THE REACTION OF THE FOWL TO PULLORINS

by

CARL ALFRED BRANDLY

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I. INTRODUCTION

The protoplasmic property of response or reaction to stimuli is the basis of cellular physiology and pathology. Local cellular response to specific antigenic stimuli in its significance in disease diagnosis was first recognized by (1) von Pirquet in 1905 in relation to the disease tuberculosis.

Certain extraneous substances (proteins or closely related substances) brought in contact with normal tissue cells alter the reaction capacity of these cells. This changed reaction capacity is characterized in many cases by the appearance in the blood stream of specific bodies to which the name "antibodies" has been applied. In other cases, however, profound specific changes in cell reaction may be developed which are fundamentally analogous to the former except that no determinable circulating antibodies can be detected. These latter changes in cell reaction are included in the various types of allergy and hypersensitivity.

The physical and chemical nature of the inciting antigen apparently determines whether circulating antibodies may or may not be formed.

Infectious disease presents a specific instance in which the phenomenon of cell reaction is set in motion, but is complicated beyond similar processes because the antigenic agent multiplies in and parasitizes the body of the host.

Specific circulating antibodies may be demonstrated during or following attacks of many infectious diseases. Allergy manifested by skin hypersensitivity to the inciting antigen may exist simultaneously in the same individual or alone.

Of the specific bacterial infectious diseases affecting the domestic fowl, that known as "pullorum disease" (bacillary white diarrhea) results in many instances in the manifestation of a cutaneous hypersensitiviteness detectable by the intradermal injection of cultures of Salmonella pullorum or some of its products. Circulating antibodies are also commonly present in these same affected or recovered individuals.

Control and eradication of pullorum disease depends primarily upon detection of the mature "carrier birds, i.e., those harboring S. pullorum infection.

The agglutination test, which depends upon the active clumping of suspensions of S. pullorum organisms by the circulating agglutinins, was first applied in a practical way to pullorum disease by Jones in 1913. In spite of many subsequent improvements and modifications the test possesses certain technical and practical difficulties in its application to this disease that are not readily overcome.

The intradermal test, which depends on the specific response of cellular allergy, was first applied to pullorum disease by Ward and Gallagher in 1918. It obviously is not subject to the same major difficulties and limitations affecting the agglutination test. However, difficulties in preparing an intradermal agent (pullorin), non-toxic to normal skins but effective in eliciting cutaneous reactions in infected individuals, have been repeatedly encountered in the original and much of the later work.

The foregoing studies were conceived with the practical object of developing a satisfactory pullorin for detecting "carriers" of pullorum disease. The fundamental investigations must therefore, obviously, be concerned with the reaction of the domestic fowl to pullor-

in.

The agglutination test and in some cases the clinical findings were used as a basis or standard of comparison to measure the relative efficiency of the various "pullorins" used.

II. REVIEW OF LITERATURE

A review of the literature dealing with observations germane to these studies may to the best advantage be divided into: (1) a brief consideration of the cause, significance, and manifestations of cutaneous hypersensitivity of infection and some general aspects of the nature of the bacterial substances used for eliciting specific skin reactions; and (2) a summarized review of some previous work on the intradermal test for pullorum disease including the type of products used, procedure of application or administration and interpretation and evaluation of results.

1. Cutaneous Hypersensitiveness of Infection

In 1905 von Pirquet (1) published his epoch making observations on cutaneous hypersensitiveness to which he and Schick (2) applied the tern "allergy"

(allos, "altered"; ergia, "reactivity"). This was the point of departure which led von Pirquet in 1907 to the discovery of the cutaneous tuberculin reaction.

Krause (3), 1916, in studying skin reactivity to tuberculin concluded that; (1) cutaneous hypersensitiveness is never present without a focus of infection: (2) it appears coincident to establishment of a focus; (4) cutaneous hypersensitiveness varies with the virulence of the invader; (5) it is never entirely lost except in pregnancy or intercurrent disease; (6) it is increased by reinfection; (7) it is diminished or completely wiped out during a period of general tuberculin reaction; and (8) he suggests that tissue hypersensitiveness may be a function of immunity to reinfection.

Gelien and Hamman (4) made many observations on cutaneous hypersensitivity in man and concluded that hypersensitiveness falls to a lower or to the original level as disease subsides and the individual recovers. If tuberculosis remains active a high degree of cutaneous hypersensitiveness is maintained but when the body resistance is overcome and disease overwhelms it, all evidence of hypersensitiveness is lost. Their contention that cutaneous hypersensitiveness is always evi-

dence of infection seems to be amply substantiated by their experiments.

Fleischner, Meyer and Shaw (5) report the results of an intensive study of cutaneous hypersensitivity on large numbers of guinea pigs inoculated with certified milk. Sensitivity to tuberculin and abortin was manifested only in diseased pigs. Guinea pigs injected repeatedly with dead cultures of abortus, typhoid and paratyphoid organisms and with abortus protein and old bovine tuberculin, developed a high titre agglutinating serum except in the case of tuberculin. Intradermal tests were all negative indicating that cutaneous hypersensitiveness cannot be produced in this manner. They concluded that in guinea pig cutaneous hypersensitiveness is always a sign of infection and never a sign of immunity and that these experiments establish for guinea pigs a definite biologic law. Further, that conditions which will sensitize an animal in an anaphylactic sense will not necessarily sensitize its skin.

Michael & Beach (6) in studying the pullorin reaction in chickens were unable to bring about specific skin reactivity in 51 cockerels injected with 1 cc each of a suspension of live S. pullorum organisms. Agglu-

tinin production was detected in some of the cockerels but in no case was S. pullorum isolated on autopsy and culture of these birds. These results indicate that no focus of infection resulted from injection of S. pullorum and failure to manifest skin hypersensitiveness was in accord with Krause's observations.

In reviewing the work on the nature of the tuberculin reaction Calmette (7) concluded that it cannot be regarded either as a phenomenon of anaphylaxis or as an anaphylatoxin reaction but that it results from the lytic action on the part of certain substances, contained in the body fluids of subjects infected with the bacilli,upon the tuberculin; and this lytic action gives rise to the formation of a specific product, both toxic and fever producing, which is the essential factor in general or local tuberculin reactions.

The von Pirquet test as described by Calmette (7) utilizes the introduction of a small drop of tuberculin into the skin through a simple scarification or pricking. If the subject is tuberculous there appears after 10 to 24 hours, occasionally a little later, a very characteristic papule or swelling of purplish red color. This papule remains visible for several days and then dis-

appears without leaving any trace. No effect from the same inoculation is produced in subjects free from tuberculous lesions.

Moore (8) observed that a positive reaction from the intracutaneous use of tuberculin in cattle is indicated by a thickening (swelling) which may be either edematous, or hard and inflamed. The swelling was recognized in some cases by the sixth hour but generally may not be clear until about the twelfth hour. He observed that the swelling continued to increase in size for two or three days.

Mantoux, according to Calmette (7), described a positive intradermal reaction as consisting of a white or pink edematous infiltration of the dermis, accompanied often by a hemorrhagic suffusion. It obtains complete development 48 hours after inoculation.

Long (9) states that injection of dilute tuberculins into the skin of healthy animals produces no grossly visible change but microscopically, a transient influx of a few leucocytes is observed but within a few hours complete restoration to normal is effected.

A similar injection into the skin of a tuberculous animal within eight to twenty-four hours produces an

acute local inflammation at the site of injection. A red raised area with central blanching, 1-2 cm in diameter, appears, which is of firmer consistency than the surrounding skin. If the tuberculin solution is concentrated in active principle or the individual is exceptionally sensitive, central necrosis occurs. Microscopically, the capillaries show dilation and engorgement, and an intense infiltration of large mononuclear and small polynuclear leucocytes is observed. There is an increase in the intercellular fluid, and some coagulated fibrin may be present. Long cites the work of Petersen and Levinson who demonstrated an increase in capillary permeability in sensitized tissues which appears to be fundamental to the reaction.

Van Es and Schalk (10) used tuberculin prepared from the avian type of organism on fowls. Intradermic inoculation of 0.05 to 0.07 cc of 50 percent avian tuberculin in physiological salt solution was followed by a perceptible local reaction in 24 hours. It increased during 48 hours to disappear in 72 hours.

In studying the intradermal tuberculin test in cattle the British Medical Research Council (11) measured the increase in thickness of the skin in reacting

and non-reacting individuals in order to determine the standard limits of reaction. Both single and double intracutaneous injection tests were employed. The average increase in skin thickness in 18 reactors was approximately 20 mm greater (four times normal thickness) than in 120 non-reactors.

They emphasize that measurement of the skin, although very valuable, does not convey as true an impression of the reaction as can be obtained by careful palpation of the inoculated area. The general characters of heat, tenderness, edema and the "feel" of the swelling are almost invariably the deciding factors inasmuch as the soft ill-defined edema, so characteristic of a positive reaction is not adequately shown by the calipers.

Modifications of the double intradermal test according to some American workers were employed. A preliminary or sensitizing dose was followed in 48-72 hours by a test dose introduced by slight pressure with the point of the needle in the center of the swelling. Readings were made before injection of the test dose and again in 24-48 hours following it.

In some tuberculous animals the reaction to the

first injection of tuberculin was so marked that a second or test dose was unnecessary and undesirable. In several others which showed a definitely positive reaction following the first injection a second dose was given to demonstrate the rapid increase of signs and symptoms as a result of it.

In both normal and tuberculous animals a rapid swelling follows soon after injection, usually reaching its maximum in three hours - no animals showed a maximum early reaction later than the sixth hour. In normal animals the fall to slightly above normal was rapid usually being complete after 48 hours.

Specific skin hypersensitiveness in eight tuberculous animals was manifested by a maximum swelling at an average time of 22-25 hours. The shortest time was nine hours and the longest 48 hours. As convincing as the delay in the development of the swelling was its persistence. In some animals receiving the single skin injection, it persisted almost undiminished in size for 99 hours. In the double intradermal test an earlier maximum swelling in tuberculous animals was obtained. It persisted for 60 hours at which time observations discontinued. In non-tuberculous animals the test dose

may cause the skin to swell to twice its normal thickness but the character of the swelling is hard, cool, non-sensitive and sharply circumscribed.

The above observations led to the conclusion that the diagnostic readings should not be made too early, not before 12 hours but better at 24 hours while the difference in normal and reacting animals is usually greater at 36 to 48 hours.

It was also observed that discrepancies in the intradermal test could be explained only by assuming that the injections were too deep or too shallow. However, as long as the tuberculin was introduced into the dermis the deeper the injections the more severe the swelling. Consequently, in thick skins interpretation of the reaction was facilitated if the injection was made deep.

The results obtained from testing numerous herds subjected to ophthalmic or subcutaneous tests indicated that the intradermal test was uninfluenced by previous, and in several cases frequent subcutaneous tests. However, slight intradermal reactions occurred occasionally in heavily infected animals.

In an effort to confirm the work of Holtum on a

double intradermal test for Bang's abortion disease, Edgington and Broerman (12) made, among others, the following tentative deduction; a single intradermal injection gives as satisfactory results as a double injection.

(a) Intradermal Diagnostic Agents. With reference to the nature of complex antigenic agents, Zinsser and Mueller (13) state that "the bacterial cell consists largely of socalled "nucleo-protein" material probably in combination with the carbohydrate structures which Avery & Heidelberger term "specific soluble substances". The latter are not antigenic although they exhibit specific immunological reactivity in vitro. True antigens are related to the nucleo-proteins.

Exhaustive chemical studies on tuberculin, the agent first used to detect cutaneous hypersensitiveness of infection, bear out the original contention of Koch that this substance is protein in nature (Esmong R. Long) (14). Purified and unpurified filtrates of Mycobacterium tuberculosis grown in broth are the chief sources of tuberculin. Autolytic products or organisms which autolyze readily (pneumococcus, meningococcus, and some others) exhibit antigenicity and

have been applied by several investigators for testing skin reactivity.

Other bacterial substances used for eliciting skin hypersensitiveness of infection are prepared according to the general methods of obtaining bacterial nucleo-proteins (15): (a) from centrifugated broth cultures as in the work of Avery and Heidelberger, Lancefield, and others; (b) from the solution of the cell as employed by Schoenholz and Meyer(16) and others; (c) from ground dried bacteria grown on agar (15) Zinsser (Avery and Morgan); and (d) the cell washings of agar cultures termed "ecto-antigens" by Ferry and Fischer (17) and proved by them to be highly antigenic were adapted to intracutaneous tests by Bushnell (20).

Zinsser and Muller (15) describe the principles of manipulation in processes (a) and (c) as being roughly the same. Extraction with slightly alkaline salt solution, usually about N/100 NaOH is followed by clearing the extract of suspended particles by centrifugation or Berkefeld filtration. A considerable quantitative loss of nucleo-protein by filtration results because of its colloidal nature. The extract is then precipitated with acetic acid, excess acidity

being avoided. On centrifugation, the sediment is redissolved in alkali and for further purification may be reprecipitated. Repetition of this procedure always leads to a considerable loss of material. This opinion of Zinsser and Mueller is consistent with Wells' statement as quoted by them that the loss of substance is probably due to denaturation of the albumins present in the original precipitate.

Washed and unwashed organisms grown on agar and in broth have also been used by numerous workers including Reichel and Harkins (18) in intracutaneous tests for detecting hypersensitiveness of infection.

2. Summary of Pullorin Investigations

A resume of literature dealing with the intradermal pullorin test since Ward and Gallagher in 1917 first proposed it, reveals that the use of a variety of S. pullorum products applied somewhat differently and interpreted, and evaluated according to several criteria has resulted in considerable confusion.

A summary of some of the work on this problem as presented in the following table may be of value in order to minimize duplication, to suggest preferable pro-

cedures and to determine the most efficient standard of comparison of results obtained.

Table I. Summary of some pullorin investigations

Worker and date	Type of pullorin	Amount and method of inoculation	Time of readings	Number birds	Basis of comparison: Agglutination test	Gross lesions	Autopsy and culture	Chick mortality	Correlation	Conclusions
Ward and Gallagher 1917 (19)	Broth cultures	0.2 cc as near edge of wattle as possible	24-34 hrs.	--	+	+			70%	Equal to agglutination test.
Scherago and Benson 1919 (21)	(a) Broth cultures	(a) ""	24-34 hrs	--	±					85% become agglutination reactors. Great lack of specificity detects large percent but not all
Fuller 1923 (22)	(a) Broth cultures	(a) ""	" "	--		+		+		Detects large percent of infected birds--only very small percent of non-carriers react.
Dalling, Allen and Mason 1925 (23)	(a) Broth cultures	(a) ""	" "	89	+				82%	None drawn
Dalling, Mason and Gordon 1927 (24)	(a)	(a) ""	" "	645				+	75.1%	Intradermal test may be useful. Condemn greater percent of hens than agglutination.
Broerman 1927 (25)	Washed cell suspension killed by 0.5 phenol	As near border of wattle as possible	28-30 hrs.	81	+	+R				Test of value in detecting infected fowl.
Gwatkin 1927 (26)	Commercial			406	+	+	+		91.6%	Agglutination test more satisfactory.
1928 (27)	Precipitated commercial			112	+					Agglutination test more satisfactory than pullorin.
Graham and Tunnicliff 1927 (28)	Precipitated Experimental flock ..... Farm flock	Intracutaneous essential.....		27,816						..... Pullorin detected not less than 70% and often more blood reactors.
Edwards and Hull 1927 (29)	Precipitated (commercial)	1/25 cc intradermally as near ventral border of wattle as possible	24 hrs		+				78.3% Association coefficient 56	Agglutination test much more reliable than intradermal test used.
Stafseth and Thorp 1928 (30)	Commercial	According to directions			+		+R			Decidedly inferior to agglutination test.
Bushnell 1927 (20)	Ecto Commercial	Intracutaneously sufficient to produce small lump size of grain of wheat	24 hrs	847	+				85.1%	Not a satisfactory substitute for agglutination test.
Bushnell and Brandly 1928 (31)	Precipitated cellular Ecto digest	0.5 to 0.1 cc intradermally	24 hrs	113	+		+		Agglutination and pullorin 71.6% culture and pullorin 76.5%	Cannot be recommended to replace agglutination test.
				946	+				75	
Bushnell and Brandly 1928 (32)	Ecto commercial 1.Cell suspension. 2.Precipitated	About 1/10 cc intradermally in wattle		3,700	+		+ (95 birds)		78.8	Cannot be recommended to replace agglutination test. Possibility of developing a satisfactory pullorin.
Michael and Beach, J.R. 1929 (6)	"cell solution"			85	+		+		98.8%	
				143	+				99.3	
Edgington and Broerman 1929 (33)	"digest"						+	+	(c)	
Beach, Holmes and Strange 1920 (34)	Broth culture filtrate concentrated. Cellular	2 to 3 drops into skin of wattle		147	+					Cellular not satisfactory. Filtrate pullorins detected 12.3% more. S. pullorum culture positive birds than agglutination test.

(a) Method of Ward and Gallagher.

(c)

Group	Hens	Reaction	Chick Mortality	S. pullorum recovered
I	24	A+P+	43.6	95.6
II	20	A+P-	60.6	88.1
III	27	A-P-	2.6	0.0

(R) Only pullorin reactors examined.

(a) Recapitulation. The S. pullorum products, or pullorins employed, consisted of: (1) entire broth cultures of various ages; concentrated and unconcentrated; killed by heat or chemicals; (2) broth culture filtrates concentrated and unconcentrated; (3) washed agar cultures of different ages destroyed by heat and chemicals; (4) powdered pullorins prepared by precipitating liquid cultures of S. pullorum; (5) pullorins prepared from synthetic media; (6) agar culture washings or "ecto" pullorin; (7) filtered and unfiltered, acid precipitated or unprecipitated solutions resulting from digestion or dissolution of the pullorum cell with alkali.

The quantity of pullorin introduced intradermally in the investigations here summarized varied from 0.04 cc to 1 cc. The quantity was also indicated by the size or nature of the swelling it produced immediately on injection. Some were described as being "enough to produce in the skin of the wattle a temporary white lump or swelling the size of a grain of wheat"and, "a small white bleb which remained on the wattle was considered ideal".

The injections were in general designated as being made "into the skin of the wattle" or "into the layers

of the skin of the wattle" although some were made "as near the edge of the wattle as possible", indicating that they may sometimes have been subcutaneous.

Final readings of results were made as late as 34 hours after injection while others were taken as early as 20 hours following this procedure.

Results were reported on experimental flocks or groups varying in number from 39 to 3700 birds, while results of field tests were recorded on as many as 27,816 birds in 172 farm flocks.

Experimentally the agglutination test was used most frequently as a basis of comparison of efficiency. Gross anatomical lesions on autopsy were alone seldom employed as a standard of efficiency. Bacteriological culture of typical lesions or of apparently normal ovaries was utilized in determining the presence or absence of infection in some birds. The incidence of chick mortality before and after testing was used to some extent as a measure of the value of the pullorin test. One or a combination of two or more of these procedures for comparing results was employed in some of the various experiments recorded.

Numerical correlation of the results of compara-

tive tests was expressed in percentage agreement and in the terms of the Yule formula.

A majority of the workers conclude that the intradermal pullorin test is inferior to the agglutination test. A few state that it is equal to the agglutination test or even superior to it. The observation that the pullorin test may be developed to a satisfactory diagnostic procedure was expressed by workers not convinced of its efficiency as applied by them.

A number of salient considerations observed by workers not readily tabulated but of sufficient significance to warrant emphasis are recorded below.

Ward and Gallagher were unable to elicit a reaction by ophthalmic, palpebral and subcutaneous tests with pullorins prepared from broth cultures. These workers observed that a transient edematous condition of the injected wattle of both healthy and S. pullorum infected birds appeared within three hours following injection. This condition, however, rapidly disappeared according to them.

Scherago and Benson critize the test because in their hands it not only showed a lack of specificity, but 85 percent of the birds tested within pullorin be-

came positive reactors to the agglutination test regardless of their reaction to the original tests.

Broerman interpreted a slight thickening of the wattle at 28-30 hours as a doubtful test and a perceptible swelling as a positive reaction. A large percent of doubtful birds were recorded by him. He also observed that the injection of pullorin did not cause birds previously negative to the agglutination test to become positive.

Graham and Tunnicliff observed that powdered (precipitated) pullorins gave better results than those consisting of broth cultures, washed agar cultures, and pullorins prepared from synthetic media cultures. The former were superior in keeping qualities. All lots of pullorin were subjected to a potency test on 15 birds. These authors in further studies emphasize the sources of variation in pullorin tests as related to injection, potency and standard of interpretation. Failure of birds showing gross ovarian lesions and positive bacteriological findings to react to pullorins was mentioned as indicating probable infection with strains of low antigenicity. It was also pointed out that some non-reacting fowls have yielded S. pullorum

upon bacteriologic culture of the ovaries while the absence of macroscopic lesions is not necessarily indicative of freedom from the disease. These investigators as well as Bushnell and others (35) have found that pathologic changes in the ovaries are not in all cases related to S. pullorum but may be due to various other microbic pathogens. Retrogressive changes of the ovary such as those following cessation of egg production have obviously at times been mistaken for gross lesions induced by S. pullorum.

Michael and Beach observed the precaution of conducting toxicity tests before applying various pullorins to experimental flocks. Experimental pullorins with the greatest concentration of active substances, but yet non-toxic could thus be utilized.

Edwards and Hull observed considerable lack of uniformity in three lots of commercial pullorins from the same source as manifested by differences in solubility and color. They considered all soft edematous swellings at the end of 24 hours after injections as positive and all hard swellings as negative.

Stafseth and Thorp conducted the tuberculin and pullorin tests simultaneously in the same flocks

and concluded that the former was highly satisfactory while the latter was very erratic.

(b) Discussion and Criticism. In a critical consideration of the work on the intradermal pullorin test here summarized various sources of error and inconsistency may be pointed out.

Some old broth cultures of S. pullorum whether freed of the organisms or not may be expected to be more or less toxic. The inference that they should be no more toxic (and therefore incapable of causing non-specific tissue reactivity) than tuberculin prepared in the same way does not seem to be well drawn because the tubercle bacillus produces little or no endotoxin while the Salmonella are primarily members of the endotoxic group. Concentrated pullorins of this nature containing as much as 50 percent glycerin will probable elicit a non-specific skin reaction with considerable frequency.

In most of the investigations no mention of testing pullorins for potency or toxicity was made. This omission would constitute a serious source of error and would account for instances of great lack of uniformity of the various lots of pullorin as described

by Edwards and Hull. Manifestations of antigenicity by certain pullorins, in that they cause birds previously negative to the agglutination test to become positive as observed by Scherago and Benson, is of particular significance when the agglutination test is used as a standard of comparison.

Although a direct quantitative relationship between the amount of pullorin introduced and the response of the tissues may not obtain, it has been observed that the injection of almost any material in quantities as large as one cc directly into the skin or subcutaneous tissues of the wattle of the fowl may produce a traumatic swelling which may persist for some time.

Discrepancies in some of the results may be attributed to failure to make the injections at a proper depth, either too deep or too shallow. In general, subcutaneous injection gives in infected animals a subdued reaction or one which may be imperceptible externally. If injections are too superficial the injected material may escape.

It appears that in some of the studies the time of taking final readings of results for interpretation

was selected without careful determination of the period of maximum specific reaction. Interpretation of a large percent of tests as doubtful, as in the experiments of Broerman, would warrant the questioning of his conclusion that "the test is of value in detecting infected fowl".

Observations that any swelling should be taken as evidence of a positive reaction; that all hard swellings should be interpreted as negative tests and all soft edematous swellings as positive reactions would result in errors if no standard limits of increase in size of the wattles of normal and infected birds were established. Trauma alone may cause more or less persistent swellings of varying consistency. A considerable variation in reaction in thin skins and thick skins must be considered. The state of vascular nutrition as evidenced in producing and non-producing hens frequently has a significant influence on the type and extent of reaction.

Observations and definite conclusions on the results of testing only a very limited number of fowls may usually be greatly discounted because of normal variation.

As a basis or standard of comparison the agglutination test cannot by any means be considered perfect. However, its value as a measure of eliminating infection from a flock as indicated by a decrease in or the entire elimination of chick mortality has been repeatedly demonstrated. The agglutination test is the only criterion that can be utilized in many cases.

Reduction in incidence of chick losses from pullorum disease following application of the pullorin test would seem the most satisfactory measure of its efficiency although in flocks not under strictly controlled conditions a source of error in the entrance of outside infection may cause serious discrepancies.

Diagnosis of pullorum infection by gross lesions on autopsy, without bacteriological confirmation, must obviously be eliminated as a standard for measuring the efficiency of the pullorin test. Bacteriological examination of diseased as well as apparently normal ova may occasionally fail to isolate S. pullorum even though it is present while the focus of infection may remain undetected in some other tissue of the body.

III. EXPERIMENTAL

1. General Plan

The general plan of conducting these studies included the testing of a number of farm flocks and of several experimental flocks belonging to the Poultry Husbandry and Bacteriology Departments. This allowed the testing of the various pullorins on a sufficient number of birds to indicate their relative merits. All the birds were subjected to the pullorin and agglutination tests simultaneously and in some cases the birds were subsequently destroyed for autopsy and bacteriological examination.

The agglutination test was adopted as the only general practical standard of comparing the results of the pullorin tests under the prevailing conditions. Complete agglutination in a serum dilution of one to 25 or greater was considered a positive reaction.*

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* Technique of agglutination tests:- S. pullorum cultures of the "S" type were used in preparing the antigen for all of the tests. Forty-eight hour growths of these cultures on Kolle flasks of agar were suspended in 0.85 percent sodium chloride solution, centrifugated, washed twice and resuspended in 0.5 percent phenolized physiological saline to a density of 50 times that of tube one, McFarland's nephelometer. This undiluted antigen was used for plate tests while for tube tests it was diluted with

The pullorin was injected at the lower border of the wattle with a tuberculin syringe carrying a 26 gauge needle. No attempt was made to introduce a definite quantity of pullorin into the wattle of all birds because of individual variation in the thickness of the wattle and the texture of the skin. An attempt was made to inject sufficient material to produce a temporary white swelling (due to pressure anemia) about the size of a large kernel of wheat. The reactions were recorded according to the thickness of the swelling as compared to the normal wattle, i.e., 2x indicates a swelling or increase in thickness to two times the normal.

The pullorins used in the studies consisted of five types: (1) Broth cultures and filtrates; (2) Alcohol precipitates; (3) Cellular; (4) Cell washing or "ecto"; (5) Alkali "digest" or "solution".

Some were obtained from outside sources but the ma-

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75 to 100 parts of 0.3 percent phenolized physiological saline solution and then adjusted to pH 7.8 to 8.0 NaOH. The tube tests were incubated for 24 hours at 37°C after which readings were made. The results of the plate tests were read within two minutes after mixing the ingredients.

jority were prepared in our laboratory.

In those birds which were destroyed for autopsy and bacteriological culture, material from all ovaries, whether they showed lesions or not, was plated on 10 percent hemolyzed blood agar. Lesions in other tissues as well as the pericardial sac and testes in male birds were cultured. For identification isolated colonies from plates showing growth were transferred to glucose, lactose, maltose and sucrose broth containing brom thymol blue as an indicator. Those that produced acid with or without gas in glucose only were considered S. pullorum.

2. Basic Studies

A review of the literature pertaining to the pullorin test leaves the impression that certain primary considerations, particularly those pertaining to the intradermal reaction have been inadequately appraised and treated. Other fundamental factors related to the problem seem to warrant further attention and consequently, basic studies dealing with the following phases were carried out: (a) The intradermal injection; (b) The reaction to "intradermal" injection; (c) Specificity of reaction; (d) Comparison of the pullorin reaction to the agglutination titer and autopsy findings (e) Effect of repeated tests;

(f) Toxicity and potency tests; (g) The double intradermal test; (h) The ophthalmic test.

(a). The Intradermal Injection. Determinations of the thickness of the wattles of a group of apparently normal mature hens of the Single Comb White Leghorn and Rhode Island Red breeds gave the following average measurements.

Group I. S.C.W.L..... 0.043 inches (1.09 mm).

Group II. R. I. Red..... 0.0481 inches (1.21 mm).

Histological sections of normal wattles indicated that the thickness of the average skin was only about one-fourth to one-third of the thickness of the wattle. This suggested that intradermal introduction, the assumed desideratum in injecting pullorins, may fail to be accomplished by the present methods of procedure because of the extreme thinness of the dermis.

In order to determine the exact position of materials injected into the tissues in the usual manner employed in the pullorin test, three mature birds were injected with suspensions of sterile India ink. A 26 gauge needle attached to a tuberculin syringe was utilized for making the injections. Immediately on introducing the ink the birds were killed and the wattles excised and prepared

for histologic sectioning and staining.

Examination of these sections revealed that the injected material was largely introduced subcutaneously. Serial sections showing the point of insertion of the needle indicated that a minute quantity of the material was deposited in the dermis during initial puncture or during withdrawal of the needle following injection. It appeared also that material injected subcutaneously because of pressure may, to some extent, pass into the dermis immediately on withdrawal of the needle and before the natural contractibility of the tissues closes the puncture. This occurs particularly in the pars reticularis and in those cases where injection into the pars reticularis may be accomplished the material may reach the pars papillaris in a similar manner. If the reaction is truly a dermal one, it must be assumed that minute quantities of pullorin may produce a marked reaction. Because of the highly vascular structure of the subcutaneum, rapid absorption and removal of pullorin by the general circulation must be expected and consequently, it can scarcely be considered possible that the pullorin remains long enough in close proximity to the dermis or that enough of it is absorbed from the subcutaneous

area to produce a significant reaction.

Figure 1 represents a microscopic cross section of a wattle injected with India ink, and the 26 gauge needle in position. These drawings were made by the aid of the camera lucida and are reproduced to scale.

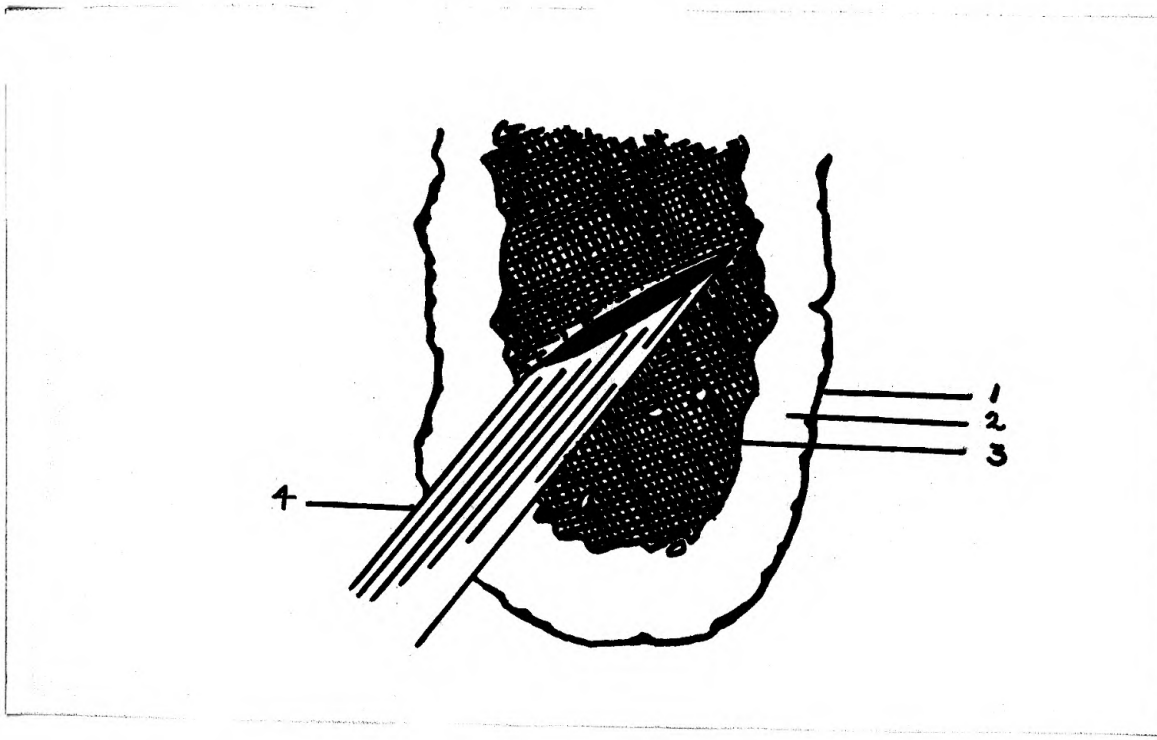

Figure 1. Drawing of cross section of injected wattle.

1. epidermis; 2. dermis; 3. India ink in subcutaneous tissues. 4. Twenty-six gauge needle in position during injection.

Observations on the influence of the depth of the wattle injection on the severity of the reaction were made as follows:

One group of 43 birds harboring 26 reactors to the agglutination test were injected with two lots of "Ecto" pullorin. Neither of these products appeared to be very active because only 13 of the 26 agglutination-positive birds reacted to the pullorin test. The association coefficient between simultaneously conducted agglutination and pullorin tests on this flock was 0.40 and the percentage correlation was 74.4. An effort was made to introduce the pullorin in such a manner and in such amounts as to produce a temporary skin thickening approximately the size of a grain of wheat. When the injection was made at the desired depth a transient blanching of the skin due to pressure was obtained over the wheat-grain size area.

It was considered that the injections were satisfactory as to depth in all except seven cases. In these seven birds, four agglutination positive and three agglutination negative hens, the injections were made deep, i.e., they were sufficiently deep so that no temporary blanching of the local skin area was produced although

the desired wheat-grain swelling was obtained. No observations on the influence of shallow injections were recorded because too much loss of fluid occurred immediately and, therefore, the injection was repeated at another point or points until a satisfactory result was obtained.

The effect of the depth of the injections may be observed by considering the following data.

Table II. Results of deep injection of pullorin.

Pullorin	Bird number	Agglutination titre	Pullorin reaction
17	1	2560	4+
17	2	160	2+
17	3	320	3+
18	4	320	3+
17	5	0	-
18	6	0	-
17	7	0	-

Twenty-four hour readings.

Of the balance of the birds which gave positive pullorin reactions, only two showed 3+ reactions, while none manifested 4+ reactions. Furthermore, 11 agglutination positive birds injected at the usual depth failed

to react to the pullorin. Deep injections into uninfected birds apparently does not cause any more readily perceptible swelling than ordinary depth injections.

These results are in accord with the observations on the intradermal tuberculin test made by the British Medical Research Council (11) that the deeper the injections, so long as they are not subcutaneous, the more marked the reaction manifested. However, it is obvious that many injections into the wattle are subcutaneous and relatively few are intradermal. This might indicate as previously stated, that a small quantity of pullorin through contact or absorption caused more of a reaction in deeper areas than in the more superficial tissues.

(b) Reaction to Intradermal Injection. The study of the response of the skin and underlying tissues as manifested by local reaction to pullorin was approached in the following manner.

(1) Infected and Non-infected Fowl. "Ecto" and "digest" pullorins prepared by the general methods described under 1, Section III were injected into the wattles of four non-infected hens and six infected ones. Failure to react to either of two successive monthly agglutination tests in 1-25 dilution of serum was the basis of consider-

ing the birds free from S. pullorum infection. The birds of the "infected" group had given repeated positive agglutination tests at irregular intervals during the preceding six months period. These latter birds had likewise been consistently positive to the pullorin test during this period.

The persistence and nature of the reaction (swelling) as recorded in the following table may be considered indicative of the local response of infected and non-infected fowl to pullorin.

Table III. Reaction to intradermal injection of pullorin.

Number of bird	Aggl. titre	Maximum early reaction*	Maximum late reaction*	Nature of swelling	Pullorin test results
1	0	4	6	FT	Negative
2	0	2	10	FT	"
3	0	4	8	FT	"
4	0	5	6	FT	"
5	100	4	26	H	Positive
6	400	3	22	SE	"
7	100	2	22	SE	"
8	100	2	22	SE	"
9	50	4	22	SE	"
10	400	2	26	SE	"
Average for infected birds	--	2.66	23.3	--	
Average for non-infected birds	--	3.75	7.5	--	

*Hours following injection.
SE Soft, edematous.
H Hard
FT Firm, tense.

No observations were made on the above birds later than the thirty-first hour because at this time they were again injected for the double intradermal test. However, observations on the persistence of the reaction in an experimental flock of agglutination reacting and non-reacting birds are recorded below.

Table IV. Persistence of the pullorin reaction (swelling).

Number of birds in flock	47
Number pullorin reactors	38
Percent agreement between pullorin and agglutination tests	87.23
Number birds showing same reaction at 24 and 48 hours	14
Number birds showing decrease in reaction at 48 hours	18
Number birds showing increase in reaction at 48 hours	1
Number birds negative at 24, positive at 48 hours	2
Number birds positive at 24, negative at 48 hours	3

The data in Table IV would indicate that the "intradermal" injection of pullorin causes a response similar to that produced by tuberculin, although a number of differences may be pointed out. The non-specific reaction

which is manifested by uninfected as well as infected birds reaches its maximum development in approximately three to four hours. This swelling is usually firm and tense to the touch. The infected birds, however, developed what must be considered a late specific reaction while the uninfected group showed a rapid return to normal after the seventh to the eighth hour, which was complete or nearly complete, before the twenty-second hour. The specific reaction is usually softer and more edematous than the primary one.

Although no readings were made during the interval of 12.5 to 22 hours following injection, the results of later readings indicate that diagnostically significant reactions are obtainable at an average time of 22.5 hours. The data in Table III suggest that the reaction in the majority of cases is more marked at 24 than at 48 hours and that the persistence of the swelling resembles that obtained in the tuberculin reaction. Persistent sensitive swellings either hard, or soft, and edematous, must be considered diagnostically significant if the possibility of traumatic injury or infection has been eliminated.

(2) Macroscopic Changes. As indicated in Table III, a rapid early swelling of non-specific nature resulting

from transient local tissue irritation follows the injection of pullorin. In testing buffer, formalin, and glycerin solutions for toxicity as recorded in later observations, the same type of reaction is induced. These swellings are usually soft and hot, obviously manifesting all the symptoms of acute inflammation except that rubor is not readily observed in the already "red" skin of the wattle.

The texture and extent of the late specific swelling varies in the majority of fowls from a relatively firm, circumscribed swelling to a soft, diffuse swelling which may in exceptional cases involve much of the integument of the lateral surface of the head. On palpation, the swellings do not always show evidence of increased "heat" of inflammation but may feel cool to the touch. A smoothness of the skin surface characteristic of edema is readily perceptible. Occasionally an individual such as bird No. 5 in Table II shows a hard swelling which must be considered atypical. The injected skin of the wattle of this bird seemed rather thick and harsh to the touch and manifested a general lowered state of nutrition characteristic of non-producing hens.

(3) Microscopic Changes. Observations of histo-

logic sections of the normal and "reacting" wattles of certain fowls including bird No. 10 listed in Table II revealed a marked increase in intercellular fluid in the dermis and underlying tissue involving the entire thickness of the wattle. Considerable dilation of the blood vessels and a marked leukocytic infiltration especially of the perivascular areas was observed. No evidence of fibrin deposition as recorded by Long in tuberculin positive skins was detected.

(4) Limits of Reaction. Although it was recognized that the "feel" of the swelling, or reaction, perhaps affords the most satisfactory criterion of interpretation, it was deemed advisable to record observations on the limits of increase of thickness of the wattles of infected and non-infected birds.

The average thickness of the wattles of the White Leghorn and Rhode Island Red breeds as previously recorded allowed calculations to be made on the deviation from the normal thickness of wattles of infected and non-infected fowl following injection of pullorin.

The measurements were taken with a dial micrometer especially adapted to this work. This type of instrument obviates much of the error incident to the use of ordin-

ary calipers.

Figure 2 is a photograph of this instrument. The front view shows the indicator set at the point of average thickness of the wattles of two common breeds of chickens employed in these studies.

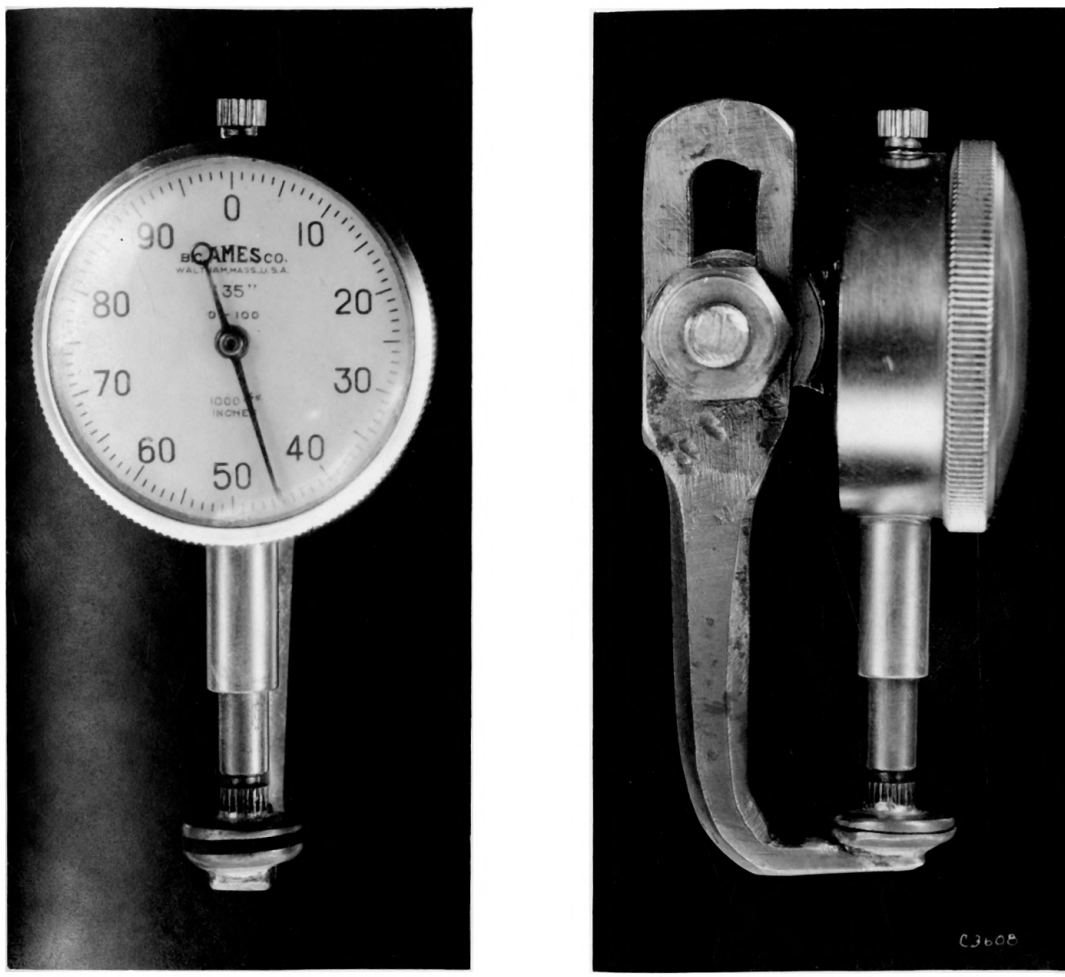

Figure 2. Micrometer especially adapted for measuring wattles.

The following table includes measurements taken at various intervals following intradermal pullorin injection of a group of reacting and non-reacting individuals.

Table V. Limits of swelling and nature of reaction in pullorin positive and negative fowls

Time of readings	Number of bird and measurement of wattle									
	1	2	3	4	5	6	7	8	9	10
Pre-injection	.035*	.052	.039	.038	.065	.047	.059	.047	.046	.047
Post-injection										
1 minute	.045	.075	.047	.065	.081	.055	.080	.072	.068	.060
1 hour	.046	.069	.046	.063	.097	.079	.090	.084	.083	.067
2 hours	.047	.078	.060	.069	.111	.085	.101	.095	.106	.070
3 "	.046	.078	.068	.073	.107	.095	.100	.091	.125	.067
4 "	.058	.071	.073	.073	.112	.083	.098	.093	.137	.077
5 "	.045	.069	.073	.079	.100	.087	.080	.085	.125	.083
6 "	.052	.067	.065	.072	.100	.085	.080	.088	.113	.085
7 "	.046	.075	.070	.071	.096	.085	.082	.081	.103	.085
8 "	.048	.076	.070	.071	.101	.090	.081	.080	.105	.080
10 "	.050	.077	.065	.065	.096	.085	.084	.086	.101	.079
12.5 "	.045	.061	.055	.064	.090	.086	.079	.070	.096	.077
22 "	.040	.050	.040	.050	.090	.099	.089	.100	.105	.098
24 "	.050	.054	.385	.045	.085	.093	.078	.089	.104	.097
26 "	.050	.052	.039	.042	.092	.065	.065	.075	.095	.102
31 "	.035	.052	.036	.046	.077	.049	.050	.075	.070	--
Interpretation	-	-	-	-	+	+	+	+	+	++

* Wattle thickness recorded in inches.
\- Negative to test.
\+ ++ Positive to test.

The measurements recorded show that the early swelling in non-infected as well as infected individuals may exceed in size that of the later specific reaction in infected fowl. However, as previously shown, the increase in thickness fails to indicate the true nature of the swelling inasmuch as early swellings were palpably firmer than the later reactions. The fact that only one of this limited group of birds gave a reaction greater than a one plus as interpreted by palpation may indicate that the differences obtained in this experiment between infected and non-infected fowls is not as great as the average. However, in this group the average swelling for infected fowl on taking the diagnostic reading at 22.5 hours following injection was 0.0087 inch as compared to 0.0458 inch for the infected lot. The increase in thickness in the infected birds corresponds approximately to the thickness of a normal wattle which is interpreted as a one plus reaction.

The reaction, manifested by swelling, is indicated by the curves for the infected and non-infected groups as plotted in Figure 3.

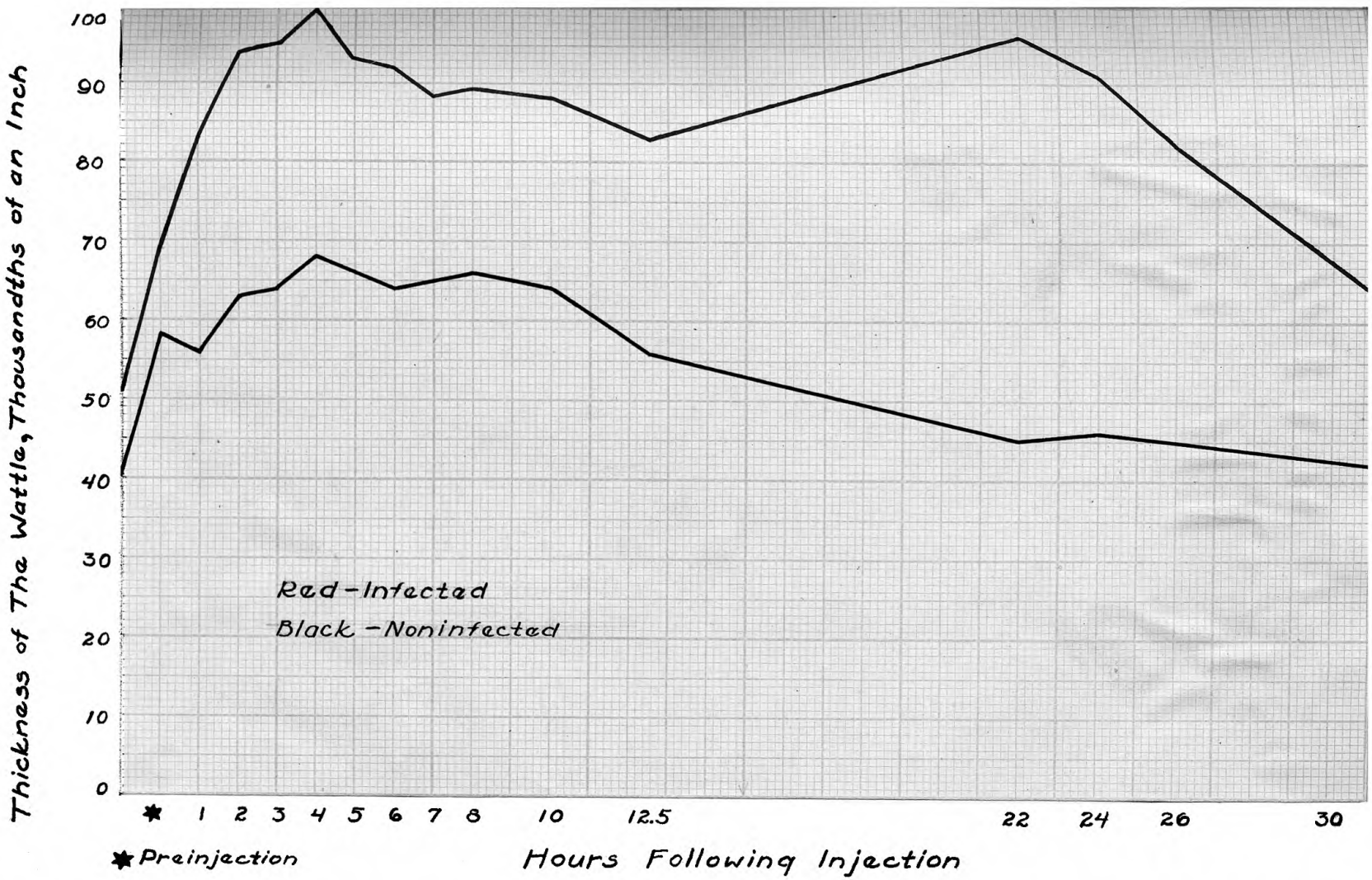

Fig.3. Reaction Curve of Infected & Non Infected Fowl as Measured By Swelling of Wattle

The average swelling of the infected and non-infected group is well illustrated by the plotted curves. The initial or non-specific swelling in the infected group was somewhat greater than in the non-infected lot but no significant differences could be observed by palpation alone. The increase in thickness of the wattle of infected fowls from the twelfth to the twenty-second hour is obviously a manifestation of specific reaction due to hypersensitiveness of infection and is in direct contrast to the decrease in swelling or size in non-infected birds. The swellings in both groups had shown a marked fall at the thirty-first hour reading although the infected group had not approached the normal thickness to the same extent the healthy group had. Whether this decrease in swelling was later followed by another increase in some individuals as indicated by the results obtained in the studies summarized in Table IV was not determined. It is probable that the swelling may be more persistent in response to different types of pullorins although the presence of a significant swelling at 22 to 24 hours following injection warrants the observation that the specific reaction is persistent as compared to the non-specific early reaction.

(c) Specificity of Reaction. Because closely related organisms manifest common, as well as highly specific antigenic factors, pullorins prepared from various strains of S. pullorum and, similar products made from closely related genera and species of organisms were tested.

A breeding flock of 104 Rhode Island Reds negative to the agglutination test was tested with "digest" and "cellular" pullorin. Five, or 5.68 percent, pullorin "reactors" were detected in a total of 88 birds tested with "digest" pullorin. In the remaining 16 birds of the flock tested with "cellular" pullorin seven, or 43.75 percent, gave positive reactions.

These results seemed to indicate either a high comparative toxicity or a great lack of specificity, particularly in the "cellular" pullorin.

To determine if the reactions, particularly to the cellular pullorins, may have been induced by the presence of a common antigenic or toxic factor of the "colon-intermediate" group, the entire flock was tested 11 days later with a similar "digest" product prepared from a pathogenic strain of Escherichia coli of avian origin.

On reading the tests 22 hours following injection only six, or 5.75 percent, of the 184 birds showed per-

ceptible swellings of the wattle, and these were not typically edematous as in the case of reactions to pullorin, but were rather firm. In no bird did the swelling exceed a one plus reaction and of this group of six only three had previously reacted to pullorin. Two of these three birds had shown a response to the highly non-specific or toxic cellular pullorin. Later tests with "cellular" pullorin in birds positive to the agglutination test gave a very low correlation which indicated rather a non-specificity of such products.

In testing the relative efficiency of "digest" pullorins prepared from different strains of S. pullorum, a similar digest product prepared from the closely related fowl typhoid organism, S. gallinarum was included in the comparative tests.

In a group of 18 fowls composed of 16 agglutination-positive and two agglutination-negative birds, tested with digest "pullorin" and "gallinarin", the percentage agreement between the former and the agglutination test was 55.5 while the "gallinarin" gave an agreement of 44.0 percent. The factor of normal variation may readily account for these differences in results although they may not point to a species specificity. As there appeared to be no advantage in using S. gallinarum in preparing pro-

ducts to detect S. pullorum carriers no further consideration was given this organism. These observations would, however, emphasize the close relationship of the two organisms, already recognized in serological tests, and would indicate that from a practical standpoint "pullorin" may be valuable in detecting carriers of fowl typhoid so well as those of pullorum disease.

A preliminary test on the relative merits of pullorins prepared from various strains of S. pullorum, which was later continued, included the following procedure and results:

Digest pullorin 33 was made from an old stock strain culture, No. 92, isolated from the ovary of a hen. Pullorin 34 was prepared identically from two strains, 4645 and 4713, freshly isolated from acute pullorum disease in chicks. Strain 92 was typically anaerogenic on glucose while the other two were aerogenic.

Tests with these two products on a flock of 83 birds gave the following correlation with the agglutination test.

Table VI. Results of tests with pullorins prepared from different strains of S. pullorum.

Pullorin	Association coefficient	Percent agreement
33	0.84	78.7
34	0.68	76.5

These results show a similar agreement with the agglutination test and were not considered evidence of species or type specificity. However, in later experiments stock culture No. 92 and other strains isolated from mature birds were used in the preparation of pullorins. Unfortunately, no tests were made with "autogenous" pullorins, i.e., products prepared from cultures isolated from the flock to be tested. Because the experimental reactor flock of the Department was collected from a number of widely separated sources, it was not considered satisfactory for this purpose. However, no other farm or experimental flock that may have harbored infection of a single strain was available for testing.

(d) Comparison of the Pullorin Reaction to the Agglutination Titre and Autopsy Findings of Various Birds.

Although a direct quantitative relationship between various types of antibodies and reactivity to pullorin may not obtain in the same individuals the following data appears to be significant.

Table VII. Comparison of pullorin reaction and agglutination titre.

Agglutination titre	Pullorin positive	Pullorin negative	Total	Percent positive
0	1	3	4 *	25.0
1-20	1	3	4	25.0
1-40	8	3	11	72.7
1-80	10	3	13	76.9
1-160	12	1	13	92.3
1-320	8	1	9	88.8
1-640	2	0	2	100.0
Totals	42	14	56	75.3

* These four birds were negative to the agglutination test.

From "Some experiments on the control of bacillary white diarrhea". L. D. Bushnell and C. A. Brandly, Jour. Amer. Vet. Med. Assn. Vol. 74, No. 4, Mch. 1929, p 450.

These results indicate that there is a much higher correlation between pullorin and agglutination tests in

birds having a high serum titer. This situation would point to similar shortcomings in the two tests because it has frequently been recognized that one of the chief limitations of the agglutination test in its practical application is failure to detect birds having a low serum titer.

A study of the cultural results of autopsy and the reaction to pullorin on two flocks of birds is given in the following table. The pullorin test was made two days before autopsy and agglutination tests in 1-20 dilution made at the same time showed that all birds were reactors. Flock No. 1 was tested with fresh "digest" pullorin and Flock No. 2 with "ecto" pullorin.

Table VIII. Comparison of pullorin reaction and cultural findings on autopsy.

Flock number	Birds examined	Birds positive to pullorin		Birds showing lesions		Percent positive to pullorin and showing lesions	S. pullorum isolated.		Percent positive to pullorin and S. pullorum culture
		No.	%	No.	%		No.	%	
1	51	36	70.6	40	78.4	90.0	25	49.0	69.4
2	62	45	72.6	53	85.5	84.9	37	59.7	82.2
	113	81	71.6	93	82.3	87.1	62	54.9	76.5

This data shows that about 80 percent of the birds which show lesions of the disease reacted to the pullorin tests, while from about one-half of the agglutination reactors it was possible to isolate S. pullorum in pure culture. It indicates also that the pullorin and agglutination tests show about the same correlation as the positive cultural results and the pullorin reaction and, as the pullorin reaction and gross lesions on autopsy.

Positive to pullorin and positive to agglutination	71.6%
Positive for S. pullorum and to pullorin	76.5%
Positive for lesions and positive to pullorin	87.1%
Positive for S. pullorum and positive to agglutination	54.9%

The much greater correlation between the pullorin test and positive S. pullorum findings than the agglutination test and S. pullorum culture would indicate that the pullorin test is more satisfactory than the agglutination test. However, when attention is called to the fact that the cultural examination included only culture of the ovaries and in some cases of only one or two diseased ova the results may not be as significant. Failure to isolate S. pullorum from some cases may have been due to discrep-

ancies in technique and therefore the positive cultural findings should not be considered a satisfactory comparative standard. The work of Broerman (33) and others would indicate that S. pullorum infection in chicks is a better criterion for comparison than any other method, and in these experiments the agglutination gave superior results as compared to the pullorin test.

(e) Effect of Repeated Tests. To determine if one or more pullorin tests resulted in desensitization to early subsequent injections the following procedure was employed. A flock composed almost entirely of birds reacting to the agglutination test was pullorin tested ten different times during a six months period. The results in each of 17 individuals typically positive to pullorin on the first or second test are given in the appended table. The agglutination titre of the serum of these birds before and following the ten tests is also recorded.

Table IX. Results of frequent consecutive tests with pullorin

Bird number	5-20 Agglutination titer	Date of test and results										11-8 Agglutination titre
		5-20	5-30	6-15	6-20	7-2	7-20	7-30	9-7	10-2	11-8	
163	400	2	3	-	3	1	2	1	3	2	2	200
838	50	2	3	-	2	-	2	0	2	3	2	100
142	800	1	3	-	2	0	2	2	2	2	2	100
850	400	2	3	-	1	2	3	2	3	1	0	100
847	50	3	3	-	3	3	1	-	3	1	2	50
116	400	2	2	-	2	2	2	2	3	1	1	400
114	800	3	3	3	3	2	2	1	3	2	1	400
58	100	4	4	3	4	2	3	0	3	1	2	100
845	800	2	1	1	3	2	2	3	2	2	2	200
121	100	1	3	2	3	2	0	0	2	2	1	200
837	50	2	2	2	1	0	2	0	3	2	1	50
844	400	0	1	1	3	1	1	2	3	1	1	200
42	200	2	2	1	3	0	-	0	1	2	1	200
146	400	0	2	1	1	2	-	3	2	2	2	100
837	400	-	2	1	1	3	2	2	3	2	1	200
76	200	-	2	2	2	0	1	3	3	2	0	100
89	800	-	2	2	1	-	2	2	3	1	1	800

- Not tested.
0 Negative to test.
1,2,3,4, - Indicates thickness of swelling as compared to normal wattle.

This data indicates that no perceptible or permanent desensitization followed the use of pullorins although a few irregular negative tests were obtained. Some discrepancy due to the use of different experimental pullorins may be expected inasmuch as seven different lots were used. In those that were used for more than one test the significant effect of aging on these particular "digest" pullorins as noted later in these studies cannot be overlooked in appraising slight discrepancies in reactions. From the results obtained we may conclude that pullorin injection repeated at frequent intervals had little effect on the agglutinating titre of the serum.

(f) Toxicity and Potency Tests. In order to eliminate the use of experimental pullorins that were primarily toxic to uninfected birds, or impotent in eliciting a reaction in infected individuals, the following general procedure was adopted: At least one day previous to the time flock tests were begun the pullorin was tested for toxicity and potency by injecting it into the wattles of a limited number of known positive and negative birds. In certain cases it was desirable that the vehicle for the pullorin be tested for toxicity before adding the active principle from the pullorum organisms. The following

table gives a typical toxicity test of certain compounds before use as a vehicle for pullorin and of toxicity and potency tests of certain pullorins.

Table X. Toxicity test of buffered mixtures and glycerin solutions employed in preparing pullorins.

Buffer mixtures*			Glycerin solution in 0.85% NaCl		
pH	Bird	Results	Percent glycerin	Bird	Result
5.2	5	No swelling	5	1	No swelling
5.4	7	" "	10	2	" "
5.6	18	" "	15	3	" "
5.8	12	" "	20	4	" "
6.0	6	" "	30	5	Slight "
6.2	4	" "	50	6	" "

* Potassium hydrogen phthalate and NaOH.

Table XI. Potency and toxicity test of digest pullorins

Bird number	Agglutination test	Pullorin test		Bird number	Agglutination test	Pullorin test	
		Pullorin 42	Pullorin 43			Pullorin 44	Pullorin 45
12	+	1+	0	16	+	2+	1+
13	+	1+	0	17	+	3+	2+
14	+	2+	1+	18	+	0	1+
15	+	3+	2+	19	+	2+	1+
1	-	0	0	7	-	0	0
2	-	0	0	8	-	0	0
3	-	0	0	9	-	0	0
4	-	0	0	10	-	0	0
5	-	0	0	11	-	0	0
6	-	0	0				

1+,2+,3+ Indicates thickness of swelling as compared to normal wattle.
0 Negative to pullorin test.
+ Positive to agglutination test
- Negative to agglutination test.

These particular tests showed that 30 and 50 percent glycerin solutions may alone produce a slight thickening of the skin although not a swelling which might be considered a one plus reaction to pullorin.

The tests on four lots of pullorin indicated that they were non-toxic to apparently non-infected fowls, while pullorin No. 43 apparently contained more of the pullorin-active principle than the others.

(g) Double "Intradermal" Test. The merits of the double "intradermal" test as compared to the ordinary single injection test were briefly studied by reinjecting the group of ten birds employed in studying the nature and limits of the pullorin reaction. (See Tables III and V).

After taking final readings on the single injection test all of the birds except No. 10 were reinjected with the same pullorin by introducing the needle into the center of the swelling (if any remained) and by gentle pressure on the syringe expelling about the same amount of pullorin as ordinarily used. Where no swelling had persisted, the injection was made as near as possible into the same area previously injected.

The following data indicates the time of readings, size of reaction, etc.

Table XII. Results of double intradermal test.

Time of readings	Number of bird and measurement of wattle								
	1	2	3	4	5	6	7	8	9
Pre-injection	.035	.052	.036	.046	.077	.049	.050	.075	.070
Post injection									
1 minute	.038	.059	.053	.046	.077	.073	.073	.091	.077
2 hours	.038	.088	.050	.050	.093	.089	.095	.094	.100
5 "	.038	.097	.042	.045	.092	.082	.085	.096	.078
16 "	.036	.081	.039	.049	.091	.086	.073	.079	.066
22 "	.035	.071	.037	.046	.089	.069	.051	.068	.075
Interpretation of reaction	0	+	0	0	+	0	0	0	0
Reaction to single injection	0	0	0	0	+	+	+	+	+

Swellings recorded in inches.
Interpretation made by palpation of wattle.

The data recorded in Table XII indicate that the double injection test is not nearly so satisfactory as the single injection method. Bird No. 2 previously negative to the pullorin as well as the agglutination test gave a positive test as determined by palpation of the wattle. Furthermore, only one bird No. 5 that had reacted to the single injection was classified as a reactor following double injection.

On observing the wattles of the agglutination positive birds at 16 and 22 hours a loss of tone generally accompanied a decrease in swelling. This was in direct contrast to the increase in size as recorded by the micrometer following the first pullorin injection.

Inasmuch as all interpretations of test results were made on the basis of the "feel" of the wattle to the touch discrepancies may appear to be present in some cases if the measurements of the wattle are taken as an indicator. Bird No. 6, classed as negative, shows a greater swelling from the normal at 22 hours than does No. 5 which was classed as pullorin positive. However, on palpating the wattle of No. 5 it seemed to possess more tone and the edema, manifested by smoothness of the skin

surfaces, was less diffuse than in the case of No. 4. The character of edema and swelling of a more or less diffuse yet not perceptibly atonic nature was obviously the basis of a positive interpretation.

A loss of tone may be the result of over stimulation of the tissues or transient desensitization to a product which may be highly toxic only because of very rapid early splitting.

The positive reaction in bird No. 2 may apparently have been infection or some type of injury other than that due to pullorin injection because none of the balance of the agglutination negative individuals became positive to pullorin.

(h) The Ophthalmic Test. In order to determine if any perceptible or significant reactions followed the conjunctival instillation of pullorins and to duplicate the work of Ward and Gallagher (19) with types of pullorin other than those used by them, a group of 53 birds was tested with digest pullorin on May 20, 1929. A drop of the pullorin used for wattle injection was allowed to fall into the bird's eye as the head was held in a horizontal position. On subsequent observation in 22 hours a perceptible change from the normal was observed only

in one bird. The treated eye showed general congestion of the conjunctiva with a slight serous exudate but the lesion in this particular individual was not considered as a probable specific ophthalmic reaction to pullorin inasmuch as the "test" dose given at this time (24 hours following the "sensitizing" dose) did not enhance the reaction. None of the other birds showed any perceptible reaction on observation at 24 hours following the introduction of the "test" dose.

3. Tests With Pullorins From Other Sources.

In the earlier experiments various types of pullorins from commercial sources and from Experiment Stations were used for comparative study in field and experimental flocks. The results of the test with these products, summarized in the following table, indicates their efficiency as compared to the agglutination test. The merits of these agents as compared to pullorins prepared in our laboratory are recorded in Section 4.

The C pullorins were purchased from commercial biological firms and the E. pullorin was obtained from Dr. Robert Graham of the Illinois Experiment Station. The precipitated pullorins were apparently similar al-

cohol precipitate products of broth cultures of S. pullorum. The cellular pullorins were saline suspensions of S. pullorum. Pullorin C 5, on staining yielded typical gram negative rods resembling S. pullorum and the turbidity of the suspension approximated that of tube one, McFarland's nephelometer.

Table XIII. Results of tests with pullorins from other sources

Type pullorin	Number tests made	Flock number	Percent agreement with agglutination
E 1 - Precipitated	62	6	69.31
C 2 - Precipitated	60	6	80.00
C 3 - Cellular	133	13	76.70
C 4 - Precipitated	143	15	60.80
C 5 - Cellular	203	20	87.60
C 6 - Broth culture	121	18	52.1
Total	722		72.0

In the use of these pullorins the highest percent agreement with the agglutination test was obtained with a cellular pullorin while one lot of the precipitated product gave the next highest correlation. The fact that the best results were obtained in flocks with a low proportion of agglutination reactors makes these figures less significant because any non-toxic product low in or entirely lacking in potency would give a relatively high correlation.

4. Tests With Pullorins Prepared in Our Laboratory

In the pullorin tests recorded in these studies three types of pullorins were used, namely, ecto, cellular and digest. Included with the digest pullorins is one lot of a product of similar nature prepared in a manner almost identical to that employed by Michael and Beach (6) and termed by them "cell solution" pullorin. Modifications of a general procedure for the preparation of each type are recorded in the following descriptions. Additional manipulation of the product in its manufacture are also described.

To prepare these products certain strains of S. pullorum of the S type were grown on plain or modified agar

at 35-37C for a period of 24-72 hours. Immediate suspension of the cultures in sterile physiological salt solution or distilled water was followed by centrifugalization. The centrifugated cells were then treated according to the general method employed for the type of pullorin to be prepared.

(a) Preparation of Ecto Pullorins. These products were prepared according to the method described by Bushnell and Hudson for ecto-antigen (36). These antigens were first used as pullorins by Bushnell (20).

Preliminary tests with these products showed that they were not highly antigenic as determined by repeated intravenous injection into rabbits and chickens and subsequent serological tests.

After centrifugation of the suspension of S. pullorum growths on Kolle flasks of nutrient agar medium, the supernatant fluid was decanted and a quantity of fresh sterile physiological saline was added to each tube. Suspension and "washing" of the cells was accomplished by repeatedly aspirating into and expelling the tube contents from a 10 cc glass pipette fitted with a rubber bulb. The suspension in each tube was drawn into and expelled from the pipette at least 50 times. The suspension was

again centrifugated and the first washing discarded. The washing process was repeated and the various washings were transferred to separate flasks, sterilized and tested to determine their value as pullorins.

Unless other-wise indicated in the following description the ecto-pullorins used in these studies were the washings obtained from a suspension of nine parts of saline, and one part of cells. Sterilization was accomplished by autoclaving at 20 pounds pressure for 12 minutes. Cultures 144, 192 and 137 were used in equal proportions in preparing these products.

Pullorin 9 - 2nd washing.
Pullorin 15 - 3rd washing.
Pullorin 16 - 3rd washing - (14 vol. saline + 1 vol. cells).
Pullorin 17 - 4th washing
Pullorin 18 - 3rd washing - (4 vol. saline + 1 vol. cells).
Pullorin 19 - 4th washing
Pullorin 20 - 2nd washing - (14 vol. saline + 1 vol. cells).
Pullorin 21 - 4th washing - (4 vol. saline + 1 vol. cells).
Pullorin 22 - 5th washing.
Pullorin 23 - 3rd washing - (4 vol. saline + 1 vol. cells).
Pullorin 30 - 3rd washing - (3 vol. saline + 1 vol. cells).

The process of autoclaving the ecto-pullorins rendered them slightly more cloudy and a fine precipitate was formed in practically all of the products. No effort

was made to separate the sediment.

In preliminary tests sterilization by filtration or by the addition of 0.5 percent carbolic acid did not enhance the potency or efficiency of the pullorins over those sterilized by heat.

Concentration by partial or complete evaporation at atmospheric or at reduced pressure at a temperature of 50 to 60C did not produce a better agent than the usual unconcentrated product.

Subjection to repeated alternate freezing and thawing of the cell suspension previous to centrifugalization failed to increase the potency of resulting ecto-pullorins as compared to those not treated in this manner.

The results of ecto pullorin tests listed in Table XV failed to show that early washings were more efficient than later washings and also that a decrease in the proportion of saline to S. pullorum cells did not enhance the potency or value of ecto pullorins. This apparent failure to concentrate the reactive principle was obviously also encountered in the preliminary efforts at concentration by evaporation. The apparent low or negligible toxicity of the ecto pullorins for the tissues of birds negative to the agglutination test did not over-

balance the discrepancies resulting from doubtful or indefinite reactions elicited in birds positive to the agglutination test and, consequently, the preparation and testing of cellular and digest pullorins was begun.

(b) Preparation of Cellular Pullorins. In the preliminary and supplementary work pertaining to these studies, cellular pullorins from commercial sources as described under 3, Section III, as well as cellular pullorins prepared in our laboratory were employed.

In the preparation of the cellular pullorins used early in these studies various concentrations of washed S. pullorum cells in saline were sterilized by heat, formalin, phenol and chloroform. As the chemically treated products did not prove to be superior to those subjected to heat only one lot of the latter product was used in the tests recorded in Table XV.

Pullorin 31 - 24 hour culture S. pullorum 137, washed twice and diluted with saline to a density of tube five McFarland's nephelometer. Sterilized by subjection to chloroform vapor for 30 hours at 37C.

Pullorin 59 - 24 hour culture S. pullorum 137, washed twice and one part cells added to nine parts of pH 5.0 buffer (potassium hydrogen phthalate - NaOH) solution, autoclaved at 15 pounds pressure for 10 minutes.

Pullorin 60 - same as 59 except that diluent was pH 6.0 buffer (phthalate - NaOH) solution.

The high percentage of reactions elicited with these products in birds negative to the agglutination tests as determined in the basic studies on this problem and in the tests with commercial products did not seem to warrant further use of cellular pullorins.

The cellular pullorins although lacking the apparent specificity of the ecto pullorins elicited a much more marked reaction of the tissues than the latter.

(c) Preparation of Digest Pullorins. S. pullorum cultures, that had been washed in saline two or more times, were treated with alkalies of various concentration, the "digest" being centrifugated in some cases and the supernatant fluid added to a diluent or, the entire "digest" was diluted to make the finished pullorin. Modifications as listed below include changes in the proportion of washed cells and alkali or "digesting" agent, changes in the length of time of alkali treatment, the use of a wide variety of diluents of the digested pullorum cells and the employment of different means of sterilization and preservation.

A brief description of the method of preparation of the 80 digest pullorins used in this work is included in Table XIV.

Table XIV. Preparation of digest pullorins.

Pullorin number.	S. pullorum strain.	Concentration of washed cells before digestion.	Digesting agent, proportion of cells to digest and time of digestion	Diluent and proportion of digest to diluent.	Sterilization.	Additional treatment
24	92	Undiluted	N/10 NaOH, 1 part to 2 NaOH 15 minutes	Saline, 1 SD+19	(b) Autoclaved	
32	92	Diluted 1x	N/10 NaOH, 3-5 15 minutes	Saline, 1 SD+10	"	
33	92	"	N/10 NaOH, 1-6 10 minutes	Saline, 1 + 10	"	
34	4713 4645	"	"	"	"	
35	92 137 144	(a) Diluted 6x	N/10 NaOH, 1-6 20 minutes	"	"	
36	"	"	N/10 NaOH, 1-6 30 minutes	"	"	
38	92 137 4713 4645	"	N/20 NaOH, 1-6 40 minutes	"	"	
39	"	"	N/10 NaOH, 1-6 20 minutes	"	"	
40	"	"	N/10 KOH, 1-6 20 minutes	"	"	
41	"	"	N/10 NaOH, 1-6 20 minutes	"	"	
42	"	Diluted 3x	N/10 NaOH, 1-6 20 minutes	"	"	
43	92 137	Diluted 3x	N/10 KOH, 1-6 20 minutes	Saline 1 D+9	"	
46	137	Diluted 6x	N/10 NaOH, 1-6	Distilled water 1 D + 9	"	
47	137	"	"	Locke's solution 1 D ± 9	"	
48	137	"	"	Distilled water 1 D + 9	"	Adjusted to pH 6.0 with CH_3COOH after autoclaving
49	137	"	"	Distilled water + chicken serum (5%) 1 D + 9	"	Adjusted to pH 7.0 and serum added after autoclaving.
50	137	"	"	Distilled water + Gelatin (5%) 1 D + 9		Adjusted to pH 7.0 and gelatin added after autoclaving.
53	137 92	Diluted 1x	N/10 NaOh, 1-6 30 minutes	pH 5.0 buffer 1 D + 9	"	
54	"	"	"	pH 6.0 buffer 1 D + 9	"	
55	"	"	"	pH 3.0 buffer (Precipitate)	"	Precipitate washed 3x and redissolved in pH 8.0 saline.
56	"	"	"	pH 4.0 buffer (Precipitate)	"	"
55A	137	Undiluted	N/10 NaOH, 1-6 2 minutes	pH 5.0 buffer 1 D + 9	"	
56A	"	"	N/10 NaOH, 1-6 5 minutes	"	"	
57	"	"	N/10 NaOH, 1-6 10 minutes	"	"	
58	"	"	N/10 NaOH, 1-6 30 minutes	"	"	
61	"	"	N/10 NaOH, 1-6 7 minutes	pH 5.0 buffer 1 D + 9	"	
62	"	"	"	pH 6.0 buffer 1 D + 9	"	
63	"	"	N/10 NaOH, 1-6 7 minutes	pH 3.0 buffer 1-9 (precipitate)	"	Precipitate washed and diluted 0.5% HCL to original volume.
64	"	"	"	pH 4.0 buffer 1-9 (precipitate		Precipitate washed with 6.0 buffer to original volume.
65	"	(m) "	N/10 NaOH, 1-6 10 minutes	pH 4.6 b[illegible] 1-9	"	
66	"	(m) "	"		0.5% formalin	
67	"	(m) "	"	pH 5.0 buffer 1-9	Autoclaved	
68	"	(m) "	"	"	0.5% formalin	
69	"	(m) "	"	pH 5.6 fubber 1-9	Autoclaves	
70	"	(m) "	"	"	0.5% formalin	
71	"	(m) "		pH 6.0 buffer 1-9	Autoclaved	
72	"	(m) "	"	"	0.5% formalin	
82	"	(m) "	"	pH 5.0 buffer 1-9	autoclaved	
83	"	(m) "	"	"	Autoclaved 15 lbs-10 minutes + formalin	
84	"	(m) "	"	"	Autoclaved + 0.3% formalin	
85	"	(m) "	"	"	Autoclaved + 0.4% formalin	
86	"	(m) "	"	"	0.2% formalin	
87	"	(m) "	"	"	0.3% formalin	
88	"	(m) "	"	"	0.4% formalin	
88A	"	(m) "	N/10 NaOH, 1-6 10 minutes	pH 5.0 buffer 1-19	Autoclaved	
89	"	(m) "	"	"	Autoclaved + 0.2% formalin	
90	"	(m) "	"	"	Autoclaved + 0.3% formalin	
91	"	(m) "	"	"	Autoclaved + 0.4% formalin	
92	"	(m) "	"	"	0.2% formalin	
93	"	(m) "	"	"	0.3% formalin	
94	"	(m) "	"	"	0.4% formalin	
95	"	"	"	(x) pH 6.0 buffer + 5% glycerin 1-14	0.3% formalin	
96	"	"	"	(x) pH 6.0 buffer + 10% glycerin 1-14	"	
97	"	"	"	(x) pH 6.0 buffer + 15% glycerin 1-14	"	
98	"	"	"	(x) pH 6.0 buffer + 20% glycerin 1-14	"	
99	"	"	"	(x) pH 6.0 buffer + 30% glycern 1-14	"	
100	"	"	"	(x) pH 6.0 buffer + 50% glycern .-14	"	
101	"	"	"	(x) pH 6.0 buffer + 50% glycern 1-14	Autoclaved	
102	"	"	"	(o) pH 6.0 buffer 1-14	0.3% formalin	
104	137 40	"	N/10 NaOH, 1-6 8 minutes	pH 7.0 buffer + 10% glycerin 1-14	0.2% formalin	
105	"	"	"	"	0.3% formalin	
106	"	"	"	pH 6.0 buffer + 10% glycerin 1-14	0.3% formalin	
107	"	"	"	pH 6.0 buffer + 50% glycerin 1-14	0.3% formalin	
108	"	"	"	"	0.2% formalin	
110	"	"	"	pH 6.0 buffer + 50% glycerin 1-14	Autoclaved	
111	"	"	"	(o) pH 6.0 buffer + 50% glycerin 1-14	0.2% formalin	
112	"	"	"	(o) pH 6.0 buffer + 10% glycerin 1-14	0.3% formalin	
113	"	"	N/10 NaOH, 1-3 5 minutes	pH 5.4 buffer 1+9	0.25% formalin	
114	"	Diluted 1x	"	pH 5.4 fubber 1+14	"	
115	"	"	"	pH 5.4 buffer 1+19	"	
116	"	"	"	pH 5.4 buffer 1+24	"	
117	"	"	"	pH 5.4 buffer 1+29	"	
118	"	"	N/10 NaOH, 1-6 4 minutes	pH 5.4 buffer 1+24	"	
119	"	"	"	pH 5.8 buffer 1+24	"	
120	"	"	"	pH 6.2 buffer 1+24	"	
121	"	"	"	pH 8.3 buffer 1+19	"	
122	"	"	"	"	Filtered through Berkefeld V	
123	"	"	"	Saline 1+19 Adjusted pH 7.1 with HCl	0.25% formalin	
124	"	"	"	"	Filtered through Berkefeld V	

(a)- Autoclaved at 15 lbs. pressure for 10 minutes.
(b)- 22 day stock antigen in 0.5% phenolized saline
(c)- Formalin added and pullorin held at 37C for 12 hours and then tested for sterility.
SD - Digest supernatant.
(o)- KH_2PO_4 - Na_2CO_3 buffer mixture.
(m)- Pullorins 65-94 grown on special agar.
(x)- KH_2PO_4 - NaOH buffer mixture.
All pH 3.0 buffers potassium hydrogen phthalate-HCl mixtures.
All pH 4.0 - 6.2 buffers potassium hydrogen-NaOH mixtures.

Pullorin 125 was prepared from 24 hour S. pullorum cultures (137 and 140) which had been washed twice in saline and the sedimented cells diluted with distilled water to a turbidity reading of 2 mm. on the Gates nephelometer. The suspension was then adjusted to pH 9.1 with N/10 NaOH, shaken vigorously for two hours at room temperature and then adjusted to pH 7.0 with N/10 HCl. The material was then alternately frozen and thawed six times by melting in warm water after freezing in the chamber of an electric refrigerator. Following this process the material was allowed to stand at ice box temperature for 24 hours and then filtered through a Berkefeld candle. Sterility test taken at this time was negative.

The earlier tests with digest pullorins indicated that although they were more toxic in fowls negative to the agglutination test than were the ecto pullorins, they possessed the distinct advantage of eliciting more definite and better marked reactions in birds reacting to the agglutination test than did the ecto products.

The encouraging results obtained on the preliminary tests with digest pullorins led to numerous attempts to

stabilize and improve these products. The results of the tests with each pullorin are given in Table XV.

The results obtained in comparing pullorins prepared from different strains of S. pullorum bore out the observations on this question made in the basic studies (C 2, Section III). No significant differences were detected in the results of various tests with pullorins prepared from one or a combination of S. pullorum cultures of aerogenic and anaerogenic types including numbers 92, 4713, 4645, 137, 144 and 40.

The effect of the medium upon which the S. pullorum cultures were grown as related to the efficiency of the pullorin prepared from these cultures was considered and consequently the organisms used in pullorins 65 to 94 inclusive were grown on a special "salts" agar medium. This medium was prepared by mixing the following ingredients, autoclaving the mixture to bring about solution and then adjusting to pH 7.4 before placing in Kolle flasks for final sterilization.

Meat extract	3 gms.
Bacto pepton	20 gms.
Bacto agar	20 gms.
Distilled water	1 liter.
Ammonium (diabasic) phosphate	0.6 gm.
Potassium citrate	1.6 gm.
Ferric citrate (soluble)	0.3 gm.
Potassium bicarbonate	0.5 gm.

No significant differences in the efficiency of the pullorin based on cultures grown on the special media and on ordinary meat extract agar were observed. However, the special agar produced somewhat heavier growth in the usual 24 to 48 hour period.

To determine the value of certain alkalies for the digestion of the S. pullorum, NaOH, KOH and $Na_2 CO_3$ were employed in various strengths and for different periods of time. Sodium carbonate was found to be decidedly inferior to the other two agents. Potassium hydroxide showed no advantages over sodium hydroxide and consequently the latter product was used almost exclusively. A N/10 NaOH solution in the proportion of six volumes to one volume of cells for a period of five to ten minutes gave better results than more dilute or more concentrated solutions allowed to act for longer or shorter periods of time. Efforts at concentration of the active principle of pullorin were no more successful than in the case of the ecto pullorins while in the experiments with the digest products primary toxicity of too greatly concentrated digests was encountered.

In the attempts to elicit reactions with pullorins sufficiently dilute to prevent non-specific reactions it

was necessary to consider stabilization of the finished products.

The rapid deterioration of digest pullorins employing physiological saline as a diluent indicated that digestion was not completely inhibited on dilution but that it continued until complete denaturation or destruction of the reactive pullorum antigen was effected. The effect of adjusting the diluted digest to the neutral point or to an acid reaction was studied in numerous products but no great increase in stability was observed. The use of buffer solutions of various pH value indicated that they were more effective than saline or other diluents in preventing rapid deterioration in potency. The addition of glycerin and formalin to prevent the injected pullorin from being absorbed too rapidly was apparently not uniformly successful or desirable.

In addition to the local styptic effect of very dilute formalin solutions this agent in low dilutions readily sterilized the product. However, no great differences in the results of heat killed and formalin killed pullorins is detectable in the data recorded.

(d) Flock Test Results. A summary of the results obtained from the use of 93 pullorins on 161 groups of birds 46 flocks over a period of three years are recorded in

Table XV. The comparisons with the agglutination test are listed in percent agreement with the latter test. Only the results with pullorins prepared in our laboratory are included in this table.

Table XV. Comparison of pullorin and agglutination tests.

Date	Pullorin	Number of tests made	Flock number	Percent agreement
7-18-27	E9	105	1	95.7
9-8-27	E16	51	2	72.5
9-9-27	E16	79	3	97.4
8-2-27	E15	80	4	80.0
10-5-27	E17	100	5	92.0
10-10-27	E17	43	5A	74.4
12-13-27	E18	92	7	71.7
12-13-27	E19	116	8	82.7
12-13-27	E20	161	9	78.4
12-20-27	E21	581	10	72.5
1-6-28	E22	66	11	50.0
1-11-28	E23	148	12	54.5
1-12-28	E23	133	14	83.5
1-12-28	24	25	16	72.0
1-12-28	E23	143	17	74.1
2-5-28	32	112	19	76.1
1-25-28	30	375	20	92.0
1-25-28	C31	72	20	79.1
1-25-28	32	485	20	85.7
10-4-28	33	33	21	78.7
10-4-28	34	47	22	76.5
10-10-28	34	43	23	97.6
10-10-28	33	52	23	100.0
10-14-28	34	38	24	65.8
10-14-28	33	41	24	80.4
11-13-28	35	73	26	84.9
"	36	34	26	91.1
11-17-28	36	84	27	84.0
"	36	131	27	83.2
11-19-28	38	34	28	79.4
"	40	34	28	85.3
"	39	79	28	86.0
"	41	79	28	87.3
11-20-28	39	74	29	89.1
"	41	74	29	90.5
"	40	48	29	89.6
"	38	48	29	89.6
11-30-28	42	152	30	80.2
"	43	186	30	89.7
12-1-28	43	38	31	89.4
"	42	90	31	94.4
5-20-29	46	9	32	66.6
"	47	11	32	36.6
"	48	12	32	75.0
"	49	12	32	66.6
5-29-29	50	8	32	50.0
"	54	30	32	80.0
"	53	22	32	81.8
6-16-29	53	21	33	47.6
"	54	23	33	39.1
"	55	6	33	33.3
"	56	6	33	16.6
6-21-29	55A	22	34	90.9
"	56A	22	34	95.4
"	57	22	34	95.4
"	58	22	34	95.4
"	C59	18	34	[illegible]
"	55A	8	35	75.0
"	56A	8	35	87.5
"	57	8	35	87.5
"	58	10	35	90.0
"	C59	12	35	75.0
"	C60	8	35	75.0
7-21-29	55A	9	36	77.7
"	56A	8	36	62.5
"	57	8	36	75.0
"	58	8	36	75.0
"	C59	8	36	62.5
"	C60	4	36	100.0
"	61	8	37	87.5
"	62	8	37	75.0
"	63	10	37	80.0
"	64	11	37	100.0
7-30-29	61	7	38	85.7
"	62	9	38	77.7
"	63	13	38	61.5
"	64	12	38	50.0
8-7-29	65	4	39	75.0
"	67	3	39	40.0
"	69	7	39	85.7
"	68	7	39	100.0
"	70	5	39	80.0
"	71	5	39	80.0
"	72	5	39	100.0
9-8-29	72	11	40	54.5
"	70	12	40	66.6
"	68	13	40	84.6
"	66	14	40	71.4
"	71	15	40	93.3
"	69	15	40	86.6
"	67	14	40	92.8
"	65	15	40	93.3
9-20-29	82	25	42	43.4
"	83	19	42	73.1
"	84	21	42	71.4
"	85	17	42	58.8
"	86	19	42	52.6
"	87	21	42	66.6
"	85A	23	42	56.5
"	88	17	42	76.4
"	89	18	42	61.1
"	90	18	42	50.0
"	91	21	42	66.6
"	92	18	42	61.1
"	93	18	42	55.5
"	94	21	42	66.6
10-3-29	71	12	43	83.3
"	72	12	43	75.0
"	84	15	43	80.0
"	86	13	43	76.9
"	87	12	43	83.3
"	88	13	43	69.2
"	90	15	43	66.6
"	92	13	43	53.9
"	93	12	43	66.6
"	94	13	43	84.6
10-4-29	72	32	44	56.2
"	93	47	44	76.5
"	95	23	44	73.9
"	96	21	44	85.7
"	97	19	44	52.6
"	92	20	44	45.0
"	99	25	44	56.0
"	100	37	44	62.1
"	101	20	44	70.0
"	102	49	44	48.9
10-17-29	104	15	45	86.6
"	105	18	45	94.4
"	106	18	45	83.3
"	107	18	45	44.4
"	108	19	45	52.6
"	110	19	45	78.9
"	111	16	45	62.5
"	112	40	45	77.5
10-31-29	113	30	46	80.0
"	114	30	46	80.6
"	115	30	46	90.0
"	116	30	46	90.0
"	117	45	46	93.3
10-31-29	113	30	47	80.0
"	114	30	47	96.6
"	115	30	47	90.0
"	116	30	47	90.0
"	117	32	47	90.0
11-8-29	115	49	48	40.8
"	116	31	48	74.1
"	117	33	48	66.6
11-15-29	118	28	48	78.5
"	119	28	49	89.3
"	120	28	49	75.0
2-7-30	121	63	50	87.5
"	122	29	50	100.0
"	123	57	50	88.3
"	124	108	50	94.2
"	125	69	50	98.5
2-9-30	121	28	51	38.3
"	122	37	51	51.3
"	123	24	51	70.8
"	124	53	51	47.1
"	125	36	51	38.8

Total lots of pullorins used	93
Total number tests made.....	7093
Total flocks tested.....	46
Agreement with agglutination tests.....	81.08

In consulting the data in Table XV a wide variation in flock test results is observed. The variation with ecto pullorins ranges from as low as 50 percent agreement with the agglutination test in flock 11 (pullorin 22) to 97.4 percent correlation in flock 3 (pullorin 16). Flock 3 harbored only one reactor to the agglutination test (2.6%) while in flock 11, 33 birds or 82.5% reacted to the agglutination test. No reactors to the pullorin test were detected in flock 3 while in flock 11, 40 reactors (60.6%) to the agglutination test were detected. These facts would point to a discrepancy in ecto pullorin tests similar to that observed in the use of the pullorins obtained from sources outside of our laboratory.

The results obtained with three lots of cellular pullorins, numbers 31, 59 and 60, show a considerable variation in different flocks. The apparent non-specificity or toxicity of cellular products as observed in the basic studies on specificity (2, C, Section III) seemed to be manifested to a lesser extent in these cellular products.

The wide range of variation obtained in testing with the same and different lots of digest pullorin may be accounted for, to a large extent, by the instability of these

products. At the same time the greater correlation between the digest pullorin and agglutination tests in flocks harboring only a low percent of agglutination reactors may be pointed out. This circumstance was also observed in tests with ecto pullorins obviously low in potency. Instability of the digest products as manifested by a rapid loss of strength on aging is readily observed in some of the flock test results recorded in Table XV. The low correlation obtained with products showing a marked loss of potency has lowered the average for digest pullorins.

It was particularly because of discrepancies due to instability of the digest pullorins that the various modifications in preparing these products as described 4, c Section III were carried out. A relatively high efficiency was obtained in early tests with these products although the results as expressed in percent agreement with the agglutination test frequently do not indicate the true value of the pullorin. An example of probable loss of potency is observed in the results with pullorins 33 and 34. These products were prepared the day before they were used first on flocks 21 and 22. In flock 21 which harbored 29 agglutination reactors the correlation with pullorin 33 was 78.7 percent. In flock 22 which showed 40 agglutination reactors the agreement with pullorum 34 was 76.5 per-

cent. On using these pullorins six days later in flock 23 the agglutination test correlation with 33 and 34 was 100 and 97.6 percent respectively. However, no reactor to either test was detected in either group of flock 23. Four days following the second test of these pullorins they were used in flock 24 with a resulting correlation of 80.4 percent for pullorin 33 and 65.8 percent for pullorin 34. This flock harbored 15.7 percent fewer reactors than the two flocks tested with the fresh pullorin while the correlation in the case of the fresh products was about the same as in the 11 day old product. Potency tests with digest pullorins eliminated numerous products that showed an almost complete loss of potency in 24 hours following preparation.

IV. SUMMARY AND DISCUSSION

The basic studies on the application of the pullorin test and the reaction of the fowl to pullorin indicates that these phases of the problem have previously not been given adequate consideration. Histologic sections of wattles immediately on injection and of normal and "reacting" wattles have shown the immediate fate of the injected material and the nature and extent of the response of the

tissues to pullorins. A summary of the results of these experiments may indicate that further studies of methods of introducing pullorins into the skin, with careful interpretation of results, may accomplish much that efforts directed largely at the preparation of satisfactory pullorins has failed to accomplish. The influence of the depth of the injection on the severity of the reaction has further emphasized the desirability of a method of introduction or injection that will allow more uniform application.

The results of the tests with pullorins employed in this work indicate that readings for the interpretation of results should be taken during the 20th to the 24th hour after injection. A double phase in the pullorin reaction which resembled in its essential features the single injection intradermal tuberculin test in the bovine may indicate that differences are due to the nature of the pullorin as compared to tuberculin and to differences of the tissues of the two species. This active response to irritation may occasionally result in swellings due to trauma being interpreted as reactions to pullorin. In certain flocks it has been observed that trauma resulting from handling fowl in wire catching crates has caused swellings indistinguishable from those following the injection of pullorin.

Although the measurement of the increase in size of the wattle of non-reacting and reacting birds failed to establish probable limits of swelling or reaction it brought out the fact that a considerable swelling of the wattle may occur without it being perceptible to the touch. However, the measurement of the wattle swelling does not indicate the true nature of the reaction and consequently the important characters of smoothness and tenseness of the skin, heat, edema and "feel" of the wattle can be detected only by palpation. These observations on the interpretation of the pullorin test are in accord with those made by the British Medical Research Council on the intradermal tuberculin test.

By applying toxicity and potency tests to diluents and completed pullorins certain unsatisfactory results and products were readily eliminated. The results of the potency tests suggest the possibility of standardization by the same method.

The results of the double injection wattle test with the pullorins used would not warrant observations as to the value of the test because only a few fowls were tested and no duplicate tests with other pullorins were included in this study. The failure to obtain a perceptible

ophthalmic reaction at 18 to 24 hours following conjunctival instillation bears out the observations of Ward and Gallagher on this point.

In summarizing the results of the tests with the three types of pullorins prepared in our laboratory the following percent correlations with the agglutination test were obtained.

1. Pullorins from outside sources (722 tests). 72.0%
2. Pullorins prepared in our laboratory (7093) 81.0%
   a. Ecto pullorins (2333 tests)..... 82.7%
   b. Cellular pullorins (120 tests)..... 95.0%
   c. Digest pullorins (4640 tests)..... 78.9%

These comparative results would seem to demonstrate the superiority of ecto pullorins. These products manifest only a low toxicity, they are easy to prepare and are quite stable but they possess the distinct disadvantage of eliciting many doubtful or indefinite reactions.

The digest pullorins give the next highest correlation. Satisfactory stabilization was not accomplished by themethods employed in these studies but because of the property of eliciting well marked reactions in many agglutination reacting fowls which do not react to ecto pullorins the value of the digest products may be greater than these results indicate. As with the ecto pullorins adequate stand-

ardization of the digest pullorins was not accomplished and consequently the conparative significance of primary toxicity remains undetermined.

The data on cellular pullorins would indicate that these products are not very satisfactory. A suspension of S. pullorum cells, although carefully washed to remove extraneous material, may be considered a relatively crude product. On the other hand, adulteration of the reactive pullorum antigen, a probable result of manipulation in attempts at purification, is not imminent.

Although these results may indicate the relative merits of the various types or groups of pullorins, it must not be overlooked that the agglutination test is by no means a perfect or near perfect standard. It is generally recognized that the agglutination test applied at successive intervals together with proper sanitation and management will eliminate chick mortality from S. pullorum infection. Consequently, any test which shows complete correlation with the agglutination test should equal it in efficiency. On the other hand, the pullorin test has not been shown definitely to be as effective in eliminating chick mortality. Broerman (33) used digest pullorins of the type described here and found that pullorum disease occurred in chicks from a group of hens neg-

ative to pullorin while no S. pullorum infection was detected in chicks from a group of hens negative to the agglutination test.

V. CONCLUSIONS

1. The very thin skin of the wattle of the fowl precludes the possibility of intradermal injection in many cases, with the methods generally employed.

2. The reaction to a single "intradermal" injection of digest pullorin is manifested by a non-specific early swelling in infected as well as non-infected fowl which reaches its maximum size at three to four hours following injection and a later specific reaction which attains its maximum size at an average time of 22.5 hours.

3. The character of soft edema with more or less severe swelling as detected by the touch is a more satisfactory criterion for interpretation of the reaction to pullorin than the increase in the thickness of the wattle as measured with the micrometer.

4. Macroscopically and microscopically the pullorin reaction resembles the tuberculin reaction as manifested in birds and other species.

5. No group activity is manifested in local tissue reactions to antigens prepared from Escherichia coli and

Salmonella pullorum. The close relationship of S. pullorum and Salmonella gallinarum may be demonstrated by tests with "intradermal" agents prepared from them.

6. Frequent repeated tests with digest pullorins does not cause significant desensitization of reactive individuals.

7. Tests for ophthalmic sensitivity to pullorin confirmed the observations of Ward and Gallagher that a response or reaction is seldom manifested.

8. The ecto and digest pullorins used in these studies were more satisfactory than other types prepared in our laboratory or obtained from other sources.

9. In an effort to develop a satisfactory pullorin sources of variation in the test including methods of application and standards of interpretation and comparison must be given major consideration.

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