THE PRODUCTION OF AGGLUTININS IN THE ANIMAL BODY BY THE INOCULATION OF SUBSTANCES OTHER THAN PRODUCTS OF BACTERIAL ORIGIN.¹

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There is to-day among observers working along the lines of immunity a tendency to no longer consider a given anti-body and the factors producing it as a compact inseparable entity following the laws of protoplasmic continuity, but as a complex body that may be split up into component parts, which may exhibit individuality of structure and independence of action. This view of the subject has been given an impetus by the work of Vaughan and later by Obermeyer and Pick. Vaughan derived split products from bacterial proteids and egg-albumen, representing a poisonous and non-poisonous portion. Obermeyer and Pick found by iodizing protein they so changed it that when the iodized portion was inoculated into animals only non-specific precipitins were formed. This led them to believe that specificity in this case was due to the aromatic radical which was changed by iodization.

The following work was begun in 1905 with hope of determining through the nature of substances having the power to produce agglutinins in the animal a more intimate knowledge of the antibodies and their elaboration in the animal economy. This in a measure was realized. As the work progressed the fact developed that, as far as tested, certain molecules or radicals containing such molecules, are always present in the substances, which induce an increase of agglutinin production; this fact strongly suggests the possibility that these molecules are responsible, for one feature at least, of this phenomenon. The observations arrange themselves under the following heads: (1) Organized ferments, (2) unor-

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ganized ferments, (3) metabolic products, (4) putrefactive products, (5) inorganic substances.

Organized Ferments.—Ballner and Sagasser in 1904 succeeded in producing, artificially, agglutinins for *Bacillus typhosus* by inoculating animals with red yeast cells (Rosa hefe), but the yeast cells themselves were not agglutinated by the serum. Acting upon this suggestion of Ballner, I inoculated rabbits with living cultures of brewer's yeast. After four or five inoculations the sera of these rabbits were tested with the following organisms:

1. *B. dysenteriae*, 3 strains (Shiga, Flexner Manila, Park Mt. Desert).
2. *B. typhosus*, 2 strains (Pfeiffer, Mt. Sinai).
3. *B. Coli*, 2 strains (Laboratory, Colon X).
4. *B. paratyphosus*.
5. *B. pyocyaneus*.
6. *B. proteus vulgaris*.
7. *S. cholerae*.
8. *Pneumococcus*, one strain.
9. *Streptococcus*, one strain.
10. *B. mallei*.

The result of inoculations with yeast cell may be shown in the serum of a young goat by the following table.

TABLE I.

Normal Serum				After 8 Inoculations.					
	10	20	50	10	20	50	100	200	500
Shiga	—	—		—	—				
Flexner Manila	+	+	—	++	++	++	++		
Park Mt. Desert	—	—				—			
Pfeiffer	+	—		++	++	++	++		
Colon	+	—		—					

After 13 Inoculations.							After 18 Inoculations.						
	10	20	50	100	200	300	10	20	50	100	200	500	1000
Shiga	—						—						
Flexner Manila	++	++	++	++	++	+	++	++	++	++	++	++	++
Park Mt. Desert		—	—	—	—			—					
Pfeiffer	+	+											
Colon	—						—						

Several rabbits inoculated with yeast cells also gave the same results as the goat. The serum of these rabbits when tested showed a steady increase after inoculation, for the Flexner Manila strain of *B. dysenteriae*, an increase for *B. typhosus* and sometimes for

Colon X strain of *B. coli* with the subsequent disappearance of the two latter. This disappearance of the agglutinins for *B. typhosus* and *B. coli* would seem to indicate one of two things. First, the normal cells of the rabbit possess a greater potentiality for the manufacture of Flexner Manila agglutinins, on account of the cell being subjected to a stronger and more specific stimulation in this direction and therefore when influenced by non-specific substances the cell forms agglutinins for this organism in preference to others because of this stronger initial stimulation. As the inoculations proceed the increment of production for the Flexner Manila strain becomes more marked and finally the entire energies of the cell seem to be occupied in forming these agglutinins to the exclusion of the others.

On the other hand the possibility suggests itself that the stimulating agent may not be as wholly non-specific as generally considered; this phase would then agree with that seen when definite specific organized agents such as bacteria or their products are used, stimulation of common agglutinins being induced. Common agglutinins may continue throughout to be formed to an equal degree along with the specific, or may disappear almost entirely after long immunization. This disappearance or persistence seems to depend somewhat upon the relation of the heterologous organisms to the homologous. The more nearly the species are related the more persistent the common agglutinins.

I have not been able to identify the Rosa hefe used by Ballner and Sagasser. The red yeast mentioned in most works on fermentation is, strictly speaking, a torula and not a true yeast. Several rabbits were inoculated with the cells of the so-called red yeast obtained from the air. This did not give rise to an increase in the production of agglutinins, though later these same rabbits responded to inoculation of brewer's yeast cells. Several strains of brewer's yeast, differing in their action upon beerwort, were tested without presenting any appreciable variation in results. The brewer's yeasts were obtained in pure cultures from the laboratory of the Brewer's Academy, New York City, and were cultivated on 10 per cent. beerwort agar. Living cells were used. Beerwort alone inoculated into rabbits was without effect.

The serum of the young goat mentioned above was subjected to the exhaustion test with *B. typhosus*, the Flexner Manila organism and living brewer's yeast cells—and cells of the torula. The agglutinins for Flexner Manila were completely exhausted by this organism. *B. typhosus* and the yeast cells absorbed all but five per cent. of the Flexner Manila agglutinins. This amount not absorbed approximately represents the amount of normal agglutinins present in the serum, which on account of its more specific nature would resist the action of purely non-specific agents.

Unorganized Ferments.—The possibility that other enzymes might bring about this increase in the animal of preëxisting agglutinins suggested the use of the unorganized ferments, diastase, pancreatin and invertin. The results obtained by the inoculation of these substances coincide with those obtained by the inoculation of yeast cells.

The agglutinins were in each case increased from 1 : 50 to 1 : 500 after five or six, weekly, inoculations. The enzymes, however, failed to absorb the agglutinins thus raised from the homologous serum.

Products of Metabolism.—Nuclein as a component part of the yeast cell was first used. Vaughan obtained a certain amount of protection against the pneumococcus in guinea pigs by the inoculation of nuclein previous to the pneumococcus infection. The nuclein was used in the form of a nucleoproteid from the pancreas, for which I am indebted to Dr. Levene of the Rockefeller Institute for Medical Research. The other products used of the metabolic group were lecithin and proteoses from egg. The same increase of agglutinins for Flexner Manila type of *B. dysenteriae* followed the inoculation of these substances as with yeast and enzymes. There was the initial rise for Flexner Manila type, *B. typhosus* and *B. coli*, with the subsequent dropping out of the two latter and a continued rise of the former as the inoculations were continued.

Products of Putrefaction.—The products of putrefaction tested were indol, skatol, ethyl mercaptan and phenol. I am indebted to Dr. C. A. Herter for the indol, skatol and mercaptan, and also for some valuable suggestions as to their use.

Ethyl mercaptan in one per cent. solution produced an increase of agglutinins in two rabbits after the fifth inoculation and followed the same course as in the cases where the enzymes and metabolic products were used. A control rabbit on injection of ethyl alcohol did not show an increased production of its normal agglutinins. Indol and skatol were without effects, as I expected, on account of the readiness with which they combined with the preformed sulphates and were excreted. Phenol, however, did enter into systemic relation with the organism and toxic effects were demonstrated in the animals inoculated, but a rise of agglutinins did not occur after a number of inoculations, the animals finally dying from excessive abscess formation.

At this point two factors present themselves as possible influences in causing an increase in agglutinins. The first is the increased production and destruction of leucocytes and the second is the fact that the substances bringing about this increase of agglutinins with the exception of the enzymes, concerning whose structure little is known, all possess a formula containing phosphorus or sulphur molecules, while those failing to effect an increase do not possess either element.

First the effect of the inoculations of these substances upon the leucocytes may be considered. Dieudonné claims that animals possessing agglutinins for a certain organism may have the amount of agglutinins increased by the inoculation of exciters of leucocytes such as aleuronat and hetol. Aleuronat in our hands brought about an increase of the initial agglutinins similar to that caused by the substances used in the preceding experiments. Hetol was not used.

To test the effect of our inoculations upon leucocytes, blood counts were made upon normal rabbits, rabbits inoculated with substances increasing the agglutinins, and substances which did not affect the agglutinins. The blood counts exhibit the same irregularity in the normal rabbit and in the inoculated rabbits irrespective of the occurrence or non-occurrence of an increase in agglutinins. This irregularity is in accordance with the statement of Brinckerhoff that the number of leucocytes per millimeter in the peripheral blood of the rabbit is constantly changing. It would appear from this irregularity that leucocytes could not account for the increased

production. The action of aleuronat should, therefore, be ascribed to its behavior as a proteid or derivative of a proteid rather than as an excitor of leucocytosis.

Inorganic Salts.—To follow more in detail the suggestion developed by the fact that phosphorus and sulphur were the elements possessed in common by the augmentors of agglutinins, several soluble inorganic salts of sulphur and phosphorus were tested, and their action was controlled as far as practical by salts containing the same base but not the same radical.

Several rabbits were inoculated with sodium phosphate, sodium sulphate, calcium and potassium phosphate. Control rabbits were inoculated with sodium chloride, calcium chloride, and potassium chlorate. A young goat was inoculated with sodium sulphate and a control goat with sodium chloride. The sera of the rabbits and the goat receiving the sulphur and phosphorus compounds showed the characteristic increase of agglutinins for the Flexner Manila strain of *B. dysenteriae*. The agglutinins for *B. typhosus* and *B. coli* were only slightly stimulated in a few instances. The agglutinins of the control animals inoculated with the salts that did not contain the sulphur or phosphorus molecules were unaffected. The doses varied according to the toxicity of the salts used and the concentrations ranged from one tenth normal to twenty-five per cent. solutions. The concentration of the solutions had no apparent effect upon the results. It is interesting to note that Vaughan found the phosphorus in the non-toxic portion of the split products which is the part that gives immunity.

A question arises as to the character of the action of these substances. Do they merely stimulate to greater activity a specific function of the cell already established or do they possess something in common with the bacteria which admits of their initiating specific action in some degree? An attempt was made to answer this question in the following manner.

Two rabbits each having normal agglutinins for the Flexner Manila type of *B. dysenteriae* up to 1 : 50, but none that were appreciable for Shiga or Park Mt. Desert types, were inoculated one with the Shiga type and the other with the Park Mt. Desert organism. After three inoculations the sera of these rabbits aggluti-

nated their homologous organisms in dilutions of 1:100. The index for the Flexner Manila type remained unchanged. The bacterial inoculations were then stopped and sodium sulphate substituted in one rabbit and diastase in the other. After four or five treatments the sera from both rabbits were tested. The agglutinins for the Flexner Manila organism were increased up to 1:500, while those for Shiga and Park Mt. Desert type remained unchanged and later disappeared.

These two experiments are not sufficient, however, to prove or disprove the assumption that the action is one of augmentation and not of initiation. I have mentioned the foregoing question because it suggests several points to be taken into consideration in answering it. First, the influence that has brought about the normal agglutinins in the rabbit for Flexner Manila has been acting practically during the adult life of the animal, while the influence of the Shiga or Park Mt. Desert organisms has only been exerted for a comparatively short period of time; hence the function of the cell to form the Flexner Manila agglutinins would be more permanent than for the other two organisms. Now when the rabbits are inoculated with strong specific substances as Shiga or Park Mt. Desert the cell responds accordingly as long as this stimulus is kept up—but upon removal of this influence and the substitution of a, presumably, non-specific stimulus, then the cell by preference responds in the direction of the more accustomed function of forming agglutinins for Flexner Manila. Another point to be considered is the fact that after withdrawal of the specific influences of Shiga and Park Mt. Desert the cause producing the normal agglutinins for Flexner Manila in the rabbit continues to act, thus adding its influence to the stimulus of the non-specific sodium sulphate inoculations and so determining the direction of the activity of the cells.

At this stage of the present investigation biological rather than physical laws seem to offer the most probable explanation of the production or augmentation of agglutinins which has been described; perhaps stimulation of certain cell activities occurs because some necessary element which enters into the cell or acts as a ferment adjuvant is provided.

The work of several authors on ferments is suggestive by anal-

ogy. Bertrand found that manganese was present in laccase and activity of laccase was proportional to the amount of this salt present.

Calcium salts are found to be essential to enzymes which cause clotting and Magnus has given the name of co-ferment to those substances, but this term has been rejected by Harden and Young. Harden and Young found that boiled yeast which was incapable of initiating fermentation alone when added to unboiled yeast increased its action to a considerable extent. They found this increase due to the presence of arsenates which brought about the same results as the phosphates.

It is too early to draw definite conclusions concerning the effect of various substances upon the production of agglutinins and the suggestions that have been offered are tentative, pending further work which is in progress.

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