Table 1. Activation energies of the complexes studied

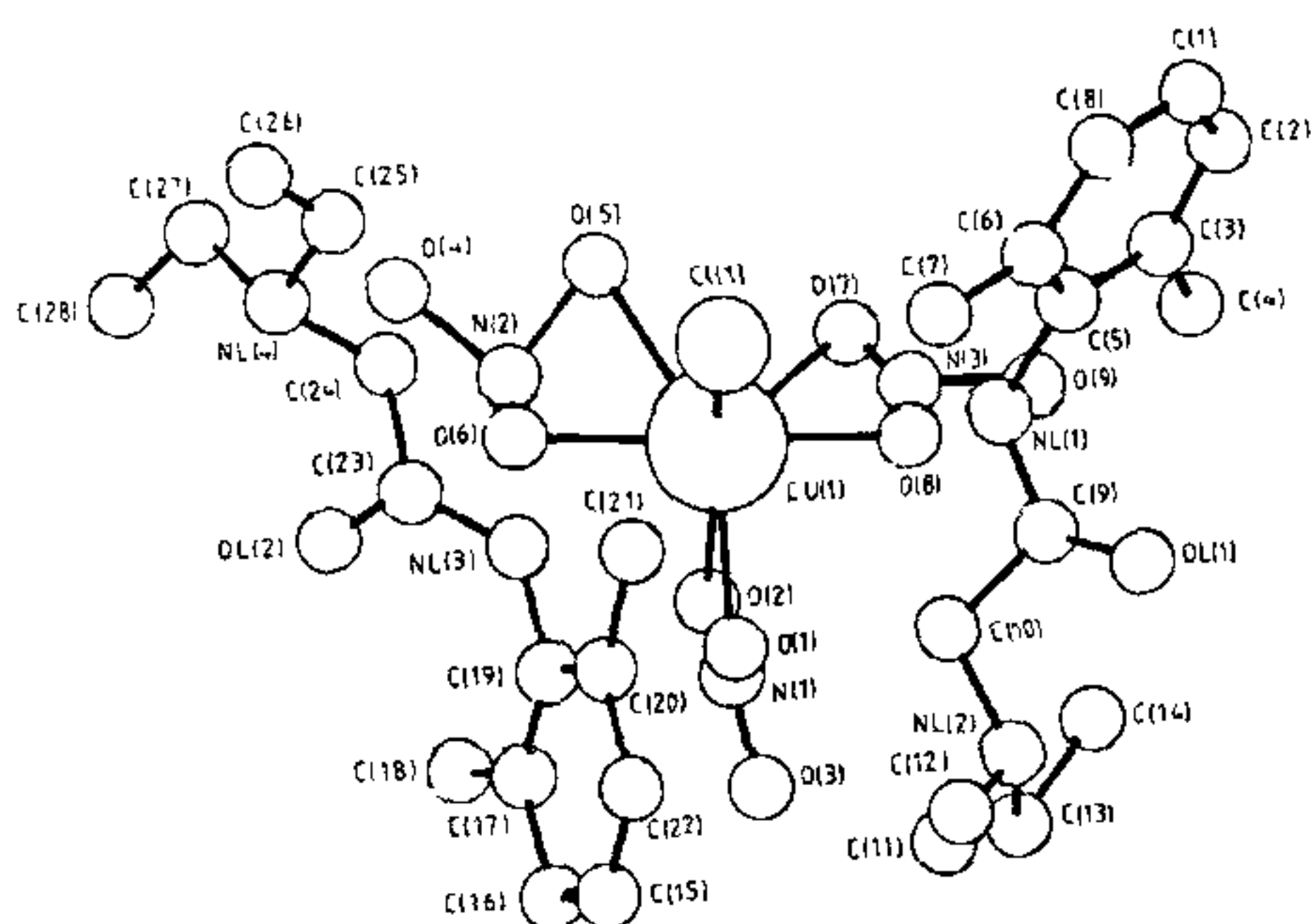| Complex	Slope of log(R) vs 1/T plot	Correlation coefficient	Activation energy (eV)
CRL	2968 54	0 80	0 5892
CUNL (388–468 K)	4655 50	0.97	0.9240
CUNL (234–279 K)	2541.87	0 98	0 5045
MNL	16585 10	0.97	3.2916

Figure 2. Projection of the molecule (CUNL) on the best plane

The resistance measurements of the complexes under study were carried out on single crystals using the two-probe technique with silverized contacts. Low-temperature measurements have been done for CUNL complex using bath-type liquid-nitrogen cryostat in the range 230–300 K. High temperature measurements for all the complexes were made in the range 300–470 K. Keithley 616 digital electrometer having an accuracy of ± 5% of the reading was used. Temperature controller DRC80C from Lake Shore Cryotronics Inc. was used to measure low temperatures. High temperatures were measured with chromel–alumel thermocouple (K type) with a digital temperature controller having an accuracy of ±1% of the reading

The resistance of the CUNL complex in the low-temperature region ranges from 10^3 to $10^6 \Omega$ and the resistance of the complexes CRL, CUNL, MNL in the high temperature region ranges between 10^3 and $10^6 \Omega$. The degree of fall of resistivity decreases in the order MNL, CRL, CUNL. An exponential variation of the resistance with temperature is observed in all the complexes according to the equation $R = R_0 \exp(-E_a/kT)$, where E_a is the activation energy and k the Boltzmann constant. This is evident from the log(R) vs 1/T plots shown in Figure 1. The activation energy values of the complexes are given in Table 1. The complex CUNL shows appreciable

conductivity in two regions, one below room temperature and the other above room temperature. The structural analysis of this compound reveals an uncommon seven coordination for the copper atom. It is bound to seven atoms – six oxygen atoms of the three nitrate groups and one chlorine atom. The structure of the complex at room temperature is shown in Figure 2 (ref. 5). It may be surmised that this behaviour in conductivity may be due to a structural phase transition at a lower temperature. This aspect is being currently investigated.

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A 20-kD mitochondrial protein is associated with cytoplasmic male sterility (CMS) in *Capsicum annuum* L.

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Mitochondria from normal and cytoplasmic male sterile (CMS) lines of *Capsicum annuum* L. were isolated and allowed to synthesize proteins in the

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presence of ^{35}S methionine. Fluorography revealed an additional polypeptide of 20 kD in CMS plants. Sodium-dodecyl-sulphate–polyacrylamide gel electrophoresis of anther proteins showed the absence of 20 kD protein during pollen mother cell (PMC) and subsequent stages of pollen abortion in CMS line. A correlation has been made between additional 20 kD protein synthesized by CMS mitochondria and the absence of the same molecular weight protein in CMS line during microsporogenesis, with pollen abortion.

PLANT mitochondrial genomes of higher plants are large and vary in size from 200 kb in Brassicaceae to 2500 kb in Cucurbitaceae. These are in contrast to mammalian and fungal mitochondrial genomes, where they are 15–18 kb and 18–78 kb, respectively. Like mammalian and fungal genomes, plant mitochondrial genome possesses three large subunits of cytochrome c oxidase and cytochrome b, several subunits of the ATP synthase complex and the NADH dehydrogenase, a number of tRNAs and the two large ribosomal RNAs. Apart from these, the plant mitochondrial genome also contains specific genes not encoded in mammalian or fungal genomes. These genes include coding regions for a 5S ribosomal RNA, the alpha subunit of the ATPase and, in some plants, the genes for proteins of the small ribosomal subunits, rps 12, rps 13 and rps14 (ref. 1). The number of polypeptides encoded by plant mitochondrial genomes is not clear. Levings and Brown[2] estimated 20 polypeptides, which form less than 10% of the total mitochondrial protein.

The involvement of plant mitochondria in cytoplasmic male sterility (CMS) is well documented[3]. CMS can arise when the nucleus of one species is backcrossed into the cytoplasm of a closely related species and results from mitochondrial mutation or incompatible interactions between nucleus and mitochondria[4]. Since CMS is utilized in plant breeding for hybrid production, tremendous interest has been generated to understand the basic mechanism of sterility as well as the interrelationship between mitochondria and nucleus. It is well known that certain nuclear fertility genes (Rf) are able to correct this abnormality, leading to the restoration of fertility. It has been shown in maize, Petunia, Sorghum and other plants that mutant mitochondria from CMS lines synthesize certain specific proteins which may interfere in pollen development. In maize, mitochondria from CMS-T synthesize a 13 kD protein which is absent in normal, CMS-C and CMS-S lines[5,6]. While the mitochondria from CMS Petunia encode an additional 25 kD protein[7], mitochondria from CMS Sorghum synthesize a 42 kD protein[8]. However, the functional relationship of mitochondrial proteins and pollen abortion remains to be established. Hence, the main objective of the present study is to characterize the proteins synthesized by the isolated mitochondria as well as anthers from normal and CMS

lines. Any differences in their profile can be related to pollen abortion in Capsicum.

The Freshno CMS and its normal (isonuclear maintainer) lines of Capsicum (Capsicum annuum L.) were obtained from the University of Agricultural Sciences, Dharwad, India. The method used for isolation of mitochondria was based on the procedure of Leaver et al.[9] with little modifications. Ten to fifteen grams of green leaves were used for this study. The leaves were thoroughly washed in sterile distilled water and homogenized in an ice-cold medium containing 0.4 M mannitol, 1 mM ethylene glycol bis(-aminoethylether)-N,N-tetraacetic acid (EGTA), 2 mM morpholinopropanesulfonic acid (MOPS), 0.1% (w/v) bovine serum albumin (BSA), 9 mM 2-mercaptoethanol and 1% polyvinyl pyrrolidone (PVP). The homogenate was filtered through four layers of muslin cloth and centrifuged at 5000 rpm for 10 min in a Sorvall RC 20 centrifuge. The resultant supernatant was again centrifuged for 10 min at 5000 rpm. The mitochondria were pelleted from the supernatant at 12,000 rpm for 15 min and washed in 20 ml of wash medium containing 0.4 M mannitol, 0.1% BSA, 1 mM EGTA and 5 mM MOPS (pH 7.8). The mitochondria were pelleted again at 12,000 rpm for 15 min. To purify the mitochondrial fraction further, the pellet was resuspended in 5 ml of wash medium and loaded on to a continuous, self-generating percoll gradient. The gradient solution contains 30% (w/v) percoll, 0.4 M mannitol, 10 mM tricine (pH 7.2), 1 mM EGTA and 0.1% (w/v) BSA. The gradient was centrifuged at 20,000 rpm for 45 min. The mitochondria, which formed a distinct buff-coloured band below the green chloroplasts, were collected and diluted with suspension medium (0.4 M mannitol, 10 mM tricine (pH 7.2), 1 mM EGTA) and centrifuged at 12,000 rpm for 15 min. This step was repeated twice to remove the percoll. The mitochondrial pellet was resuspended to a concentration of 5–20 mg/ml in 0.4 M mannitol. 10 mM tricine (pH 7.2), 1 mM EGTA. The purified mitochondria (250–600 µg of protein) were incubated in microfuge tubes in a final volume of 250 mM mannitol, 90 mM KCl, 10 mM MgCl$_2$, 10 mM tricine buffer (pH 7.2), 5 mM potassium phosphate (pH 7.2), 1 mM EGTA, 25 mM of 19 amino acids except methionine, 9 mM dithiothreitol, 1 mM GTP and L (^{35}S) methionine, 5–20 µCi In addition, incorporation was carried out using 10 mM succinate, 2 mM ADP, 2 mM GTP and 30 mM MgCl$_2$. Cycloheximide (5 µg/ml) was included to inhibit the activity of any contaminating cytoplasmic ribosomes and erythromycin (100 µg/ml) was included to inhibit any contaminating plastid ribosomes. The time course of the reaction was followed by removing 5 µl of aliquot onto 1.5 cm disks of Whatman 3 MM filter paper The disks were dried in a stream of air for 30 s before immersion in ice-cold 10% trichloroacetic acid (TCA) for at least 15 min. Unincorporated radioactivity was removed by

washing in 5% TCA at 90°C for 15 min, in 5% TCA at room temperature for 5 min (four times), in ethanol–ether (1:1) at 37°C for 5 min and finally in ether at 37°C for 10 min. The disks were dried and the radioactivity incorporated into the protein was estimated in a scintillation counter. After 90 min of incubation, the incorporation of ^{35}S methionine was stopped by the addition of 1 ml of ice-cold arresting medium (10 mM unlabelled methionine, 0.4 M mannitol, 10 mM tricine (pH 7.2) and 1 mM EGTA). The mitochondria were pelleted at 12,000 rpm for 5 min in an Eppendorf microcentrifuge and stored at −70°C. The pelleted mitochondria were solubilized in 100 μl of sample buffer containing 60 mM Tris–HCl (pH 6.8), 2% SDS, 10% sucrose, 10% 2-mercaptoethanol and boiled for 5 min at 100°C. The proteins were analysed by SDS-PAGE[10] in 12% or 15% gel. Samples containing 50,000 cpm of labelled proteins were loaded in each lane. The co-electrophoresed molecular weight markers were: 2-lactalbumin (14.2 kD), trypsin inhibitor (20.1 kD), trypsinogen (24 kD), carbonic anhydrase (29 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), albumin egg (45 kD) and albumin bovine (66 kD). After electrophoresis, the gels were stained with Coomassie brilliant blue or silver nitrate (AgNO$_3$). Fluorography was carried out according to Chamberlain[11] using the fluor sodium salicylate. Prior to drying, the gel was treated with the solution of sodium salicylate for 25 min. The gel was dried onto Whatman 3 MM paper and exposed to Indu X-ray film for 2 weeks.

For characterization of proteins during micro-sporogenesis, the anthers were separated according to their stages. Each flower bud contains five anthers at the same developmental stage. One anther was removed for stage determination and the rest of the anthers were utilized for SDS-PAGE of proteins. The different stages recognized cytologically were: (i) sporogenous, (ii) pollen mother cells, (iii) tetrads and (iv) microspores/aborted anther. The anthers were homogenized with a glass rod homogenizer in a solubilization buffer containing 0.1 M Tris–HCl, pH 7.4, 5 mM 2-mercaptoethanol, 5 mM EDTA and 5% polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 8000 rpm for 15 min at 4°C. The supernatant was precipitated with TCA at a final concentration of 10%. After 30 min in an ice bath, the precipitants were collected by centrifugation, washed twice with prechilled 90% acetone and once with 100% acetone. The precipitants were dried and stored at −20°C. SDS-PAGE was carried out at room temperature with a slightly modified Laemmli buffer system[10] using 75 mm slab gel with 4% stacking gel and 8–16% gradient-resolving gel. The anther pellets were resuspended in a sample buffer consisting of 62.5 mM Tris–HCl (pH 6.8) 2% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol and heated at 100°C for 5 min and centrifuged. The

supernatant was recentrifuged and the protein content determined[12] with BSA as a standard. Before electrophoresis, the proteins were precipitated again with ice–cold acetone, redissolved in the sample buffer and boiled again for 5 min. Electrophoresis was carried out with a constant current of 15 mA for 12–14 h. The co-electrophoresed molecular weight markers were the same as those described above. After electrophoresis, the gel was removed and fixed for 6 h with acetic acid/methanol/water (10:40:50, v/v/v). Subsequently, the gel was stained overnight with 0.2% Coomassie brilliant blue in acetic acid/methanol/water (10:25:65, v/v/v) and destained in acetic acid/methanol/water (10:25:65, v/v/v).

Mitochondria prepared from green leaves of normal and CMS *Capsicum* are allowed to incorporate ^{35}S methionine by the procedure described above. By following the above procedure, we obtained coupled mitochondria (data not shown), which is a prerequisite for mitochondrial protein synthesis. The kinetics of incorporation is comparable for normal and CMS lines and is linear over 90 min. Cycloheximide (5 μg/ml), which inhibits protein synthesis from cytoplasmic ribosomes, does not perceptibly modify this incorporation. Although erythromycin (100 μg/ml) is a potent inhibitor of plastid translation, it causes only a
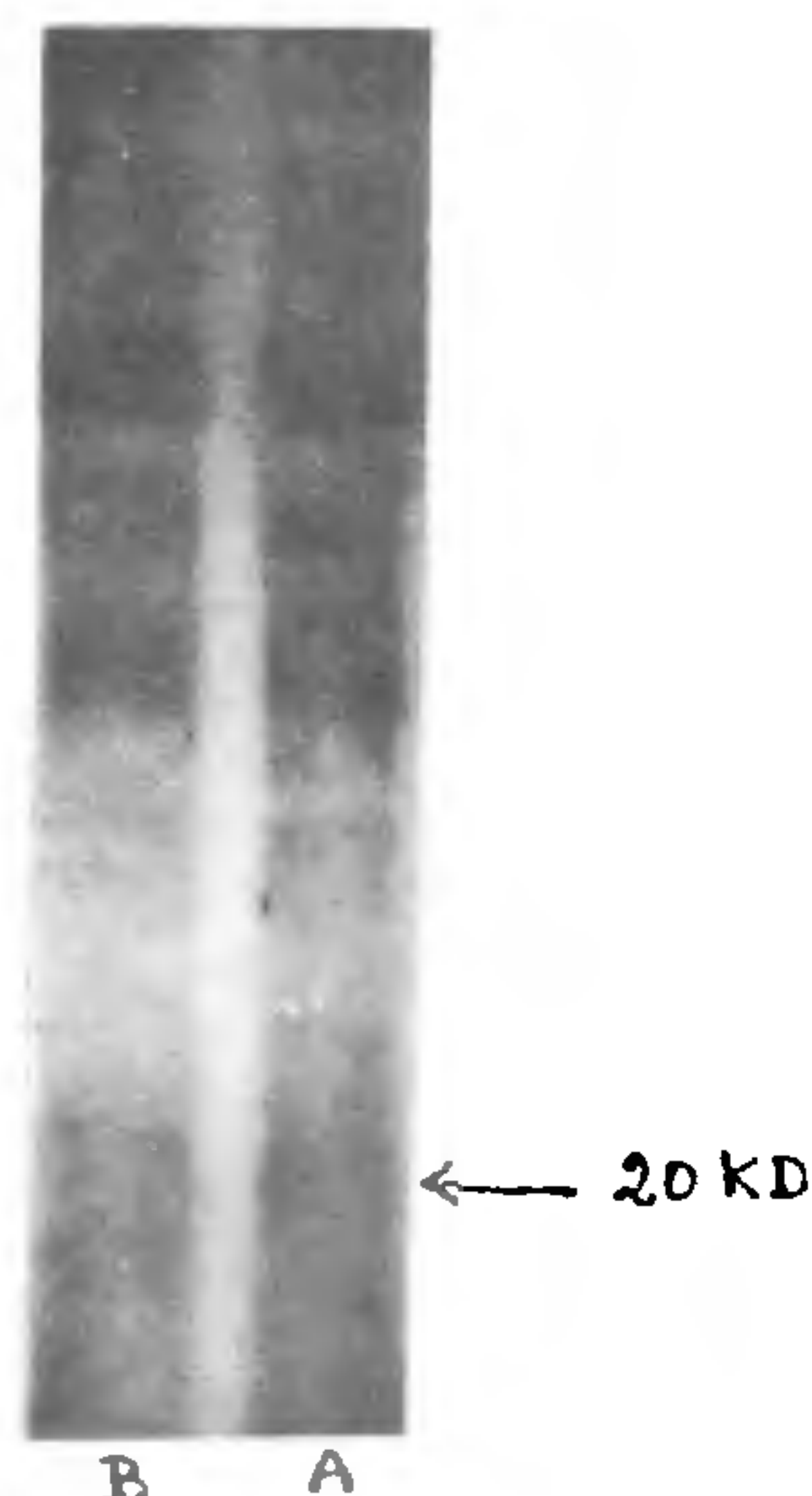

Figure 1. Fluorogram of mitochondrial protein synthesis from normal and CMS lines of *Capsicum annuum*. Note the characteristic additional protein of 20 kD in CMS line, which is absent in normal line (B, Normal line; A, CMS line)

small reduction in the label incorporation into mitochondria and has little effect on the pattern of protein synthesis. The labelled proteins synthesized by isolated mitochondria are fractioned by polyacrylamide gel electrophoresis and detected by fluorography. Profiles of the polypeptides synthesized by mitochondria from normal and CMS leaves are shown in Figure 1. About 20 polypeptides are synthesized by normal and CMS lines. A comparison of the polypeptides synthesized *in vitro* by normal and CMS mitochondria reveals an additional protein of apparent molecular weight 20 kD in sterile line. The mitochondrially synthesized polypeptides are low in abundance and are not easily detectable by protein staining with silver nitrate.

SDS-PAGE of anther proteins shows that numerous polypeptides are present during microsporogenesis (Figure 2). No differences are observed during the sporogenous stage in normal and CMS anthers. However, striking deviation between normal and CMS line becomes vivid during the PMC stage, where a protein of apparent molecular weight 20 kD is absent in sterile line. The same trend continues during subsequent stages of abortion of microspores. Apart from this, we could not detect any other differences between the banding patterns of normal and CMS anthers.

In the present study, an attempt has been made to characterize the (i) mitochondrial gene expression between normal and CMS lines of *Capsicum* and (ii) anther
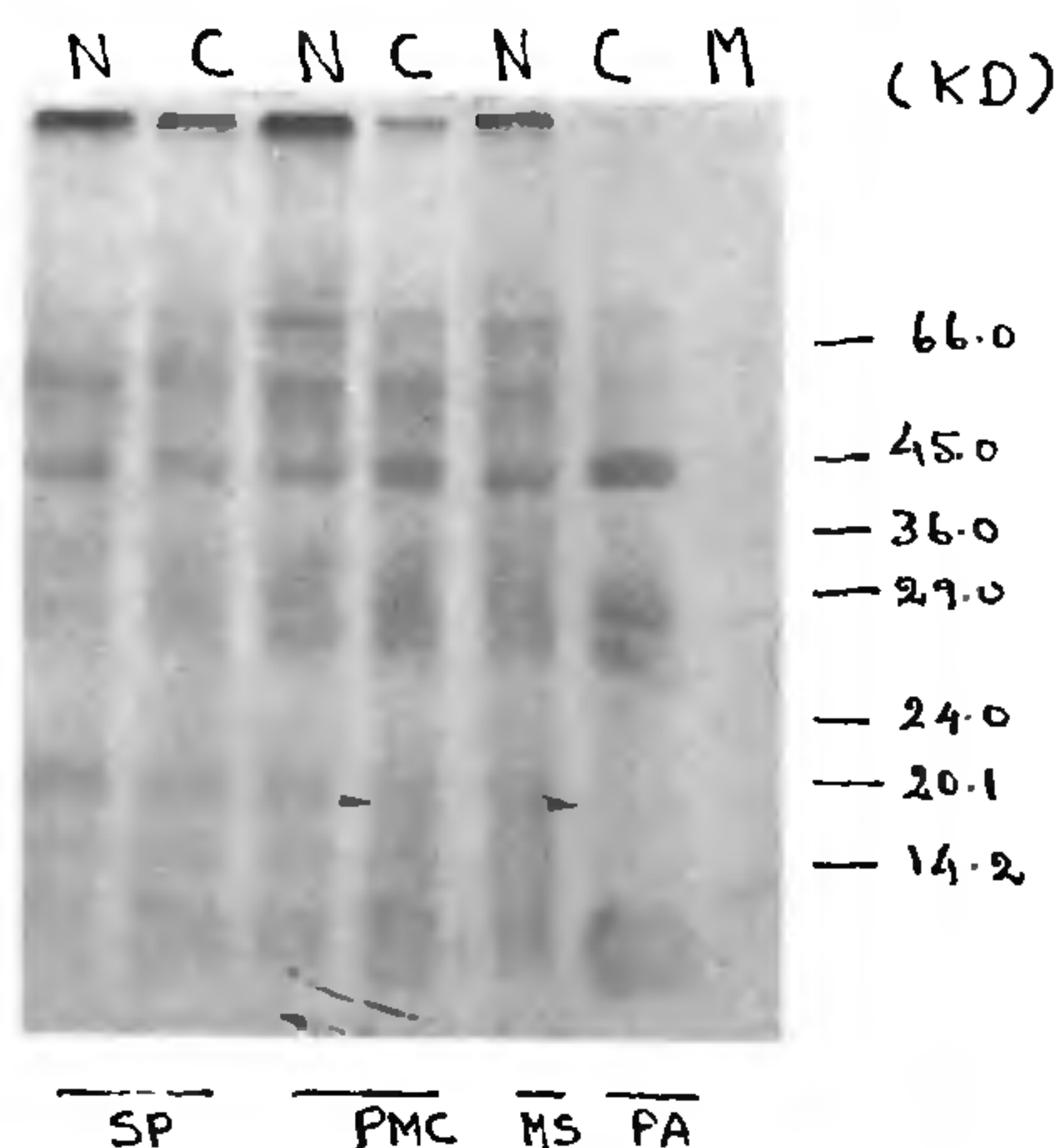

Figure 2. SDS-PAGE of anther proteins from normal and CMS lines of *Capsicum annuum* at different developmental stages Note the absence of 20 kD protein (arrow) in CMS line during PMC and completely aborted anther. Molecular weights in × 10³ daltons of marker proteins are indicated in right side (N, normal line; C, CMS line, M, molecular weight markers, SP, sporogenous stage; PMC, pollen mother cell stage, MS, microspore stage; PA pollen aborted stage in CMS line)

proteins at different developmental stages of normal and CMS lines, an essential part of an investigation into the mechanism of male sterility, which is entirely lacking in *Capsicum*. The mitochondrial translation products reveal the presence of about 20 polypeptides found in other CMS systems like maize, *Sorghum*, *Brassica* and *Nicotiana*[2,13] In *Capsicum*, we have observed the presence of a characteristic polypeptide of 20 kD in CMS line but not in normal line. Similarly, in maize, CMS-T mitochondria synthesize a 13 kD polypeptide which is absent in normal, CMS-C and CMS-S mitochondria. On the contrary, a polypeptide of M_r 21,000 reported in normal, CMS-C and CMS-S mitochondria is not detectable in CMS-T (refs 5, 6). It has been shown that the 13 kD protein in CMS-T is encoded by the gene designated as *T-urf 13*, which is a component of the inner mitochondrial membrane. The *urf 13* is not specifically bound to any particular inner membrane component but is associated with different complexes[14]. An antiserum prepared from a chemically synthesized oligopeptide, based on the deduced amino sequence encoded by *T-urf 13*, that detected *urf 13*, has identified this 13 kD protein in CMS-T. In *Sorghum*, mitochondria of CMS plasma type 9E lack a 38 kD protein, which is a characteristic of other CMS sources, and produces a new 42 kD protein which immunoprecipitates with an antiserum raised against yeast cytochrome c oxidase subunit I and the rearrangements have altered Cox I transcription and translational termination[8]. It is observed in *Petunia* that a gene *Pcf* is associated with CMS and its sequences are derived from *atp9*, *Cox II* and an unidentified reading frame, *urf S* This *Pcf* gene encoded a 25 kD protein which is antigenically related. A variation in mitochondrial translation products is also found in *Vicia faba*[15], *Beta vulgaris*[16], *Nicotiana tabaccum*[17,18] and *Triticum aestivum*[17]. In *Capsicum*, we have observed that the tapetum remains appressed onto the meiocytes and microspore tetrads and, consequently, no locular cavity enlargement takes place, resulting in pollen abortion[19]. The delayed dissolution of callose is also associated with pollen abortion[19]. In the present study, the first biochemical deviation in anther, as evidenced by SDS-PAGE, is observed at pollen mother cell (PMC) stage, where the 20 kD protein is absent in CMS line, and this difference is maintained till the completion of pollen abortion. Perhaps this 20 kD protein in the anther is essential for the enlargement of locular cavity and normal synthesis of an enzyme callase, which is synthesized from tapetum and degrades the callose layer surrounding microspore tetrads, and hence its absence triggers abnormality leading to pollen degeneration. It is interesting to note from our study that the CMS mitochondria synthesize an additional 20 kD protein and the absence of the same protein (based on molecular weight) in anther leads to pollen abortion. At present, we are not able to establish direct functional relationship of

the polypeptide 20 kD, synthesized by CMS mitochondria, and its effect on microsporogenesis. Nevertheless, it can be inferred that the additional 20 kD mitochondrial protein may inhibit 20 kD protein in anther, leading to pollen abortion. This observation is consistent with the report on the involvement of mitochondrial gene in anther development in *Nicotiana*[20] and structural abonormalities of mitochondria during pollen abortion in maize CMS-T[21], wheat[22], sugarbeet[23], rice[24] and *Brassica*[25]. It has been shown that the protein 13 kD of CMS-T maize is present in the mitochondria of tapetal cells even after the mitochondria have begun to degenerate[26]. Similarly, the expression of *Pcf* gene is greatest in the anthers of CMS *Petunia*[27].

All the studies, including the present one, describe the mitochondrial translation products from the vegetative tissue. However, it has been documented that mitochondria from different organs synthesize different proteins[28], apparently in response to altered cellular milieu or environmental stimuli. Hence, for a better understanding of cytoplasmic male sterility, mitochondrial translation studies should be carried out in anthers where pollen abortion takes place at different developmental stages.

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Homorepeats of amino acids in proteins – A database search

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Occurrence of individual amino acids and their homorepeats has been computed from the available protein sequence database. Significant numbers of homorepeats, up to a maximum of hexamers, were found for most amino acids. Preliminary results indicate that the patterns of percentage occurrence of individual amino acids are similar to those of their homodimers. Higher orders, however, show deviations in the predominance profile. In the case of valine and isoleucine, the frequency of occurrence of homorepeats (beyond trimers) goes down considerably, in sharp contrast to their predominance as individual entities. Tryptophan was not found beyond homotrimeric repeats and tyrosine and cysteine were not found in tandems beyond pentamers. Interspecies distribution profiles reveal some interesting deviations. The cysteine content in *E. coli* proteins was about 50% lower compared to human proteins. Very few *E. coli* proteins have higher-order repeats and the functional importance of these higher-order repeats has been analysed. Plant proteins have very high glutamine tandems in contrast to intermediate frequency of single glutamine occurrences. This suggests a preferential selection of some amino acids and their tandems in the course of evolution to suit diverse functional and structural requirements.

PROTEIN structure is primarily a reflection of its amino acid sequence[1,2]. Automated protein-sequencing methods have generated a vast repertoire of protein sequences[1,3]. These data are made available in various computerized protein sequence databanks[4]. The number of proteins sequenced exceed manyfold those with defined three-dimensional structures solved by X-ray crystallography and NMR