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THE SYNTHESIS OF BACTERIOPHAGE ϕX174-NEUTRALIZING PROTEIN IN A SUBCELLULAR SYSTEM OBTAINED FROM THE SPLEEN OF ϕX174 IMMUNIZED RATS

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SUMMARY

The microsomal fraction of rat spleen was isolated at varying lengths of time after the application of a "booster"-injection of bacteriophage ϕX174 to the animal. Together with the "pH 5-enzyme" fraction, which is obtained routinely from the 100 000 $\times g$ spleen-supernatant of non-immunized rats, it formed the subcellular system which was tested for its ability to synthesize ϕX174-neutralizing protein. In a large number of experiments an increase was found in the concentration of ϕX174-neutralizing protein upon incubation of the subcellular systems under suitable conditions. The results obtained strongly suggest that this increase is due, at least partly, to true synthesis of this protein, as it could be shown to be ATP-dependent, ribonuclease-, and puromycin-, sensitive and also dependent on the time of incubation of the subcellular system.

INTRODUCTION

During the last decade, the results of numerous studies on the incorporation of ^{14}C-labelled amino acids into total protein by so-called "cell-free" or "subcellular" systems have led to a substantial increase of our knowledge on the process of protein biosynthesis. But the synthesis of specific functional proteins, *e.g.* enzymes, has so far been shown to occur in only a few subcellular systems obtained from bacteria (see *e.g.* Refs. 1–4).

The only mammalian systems which are reproducibly active in this respect are the subcellular systems from reticulocytes synthesizing haemoglobin[5–7] and from rat-liver synthesizing serum albumin[8]. Making use of the high sensitivity with which the concentration of bacteriophage ϕX174 can be determined, an attempt was made to obtain a subcellular system which would synthesize phage ϕX174-neutralizing

Abbreviations: Ab, antibody; Ag, antigen; PEP, phosphoenol pyruvate; pfu, plaque forming unit.

protein. The minute amounts of newly formed protein in such a system make it very difficult to present unambiguous evidence for the synthesis of one chemically well-defined protein *de novo*. Therefore, it may be better to speak of phage-neutralizing protein rather than of phage-antibody when synthesis in a subcellular system is concerned. However, the results obtained in our introductory experiments with such a system seem to look promising enough to justify their publication.

MATERIALS AND METHODS

Reagents and solutions

Ribonuclease (EC 2.7.7.16) and trypsin (EC 3.4.4.4) were purchased from Sigma Chemical Cy, St. Louis, Mo. (U.S.A.), lysozyme (EC 3.2.1.17) from Fluka A.G., Buchs (Switzerland) and the sodium salts of adenosine and guanosine 5'-triphosphate from Pabst Laboratories, Milwaukee, Wisc. (U.S.A.).

Yeast RNA, PEP and PEP kinase (EC 2.7.1.40) were obtained from Boehringer u. Soehne GmbH, Mannheim (Germany). The inorganic chemicals used were purchased from the British Drug Houses, Poole (Great Britain). Puromycin was a gift of Professor Dr L. BOSCH at Leyden.

The homogenization and incubation mixture for the subcellular systems contained 0.01 M $MgCl_2$, 0.025 M KCl, 0.01 M $KHCO_3$ and 0.25 M sucrose in a 0.035 M Tris buffer (pH 7.8).

For every experiment, the ATP-generating system was freshly made by dissolving, at 4°, 25 mg of ATP, 25 mg of PEP and 5 mg of GTP in 2.3 ml of medium and subsequently adjusting the pH to 7.8 with 1 N KOH. Afterwars, 0.05 ml of PEP kinase was added. From this "ATP system" samples of 0.2 ml each were used for each test tube that needed ATP, the final concentrations in 1 ml of incubation mixture being: 3.3 μmoles ATP, 4.6 μmoles PEP, 0.8 μmole GTP and 42 μg of PEP kinase.

The growth medium for the preparation of the phage was made according to Fraser and Jerrel and contained per litre:

$Na_2HPO_4 \cdot 2H_2O$, 13.6 g; NH_4Cl, 1.0 g; KH_2PO_4, 4.5 g; $MgSO_4 \cdot 7H_2O$, 0.62 g; casamino acids (Difco), 15.0 g; glycerol, 30.0 g; 1 % gelatin, 1.0 ml; 1 M $CaCl_2$, 0.3 ml; 50 % (w/v) glucose, 10.0 ml.

The solutions of $CaCl_2$ and glucose were sterilized separately and added afterwards.

Preparation of stock suspensions of high titre-phage ϕX174

The preparation of phage suspensions[9–12] containing about 10^{11} viable particles per millilitre was carried out as follows: 900 ml of Fraser and Jerrel medium, making up the content of a culturing vessel as pictured in Fig. 1 were incubated at 37° after inoculation with 100 ml of a culture of *Escherichia coli* C which was 18 h old. Compressed air (1 l/min) was led through a wash bottle filled with sterile cotton plugs, into the culturing vessel. The outlet was connected to another wash bottle filled with 50 ml of 0.1 N KOH in order to prevent the infection of the incubation stove with ϕX174 phage after the inoculation of the cell-suspension. (At about the

time that the suspension is ready for harvesting, aeration causes considerable foaming: phage particles escaping with foam bubbles from the outlet are then inactivated in the KOH.)

When the absorbancy of the *E. coli* host cell culture was 0.8 at 660 mμ (corresponding to about 10^9 bacterial cells per millilitre) a suspension of φX174 phage was added with a multiplicity of 0.01. This means that, in the culture, a concentration of one phage particle per 100 *E. coli* cells, or of 10^7 active φX particles per millilitre must be obtained.

Every 20 min the $E_{660\ m\mu}$ of the inoculated culture was measured. The culture was harvested by centrifugation in the cold at 3500 × *g* for 60 min as soon as the initial rise in absorbancy levelled off (after 1–2 h).

The fraction containing the debris of *E. coli* cells and ghosts with φX174 particles attached to them was taken up in 30 ml of a solution of borate buffer at pH 7 (300 ml of sodium borate solution, saturated at 4° was brought to pH 7 with 3 N HCl, 4 ml 0.1 M EDTA being added afterwards).

The φX particles were detached from the cell-remnants by the addition of 4 ml of 0.9 % NaCl solution containing 1.25 mg of lysozyme and 1.25 mg of trypsin. After incubation for 1 h at 37°, the suspension was allowed to stand overnight at 4°. Subsequently, it was spun for 30 min at 3000 × *g* and the number of pfu/ml was estimated in the clear supernatant. The suspension of phage was stored at −16° until used.

Attempts to improve the yield of phage φX174 by salting out or by ultracentrifugation of the bacterial lysate were unsuccessful. As the suspension of phage thus obtained was to be applied only intraperitoneally, further purification[10] was considered not to be necessary.

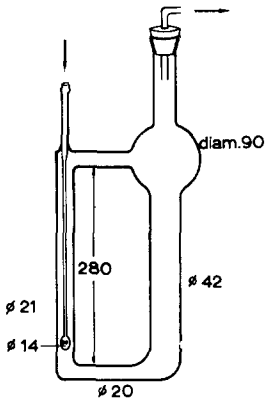

Fig. 1. Culturing vessel for *E. coli* and φX174-phage (outer diameters are given in mm).

Phage determinations

To estimate the amount of plaque-forming units[13] in a suspension of φX174 phage, adequate dilutions (*e.g.* 10^2, 10^3, . . . fold) of this suspension were made in 0.9 % NaCl solution.

Samples of the diluted phage suspensions (0.1 ml each in volume) were added

to about 6 ml of a molten nutrient-agar (0.7 %) at 45°, containing 0.1 ml of a culture (3–4 h old) of *E. coli* C. The contents of the tube were mixed and poured on to a solidified, and dried, layer of nutrient-agar (2 %) in a Petri-dish. After solidification of the upper agar layer, the plates were dried (for 1 h) and cultured (for 4 h) at 37°.

Clearly visible plaques were counted and the concentration of phage which was present in the original sample was calculated.

If desired, the assay plates can be incubated overnight. In this case layers of the following composition should be used:

top layer: 0.7 % agar, 0.5 % peptone, 0.3 % beef extract; bottom layer: 1 % agar, 1 % tryptone, 0.5 % yeast extract and 1.2 % glucose.

Immunization of the rats

Rats were immunized against ϕX174 by one intraperitoneal injection of 1 ml containing 10^{11} pfu, followed by a "booster"-injection with a dose of 10^{10} pfu after a month, or longer. The k_{pm}-values in the serum (see the next section) varied from 1000 to 1400 min^{-1} during 1, 2, 3, 4 and 5 days after the booster and jumped to 2800 min^{-1} on the sixth day. This observation on the first rise in the phage-neutralizing activity of the serum is in rather good agreement with the data presented in Table I and Fig. 3, which were obtained in subcellular systems using pH 5-enzyme fractions prepared from the 100 000 $\times g$ supernatant of spleens from immunized animals. Routinely, subcellular splenic systems were prepared 7–8 days after the application of the booster injection to the animals.

The determination of the phage-neutralizing capacity of antisera and subcellular systems

From our data as illustrated in Fig. 2, it can be concluded that the neutralization of active ϕX174 particles (Ag) by an excess of antibody (Ab) protein follows first order kinetics with respect to the concentration of particles. Moreover, the validity of Eqn. 2 which was tested in a four-fold antiserum dilution series proves that this reaction is also first order in the antibody concentration and, consequently, can be described as:

$$\mathrm{Ag} + \mathrm{Ab} \xrightarrow[k_{b1}]{} \text{inactivated complex}$$

When the rate of inactivation is measured in the presence of an excess of Ab, a pseudo monomolecular rate constant (k_{pm}) can be determined, to which applies:

$$k_{pm} = k_{b1}[\mathrm{Ab}] \tag{1}$$

Taking a dilution factor (D) of the antiserum into account, the integrated value of k_{pm} can be calculated to be[13,14]:

$$k_{pm} = \frac{D}{t - t_0} \ln \frac{[\mathrm{Ag}]_0}{[\mathrm{Ag}]_t} \tag{2}$$

$[\mathrm{Ag}]_0$ and $[\mathrm{Ag}]_t$, the antigen concentrations at the beginning (t_0) and at the time t of the inactivation reaction were determined by counting the pfu as described before.

The procedure was as follows: From an antiserum, appropriate dilutions (100-, 500-, ... fold) were made in 0.9 % NaCl solution. From a stock suspension of the phage, a dilution containing about $4 \cdot 10^6$ pfu/ml was made in a solution of 0.9 % NaCl. A sample (0.1 ml) of this diluted phage-suspension was added to a standard amount (9.9 ml) of the diluted test-serum and incubated at 37°. At 20-min intervals, samples of 0.5 ml each were taken from the incubation mixture and added to 4.5 ml of a solution of 0.9 % NaCl; finally, samples (0.1 ml each) of this dilution were plated by the agar-layer-technique as described above. From the phage counts and the dilution factor of the serum the k_{pm} values were calculated according to Eqn. 2: from the data as presented in Fig. 2, the accuracy of the k_{pm} values could be estimated to be about 5–10 % when 100–500 pfu are counted in each phage ϕX174 determination.

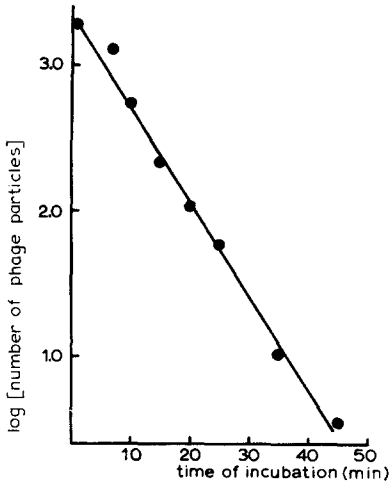

Fig. 2. Time-course of phage ϕX174-inactivation at 37° by a diluted rat antiserum. Experimental conditions as described in the text.

In the experiments with the subcellular systems, samples were taken from the incubation mixture at different times, centrifuged at 1500 × *g* in the cold, and diluted 20- or 50-fold with a solution of 0.9 % NaCl to a final volume of 10 ml. Then 0.1 ml of a diluted phage-suspension ($4 \cdot 10^6$ pfu) was added. After 1, 21 and 41 min of incubation at 37°, samples 0.5 ml each in volume were added to 4.5 ml of a solution of 0.9 % NaCl: finally, samples (0.1 ml) of this diluted test solution were plated and counted as described before. Subsequently, k_{pm} was calculated according to Eqn. 2.

The percentage "increase" in the concentration of phage ϕX174-neutralizing protein in the complete subcellular system as compared with the blank systems (−ATP and +ATP+ribonuclease (Tables I and II)) as well as in the time-course experiment (Fig. 4) was calculated with the aid of Eqn. 1.

When, for example, the "percentage increase", *i.e.* the percentage difference from that in a blank or control system in the concentration of phage-neutralizing protein after the incubation of a complete subcellular system was to be calculated,

this was carried out as follows:

$$\text{percentage increase} = 100 \times \frac{k_{\text{pm, complete}}}{k_{\text{pm, blank}}} - 100 \quad (3)$$

That this "percentage increase" reflects a real time-dependent increase, is apparent from the results obtained in a time-course experiment (Fig. 4) where it was calculated according to:

$$\text{percentage increase at time } t = 100 \times \frac{k_{\text{pm}, t}}{k_{\text{pm}, t_0}} - 100 \quad (4)$$

Preparation of the subcellular systems

Phage-sensitized rats were sacrificed by a blow on the head, or by decapitation, after chloroform-anesthesia. The spleen was quickly taken out, put into an ice-cold medium and weighed. The organ was cut into pieces with scissors and homogenized in 2.5–3 times its weight (in grams) of homogenization medium. A few (3–5) strokes with a motor driven teflon pestle of a loosely-fitting Potter-Elvehjem homogenizer were considered to be the least harmful for the protein-synthesizing entities. This and all further manipulations were done in the cold.

The homogenate was centrifuged for 10 min at 15 000 $\times g$ in a P.R. "International" centrifuge. From the resultant supernatant, the microsomal pellet was spun down in 60 min at 100 000 $\times g$ in a SW 39 rotor of a Spinco ultracentrifuge (model L).

Subsequently, this 100 000 $\times g$ microsomal pellet was carefully re-suspended in 1 ml of incubation medium by intermittent stirring and standing in the cold for 0.5 h. The turbid microsomal suspension was then diluted with medium in such a way that it contained about 5 mg of microsomal RNA/ml as estimated by its extinction at 260 mμ.

From the supernatant (100 000 $\times g$) of the spleen of sensitized or normal rats, the "pH 5-enzyme" fraction (containing amino acid-activating enzymes and transfer RNA's[15] was precipitated by lowering the pH of the solution to 5 by means of 0.1 M acetic acid and by centrifuging at 1500 $\times g$ for 10 min. The precipitate was taken up in 1 ml of medium and titrated carefully to pH 7.8 with 0.1 M KOH. Routinely, this pH-5 enzyme fraction was "washed" by another isoelectric precipitation cycle.

Subcellular systems were composed of 0.2-ml samples (containing 0.5–1 mg of RNA) of spleen microsomes, 0.2 ml (1–2 mg of protein) of "pH 5-enzyme" fraction and 0.2 ml of the ATP-generating system. The mixture was then brought to a final volume of 1 ml with incubation medium. The incubation was carried out for varying periods of time (30 min in routine experiments) at 37°. In the beginning, "pH 5-enzyme" fractions were prepared from the 100 000 $\times g$ supernatant of the spleen of immunized rats. As it appeared that this fraction contained rather large amounts of ϕX174-neutralizing protein (see Table I and Fig. 3), the "pH 5-enzyme" fraction was isolated from the spleen of non-immunized rats in later experiments: as could be expected, the k_{pm} values and, consequently, the amounts of ϕX174-neutralizing protein present in the control systems decreased considerably (Table II).

RESULTS AND DISCUSSION

As we were most interested in obtaining a reproducibly active system, a number of experiments was first carried out, which indicated that an ATP-dependent "increase" in the ϕX174-neutralizing capacity occurs upon incubation of the subcellular system.

The results of these introductory experiments which were performed with subcellular splenic systems that were prepared entirely from (2–4)ϕX174 boosted rats at various times after the application of the challenging dose are presented in Table I and Fig. 3.

TABLE I

THE ATP-DEPENDENT "INCREASE" IN THE AMOUNT OF ϕX174 NEUTRALIZING PROTEIN

Incubation, at 37° for 30 min, of subcellular systems of rat-spleen obtained at various days after the application of the booster injection; k_{pm} values and "increase" were calculated according to Eqn. 2 and Eqn. 3. Incubation media and procedures are as described in the text.

Days after booster	k_{pm}-*values* (min^{-1})		$\frac{k_{pm},\ complete}{k_{pm},\ blank}$	*Increase with regard to control without ATP* (%)
	Complete system	*Same system, −ATP*		
+1	1.0	0.7	1.4	40
+2	0.3	0.1	3	200
+3	4.0	1.9	2.1	110
+4	1.8	1.6	1.1	10
+4	2.9	2.0	1.4	40
+5	1.9	1.4	1.4	40
+6	8.7	6.3	1.4	40
+7	26	—	—	—
+9	25	20	1.2	20

Firstly, it was found that an ATP-dependent "increase" in the amount of ϕX174 neutralizing capacity occurs consistently upon incubation for 30 min at 37°.

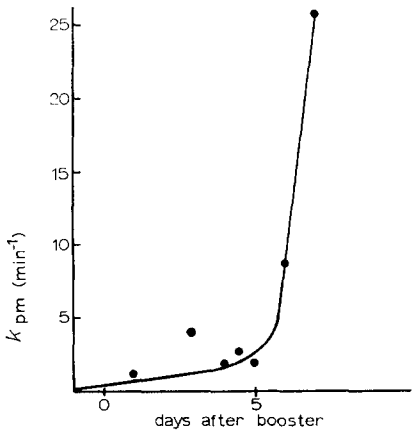

Fig. 3. K_{pm}-values in complete subcellular rat-spleen systems prepared at various days after the application of the booster dose of phage ϕX174: the data plotted were taken from Table I.

Moreover, the rise in the k_{pm} values of the subcellular systems which is observed between the 5th and 6th day after the application of the booster injection seems to agree rather well with the rise in the amount of serum antibody observed on the 6th day as mentioned in the previous section. As this rise may reflect an increase in the antibody-synthesizing activity of the organ, in subsequent experiments the rat-spleens were obtained from 7–8 days after the application of the booster injection. Furthermore, in order to reduce the high k_{pm} values of the control systems, the "pH 5-enzyme" fraction was prepared routinely from the 100 000 $\times g$ supernatant obtained from the spleen of non-immunized rats.

Once it appeared that an ATP-dependent "increase" of the ϕX174-neutralizing capacity occurs reproducibly on incubation of the complete subcellular systems, we tried to obtain experimental evidence that this increase is not only due to an ATP-dependent release of nascent phage-neutralizing protein from the microsomal fraction. Since it was found, recently[16], that the "solubilization" of nascent protein from rat-liver ribonucleoprotein particles is stimulated by both ATP and ribonuclease, whereas protein synthesis *de novo* should be inhibited by the action of this enzyme, a second series of experiments was carried out. In these experiments, the "increase" in phage-neutralizing protein in complete systems was compared with two control systems, one containing no ATP-system, and one with the ATP-system, to which ribonuclease (50 μg/ml) was added. The results of these experiments are listed in Table II: the observed "increases" in the amount of phage-neutralizing protein upon incubation of the complete subcellular system as compared with the control systems proved to be not only ATP-dependent but also ribonuclease sensitive.

As these findings strongly suggested that the observed increases in phage-neutralizing capacity could not be due to a "release" or "solubilization" of nascent, phage-neutralizing protein only, an experiment was carried out to follow the time

TABLE II

"INCREASE" IN ϕX174-NEUTRALIZING PROTEIN

Incubation at 37° of subcellular spleen systems prepared 7–8 days after the booster: the incubation time was 30 min, the "pH 5-enzyme" fraction was prepared from the spleen of non-immunized rats. In the last two experiments listed, the "pH 5-enzyme" fraction was replaced by the 100 000 $\times g$ supernatant fraction and the system was incubated for 2 h. Incubation medium and procedures are as described in the text.

k_{pm}-values (min^{-1})			k_{pm}, complete / k_{pm}, blank 1	k_{pm}, complete / k_{pm}, blank 2	Increase over control (%)	
Complete system k_{pm}, complete	Same without ATP k_{pm}, blank 1	Same with ribonuclease k_{pm}, blank 2			without ATP	with ATP and ribonuclease
2.2	1.5	—	1.5	—	50	—
3.7	3.1	2.6	1.2	1.4	20	40
2.5	1.4	2.7	1.8	0.9	80	−10
3.4	2.3	2.9	1.5	1.2	50	20
1.6	1.0	0.9	1.6	1.7	60	70
1.2	0.7	0.7	1.7	1.7	70	70
1.8	1.1	1.1	1.6	1.6	60	60
5.4	4.1	3.6	1.3	1.5	30	50
5.4	3.7	4.3	1.5	1.3	50	30
1.7	1.1	1.4	1.5	1.2	50	20

course of the apparent synthesis of this protein in a complete, as well as in the two control (−ATP and +ATP+ribonuclease) systems. The results, which are shown in Fig. 4, indicate that a time-dependent, ATP-requiring and ribonuclease-sensitive synthesis of ϕX174-neutralizing protein occurs in the subcellular, rat-spleen system studied.

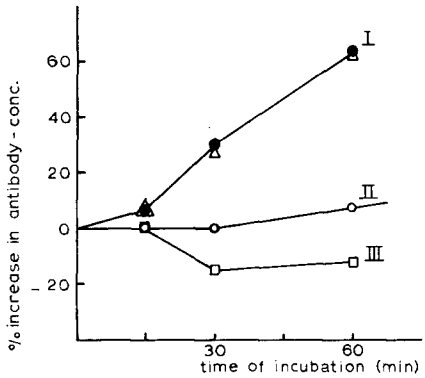

Fig. 4. Time-course of the increase in phage-neutralizing activity during the incubation at 37° of subcellular rat-spleen systems: the "pH 5-enzyme" fraction used was prepared from the spleen of non-immunized rats. Percentage increases were calculated according to Eqn. 4: conditions and procedures are as described in the text. I, complete subcellular system; II, without the ATP system; III, with ATP and 50 μg ribonuclease/ml.

This tentative conclusion is confirmed by the observed effect of puromycin, a rather specific inhibitor of protein synthesis[17], on the increase in ϕX174-neutralizing capacity during the incubation of a complete subcellular system (Fig. 5).

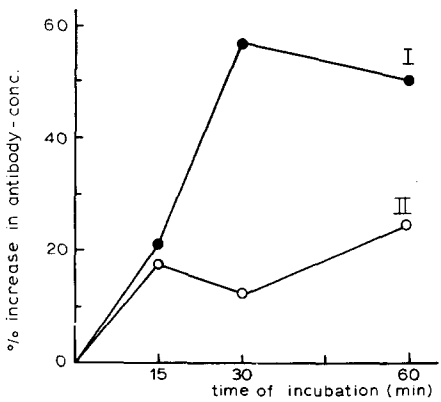

Fig. 5. Effect of puromycin on the increase in phage-neutralizing activity during the incubation, at 37°, of a subcellular rat-spleen system: the "pH 5-enzyme" fraction used was prepared from the spleen of non-immunized rats. Percentage increases were calculated according Eqn. 4: conditions and procedures are as described in the text. I, complete subcellular system, prepared 3 days after the application of the booster injection; II, same, with 80 μg puromycin/ml.

Finally, we should like to draw attention to a peculiar finding which may be of interest with regard to studies on the subcellular synthesis of a specific functional protein. In a number of experiments an attempt was made to improve the results by

using a ribosomal fraction instead of a microsomal fraction. For this purpose, spleen ribosomes were isolated in various ways, involving deoxycholate[18], deoxycholate–Lubrol[19] and isooctane[20] treatments respectively. Moreover, a ribosomal splenic fraction was prepared according to the method described recently by BLOEMENDAL *et al.*[21], without the use of any detergent. All of these preparations were active in incorporating [^{14}C]amino acids into protein[22], the latter preparation being almost as active as a similarly prepared rat-liver ribosomal fraction[23]. But not one of the subcellular rat-spleen systems containing ribosomes instead of microsomes showed any increase in ϕX174-neutralizing activity. Together with similar findings in a subcellular rat-liver system synthesizing serum albumin[8] and in a subcellular yeast-system synthesizing α-glucosidase[24] this result may be seen as an indication of some, as yet unknown, function of the microsomal membranous fraction during a late phase of protein synthesis, presumably after the formation of the peptide chain.

In conclusion, it seems justifiable to state that the introductory data reported in this paper strongly suggest that a subcellular system from rat-spleen can be used to study the synthesis of a functional ϕX174-neutralizing protein. However, also with this system, the problem of an unambiguous physico-chemical characterization of the minute amounts of newly-formed protein which was mentioned in the introduction of this paper, remains unsolved.

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REFERENCES

1 K. MARUSHIGE, T. YURA AND M. IMAI, *Biochim. Biophys. Acta*, 87 (1964) 90.
2 Y. OHTAKA AND S. SPIEGELMAN, *Science*, 142 (1963) 493.
3 H. R. WHITELEY AND M. OISHI, *Biochem. Biophys. Res. Commun.*, 13 (1963) 6.
4 G. D. NOVELLI, in H. J. VOGEL, V. BRYSON AND J. O. LAMPEN, "*Informational macromolecules*", Academic Press, London, 1963, p. 301.
5 G. VON EHRENSTEIN, B. WEISBLUM AND S. BENZER, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 669.
6 B. HARDESTY, R. MILLER AND R. SCHWEET, *Proc. Natl. Acad. Sci. U.S.*, 50 (1963) 924.
7 J. KRUH, J. C. DREYFUS AND G. SCHAPIRA, *Biochim. Biophys. Acta*, 87 (1964) 253.
8 A. VON DER DECKEN AND P. N. CAMPBELL, *Biochem. J.*, 84 (1962) 449.
9 R. C. HUANG AND J. BONNER, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 1216.
10 R. R. RUECKERT, W. ZILLIG AND K. HUBER, *Virology*, 17 (1962) 204.
11 J. W. UHR, M. S. FINKELSTEIN AND J. B. BAUMANN, *J. Exptl. Med.*, 115 (1962) 655.
12 J. W. UHR, M. S. FINKELSTEIN AND J. B. BAUMANN, *J. Exptl. Med.*, 117 (1963) 457.
13 A. ADAMS, *Bacteriophages*, Interscience, New York, 1959.
14 K. M. COWAN, *J. Immunol.*, 88 (1962) 476.
15 E. B. KELLER AND P. C. ZAMECNIK, *J. Biol. Chem.*, 221 (1956) 45.
16 T. HULTIN AND K. A. ABRAHAM, *Biochim. Biophys. Acta*, 87 (1964) 232.
17 D. NATHANS, G. VON EHRENSTEIN, R. MONRO AND F. LIPMANN, *Federation Proc.*, 21 (1962) 127.
18 J. W. LITTLEFIELD AND E. B. KELLER, *J. Biol. Chem.*, 224 (1957) 13.
19 R. RENDI AND T. HULTIN, *Exptl. Cell. Res.*, 19 (1960) 253.
20 A. O. HARTREY AND V. SCHIRREN, *Biochim. Biophys. Acta*, 61 (1962) 467.
21 H. BLOEMENDAL, W. S. BONT AND E. L. BENEDETTI, *Biochim. Biophys. Acta*, 87 (1964) 176.
22 J. H. H. VAN DER MEER, *Thesis*, University of Utrecht, 1964.
23 R. K. A. VAN WERMESKERKEN, personal communication.
24 H. P. J. BLOEMERS AND V. V. KONINGSBERGER, *Proc. Konink. Ned. Akad. Wetenschap., Ser. B*, in the press.