The amino acid sequence of the COOH-terminal cyanogen bromide fragment (residues 12 to 54) of the testis-specific basic protein of the rat has been determined. This analysis completes the primary structure of the whole protein by overlapping the sequence of the 23 residues from the NH₂ terminus previously published (Kistler, W. S., Noyes, C., and Heinrikson, R. L. (1974) Biochem. Biophys. Res. Commun. 57, 341-347). The complete sequence of this small, highly basic protein is: NH₂-Ser-Thr-Ser-Arg-Lys-Leu-Lys-Thr-5
His-Gly-Met-Arg-Arg-Gly-Lys-Asn-Arg-Ala-Pro-His-Leu-10
15
20
Gly-Val-Lys-Arg-Gly-Gly-Asn-Arg-Lys-Tyr-Arg-Lys-25
20
Ser-Ser-Leu-Lys-Ser-Arg-Lys-Arg-Gly-Asp-Ser-Ala-Asp-30
35
40
45
Arg-Asn-Tyr-Arg-Arg-Asp-His-Leu-COOH.

The adult testis of a number of mammalian species, including man, contains a small and highly basic protein of markedly restricted amino acid composition (1, 2). This protein is readily extracted in soluble form by homogenization of the testis in dilute mineral acid (0.2 M H₂SO₄) and may be identified after further treatment of such extracts as a discreet stained band resulting from polyacrylamide gel electrophoresis under appropriate conditions (1, 2). In the rat this protein could not be detected in extracts prepared from a large number of other organs, and therefore, it has been assigned the provisional designation “testis-specific” (1, 3). The testis-specific basic protein has been purified from both rat and man, and in each case it was found to be rich in arginine, lysine, glycine, and serine, but to lack six of the amino acids commonly found in proteins, namely, glutamic acid, glutamine, cysteine, isoleucine, phenylalanine, and tryptophan (1, 2).

Although the function of this testicular protein is not known, several lines of evidence indicate that it is associated with the spermatogenic function of the male gonad rather than its role as an endocrine organ. Briefly, under a variety of conditions the presence of the testis-specific basic protein correlates with the occurrence of developing haploid cell types (spermatids) in the seminiferous tubules rather than with the functional state of the androgen-secreting interstitial cells of Leydig (1, 2). Despite this apparent association of the testis-specific basic protein with the development of spermatids, in the rat this protein was undetectable in epididymal spermatozoa, the end product of spermatid maturation (1). Its absence from spermatozoa and its distinctive amino acid composition distinguish it from another class of small and basic proteins associated with spermatogenesis, the basic chromosomal proteins found in association with the DNA of sperm headpieces (1, 4-6).

Because of the wide occurrence of this testicular protein among placental mammals and because of its possible utility as a specific gene product for the study of spermatogenesis, we have been prompted to determine its primary structure. In a previous report (3) the testis-specific basic protein of the rat was shown to consist of a polypeptide chain of 54 residues with a calculated mass of 6200 daltons. The first 23 residues from the NH₂ terminus were identified by automated Edman degradation (3). In addition it was shown that the protein can be cleaved by cyanogen bromide at the single methionyl residue located at position 11 to yield two fragments that can be isolated in pure form by gel filtration (3). In the current paper we present the results of studies that allow the determination of the remainder of the sequence of the rat testis-specific basic protein.

EXPERIMENTAL PROCEDURES

Materials—The testis-specific basic protein was purified as described previously (1, 3). From a typical preparation, the yield of material judged to be pure on the basis of polyacrylamide gel electrophoresis was roughly 25 mg from 900 g of rat testis.
Chymotrypsin (Code SDS) and trypsin (Code TR1PCK) were obtained from Worthington Biochemical Corp., Freehold, N. J. Carboxypeptidase A and thermolysin were purchased from Boehringer Mannheim, New York, and Sigma Chemical Co., St. Louis, Mo., respectively.

Pyridine and N-ethylmorpholine were distilled over ninhydrin (1 g per liter). Phenylisothiocyanate, N,N-dimethylallylamine, anhydrous heptafluorobutyric acid, benzene, ethyl acetate, and butyl chloride were highly purified reagents from Beckman Instruments (Palo Alto, Calif.) for use in automated Edman degradation. Hydroiodic acid (57.7% containing hypophosphorous acid as preservative) was obtained from Fisher Chemical Co., Pittsburgh, Pa. Citraconic anhydride was from Aldrich Chemical Co., Milwaukee, Wis., and cyano bromide was from Pierce Chemical Co., Rockford, III. Sephadex and Bio-Gel chromatography materials were obtained from Pharmacia Fine Chemicals, Piscataway, N. J., and Bio-Rad Laboratories, Richmond, Calif., respectively. All other chemicals were of reagent grade.

Amino Acid Analysis—Samples were hydrolyzed in redistilled constant boiling HCl in vacuo at 110°C for 24 hours. Peptides eluted from paper were frequently hydrolyzed in a solution containing 6 N redistilled phenol and 99% redistilled constant boiling HCl. Phenylthiocarbamylated peptides were redissolved in 0.5 ml of 0.01 M glacial acetic acid, and the acidified mixture was then lyophilized. In some cases the peptides eluted from paper were separated from the bulk of the ultraviolet and/or visible light-absorbing impurities eluted from paper by passage over a column (0.9 X 50 cm) of Bio-Gel P-2 equilibrated and developed with 0.1 M NH₄HCO₃. Six peptides (Th I, Th II, Th IIA, Th IIB, Th V, Th VIl) were obtained in pure form in this manner. Peptide Th V initially contained substantial amounts of peptide Th IIB. These two fragments were resolved by gel filtration on Bio-Gel P-2 as described above.

Peptides Th III, Th IV, and Th VI were not resolved by the chromatographic procedure, but together constituted the most slowly migrating ninhydrin-positive area (see Fig. 2). These three peptides were resolved by high voltage electrophoresis. The mixture obtained from preparative chromatography was applied along a 17-cm line on a sheet of Whatman No. 1 chromatography paper. Descending chromatography in the 1-butanol-pyridine-acetic acid-water system as described above was carried out for 24 hours during which time the solvent front migrated approximately 47 cm from the origin. Peptides were located by staining strips cut from the edges of the zone of migration with ninhydrin. The peptides so detected were eluted with 5% acetic acid, and the resulting solutions were lyophilized. In some cases the peptides eluted from paper were separated from the bulk of the ultraviolet and/or visible light-absorbing impurities eluted from paper by passage over a column (0.9 X 50 cm) of Bio-Gel P-2 equilibrated and developed with 0.1 M NH₄HCO₃.

Amino acid analysis was performed in a Beckman 121A amino acid analyzer. In some instances, assignments made by amino acid analysis were confirmed by the sequence analysis methods described below.

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Chromatography of the phenylthiohydantoins (16) on plates of silica gel (Eastman 13181 with fluorescent indicator) was not resolved by the method described for the material eluted from those areas with no overlap.

Trypsin—Trypsin digestions were performed on one of the thermolysin peptides following citraconylation (9). Peptide Th VI (800 nmol) was dissolved in 2 ml of 0.1 sodium borate at pH 8.5. Twenty-five microliters of the citraconylated thioprotein was added gradually over a period of several minutes at room temperature while the sample was being maintained at 8 and 50°C by the dropping addition of 1 N NaOH. The peptide was then separated from excess reagents by passage through a column (0.9 X 50 cm) of Bio-Gel P-2 equilibrated and developed with 0.1 M NH₄HCO₃. Fractions containing peptide were lyophilized, the dry residue was dissolved in 2 ml of 0.1 M N-ethylmorpholine acetate at pH 8.5, and 10 µg of trypsin was added. After incubation for 4 hours at 31°C, digestion was terminated by the addition of 200 µl of glacial acetic acid. The acidified solution was allowed to stand for 16 hours at room temperature to effect removal of the citraconyl blocking groups. The solution was then reduced in volume under vacuum and lyophilized.

Analytical Scale Cleavage—Digestion of the intact protein with chymotrypsin was carried out at 8°C with 10 µg of enzyme and 200 µg of testis-specific basic protein in a 200-µl volume of 0.1 M N-ethylmorpholine acetate buffer at pH 8.5. Samples of 50 µl were removed at appropriate times and the digestion stopped by mixing with 5 µl of glacial acetic acid followed by lyophilization. Hydrolysis with thermolysin was done in a similar fashion except that the buffer solution contained 3 mM CaCl₂ and a ratio of 1 µg of enzyme was used for 185 µg of intact protein. In the case of trypsin, 300 µg of intact protein were digested with 1 µg of enzyme under the same conditions described for chymotrypsin.

Sequence Analysis—Automated Edman degradation (10-12) was performed in a Beckman Protein-Peptide Sequencer (model 880-B). All runs were made with the "Fast Peptide Program" No. 80872 of the manufacturer, characterized by a volatile coupling buffer containing N,N-dimethylallylamine. The phenylthiohydantoins liberated after each cycle were identified and quantitated as such or as the trimethylsilyl derivatives by gas chromatography (13, 14) on a Beckman GC-4B unit. Alternatively, the residues liberated were converted back to the parent amino acid (or to alanine in the case of serine) by hydrolysis in 5 N NaOH at 105°C and the resulting samples were fed to the amino acid analyzer. In some instances, assignments made by these quantitative procedures were confirmed by thin layer chromatography of the phenylthiohydantoins (16) on plates of silica gel (Eastman 13181 with fluorescent indicator). A ninhydrin spray reagent for color differentiation of various phenylthiohydantoins (17) was also employed for selected samples.

Manual Edman degradation was performed according to the
semi-micro procedure of Peterson et al. (18), and the liberated residues were identified as described above.

Assignments of COOH-terminal sequences were made by the rate and stoichiometry of release of amino acids from peptide or protein samples during hydrolysis with carboxypeptidase A. Enzyme crystals were washed with water and brought into solution by method 1 of Ambler (19). Digestion was performed in 0.2 M N-ethylmorpholine acetate adjusted to pH 8.5 or, when lysine was the anticipated COOH-terminal residue, in 0.3 M N-ethylmorpholine acetate at pH 9.0. Digestion was terminated by freezing followed by lyophilization. Amino acids released were identified by applying samples of the digest dissolved in sodium citrate buffer directly to the amino acid analyzer.

**RESULTS**

In an earlier communication we reported the amino acid sequence of the first 23 residues of the testis-specific basic protein from the rat (3). Furthermore, it was shown that treatment of the intact protein with cyanogen bromide results in cleavage of the polypeptide chain at the single methionyl residue at position 11 to produce two fragments that are easily isolated in pure form by gel filtration (3). The remainder of the sequence of this 54-residue protein has been elucidated by a combination of methods including digestion with carboxypeptidase A, isolation and analysis of thermolysin fragments, and automated Edman degradation of the COOH-terminal cyanogen bromide fragment (CNBr II) from this molecule.

**Hydrolysis with Carboxypeptidase A**—Digestion of the intact protein with carboxypeptidase A at 34°C resulted in the rapid and nearly quantitative release of leucine (Fig. 1). Histidine and serine were released at an identical rate and considerably more slowly than leucine. No other amino acids were liberated over the course of a 2-hour incubation. A similar digestion carried out at 4°C allowed more precise observation of the rate of release of leucine, but, again, the rates of appearance of histidine and serine were indistinguishable. The COOH-terminal structure of the intact protein was thus established as: -(Ser,His)-Leu.

**Isolation and Analysis of Thermolysin Fragments**—Trial digestion of the intact protein with either trypsin, chymotrypsin, or thermolysin demonstrated that the protein is readily cleaved at numerous sites by all three proteolytic enzymes. Digestion with thermolysin gave rise to a reproducible set of fragments that appeared to map without overlap in the analytical system described under “Experimental Procedures” (Fig. 2). The mapping behavior of these fragments suggested that they could all be obtained in pure form by a two-step procedure involving paper chromatography followed by high voltage electrophoresis. Fragmentation with thermolysin was therefore carried out on a preparative scale, and indeed it was possible to obtain all of the principal peptides in good yield (Table I).

Five of these peptides (Th I, Th II, Th III, Th III B, Th IV) appear in the sequence previously reported (3) for the NH2-terminal 23 residues (cf. Fig. 6). The position of Th IV was readily established, since it contained the single valine in the protein, a residue already identified at position 23 (3). Of the three remaining peptides, only Th VII contained both a leucyl and a histidinyl residue (Table I), and in view of the carboxypeptidase A digestion described above, it was placed at the COOH terminus of the protein.

**Partial Sequence Analysis of CNBr II**—Automated Edman degradation of CNBr II (490 nmol) resulted in unambiguous identification of each residue liberated for the first 17 cycles. Due primarily to incomplete coupling and/or cleavage of the proline encountered on the eighth cycle, a severe overlap problem developed during the subsequent degradation. Hermanson et al. (14) have noted the generation of overlap accompanying automated Edman degradation of prolyl residues, and they found a satisfactory solution to consist of a temperature increase during the coupling phase of the Edman cycle. Accordingly, a second run was made with CNBr II (650 nmol), and the temperature for the coupling phase of cycle 8 was raised from the usual 52°C to 66°C. Unfortunately, this procedure did not increase the recovery of proline on the correct cycle, and the ensuing overlap was as severe as that encountered previously.

Because of the overlap problem described, it was generally impossible to place with confidence such recurrent components of this protein as lysine, arginine, and serine after the 17th cycle. Conversely, despite large gaps in the sequence analysis, the

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**Fig. 1.** Release of amino acids from the rat testis-specific basic protein by carboxypeptidase A. Intact protein (80 nmol) was digested with 50 μg of carboxypeptidase A in 1 M N-ethylmorpholine acetate at pH 8.5 as described under "Experimental Procedures." Incubation was at 34°C. The amount of sample removed at each time point corresponded to 10 nmol of protein. At 120 min the yield of leucine was 9.3 nmol or 93%. Leucine, •; histidine, △; serine, ○.
identification of certain amino acids could still be made with a high degree of confidence until the 39th cycle. The partial sequence thus generated is given in Fig. 3, and the quantitative nature of the degradation process, as illustrated by the recovery of representative residues, is depicted in Fig. 4. The sudden introduction of overlap at the proline encountered on the 8th cycle is evident, and the gradual development of this overlap to the point where maximum recovery of a given residue occurred is shown. Despite the recovery of these latter residues as a broad peak encompassing several cycles, it is clear that the correct assignment of the residues indicated in Fig. 4 most probably corresponds to the cycle on which the amino acid in question first appeared significantly above its background level.

The numbering of residues refers to their position in the intact protein, with the remaining two necessarily located distal to that point. The location of tyrosyl residues at positions 32 and 46 of the intact protein completed a skeletal outline of the sequence generated agrees exactly with that previously established for the NH2 terminus of the intact protein (3).

**TABLE I**

Composition of peptides derived by digestion of intact testis-specific basic protein with thermolysin

| Amino acid | Peptide | Total Resides |
|------------|---------|---------------|
|            | ThII    | ThII A        | ThII B | ThIII | ThIV | ThV | ThVI | ThVII |
| Aspartic acid | 0.81 (1) | 0.95 (1) | 0.91 (1) | 1.02 (1) | 2.90 (3) | 4 |
| Threonine | 1.66 (2) | 0.99 (1) | 1.03 (1) | 1.89 (2) | 1.79 (2) | 0.96 (1) | 8 |
| Serine | 0.98 (1) | 2.11 (2) | 2.27 (2) | 1.12 (1) | 2 |
| Proline | 0.98 (1) | 0.96 (1) | 1.02 (1) | 2 |
| Glycine | 0.90 (1) | 1.03 (1) | 0.76 (1) | 1 |
| Alanine | 0.97 (1) | 1.05 (1) | 1.02 (1) | 2 |
| Valine | 1.00 (1) | 1.00 (1) | 2.00 (2) | 10 |
| Methionine | 0.93 (1) | 0.93 (1) | 0.9 (3) | 11 |
| Leucine | 1.00 (1) | 1.00 (1) | 2.93 (3) | 12 |
| Tyrosine | 0.97 (1) | 0.96 (1) | 2.09 (2) | 13 |
| Histidine | 1.00 (1) | 1.00 (1) | 1.01 (1) | 54 |
| Lysine | 0.90 (1) | 1.03 (1) | 0.95 (1) | 5 |
| Arginine | 0.97 (1) | 1.05 (1) | 1.02 (1) | 3 |
| Total Resides | 5 | 5 | 3 | 2 | 1 | 2 | 3 | 2 | 1 |
| Yield (%) | 79 | 50 | 29 | 15 | 87 | 43 | 85 | 87 | 48 |

Arg-Arg-Gly-Lys-Asn-Arg-Ala-Pro-His-Lys-Val-Lys-Arg-Gly-Gly-Ser-Lys-(Arg,Lys)-
12 15 20 25 30
Tyr-Ser-Leu-Leu-Asx-Asx-Ala-Tyr
35 38 41 45 50

Fig. 3. Partial sequence derived by automated Edman degradation of CNBr II. The sequence is derived from two runs on CNBr II as described in the text. Other details may be found in the legend to Fig. 4. The numbering of residues refers to their position in the intact protein.

**Sequence of Th V**—The peptide (20 nmol) was treated with 2.5 μg of carboxypeptidase A for 5 hours at 34°C. Serine (11.7 nmol) and lysine (3.2 nmol) were the only amino acids released. The peptide (500 nmol) was then subjected to four cycles of automated Edman degradation. Identification of the residue released in each case was made by direct hydrolysis of the thiazolinone derivatives with HI for conversion back to the parent amino acid. The following residues were identified: Tyr (162 nmol)-Arg (36 nmol)-Lys (120 nmol)-Ser (as alanine, 34 nmol), leaving serine as the COOH-terminal residue by difference (Table I). This peptide therefore comprises residues 32 to 36 in the intact protein (cf. Fig. 6) since the tyrosyl and seryl residue had been placed previously during the analysis of CNBr II (Fig. 3).

**Sequence of Th VI**—The tridecapeptide (6.5 nmol) was treated with 1.3 μg of carboxypeptidase A for 7 hours at 37°C. Approximately 3 nmol of either serine or asparagine, which coelute on the analyzer, were recovered. Manual Edman degradation was performed on 300 nmol of this peptide, and the liberated residues were identified by a combination of the techniques described under "Experimental Procedures." The following sequence was established with reference to the known composition (Table I): Lys-Ser-Arg-Lys-Arg-Gly-Ax-Ser-Ala-(Arg, 2Asx). The sequence allowed unambiguous interpretation of the results.
of the carboxypeptidase digestion as indicating a COOH-terminal asparaginyl residue.

To obtain shorter fragments of this peptide, lysine residues were first rendered resistant to tryptic digestion by derivatization with citraconic anhydride. After digestion of the citraconylated peptide, mapping of the tryptic fragments indicated that despite the 3 arginyl residues in this peptide, it had been split into only two fragments. These two peptides, T(c)1 and T(c)2, were separated by high voltage electrophoresis. The compositions of the two fragments presented in Table II indicated that the susceptible arginyl residue was at position 4 of the parent peptide, Th VI. Analyses of both fragments were in accord with the sequence thus far determined.

Following removal of the citraconyl blocking groups, further fragmentation of peptide T(c)2 was effected by treatment of 460 nmol of the peptide with 20 µg of trypsin in 1 ml of 0.1 M N-ethylmorpholine acetate at pH 8 containing 2 mM CaCl₂ for 16 hours at 34°. Mapping of the digest delineated six ninhydrin-positive spots, two of which gave distinctive color reactions. An anionic peptide at pH 6.4 gave a yellow color suggesting an NH₂-terminal glycyl residue while a neutral fragment gave a brown color identical with that developed by free asparagine. Five of the six fragments were isolated by a combination of high voltage electrophoresis and paper chromatography. The compositional analyses of these fragments given in Table III allowed the determination of the complete sequence of T(c)2 (Fig. 5). Fragment T2 was identified as asparagine based on amino acid analysis before and after hydrolysis, thus confirming the preliminary assignment on the basis of ninhydrin color. In view of the specificity of trypsin and the composition of T1 in conjunction with the previously established partial sequence of Th VI, the COOH-terminal 3 residues of Th VI must be -Asp-Arg-Asn-. The identification of the 2 aspartyl residues in T1 is based on the electrophoretic mobility of peptide fragments T1, T3, and T4. It is apparent that even after a prolonged digestion with trypsin, cleavage at the NH₂-terminal Lys-Arg sequence is incomplete. To complete the recovery of fragments from this tryptic digestion, free lysine should be identified. In fact, the sixth ninhydrin-positive spot identified during mapping of the digest had the mobility expected for free lysine.

Sequence of Th VII—The COOH-terminal thermolysin peptide (300 nmol) was subjected to five cycles of manual Edman degradation. The product of each of the first three cycles was identified unambiguously to yield the partial sequence Tyr-Arg-Ser-. Since the COOH-terminal residue is known to be leucine (Fig. 1), the location of histidine at the fourth position in the peptide may be made confidently by difference. Thus the sequence is Tyr-Arg-Ser-His-Leu.

Complete Sequence—The results of this investigation are
summarized in Fig. 6, which gives the complete sequence of the testis-specific basic protein of the rat.

DISCUSSION

In the sequence analysis of the rat testis-specific basic protein, automated Edman degradation of both the intact protein (3) and the large COOH-terminal cyanogen bromide fragment CNBr II provided a continuous sequence for over half the length of this small protein. In addition, this technique served to identify a sufficient number of key residues in the COOH-terminal region of the protein to permit alignment of all the peptides derived from the intact protein by thermolysin digestion. Because of the unusually restricted distribution of hydrophobic residues in this protein, thermolysin proved an ideal agent for its dissection. Quantitative cleavages were obtained at each internal hydrophobic residue. The sole exception to this pattern, partial cleavage between Thr 8 and His 9, is an example of the occasional susceptibility to thermolysin of the bonds on the amino-side of histidinyl residues (20, 21). These thermolytic peptides were obtained in very high yields considering the use of paper methods during purification. This is probably due to the unusually hydrophilic character of these fragments.

The complete sequence of this protein (Fig. 6) reveals that both the numerous basic residues and the relatively less common hydrophobic residues are rather evenly distributed along the length of the molecule. While there is a tendency for basic residues to occur in groups of two or three, the hydrophobic amino acids invariably occur alone, and, other than the COOH-terminal leucine, are followed by a basic residue. The 2 aspartyl residues, constituting the only acidic amino acids in the protein, both occur within the 4-residue sequence commencing at position 41. Their presence along with a relative scarcity of basic residues gives the COOH-terminal 10 or so residues a neutral character despite the overlapping basicity of the remainder of the molecule. Also worthy of note is the existence of an internal homology between two pentapeptide sequences, specifically residues 10 to 14 (Gly-Met-Arg-Arg-Gly) and residues 22 to 26 (Gly-Val-Lys-Lys-Gly).

The testis-specific basic protein has also been isolated in pure form from the human testis (2). Nothing is yet known of the sequence, but the amino acid composition is in accord with only two substitutions of a conservative nature differentiating the protein from mouse was claimed to be present in spermatozoa (22). Recent evidence1 indicates that mouse spermatozoa, like those of several other eutherian mammals (1, 5, 6), contain a class of basic chromosomal proteins that is readily distinguishable from the testis-specific basic protein, both by a great excess of arginine over lysine and by a high content of half-cystine. While it is possible that the association of the testis-specific basic protein with developing gametes may be transitory in some species (rat and man) but more permanent in others (mouse), the findings mentioned above indicate that the characterization of the basic protein from mouse testis and spermatozoa as “mouse protamine” (22) is perhaps premature.

Although nothing is yet known about the function of the testis-specific basic protein, recent experiments2 are in accord with the association of this protein with chromatin prepared from detergent-washed testicular nuclei. Since the distinct possibility of a chromosomal origin is raised, it is appropriate to inquire whether the testis-specific basic protein bears a structural relationship to any of the well known basic chromosomal proteins of eucaryotic cells, the histones (23). Full sequences have now been determined for representatives of almost all of the established histone classes (24-29). Assuming that rat histones, with perhaps the exception of some members of the lysine rich family, will be very nearly identical with those of other species, the examination of the published structures indicates that the rat testis-specific basic protein could not be derived from any of them by degradation. This finding confirms a conclusion drawn earlier (1) on the basis of less direct evidence. In addition, it appears that an evolutionary relationship, if any, between the testis-specific basic protein and one or another of the histones must be relatively distant. The sequence of the testis-specific basic protein shares no substantial regions of overlap with any of them.

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