Medicine. — *Investigation into the phage content of phage-containing globulin repeatedly precipitated by means of ammonium sulphate.* By P. C. FLU. (Communicated by Prof. J. V. D. HOEVE.)

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During researches on the rôle of the cation Mg^{++} in the lysis of bacterium megatherium by the corresponding bacteriophage, I was able to demonstrate that the phage in a magnesium-free medium cannot dissolve bacterium megatherium.

However, in such a medium phage is adsorbed by the living megatheria as well as by those killed at 60° C. Likewise the protein isolated from the microbes appeared to be able to do this.

A method enabling me to obtain globulins from bacterium megatherium is the following :

The microbes are cultivated in Roux's flasks on 1 % of peptone agar (water 100, peptone 1, agar 2 to 3 %, p_H 7.5). The 18 hours old culture layer is suspended in 40 cc of distilled water. In order to obtain c. 1 gr. of globulin, at least 2 L. of suspension are required. The suspension in well closed flasks is placed in the ice-box for 18 hours. Then it is filtered, first through cotton wool, subsequently through „papier Chardin" and finally through „papier Chardin" prepared by means of infusorial earth.

The result is a clear yellow or brownish yellow liquid which, adjusted to p_H 6.5, is cooled until c. 4° C., after which treatment an equal amount of concentrated ammonium sulphate solution is slowly added.

After the mixture has been kept at 4° C. during a night, a flocculated precipitate has been formed which is collected and dissolved in 0.9 % of NaCl. From this solution the globulin is again precipitated by means of concentrated ammonium sulphate solution and this treatment is once more repeated. After collecting the precipitate in as little liquid as possible, a syrupy mass is obtained which during 24 hours is dialysed opposite distilled water. A collodion film is used as the dialyser membrane.

The content of the dialyser case is first dried in the apparatus of Faust Heim and then in the exsiccator in vacuo. In an agate mortar the dried mass may now be rubbed to a fine powder. The quantity of globulin amounts to c. 1 gr.

STANLEY's communication, that he had succeeded in isolating from parts of plants infected with mosaic virus a crystalline protein possessing the properties of this virus, induced me to make some experiments with globulin from megatherium. The results are briefly communicated below.

100 mgr. of the dry powdered globulin, which itself as a repeated

examination showed is not able to produce the phage, is dissolved in 10 cc of 0.9 % NaCl and mixed with 5 cc of a suspension of megatherium phages. The phage suspension is obtained by suspending a 16 hours old culture of bacterium megatherium 899 lysogen in 10 cc of distilled water and by filtering this suspension through a porcelain filter (Chamberland L_3).

To the mixture of phage and globulin 5 cc of concentrated ammonium sulphate solution is added. After 6 hours in the icebox it is centrifugated, the precipitate dissolved in 5 cc of 0.9 % NaCl and filled up to 30 cc by means of concentrated ammonium sulphate solution.

Further investigation consists of, each time after 6 hours or more in the ice-box, centrifugating these 30 cc and subsequent determination of the phage content in 0.05 cc of the liquid both of the centrifugate and of the liquid above it.

For the determination of the phage content of the gelatinous centrifugate, this is dissolved in 5 cc of 0.9 % NaCl. After removing 0.05 cc, it is again filled up till 30 cc with concentrated ammonium sulphate solution.

This treatment is repeated 20 times. After the 20th time there is a large number of phages in the liquid, in the centrifugate even an enormous number. On investigation of 0.05 cc of the centrifugate this number appeared to be always infinitely large.

In the table below the results of some determinations are given :

Number of times examined	Number of phages	
	In 0.05 cc of centrifugate	In 0.05 cc of the liquid above the centrifugate
1st	Total corrosion of the microbial layer	~ [1]
2nd	id	~
6th	id	~
9th	id	1688
12th	id	815
15th	id	50
18th	id	641
20th	id	~

[1] ~ = a countless number of phages.

The phage content of the liquid above the centrifugate decreases gradually. This is certainly due to the fact that after repeated precipitations this liquid possesses less dissolved phage-containing globulin. The total number of the phages originally present in the liquid decreases also by removal of material for examination, pouring off the liquid above the

centrifugate, which causes millions of phages to be lost, and the inevitable loss of part of the centrifugate while the liquid is flowing away.

In the same way as was described above globulin is prepared from a lysogenic megatherium strain. Only here the filtrate of the paper filter with infusorial earth is passed through Chamberland L_3 filters in order to ensure absence of bacteria from the material.

The dialysis, however, is omitted since an investigation showed that the sulphate ion in concentrations from 0.5 to 0.01 N. destroys bacteriophage megatherium.

The globulin is therefore obtained by sharp centrifugation of the floccules collected in a small quantity of 0.5 concentrated ammonium sulphate solution.

It may then be used immediately for examination.

If we want to liberate it as much as possible from the salt solution, the latter may be poured off, after which distilled water is poured on the precipitate and quickly poured off again.

The drying can take place in the exsiccator in vacuo.

Of this globulin also c. 100 mgr. is dissolved in 15 cc of 0.9 % NaCl and then mixed with concentrated ammonium sulphate solution *aa*, but as a matter of course inentional addition of phage does not take place, the microbe itself supplying the phage.

For each stage of the treatment which is applied to obtain the globulin, the phage content of the products is determined. The result is given below :

Nature of the examined product	Number of phages per cc
Filtrate through "paper with infusorial earth" of the microbial emulsion	67.200
Suspension of the precipitated globulin in 200 cc of liquid	60.000.000
Solution of 100 mgr. of globulin in 5 cc of 0.9 % NaCl	510.000.000

Consequently the ammonium sulphate appears to be able to precipitate the phage together with the globulins from the liquid.

On examination of the phage-containing globulin by the method described above, it becomes apparent that now also, after more than 20 times repeating the treatment, the phage content of the precipitated globulin is high, much higher than the most strongly concentrated phage solution that may be obtained from lysogenic germs.

When the phage-containing globulin is dissolved in water of p_H 8 and it is tried according to STANLEY's method to produce a crystalline precipitate, this attempt will fail, but an amorphous precipitate is formed containing the phages.

The possibility that the globulin would be extracted from the culture medium may be practically excluded, for the cultivation took place on agar to which 1 % of peptone was added. In order to exclude this possibility completely the megatherium strain 338 which is sensitive to phage was subjected to lysis in a synthetic medium.

As synthetic medium was taken the Uschinsky adjusted to p_H 7.2 and of the following composition :

Magnesium sulphate	0.2 gr.
Calc. chloride	0.1 gr.
Sodium asparagine	3.4 gr.
Ammonium lactate	6.0 cc
Sec. Potassium phosphate	2.0 gr.
Sodium chloride	5.0 gr.
Glycerin	30.0 cc
With distilled water to	1000.0 cc

This medium was infected with bacterium megatherium 338 lysabel, suspended in Uschinsky and with bacteriophage antimegatherium suspended in distilled water.

Altogether 4 L. of medium were used. After 24 hours in the incubator at 37° C. the lysed mass was first filtered through ordinary Chardin paper and afterwards through Chardin paper prepared with infusorial earth.

The clear filtrate was mixed with an equal amount of concentrated ammonium sulphate solution. Turbidity sets in but does not result in the formation of large floccules, as was the case in the two experiments described above; not even after 24 hours in the ice-box.

By filtration of the eight Liters of liquid through the a-filter of Chardin paper it is possible to collect the finely divided precipitate. Maceration of this filter in 200 cc of 0.9 % NaCl solution yields a more concentrated solution of the globulins which is adjusted to p_H 6 and from which the ammonium sulphate solution now produces a flocculated precipitate which may be collected by centrifugation of the liquid. Altogether, by estimate, I obtain 50 mgr. of the globulins which, in order to prevent a further loss, are not dialysed but are used immediately for the experiment. This is carried out by the method described above.

The result of the investigation on phages of the liquids obtained at the various phases of treatment follows below :

	Dilution	Phages in 0.1 cc of the dilution
A. Liquid after filtration through infusorial earth and immediately after mixing with ammonium sulphate.	1/100 1/1000 1/10000	$\simeq$ $\simeq$ 1768

	Dilution	Phages in 0.1 cc of the dilution
B. Liquid which after mixing with amm. sulph. was placed in the ice-box for 24 hours and in which numerous small floating floccules have been formed.	1/100	1155
	1/1000	139
	1/10000	28
C. The 200 cc of 0.9 % NaCl solution used for maceration of the filtration paper through which liquid B was filtered.	1/100	846
	1/1000	101
	1/10000	19
D. The 30 cc of concentrated amm. sulph. solution from which the globulin was precipitated by centrifugation in the first experiment of the precipitation series.	1/100	~
	1/1000	1468
	1/10000	390
	1/100000	50
	1/1000000	12
E. The globulin and phage-containing precipitate from liquid D which was dissolved in 5 cc of 0.9 % NaCl solution.	1/100	≃
	1/1000	≃
	1/10000	~
	1/100000	4496
	1/1000000	455

≃ = complete corrosion of the bacterial layer.
~ = a countless number of phages.

After the globulin precipitate from (E) has been dissolved and precipitated 20 successive times and subsequently still phage is observed in the globulin precipitate as well as in the liquid above it, examination of these two components shows the following :

	Dilution	Phages in 0.1 cc of the dilution
Liquid above the globulin precipitate from the tube of the 20th precipitation.	1/100	850
	1/1000	73
	1/10000	4
	1/100000	0
	1/1000000	0
The globulin precipitate of the 20th precipitation dissolved in 5 cc of 0.9 % NaCl solution.	1/100	~
	1/1000	~
	1/10000	510
	1/100000	44
	1/1000000	7

These results show that together with the globulins the phages may be flocculated out of solutions of phage-free globulins from megatherium, purposely mixed with bacteriophage megatherium, as well as those of globulins from lysogenic megatherium stems, which immediately after

preparation contain the phage, and liquid synthetic mediums in which lysis has taken place by means of phage.

The globulin which by artificial means contains phage behaves in the same way as the globulin containing phage, so to speak, „by nature".

It may be that the phage is adsorbed by the globulin or that globulin and phage are precipitated by equally strong solutions of ammonium sulphate.

In any case they show that from a mixture of globulins and phage these two substances cannot be separated by repeated precipitation with ammonium sulphate or by means of other precipitants.

The method followed in order to obtain the crystalline substance, considered by STANLEY as the virus of the mosaic disease, is described by him as follows :

I quote literally from his publication [1]).

"The crystalline protein described in this paper was prepared from the juice of Turkish tobacco plants infected with tobacco-mosaic virus. The juice was brought to 0.4 saturation with ammoniumsulfate and the precipitated globuline fraction thus obtained was removed by filtration. The dark brown globulin portion was repeatedly fractionated with ammonium sulfate and then most of the remaining color was removed by precipitation with a small amount of lead subacetate at p_H 8.7. An inactive protein fraction was removed from the light yellow colored filtrate by adjusting to p_H 4.5 and adding 2 % by weight of standard celite.

The celite was removed, suspended in water of p_H 8 and the suspension filtered. The active protein was found in the colorless filtrate. This procedure was repeated twice in order to remove completely the inactive protein. Crystallization was accomplished by adding slowly, with stirring, a solution containing 1 cubic centimeter of glacial acetic acid in 20 cubic centimeters of 0.5 saturated ammonium sulfate to a solution of the protein containing sufficient ammonium sulfate to cause a faint turbidity. Small needles about 0.03 mm long appeared immediately and crystallization was completed in an hour. Crystallization may also be caused by the addition of a little saturated ammonium or magnesium sulfate to a solution of the protein in 0.001 N. acid. Several attempts to obtain crystals by dialysing solutions of the protein gave only amorphous material. To date a little more than 10 grams of the activated crystalline protein have been obtained".

Although the details are different, I am inclined to think that the principle I applied to the isolation of the globulins and to the further treatment of the phage-containing globulins is the same as that followed by STANLEY.

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[1]) Isolation of a crystalline protein possessing the properties of Tobacco-mosaic virus, by W. M. STANLEY. Science, 81, 644—645, No. 2113.

However, as is apparent from my results, precipitation with ammonium sulphate cannot be regarded as a method enabling us to separate phages from globulin. On precipitation of the globulins the phages will always be carried along.

Even if I had succeeded in crystallizing the phage-containing globulins either partially or completely and even if these crystals should possess properties of the phage, yet my opinion would not have changed, since these crystals would have been obtained as well by precipitation from the protein solution.

What indeed are protein crystals ? Hardly anything else than another form in which the colloidal protein presents itself.

Now all crystallographers who studied egg albumin and other protein crystals agree that optically they present themselves as real crystals. However, the crystals are composed of colloidal particles and to this fact they owe the capacity to swell considerably in water, which ordinary crystals do not possess. Per gram of the substance they contain e.g. 0.22 grams of water [1] [2]. It is possible that in this water impurities are to be found.

Even of the most frequently and best studied egg albumin crystals it has not been proved that they are no mixed crystals, viz. built up of different colloids [3]. They retain all sorts of impurities [4].

Recrystallization repeated six times is not always sufficient to remove from the crystals impurities which may become perceptible by means of colloidal gold [5].

It is not my intention to criticize STANLEY's researches. I am not acquainted with the mosaic disease of tobacco, have never worked with the virus of this disease and consequently could not reproduce STANLEY's experiments, but I am inclined to think that neither the nature of the protein crystals nor the method by which they are formed guarantee that possible virus properties of the crystals must be ascribed to the crystals themselves and not to virus particles adsorbed by the colloids of which they are composed.

It seems advisable, therefore, to be very careful before concluding that it has been proved that a lifeless substance may possess an important property of the living matter, viz. that of independent unlimited reproduction with preservation of the individuality in a heterogeneous medium.

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[1]) S. P. L. SÖRENSEN, Proteinstudien. Hoppe Seylers Zeitschrift für physiologische Chemie, 103, 1—14.

[2]) S. P. L. SÖRENSEN und M. HOYINP, Proteinstudien. Hoppe Seylers Zeitschr. für physiol. Chemie, **103**, 211.

[3]) A. F. HOLLEMAN, Leerboek der Organische Chemie, 12th ed. (1932).

[4]) D. J. VAN ALPHEN, Leerboek der Organische Scheikunde, ...(1934).

[5]) Dr. FR. N. SCHULZ, Allgemeine Chemie der Eiweisstoffe, Stuttgart (1907).