ium phlei showed the same pattern of protection against *Staphylococcus aureus* Smith; namely, effectiveness at 5 days, but not at 1 day, post administration. Endotoxin and zymosan also gave the same results against *Streptococcus pyrogenes,* namely, protection after 1 day, but not after 5 days.

Discussion. The present study confirms previous reports. It was shown earlier that endotoxin protects mice against subsequent staphylococcal(5,6) and streptococcal(7) infections. It was also known that pretreatment of mice with zymosan induces resistance against gram-positive cocci(8).

The new findings extend our knowledge of the prophylactic spectrum of *Mycobacterium phlei* which Biozzi *et al*(9) reported to protect mice against *Salmonella enteritidis*. The observation that the recently discovered stimulant, leaves of *Maytenus laevis,* induces protection against pathogenic microorganisms supports the view that RES stimulants frequently have the capacity to augment host defense.

Summary. Intravenous administration of pulverized leaves of *Maytenus laevis* to mice induced resistance to subsequent experimental challenge with pathogenic cocci. Endotoxin, zymosan and *Mycobacterium phlei* showed more transient prophylactic activity against the same pathogens.

The authors appreciate the technical assistance of Mr. George J. McGee (K-G Laboratories, Parsippany, N. J.) and the statistical work of Mr. Neial Stasilli.

1. DiCarlo, F. J., Haynes, L. J., Sliver, N. J., Phillips, G. E., *J. Reticuloendothelial Soc.,* in press.

2. Biozzi, G., Benacerraf, B., Halpern, B. N., *Brit. J. Exp. Path.,* 1953, v34, 441.

3. DiCarlo, F. J., Haynes, L. J., Malament, S. G., Phillips, G. E., *Can. J. Biochem. Physiol.,* 1963, v41, 731.

4. Miller, L. C., Tainter, M. L., PROC. SOC. EXP. BIOL. AND MED., 1944, v57, 261.

5. Dubos, R. J., Schaedler, R. W., *J. Exp. Med.,* 1956, v104, 53.

6. Sultzer, B. M., Freedman, H. H., *ibid.,* 1962, v116, 943.

7. Michael, J. G., Massell, B. F., *ibid.,* 1962, v116, 101.

8. Kiser, J. S., Lindh, H., de Mello, G. C., *Ann. N. Y. Acad. Sci.,* 1956, v66, 312.

9. Biozzi, G., Stiffel, C., Halpern, B. N., Mouton, D., *Rev. Franc. Etudes Clin. Biol.,* 1960, v5, 876.

Received January 15, 1964. P.S.E.B.M., 1964, v116.

Observations on the Use of Avidin in Bacteriological Media.* (29200)

CHIK H. PAI AND HERMAN C. LICHSTEIN
Department of Microbiology, College of Medicine, University of Cincinnati, Ohio

Avidin is used widely as a tool in studies of the biochemical functions of biotin because of its ability to form a biologically inactive complex with the vitamin(1). It has been reported also that avidin can be used in the purification of biotin since the complex with biotin is formed in a stoichiometric manner and can be split readily by heat treatment(2).

During our studies on the physiological control of biotin synthesis in *Escherichia coli,* an attempt was made to investigate the amount of biotin synthesized by the bacterial cells in the presence of avidin with the expectation that it would remove biotin quantitatively from the growth medium and that biotin would be released into an assayable form by subsequent treatment with heat. However, it was found that avidin could not be used for this purpose because of difficulties in releasing biotin from the avidin-biotin complex when it was allowed to form in certain media. In this paper are reported some observations on the release of biotin from avidin-biotin complexes formed under various conditions.

* Supported in part by National Science Foundation and by Office of Naval Research.

Materials and methods. d-Biotin was obtained from the California Foundation for Biochemical Research, Los Angeles. Avidin

TABLE I. Release of Biotin from Biotin-Avidin Complexes Formed in Distilled Water.

Boiling time (min.)	Biotin released ($\times 10^{-1}$ μg)		% recovery† of biotin
	Avidin only*	Avidin-biotin*	
0	0	0	0
5	1.24	–	–
10	1.49	9.84	87.9
20	1.71	–	–
30	1.8	11.0	96.8
40	1.83	–	–
50	1.88	11.2	98.1
60	1.85	11.3	99.5

* Avidin (N.B.C.), 1 unit; biotin, 9.5×10^{-1} μg.
† Amount of biotin released from avidin subtracted from amount of biotin released from biotin-avidin complex.

was purchased from Nutritional Biochemicals Co., (N.B.C.), Cleveland, Ohio, and from General Biochemicals, (G.B.), Chagrin Falls, Ohio. The avidin preparations were dissolved in distilled water (1 unit/ml), sterilized by filtration through a Millipore (pore size: 0.45 μ), and kept in a freezer. Microbiological assay for biotin was performed with *Lactobacillus arabinosus* 17-5 according to the method of Wright and Skeggs(3), except that growth was determined turbidimetrically by use of a Klett-Summerson photoelectric colorimeter.

Known amounts of avidin and d-biotin were added to 200 ml of sterile reaction menstruum contained in a 500 ml Erlenmeyer flask which was then incubated at 37°C on a shaker (160 rpm). An aliquot of the reaction mixture was removed at various intervals of incubation time and boiled for 60 minutes to release biotin which was assayed microbiologically. The medium employed for the growth of *E. coli* in studies of biotin synthesis(4) had the following composition: 1 g each of KH_2PO_4, K_2HPO_4 and NaCl; $MgSO_4 \cdot 7H_2O$, 0.7 g; $(NH_4)_2SO_4$, 4 g; sodium citrate, 0.5 g; glucose, 5 g; pH, 6.8. Sterile glucose was added aseptically after the medium was autoclaved. This medium was used in these studies and is identified as basal medium.

Results. To determine the conditions necessary for maximum release of biotin from a biotin-avidin complex, avidin was allowed to react with d-biotin in aqueous solution for 30 minutes after which time the reaction mixture was boiled for various periods. As a control, a solution of avidin was boiled and assayed for biotin. No biotin activity was found in the unboiled samples and essentially complete recovery of biotin was obtained by boiling the biotin-avidin complex for 60 minutes (Table I). In agreement with other reports (5), the avidin preparation itself was found to contain a significant amount of biotin (corresponding to about 18% of its binding capacity) which was released into an assayable form by boiling.

That the conditions found optimal for release of biotin from avidin-biotin complexes formed in distilled water were not adequate for a complex which was formed in a reaction menstruum such as the basal bacteriological medium is shown in Table II. Only 58.4% of added biotin was recovered at zero time and recovery was even less (35.6%) when the mixture was incubated for 2 hours, suggesting that the dissociation of the avidin-biotin complex was interfered by some ingredient(s) of the basal medium. The poor recovery of biotin from avidin-biotin complexes formed in basal media was observed using two commercial preparations of avidin.

Attention was directed next to the effect of each ingredient of the basal medium on release of biotin by subsequent heat treatment of biotin-avidin complexes. A solution of each component was prepared in the same concentration as in the basal medium. The recovery of biotin from avidin-biotin complexes formed in such solutions revealed that release of the vitamin was affected by the ionic strength of the solutions in which biotin and avidin were allowed to combine (Table III). For example, in the ammonium sulfate solution which had the highest ionic strength, recovery of biotin was the lowest, whereas in the glucose solution, which is non-ionizable, recovery was closest to that found in distilled water.

If the effect of these ions on the subsequent liberation of biotin from avidin-biotin complexes was due to physical or chemical alteration of the protein structure, the presence of these ions might result in a loss of activity of biotin. To investigate this aspect, avidin or avidin plus biotin was incubated in the

TABLE II. Release of Biotin from Biotin-Avidin Complexes Formed in Basal Medium.

Incubation time (hr)	Biotin recovered after addition to medium of: Biotin Amt	Biotin % recovery	Avidin Amt	Avidin % recovery*	Biotin + avidin Amt	Biotin + avidin % recovery†
0	12.3‡	102	.98‡	53.9	7.66‡	58.4
2	12.4	103	.8	43.2	4.86	35.6
4	12.4	103	.8	43.2	5.8	42.1

12×10^{-1} μg of d-biotin and 1 unit of avidin (N.B.C.) were used.

* Total amount of biotin contained in 1 unit of avidin was assumed to be 1.85×10^{-1} μg (Table I).

† Unboiled samples of this mixture had residual biotin activities of 1.07, 0.58, and 0.48 $\times 10^{-1}$ μg at 0, 2, and 4 hours of incubation respectively. % recovery was calculated as:

$$\frac{\text{biotin released} \times 100}{\text{biotin added} + \text{biotin contained in avidin} - \text{residual biotin}}$$

‡ $\times 10^{-1}$ μg.

TABLE III. Release of Biotin from Biotin-Avidin Complexes Formed in Various Reaction Menstruums.

Reaction menstruum	Concentration (g/l)	Ionic strength	Avidin added (unit)	Biotin released ($\times 10^{-1}$ μg)	Recovery (%)
KH_2PO_4 and K_2HPO_4	1.0 each	.0246	1	4.22	35.6
NaCl	1.0	.0171	1	6.03	50.9
$MgSO_4 \cdot 7H_2O$	.7	.0114	1	5.28	44.6
$(NH_4)_2SO_4$	4.0	.0906	1	1.8	15.2
Glucose	5.0	—	1	9.65	81.4
Sod citrate ($2H_2O$)	.5	.0102	1	6.18	52.1
H_2O	—	—	1	11.2	94.5
H_2O	—	—	0	10.1	101.0

1μg of biotin added to each solution. Incubation time: 2 hr. The avidin preparation (N.B.C.) contained 1.85×10^{-1} μg of biotin/unit.

basal medium at 37°C for 30 minutes and the residual activities of avidin tested in the presence of appreciable amounts of free biotin. As a control, distilled water was used as the incubation menstruum. No decrease in the activity of avidin was found during the incubation period when avidin and biotin were added simultaneously to either reaction menstruum (Table IV). However, when avidin alone was incubated in the basal medium it was inactivated rapidly; no activity was lost during incubation in distilled water. These results suggest that the affinity of the avidin molecule for biotin is so great and so specific that the presence of the ions did not affect the activity of avidin when biotin was present simultaneously in the menstruum, although subsequent liberation of biotin by heat treatment would be significantly inhibited (Table III).

Discussion. The findings reported here show clearly that the liberation of biotin from avidin-biotin complexes is influenced by the presence of ions in the reaction menstruum in which the complex is allowed to form. The same ions present in the glucose mineral salts medium which have detrimental effect on the activity of free avidin are, surprisingly, rather protective for avidin-biotin complexes against subsequent liberation of biotin upon heat treatment. Although the instability of avidin in dilute solutions at elevated temperature was investigated in some detail(6,7), and the sensitivity of avidin to trace metals(7) and the protective effect of biotin on denaturation of avidin(6,8) were previously reported, no information is available as to the stabilization of avidin-biotin binding by such ions. The stabilizing effect of biotin for avidin was explained by the hypothesis that the binding site of avidin would have the structure in a protein most susceptible to hydrolysis, and that the site would be stabilized by its firm combination

TABLE IV. Effect of Preincubation in Basal Medium on the Activity of Avidin.

Absence or presence of biotin during preincubation	Incubation menstruum	Activity of avidin ($\times 10^{-4}$ units) Avidin (NBC) Incubation (hr) 0	Avidin (NBC) Incubation (hr) 2	Avidin (GB) Incubation (hr) 0	Avidin (GB) Incubation (hr) 2
Absent	Distilled water	4.5	5.0	6.0	6.2
	Basal medium	2.2	1.5	2.2	1.5
Present	Distilled water	4.9	4.8	5.5	5.5
	Basal medium	4.9	4.8	5.5	5.5

Avidin (approximately 5×10^{-4} unit) was incubated with or without biotin (5.5×10^{-4} μg) in each steril menstruum.

with biotin(8). These ions, however, do not seem to act directly on the biotin binding site of avidin to cause the irreversible loss of activity, since avidin-biotin combination is not affected by the presence of the ions if the avidin and biotin are added simultaneously. The avidin-biotin complex thus formed is much more resistant to heat than that formed in the absence of the ions. The ionic agents might exert their effects by non-specifically upsetting the delicate charge balance of the protein resulting in an alteration of the protein structure so that the biotin binding sites of avidin become less susceptible to hydrolytic agents.

In view of the fact that all biological materials contain various ions which are capable of inhibiting the subsequent liberation of biotin from avidin, the prospect for the use of avidin as a tool in the purification of biotin appears to be discouraging. This is complicated further by the fact that commercial preparations of avidin contain significant amounts of biotin which will affect both the quantitative and qualitative recovery of the vitamin.

Summary. Data were presented on the quantitative release of biotin from avidin-biotin complexes formed under several conditions. The presence of various ions was found to interfere with the binding and release of biotin.

It is a pleasure to thank Miss Toby Kabakoff for excellent technical assistance in this work.

1. Ochoa, S., Kaziro, Y., *Fed. Proc.*, 1961, v20, 982.
2. Eakin, R. E., Snell, E. E., Williams, R. J., *J. Biol. Chem.*, 1941, v140, 535.
3. Wright, L. D., Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1944, v56, 95.
4. Pai, C. H., Lichstein, H. C., *Biochem. Biophys. Acta*, 1962, v65, 159.
5. György, P., Rose, C. S., *Science*, 1941, v94, 261.
6. György, P., Rose, C. S., Tomarelli, R., *J. Biol. Chem.*, 1942, v144, 169.
7. Fraenkel-Conrat, H., Snell, N. S., Ducay, E. D., *Arch. Biochem. Biophys.*, 1952, v39, 80.
8. ———, *ibid.*, 1952, v39, 97.

Received January 14, 1964. P.S.E.B.M., 1964, v116.

Progressive Descending Influenza Infection in Mice Determined by Immunofluorescence. (29201)

D. P. NAYAK, G. W. KELLEY, G. A. YOUNG, AND N. R. UNDERDAHL*
Department of Veterinary Science, University of Nebraska, Lincoln

Hers(1) reviewed studies of influenza virus by immunofluorescence (IF) in tissue cultures and embryonating eggs and indicated that few studies have been made on pathogenesis of influenza by IF in adapted or natural hosts. Liu(2) studied human influenza

* Published with approval of the director as paper no. 1474 Journal Series, Nebraska Agri. Exp. Station. This investigation was supported by U.S.P.H.S. grant.