Institute of Pathology (Director: Prof. P. ENDES),
University Medical School, Debrecen

THE GRANULATED CELLS OF THE JUXTAGLOMERULAR APPARATUS

II. HISTOCHEMICAL TESTS FOR AMINO ACIDS

SZ. GOMBA, MARGIT SOLTÉSZ and P. ENDES

(Received May 30, 1962)

In a previous paper [17] we have reported on our preliminary histochemical studies concerning the granules in the cells of the juxtaglomerular apparatus. The granules were found to be composed of protein and carbohydrate, and it was conjectured that they contained also lipids. The present histochemical investigations were undertaken to determine the amino acid spectrum of the protein component of the granules.

Material and method

The kidneys of albino mice from our laboratory stock were used. The material was usually fixed in formol neutralized with a 10 per cent solution of calcium carbonate, and then embedded in paraffin; frozen sections were examined in a few instances only. The histochemical procedures applied were as follows.

1. Millon's reaction, as modified by BAKER [3].
2. Glenner's nitrosophenol reaction for tyrosine [13].
3. Glenner—Lillie's diazotization-coupling reaction for tyrosine [16].
4. Tetrazone reaction with Fast blue B [8]. The concentration of the tetrazonium salt was 0.1 per cent.
5. Dimethylamino benzaldehyde nitrite (DMAB) reaction according to ADAMS [1]. We prolonged the time of treatment from the original 60 seconds to 90 seconds in respect of both reagents.
6. Glenner's rosindole reaction [14].
7. Post-coupled benzylidene reaction [15] with 1-amino-2-naphthol-4-sulphonic acid.
8. Dinitrofluorobenzene (DNFB) reaction [8].
9. Alkaline tetrazolium reaction with neotetrazolium [29].
10. 2,2'-dihydroxy-6,6'-dinaphthyl-disulphide (DDD) according to BARRNETT—SELIGMAN [7]. Reduction of the disulphide groups was effected by means of 10 per cent sodium thioglycolate, as described by LILLIE [21].
11. Sakaguchi II reaction (as modified by Baker), for arginine [29].
12. Pretreatment with dinitrofluorobenzene (DNFB): 1 per cent solution of DNFB in 90 per cent alcohol; the solution was 0.01 M for sodium hydroxide. Duration of treatment 24 hrs at room temperature. Control in alcohol only.
13. Benzoyl treatment, 10 per cent pyridine benzoyl chloride solution, 24 hrs at room temperature. Control in pyridine only.
14. Iodine treatment, 0.78 N iodine solution in 96 per cent alcohol, 72 hrs at room temperature. Control in alcohol only. Besides, after LANDING [22]: in a solution containing 0.3 per cent iodine and 0.6 per cent potassium iodide (pH = 10), 24 hrs at room temperature.
15. Potassium persulphate treatment ($K_2S_2O_8$), with a 2 per cent aqueous solution 20 hrs at room temperature. Control in distilled water only.
16. Peracetic acid and performic acid treatment with Pearse's solutions [29] for 24 hrs with the former, 90 minutes with the latter, at room temperature.

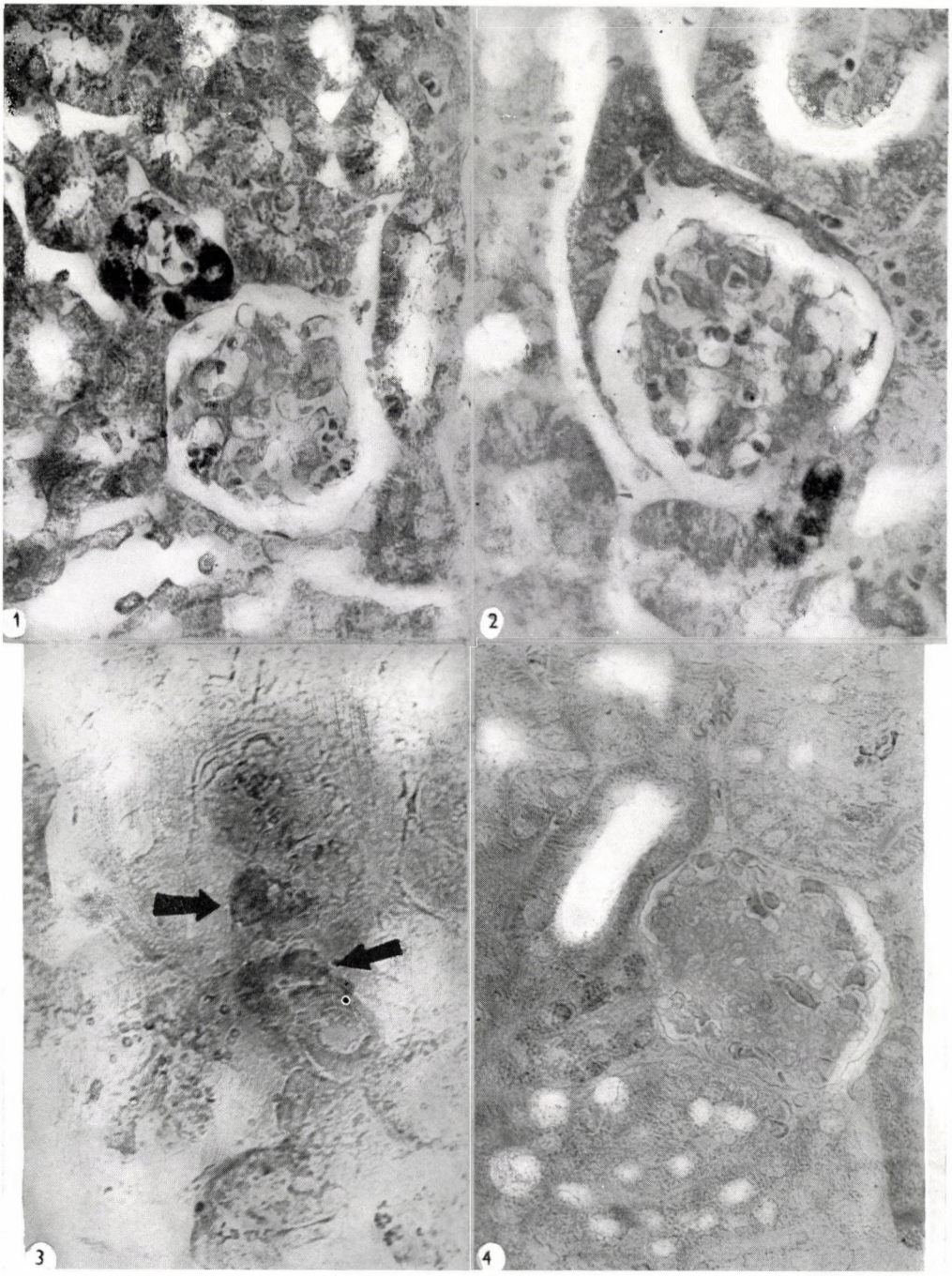

Fig. 1. Glenner—Lillie's resorcin-copperchelate method for tyrosine. Note juxtaglomerular cells filled with positively reacting granules in the wall of the transversely sectioned afferent arteriole. × 400

17. Deamination, with equal parts of 10 per cent acetic acid and 5 per cent sodium nitrite according to PEARSE [29] 24 hrs at + 4° C.

We covered the sections with Canada balsam or, if necessary, with a mixture of glycerol and gelatin, and examined them immediately after the reactions.

Results

The results are listed in Table I. It can be seen that the Millon positivity of the granules, demonstrated in our earlier paper disappeared on treatment with benzoyl chloride and iodine, but persisted after DNFB treatment. The Millon positivity of the juxtaglomerular cells was restricted to the granules which dis-

Table I

Millon's test—Baker's modific.	+
Jodination + Millon's test—Baker's modific.	−
Dinitrofluorobenzoylation + Millon's test—Baker's modific.	+
Performic acid + Millon's test—Baker's modific.	+
Glenner's nitrosophenol test for tyrosine	+
Glenner—Lillie's diazotization coupling reaction for tyrosine	+
DMAB nitrite reaction (Adams)	+
Performic acid and peracetic acid treatment + DMAB nitrite r.	−
$K_2S_2O_8$ + DMAB nitrite reaction	−
Dinitrofluorobenzoylation + DMAB nitrite reaction	+
Benzoylation + DMAB nitrite	−
Jodination + DMAB nitrite reaction	−
Rosindole reaction (Glenner)	+
Post-coupled benzylidene reaction	+
Tetrazonium reaction with Fast Blue B	+++
Jodination + tetrazonium reaction	+
Dinitrofluorobenzoylation + tetrazonium reaction	+
Benzoylation + tetrazonium reaction	−
Dinitrofluorobenzene reaction	+
Deamination + dinitrofluorobenzene reaction	+
Tetrazolium reaction with neotetrazolium	+
DDD reaction (Barrnett—Seligman)	−
10 per cent sodium thioglycolate + DDD reaction	−
Sakaguchi II reaction—Baker's modific.	−

Fig. 2. Juxtaglomerular cells giving positive tetrazonium reaction with Fast blue B in the glomerular hilus. × 400

Fig. 3. Darkly stained granulated juxtaglomerular cells in two confronting hili. Adams dimethylaminobenzaldehyde reaction. × 200

Fig. 4. Alkaline tetrazolium reaction with neotetrazolium. Juxtaglomerular cells filled with positive granules in the wall of the longitudinally sectioned afferent arteriole situated in the hilus of the glomerulus

played a pale brownish-red colour. The colour was not very distinct, but at a magnification by 150 the juxtaglomerular apparatus was well distinguishable from its surroundings. Millon's reaction is regarded as absolutely specific for tyrosine in animal tissues; it is generally positive for phenols in vitro and the reagent gives a red colour with tryptophan. Tyrosine ceases to be positive with Millon's reagent after pretreatment with benzoyl or iodine. DNFB reacts with tyrosine and does not react with tryptophan. A positive response to Millon's reagent persisting after DNFB treatment seems, therefore, to be contradictory. Yet, GLENNER and LILLIE [16] observed an essentially similar phenomenon in the course of diazotization-coupling reactions for tyrosine in which a process of chelation occurs as it does in Millon's reaction. It is possible that the dinitrophenyl radical is displaced by the chelate bridge formed in the reaction. With a view to ascertaining that the Millon positivity was due to the presence of tyrosine, we repeated the reaction after performic acid treatment; it was positive also in this case. That GLENNER's nitrosophenol reaction [13] (which demonstrates the presence of tyrosine by the formation of an ortho-nitrosotyrosine-metalchelate complex) proved to be likewise positive should be regarded as a further argument in favour of the presence of tyrosine. The granules gave a positive reaction on GLENNER—LILLIE's diazotization-coupling [16], whether or not alphanaphthol was coupled or a resorcin-copperchelate complex was formed.

Coupled tetrazonium gives a positive reaction with histidine, lysine, tyrosine, tryptophan, arginine and cysteine. The granules displayed a red colour with this method, a phenomenon which may have been due in the given case to the presence of tyrosine, tryptophan or histidine, since no other possible amino acid was demonstrable with the direct method. The colour reaction is considerably weakened by pretreatment with DNFB, potassium persulphate, performic acid or peracetic acid, it disappears on benzoylation and is resistant to iodination. Literary data are contradictory concerning the behaviour of the tetrazonium reaction following pretreatments of the said nature. The tetrazonium reaction for tryptophan is, according to DANIELLI [9], selectively blocked by performic acid, so that the considerable weakening of the colour reaction after performic acid pretreatment points to the presence of tryptophan. Negative Millon, negative DMAB and positive tetrazonium reaction following iodination probably point to the presence of histidine, but literary data are rather controversial in this respect.

The fact that the granules entered into reaction with DMAB is considered the most noteworthy result of our investigations. The granules stained blue, the juxtaglomerular cells were sharply outlined, and their colour was more intensive than that of any other part of the kidney. The juxtaglomerular apparatus could well be recognized with $30\times$ magnification. The reaction was strongest in the granules, but even the cytoplasm gave an uneven

patchy colour reaction, a phenomenon presumably due to a damage of the granules caused by the acid treatment. The colour effect was more pronounced in formalin-fixed frozen sections than in material embedded in paraffin. If fixation in formalin lasts 24 instead of the usual 6 hours, the diffuse pale blue staining of the renal tissue disappears, while the colour of the juxtaglomerular apparatus undergoes no change, so that contrasts become more pronounced. Since it is only with tryptophan that this method gives a blue colour in the tissues, the procedure is quite specific. Pretreatment with performic acid renders the reaction negative, since performic acid selectively oxidizes tryptophan. The blue staining is likewise inhibited by pretreatment with potassium persulphate, iodine, or benzoyl chloride, while pretreatment with DNFB produces no effect. The fact that the rosindole reaction was positive seemed to corroborate the result of the DMAB reaction. A comparison of these three reactions for tryptophan shows that ADAMS' method [1] gives the most intensive colour effect.

Using material fixed in formol, CARNOY's fluid and alcoholic trichloroacetic acid, we provoked the tetrazolium reaction and BARRNETT—SELIGMAN's DDD reaction for the demonstration of possible sulphurous amino acids. The last-named method gave negative results whether or not it was applied alone or after reduction with 10 per cent sodium thioglycolate. On the other hand, the granules assumed a vivid red colour after treatment with alkaline tetrazolium. Structures containing cystine, cysteine, lipids, lipofuscin or reducing sugars give, according to PEARSE [29] a positive reaction with this method, and also intracellular enterochromaffin granules and adrenochromes may do so. The result of the DDD reaction, further the negative results of the HILLARP—HÖKFELT and hexamethylenetetramine reactions, as described in our previous paper [17], make it probable that the positive result of the alkaline tetrazolium reaction is due to the presence of lipids or reducing sugars. This problem requires further investigation.

SAKAGUCHI's reaction was negativ in our earlier experiments; for the sake of completeness, we repeated the reaction in the present investigations with BAKER's modification, and the result was negative again.

Discussion

Recent literature contains numerous investigations made with a view to ascertaining the physiological and pathological significance of the intracellular granules of the juxtaglomerular apparatus. The results of these investigations justify the conclusion that renin is produced by the cells of the juxtaglomerular apparatus. BING [4] concluded on the evidence of kidney microdissection that renin was produced in the immediate vicinity of the glomeruli.

DEMOPOULOUS et al. [10] found the renin activity of renal extracts to be directly proportional to the juxtaglomerular index. EDELMAN and HARTROFT [12] selectively stained the granules by means of fluorescein-labelled antirenin. DENGLER and REICHEL [11] located renin activity in the mitochondrial fraction by the ultracentrifugation of kidney homogenate. None of these investigations threw light on the chemical composition of the juxtaglomerular granules.

HARADA's results [18, 19] and our own histochemical investigations showed that the granules were chiefly composed of protein and carbohydrate, and that they presumably contained lipids. Lipid was demonstrated by MCMANUS by means of Sudan black. The results of HESS and PEARSE [20] were more decisive; by histochemical methods they demonstrated mitochondrial alpha glycerophosphate dehydrogenase activity in the granulated cells, and observed a linear relationship between the degree of activity and the renin contents of the kidney. While these observations meant a valuable contribution to our knowledge concerning the metabolism of juxtaglomerular cells, they presented no new data on the chemical composition of the granules.

Our present investigations have pointed to the presence of a considerable amount of tryptophan in the granules. Further investigations will have to decide whether and how far the role of tryptophan is decisive in the composition of the granules. That alkaline tetrazolium reaction proved the presence of a reducing substance in the granules is a significant fact. In view of the findings of MCMANUS, and in consideration of our above results, the reducing substance in question originates most probably from lipid matter, although the PAS-positive granules may contain reducing sugars.

Let us note in connection with the negative result of the DDD reaction that certain histochemically satisfactory fixing agents as, for instance, Carnoy's fluid and alcoholic trichloroacetic acid, induce grave changes in the juxtaglomerular cells, so that only formol-fixed sections could be used for the reaction. Similar damaging effects were observed in connection with the slow or rapid (liquid air) cooling of the kidney. Relying on the results of our investigations in progress, we regard fixation by formalin as the procedure that causes the least damage to the structure of juxtaglomerular cells.

MCMANUS [24], OBERLING [30], HESS and PEARSE [20] claim that the macula densa and the juxtaglomerular complex constitute a functional unity. Our observations have not confirmed this theory.

Summary

Histochemical tests have shown that the granules in the cells of the juxtaglomerular apparatus of albino mice contain tyrosine and a significant amount of tryptophan. They probably contain histidine as well, but its presence needs further proof. The reactions failed to demonstrate the presence of sulphurous amino acids and arginine. The result of the alkaline tetrazolium reaction allows to conclude that the granules contain a reducing substance that may be of lipoid origin or consist of reducing sugars.

REFERENCES

1. ADAMS, C. W. M.: (1957) A p-dimethylaminobenzaldehyde nitrite Method for the Histochemical Demonstration of Tryptophan and Related Compound. J. clin. Path. **10,** 57. — 2. ADAMS, C. W. M.: (1960) A Histochemical Method for Tryptophan Applicable to Formalin Fixed Tissues. J. Path. Bact. **80,** 442. — 3. BAKER, J. R.: (1956) The Histochemical Recognition of Phenols, especially Tyrosine. Quart. J. micr. Sci. **97,** 161. — 4. BING, J., KAZIMIERCZAK, J.: (1960) Renin Content of Different Parts of the Periglomerular Circumference. Acta path. microbiol. scand. **50,** 1. — 5. BARNARD, E. A.: (1960) The Cytochemistry of Proteins. New Approaches in Cell Biology. Academic Press, New-York, 145. — 6. BARRNETT, R. J.: (1953) The Histochemical Distribution of Protein-bound Sulfhydril Groups. J. nat. Cancer Inst. **13,** 905. — 7. BARRNETT, R. J., SELIGMAN, A. M.: (1952) Histochemical Demonstration of Protein-bound Sulfhydril Groups. Science, **116,** 323. — 8. BURSTONE, M. S.: (1955) An Evaluation of Histochemical Methods for Protein Groups. J. Histochem. Cytochem. **3,** 32. — 9. DANIELLI, J. F.: (1950) Studies on the Cytochemistry of Proteins. Cold. Spr. Harb. Symp. quant. Biol. **14,** 32. — 10. DEMOPOULOUS, H., KALEY, G., ZWEIFACH, B. (1960) The Histologic Distribution of Renin in the Kidneys of the Rat and Rabbit. Amer. J. Path. **37,** 443. — 11. DENGLER, H., REICHEL, G.: (1960) Untersuchungen zur intrazellulären Localisation der Renin- und Hypertensinase-Activität. Experientia (Basel) **16,** 36. — 12. EDELMAN, R., HARTROFT, P. M.: (1961) Localisation of Renin in Juxtaglomerular Cells of Rabbit and Dog through the Use of the Fluorescent Antibody Technique. Circulat. Res. **9,** 1069. — 13. GLENNER, G. G.: (1959) A Nitrosophenol Reaction for Tyrosine and Related Compounds in Tissue Sections. J. Histochem. Cytochem. **7,** 423. — 14. GLENNER, G. G. (1957): The Histochemical Demonstration of Indole Derivatives by the Rosindole Reaction of E. Fischer. J. Histochem. Cytochem. **5,** 297. — 15. GLENNER, G. G., LILLIE, R. D.: (1957) The Histochemical Demonstration of Indol Derivatives by the Post-coupled p-dimethylamino-benzylidene Reaction. J. Histochem. Cytochem. **5,** 279. — 16. GLENNER, G. G., LILLIE, R. D.: (1959) Observations on the Diazotization-coupling Reaction for the Histochemical Demonstration of Tyrosine: Metal Chelation and Formazan Variants. J. Histochem. Cytochem. **7,** 416. — 17. GOMBA, SZ., SOLTÉSZ, M., ENDES, P.: (1962) Studies of the Granulated Cells of the Juxtaglomerular Apparatus. I. Acta morph. Acad. Sci. hung. **11,** 189. — 18. HARADA, K. (1954): Histochemical Studies of the Juxtaglomerular Apparatus. Rev. belge Path. **23,** 311. — 19. HARADA, K.: (1956) Selective Contrast Staining of Granules in the Juxtaglomerular Complex. Stain Technol. **31,** 207. — 20. HESS, R., PEARSE, A. G. E.: (1961) Mitochondrial alphaglycerophosphate dehydrogenase Activity of Juxtaglomerular Cells in Experimental Hypertension and Adrenal Insufficiency. Proc. Soc. exp. Biol. (N. Y.) **106,** 895. — 21. KISZELY, GY., BARKA, T.: (1958) Gyakorlati mikrotechnika és hisztokémia. Medicina, Budapest. — 22. LANDING, B. J.: (1956) Selective Demonstration of Histidine. Stain Technol. **31,** 197. — 23. LILLIE, R. D.: (1956) The p-dimethylaminobenzaldehyde Reaction for Pyrroles in Histochemistry: Melanins, Enterochromaffin, Zymogen Granules, Lens. J. Histochem. Cytochem. **4,** 118. — 24. MCMANUS, J. F. A.: (1957) Further Observations on the Glomerular Root of the Vertebrate Kidney. Quart. J. micr. Sci. **88,** 39. — 25. MCMANUS, J. F. A.: (1955) Applications of Histochemistry to Elucidation of Kidney Structure and Function. J. Histochem. Cytochem. **3,** 243. — 26. MADDY, A. H.: (1961) 1-fluoro-2 : 4-dinitrobenzene as a Cytochemical Reagent. I. Histological Applications. Exp. Cell Res. **22,** 169. — 27. MADDY, A. H.: (1961) 1-fluoro-2 : 4-dinitrobenzene as a Cytochemical Reagent. II. The Chemical Basis of the Reaction with Cell Nuclei. Exp. Cell Res. **22,** 181. — 28. MARSCHALL, J., WAKERLIN, G. E.: (1948) Purification and Possible Histochemical Localisation of Renin. Fed. Proc. **7,** 78. — 29. PEARSE, A. G. E.: (1960) Histochemistry. Churchill. London. — 30. OBERLING, CH. H.: (1960) Comp. R. Soc. Biol. Paris. **250,** 929.

UNTERSUCHUNG DER GRANULIERTEN ZELLEN DES JUXTAGLOMERULÄREN APPARATES II. HISTOCHEMISCHE UNTERSUCHUNG DER AMINOSÄUREN

SZ. GOMBA, M. SOLTÉSZ, P. ENDES

Bei der histochemischen Untersuchung der Aminosäuren in den granulierten Zellen des juxtaglomerulären Apparates von Mäusen wurde festgestellt, daß die Körnchen Tyrosin und eine verhältnismäßig große Menge Tryptophan enthalten. Es wird angenommen, daß sie auch Histidin enthalten, doch ließ sich dies nicht eindeutig beweisen. Schwefelhaltige Amino-

säuren konnten nicht nachgewiesen werden, und die Anwesenheit von Arginin konnte gleichfalls ausgeschlossen werden. Das Resultat der alkalischen Tetrazoliumreaktion läßt darauf schließen, daß die Körnchen einen reduzierenden Stoff, vermutlich Lipoid oder reduzierenden Zucker enthalten.

ИССЛЕДОВАНИЕ ГРАНУЛИРОВАННЫХ КЛЕТОК ЮКСТАГЛОМЕРУЛЯРНОГО АППАРАТА. II. ГИСТОХИМИЧЕСКИЕ ИССЛЕДОВАНИЯ АМИНОКИСЛОТ

С. ГОМБА, М. ШОЛТЕС, П. ЭНДЕШ

Авторы проводили гистохимическое исследование гранулированных клеток юкстагломерулярного аппарата мышей и нашли, что зернышки содержат тирозин и относительно большое количество триптофана. Предполагается, что они содержат также гистидин, но доказать это не удалось. Не удалось доказать и присутствия аминокислот с содержанием серы; наличие аргинина также можно исключить. Результат реакции с щелочным тетразолом позволяет сделать вывод, что зернышки содержат восстановитель, которой, по всей вероятности, представляет из себя липоид или восстановительных сахар.

Dr. Szabolcs Gomba
Dr. Margit Soltész
Dr. Pongrác Endes
Debrecen, Kórbonctani Intézet, Hungary

Printed in Hungary

A kiadásért felel az Akadémiai Kiadó igazgatója Műszaki szerkesztő: Farkas Sándor
A kézirat nyomdába érkezett: 1963. VII. 4. — Terjedelem: 10 (A/5) ív, 57 ábra

63.57402 Akadémiai Nyomda, Budapest — Felelős vezető: Bernát György