THE ANTIBACTERIAL ACTIVITY OF THE EGG WHITE PROTEIN CONALBUMIN

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Conalbumin is an egg white protein that inhibits growth of microorganisms by forming a pink-colored, remarkably stable complex with iron. Although the antibacterial activity of egg white has long been known, the existence of such an iron binding agent was not recognized prior to the report of Schade and Caroline in 1944. In 1946 conalbumin was identified as the agent responsible for the antibacterial activity due to iron binding and for the pink discoloration of egg white containing iron (Schaible and Bandemer, 1946; Alderton, Ward, and Fevold, 1946). Warner and Weber (1951) have recently reported the preparation of conalbumin in crystalline form. Interest in conalbumin has been stimulated by its close similarity to siderophilin, the β_1-metal-binding globulin from mammalian plasma (Fiala and Burk, 1949; Schade, Reinhart, and Levy, 1949).

Recent studies at this laboratory concerned the chemical groupings in conalbumin responsible for its iron binding properties and the mechanisms by which a test organism, *Micrococcus pyogenes* var. *albus*, is capable of delayed growth in the presence of inhibitory amounts of conalbumin (Fraenkel-Conrat and Feeney, 1950). It was postulated that the organism may overcome inhibition by utilizing the free iron in equilibrium with the iron-conalbumin complex and not by destroying the protein or by its own adaptation to an iron deficiency. These studies have since been extended and one phase has been recently reported (Feeney, 1951). In this recently reported phase it was found that inhibition by conalbumin could be largely prevented by the addition of 8-hydroxyquinoline and that this prevention was intimately related to the iron and cobalt contents of the medium.

The present report presents results of the main phase of the extended studies and describes efforts to further characterize the mechanism of action of this protein on several organisms. The investigations include studies on the influence of variations in cultural conditions and comparisons with several nonprotein metal-ion complexing agents and the iron-complexing, synthetic, protein derivative, hydroxylamido ovomucoid (Fraenkel-Conrat, 1950).

METHODS

Cultures. The culture of *Micrococcus pyogenes* var. *albus* was supplied by A. L. Schade from the collection of the Overly Research Foundation and was the one employed in our recent investigations (Fraenkel-Conrat and Feeney, 1950; Feeney, 1951). *Bacillus subtilis* was the strain employed for subtilin production

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(Feeney and Garibaldi, 1948), ATCC 6633. *M. conglomeratus* was the strain *MY* used for antibiotic assay of subtilin. *M. lysodeikticus* was the strain routinely employed in this laboratory for the bacteriolytic assay of lysozyme (Alderton and Fevold, 1946). The four lactic acid bacteria were: *Lactobacillus casei E*, strain ATCC 7469; *Leuconostoc mesenteroides*, strain ATCC 8042; *L. arabinosus*, strain ATCC 8014; and *Streptococcus faecalis*, strain ATCC 8043. The *Pseudomonas fluorescens* culture was supplied by F. W. Lorenz of the University of California and was a provisionally identified isolate from spoiled (sour) eggs. The unidentified gram negative rod was isolated from spoiled eggs. It produced no pigment, grew on the Sy medium described below, and grew well between 15 and 25 C but not at 37 C. The strains of *Proteus vulgaris* and *P. aeruginosa* were stock cultures of this laboratory.

Unless otherwise noted, results reported were obtained with *M. pyogenes* var. *albus* as the test organism.

Media. Two media were employed for the majority of these studies. Medium PI, identical to the medium employed in the first report of this series (Fraenkel-Conrat and Feeney, 1950), and employed for certain of the earlier parts of this study, consisted of 0.40 per cent bacto-peptone (Difco) and 0.12 per cent bacto-beef extract (Difco) adjusted to a pH of 7.6. Medium PII consisted of medium PI supplemented with 0.50 per cent disodium phosphate and 0.03 per cent citric acid. It was identical to the medium employed in the recently reported phase of these investigations (Feeney, 1951), and the same samples of ingredients were employed for its preparation. Chemical analyses showed that the medium as diluted in the tests contained 3.6 μM iron, 0.27 μM copper, and approximately 0.17 μM cobalt. As will be noted below, the addition of either the citrate or phosphate to medium PI or both (to give medium PII) did not influence significantly the results obtained at pH 7.6. Unless otherwise noted medium PII was employed for all studies.

The synthetic medium, Sy, employed for some of the studies with the gram negative rods, had the following composition: asparagine monohydrate, 0.10 per cent; Na_2SO_4, 0.02 per cent; Na_2HPO_4, 0.05 per cent; and a salt mixture. This salt mixture consisted of the chlorides of the following cations in amounts to give the concentrations in the medium as indicated in parts per million: K, 40; Mg, 10; Mn, 1.0; Zn, 1.0; Co, 0.10; and Cu, 0.10. The salt mixture was sterilized separately and added after sterilization.

Proteins and reagents. The conalbumin was similar to preparations employed in other recent studies at this laboratory (Fraenkel-Conrat and Feeney, 1950; Feeney, 1951). In calculating the molar concentrations employed, the older value of 87,000 for the molecular weight (Bain and Deutsch, 1948) was employed rather than the newer value of 76,000 for crystalline material (Warner and Weber, 1951). One gram mole of these preparations bound 1.9 to 2.0 gram atoms of iron as measured by chemical binding tests (Fraenkel-Conrat and Feeney, 1950). Analyses for residual iron binding activities in cultures containing conalbumin were performed as follows: the cultures were pooled and centrifuged and the cells washed with water. The supernatants and washings were combined,

acidified to pH 4.5, and dialyzed against several changes of 0.01 M citric acid. After the dialysis residues were concentrated by evaporation, iron binding activities were determined by the chemical method.

The hydroxylamido ovomucoid was prepared by Heinz Fraenkel-Conrat by his method (Fraenkel-Conrat, 1950). In the absence of a known molecular weight, the molecular weight of ovomucoid, 29,000 (Lineweaver and Murray, 1947), was employed. This derivative will usually be referred to as HAO for convenience.

The conalbumin, and for certain experiments the HAO, was sterilized by filtration through Seitz filters and added to the medium after sterilization. Total nitrogen or dry weights were always determined on the sterile filtrates to correct for the appreciable losses encountered from filtration. HAO was not usually sterilized separately. It was added to the medium before sterilization unless otherwise indicated.

All salts and reagents were of reagent grade with the exception of the hydroxylamine hydrochloride, which was practical grade.

General bacteriological techniques. The general bacteriological procedures were as previously described (Fraenkel-Conrat and Feeney, 1950). Growth tests were conducted in 5 ml volumes of media. Cultures and inocula were grown at 37 C, with the exception of the culture of *P. fluorescens* and the unidentified gram negative rod which were grown at 25 C. Incubation times were those indicated or the approximate minima necessary to obtain maximum growth in the control tubes containing no inhibitor. Inocula on the peptone media were incubated for 18 to 24 hours; on the Sy medium, for 40 to 48 hours. These were prepared for use by diluting 1 to 100 or 1 to 10,000 in saline and 0.1 ml of the saline dilution was employed per 5 ml of test medium. Tests performed with 1 to 100-fold dilutions are so indicated.

The dialysis tubes employed for the studies of interrelationships when conalbumin and organisms were separated by a membrane were prepared and employed as recently described (Feeney, 1951).

All experiments were performed in duplicate or triplicate. Growth was measured by turbidity measurements on the Klett-Summerson photoelectric colorimeter (Feeney, 1951) or by visual estimates of relative growth. The visual estimates were employed for most all experiments with *B. subtilis* and *M. lysodeikticus* and with all organisms in experiments employing the dialysis tubes. They also were employed frequently for experiments in which the differences in growth between tubes were large. Where the differences were small, turbidity measurements were usually made.

RESULTS

Inhibition of growth by conalbumin. When conalbumin was added to media in stoichiometric excess of the iron found in the media by chemical analysis, inhibitions of growth of most organisms studied were observed. Inhibition was evidenced with most organisms by an increase in the lag period and by decreased rates of growth after growth had finally begun. With some organisms, complete

inhibition during the periods observed occurred with concentrations of conalbumin only slightly in stoichiometric excess of the iron, while with others only a slight increase in the lag period occurred even with concentrations stoichiometrically 10 to 50 times the iron in the media. In addition, growth usually was less diffuse in the presence of excess conalbumin than in its absence. This effect was easily observed in the earlier stages of the growth of *M. pyogenes* var. *albus*. In the later stages, excess conalbumin frequently caused autolysis to occur more readily.

The concentration of conalbumin theoretically necessary to complex with the iron in media PI and PII was 1.8 to 1.9 μM, as calculated on a total iron content of 3.6 to 3.8 μM in these media (Feeney, 1951) and on the basis of one molecule of conalbumin complexing with 2 atoms of iron (Fraenkel-Conrat and Feeney, 1950). However, under the more optimum conditions for inhibition and with the more sensitive organisms, partial inhibition was obtained with concentrations of conalbumin slightly less than this amount, or 1.4 to 1.6 μM. No attempts were made to differentiate by analytical procedures between the total amount of iron in a medium and the available iron (that possibly lesser amount available to the organisms and possibly also available for complex formation with the conalbumin).

The results of one comparative experiment with *M. pyogenes* var. *albus* and *P. aeruginosa* are presented graphically in figure 1. A difference in response of these organisms is evident. Several typical experiments with other organisms studied are presented in table 1. The sensitivity of *M. lysodeikticus* evident in table 1 was found in 7 other experiments with this organism. The 4 gram negative rods were all inhibited but, even at high concentrations of conalbumin, complete inhibition was not obtained with these organisms. For example, in an experiment with *P. aeruginosa* visible growth was evident at 48 hours of incubation with a conalbumin concentration of 46 μM, a concentration approximately 20-fold the concentration of iron. Unsatisfactory results were obtained in several experiments with the lactic acid organisms. Growth on medium PII was very slight with *L. arabinosus* and poor with *L. casei E* and *L. mesenteroides* within 48 hours. *S. faecalis* gave moderate growth as early as 16 hours of incubation, and no inhibition was evident with 11.6 μM conalbumin.

The effect of conalbumin on a simple and iron low medium was studied with 3 gram negative rods. The iron requirements of these organisms have been extensively studied, and they grow well on simple media (Waring and Werkman, 1943). The results of one experiment with *P. fluorescens* on medium Sy are presented graphically in figure 2. As little as 0.023 μM conalbumin gave a definite inhibitory effect, but approximately 50 times as much (1.2 μM) still allowed for delayed growth. Results similar to those of figure 2 were obtained in 6 other experiments with *P. aeruginosa* and *P. fluorescens* on medium Sy. In one experiment with the unidentified gram negative rod, this organism was inhibited by 0.04 μM conalbumin in a similar fashion as was *P. fluorescens*.

Observations on the production of the blue-green pigment by *P. aeruginosa* and the fluorescent pigment by *P. fluorescens* were made throughout these

studies. After 24 to 48 hours of incubation neither of these organisms produced significant amounts of pigment on media PI or PII. However, the addition of

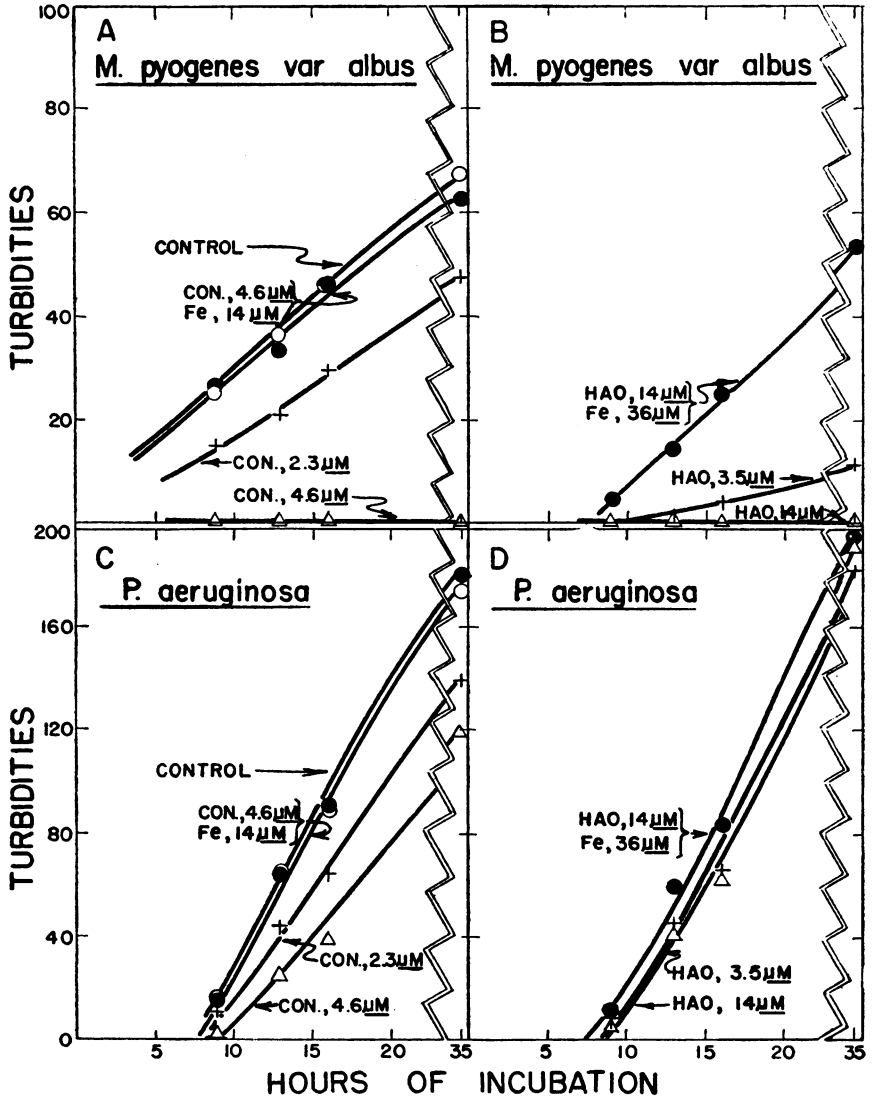

Figure 1. Inhibition of growth of *Micrococcus pyogenes* var. *albus* and *Pseudomonas aeruginosa* by conalbumin and hydroxylamido ovomucoid. Symbols CON and HAO refer to conalbumin and hydroxylamido ovomucoid, respectively. Controls were the same for A and B and for C and D and are graphed only in A and C, respectively.

4.6 μM conalbumin greatly increased pigment production, while the further addition of 11 μM iron decreased the pigment production to that observed without conalbumin. On the synthetic medium, Sy, good pigment formation

TABLE 1

Inhibition of growth of several bacteria by conalbumin and hydroxylamido ovomucoid

EXPERIMENT	ORGANISM* STUDIED	RELATIVE GROWTH† WITH PROTEIN; ADDITIONS AND AFTER HOURS OF INCUBATION INDICATED							
		With conalbumin				With HAO‡			
		Amount added	Growth after hours			Amount added	Growth after hours		
			15	34	82		15	34	82
		$\mu M/l$				$\mu M/l$			
A	*M. lysodeikticus*	0	3+	4+	4+				
		1.4	1+	2+	4+	0.3	3+	4+	4+
		1.8	0	tr	2+	1.0	1+	3+	4+
		2.3	0	tr	tr	1.4	tr	1+	4+
		4.6	0	0	0	1.7	0	1+	3+
		4.6§	1+	2+	4+	1.7§	3+	4+	4+
			17	41	120		17	41	120
B	*M. conglomeratus*	0	2+	4+	4+				
		2.4	tr	3+	4+	1.1	0	2+	4+
		7.2	0	1+	3+	4.6	0	0	4+
		7.2§	3+	4+	4+				
			10	16	24		10	16	24
C	*B. subtilis*	0	2+	4+	4+				
		3.8	tr	1+	4+	1.7	1+	3+	4+
		7.6	0	1+	4+	7.0	tr	2+	4+
		7.6§	2+	4+	4+	7.0§	2+	4+	4+
			16	33	88		16	33	88
D	*B. subtilis*	0	4+	4+	4+				
		19	tr	1+	1+	35	0	0	tr
		19§	4+	4+	4+	35§	1+	4+	4+
	P. aeruginosa	0	4+	4+	4+				
		19	2+	4+	4+	35	1+	4+	4+
		19§	4+	4+	4+	35§	4+	4+	4+
			16	21	40				
E	*P. vulgaris*	0	2+	4+	4+				
		4.6	1+	4+	4+				
		4.6§	2+	4+	4+				

* Inocula for experiments A–D were 0.1 ml of a 1 to 100 dilution of a 24 hour broth into saline. Inocula for experiment E were 0.1 ml of a 1 to 10,000 dilution.

† Growth was estimated visually and reported as tr = trace and 1+ to 4+ = gradations from slight to maximum.

‡ Hydroxylamido ovomucoid added to media before sterilization of tubes.

§ Iron added in following amounts and where indicated: experiment A, 18 μM with both conalbumin and HAO; experiment B, 20 μM with conalbumin; experiment C, 22 μM wth conalbumin and 36 μM with HAO; experiment D, 36 μM with conalbumin and 140 μM with HAO; and experiment E, 22 μM.

occurred without adding conalbumin, but relationships similar to those observed on media PI and PII were obtained after supplementing the medium with iron. This agrees with the observed growth increments obtained by adding iron to medium Sy (figure 2).

From the above and other results it was concluded that the relative sensitivity to conalbumin of the organisms studied on media PI and PII was as follows: *M. lysodeikticus* (the most sensitive) > *M. pyogenes* var. *albus* and *M. conglomeratus* > *B. subtilis* > the 4 gram negative rods.

Inhibition of growth by hydroxylamido ovomucoid. Hydroxylamido ovomucoid (HAO) inhibited the growth of most of the organisms tested in concentrations

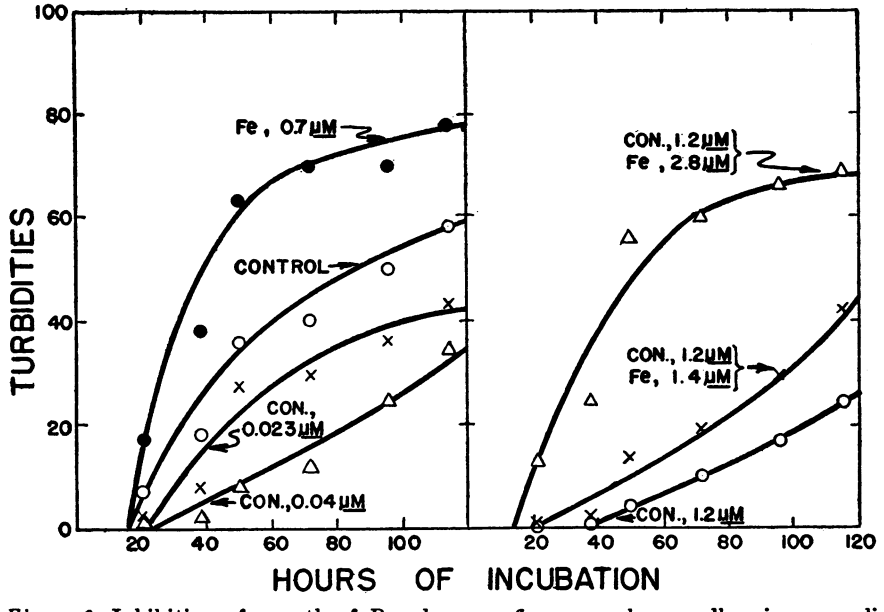

Figure 2. Inhibition of growth of *Pseudomonas fluorescens* by conalbumin on medium Sy (synthetic). Symbol CON refers to conalbumin.

roughly equivalent to those of conalbumin (figure 1 and table 1). With *M. pyogenes* var. *albus*, HAO and conalbumin were closely similar in their effects, while with *P. aeruginosa*, HAO was less effective (figure 1). Further similarities in the effects of these two iron binding proteins were noted in experiments with *M. pyogenes* var. *albus* and *M. lysodeikticus* in which mixtures of the proteins were tested. The inhibitory effects obtained were equivalent to the sums of the inhibitory effects contributed by each component. Pigment formations by *P. aeruginosa* and *P. fluorescens* were influenced by HAO in a similar manner as by conalbumin. However, at least one important difference between conalbumin and HAO was noted. HAO could be added to the medium prior to sterilization with only a small (less than 20 per cent) loss of activity occurring during sterilization. This heat stability confirms the studies on effects of heating on chemical binding (Fraenkel-Conrat, 1950).

The specificity of iron for counteracting the inhibition by HAO was investigated in only one experiment. In this experiment, 1.0 μM HAO was not inhibitory, 1.8 μM HAO was strongly inhibitory, and 1.8 μM HAO plus 2.2 μM Fe was not

TABLE 2

Effects of various metal complexing reagents in presence and absence of conalbumin

EXPERIMENT	ADDITIONS			RELATIVE GROWTHS* AFTER HOURS OF INCUBATION INDICATED		
	Reagent		Conalbumin			
	Name	Amount		16	41	65
		μM/l	μM/l			
A	—	0	0	3+	4+	4+
	—	0	1.6	1+	4+	4+
	—	0	2.3	tr	3+	3+
	Hydroxylamine	14	0	3+	4+	4+
	Hydroxylamine	140	0	3+	4+	4+
	Hydroxylamine	14	1.6	1+	4+	4+
	α,α′-Dipyridyl	6.4	0	3+	4+	4+
	α,α′-Dipyridyl	64	0	3+	4+	4+
	α,α′-Dipyridyl	6.4	1.6	1+	4+	4+
	8-Hydroxyquinoline	6.9	0	0	0	0
	8-Hydroxyquinoline	6.9	1.6	3+	4+	4+
	1,10-Phenanthroline	5.5	0	3+	4+	4+
	1,10-Phenanthroline	55	0	0	0	0
	1,10-Phenanthroline†	55	0	0	0	2+
	1,10-Phenanthroline	5.5	1.6	0	2+	1+
				16	48	
B	—	0	0	3+	4+	
	—	0	1.6	2+	4+	
	1,10-Phenanthroline	5.5	0	2+	4+	
	1,10-Phenanthroline	11	0	2+	4+	
	1,10-Phenanthroline	22	0	0	tr	
	1,10-Phenanthroline‡	22	0	2+	4+	
	1,10-Phenanthroline	1.1	1.6	2+	4+	
	1,10-Phenanthroline	5.5	1.6	tr	4+	
	1,10-Phenanthroline	11	1.6	0	3+	
	1,10-Phenanthroline§	11	1.6	2+	4+	

* Test organism was *Micrococcus pyogenes* var. *albus*. Inocula were 0.1 ml of a 1 to 10,000 dilution of a 24-hour broth culture in saline. Growth was estimated visually and reported as tr = trace and 1+ to 4+ = gradations from slight to maximum.

† 101 μM iron added.

‡ 72 μM iron added.

§ 36 μM iron added.

inhibitory at 16 hours of incubation. The following ions added as salt mixtures to give the μM concentrations indicated did not counteract the inhibition given by 1.8 μM HAO: Cu 3.3, Co 3.4, Mo 2.0, Mn 3.6, and Mg 8.0.

Inhibition by several nonprotein metal-ion complexing agents. The supplementary inhibitory effects given by mixtures of conalbumin and HAO suggested that

similar effects might be obtained with mixtures of conalbumin and various other iron binding agents. The results of several experiments on this subject are given in table 2. Hydroxylamine and α,α'-dipyridyl did not inhibit growth when tested singly or affect inhibition when tested as a mixture with conalbumin. *o*-Phenanthroline (1,10-phenanthroline) and oxine (8-hydroxyquinoline) both inhibited growth when tested singly and materially influenced growth when tested as mixtures with conalbumin. The effects of these latter two substances when tested as mixtures with conalbumin were, however, the opposite. *o*-Phenanthroline supplemented the inhibitory action of conalbumin while oxine appeared to counteract, or reverse, the inhibitory action of conalbumin. This one result with oxine is included here for illustrative purposes only since details of the studies with oxine and interrelationships with cobalt have been recently described (Feeney, 1951). Extensive tests showed that the relative order of sensitivities of the organisms of this study to *o*-phenanthroline was similar to their order of sensitivities to conalbumin. However, the inhibitory effects of high levels of *o*-phenanthroline were only partially prevented by the addition of iron, while the effects of high levels of conalbumin were completely prevented by the addition of iron.

Low concentrations of citrate (<0.05 per cent) did not significantly affect inhibition by conalbumin at a pH of the medium of 7.6, but citrate caused a slight decrease in the inhibition at pH values above 8.0.

Effect of pH of the medium. It has been previously noted that the chromogenic activity of conalbumin is slightly greater at pH values above 7.6 (Fraenkel-Conrat and Feeney, 1950). This indication of a greater stability of the iron-conalbumin complex at higher pH values has been substantiated further by chemical-equilibrium studies on mixtures of conalbumin, iron, and another iron binding agent such as *o*-phenanthroline or ethylenediamine tetraacetate at pH values of 7, 8, 9, and 10. Such slightly greater stabilities of the iron-conalbumin complex at higher pH values should cause increased growth inhibitory activities at higher pH values of media, particularly if growth occurs by utilization of the free iron in equilibrium with the complex. This was shown with 1.4 and 2.3 μM conalbumin in experiments in which the pH of the medium was adjusted to 7.3, 7.7, 8.0, and 8.3. At both concentrations the degrees of inhibition obtained were greater at each successively higher pH. Results at pH 7.3 and 8.3 are given in figure 3. Similar effects were obtained when the pH of the medium was adjusted before and after sterilization and in the presence or absence of citrate and phosphate. Although citrate caused a slight decrease in inhibitory activity at higher pH values, this decrease was much less than the increase in activity caused by increasing the pH. Similar results, but to varying degrees, were obtained with 3 other organisms tested: *P. aeruginosa*, *B. subtilis*, and *M. lysodeikticus*.

This greater activity of conalbumin at higher pH values also suggested that the greater activity might be related to the bicarbonate-carbonate systems in the medium since some investigators have postulated that carbon dioxide and oxygen are required for the formation of the iron-conalbumin complex

(Schade, Reinhart, and Levy, 1949). However, addition (after sterilization) of 23 or 230 μM bicarbonate to the medium did not influence the inhibition given by 1.6 or 2.3 μM conalbumin at pH 7.6. Medium PI and an inoculum diluted 1 to 100 were employed.

Effects of various other additions to medium. Of a number of nutrilites tested[2] only riboflavin influenced inhibition by conalbumin, and this influence was to increase the inhibition (table 3). Concentrations of 2 μg per ml (5.3 μM) gave a pronounced effect, and higher concentrations up to 20 μg per ml were progressively, although slightly, more effective. Similar results were obtained when HAO was substituted for the conalbumin. That this effect of riboflavin was not caused by a nutritional imbalance, at least one involving the other nutrilites studied,[2] was shown by experiments in which the nutrilites were added together

TABLE 3

Effects of riboflavin and sucrose on inhibition of growth by conalbumin

ADDITIONS*	RELATIVE GROWTHS† AT pH AND AFTER HOURS OF INCUBATION INDICATED					
	pH 7.6			pH 8.1		
	18	41	65	18	41	65
None	4+	4+	4+	3+	4+	4+
Conalbumin	tr	4+	4+	0	1+	2+
Riboflavin	4+	4+	4+	4+	4+	4+
Conalbumin plus riboflavin	0	1+	1+	0	0	0
Sucrose	>4+	>4+	>4+	>4+	>4+	>4+
Conalbumin plus sucrose	>4+	>4+	>4+	0	3+	>4+
Riboflavin plus sucrose	>4+	>4+	>4+	>4+	>4+	>4+
Conalbumin plus sucrose plus riboflavin	0	>4+	>4+	0	4+	>4+

* Concentrations of conalbumin, riboflavin, and sucrose where added were, respectively: 3.5 μM, 10 μg/ml (26.5 μM), and 4 mg/ml.

† Test organism was *Micrococcus pyogenes* var. *albus*. Inocula were 0.1 ml of 24-hour broth culture diluted 1 to 100 in saline. Growth was estimated visually and reported as tr = trace and 1+ to 4+ = gradations from slight to maximum.

with varying mixtures of riboflavin and conalbumin. Similar inhibitory effects were obtained in the presence and absence of the other nutrilites. A preparation of the dinucleotide[3] of riboflavin was tested at a concentration of 10 μg per ml with 2.3 μM conalbumin. An effect was obtained, but it was less than that given by 4 μg per ml riboflavin itself.

The effects given by riboflavin suggested that changing the redox potential

[2] Addition of the following mixture of nutrilites in the amounts indicated, expressed as μg per ml, did not influence inhibition by 1.8 μM conalbumin (on medium PI and with an inoculum of 1 to 100): thiamin 20, calcium pantothenate 20, pyridoxine 10, pyridoxamine 10, pyridoxal 4, nicotinic acid 10, nicotinamide 10, *para*-aminobenzoic acid 1.0, folic acid 0.1, biotin 0.1, ascorbic acid 1,000, and glutamine 5.0.

[3] The preparation of dinucleotide was kindly supplied by Dr. Henry A. Lardy and was stated to contain 40 per cent flavin adenine dinucleotide and approximately 1.0 per cent riboflavin.

of the medium might influence inhibition by conalbumin. However, negative, uninterpretable, or irrelevant results were obtained in extensive studies of the inhibition by conalbumin in shallow layer cultures, in anaerobic jars, and with media to which was added sodium thioglycolate, methylene blue, or ascorbic acid. These studies were performed in medium PI and with an inoculum diluted 1 to 100.

The effects given by riboflavin also suggested an important significance to the earlier observation of Bain and Deutsch (1948) that conalbumin is a flavoprotein. However, a nonspecific action was indicated by the report of Albert (1950) that riboflavin may form a complex with iron. The fact that the

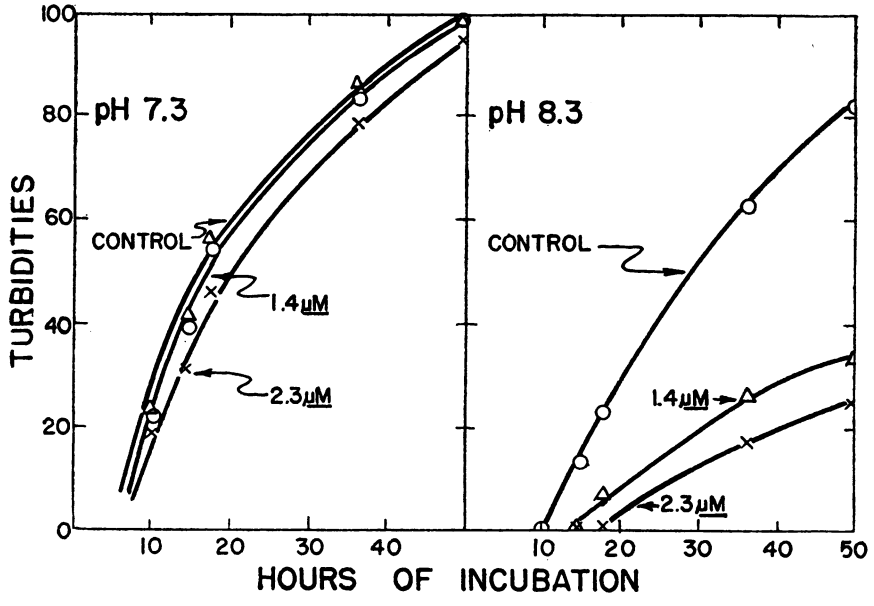

Figure 3. Effect of pH of medium on inhibition of growth of *Micrococcus pyogenes* var. *albus* by conalbumin. Concentrations indicated are for added conalbumin.

dinucleotide of riboflavin was less active than riboflavin itself also indicated a nonspecificity. The effects of riboflavin, therefore, were considered as supplementary iron bindings.

In contrast to the increases in inhibitory activity obtained by increasing the alkalinity of the medium or addition of riboflavin were the effects obtained by adding sucrose to the medium. Sucrose decreased the inhibition by conalbumin. This effect was evident at pH 7.6 and 8.3 and in the presence or absence of riboflavin (table 3). It was not due to contamination with iron as was shown by chemical analyses for iron and by observations of the inhibitory effects given by different levels of conalbumin.

Recovery of conalbumin activity from cultures. The observation that growth of *M. pyogenes* var. *albus* in the presence of inhibitory amounts of conalbumin occurs before any significant loss of iron binding activity occurs (Fraenkel-

Conrat and Feeney, 1950) has now been extended to include *B. subtilis* and *P. aeruginosa*. In one of several experiments 19.6 μM conalbumin was added to replicate tubes. Four of these tubes were inoculated with *B. subtilis* and two with *P. fluorescens*. After incubation for 14 hours each set was pooled. Klett readings of cell turbidities were 12 for the *B. subtilis* cultures and 21 for the *P. fluorescens* cultures. Chemical determinations for iron binding activities showed that the amounts of activities remaining were equivalent to a conalbumin concentration of 17.7 μM for the *B. subtilis* cultures and 19.5 μM for the *P. fluorescens*, or recoveries, respectively, of 90 and 99 per cent. Thus, growth was initiated when the cultures contained approximately 9 to 10-fold the concentration of conalbumin theoretically required to bind the iron in the medium. In other experiments, in which the cultures were incubated for prolonged periods or rapid growth was obtained by adding iron, recoveries of 58 to 90 per cent were obtained.

Effects of separating conalbumin and bacteria by a dialysis membrane. These experiments were performed with the dialysis tubes and by the procedures recently described (Feeney, 1951). As previously found with *M. pyogenes* var. *albus*, *M. lysodeikticus* was inhibited when the organisms and conalbumin were physically separated by a dialysis membrane. In three such experiments with *M. lysodeikticus*, the conalbumin, 4.6 μM as based on the total volume of medium inside and outside of bag, was added inside the bag, the organisms were inoculated outside of the bag, and the medium was PII adjusted to pH 8.1.

The effects given by riboflavin and sucrose were also studied in the dialysis tubes. It was found that when the organisms and conalbumin were physically separated, the effects of riboflavin and sucrose were more or less similar to the effects obtained under the usual cultural conditions.

Still other experiments were performed in which small amounts of conalbumin were added outside the bag in the dialysis tubes (that is, in the section with the organisms). In six such experiments, the conalbumin concentrations, as calculated on the total volume of medium, were 4.6 μM inside the bag and varied from 0.0092 to 0.92 μM outside the bag. These relatively small concentrations of conalbumin outside the bag all caused increases in the lag period as compared to controls where the conalbumin was added only inside the bag.

DISCUSSION

Our studies on the antibacterial activity of conalbumin were initiated in an effort to explain how microorganisms grow in the presence of amounts of conalbumin in stoichiometric excess of the iron in the medium. Although the mechanism by which such growth occurs has not been fully explained, the results appear to support best a simple interpretation based on utilization of iron in equilibrium with the iron-conalbumin complex. Thus, as organisms requiring iron grow and assimilate the free iron in solution, the complex would dissociate to supply a low but continually replenished level of iron. In this connection, it has long been known that under certain conditions the addition of metal-ion sequestering agents to media may be beneficial, rather than detrimental, possibly

by preventing precipitations and consequent poor availability of the metal ions. A case in point is the recent report by Jacobson (1951) that the strong complexing agent, ethylenediamine tetraacetate, can be employed for maintenance of iron supply in nutrient solutions for plant growth.

Perhaps the reason why inhibition occurs is as important as the reason why growth occurs. One explanation might be that the concentration of free iron in equilibrium with the complex is too low to satisfy definite requirements for iron. Based on this explanation, inhibition would be caused by an iron deficiency, either as a result of an inability of the organism to assimilate iron at such a low concentration or, possibly, as a result of too slow a rate of dissociation of the iron-conalbumin complex. However, the results of an associated study suggest still another explanation, one involving ionic imbalances (Feeney, 1951). Based on this latter explanation, inhibitions of growth would result from the toxic effects of metal ions such as zinc, cobalt, and copper due to the relatively low concentration of free iron in the medium.

Many closely related questions can be raised in the interpretations of these results. Of paramount importance are the essentiality of iron and the possibility of alternate metabolic pathways. That very low iron requirements may exist in extensively purified media is indicated by the results of Rubbo, Albert, and Gibson (1950), while our studies (Feeney, 1951) suggest that the possibility of metal-ion imbalances must be carefully considered in determining mineral requirements. Thus, when it is found that the addition of a particular metal ion to a given medium is necessary to obtain growth, the possibility should be considered that growth might have been originally inhibited because of a metal-ion imbalance and that the addition of the metal ion might have merely adjusted the ionic relationships to one favorable for growth. Also of importance would be further information on the mode of binding of iron by conalbumin and the antagonistic effects of 8-hydroxyquinoline and conalbumin (Feeney, 1951).[4]

Although it has been demonstrated that part of the antibacterial activity of egg white is due to its iron binding activity (Schade and Caroline, 1944), the importance of the contribution of conalbumin to the total antibacterial activity of egg white and possible interrelationships with the other recognized antibacterial proteins, lysozyme and avidin, are unknown. The conditions in egg white, however, suggest an important role for conalbumin. Both the high alkalinity, greater than pH 9.0, and very low iron content (Schaible and Bandemer, 1946) usually encountered in egg white are conducive to strong inhibitory activity. Indirect evidence is given by reports showing that certain gram negative rods, which are among the less sensitive to conalbumin, are very common contaminants of eggs (Haines, 1939). Variations in the antibacterial activity of the egg white in infertile eggs may be related to passage of iron, and other

[4] Recent studies at this laboratory have shown that when higher levels of oxine and cobalt are employed than were previously employed (Feeney, 1951), a relationship between oxine and cobalt more similar to that reported by Rubbo *et al.* (1950) was found. In these studies approximately one atom of cobalt was required per molecule of oxine, which is still twice the theoretical amount (Rubbo *et al.*, 1950).

minerals, from the yolk to the white but do not appear to be related to deteriorative changes in the conalbumin. Recent studies have shown that conalbumin is quite stable in egg white and eggs in the absence of microbial invasion and under normal storage conditions (Feeney *et al.*, 1952). More significant, perhaps, is the role conalbumin plays in variations encountered during the commercial practice of fermenting egg white prior to drying. In processes depending upon growth of the microorganisms, the inadvertent introduction of metal ions should extensively influence the type and rate of fermentation.

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SUMMARY

A study was made of the inhibitory activity of conalbumin, the iron binding egg white protein, on the growth of several organisms and on the influence of various factors on this inhibitory activity. Large differences were found in the sensitivities of different organisms to conalbumin. These differences were in part similar to those found in comparative studies with two other metal-ion complexing materials, *o*-phenanthroline and hydroxylamido ovomucoid. Possible mechanisms of action were discussed.

REFERENCES

ALBERT, A. 1950 The metal-binding properties of riboflavin. Biochem. J., **47**, xxvii.

ALDERTON, G., AND FEVOLD, H. L. 1946 Direct crystallization of lysozyme from egg white and some crystalline salts of lysozyme. J. Biol. Chem., **164**, 1–5.

ALDERTON, G., WARD, W. H., AND FEVOLD, H. L. 1946 Identification of the bacteria-inhibiting iron-binding protein of egg white as conalbumin. Arch. Biochem., **11**, 9–13.

BAIN, J. A., AND DEUTSCH, H. F. 1948 Separation and characterization of conalbumin. J. Biol. Chem., **172**, 547–555.

FEENEY, R. E. 1951 The antagonistic activities of conalbumin and 8-hydroxyquinoline (oxine). Arch. Biochem. and Biophys., **34**, 196–208.

FEENEY, R. E., AND GARIBALDI, J. A. 1948 Studies on the mineral nutrition of the subtilin-producing strain of *Bacillus subtilis*. Arch. Biochem., **17**, 447–458.

FEENEY, R. E., DUCAY, E. D., SILVA, R. B., AND MACDONNELL, L. R. 1952 Chemistry of shell egg deteriorations: the egg white proteins. Poultry Sci., **31**, 639–647.

FIALA, S., AND BURK, D. 1949 On the mode of iron binding by siderophilin, conalbumin, hydroxylamine, aspergillic acid, and other hydroxamic acids. Arch. Biochem., **20**, 172–175.

FRAENKEL-CONRAT, H. L. 1950 Comparison of the iron-binding activities of conalbumin and hydroxylamidoproteins. Arch. Biochem., **28**, 452–463.

FRAENKEL-CONRAT, H. L., AND FEENEY, R. E. 1950 The metal binding activity of conalbumin. Arch. Biochem., **29**, 101–113.

HAINES, R. B. 1939 Microbiology in the preservation of the hen's egg. Special report no. 47, Department of Scientific and Industrial Research, Great Britain. Food Investigation Board, H. M. Lord Stationery Office. 65 pp.

JACOBSON, L. 1951 Maintenance of iron supply in nutrient solutions by a single addition of ferric potassium ethylenediamine tetra-acetate. Plant Physiol., **26**, 411–413.

LINEWEAVER, H., AND MURRAY, C. W. 1947 Identification of the trypsin inhibitor of egg white with ovomucoid. J. Biol. Chem., **171**, 565–581.

RUBBO, S. D., ALBERT, A., AND GIBSON, M. I. 1950 The influence of chemical constitution on antibacterial activity. Part V: The antibacterial action of 8-hydroxyquinoline (oxine). Brit. J. Exptl. Path., **31**, 425–441.

SCHADE, A. L., AND CAROLINE, L. 1944 Raw hen egg white and the role of iron in growth inhibition of *Shigella dysenteriae*, *Staphylococcus aureus*, *Escherichia coli*, and *Saccharomyces cerevisiae*. Science, **100**, 14–15.

SCHADE, A. L., REINHART, R. W., AND LEVY, H. 1949 Carbon dioxide and oxygen in complex formation with iron and siderophilin, the iron-binding component of human plasma (1,2,3). Arch. Biochem., **20**, 170–172.

SCHAIBLE, P. J., AND BANDEMER, S. L. 1946 Composition of fresh and storage eggs from hens fed cottonseed and non-cottonseed rations. V. Cause of discoloration. Poultry Sci., **25**, 456–459.

WARING, W. S., AND WERKMAN, C. H. 1943 Iron requirements of heterotrophic bacteria. Arch. Biochem., **1**, 425–433.

WARNER, R. C., AND WEBER, I. 1951 The preparation of crystalline conalbumin. J. Biol. Chem., **191**, 173–180.