THE REQUIREMENT OF OVALBUMIN FOR THE GROWTH OF GROUP A HEMOLYTIC STREPTOCOCCUS IN A SYNTHETIC MEDIUM*

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The culture of Group A hemolytic streptococcus in defined media is a problem which has interested many workers for a number of years. In addition to complex requirements as to amino acids, vitamins, and purine-pyrimidine bases, Group A streptococci require the presence of material such as blood serum or tissue extract for vigorous growth from small inocula, capable of repeated subcultivation.

A possible indication of the chemical nature of the active principal was obtained by Woolley (1) who showed that a non-dialyzable, water-soluble material from liver extract was required for the growth of several strains of Group A hemolytic streptococci. Further investigation (2) with *Lactobacillus casei* led to the conclusion that the active principal was peptide in nature. The material was termed strepogenin. In contrast to *L. casei*, the growth of *Streptococcus hemolyticus* was not induced by a number of di- and tripeptides (3), refer to reference 4 for a recent review).

Several workers (5, 6) have employed either enzymatic or acid digests of casein as a nitrogen source for the cultivation of Group A streptococci in defined media. However, these media have not been successful in promoting luxuriant growth when continuous transfer is made with a small inoculum. In view of these facts, it is questionable whether Group A streptococci possess an absolute growth requirement for peptides or proteins.

It is the purpose of this report to demonstrate that crystalline ovalbumin, containing free sulfhydryl (SH) groups, fulfills the requirement for growth of Group A streptococci from small inocula in a synthetic medium. No other crystalline proteins or peptides have as yet been found to be active under the same conditions. The presence of C^{14}-labelled ovalbumin in the synthetic medium resulted in a distribution of the isotope between the streptococcal cells and the culture fluid.

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Methods

Preparation of Inoculum

Stock blood agar cultures were transferred to brain-heart infusion broth (7) and the latter incubated 18 hours at 37°C. 1 ml. aliquots of the culture were pipetted into small tubes, quickly frozen by means of a dry ice–cellosolve mixture and stored at −60°C. For daily use a tube of culture was thawed, and 0.4 ml. used to inoculate 10 ml. brain-heart infusion broth. The latter was incubated at 37°C. for 6 hours, centrifuged, the cells washed twice in distilled water and diluted (approximately 50 times) to give an optical density at 550 mμ of 0.05. One drop (20/ml.) was used per 10 ml. synthetic medium. This inoculum has been further diluted one thousand times and the cell mass produced in the medium to be described was 95 per cent of that obtained with the same inoculum before dilution.

TABLE I

Basal Synthetic Medium for Growth of Group A Streptococci

Quantities given are in mg./10 ml.

DL-alanine	4.0	Na_2HPO_4	67.0
L-arginine·HCl	4.0	KH_2PO_4	5.4
L-cystine	3.0	$MgSO_4 \cdot 7H_2O$	0.5
Glycine	4.0	$FeSO_4 \cdot 7H_2O$	0.05
DL-histidine	4.0	$MnCl_2 \cdot 4H_2O$	0.025
DL-isoleucine	4.0	$ZnSO_4 \cdot 7H_2O$	0.01
L-leucine	4.0	Adenine	0.05
L-lysine·HCl	4.0	Uracil	0.05
DL-methionine	4.0	Glucose	100.0
DL-phenylalanine	4.0	Glutamine	1.0
L-proline	2.0	Nicotinic acid	0.012
DL-serine	8.0	Pyridoxal·HCl	0.008
DL-threonine	4.0	Ca pantothenate	0.010
DL-tryptophane	4.0	Thiamin·HCl	0.006
L-tyrosine	4.0	Riboflavin	0.006
DL-valine	4.0		

Synthetic Culture Medium

The medium used is based on that developed by Slade *et al.*, (6, 8). Certain changes were made in the original basic formula during the present investigation. These are shown in Table I, which presents the composition of the basal synthetic medium. All substances, both organic and inorganic, have been found to be essential for maximum growth from small inocula in the presence of crystalline egg albumin. Growth occurred in the absence of either adenine or thiamine, and either Mn^{++} or Fe^{++}. However, the cell yield was approximately one-half maximum if one of these substances was lacking. For this reason these substances have been included in the basal medium.

With the exception of adenine, uracil, cystine, inorganic salts, and the vitamins shown in Table I, the medium was prepared daily from the dry state. Solutions of the other ingredients were added and the pH adjusted to 7.6 with N NaOH. The medium was tubed in 10 ml. volume in duplicate and held in free flowing steam for 10 minutes. None of the materials used in this work have been sterilized by steam pressure. All tubes were incubated at 37°C. in a water bath with appropriate controls.

Microscopic examination of the culture fluid after growth showed the presence of amor-

phous material in addition to the bacterial cells. The material was removed by washing the cells from each tube on the centrifuge with 10 ml. 0.05 M veronal (pH 8.6). The process was repeated twice and the cells then suspended in 10 ml. water. The optical density was read at 550 mμ.

The egg albumin used was a powder preparation obtained from Merck and Co., Rahway, New Jersey.

TABLE II

Serological Type and Source of Group A Streptococci Employed

Designation	Serological type	Source
B-236	1	Throat cultures, Municipal Contagious Disease Hospital, Chicago
B-62	11	
B-60	12	
B-64	26	
B-48	36	
Nass	12	Dr. C. H. Rammelkamp, Western Reserve University, Cleveland
Molnar	4	
C 440	12	Dr. A. T. Wilson, du Pont Institute, Wilmington
Blackmore	11	Dr. E. W. Todd, (deceased), London
SF 42	12	
C 203	3	Dr. A. W. Bernheimer, New York University, New York
C 203u	3	
S 43	6	Dr. R. Lancefield, The Rockefeller Institute, New York
5797	8	Dr. S. Elliott, The Rockefeller Institute, New York
T 19	19	Dr. J. Seal, Naval Training Command, Great Lakes
N 19	19	Dr. A. F. Coburn, The Rheumatic Fever Research Institute, Chicago

Cultures Used

All the organisms were members of the Lancefield Group A and were hemolytic when grown in sheep blood agar. The cultures were of the following serological types: 1, 3, 4, 6, 8, 11, 12, 19, 26, and 36. The majority of the work was done with strain N19 (type 19) obtained during World War II from a patient with scarlet fever. Table II lists the cultures and their sources.

Treatment of Proteins

Heating.—All proteins were heated in a 0.25 per cent aqueous solution (50 ml.) in free flowing steam for 10 minutes and cooled in tap water without shaking. Egg albumin solu-

tions developed a slight haze in the heating process. The optical density reading of this solution at 280 mμ after centrifuging showed a reduction of only 15 per cent. In routine use ovalbumin was steamed in the basal medium. No haze developed in this case. Crystalline bovine serum albumin was also treated in the same manner.

Irradiation.—Egg albumin (0.25 per cent in water) was irradiated for 72 hours in a 1 cm. quartz cell at a distance of 10 cm. with a Hanovia ultraviolet lamp. A slight haze developed during this treatment.

Shaking.—Egg albumin (0.25 per cent in water) was shaken under an atmosphere of nitrogen in a Mickle tissue disintegrator for 3 hours. Approximately one-half the ovalbumin (based on dry weight) was made insoluble by this treatment so the solution was centrifuged before testing in the synthetic medium.

Iodoacetate Treatment.—To a Seitz-filtered 0.25 per cent heated egg albumin solution was added iodoacetate (adjusted to pH 7) to give a final concentration of 0.015 M. This mixture was held in the ice box for 24 hours, dialyzed in running tap water several hours and overnight in distilled water. The possibility that this albumin solution was inhibitory

TABLE III

Effect of Heated Egg Albumin on the Reduction of 2,6,-Dichlorophenol Indophenol

Tube No.	Unheated egg albumin	Heated egg albumin	Optical density	Optical density
	ml.	*ml.*	660 mμ	Δ
1	1.0	—	0.355	—
2	2.0	—	0.350	—
3	3.0	—	0.355	—
4	—	1.0	0.290	0.065
5	—	2.0	0.200	0.150
6	—	3.0	0.140	0.215

to the growth of strain N19 was checked in brain-heart infusion broth. No inhibition occurred.

Aeration.—Air was forced through a sintered glass disc (coarse) into a heated egg albumin solution (0.25 per cent) at room temperature for 4 hours.

Acid Hydrolysis.—Egg albumin (500 mg.) was hydrolyzed in either 2 N or 12 N HCl in sealed vials at 121°C. for 4 hours. The vials were opened and evaporated to dryness under vacuum in a desiccator containing solid NaOH and $CaCl_2$. The contents of the vials were suspended in water and evaporated again in a similar manner. The drying process was then repeated four times on a steam bath. The material was adjusted to pH 7.5 and to 10 mg./4 ml. (based on original weight), treated with activated charcoal (darco), and filtered.

Determination of Reducing Power.—A procedure for determining reducing power has been developed based on the method of Todrick and Walker (9) in which the reduction of 2,6-dichlorophenol indophenol[1] to the colorless state was measured at 660 mμ. A 1/25 dilution of a 0.1 per cent solution of DPI was used. The procedure is as follows: 1, 2 and 3 ml. of heated and unheated 0.25 per cent solutions of protein were pipetted into several tubes. The volume was made to 3 ml. with water. 1 ml. DPI was added to each and after 10 minutes the optical density was read. A plot of the differences in optical density between the tubes containing heated and unheated protein resulted in a straight line. A typical set of values obtained with egg albumin is given in Table III.

[1] DPI, 2,6-dichlorophenol indophenol.

Preparation of Ovalbumin

Precipitation of Crystalline Ovalbumin from Egg Albumin Powder.—10 gm. crude egg albumin powder was dissolved in 200 ml. water and clarified by centrifugation. The pH was adjusted to 5.0, and solid $(NH_4)_2SO_4$ was added to give 40 per cent saturation. The precipitate was discarded and the pH adjusted to 4.7. The sulfate concentration was increased until the solution became hazy. The solution was allowed to stand 24 hours and the precipitate removed by centrifugation. This crystallization procedure was repeated twice; the crystalline precipitate was dissolved in water, dialyzed overnight, and lyophilized.

Preparation of C^{14}-Labelled Ovalbumin.—5 ml. $NaHCO_3$ (containing 1 mc. C^{14}) was injected intraperitoneally into a laying hen and 3½ hours later the injection was repeated. The animal was then held an additional 20 hours. The oviduct (35 gm.) was removed, cut into small pieces, and 100 ml. water added. The mixture was agitated in a Waring blendor for 3 minutes and the pH then adjusted from 6.2 to 7.5 by the addition of 0.1 N NaOH. The agitation was continued for another 3 minutes. The mixture was centrifuged at 20,000 *g* for 10 minutes and the supernate poured off. The precipitate was extracted 5 times with 80 ml. portions of water. The extracts were combined and the ovalbumin crystallized as given in the preceding section. At least 6 precipitations were necessary in order to obtain an ovalbumin preparation which showed the characteristic electrophoretic pattern. The final material was dialyzed for 48 hours and lyophilized. Dialysis removed the C^{14} which could be released by ninhydrin treatment (10). The presence of traces of contaminating protein cannot be ruled out; it is unlikely however that they exerted a significant effect on the results obtained. Ovalbumin prepared in this manner has been reported to be non-uniformly labelled with respect to aspartic acid and glutamic acid (11). It possessed a C^{14} content of 260 C.P.M./mg.

Determination of Ovalbumin.—Ovalbumin was estimated by the method of Sutherland *et al.* (12). Crystalline material (Armour) was used as a standard in the procedure.

Paper Electrophoresis

The apparatus used was similar to that described by Durrum (13).[2] Three inch wide strips of Whatmann 3 MM paper were run for 4 hours at room temperature in veronal buffer (pH 8.6, $\mu = 0.10$) at an initial current of 4 to 5 milliamperes. Crystalline ovalbumin (150 μg.) moved 6 to 7 cm. under these conditions. Brom-cresol green was used as staining agent.

RESULTS

Activation of Ovalbumin.—A study in this laboratory of proteins from many sources showed that under certain conditions ovalbumin was active in permitting growth of Group A streptococci. Growth was obtained when the ovalbumin was sterilized in the culture medium by heating to 100°C. for 10 minutes. In contrast, it was found that ovalbumin sterilized by filtration was completely inactive when added to the heat-sterilized basal medium. Further investigation disclosed that ovalbumin was converted from an inactive to an active state when heated. Activation occurred either in separate solution or in the basal medium. The effect is illustrated by the data in Table IV. Egg albumin powder or crystalline ovalbumin were equally effective in permitting growth. In the

[2] The authors are indebted to Dr. J. W. Hahn of this Institute for use of the apparatus constructed by him.

absence of albumin no growth occurred on extended incubation even though the number of cells in the inoculum was increased tenfold. In the presence of unheated albumin slight growth was occasionally found. This growth was equivalent to 10 per cent or less of the maximum response. Full growth could be induced in the presence of heated albumin when either the basal mixture of amino acids (Table I) was present or when this mixture was replaced with an enzymatic digest of casein.[3] The inorganic ash of ovalbumin produced no growth response. The 2 N and 12 N acid hydrolysates were also inactive. The data in Table IV illustrate as well the excellent agreement in growth response between duplicate tubes commonly found with this medium.

Growth Response.—A typical growth response curve to heated ovalbumin is given in Fig. 1. No further significant growth occurred on extended incubation.

TABLE IV

Effect of Egg Albumin on the Growth of S. hemolyticus (Strain N19)

No.	Additions to basal medium	Optical density
1	None	0, 0
2	EA, unheated	0, 0
3	EA, heated in separate solution	0.340, 0.335
4	EA, heated in basal medium	0.360, 0.370
5	EA, filtered, unheated	0, 0
6	EA, filtered, heated	0.325, 0.330
7	Crystalline EA, unheated	0, 0
8	Crystalline EA, heated	0.340, 0.350

1 mg. albumin/ml. used in each case.
EA, egg albumin.

Approximately 75 per cent of the total response was obtained in 10 hours, an indication that optimum conditions prevailed from the time of inoculation. Strain N19 was subcultured ten times in this medium without decrease in growth response. Fifteen strains representing ten different serological types among the Group A streptococci were tested. Each strain grew well except C440 (type 12), which was poorly hemolytic on sheep blood agar and also difficult to cultivate in beef heart infusion broth. In addition this strain possessed a weak constitutive arginine dihydrolase enzyme system and did not show an adaptive response to arginine as did other typical Group A streptococci (4).

It can be seen in Table III that the heat treatment as used in this study produced ovalbumin which possessed the ability to reduce DPI rapidly. Unheated albumin was not able to reduce DPI. The biological activity of the heated albumin was removed by means of: (*a*) oxidation by aeration through

[3] Bacto-casitone; pancreatic digest of casein obtained from Difco Laboratories, Detroit.

a sintered glass disc (coarse) or (*b*) exposure to an alkylating agent, iodoacetate. The activity of aerated albumin was restored by a second heating period.

Shaking ovalbumin under nitrogen resulted in a preparation which would partially replace heated albumin. Furthermore, on heating the shaken material an increase in growth of only 20 per cent was obtained, instead of the complete activation produced by heating untreated ovalbumin.

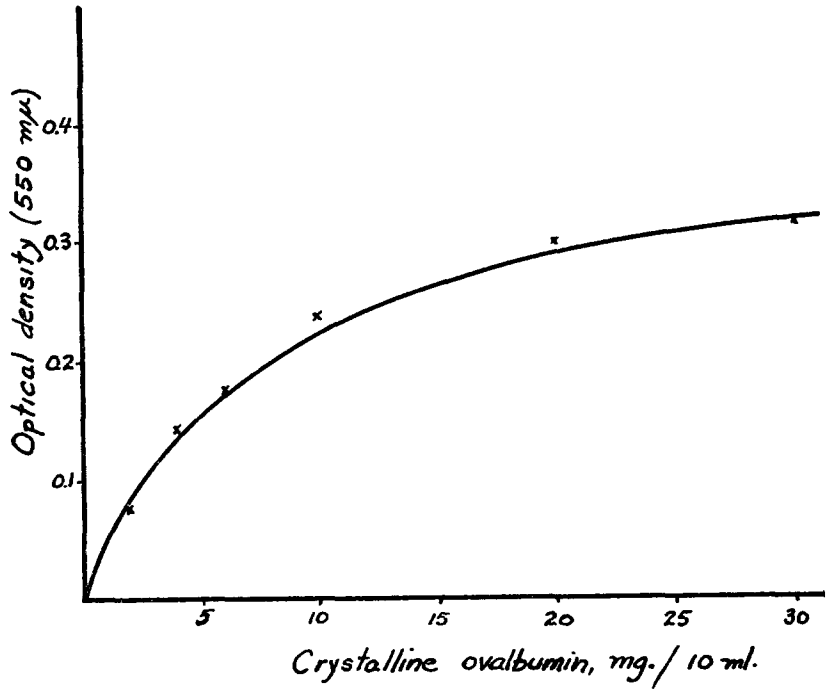

FIG. 1. The effect of crystalline ovalbumin (heated) on the growth of *S. hemolyticus* (N19).

Ultraviolet irradiation for 4 hours resulted in albumin which partially reduced DPI and also produced a partial growth response of N19. Further irradiation for 72 hours did not produce a material which would completely replace heated albumin.

Ovalbumin and SH Groups.—It is known that denatured (heated) ovalbumin contains free sulfhydryl (SH) groups (14, 15). The behavior of heated ovalbumin suggested that its activity for the streptococcus was due, in part, to a reducing effect.

However, addition of ascorbic acid, sodium bisulfite, glutathione, or cysteine to the basal synthetic medium did not result in growth. In the presence of suboptimum quantities (0.10 per cent) of ovalbumin (Fig. 2), the reducing agents glutathione, ascorbic acid, and cysteine (not shown) increased total growth whereas thioglycolate had no effect. The addition of

these compounds in the same quantities in the presence of 0.15 per cent ovalbumin had little effect.

The above results indicated that heated ovalbumin was acting as a reducing agent in the culture medium, but that it was not capable of being replaced by simple reducing agents. Thus other functions of ovalbumin were looked for.

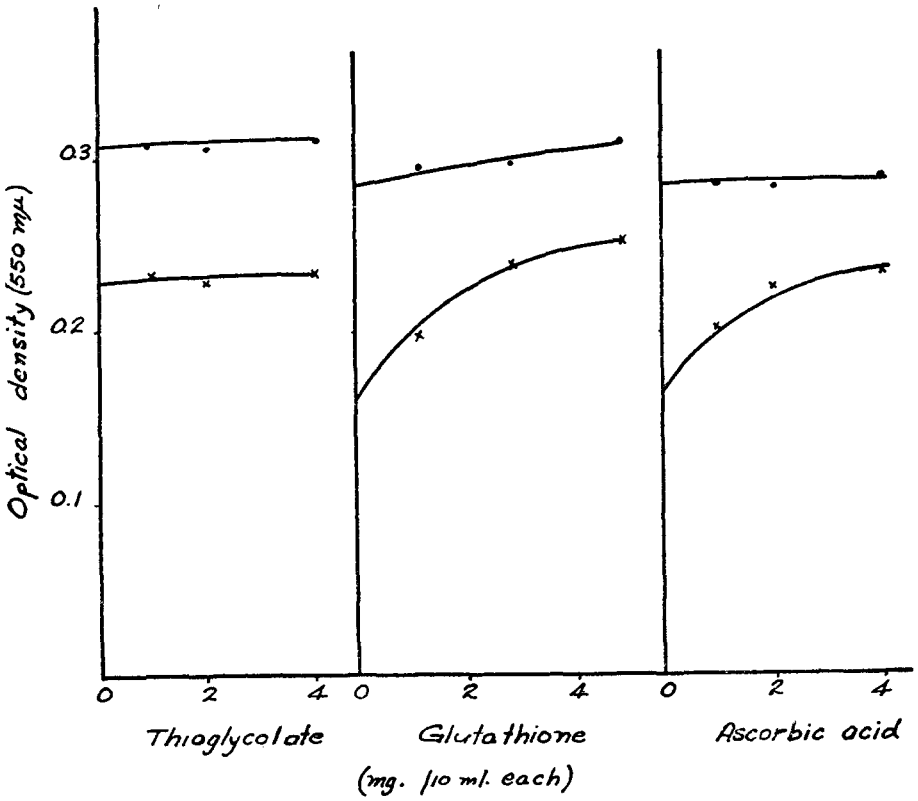

FIG. 2. The effect of reducing agents on the total growth of *S. hemolyticus* in the presence and absence of ovalbumin. Solid dots (.), 1 mg./ml. ovalbumin present in each case; crosses (x), 0.5 mg./ml. ovalbumin present in each case. No growth was obtained in the absence of ovalbumin.

The following example indicates the necessity for ovalbumin protein as well as SH groups for streptococcal growth. A commercial crystalline egg albumin was found to be inactive on heating in the synthetic culture medium. Further investigation showed that with this particular preparation, SH groups were not exposed on heating, as judged by the lack of reduction of DPI. Several other preparations from the same source behaved in a similar manner.

Consideration was also given to the possibility that ovalbumin acted as a

chelating agent and that this property was removed by the heating process. A 10-fold increase however, in the concentration of inorganic salts in the presence of unheated ovalbumin did not produce growth.

It also seemed possible that heated albumin possessed the ability to remove a growth inhibitor present in the basal medium.

Six tubes of basal medium containing 10 mg. ovalbumin/10 ml. tube were steamed and held 18 hours at 37°C. The tube contents were combined and the pH lowered to 4.7 with 5 N HCl. The precipitated ovalbumin was removed by centrifugation, the pH of the supernate raised to 7.6, and the solution sterilized by filtration. The medium was tubed in 10 ml. quantities. To the 1st pair was added 10 mg. each unheated ovalbumin, to the 2nd pair 10 mg. each heated ovalbumin, and the 3rd pair served as controls. The tubes were inoculated with N19 and incubated 18 hours at 37°C. Vigorous growth occurred in the presence of heated ovalbumin; the other two pairs were negative.

These results make it unlikely that the activity of heated albumin is due to removal of an inhibitor from the basal medium.

Activity of Other Proteins.—The following purified proteins have been tested in both the heated and unheated states: crystalline preparation of pepsin, insulin[4], trypsin, chymotrypsin[5], and ACTH[6]. In each case there was no significant growth response. Crystalline bovine serum albumin[7] (15 mg./10 ml.) produced a 20 per cent response in 17 hours which did not increase on extended incubation.

A number of crude proteins have been tested in the heated and unheated states. None of these showed any activation on heating.

No growth was obtained in the presence of rennin, gelatin, or urease; liver fraction L gave a 10 per cent response in 18 hours, no further increase was noted in 41 hours. The following peptides were found to have no effect: anserine, carnosine, salmine, DL-alanylalanine, DL-alanylglycine, glycylalanine, glyclyglycine, glycylglyclglycine, glycyl-L-leucine, glycyl-L-tryptophane, glycyl-L-tyrosine, glycylvaline, L-leucyl-L-tyrosine, DL-leucylglycine and DL-leucylglycylglycine. Strepogenin[8] (30 mg./10 ml.) produced no growth until about 18 hours, when growth began and reached an optical density of 0.10 at 24 hours. No further growth occurred. Crystalline B_{12} and coenzyme A were inactive.

Utilization of Ovalbumin.

Duplicate tubes containing 20 ml. culture medium/tube were used to follow the removal of protein from solution during growth of the culture. 20 mg. ovalbumin was present in each tube. Equal aliquots were removed from each tube at various times and combined. The cells were removed by centrifugation and the supernatant was dialyzed overnight to

[4] We are indebted to Eli Lilly and Company, Indianapolis for this material.

[5] Twice recrystallized contained 50 per cent $MgSO_4$; obtained from Worthington Chemical Co., Freehold, New Jersey.

[6] Control J 19907, Armour and Company, Chicago.

[7] Fraction VI, lot PI; obtained from Armour Laboratories, Chicago.

[8] Lot 21951, obtained through the courtesy of Dr. L. K. Wright, Sharp & Dohme, Inc., Philadelphia.

remove tyrosine and tryptophane before determination of the protein present. No glutathione was present in this experiment. Protein was determined by the biuret method (12).

A microscopic examination of the centrifuged cells (strain N19) revealed that amorphous material was present with the cells. The optical density of the suspension at optimum growth was about 0.60. The amorphous material was removed by washing the cells three times with an equal volume of 0.05 M veronal buffer (pH 8.6). The optical density of the washed cells was then 0.37. Paper electrophoresis showed that the veronal wash contained ovalbumin. The protein content of the solution was equivalent to 20 to 30 per cent of the original protein (based on ovalbumin). The culture supernatant contained 15 to 25 per cent of the original protein. Consequently, about one-half of the original protein could not be accounted for.

It has been found that the precipitation of protein could not be prevented by control of pH. The usual pH change which occurred during growth was 7.8–6.0. Precipitation began at a pH of about 7.2. The titration of uninoculated medium with HCl showed that a pH of 4.9 was necessary in order for the precipitation of ovalbumin to begin.

Fig. 3 shows clearly that the protein content of the culture fluid decreased sharply during the growth cycle. 70 per cent of the growth occurred during the period of most rapid decrease in protein content. A small increase in protein occurred between the 8th and 25th hour. The increase probably reflects synthetic activity in the culture.

The above results suggested that removal of the protein was due in part to utilization of ovalbumin by the streptococci as an essential process of growth of the culture. In order to obtain more concrete evidence on this point, it was decided to prepare ovalbumin labeled with C^{14}. The use of such ovalbumin would give information on (*a*) the incorporation of carbon from ovalbumin into the bacterial cells, and (*b*) the conversion of the carbon of ovalbumin into dialyzable and non-dialyzable forms.

C^{14} ovalbumin was steamed in the basal medium at a level of 13 to 15 mg./10 ml. The reducing ability of this albumin was equivalent to 55 per cent of that of egg albumin powder, consequently glutathione (3 mg./10 ml.) was added in order to obtain maximum growth.

The cells were separated from the culture fluid by centrifugation at the conclusion of the incubation period. The cells from 10 ml. culture fluid were then washed four times on the centrifuge with about 5 ml. 0.05 M veronal each, followed by two washings with 0.1 per cent non-isotopic crystalline ovalbumin in veronal. The cells were then made to a convenient volume and an aliquot removed for C^{14} count. The veronal wash and the ovalbumin-veronal wash were kept separate and C^{14} counts were made on each.

The culture fluid was dialyzed overnight in distilled water. The dialysate and the non-dialyzable fraction were evaporated in cellophane sacs in a stream of air at room temperature. C^{14} counts were made on each.

Two experiments are given in Table V. After growth of strain N19 (no other strain was tested) 63 to 72 per cent of the recovered C^{14} was associated with the bacterial cells. This carbon was not removed from the cells by repeated washing. The data also show that about 25 per cent of the C^{14} was present in the culture fluid after growth and that three-fourths of the isotope was in a

non-dialyzable form. A small quantity of C^{14} (about 5 per cent) was found in the wash water of the cells.

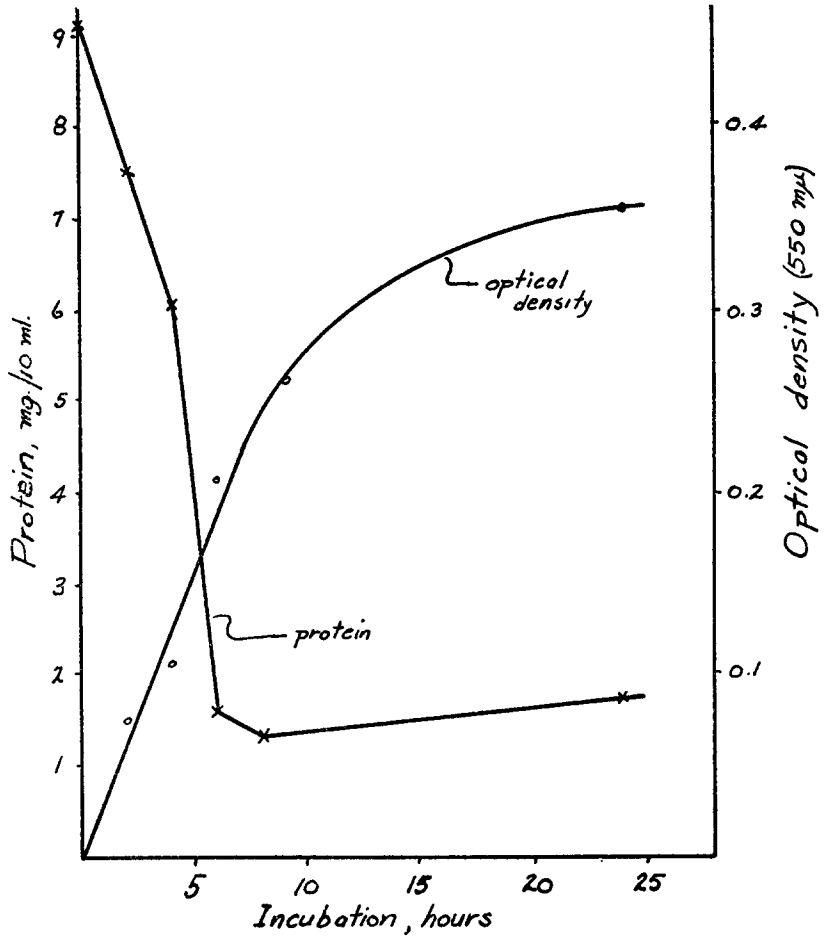

FIG. 3. The rate of growth and rate of removal of protein from the synthetic medium by *S. hemolyticus* (N19).

In view of the C^{14} present in the non-dialyzable residue of the culture fluid it was of considerable interest to determine whether the solution contained ovalbumin.

The material (Table V, Experiment 2) was concentrated approximately 15 times by pervaporation and examined by paper electrophoresis. No ovalbumin was found. Material was present however, which migrated about two-thirds of the distance travelled by crystalline ovalbumin. The material formed a color with brom-cresol green. The protein determination (12) showed that approximately 7 mg. protein (based on ovalbumin) was pres-

ent. It is apparent, in this case at least, that the biuret method of protein determination cannot be taken as an absolute value of the ovalbumin present. The method does indicate however, that protein and/or peptide is present in the non-dialyzable fraction of the culture fluid. The activity of peptides in the biuret method is based on the finding that 1 μg. glutathione is equivalent to 0.15 μg. crystalline ovalbumin.

These results indicate that the protein present in this fraction is probably composed in part, of proteins and peptides arising from the degradation of ovalbumin.

The veronal wash of the streptococcal cells (Table V, Experiment 2) was found to contain protein based on the biuret method equivalent to 15 mg.

TABLE V

Distribution of C^{14} from Ovalbumin during Growth of S. hemolyticus (N19)

Culture fraction	Experiment 1		Experiment 2	
	Total C^{14}	Recovery, per cent C^{14} in all fractions	Total C^{14}	Recovery, per cent C^{14} in all fractions
	C.P.M.		C.P.M.	
Cells	1800	63	8600	72
Culture fluid				
(*a*) dialyzable	130	5	840	7
(*b*) non-dialyzable	750	26	1950	17
Cell wash	175	6	470	4
Total C^{14} per cent recovered	85		101	

C.P.M., counts per minute.

Experiment 1 contained 3380 C.P.M. C^{14} (13 mg. ovalbumin in 10 ml.) and Experiment 2 contained 11,700 C.P.M. C^{14} (45 mg. ovalbumin in 30 ml.).

ovalbumin. The C^{14} present was only 4 per cent. This indicates that the bulk of the protein is very likely composed of non-labelled amino acids. Degradation of the C^{14} ovalbumin to yield proteins of lower molecular weight would very likely have resulted in a higher C^{14} content.

Non-Utilization of Ovalbumin by Cell Suspensions and Cell-Free Extracts.— The above results suggested that the degradation of ovalbumin may occur in cell suspensions of streptococci or in cell-free extracts.

To check this possibility, strain N19 was grown for 6 hours in the basal synthetic medium plus ovalbumin, and in brain-heart infusion broth, the cells removed from each medium by centrifugation, washed twice in water, and suspended in 0.25 per cent heated ovalbumin at pH 6.0, 6.5, 7.0, 7.5, and 8.0, and a final cell concentration of 10 per cent (wet weight). The mixtures were held 2 hours at 37°C. with frequent shaking, centrifuged, and the protein in the solution determined after dialysis overnight. There was no change in the protein content of the system when compared with the controls.

Similar experiments were performed with cell-free extracts of N19 which had been prepared by shaking with ballotini beads in a Mickle disintegrator. There was no change in the protein content of the mixtures.

Precipitin tests on culture fluid using antisera[9] to the precursor and active protease of Elliott (16) have given negative results.

DISCUSSION

It has been established by a number of workers that the SH groups of ovalbumin are "unmasked" during heat treatment (see reviews 14, 15). Shaking and irradiation produced similar results. The majority of the SH groups of the protein were exposed in the process. No other protein compares quantitatively with ovalbumin in this respect.

It seems clear that under the present conditions heating, irradiation, and shaking produced ovalbumin with free SH groups. Such albumin was required for the growth of group A streptococci. Untreated albumin was inactive. The activity of heated albumin was removed by oxidation; the activity could be restored by a second heat treatment. Treatment with an alkylating agent also removed the activity of heated albumin. Commercial crystalline ovalbumin preparations which were not able to reduce DPI did not permit growth.

The above findings indicate that the SH groups of ovalbumin play a significant role in the activity of the protein during growth of *S. hemolyticus*. Of importance is the ready availability of these groups and their presence in sufficient quantity. Similar conditions may be required for the growth of the group A streptococcus in nature.

It is also evident from the present results that the SH groups must be a part of the protein molecule in order for the latter to promote growth of the streptococcus. This is illustrated by the inability of glutathione plus untreated albumin to replace heated albumin. Furthermore, the activity of heated, oxidized albumin could not be restored by the addition of glutathione.

No other protein or peptide produced a typical growth response under the present conditions. Crystalline bovine serum albumin and strepogenin produced a 10 per cent response on extended incubation.

Earlier work (17) showed that glutathione reduced the lag period required for growth in the presence of a non-dialyzable preparation from the pancreatic digest of casein. In the present experiments the growth rate in the presence of the optimum concentration of ovalbumin was not affected by the addition of glutathione. This is most likely due in part, to an adequate supply of SH groups in a more readily available form. Of interest is the report (18) that the cystine residue of oxytocin is required for the growth of *L. casei*. All the peptides of insulin which were active under the same conditions also contained cystine. A

[9] Obtained through the courtesy of Dr. S. D. Elliott, The Rockefeller Institute for Medical Research, New York.

material which appeared to be a protein has been reported as essential for the growth of *Lactobacillus bulgaricus* (19). All crystalline proteins tested were inactive. It is not known whether heated ovalbumin was tested.

Ovalbumin was rapidly removed from the culture fluid during growth. Approximately one-half of the original protein content of the medium could not be accounted for. The results obtained with the C^{14}-labelled ovalbumin indicate that the ovalbumin was degraded into units of lower molecular weight which became associated with the streptococcal cell during growth of the culture. The results also indicate that the protein which precipitates during growth is not composed of "ovalbumin units" but rather is composed largely of non-labelled amino acids.

It should be noted that crystalline ovalbumin contains polysaccharide consisting of mannose and glucosamine, together with an unidentified nitrogenous constituent (20). The relationship of this group to the activity of ovalbumin in the present experiments is unknown.

The growth of Group A hemolytic streptococcus from small inocula has been obtained for the first time under fairly well defined conditions. Ovalbumin, although not chemically defined, can be obtained in crystalline form and is readily available. An opportunity is thus at hand to obtain definitive information on the activity of protein in the growth processes of these organisms. Further investigation is necessary before an outline of the mechanism of this action can be drawn. Work is in progress on the preparation of S^{35}-ovalbumin possessing a high specific activity. The isotopic albumin used in the present experiments was not of sufficiently high specific activity for identification of the components which contained the isotope.

SUMMARY

Crystalline ovalbumin, when heated under controlled conditions, promoted growth of *Streptococcus hemolyticus* in a synthetic medium. A similar activation of albumin also was produced by ultraviolet irradiation or by shaking under nitrogen. Untreated albumin failed to permit growth under the same conditions. The activity of treated albumin was destroyed by aeration or by reaction with iodoacetate.

The data suggest that the activity of ovalbumin for the streptococcus depends on the presence of "exposed" SH groups. However, other sulfhydryl-containing compounds (thioglycolate, glutathione, cysteine) could not replace ovalbumin.

A number of crude and crystalline proteins, and di- and tripeptides were inactive. Strepogenin and crystalline bovine serum albumin produced a weak growth response on extended incubation.

Approximately 50 per cent of the original protein in the culture disappeared during growth. Ovalbumin labelled with C^{14} was added to the synthetic me-

dium. After growth of the culture the isotope was found associated with the cells and in the culture fluid in both dialyzable and non-dialyzable forms.

Under the conditions described, luxuriant growth of 15 strains (10 serological types) of Group A streptococci was obtained in 10 hours from small inocula. The growth was capable of repeated subcultivation.

Note Added in Proof:—Several experiments in which strain N19 was cultured in the basal medium containing S^{35}-labelled ovalbumin (180,000 C.P.M/mg.) resulted in the location of 55 to 70 per cent of the isotope in the streptococcal cells. Sonic oscillation of a washed aqueous suspension of these cells released about 80 per cent of the isotope into the soluble fraction of the sonic extract. Further investigations are in progress.

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