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The Biology of Oidium Albicans with Special Reference to Mycelial Production.

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A Thesis presented in partial fulfillment of the Requirement for the degree of Doctor of Philosophy.

I.

General considerations of the biology and classification of the Oidia.

Oidium (Monilia, Endomyces, Saccharomyces) albicans is generally accepted as the specific cause of thrush or parasite stomatitis, a disease of wide distribution.

The organism has been found associated, however, with a variety of conditions - general infection involving the skin, nails and lungs[1] (Christensen, Am. Jour. Child. Diseases, 26: 3, p.460, 1923); normal saprophite of the stomach[2] (Moppert and Kagan, Revue Médicale de la Suisse Romandie. 42: 8, p.505, 1923); gastric contents in cases of duodenal ulcer[3] (Marko, Beitrage z. klinischen Chirurgie 130: p.549, 1924); laryngeal involvement[4] (M. Maciuceanu-Manoël, Arch. de Méd. des Enf. 28: 4, p.231, 1925); intestinal tract[5] (Le Dantec. Compt. rend. de la Soc. de Biol. Vol XIV, No.21, p.1066, 1908) [6] (Bahr, P. H. Brit. Med. Jour. Vol.2: p.171, 1914); infection of skin and chronic paronychia[7] (Shelmire, Arch. Dermat. and Syph. 12: 6, p.779, 1925); intradigital saccharomycosis[8] (Zwick, personal communication)

In the lesions of thrush the organism is found most frequently in the forms of hyphae or mycelia. The elongated cells or filaments range from 30 to 40 microns in length and from 2 to 5 microns in width.

They are subject to considerable variation in size and shape. Reproduction occurs by the addition of new hyphae (apical growth) or by division of a single mycelium. In both cases the mycelia are separated by distinct septa in which respect the organism closely resembles the higher fungi. Reproduction may also occur by budding at the septa. The paper deals with the methods used in an attempt to control and fix mycelial and yeast form propogation.

The yeast forms are as widely distributed as are the mycelial types. They are consistently found in cultures upon the common laboratory media made from the lesions of the disease. The yeasts vary little in size. They are globular or ovoid in shape and range from 2 to 3 microns in diameter. They usually reproduce by budding but at times by hypha and division. Asca have not been observed.

Both types of cells stain readily with ordinary aniline dyes. Archibald's stain is very efficient.

Upon the common laboratory mediums (optimum reaction P_H7-7.6.5) the organism grows in large white glistening colonies, the surfaces being slightly convex and with regular edges. (Fig. 4)

Under these conditions, reproduction invariably occurs by yeast formation, special methods being required to grow the mycelial forms.

Recent researches have brought to the light the probability of the existance of multiple species of the "thrush fungus." [9] Castellani (Castellani, Annals. de l'Inst. Pasteur 1916. Vol. 30. P. 149) has shown by means of fermentative reactions that several strains of Monilia albicans differ in their ability to ferment sugars as shown in table 1.

Classification.

The true relation of Oidium albicans to the Saccharomyces is as unsettled as is the relation of the yeasts to the fungi.

In the vegetable kingdom, the fungi are embraced by the two phyla, the Schizomycetes and the Thallophyta. In the phylum Thallophyta are included the algae, the division fungi and the lichens. The division fungi are grouped into the following classes: the Phycomycetes, the Ascomycetes and the Hyphomycetes or the "Fungi Imperfecti".

The relationship of the Ascomycetes and the Hyphomycetes to each other has been for some years the center of attack of conflicting opinions The morphological variations of the organisms in these classes and the presence or absence of the ability to produce asci have been the sole means of differentiation.

In the Class Ascomycetes are included the organisms that reproduce by means of asci and ascospores. In this class belong the Saccharomyces or the "true yeasts".

The Hyphomycetes includes all those species which, chiefly, either because of morphology or the failure of asci to be observed, can not be included among the Ascomycetes.

The Family Saccharomycetes includes those genera represented by unicellular fungi, which multiply by budding, at times by partition, and regularly by the production of asci. The asci as "spore cases" that usually contain from four to eight ascospores. The ascospores have the ability to germinate to a vegetative cell. These organisms are the so-called "true yeasts" and can be further classified by biochemical and physiological differences.

The Hyphomycetes on the other hand include all those genera which have not been seen to produce asces. The organisms of this group are very frequently found as mycelia. The species of this class are not so easily differentiated by biochemical or physiological methods. The classification depends mostly upon morphological differences which in many respects may closely resemble those of the true yeasts or higher fungi.

The place of the Oidia among the fungi is questionable. Because of a failure to produce asces it can not be classified under the family Saccharomycetes and taken its place by general agreement under the Hyphomycetes.

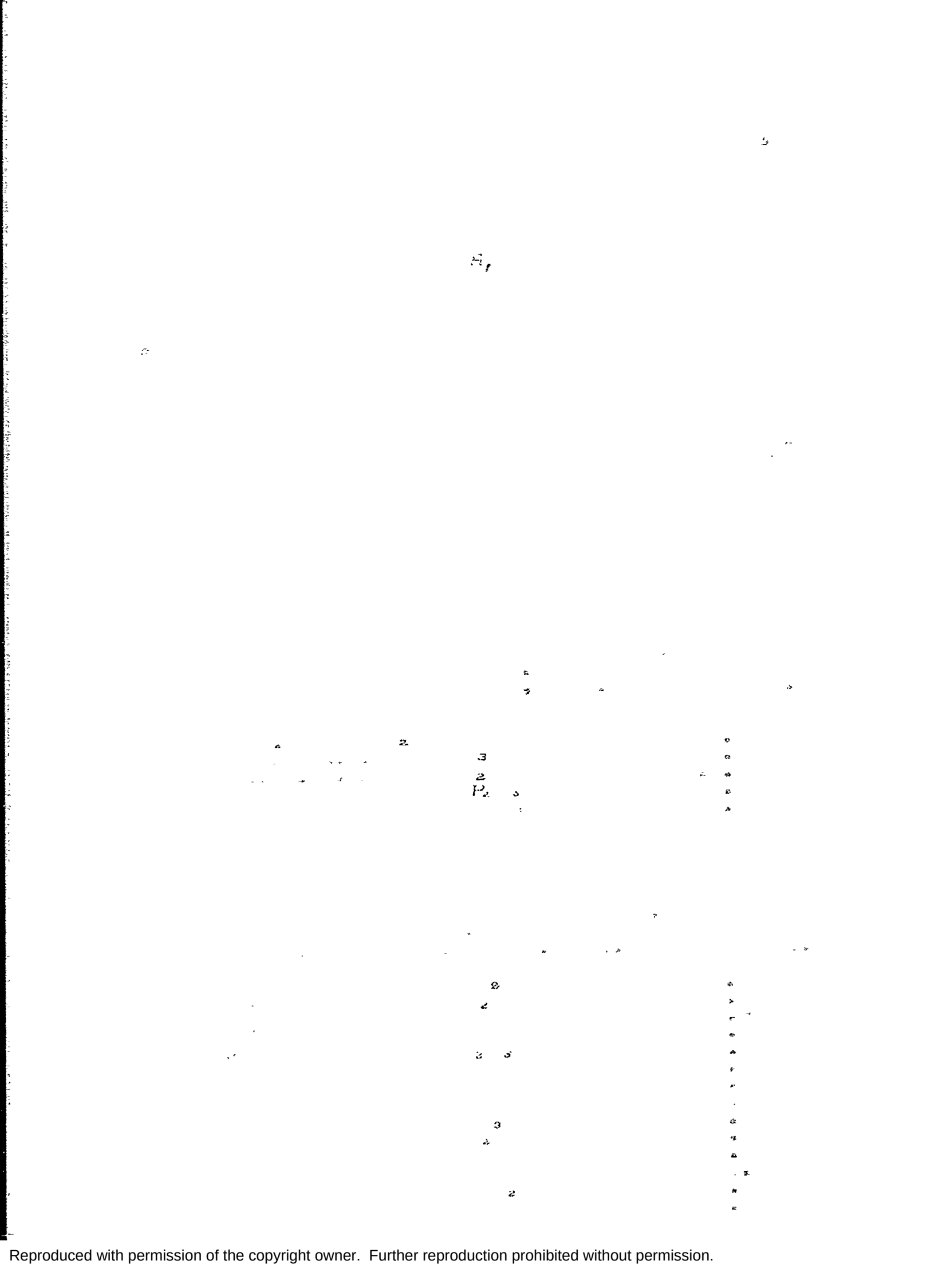
The genus has been subjected to various names and descriptions. The differences in opinion have been based upon the dual yeast-mycelial role of propogation.

Oidium albicans was discovered by Robin[10] (Draper - Jour. Infect. Dis. 34, No.6, p.631, June 1924)

The ability of Oidium albicans to change at the slightest provocation from the mycelial type of cells as isolated from thrush lesions to yeasts has long been regarded as an interesting phenomenon.

The opinions of the earlier workers concerning this change have been set forth in an earlier paper.[10]

The question of classification of this species revolves around that set forth by Guilliermond.[11] (Guilliermond, Tanner, Yeasts p.131)



An unexpectedly high proportion of Phosphorus was found to be present. It is significant in view of the fact that earlier experiment reported later in this paper had demonstrated the best mycelial growth when Phosphorus was present.

Adaptation of Mycelia to Peptone Solution.

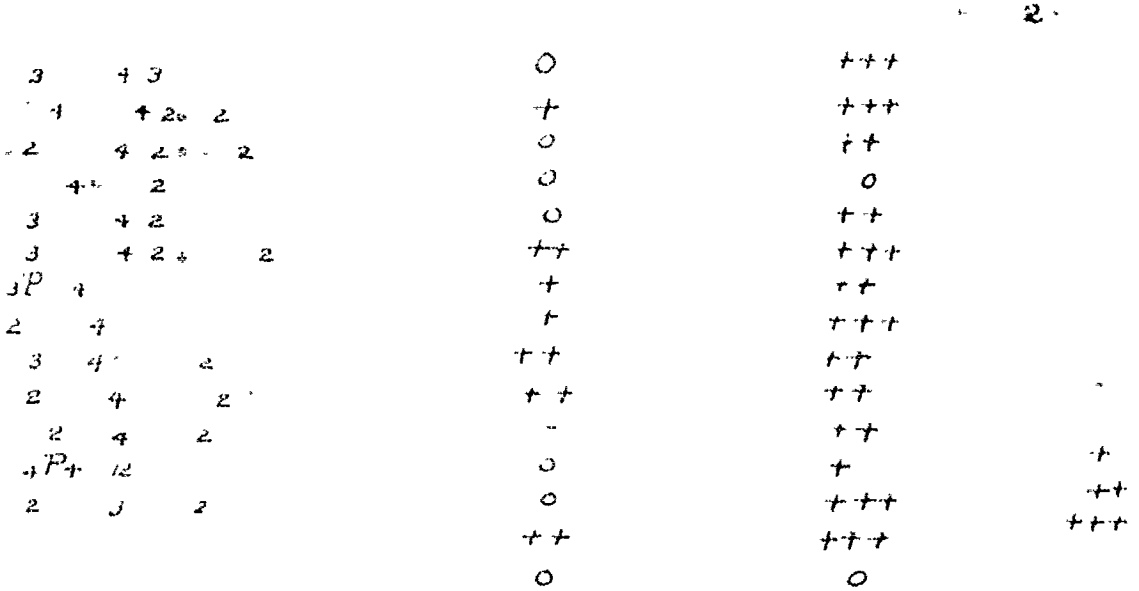
OA_2 a second strain of Oidium Albicans was isolated from a case of thrush in February 1925. In all previous experiments 1% Peptone solution had been used as a control medium. Under ordinary circumstances mycelial cells did not develope in this medium.

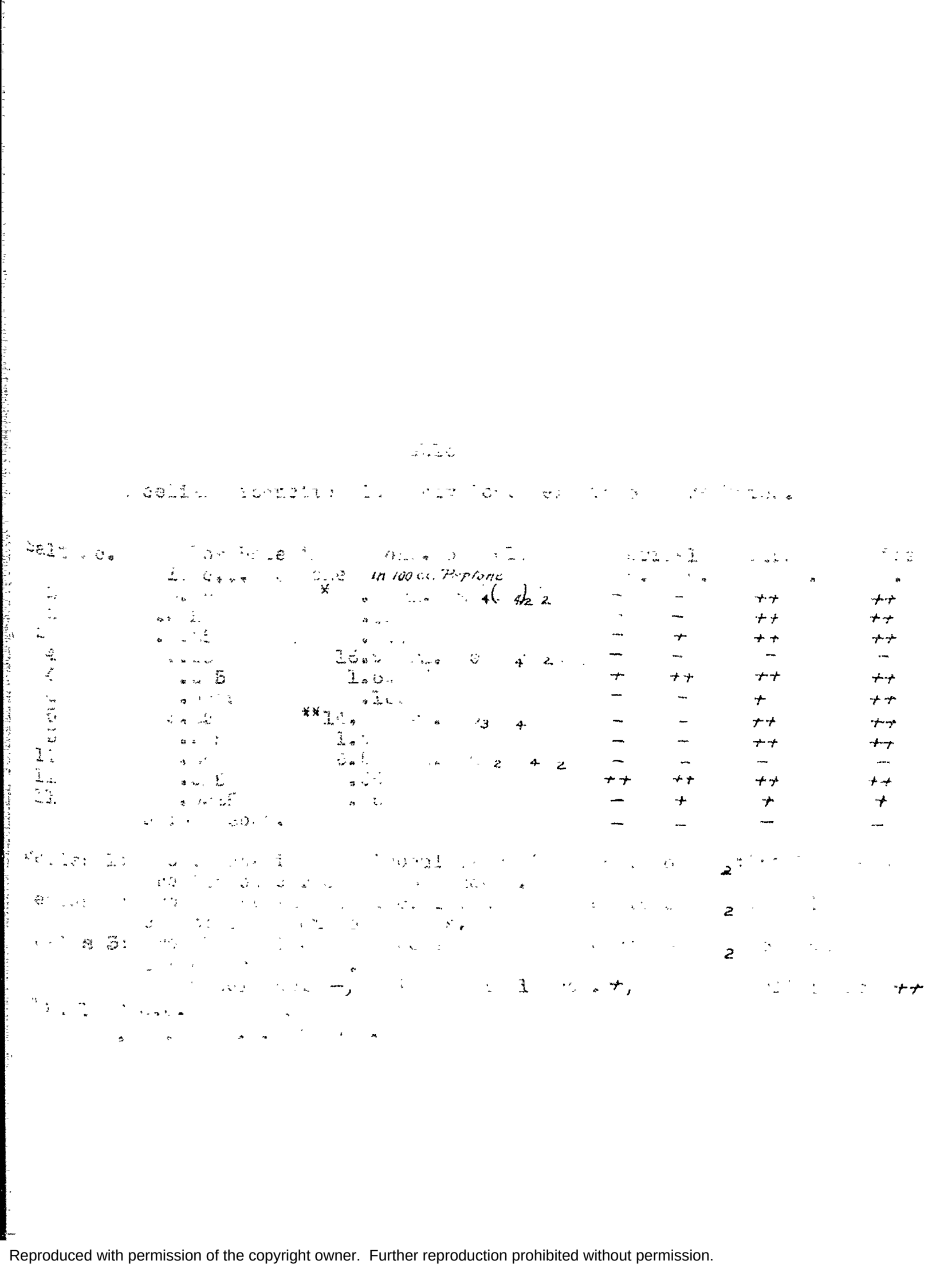
As a further check upon the value of Peptone as a control medium the two strains, OA_1 and OA_2 were grown simultaneously in Peptone solution for 35 days. Transplants were made daily or as soon as abundant growth appeared. OA_1 after 32 days developed mycelia. Further subcultivation increased the abundance of mycelial production. OA_2 at no time developed mycelia. Both organisms were grown under parallel conditions in Wittes' and in stock Peptone (Parke-Davis). OA_1 developed hyphae in Wittes after 30 days. OA_2 failed to do so.

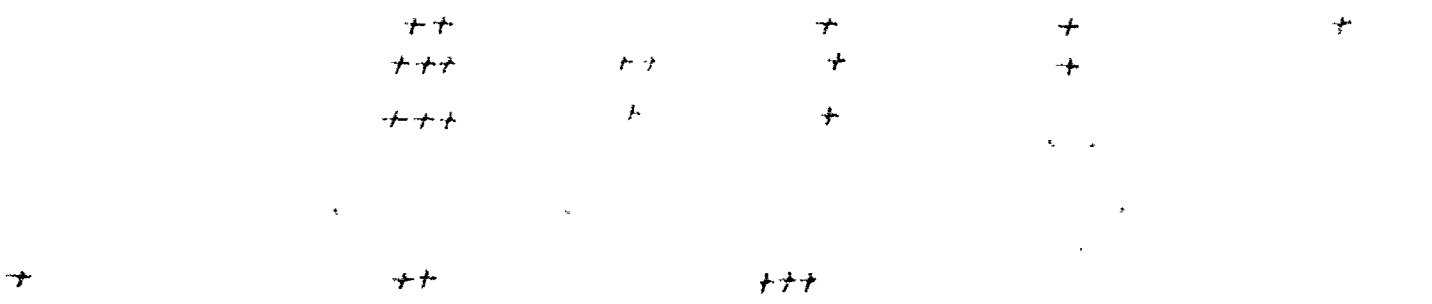
Influence of Various Metals and their Phosphate Salts upon Mycelial Production.

Since the factor that influenced the production of mycelia in carrot infusion appeared to be in the ash of carrots, solutions of percent weight in Peptone solution of the following salts were prepared: KSCN, $Fe_2(SO_4)_3$, $AgNO_3$, $Si(NO_3)_2$, $CuBr_2$, HgI, Pb acetate, $HgCl_2$, $SnCl_2$, and Peptone solution as a control.

Strain OA_2 which did not produce mycelia in Peptone solution was used. A slight production of mycelia in a solution of Ferric sulphate (1:1,000,000) led to a experiment using various soluble salts of iron.







I have shown that mycelia develope in carrot infusion at a given distance below the surface of the medium[10] (Draper - Jour. Infect. Dis. 34, No.6, p.631, June 1924) The same conditions prevail in the cultivation of the organisms in agar.

Test tubes containing 10 cc. of cooled melted agar, P_H 6.5, to which had been added 2 cc. of one-half molar solutions of various phosphate salts, were inoculated with yeasts of OA_1 and OA_2. The contents of the tubes were poured into Petri dishes and allowed to solidify. A layer of sterile agar was superimposed upon the seeded layer. The plates were incubated at 37.5 degrees C. In 24 hours colonies showing mycelia were visible with a 16 m m lens, and rapidly developed to where the mycelia were visible to the eye. If the seeded layer was not covered by a layer of agar the surface colonies developed yeast forms rapidly and apparently this growth interferred with the development of the deeper colonies. If one seeded the plates with mycelial threads colonies of this type appeared rapidly. Thirteen soluble phosphates were used. Of these those of Ca, Fe, Pb, Mn. and Mg were most favorable, while those of K and Cr gave unfavorable growth.

It would appear that phosphates in the presence of a suitable Oxygen tension do influence mycelial production, As will be shown below under the respiration experiments CO_2 also seems to play a role.

Appearance of "Type" Colonies.

Although the hyphae growth in various phosphate agars under partial tension was general, their mode of development differed so much as to yield entirely different types of colonies.

For the purpose of studying more in detail the factors influencing this variation, the salts that supported three types of growth, respectively, were selected: iron phosphate, sodium phosphite and carrot infusion.

Carrot infusion gave rise to what will be hereafter termed as "Type I" colonies (Fig. 1) They may be described as being colonies consisting of a multitude of yeasts from which proliferate hair-like filaments after five to seven days of incubation. Subcultivation of such colonies was accompanied by a decrease in the time necessary for the production of mycelia.

Iron phosphate agar gave rise to "Type II" colonies (Fig. 2) The early appearances of such colonies are similar to those of Type I but after 6-8 days small tufts of mycelia shoot out from the sides of the colony, which appear like tufts of grass. These tufts grow into long single hyphae which, in turn, give rise to lateral branching at an angle of about 30 degrees. The longitudinal hypha along with its branches takes on a *frond*-like appearance.

Colony "Type III" (Fig. 3) develops in sodium phosphite agar. It differs in that it is usually slower in development. After 10 days of incubation spine-like projections proliferate into the medium from a central mass of yeast cells. These projections are characterized by a single longitudinal filament but differ from Type II in the production of yeasts along the axis instead of mycelia. A 15 day old colony resembles much the shape of a star fish. It is usually from 1/4 to 1/2 the size of the other types. Repeated experiments showed that the Types developed consistantly in their given mediums.

Respiration of Oidium albicans with Special Reference to Mycelial Production.

Plate cultures of Oidium albicans in 10 cc. of agar, to which had been added 2 cc. of a half molar solution of calcium monophosphate grown aerobically, supported the growth of colonies throughout the depth of the medium. Upon the surface yeast colonies only developed. At the bottom of the medium and upon the inner surface of the dish yeast colonies likewise developed. After 5 days incubation mycelial threads proliferated from the deepest colonies up into the medium. Colonies midway between the surface and the bottom of the medium developed hyphae. This phenomenon suggested the use of partial tension methods.

An attempt was made to determine, if possible, the exact CO_2- O_2 equilibrium necessary for the best mycelial development. Cultures were made upon the surfaces of various phosphate agar plates in pairs. One series was incubated aerobically. The other series in a dessicating jar containing some CO_2. Little difference was noted in the amount of mycelial growth with the exception that under CO_2 the mycelial development occurred about 24 hours earlier than in the aerobic series.

Mixtures of CO_2 and air in proportions of 1:5, 2:4, 3:3, 4:2 and 5:1, as roughly determined by volumetric methods, were placed over agar slant cultures, containing no added phosphates. The mixtures of one part air to five parts of CO_2, growth was accelerated. After 7 days mycelial growth appeared in all cultures as shown in Table 8.

In another experiment N_2 was mixed with air in the same proportions as in the CO_2 experiment. Here growth and mycelial production occurred early in a mixture of 19 parts of N_2 and 1 part of air; but in 19 parts of air and 1 part of N_2 no mycelia were produced; all were yeasts. Evidently if reduced O_2 tension and CO_2 influence the production of mycelia they exert this influence chiefly at the beginning of growth, and before the growth of the microorganisms has altered the media.

It now appears that mycelial production of Oidium albicans depends upon either or both of two factors. The presence of soluble non toxic phosphates and a low O tension or this combined with a high CO_2 tension and of favorable tensions of O_2 - CO_2 or O_2 - N_2.

May not the disturbance of this delicate balance be accountable for pleomorphism of Oidium albicans?

Part III.

Experiments relating to Type Colonies of Oidium albicans and an attempt to fix such colonies upon various mediums.

The different types of colonies produced by Oidium albicans have already been discussed. At the time that these types were observed no particular emphasis was placed upon them other than that they represented mycelial production of the organism upon agar containing phosphates.

Pleomorphism is not an unusual observation as many organisms have been noted to produce under certain conditions colonies that were different in appearance from those usually seen.

Several investigators have by different methods successfully bred and fixed type colonies. An example of this is the work of Jordan,[13] (Jordan, J. A. M. A. 86: 6 p. 177) upon the "rough" and "smooth" types of colonies upon the Paratyphoid bacillus. By selection of a single cell he was able to fix a type.

The relatively consistant production of totally different types of colonies upon certain mediums by Oidium albicans suggested the following work.

In dealing with three types of colonies upon three mediums it is wise to bear the following relationships in mind.

1. In Carrot Infusion, (C. I.) agar the majority of colonies are Type I (Fig. 1)
2. In M/2 Ferric Phosphate (#4) agar the majority of colonies are Type II. (Fig. 2)

3. In M/2 Sodium Phosphite agar (#13) the majority of colonies are Type III. (Fig. 3).

Can a given type be reproduced in 100% purity upon its specific medium by means of selection of type cells?

Can one type be fixed either temporarily or permanently by means of chemical or physical agents upon the medium that usually supports another type?

Experiments in producing a pure type colony strain by selection and passage upon its specific medium:-

Yeasts of a 24 hour old carrot culture of Oidium albicans, OA_1, were seeded in agar mediums containing Carrot Infusion, #4 (Iron) and #13 (Sodium) respectively and incubated under the partial tension conditions previously described, a layer of sterile agar being superimposed upon the inoculated agar.

In each plate there were developed many colonies that were characteristic in type, in 5-8 days, for their specific mediums. Such type colonies were fished and seeded upon slants of media corresponding to the original mediums on which they originally had appeared.

Subcultures were made upon the same mediums daily for a period of 35 days. The organisms, as yeasts, were again seeded into partial tension plates of the same medium and the percentage of true type colonies computed. There appeared to be no marked difference in the percentage before the subcultivation and the percentage recorded after it. However, as usual, Type I, Type II, and Type III colonies were always in majority upon C.I. #4 and #13 respectively.

Transferring and Culturing Type Colonies "in toto".

Very young type colonies were cut from their respective mediums and introduced into cooled melted agar and shaken to disperse the mycelial cells as much as possible. The suspensions were diluted and replanted upon the mediums in the same manner as was described in the preceeding experiment. It is obvious that the sole difference in the two experiments was the use of mycelial cells in the latter for seeding the plates.

Seventy-five percent of the colonies that developed in C. I. agar were of Type I and the remaining percent included Type II, Type III and yeast colonies. The growth upon #4 medium was unusually rapid and for the first time showed approximately 100% true Type II breeding. The colonies on #13 medium were slow in developing. Due to this retarded growth the percentage of Type colonies could not be determined. The experiment was repeated with similar results. It thus appears that if partial tension plates are inoculated with mycelia of a type colony the percentage of true typesdeveloped is greater than if they are inoculated with yeasts. Is the tendency to vary more inherent in the yeast forms of a given strain than in the mycelia of the same strain?

By means of selection, a given strain produces in #4 medium approximately 100% colonies true to type. (Fig. 5). If, however, this strain is then cultured ~~upon~~ in C. I., all Type II characteristics are lost in favor of Type I. It is thus not possible to fix colony characteristics permanently by continued cultivation upon a single medium.

Experiments on the fixation of types by means of heat:-

The well known experiments of Hansen on the use of heat to fix type variants among the yeasts, and those of Pasteur on the anthrax bacillus

for 35 minutes. A third heating further increased its sensitivity to heat, this strain being killed in 35 minutes but not in 30 minutes. A fourth heating of such subcultures reduced the killing time to 30 minutes, growth occurring after 25 minutes. No higher degree of sensitivity could be reached. This latter strain will be designated S 25-75 to differentiate it from the original S 40-75.

An interesting fact presents itself in the observation that a heated strain (S 25-75) grown upon C. I. agar and transplanted into #4 medium will produce colonies retaining Type I characteristics and not reverting to Type II as is the case with unheated strains. Such results pointed to a permanent fixative of colony characteristics by heat regardless of the medium upon which it was subsequently grown.

These results were checked in the following manner:
Two suspensions were prepared of OA in carrot infusion. One suspension was heated to 75 degrees for 25 minutes. The other was not heated and was used as a control. Both suspensions were plated in C. I. agar by the usual partial tension method. Type I colonies predominated in both subcultures. The suspensions were also seeded into #4 medium in which the usual Type II colonies developed. Transplants of subcultures of the heated and unheated strains from C. I. agar to #4 medium were accompanied by the retension of the Type I characters in many of the colonies of the heated strain (Fig. 6), and by the reverting to Type II characteristics of the colonies of the unheated strain. (See Figures)

When the unheated strain was plated in exactly the same way most of the colonies grew as Type II colonies as was to be expected (Fig.5)

Those experiments were repeated three times, and the fixation effects of the heating may be said to last for at least six weeks. It is too soon to say whether the fixation is permanent or not.

In the above experiment we apparently fixed Type I colony in Type II medium (#4). Some experiments which are incomplete seem to show that similar results may be expected in the fixation of Type II colony on Type I medium (C. I.). Here, however, difficulties have been encountered in the fact that the heated cells of Type II colonies do not grow out under the partial tension conditions used above. They may be made to appear, however, by inoculating the surface of the seeded plate with yeasts of the same strain, and when they grow they develope as deep Type II colonies.

Part IV.

Summary and Conclusions.

Yeasts of Oidium albicans, when grown in carrot infusion, developed as mycelia below a certain level in the test tube. The ash of carrots contained some substance that influenced such mycelial growth. Analyses of the ash showed that it contained a large amount of Phosphorus.

Various phosphate-containing salts, in appropriate concentrations in 1% Peptone, supported the hyphae growth.

The organisms grew in meat infusion agar as mycelia if a small amount of inorganic phosphate or carrot infusion was added, and they were cultivated under special partial tension conditions.

Various strains of the organism differed in their biochemical and physiological activities. Cells of the same strain when grown in different phosphate mediums developed "Type" colonies that were specific for their respective mediums. Three such types were observed.

A Type grown in its specific medium over a considerable period of time did not retain its Type characters when transplanted into a medium specific for another type.

Exposure of cells from a given type colony to an appropriate amount of heat tended to fix the type colony characters insofar that they were retained when they were seeded in a medium which usually supported another type. By this method Type I was fixed upon P4 medium, and Type II was partially fixed upon C. I. medium. The degree of permanence of