azoproteins to mice are presented. The azoprotein granules seen intracellularly in the tissues of such animals are present predominantly in the mitochondrial fraction. Evidence that such granules are not labeled mitochondria is presented, and the significance of the variation in the results of different staining technics on mitochondrial fractions is discussed with regard to the homogeneity of these preparations.

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A Semi-Synthetic Medium for Labelling Hemolytic Streptococci with Radioactive Phosphorus.* (19165)

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Although several "synthetic" media for the cultivation of group A hemolytic streptococci, such as those of Bernheimer *et al.*(1), Christensen(2), Stock(3), Dole(4), and Slade and Knox(5), have been reported and appear to be satisfactory for the growth of these bacteria, they are not suitable for studies which require the incorporation of radioactive phosphorus in the bacterial cell. This is due to the fact that their buffer and amino acid nitrogen sources contribute an excessive amount of ordinary phosphorus which would compete with the radioactive form of the element in metabolic processes of the organism.

It is the purpose of the present paper to report the development of a medium which will support excellent growth of streptococci in the presence of a small, but optimal amount of phosphorus in the form of radioactive phosphoric acid.†

Materials and methods. Organisms. The present study was done mainly with group A hemolytic streptococci type 18 which was isolated from the throat of a patient with active rheumatic fever. In the later parts of this study, group A hemolytic streptococcus type 14, which is a mouse-virulent strain (S23/101/4),‡ was also used. The purity of these strains was checked by serological typing at various times during the progress of the experiments. *Inoculum.* A platinum loop, 3 mm in diameter, was used throughout the study. A loopful of an overnight culture of the streptococcus was inoculated into 10 ml of the semi-synthetic medium. The organisms were maintained at first in a serum broth culture. When equivalent growth was finally obtained with the semi-synthetic medium, it was always substituted so as to avoid any possible transfer of traces of substances in the serum broth which might stimulate the bacterial growth. *Criteria of growth.* The appearance of turbidity to the naked eye after cultivation at 37°C for 15 to 18 hours, and the presence of only Gram positive cocci in chains on smear were taken as evidences of growth. Simultaneously with each set of experiments with semi-synthetic media, a tube of serum broth was seeded to serve as a control for the viability of the organisms in the inoculum. *Sterilization of the medium.* After the solid constituents were dissolved, the pH of the solution was adjusted to 7.8; the final volume was made up with distilled water and the

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† Obtained from the Oak Ridge National Laboratory, A.E.C., Oak Ridge, Tenn.

‡ Kindly given by Dr. Rebecca Lancefield

TABLE I. Composition of the Proposed Medium.*

Enzyme hydrolyzed lactalbumin†	2 g‡
Glucose	250 mg
DL-tryptophane	10
L-cystine	10
Glutamine	5
Adenine sulphate $2H_2O$	1
Guanine·HCl·$2H_2O$	1
Uracil	1
Xanthine	1
Ascorbic acid	20
Biotin	5 μg
Folic acid	5
PABA	50
Riboflavin	100
Thiamin	100
Pyridoxine	100
Nicotinic acid	100
Ca pantothenate	100
$MgSO_4 \cdot 7H_2O$	20 mg
NaCl	4
$FeSO_4 \cdot 7H_2O$	4
$MnSO_4 \cdot H_2O$	12
Na acetate	200
KCl	50
$Na_2CO_3 \cdot H_2O$	200
K_2HPO_4	as required†
Distilled water *ad*	100 ml

* Tryptophane and cystine were first dissolved in several ml of water with the aid of a few drops of conc. HCl. Before addition of sodium carbonate to the rest of the mixture, pH of solution should be adjusted between 5 and 6 to prevent decomposition of carbonate. After pH of complete medium was adjusted to 7.8, water was added to the required volume.

† See text.

‡ The hydrolysate prepared (see text) contained about 80 mg of solids per ml.

entire medium was sterilized by filtration through a Seitz EK pad. Appropriate steps were taken to insure the sterility of the medium before it was used in experiments. *Determination of phosphorus.* Samples were digested with a mixture of equal volumes of conc. H_2SO_4 and conc. HNO_3 (analytical reagents) on a hot plate until the fluid became watery clear and all the residual NO_2 was expelled. The Fiske-Subbarow method (6) was utilized to determine the amount of phosphorus in the sample.

Experimental. Constituents of the proposed medium. Although many of the constituents of the medium used in this study are similar in kind and amount to those of other workers as referred to above, (the complete composition of the proposed medium is listed in Table I), important modifications in the nitrogen and buffer sources and minor alterations in the rest of the medium have been made. It should be noted, for example, that the present medium incorporates the same amounts of purines, pyrimidines, vitamins, and minerals which have been used with uniform success for the cultivation of lactobacilli(7). Sodium acetate was used because of its potential value as a bacterial growth-stimulant(8,9). Glutamine was included to enhance the rapid growth of streptococcus. Glutamic acid failed to replace glutamine for this purpose, as has been observed previously(10). The amino acids, tryptophane and cystine, were included to avoid their possible loss during the preparation of the protein hydrolysate. Ascorbic acid served equally well as thioglycollic acid for the maintenance of the proper O/R conditions.

A buffer other than phosphates. Two possible buffers, other than phosphate, effective at pH 7.6 or 7.8 are sodium diethyl barbiturate and borate. Sodium diethyl barbiturate was tried in amounts varying from 16 to 75 mg per 100 ml medium, but no growth was observed after 48 hours' cultivation at 37°C. Because borate is potentially toxic in the quantities needed for adequate buffering action at the pH range required, no trial was made to establish its practical value as a buffer in the present experiments. Finally, sodium carbonate was used at a level of 200 mg ($Na_2CO \cdot H_2O$) per 100 ml medium. Excellent growth of the streptococcus was obtained, and the final pH of the culture after 24 hours incubation at 37°C was 6.5. For sake of economy in this series of experiments, the organic nitrogen source was enzyme hydrolyzed casein (2 g per 100 ml medium), and phosphorus was added as K_2HPO_4 in the amount of 50 mg per 100 ml medium.

Lactalbumin hydrolysate as organic nitrogen source. 1) *Removal of phosphorus.* Although pure lactalbumin contains no phos-

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phorus, this element was found to be present as an impurity in the ordinary commercial samples of lactalbumin. The removal of this phosphorus was essential in order to obtain a "phosphorus-free" preparation. Two hundred g of lactalbumin were washed by stirring vigorously for 30 minutes with 5 volumes of 50% ethanol, followed by filtration through a Buechner funnel. After 3 such washings and filtrations, the material was washed once with 95% ethanol, filtered, and dried in a hot air oven at 90°C-100°C overnight. No detectable amounts of phosphorus could be found in 1 and 5 g samples of the refined preparations.

(2) *Preparation of enzyme hydrolysate.* To insure adequate enzymatic hydrolysis of lactalbumin, both peptic and pancreatic hydrolysis was applied. One hundred g of lactalbumin (phosphorus-free) was suspended in 1 liter of distilled water and the pH was adjusted with HCl to between 1 and 1.5. One gram of pepsin (U.S.P. reference sample) was added. The suspension was covered with a thin layer of toluene and incubated for 48-60 hr at 37°C. At the end of this period, the pH of the digest was found to be 2.5. The peptic digestion was terminated by adjustment of the pH of the preparation to 6 with NaOH, 8 g of $NaHCO_3$ was then added, and the pH further adjusted to 8. One gram of pancreatin (U.S.P.) was added and hydrolysis continued for another 48-60 hr at 37°C. No significant change in pH of the hydrolysate was observed during this period. Since certain batches of hydrolysate, which were steamed and clarified with activated carbon according to the method of Roberts and Snell(11), gave inconsistent results, the latter step of clearing with activated carbon was omitted from the present procedure. At the end of the pancreatic digestion, the pH of the fluid was brought to 3.5 with HCl and steamed for 15 minutes. Precipitates were removed by centrifugation and filtration. The filtrate was clear and light brown in color and contained about 80 mg of solids per ml. Batches of lactalbumin hydrolysate so prepared have not failed as a nitrogen source for good growth of the organism. With 100 ml of the proposed medium (Table I), containing 40 ml of this hydrolysate and 5 mg K_2HPO_4, a 15-hr culture contained about 650 million streptococci per ml (determined turbidimetrically against Brown's $BaSO_4$ standards)(12). When K_2HPO_4 was excluded from this medium, no detectable amounts of P could be found in the preparation and no growth of streptococcus was observed.

Trials of hydrolysis of lactalbumin with pancreatin alone were made. Such a product contained only about 16 mg of solids per ml and failed to support the growth of the organism. Both amino acids and acid hydrolyzed proteins (egg albumin and casein) as sources of amino nitrogen were tried but without success. When, however, liver fraction "L" was added at a level of 10 mg per tube, good growth was obtained with either amino acids or acid hydrolyzed proteins. This may serve as an evidence to emphasize the importance of a peptide-like substance, strepoginin, for the metabolism of group A hemolytic streptococci(13).

Amounts of potassium and phosphorus required for growth. It was evident that the amount of potassium became limited as K_2HPO_4 was eliminated from the medium. The use of 5 mg each of KCl and K_2HPO_4 per 100 ml of medium did not result in a maximum growth. When the level of KCl was increased to 50 mg per 100 ml medium, excellent growth was again noted. No exact determination was made as to the optimal amount of K required by the organisms for maximum growth. With the medium described in Table I, it was found in a series of experiments that as little as 3.0 mg of K_2HPO_4 (equivalent to 55.2 μg P) per 100 ml medium resulted in maximum growth of the streptococcus while with 0.5 mg K_2HPO_4 (equivalent to 9.2 μg P) per 100 ml medium, suboptimal but definite growth was observed.

Tagging of streptococcus with P32. Attempts were then made to label streptococcus

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12. Cunningham, J. and Timothy, B., *Indian J. Med. Research*, 1924, v11, 1253.

13. Wooley, D. W., *J. Exp. Med.*, 1941, v73, 229.

with radiophosphorus. Ten ml of the proposed medium (Table I) containing 0.3 mg K_2HPO_4 was used for each experiment. One hundred μc of carrier-free P32 was added to the medium. To ensure better utilization of phosphorus, 1 ml of an 18-hr culture of type 14 hemolytic streptococcus was employed as the inoculum. Cultivation was carried out at 37°C for 4 hours. The culture was heated in a boiling waterbath for 5 minutes and centrifuged. The sediments were washed 3 times with liberal amounts of distilled water and dried in a hot-air oven at 90°C overnight. Two ml of conc. HNO_3 was used to digest the dried bacteria. The excess acid was removed by heating to dryness. The solids were dissolved with 1 ml of 1:3 HCl and 0.1 ml amounts of the solution were put on small filter paper discs (2 cm in diameter). Counting was done after the paper discs were dried. It was found that only 2% of the radioactivity added was taken up by the organism. The experiment was repeated twice with 200 and 400 μc respectively. Cultivation was prolonged to 18 hr. With these 2 trials, about 20% of the radioactivity could be recovered in the organisms. It seemed therefore that a highly radioactive streptococcus could be obtained by using a larger amount of P32 and cultivating for a longer time.

Discussion. It is evident that the proposed medium was able to support excellent growth under circumstances of a controlled supply of phosphorus. It should be of value in bacteriological and immunological studies involving the use of radiophosphorus. It is also plausible that such a medium may be used to study phosphorus metabolism of other micro-organisms.

Summary. (1) A detailed procedure for preparing a "phosphorus-free" protein hydrolysate capable of supporting excellent growth of group A hemolytic streptococci in combination with other chemically defined constituents has been described. (2) This medium has been used successfully for tagging group A hemolytic streptococci with radiophosphorus.

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Lymphocytic Response of Normal Individuals to the Insulin Glucose Tolerance Test. (19166)

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Somogyi(1) has reported that glucose administered orally to normal persons 30 to 60 minutes after insulin results in the development of greater hyperglycemia than the administration of the same amount of glucose without insulin. This has been interpreted by Engel and Scott(2) to signify that "glucose magnified the normal homeostatic response to hypoglycemia." As this response is presumably mediated by the hormones of the anterior pituitary, the adrenal medulla and the adrenal cortex, Engel and Scott introduced the insulin glucose tolerance test for differentiating the normal from the hypopituitary or the hypoadrenal individual. Previous observations have shown that in the normal person either oral(3,4) or intravenous(5) glu-

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