# PROTIDES
## OF THE
# BIOLOGICAL FLUIDS
## 1959

Edited by

**H. PEETERS**

# PROTIDES OF THE BIOLOGICAL FLUIDS

PROCEEDINGS OF THE SEVENTH COLLOQUIUM
BRUGES 1959

*Edited by*

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# PREFACE

The annual Bruges Colloquia show that the study of the problems related to the structure and metabolism of proteins is each year becoming increasingly extensive and in this report of the Seventh Colloquium a wide range of topics has been covered. An attempt has been made to arrange the many papers in a logical order.

In the introductory paper "The Synthesis of Antibodies and of Proteins" a comprehensive picture of the subject is drawn by Professor SCHULTZE of Marburg/Lahn, whose contributions to protein chemistry as well as to the Colloquium are so important.

The next eleven papers deal with technical subjects. They stress the great importance that the Colloquium has for seven consecutive years attached to technical developments connected with the analytical study of protein mixtures. Immunoelectrophoresis, which appeared on the programme for the first time at the Second Colloquium in 1954, when a series of papers was presented by GRABAR and his coworkers, is now represented by several papers from various authors. The other papers in this series of more or less technical contributions, deal with chromatography.

General biochemical problems are treated next, followed by an important series of papers relating to the binding properties of proteins.

The next section deals with the nutritional aspects of proteins. A large group of lecturers discussed the present status of the carnitine problem. This nitrogenous compound belonging to the widely distributed group of betaines is only found in animals, and its origin, function and fate are still obscure.

Next, the relation of protein problems to pathology is considered, the serum protein pattern, the macroglobulins and the urinary proteins being discussed.

The Colloquium was concluded with a Round Table Discussion on *Advances in the Analysis and Pathology of Proteins and Related Substances*. In this discussion, an attempt was made to approach the many problems connected with the biochemistry of proteins, from various angles by bringing together opinions and views from various countries as widely separated as Africa, Japan and North America.

Once again the original purpose of these Colloquia—to be the annual meeting place of all those interested in protein problems and to be as far as possible the *United Nations of the Protein World*—has been maintained.

Bruges, 1959

HUB. PEETERS

## ACKNOWLEDGEMENTS

The members of the Scientific Committee of the Seventh Colloquium are glad to have the opportunity of expressing their sincere appreciation for the generous financial support given by the Provincial Government of West Flanders.

The personal interest shown by the Governor, Chevalier P. VAN OUTRYVE D'YDEWALLE, and by Mr. J. STORME, Member of the Council, were of great help to our work.

It is a pleasure to thank the Local Authorities of the City of Bruges, especially, Mr. P. VANDAMME, Burgomaster, and Mr. F. VANDENBROELE, Magistrate, as well as the President, the Very Reverend A. LOGGHE, and the Members of the Public Assistance Committee, for their collaboration in the general organization of the Congress.

We wish to express our gratitude to the Mother Superior and the Sisters of the St. Jans Hospital for their co-operation and also to Miss R. ROMMELAERE, who has been secretary to the Colloquium for seven years.

We are also indebted to many others, too numerous to mention, who gave unstintingly of their time and effort to make this undertaking a success.

The Scientific Committee of the Seventh Colloquium

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* This paper has already been published in *Clin. Chim. Acta*, 4(1959) 610.
** Summary only, the complete paper has been published in *Clin. Chim. Acta*.

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**ACADEMIC SESSION**

# THE SYNTHESIS OF ANTIBODIES AND PROTEINS*

H. E. SCHULTZE

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In the last few years biochemists have been very successful in the investigation and the chemical synthesis of active biological substances. This progress has extended to the secrets surrounding the protein, that vital source of biological and biocatalytical activity.

*The role of amino acid sequence in protein chemistry*

The work of numerous investigators has shown that the properties of proteins and their special functions as enzymes, hormones, toxins etc., depend on their chemical

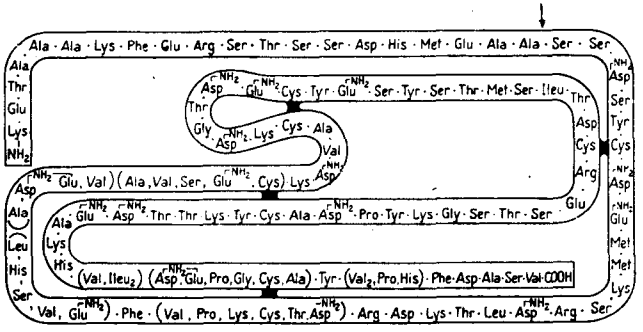


**Fig. 1. The peptide chain of the ribonuclease molecule.**

constitution and particularly on the sequence of the different amino acids contained in each protein. However, it has been shown that only a certain site of the protein molecule is responsible for a particular activity[1]. For example the sequence, aspartic acid–serine–glycine, was found to be an important constituent of the active range in the peptide chains of the proteolytic enzymes, α-chymotrypsin[2–5], trypsin[6–8] and thrombin[9]. The active centre of the ribonuclease molecule which consists of one peptide chain of 124 amino acids[10, 11] was discovered[12] in the area surrounding the 20th and 21st amino acid (Fig. 1) counted from the terminal free aminogroup.

Deviations in the sequence of the peptide chains were also noted in proteins like insulin[13] or cytochrome *c*[14–16] when prepared from different animals. In the case of serum albumins the influence of species specificity is easily detectable by determination of the end groups of the chain (Table I). The differences are especially evident in albumins produced from unrelated animals.

* This paper has already been published in *Clin. Chim. Acta*, 4 (1959) 610.

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TABLE I

END GROUP COMPOSITION OF SERUM ALBUMINS OF DIFFERENT ORIGIN

| *Species* | *$NH_2$-end* | *COOH-end* |
|---|---|---|
| Man[17] | Asp·Ala· | ·Gly·Val·Ala·Leu[19, 20] |
| Ox[17] | Asp·Thr· | Asp·Glu·Lys·Ser·Val·Thr·Leu·Ala[19] |
| Horse[18] | Asp·Thr· | Val·Ser·Leu·Ala[18] |
| Ass[18] | (Asp·Thr·) | Ser·(Val·Lys)·Leu·Ala[18, 20] |
| Mule[18] | (Asp·Thr·) | (·Leu·Ala)[18] |
| Rabbit[20, 21] | Asp· | Leu·Ala[20] |
| Sheep[20] | Asp· | (Glu·Asp·Thr)·Ser·Val·Lys·Leu·Ala[20] |
| Monkey[20] | Asp· | (Asp·Glu)·Ser·Lys·Val·Leu·Ala[20] |
| Pig[17, 20] | Asp· | |

These examples demonstrate the great importance of chemical investigations of proteins. The knowledge of the chemical constitution of the peptide elements provides us with the most reliable information not only as to the specific action, but also as to the origin of a protein. This fundamental part of protein structure, called the primary

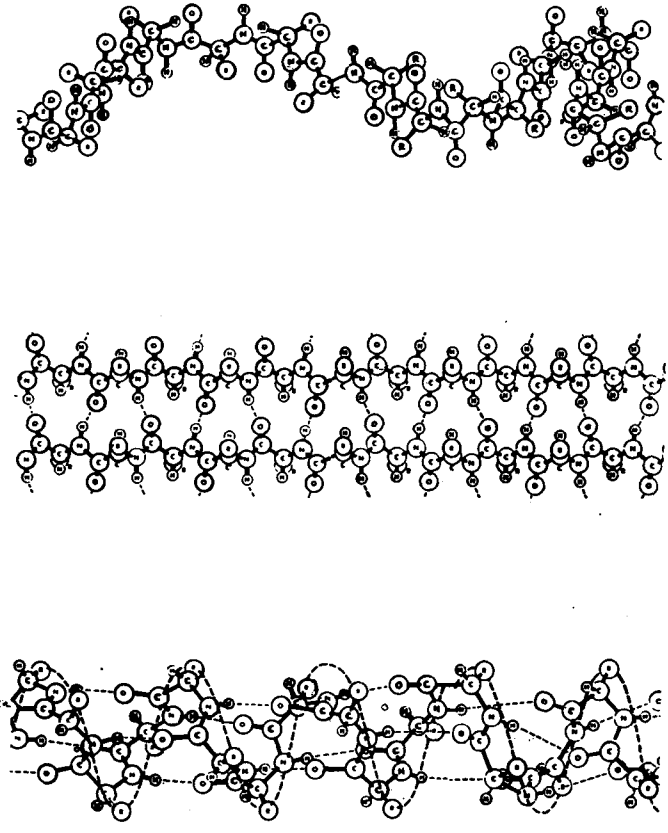

Fig. 2. Secondary structure of peptide chains[22]. α-Helix; β-configuration; arbitrary chain.

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structure, is hereditary, which means that the synthesis of peptides is controlled by genes. It determines the secondary structure (Fig. 2) of the different types of folding of the peptide chains and finally the tertiary structure (Fig. 3), which represents the three-dimensional configuration of the proteins which are usually of very high molecular weight.

The determining influence of the primary structure on the properties of the whole molecule is clearly shown by the hemoglobins Hb A, Hb S and Hb C[24]. Their differences in structure and electrophoretic behaviour arise from the fact that of the total amount of 300 amino acids of Hb A one single glutamic acid group is substituted by valine or lysine in the two other hemoglobins.

*The nature of antibodies*

The only type of protein whose biological activity is not hereditary but acquired by antigen stimulus of exogenous or endogenous origin is the antibody. Like the enzymes, proteohormones and pharmacologically efficient proteins, the antibodies

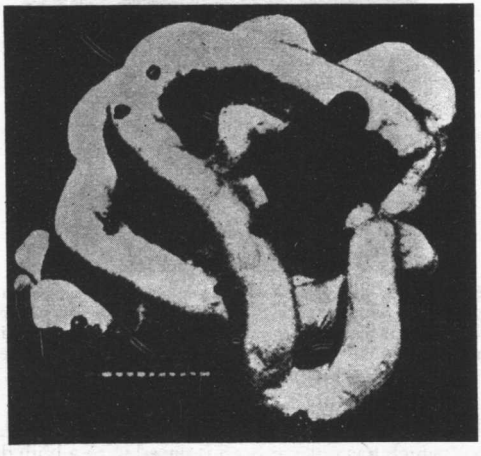

Fig. 3. Three-dimensional model of the myoglobin molecule[23].

are characterized by a high degree of specificity in their mode of reaction. Even in the early days of antitoxin discovery BEHRING found that the binding and neutralizing capacity of the diphtheria antitoxin or of the tetanus antitoxin was extended only to the homologous toxins.

According to recent investigations, the antibodies—whether derived from animals or humans—may be considered as representatives of a genetically related class of proteins. In view of their generally slow mobility and of their immunochemical behaviour (Fig. 4) it seems advisable to regard the antibodies as components of the $\gamma$-globulin system.

Most human *$\gamma$-globulin* is poor in carbohydrates and proteins with the slow sedimentation rate of $S = 7$ (mol. wt. = 156,000). In immuno-electrophoretic analysis it shows an extended precipitation band instead of the usual circular band (Fig. 4b).

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Inhomogeneity is confirmed by the chemical analysis of the slower and faster moving fractions, which differ in the content of neuraminic acid[25]. This phenomenon can be explained by the immuno-chemical relationship of several protein individuals deviating from each other in electrophoretic mobility.

Another characteristic of this fraction is the occurrence of numerous antibodies (shown in Table II).

The small $\beta_2$A-fraction, isolated by the HEREMANS[26] procedure, also shows signs of inhomogeneity as regards its behaviour in immuno-electrophoresis (Fig. 4e) and the complex composition of antibodies. The carbohydrate content of $\beta_2$A-globulins is much higher than that of the main fraction mentioned above.

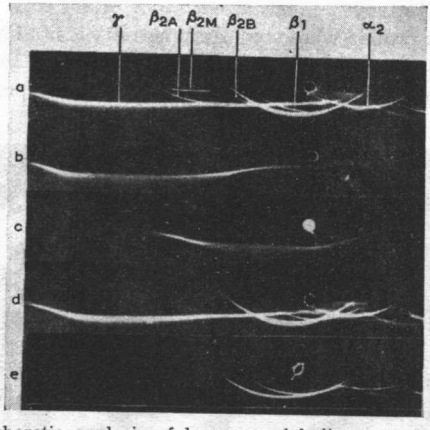


Fig. 4. Immuno-electrophoretic analysis of human $\gamma$-globulin components. (a) normal human serum; (b) normal $\gamma$-globulin components $S_{20,w} = 7$, poor in carbohydrates; (c) $\beta_2$A-globulin components $S_{20,w} = 7$, rich in carbohydrates with traces of $\beta_2$M-globulin $S_{20,w} = 18$, rich in carbohydrates; (d) normo-$\gamma$-globulinemia; (e) agammaglobulinemia. All tested with rabbit antiserum against human serum.

The macro molecular $\beta_2$M-globulin[27-30] is identical with $\gamma_1$M[31-33] ($S = 18$–$20$, mol. wt. = 900,000), which has not yet been isolated with a high degree of purity, and is also rich in carbohydrates.

Its electrophoretic mobility corresponds to that of the $\beta_2$A-globulin but, unlike the components with the sedimentation constant $S = 7$, the $\beta_2$M-globulin is altered by the action of sulphydryl compounds like cysteine or mercaptoethanol, the sedimentation constant being reduced by such treatment from $S = 18$–$20$ to $S = 7$[34-37]. This dissociation of the macro molecular component of the $\gamma$-globulin system is accompanied by a total loss of antibodies. In our experiments with $\gamma$-globulin fractions accumulated in $\beta_2$M we found a parallel increase of antibodies against polysaccharides of blood groups or bacteria (Table II). Pneumococcus-polysaccharide antibodies from horse, ox and pig[38, 39] also belong to the macro molecular type.

Finally, *properdin* belongs to the group of macro molecular antibodies. PILLEMER, BLATTBERG, NELSON and others[40-43] have shown that it has heterophilic activities (Table II) and moves in electrophoresis at the rate of the fast $\gamma$-globulins or slow $\beta$-globulins.

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The occurrence of other representatives of the $\gamma$-globulin system with specific affinities is possible. We agree with GRABAR that these $\gamma$-globulins may function as transport substances primarily for metabolites in a general physiological mechanism but secondarily also for antigenic materials of endogenous or exogenous origin. Recent experiments in the field of plant hemagglutinins[45] have revealed that the protein of

TABLE II

DISTRIBUTION OF NATURALLY ACQUIRED ANTIBODIES IN THE COMPONENTS OF HUMAN $\gamma$-GLOBULIN SYSTEM

| *$\gamma$-globulin components** | *$\gamma$-globulins low in carbohydrate*** | *$\beta_2A$-globulin**** | *$\beta_2M$-globulin* | *Properdin* |
|---|---|---|---|---|
| | Antibody activity§ | | | Antibody against: |
| Diphtheria | +++ | ++ | + | zymosan |
| Tetanus | ++ | + | ++ | levan |
| Typhoid H | ++ | + | ++ | dextran |
| Typhoid O | + | ++ | +++ | polysaccharid |
| Paratyphoid B | + | + | +++ | (shiga, coli a.o.) |
| Pertussis | + | | | stromata of |
| Poliomyelitis typ I–III | ++ | ++ | ++ | erythrocytes and |
| Isoagglutinins (anti-A a.B) | | + | +++ | organ cells |
| | | | | phagues |
| | | | | New castle virus |
| | | | | protozoes |
| | | | | rh-agglutin. |
| | | | | cold-agglutin. |
| | | | | reagins |
| | | | | lues-antib. |
| | | | | Forssman antib. |
| | | | | hemolys. (rabbit) |
| Electrophoresis§§ | 0.5–2.1 | 1.2–3.6 | | |
| Sedimentation constant§§§ | 7.1 | 7 | 18–20 | 27 |
| Hexoses | 1.4 | 3.2 | 4.2 | |
| Fucose | 0.19 | 0.22 | 0.46 | |
| Acetylhexosamine | 1.1 | 2.9 | 3.0 | |
| Acetyl neuraminic acid | 0.23 | 1.8 | 2.0 | |

* In total $\gamma$-globulin fractions: antibodies against scarlet fever, smallpox, herpes, varicella, rubella infect. and hematog. hepatitis and infect. mononucleosis.
** Free of $\beta_2$A- and $\beta_2$M-globulin.
*** Traces of $\beta_2$M-globulin.
§ Own results.
§§ pH = 8.6; $-u \cdot 10^{-5}$ cm$^2$/V· sec.
§§§ $S_{20,w} \cdot 10^{-13}$ cm/sec· dyn.

the seed of *laburnum alpinum*, which has a high binding capacity for blood group substances, also has the physicochemical characteristics of a $\gamma$-globulin. We have, therefore, to include a large group of naturally occurring proteins in our system of $\gamma$-globulins or binding globulins, and we may conclude that there is only a gradual and not a fundamental difference between the so-called normal $\gamma$-globulins and those of an acquired specificity, namely the antibodies.

The individual antibodies can naturally differ in electrophoretic mobility as well

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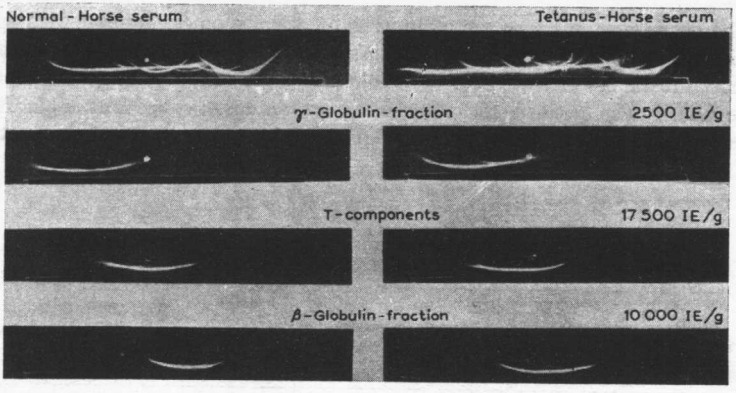


Fig. 5. Immuno-electrophoretic analysis of isolated globulin fractions of a normal and a hyperimmunised horse.

as in chemical composition[47-48]. However, no chemical difference has yet been detected between a $\gamma$-globulin with a high antibody activity and a $\gamma$-globulin of the same rate of migration with no, or only a low, antibody specificity[49].

Fig. 5 illustrates the conformity in the immuno-electrophoretic test with rabbit antiserum against horse serum, of $\gamma$-globulin fractions from a normal horse and those from a horse hyperimmunised with tetanus-toxin, both separated by zone electrophoresis. According to Table III no differences in carbohydrate content can be observed in the corresponding fractions.

The probability that the acquired immune specificity could be accompanied by a deviation—possibly only slight—in the primary structure, *i.e.* in the amino acid composition, cannot be excluded, because the complete analysis of the $\gamma$-globulin peptide chains has not yet been carried out. But in the case of rabbit $\gamma$-globulin, which consists

TABLE III

CARBOHYDRATE CONTENT OF ISOLATED GLOBULIN FRACTIONS OF A NORMAL AND A HYPERIMMUNISED HORSE

| *Protein* | *Antitoxin units/g* | *Electrophoretic mobility** | *Hexoses* | *Acetyl hexosamine* | *Acetyl neuraminic acid* |
|---|---|---|---|---|---|
| (a) Before immunisation | | | | | |
| Normal-$\gamma$-globulin | | 1.7 | 1.1 | 1.1 | 0.22 |
| Normal-T-globulin | | 4.4 | 2.1 | 1.9 | 0.89 |
| Normal-$\beta$-globulin | | 5.5 | 3.3 | 2.8 | 1.74 |
| (b) After immunisation | | | | | |
| Anti-tetanus-$\gamma$-globulin | 2,500 | 1.9 | 1.1 | 0.9 | 0.25 |
| Anti-tetanus-T-globulin | 17,500 | 3.5 | 2.0 | 1.9 | 0.69 |
| Anti-tetanus-$\beta$-globulin | 10,000 | 4.4 | 2.6 | 2.3 | 1.49 |
| Anti-diphtheria-$\gamma$-globulin | 5,000 | 1.7 | 1.5 | 1.4 | 0.39 |
| Anti-diphtheria-T-globulin | 25,000 | 3.5 | 2.2 | 1.9 | 0.71 |

* pH = 8.6; $-u \cdot 10^{-5}$ cm$^2$/V · sec.

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of a single peptide chain, the same pentapeptide sequence was found in a normal and in an antibody-containing $\gamma$-globulin[50]. HAUROWITZ' view, now generally accepted, is that the antibody specificity is caused merely by an abnormal mode of folding of the peptide chains, which results in the formation of a "combining site" complementarily adjusted to one of the "determinant groups" of the antigen molecule[51]. We have, therefore, to distinguish between the hereditary specificity of proteins based on the amino acid pattern and the acquired specificity of $\gamma$-globulins caused by alterations in the secondary or tertiary structure. This statement leads to the conclusion that the mechanism for the synthesis of the peptide chain of antibodies is the same as that for all the other proteins.

*The formation of peptide bonds and proteins in general*

The energy required for the formation of the typical peptide bonds is supplied by the following reactions:

$$\text{Amino acid} + \text{E} + \text{ATP} \rightleftharpoons \text{E} \cdot \text{AMP} \cdot \text{amino acid} + \text{PP} \quad (1)$$

$$\text{E} \cdot \text{AMP} \cdot \text{amino acid} + \text{amino acid} \rightleftharpoons \text{E} \cdot \text{AMP} + \text{peptide} \quad (2)$$

E = specific enzyme ATP = adenosine triphosphate
PP = pyrophosphate AMP = adenosine monophosphate

Generally the reaction site of this first step in protein synthesis[52] is the living cell (Fig. 6). However, the formation of peptides and even of proteins also takes place in

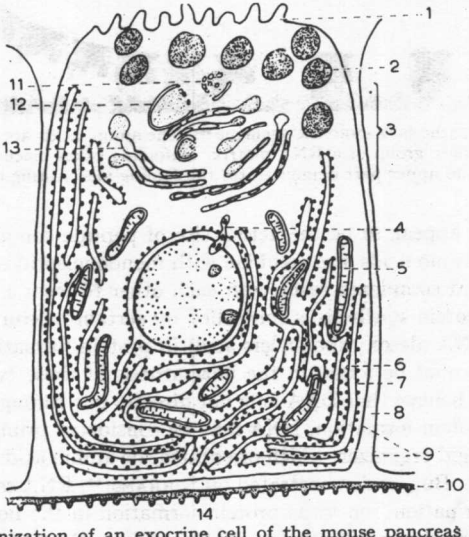


Fig. 6. Stepwise organization of an exocrine cell of the mouse pancreas (Scheme according to SJÖSTRAND[58]). 1 and 9: cell membrane (60 Å in diameter); 2: zymogen granules (store of enzymes); 3: GOLGI apparatus, consisting of double membranes with vacuoli inside; 4: breaking through of nucleolar substance (RNA); 5: mitochondrion, bordered by an outer double-edged membrane, inside with cristae (producer of energy, store of energy; dimensions $^1/_2\,\mu \times 3$ to $8\,\mu$); 6: basement cytoplasmic membranes with = 7: granula attached to one side, probable microsomes (loci of protein synthesis, dimensions $\sim$ 140 Å in diameter); 8: space between two cells; 10: basement membrane; 11–13: particles of the GOLGI apparatus, vacuoli.

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tissue slices or homogenates if the following factors are present: adenosine-triphosphate, an ATP-generating system, guanosine-triphosphate or guanosine-diphosphate, microsomes and a soluble cell extract[53–57].

The soluble enzymes and ATP have been found to effect the initial carboxyl activation of amino acids[59]. The activating enzymes are probably specific for the different amino acids[60–61]. The adenyl-amino acids are extremely reactive intermediates and exist probably only in combination with the enzyme. It is not yet clear whether the so-called activated amino acids are identical with adenyl-amino acids.

The soluble extract contains a labile form of ribonucleic acid named S-RNA[62], which has a low molecular weight. This RNA-derivative activates and binds amino acids in the presence of ATP by an ester bond to ribose[63–64]. The amino acids so bound to ribonucleic acid are subsequently transferred to microsomal protein, and this transfer is dependent upon guanosine-triphosphate. The ribonucleoprotein particles

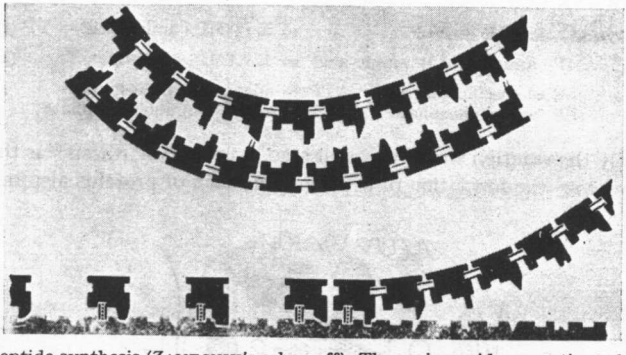

Fig. 7. Polypeptide synthesis (ZAMECNIK's scheme[68]). The amino acids are activated by ATP and bound on the phosphoric group of a RNA-matrix. Unfolding takes place after the formation of peptide bonds. The upper part demonstrates the folding and uniting of two chains.

of the microsomes appear to be the actual site of peptide condensation. It is quite possible that the amino acids also combine with mononucleotides and that these nucleotidyl–amino acid complexes react with each other to form a nucleoprotein[65]. In fact, the rate of protein synthesis is controlled by certain mixtures of nucleotides.

The role of DNA (desoxyribonucleic acid) in protein formation is not yet clear. As no correlation could be found in the biosynthesis of these two macromolecules, most investigators believe that DNA acts not directly but through RNA in the reactions leading to protein formation. DNA may be considered primarily as a template, as a more or less rigid organizer, which orientates the amino acids during the process of protein synthesis. But, as demonstrated by SCHRAMM[66], RNA can act as a template and transfer "information" on virus protein formation in the host cell. The special mechanism by which the peptide chain is unbound from the matrix and folded is not yet known[67].

Some idea may be derived from the scheme of polypeptide synthesis illustrated in Fig. 7.

A specific sequence of amino acids in the peptide chain can also be attributed to the action of a series of highly specific enzymes. The term zymosequential specificity,

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however, has been coined for an assumed kind of enzymes which continually change their specificity[69]. The older enzyme theory, which explained the transfer of specificity by a successive action in the filing of amino acids in a chain, has been refuted by experiments with isotopes. Whichever theory we prefer, the template or the enzyme theory, we have reason to believe that the specificity of a protein based on a typical sequence of the amino acids in its peptide chain is genetically determined.

With regard to the antibodies, we know that they have the same amino acid composition, the same terminal amino acids and most probably also the same amino acid sequence as other $\gamma$-globulins without the special activity, therefore we must investigate the influence of genes in the different steps of antibody formation.

*Origin of antibodies*

One of the most important discoveries about antibodies is the observation that the liver, which is the principal organ of protein synthesis, is seldom engaged in antibody production[70–72]. If antigen is injected intravenously, most of the antibody is formed in the spleen[73] or in the bone-marrow. Local injections lead to antibody for-

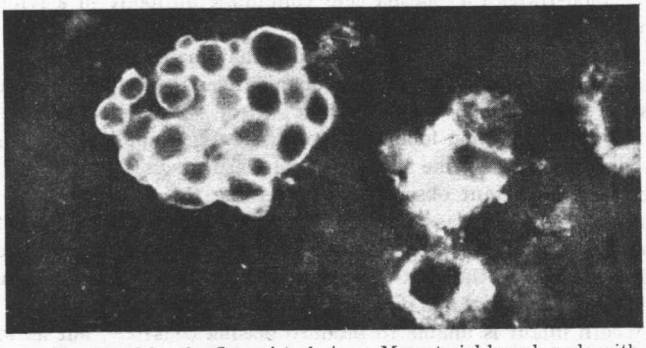

Fig. 8. Plasma cells stained by the COONS' technique. Mesenterial lymph node with plasma cells containing antibody.

mation in the regional lymph nodes[74, 75]. It is possible that antibodies synthesized by different cells have a different electrophoretic mobility and different amino acid end groups[76, 77].

FAGRAEUS[78–82] first discovered a relationship between antibody production and the appearance of plasma cells in the spleen and lymph nodes of immunised animals. She also observed an increase particularly of immature plasma cells in tissue cultures of extirpated spleen tissue from rabbits sensitized with typhoid vaccine. The origin of the plasma cells is not yet clear. Some investigators[78–83] suggest that, like the plasma cells produced in the bone-marrow of patients suffering from myelomatosis, they stem from reticulum cells, while others[83–87] regard them as transformed lymphocytes.

The histochemical identification of antibody-containing plasma cells has been much facilitated by COONS'[88–92] tracer method, using fluorescein–isocyanate, which is easily bound by proteins. As a result of an immune reaction the stained antibody is fixed by those cells containing the homologous antigen or by the cells engaged in antibody formation, if a layer of the specific antigen is artificially deposited on their sur-

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