products on the growth of Mycoplasma laidlawii. Erlenmeyer flasks contained 40 ml. basal medium supplemented with 50 μg. thymidine/ml. and: Na-RNA, 50 μg./ml. (○); degradation products of RNA by ribonuclease, 50 μg./ml. (●); nucleoside mixture (adenosine, guanosine, cytidine, uridine, 15 μg./ml. of each) (▲); 5'-ribonucleotide mixture (adenylic acid, guanylic acid, cytidylic acid, uridylic acid, 15 μg./ml. of each) (△); alkaline hydrolysate of RNA, 500 μg./ml. (▲) or 50 μg./ml. (△); 2'- and 3'-ribonucleotide mixture (adenylic acid, guanylic acid, cytidylic acid, uridylic acid, 15 μg./ml. of each) (×). Initial inoculum 4 × 10^5 viable particles/ml. Acid production determined in 8 ml. samples of cultures taken after various incubation periods at 37°C.

Figure 4 represents growth curves of Mycoplasma laidlawii inoculated into the basal medium, supplemented with 50 μg. thymidine and Na-RNA or its degradation products/ml. Best growth was obtained with undegraded RNA. Degradation of RNA to a mixture of oligonucleotides and -3'-pyrimidine mononucleotides by pancreatic ribonuclease decreased its growth-promoting activity, and alkaline hydrolysis of RNA to nucleosides-2' - and -3'-phosphates nearly abolished it. Fifty μg. of the alkaline degradation products/ml. medium were unable to support growth, while 500 μg./ml. of these degradation products enabled slow and limited growth (Fig. 4). Similar results were obtained with a mixture of the four nucleoside-2' - and -3'-phosphates obtained from commercial sources (adenylic, guanylic,
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cytidylic, uridylic acids). Mixtures of the -5'-phosphate isomers of the above mentioned mononucleotides were active in growth promotion. A mixture of the four nucleosides, adenosine, guanosine, cytidine, uridine (15 μg./ml. each) enabled growth to take place. Growth promoted by nucleosides was inferior to that promoted by undegraded RNA (Fig. 4). Degradation of RNA to nucleosides by viper venom decreased, but did not abolish, its growth-promoting activity (Fig. 5). Hydrolysis of RNA to purine bases and pyrimidine nucleotides by 1.5 N H₂SO₄ at 100° abolished its growth-promoting activity completely. Purines and pyrimidines, in several mixtures and concentrations, were inactive in growth promotion. Addition of D-ribose to the purines and pyrimidines mixture was without any effect.

![Fig. 5. The effect of degradation of RNA to nucleosides on its growth-promoting activity for Mycoplasma laidlawii. Twenty mg. of dried viper venom were added to 10 ml. of Na-RNA solution (5 mg./ml.) in 0.01 M-MgCl₂. The mixture was brought to pH 8.6 by N-NaOH and incubated at 37°. Samples for bioassay and inorganic phosphorus determination (Umbreit, Burris & Stauffer, 1957) were taken at the time intervals indicated and placed for 5 min. in a boiling water bath to stop the reaction. Test tubes contained 10 ml. basal medium supplemented with 50 μg. thymidine/ml. and 250 μg./ml. (●) or 50 μg./ml. (○) of RNA degradation products. Percentage of RNA degraded to nucleosides (×) was calculated from inorganic phosphorus determinations. Initial inoculum 10⁵ viable particles/ml. Acid production determined after incubation at 37° for 87 hr.](image)

On the basis of these results experiments were carried out to determine the nucleosides essential for the nutrition of Mycoplasma laidlawii. By the single omission method it was found that adenosine, guanosine and cytidine were required, whereas uridine could be omitted without affecting growth. Best growth was obtained when the concentration of the three nucleosides in the medium was rather high (100 μg./ml. of each); at lower concentrations growth was markedly diminished (see Figs. 4, 5).

The possibility of replacing the nucleosides by their corresponding purine and pyrimidine bases was tested. Adenosine could be replaced by adenine; while
5 μg./ml. was found to be optimal, higher concentrations showed some growth-inhibitory activity. Hypoxanthine could replace the requirement for adenosine, but was less effective than adenine. Guanosine could not be replaced by guanine. Nor could cytosine replace cytidine. The pyrimidine ring precursors ureidosuccinic acid and orotic acid were inactive. Thus the smallest fragments of RNA still capable of promoting growth of *Mycoplasma laidlawii* are adenine (5 μg./ml.), guanosine (100 μg./ml.) and cytidine (100 μg./ml.).

Experiments were also performed in order to replace each of the three nucleosides, adenosine, guanosine or cytidine, by its corresponding mononucleotide, either the -2', -3' or the -5'-isomer. Adenosine could be replaced by any of the three isomers, whereas guanosine and cytidine could be replaced only by the -5'-isomers. These results are in agreement with the observation that the mixture of -2'- and -3'-mononucleotides obtained by alkaline hydrolysis of RNA was practically inactive in growth promotion. The alkaline hydrolysate of RNA became active when its constituent mononucleotides were dephosphorylated to nucleosides by acid phosphatase.

All the above results were obtained with the partially defined medium which contained 10% (v/v) whole human serum. In order to exclude the possibility that small amounts of nucleic acid components present in the whole serum were masking the true nutritional requirements of *Mycoplasma laidlawii* to a certain degree, the serum was exhaustively dialysed. Although growth of the test organism in the medium containing the dialysis residue of the serum was somewhat inferior to that obtained in whole serum medium, it sufficed for the analysis of nucleic acid precursor requirements. Adenine, guanosine, cytidine and thymidine were found essential for growth in the dialysed-serum medium.

**DISCUSSION**

The results of the present work show that *Mycoplasma laidlawii* strain A is capable of synthesizing thymidine when folinic acid is added to the medium. Folic acid is inactive, probably due to inability of the Mycoplasma to reduce it to folinic acid, the derivatives of which are the biologically active forms of this vitamin (Rabinowitz, 1960). The amount of folinic acid required for growth of *M. laidlawii* is of the same order as that required by *Pediococcus cerevisiae*. The latter microorganism, however, is able to grow when a high concentration of folic acid (2 μg./ml.) is present in the medium (Nichol, 1959; Grossowicz & Mandelbaum, 1961), whereas even with 50 μg. folic acid/ml. no growth of *M. laidlawii* took place. Aminopterin inhibited growth of *M. laidlawii* only when its concentration was several times higher than that of folinic acid. Aminopterin is particularly effective as an inhibitor of the enzymic reduction of folic acid to the tetrahydro form. Therefore its antagonistic activity towards organisms which are unable to reduce folic acid to folinic acid is lower (Nichol, 1959; Handschumacher & Welch, 1960). The complete reversal by thymidine of growth inhibition caused by aminopterin indicates that *M. laidlawii* requires folinic acid for the biosynthesis of thymidine only, when growing in the partially defined medium of Razin & Knight (1960a).

The minimal growth requirements of *Mycoplasma laidlawii* for nucleic acid precursors were found to be the nucleosides adenosine, guanosine and cytidine.
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Of these, only adenosine could be replaced by its purine base adenine. The better utilization of nucleosides for growth of various micro-organisms, as compared with their purine or pyrimidine bases, has been described (Loring & Pierce, 1944; Nakamura, 1957; MacLeod, Hogenkamp & Onofrey, 1958; Chakraborty & Loring, 1960).

The findings of the nutrition studies indicate the presence in *Mycoplasma laidlawii* of various enzymic activities concerned with nucleic acid metabolism. This organism is capable of transforming ribonucleosides to deoxyribonucleosides. Vitamin B₁₂ is usually involved in this transformation (Wacker, Kirschfeld & Traeger, 1959; Manson, 1960). The question whether *M. laidlawii* requires vitamin B₁₂ for growth must remain open until a completely defined medium is devised for it. *M. laidlawii* is capable of deaminating cytidine to uridine, apparently by the action of a cytidine deaminase described in a variety of microbial and mammalian cells (Crosbie, 1960). The enzymic methylation of deoxycytidine or deoxyuridine, with folic acid as cofactor, apparently supplies *M. laidlawii* with the required thymidine. Another enzymic ability of this organism is the amination of hypoxanthine to adenine.

The very significant decrease in the growth-promoting activity of RNA after its hydrolysis to 2' and 3'-mononucleotides might be explained by the well-known observations that bacteria and various animal tissues incorporate mononucleotides only after dephosphorylation to nucleosides (Leibman & Heidelberger, 1955; Lesley & Graham, 1956; Lichtenstein, Barner & Cohen, 1960). *M. laidlawii* apparently lacks the phosphatases which attack the 2' and 3'-mononucleotides. Dephosphorylation of these mononucleotides to nucleosides by commercial acid phosphatase enabled good growth of *M. laidlawii*. The decrease in the growth-promoting activity of RNA after its degradation by pancreatic ribonuclease might be similarly explained by the appearance of the biologically inactive 3'-pyrimidine mononucleotides as part of the degradation products (Davidson, 1960).

A problem which as yet remains unsolved is why growth of *Mycoplasma laidlawii* should be much faster and better with undegraded RNA or oligoribonucleotides than with smaller fragments of the molecule. Are undegraded RNA or oligonucleotides capable of penetration into the Mycoplasma cell? Undegraded DNA is known to penetrate into bacterial cells (Fox & Hotelkiss, 1960) and mammalian cells (Gartler, 1960). Lately RNA was also found to be capable of penetrating into tissue cells (Amos, 1961). The thin and plastic cell envelope of the Mycoplasma (Edwards & Fogh, 1960; van Iterson & Ruys, 1960) is apt to facilitate such penetration. Oligoribonucleotides were found to serve as primers for RNA synthesis by polynucleotide phosphorylase of *Azotobacter agile*. The oligonucleotides abolished the lag period usually observed on testing the activity of this enzyme (Singer, Heppel & Hilmo, 1960). Utilization of undegraded oligonucleotides by *Mycoplasma* might similarly enhance RNA synthesis and consequently protein synthesis, thus explaining the faster growth rate observed in the presence of undegraded RNA or oligoribonucleotides.

REFERENCES


