Advances in MICROBIAL PHYSIOLOGY

VOLUME 21

Advances in

MICROBIAL PHYSIOLOGY

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Advances in MICROBIAL PHYSIOLOGY

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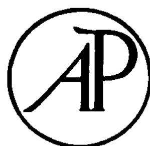
Volume 21
1980



ACADEMIC PRESS

A Subsidiary of Harcourt Brace Jovanovich, Publishers

London New York Toronto Sydney San Francisco

ACADEMIC PRESS INC. (LONDON) LTD.
24/28 Oval Road
London NW1 7DX

United States Edition published b
ACADEMIC PRESS LTD.
111 Fifth Avenue
New York, New York 10003

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British Library Cataloguing in Publication Data

Advances in microbial physiology.
Vol. 21
1. Micro-organisms—Physiology
I. Rose, Anthony Harry II. Morris, John Gareth
576'.11 QR84 67-19850

ISBN 0-12-027721-2
ISSN 0065-2911

Printed in Great Britain by
Spottiswoode Ballantyne Ltd.
Colchester and London

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Transcription in Acellular Slime Moulds

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I. Introduction

A. BIOLOGY OF ACELLULAR SLIME MOULDS

Two species of slime moulds have become widely studied in cell biology, the acellular slime mould *Physarum polycephalum* and the cellular slime mould *Dictyostelium discoideum* (Ashworth and Dee, 1975). Although roughly a thousand species of acellular slime moulds have been characterized taxonomically, the vast majority of them have not

been cultivated in the laboratory and are therefore not amenable to any detailed analysis (Martin and Alexopoulos, 1969). The first important physiological study on an acellular slime mould addressed itself to protoplasmic streaming (Kamiya, 1940; Allen and Kamiya, 1964). This study made use of rolled oats as a nutrient medium for the slime mould. The development of a semi-defined medium by Daniel and Rusch (1961) opened up the field for biochemical investigations and there appeared, from the same laboratory, the first study of DNA synthesis in *P. polycephalum* (Nygaard *et al.*, 1960). Since then about 160 published papers have addressed themselves to the nucleic acid metabolism of this organism.

The life cycle of *P. polycephalum* is shown in Fig. 1. The organism has two vegetative stages, both amenable to laboratory studies with axenic and fully defined media (Daniel *et al.*, 1963; Goodman, 1972; McCullough and Dee, 1976). In the diploid stage the organism can form huge multinucleate plasmodia containing 10^8 to 10^9 nuclei under standard laboratory conditions. The attraction of the organism at this stage of the life cycle is that nearly all nuclei pass through mitosis simultaneously (Howard, 1932). This high degree of mitotic synchrony, as well as the convenient duration of interphase (8 to 10 h under standard conditions), make the organism ideally suitable for biochemical studies of the mitotic cycle. The other vegetative stage of the organism, the amoebae, has not received anything like as much attention as the plasmodia. However, the transition from one stage of the life cycle to another, mostly induced by nutritional changes, is being investigated by many students of cell differentiation.

Several recent reviews on the biology of acellular slime moulds are available, most of them limited to *P. polycephalum*. In an excellent booklet, Ashworth and Dee (1975) give a concise introduction to the biology of both the cellular and acellular slime moulds, whereas Dee (1975) limits herself to the biology of *P. polycephalum*. Two brief reviews are concerned primarily with differentiation of *P. polycephalum* (Kleinig, 1972) and with its nuclear division cycle (Jockusch, 1975). In 1973 Hüttermann compiled several reviews on the biochemistry, physiology and genetics of *P. polycephalum* and a few closely related species. Another more extensive series of reviews is being published by Dove and Rusch (1979).

Some more specialized reviews have been published over the last few years dealing with the following topics: differentiation (Sauer, 1973),

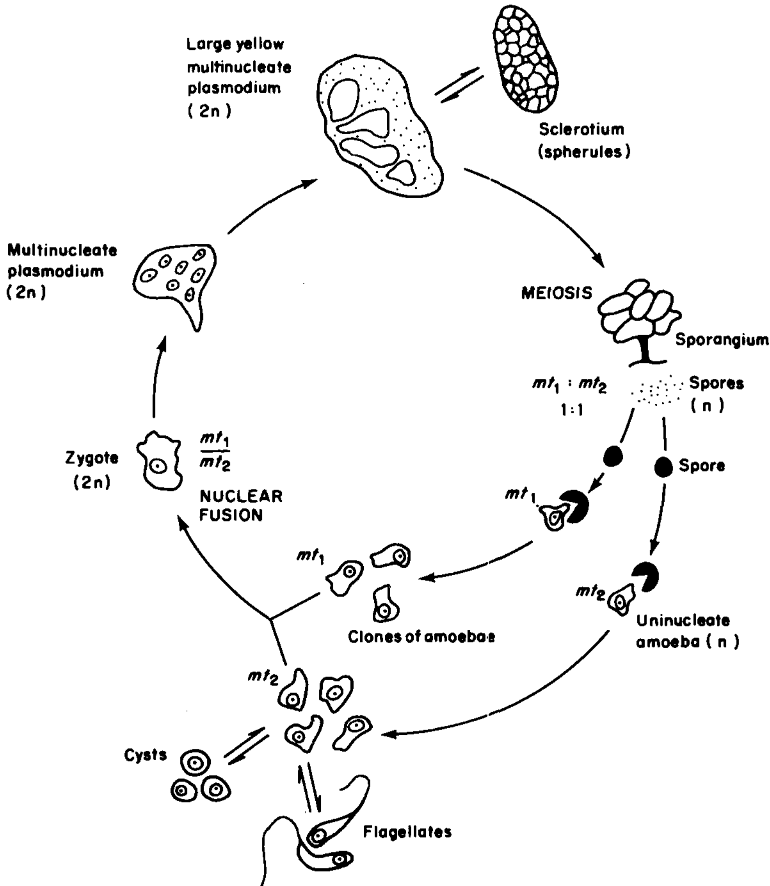

FIG. 1. Life cycle of the acellular slime mould *Physarum polycephalum*. Multinucleate yellow plasmodia can be grown in the laboratory either as microplasmodia in shake culture with liquid medium, or as a surface culture on filter paper soaked with liquid medium. Upon starvation, microplasmodia give rise to spherules while surface plasmodia transform into sclerotia. An alternate pathway of differentiation leads from diploid surface plasmodia (2n) to sporangia and haploid spores (n). In addition to starvation this process requires specific triggers, in particular visible light. Upon hatching, spores give rise to uninucleate amoebae. In heterothallic strains amoebae have to be of opposite mating type (mt_1 and mt_2) in order to go through plasmogamy and karyogamy and produce a diploid zygote. Sclerotia, spherules, spores and cysts are highly resistant to adverse external conditions. Reproduced from Ashworth and Dee (1975).

mitotic cycle (Braun *et al.*, 1977), biochemistry of the life cycle (Rusch, 1970), gene expression in the cell cycle (Sauer, 1978) and RNA synthesis in the cell cycle (Grant, 1973).

In the present review we would like to lay particular emphasis on transcription as it relates to the cell cycle and to cell differentiation of *P. polycephalum* and put less weight on the basic biochemistry, since this has recently been dealt with in considerable detail (Holt, 1979; Melera, 1979). We do this also because we feel that this series of reviews addresses itself primarily to those readers wanting to inform themselves on typically microbial aspects rather than on biochemical and molecular facets of a problem. It is of course clear that a real understanding of differentiation is only possible with a very sound knowledge of the molecules involved in this process, as well as the way they interact.

B. CULTIVATION OF ACELLULAR SLIME MOULDS

1. Physarum polycephalum

Most of the cultivation methods used today are based on the procedure developed by Daniel and Rusch (1961) and are described in more detail by Daniel and Baldwin (1964). For the preparation of synchronous surface cultures, see Guttes and Guttes (1964). In brief outline, *P. polycephalum* is grown in shake culture at 26°C in a medium containing, amongst other ingredients, tryptone, yeast extract and haematin. To prepare synchronous surface cultures many microplasmodia are allowed to fuse on a Millipore filter, which, following fusion of the microplasmodia, is soaked from below with growth medium. Several small, but useful, modifications have since been described: preparation of large surface cultures (Mohberg and Rusch, 1969), replacement of tryptone with mycological peptone to get higher incorporation rates of labelled precursors into nucleic acids (Plaut and Turnock, 1975), replacement of Millipore filters by inexpensive paper filters (Vogt and Braun, 1976) and several new media (See Section I.A).

2. Physarum rigidum

A semi-defined medium has been developed for this organism by Henney and Henney (1968). This is a liquid medium rather similar to that developed by Daniel and Rusch (1961) for *P. polycephalum*.

3. *Other Myxomycetes*

Liquid media for several other acellular slime moulds have been developed as well, but transcription in these organisms has not been studied so far. For reference consult Taylor and Mallette (1976).

II. DNA, RNA and RNA Polymerases

A. DNA

All data reviewed here pertain to *P. polycephalum*, since no observations on the DNA of other acellular slime moulds have been published. Holt (1979) has recently compiled an extensive and critical review so that we will limit ourselves to those points which are essential for the understanding of the process of transcription.

The macromolecular composition of rapidly growing microplasmodia is about 1:22.5:120 for DNA:RNA:protein (Plaut and Turnock, 1975). Three types of DNA can easily be distinguished on the basis of buoyant density (Braun and Evans, 1969): nuclear chromosomal DNA (approx. 88%), nuclear satellite DNA or ribosomal DNA (approx. 2%) and mitochondrial DNA (approx. 10%).

1. *Chromosomal DNA*

Haploid, non-replicated nuclei of *P. polycephalum* contain 0.28 pg of DNA or 63 times more than *Escherichia coli* (Mohberg, 1977; Holt, 1979). Taking the most likely haploid chromosome number to be 40, this gives 4×10^9 daltons or 6000 kilobase pairs (kb) of DNA per chromosome. In CsCl gradients nuclear DNA of several strains of *P. polycephalum* bands at a density of 1.700 ± 0.003 g/ml, corresponding to 41% guanine plus cytosine. For one strain of *P. polycephalum*, a slightly higher value has been published (1.705) which requires further confirmation (Holt, 1979). Strains with an aberrant DNA density could be of interest with respect to the fusion killing phenomenon (Carlile and Dee, 1967). Maybe DNA modification, similar to that found in bacteria, also protects DNA in acellular slime moulds. Although several DNAases have been tentatively characterized in *P. polycephalum* extracts (Polman *et al.*, 1974), none of these enzymes has so far been shown to cut DNA as specifically as do bacterial restriction enzymes. The fact that foreign DNA can get into eukaryotic nuclei, as shown recently for yeast (Hinnen *et al.*, 1978), should further encourage the search for site-specific DNAases in organisms such as the acellular slime moulds.

The complexity of a genome can be estimated by measuring the speed of re-annealing of denaturated DNA (Britten and Kohne, 1968). Although the published data are rather crude, it can be tentatively

concluded that *P. polycephalum* DNA consists of about 45% of moderately repeated base sequences and 55% of unique base sequences (Britten and Smith, 1970; Fouquet *et al.*, 1974a). A highly repeated fraction has not been found in chromosomal DNA. This distribution is rather similar to that found in mammals, whereas *Dictyostelium discoideum* has a slightly higher proportion of unique sequences, namely approximately 70% (Firtel and Bonner, 1972).

Denaturation and rapid renaturation of *P. polycephalum* chromosomal DNA leads to the appearance of "hairpin" structures (Hardman and Jack, 1977). The "hairpins" are caused by the presence of inverted repetitions, whose average length was measured to be about 340 nucleotides. In about half the "hairpins", a loop of up to several thousand nucleotides could be detected by electron microscopy. The function of these inverted sequences is not known in any organism. The sizes of individual foldback duplexes and the lengths of the sequences separating them are non-random. Separate foldback foci are spaced, on average, 7000 nucleotides apart and they appear to be distributed uniformly amongst DNA chains (Hardman *et al.*, 1979). The function of these foldback sequences is not known in any organism.

2. *Ribosomal DNA*

This DNA is present exclusively, or at least nearly exclusively, in the nucleolus as free extrachromosomal DNA. In CsCl, rDNA bands at a density of 1.712 $\pm$ 0.002 g/ml corresponding to 54% guanine plus cytosine. Per haploid unreplicated genome there are 190 free rDNA molecules, each coding for two molecules of 5.8S rRNA, 19S rRNA and 26S rRNA. The coding sequences are arranged head to head with a large central, presumably untranscribed region (Vogt and Braun, 1976; Molgaard *et al.*, 1976; Hall and Braun, 1977). The rDNA has a molecular weight of 38×10^6 daltons or 58 kb (see Fig. 2). For reviews see Braun and Seebeck (1979), Holt (1979) and Molgaard (1978).

3. *Mitochondrial DNA*

This fraction of DNA bands in a CsCl gradient at a density of 1.686 g/ml, corresponding to a guanine plus cytosine content of 26% (Braun and Evans, 1969). In contrast to the relatively small mammalian mitochondrial DNA (molecular weight 10×10^6), the DNA from *P. polycephalum* mitochondria has been assigned a molecular weight of

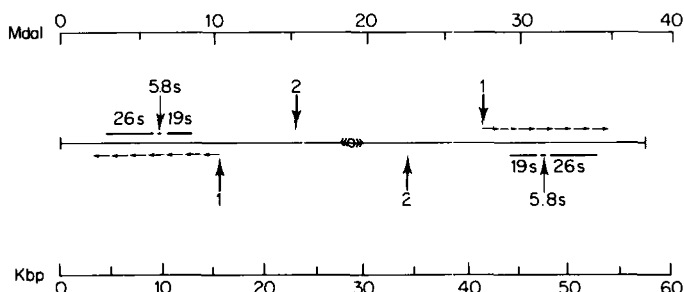

FIG. 2. This figure is a schematic representation of an rDNA molecule. It is a large palindrome (molecular weight 38 × 10^6) with a centre of symmetry (indicated by ≪0≫ and it contains two sets of ribosomal genes, one towards each end of the molecule (localization of the 5.8S genes taken from Campbell *et al.*, 1979). The dimensions of the molecule are indicated in megadaltons (upper scale) and in kilobase pairs (lower scale). The positions of the ribosomal genes are indicated by the heavy bars. Arrows (1) indicate the suspected regions of initiation of transcription which proceeds from there through the coding regions as indicated by the small arrows. The *in vivo* termination point of transcription has not as yet been determined. Arrows (2) indicate the closest possible binding sites of the regulatory phosphoprotein. The data available at present do not rule out the possibility that the binding sites for this protein may still be further away from the transcription initiation sites, i.e. closer to the symmetry centre of the molecule.

up to 37 × 10^6 (Sonenshein and Holt, 1968; Kessler, 1969). Recently Bohnert (1977) found a minority of relaxed circular molecules of 41 × 10^6 daltons amongst a majority of linear molecules. By analogy with other organisms it seems likely then that the mitochondrial DNA of *P. polycephalum* is *in vivo* a closed circular molecule, which becomes nicked during DNA extraction and preparation. This clearly needs to be confirmed by an independent method such as restriction enzyme analysis. It may be noted that *Saccharomyces cerevisiae* has closed circular DNA in its mitochondria and that this has a molecular weight of 49 × 10^6 (Borst and Grivell, 1978). Kuroiwa (1974) has presented some rather preliminary evidence suggesting that each mitochondrion of *P. polycephalum* may contain as many as 360 × 10^6 daltons of DNA, or nine genome equivalents.

B. RNA

Although there has been considerable interest in the process of transcription in acellular slime moulds for several years, individual RNA species have not been analysed in much detail. There is at least

one technical reason for this; large RNA molecules are notoriously hard to isolate intact from these organisms, presumably because of the large amount of ribonucleases produced (Braun and Behrens, 1969; Pilly *et al.*, 1978). As in other organisms, most of the RNA of the cell is involved in protein synthesis, an area which will not be dealt with in this review. It should be mentioned, however, that several methods have been described for the isolation and characterization of ribosomes (Henney and Jungkind, 1969; Hall and Braun, 1977) and polysomes (Brewer, 1972; Schwärzler and Braun, 1977) from acellular slime moulds. Again, undegraded polysomes are hard to obtain from *P. polycephalum*, particularly from macroplasmodia, and new simple methods which leave the mRNA intact should be developed.

1. *Large Ribosomal RNAs*

These have been characterized by several groups using sedimentation velocity and gel electrophoresis. The results have been reviewed in much detail by Melera (1979). Under non-denaturing conditions the two rRNAs have apparent molecular weights of 1.37×10^6 (26S) and 0.73×10^6 (19S); and under denaturing conditions, which give more reliable results, molecular weights are $1.29 \pm 0.03 \times 10^6$ and $0.70 \pm 0.02 \times 10^6$, respectively (McMaster and Carmichael, 1977). The differences between the molecular weights obtained under two sets of conditions are small and do not warrant an extensive discussion. Upon denaturation 26S rRNA releases 5.8S rRNA. This holds for *P. polycephalum* (Hall and Braun, 1977) as for other eukaryotes, where the molecular weight of the 5.8S rRNA has been determined to be 51,000 (Maden and Robertson, 1974). Clearly the size of the large *P. polycephalum* rRNA lies about halfway between that for bacteria and that for mammals, whilst the small rRNA *P. polycephalum* is marginally larger than that of mammals. Apart from its size, not much information is available on *P. polycephalum* rRNA. It has very little secondary structure as determined by electron microscopy (Gubler *et al.*, 1979): this is in contrast to the well documented secondary structure of vertebrate rRNA (Wellauer and David, 1973). The base composition of both large rRNA molecules is very similar with 24% cytosine, 23% adenine, 30% guanine and 23% uracil (Zellweger and Braun, 1971). No sequence data have so far been published. In *P. rigidum* the two

large rRNA molecules were assigned apparent sedimentation values of 26S and 18S (Henney and Jungkind, 1969).

In all eukaryotic cells studied so far, the two large rRNA molecules are cleaved from a single precursor which, at least in vertebrates, is more than double the size of the combined 18S and 28S rRNAs. In *P. polycephalum* two likely precursors have been partly characterized, but it is not clear whether the larger of the two is in fact the initial and intact transcript. In pulse-labelling experiments, both Melera and Rusch (1973a) and Jacobson and Holt (1973) demonstrated the formation of very large RNA by *P. polycephalum*, some of which appeared to give mature rRNA following an adequate chase period. A quantitative conversion, however, could not be shown. Melera and Rusch (1973a) observed a sharp peak at about 4.1×10^6 daltons, Jacobson and Holt (1973) a more heterodisperse profile of which the largest fraction had nearly the same molecular size. Melera (1979) gave a recalculated molecular weight of 4.0×10^6 for the larger precursor. The likely immediate precursor of the mature 26S rRNA has a molecular weight of about 1.9×10^6 (Melera and Rusch, 1973a) and a base composition indistinguishable from that of mature 26S rRNA (Zellweger and Braun, 1971). Grainger and Ogle (1978) have made interesting observations by electron microscopy on the putative first rRNA transcript. They examined spread chromatin, in which they could see occasional transcription units of the size expected for ribosomal cistrons. The transcribed lengths of DNA, similar in appearance to those found by Miller and Beatty (1969), were about 4.2 μm long, only slightly larger than expected from the data reviewed above. In conclusion, the two sets of data strongly suggest that a large precursor of rRNA has a molecular weight of about 4×10^6. It still needs to be shown whether this is the initial transcript carrying a 5′-triphosphate without having undergone any splicing.

2. *tRNA and Other Small RNA Species*

Of the total RNA, tRNA makes up about 13% (Braun *et al.*, 1966). By chromatography and aminoacylation, 44 different tRNA species have so far been identified in *P. polycephalum* (Melera and Rusch, 1973b; Melera *et al.*, 1974). As in other organisms, ribosomes of *P. polycephalum* have also been shown to contain a 5S RNA. This accounts for about one per cent of all RNA (Melera and Rusch, 1973b). Both tRNA and

5S rRNA are coded for by chromosomal DNA: there are approximately 1000 gene copies per haploid genome for each of the two RNA types (Hall and Braun, 1977). In 1977 Hellung-Larsen and Frederiksen showed the presence of an additional small and presumably stable RNA, with a sedimentation value of 7 to 8S. It amounts to about 0.1% of the total RNA and its function is unknown.

3. *Hn-RNA and mRNA*

These two groups of molecules are notoriously difficult to characterize in an organism, primarily because they are intrinsically heterogeneous in size, composition and function. No single mRNA with a specific function, or its precursor, has so far been studied in detail in an acellular slime mould. Studies on the *in vitro* translation of mRNA for histones are reported to be in progress (Sauer, 1978).

The cytoplasm appears to contain both polyA-plus and polyA-minus mRNA, though no data have so far been published on the latter (Sauer, 1978). Polysomal polyA-RNA may represent about 1% of cellular RNA (Sauer, 1978). It is heterogeneous in size, sedimenting at about 8S to 15S (Fouquet *et al.*, 1974b; Fouquet and Sauer, 1975). Electrophoresis under denaturing conditions apparently gave an average molecular weight of 0.45×10^6, a value well within the size range given above (Melera, 1979). Most of this RNA hybridizes to unique sequence DNA (Fouquet and Sauer, 1975). Adams and Jeffery (1978) devoted a detailed study to the polyA sequences of the cytoplasmic RNA, most of which is likely to be polysomal. Two types of sequences were discovered, differing in several properties: short oligoA sequences (averaging 26 nucleotides) are metabolically stable, while longer polyA sequences are both turned over and may also be shortened. The oligoA sequences do not arise by degradation of polyA sequences; in fact the two may not even be part of the same RNA molecules. The oligoA sequences have about the same stability as ribosomal RNA: virtually no degradation is observed during an 18 h period. By contrast, half of the polyA sequences disappear within about 4 h. Superimposed on this degradation there may be a progressive shortening of the polyA sequences. Newly made polyA sequences are about 65 nucleotides long, whilst they seem to average only about 50 nucleotides after 6 to 8 h. These authors suggest that newly made polyA sequences progressively lose up to 15 nucleotides: it

must be conceded, however, that newly made polyA-sequences are heterogeneous in length and that the longer sequences are more rapidly degraded than the shorter ones. Inhibitor studies suggest that translation is required for polyA-sequence destruction.

Not much information is available on hnRNA. Nuclear polyA-RNA appears to vary in size from 4S to 30S (Fouquet and Sauer, 1975) with an average molecular weight, as determined under denaturing conditions, of about 0.51×10^6 (Melera, 1979). Both findings may require revision since no one has yet isolated nuclei with a high degree of purity without degrading RNA. About one-third of the nuclear polyA-RNA hybridizes to reiterated DNA sequences (Fouquet and Sauer, 1975). Melera *et al.* (1979) have undertaken a preliminary study on polyadenylated RNA using electrophoresis under denaturing conditions, where the RNA came from fractions enriched either for nuclei or cytoplasm. After a 45 min labelling period, the nuclear fraction contains a higher proportion of large RNA molecules (mol. wt. $>1.37 \times 10^6$) than does the cytoplasmic fraction. This suggests that part of the polysomal mRNA may originate in a distinctly larger nuclear precursor, as is well established for mammalian cells. In other eukaryotes, such as *Dictyostelium discoideum* and *Achlya bisexualis*, the nuclear precursor of cytoplasmic mRNA appears to be nearly the same size as the mature product (Firtel and Lodish, 1973; Timberlake *et al.*, 1977). Unfortunately Melera *et al.* (1979) have, to date, neither studied the kinetics of hnRNA synthesis and transformation into cytoplasmic mRNA, nor have they been able to quantify the amounts of hnRNA since they have not made their studies with purified nuclei or polysomes but only with enriched fractions. It would be desirable in future to study specific nucleotide sequences in addition to a mixed population of molecules. The coding sequences for actin might be attractive candidates for such a study since actin is one of the most abundant proteins of *P. polycephalum*. The amino acid sequence of *P. polycephalum* actin is similar to that of vertebrate cytoplasmic actin (Vandekerckhove and Weber, 1978). Coding sequences for actin of *Dictyostelium discoideum* have been cloned in plasmids (Kindle and Firtel, 1978).

C. INHIBITORS OF *IN VIVO* TRANSCRIPTION

Over the past fifteen years, several attempts have been made to find a specific inhibitor of *in vivo* RNA synthesis in *P. polycephalum*. The

search, however, has not met with striking success. Both actinomycin C and actinomycin D are required in very high concentrations (100 to 250 μg/ml) substantially to inhibit RNA synthesis (Mittermayer *et al.*, 1966; Sachsenmaier *et al.*, 1967). Rather similar results were obtained more recently for daunomycin and toyocamycin (Fouquet *et al.*, 1975a). The same authors found, however, that 50–200 μg cordycepin (3′-deoxyadenosine)/ml very substantially inhibited RNA synthesis, particularly if plasmodia are pretreated with the drug before labelling. So cordycepin appears to be the best inhibitor available, but it is far from ideal: perhaps the effect of cordycepin could be enhanced by using more suitable media. In addition, it might be fruitful to search for derivatives of such antibiotics as actinomycin, since *in vitro*, with isolated nuclei, actinomycin is active at very low concentrations indicating that the antibiotics used so far are unable to enter the cell (Mittermayer *et al.*, 1966). It should be possible to find derivatives with better permeability properties. The fungal toxin α-amanitin has no inhibitory effect *in vivo.*

Heat treatment of plasmodia for 10 to 30 min at 41°C increases uridine incorporation into RNA, while it decreases amino-acid incorporation into protein (Bernstam, 1974). The mechanism of this differential effect is not understood. Inorganic cadmium in the concentration range around 10^{-3} *M* considerably diminishes the RNA synthesis of *P. polycephalum* plasmodia within a few hours (Sina and Chin, 1978). It is not clear from the data presented how specific is this effect, although it is clear that cadmium greatly distorts the structure of nucleoli, the organelles of rRNA synthesis.

D. RNA POLYMERASES

1. *Purification and Characterization*

Physarum polycephalum, as all other eukaryotes investigated so far, contains multiple RNA polymerases. In recent years, several laboratories have addressed themselves to the analysis of these enzymes (for recent reviews see Melera, 1979; Sauer, 1978).

The presence of two different RNA polymerase species in extracts from isolated nuclei was first demonstrated in 1973 (Hildebrandt and Sauer, 1973) and several laboratories have since confirmed and expanded those early findings (Gornicki *et al.*, 1974; Burgess and Burgess, 1974; Weaver, 1976; Smith and Braun, 1978). When a nuclear

extract is chromatographed on a DEAE-ion exchanger, the RNA polymerase activity which elutes first is entirely sensitive to a fungal toxin, α-amanitin. On the other hand, the RNA polymerase activity eluting in a later peak is resistant to this toxin. This represents an inversion of the situation observed when RNA polymerases of various other eukaryotes are chromatographed on DEAE-resins. Here the α-amanitin-resistant polymerase activity elutes first (and hence is designated polymerase I), while the α-amanitin-sensitive enzyme follows in a later peak and is designated polymerase II. For the sake of clarity, the designation of the RNA polymerases from *P. polycephalum* follows that employed for other eukaryotes so that the α-amanitin-resistant polymerase is designated polymerase I and the α-amanitin-sensitive enzyme is polymerase II, irrespective of their order of elution from the DEAE-ion exchanger. This convention is justified since the α-amanitin-resistant polymerase from *P. polycephalum* is localized in the nucleolar chromatin and is involved in the transcription of ribosomal RNA (Davies and Walker, 1977; Seebeck *et al.*, 1979) as are its counterparts in other eukaryotes. Furthermore, a comparison of the salt optima of the RNA polymerases from *P. polycephalum* demonstrates that, again in accordance with the situation in other eukaryotes, the α-amanitin-resistant polymerase has a very much lower salt optimum than the α-amanitin-sensitive enzyme (Gornicki *et al.*, 1974; Burgess and Burgess, 1974; Smith and Braun, 1978).

In addition to RNA polymerases I and II, a third RNA polymerase activity, possibly representing RNA polymerase III, has recently been discovered after DEAE-Sephadex fractionation of high-salt homogenates from total plasmodia (Ernst and Sauer, 1977; Hildebrandt and Sauer, 1976a). This activity elutes at still higher salt concentrations than do polymerases I and II and it is resistant to α-amanitin concentrations of up to 10 μg/ml but is sensitive to higher toxin concentrations. This behaviour would suggest that this third activity is in fact RNA polymerase III but, due to its instability on purification, no additional information on this enzyme is presently available. A formal demonstration that it is a nuclear polymerase is also lacking at present.

Early attempts to purify RNA polymerases from *P. polycephalum* were based on salt extractions of isolated nuclei (Gornicki *et al.*, 1974; Burgess and Burgess, 1974; Weaver, 1976). Though polymerases could be purified from such extracts to specific activities comparable to those obtained with *Escherichia coli* RNA polymerase, the overall yield was

consistently low (a typical figure being 5 μg of purified RNA polymerase from 100 ml of packed plasmodia (Smith and Braun, 1978)). An approximately ten-fold increase in yield was achieved when total plasmodia, rather than purified nuclei, were used for the initial salt extraction (Hildebrandt and Sauer, 1973; Smith and Braun, 1978). Since Hildebrandt and Sauer (1976b) have very elegantly demonstrated that RNA polymerases I and II reside exclusively in the nucleus in intact plasmodia, the increase in enzyme yield observed when total plasmodia, rather than isolated nuclei, are extracted suggests that considerable leakage of RNA polymerase from the nucleus into the cytoplasm takes place during nuclear isolation.

Fractionation of the two RNA polymerase activities from crude extracts can be achieved by ion-exchange chromatography on DEAE-Sephadex or DEAE-Sepharose. In addition to the ionized groups of the resin, the supposedly inert matrix appears to be involved in this fractionation step too. This may be caused by hydrophobic interactions, since DEAE-cellulose is ineffective in separating RNA polymerases I and II from *P. polycephalum* (Burgess and Burgess, 1974; Hildebrandt and Sauer, 1976a; S. S. Smith, personal communication). This is quite a contrast to other eukaryotic RNA polymerases where DEAE-cellulose usually is the resin of choice for this purification step. However, a similar observation has been made during the purification of RNA polymerase I from sea urchins (D. Stafford, personal communication).

A further purification step usually applied to DEAE-Sepharose-fractionated polymerases I and II is chromatography on phosphocellulose (Gornicki *et al.*, 1974; Burgess and Burgess, 1974; Hildebrandt and Sauer, 1973; Smith and Braun, 1978). Here, polymerase II again elutes ahead of polymerase I. This step usually results in rather poor yields of protein. However, a strong increase in specific activity of the polymerases, notably polyermase I, is observed, leading even to recoveries of polymerase I activities which exceed the input (Burgess and Burgess, 1974). This observation cannot be adequately explained solely by removal of contaminating protein. It suggests that inhibitors were removed which had previously contaminated the enzyme preparation. Hildebrandt *et al.* (1979) have described a nucleolar inhibitor for RNA polymerases, the removal of which during chromatography of the enzyme on a cation-exchanger could account for the observed strong stimulation of polymerase I after chromatography on phosphocellulose (see Section II.C).

An alternative method for the purification of RNA polymerase II has recently been established which results both in better yields and greater purity of the enzyme (Smith and Braun, 1978). The crude salt extract from total plasmodia is treated with polyethyleneimine, which leads to the precipitation of, amongst other proteins, RNA-polymerase (Jendrisak and Burgess, 1975). The precipitated polymerase is eluted from the pellet and the eluate is then treated with ATP and magnesium to precipitate the vast amounts of actin which otherwise seriously contaminate the enzyme preparation. The actin-free supernatant is then fractionated by chromatography on DEAE-Sephadex, followed by heparin-Sepharose. The latter step achieves a dramatic increase in activity which is, in other purification schemes, observed after phosphocellulose chromatography (see above). RNA polymerase prepared in this manner is essentially pure except for a trace of actin which is still present. However, the enzyme purified to this state is free of nucleases. The remaining traces of actin can be removed by chromatography on phosphocellulose.

2. *Subunit Structure*

The subunit structure of polymerases I and II from *P. polycephalum* is still the subject of further investigation. Earlier workers reported an extremely simple subunit structure for polymerase I as well as II. Each enzyme was thought to consist of two polypeptides of molecular weights between 100,000 and 200,000. This view now appears to have been overly simplistic, probably brought about by the fact that insufficient amounts of protein had been loaded onto the gels. More recent investigation, though confirming the presence of the large-molecular weight subunits identified earlier, have revealed the presence of several additional presumptive subunits. The most recently proposed subunit structure for RNA polymerase I suggests that it consists of five polypeptides of the following molecular weights 200,000; 135,000; 45,000; 24,000; 17,000 in the ratio 1 : 1 : 1 : 2 : 1, respectively (Gornicki *et al.*, 1974). Her molecular weight determinations agree well with those reported by other workers for the two large subunits (Hildebrandt and Sauer, 1973; Burgess and Burgess, 1974). A comparison of Gornicki's subunit structure for polymerase I with that proposed by the same laboratory for polymerase II, *viz.* four

polypeptides of molecular weights 175,000; 140,000; 24,000; 17,000 present in the ratio 1 : 1 : 2 : 1, respectively (Weaver, 1976), suggests that the two polymerases might share some of the low molecular weight subunits (24,000 and 17,000). The most recently proposed structure for polymerase II has extended the number of subunits to six and includes an additional large molecular weight subunit, i.e. polypeptides of molecular weights 215,000; 170,000; 135,000; 38,000; 25,000; 14,000 in equimolar amounts (Smith and Braun, 1978). Such an overall subunit structure is rather similar to that proposed for RNA polymerase II from a variety of plants or from calf thymus (Jendrisak and Guilfoyle, 1978; Hodo and Blatti, 1977). Though the reported accounts of subunits from polymerase II from *P. polycephalum* vary in the number of subunits found, there is a general agreement on the molecular weights of those subunits identified so far in the different laboratories. One of the large subunits which is consistently found in polymerase II by all workers (molecular weight about 140,000) might be analogous to a subunit of similar size found in RNA polymerase II from all other eukaryotic sources and which is the binding site for α-amanitin (Brodner and Wieland, 1976).

When intact polymerase II is analysed on non-denaturing gels, two major and various minor subfractions of the enzyme can be detected, though the enzyme preparation is homogeneous when analysed by ion-exchange chromatography or by electrophoresis in denaturing SDS-gels (Smith and Braun, 1978; S. S. Smith and R. Braun, in preparation). Two dimensional gel electrophoresis reveals that each of the two largest subunits found in SDS gels (molecular weights 215,000 and 170,000) probably corresponds to one or the other of the two major subforms of the intact enzymes detected on non-denaturing gels. The presence of subforms of RNA polymerase II has been observed in a variety of organisms (Dezelee *et al.*, 1976; Jendrisak and Guilfoyle, 1978; Hodo and Blatti, 1977) and they are supposed to reflect the transcriptional state of the tissue in question (Jendrisak and Guilfoyle, 1978). Heterogeneity of polymerase II from *P. polycephalum*, even though purified from plasmodia supposedly in balanced growth, is also suggested by the broad salt optimum consistently observed (Gornicki *et al.*, 1974; Smith and Braun, 1978). The various subforms of RNA polymerase II could reflect different stages of the cell cycle or else various subforms might be required for transcription of different gene categories.

3. *Summary*

The RNA polymerases of *P. polycephalum* closely resemble those found in other eukaryotes. While little is known about RNA polymerase III, the subunit structure of polymerases I and II have been investigated in some detail. Both enzymes consist of several units. Polymerase I, the enzyme involved in the transcription of ribosomal RNA, is entirely resistant to a fungal toxin, α-amanitin. Polymerase II, the enzyme which transcribes RNA destined to become mRNA, is very sensitive to α-amanitin which inactivates the enzyme presumably by binding to one specific subunit. An analysis of the subunit structure of RNA polymerase II shows heterogeneity in some of the large subunits. These variations are thought to reflect differences in transcriptional activity or specificity.

E. REGULATORY FACTORS

1. *Initiation Inhibitor*

Early work on transcription in *P. polycephalum* has led to the postulation of a nucleolar inhibitor of RNA polymerases. Such a factor was tentatively characterized as an organic polyphosphate molecule (Sauer *et al.*, 1969a; Sauer *et al.*, 1969b; Goodman *et al.*, 1969). Recently these results have been re-examined and considerably expanded (Hildebrandt *et al.*, 1979). A polymerase inhibitor has been found to be enriched in the nucleoli. It copurifies with DNA during phenol extraction, but can be separated from DNA in CsCl equilibrium density gradients due to its higher buoyant density. The inhibitor can be further purified by ion-exchange chromatography on DEAE-Sephadex and by density gradient centrifugation where the material sediments homogeneously with a sedimentation coefficient of 4S. It is composed of acid-labile and acid-resistant phosphate in a ratio of 3 : 1, and a single carbohydrate component, tentatively identified as glycerol; it does not contain significant amounts of nitrogen. The inhibitor binds tightly to free RNA polymerase, but not to template-bound enzyme nor to free DNA and therefore seems to compete with DNA for the template-binding site of the polymerase. It inhibits homologous RNA polymerase I much more strongly than homologous polymerase II or heterologous RNA polymerase I and it does not have any effect on homologous DNA polymerase. This at least partial specificity of the inhibitor for polymerase I, together with its nucleolar

localization suggests, but by no means proves, that this substance might be a negative control element in the regulatory system of ribosomal RNA transcription. A possible physiological role for this substance is also suggested by the observed fluctuations of inhibitor concentration *in vivo* during the cell cycle and development (see Sections III.B and IV.B) and more information on the structure and function of this interesting substance is eagerly awaited.

2. *Stimulatory Phosphoprotein*

A large non-histone phosphoprotein (molecular weight 70,000) which acts as a strong positive control element for the transcription of ribosomal RNA *in vitro* has recently been characterized (Kuehn *et al.*, 1979). This protein is highly phosphorylated *in vivo* by a nuclear protein kinase which is dependent on the presence of polyamines and which is strongly inhibited by cyclic AMP (Atmar *et al.*, 1978). It is localized in the minichromosomes containing the ribosomal genes (Seebeck *et al.*, 1979). In its phosphorylated form, this protein binds to one specific region of ribosomal DNA with a binding constant of approximately 10^{-10} *M*. Preliminary mapping of the binding site has shown that it is located upstream from the sequences coding for ribosomal RNA and is at least 6000 base pairs away from the start of those sequences (Fig. 2). On dephosphorylation, this protein loses its ability to bind DNA. The effect of the protein on *in vitro* transcription is equally dependent on phosphorylation: addition of purified phosphoprotein to isolated minichromosomes containing the ribosomal genes results in a five-fold stimulation of transcription, while the dephosphorylated protein has no such effect. Analysis of the RNA transcribed *in vitro* in the presence or absence of the 70,000 daltons protein demonstrates that in both cases the same sequences are transcribed, though in the presence of the protein transcription is five times more efficient.

It is at present unclear if this stimulation of transcription is brought about by a higher frequency of initiation or by some other effect (e.g. on elongation). The observation that this protein is a strong stimulator of transcription, though it binds at a specific site far removed from the coding sequences, poses an intriguing problem: if this protein-binding site is anywhere close to the initiation site of transcription, a very long precursor RNA should be predicted, which, however, has not yet been

detected *in vivo* (see Section II.B). Alternatively, this protein could facilitate the binding of RNA polymerase molecules to the DNA at a site far away from the onset of transcription. These lined-up polymerases could then slide along the DNA to the coding sequences without transcribing *en route* and initiate only further downstream. Unfortunately too little information is available at present to give substance to any of these possibilities, and certainly many other speculations on the mechanism of action of such a positive control element for a eukaryotic gene are conceivable. Regulatory proteins, such as the one described above, might in evolution be a highly conserved class of molecules and preliminary comparison of the chemical composition of this 70,000 daltons protein from *P. polycephalum* has shown striking similarities with a possibly analogous protein isolated from rat nucleoli. These two proteins from both rat and *P. polycephalum* have the same molecular weight, are both acidic proteins and have a very similar amino-acid composition (Kuehn *et al.*, 1979; James *et al.*, 1977).

3. *Elongation Factor*

While both of the control factors dealt with so far may act on the level of initiation of transcription, a possible elongation factor has recently been described (Ernst and Sauer, 1977). This elongation factor is found in the material not retained by DEAE-Sephadex during RNA-polymerase purification and appears to be a protein with an isoelectric point of about 8. Since it has not yet been purified, its molecular structure has not been determined. The factor stimulates both RNA polymerases I and II equally well if the template is double-stranded DNA; no stimulation is observed when either single-stranded DNA is used as template or with *E. coli* RNA polymerase. The extent of stimulation, when double-stranded DNA is used as template, is strongly dependent on the size of the template, decreasing rapidly with decreasing molecular weight of the DNA. The factor does not have endonuclease activity nor does it act as an RNAase-inhibitor under the conditions tested, and from the low salt optimum reported for the stimulatory effect it appears unlikely to be an unwinding enzyme. Analysis of the transcription products demonstrated an increase in the length of the chains synthesized *in vitro* in the presence of the elongation factor, thus suggesting that this factor might stabilize the

transcription complex *in vitro*. A possible *in vivo* role of this factor for transcription is suggested by the fluctuations of factor concentrations with changing transcriptional activities of the plasmodia during the cell cycle and development (see Sections III.B and IV.B).

4. *Summary*

Three factors which regulate transcription have so far been identified. They are an organic polyphosphate which acts as an inhibitor of RNA polymerase I, a presumably proteinaceous elongation factor and a highly phosphorylated non-histone protein which specifically stimulates the transcription of rRNA. While the inhibitor and the elongation factors interact directly with RNA polymerase, the stimulatory phosphoprotein binds to specific sites of the ribosomal DNA. The mechanisms of action of all three factors are currently being investigated in several laboratories.

F. *IN VITRO* TRANSCRIPTION

1. *Transcription in Isolated Nuclei*

P. polycephalum has long been recognized to afford a particularly suitable system for investigating transcription *in vitro*, using either isolated nuclei or nucleoli. The advantages of this organism for this type of study are the high natural synchrony of the mitotic cycle, the possibility of studying transcription during different phases of development and certainly also the ease with which nuclei can be prepared. Early studies have demonstrated the transcriptional activity of isolated nuclei (Mittermayer *et al.*, 1966; Cummins and Rusch, 1967) and have furthermore established that the transcriptional activity of nuclei isolated at different times during the cell cycle approximately follows the fluctuations of the transcriptional activity *in vivo* (Grant, 1972; Cummins and Rusch, 1967; Braun *et al.*, 1966: Cummins *et al.*, 1966; Zellweger and Braun, 1971; see Section III).

A more detailed investigation of the basic requirements and the action of several transcription inhibitors suggested the existence of at least two different RNA polymerases in nuclei before these enzymes were actually isolated and characterized biochemically (Grant, 1972). Isolated nucleoli were shown to contain an RNA polymerase activity which was largely resistant to the fungal toxin α-amanitin. This enzyme

was minimally active at 10 m*M* $MgCl_2$ and under low salt conditions, and no additional stimulation could be achieved by the addition of Mn^{2+} or by raising the ionic strength of the incubation medium. If, however, nuclei instead of nucleoli were analysed, a second enzyme activity became apparent. This latter was sensitive to α-amanitin and optimal conditions of activity required provision of Mn^{2+} ions and moderately high ionic strength (100 m*M* KCl). The differential effect of Mn^{2+} ions on the activity of the two polymerases in nuclei, where only polymerase II but not I was stimulated, was in apparent contrast to the ionic requirements of the two enzymes purified subsequently (Gornicki *et al.*, 1974), which are both stimulated to similar extents by Mn^{2+}. However, in the experiments of Grant (1972) with nuclei or nucleoli, addition of Mn^{2+} was always accompanied by the addition of salt to the incubation mixture. Since RNA polymerase I demonstrates optimal activity at a very low salt concentration, the stimulation of activity brought about by the Mn^{2+} ion was probably nullified by the concomitant rise of the salt concentration to supra-optimal levels.

Davies and Walker (1977) have taken up and considerably expanded these observations. The method of preparing nuclei was shown to be of crucial importance for the overall transcriptional activity of the purified nuclei. Exposure to high sucrose concentration (1 *M*) and to 10 m*M* $MgCl_2$ led to a considerable loss of activity, an observation most readily explained by the leakage of RNA polymerases out of the nuclei during isolation (Hildebrandt and Sauer, 1976b; see Section II.D). Structural integrity of the chromatin was demonstrated to be another important factor, since sonication of isolated nuclei completely abolished transcriptional activity. Overall transcriptional activity in this system has a salt optimum of 100 m*M* KCl, suggesting an overabundance of RNA polymerase II. This is confirmed by the observation that about 60% of ^{3}H-UTP incorporation can be inhibited by 16.5 μg of α-amanitin/ml. No further inhibition is observed with inhibitor concentrations up to 200 μg/ml, indicating the absence of RNA polymerase III activity (Davies and Walker, 1978). This latter observation, as well as the difficulty of detecting RNA polymerase III during enzyme purification (see Section II.D), again suggests that this enzyme is either very easily lost from nuclei during purification or that, in *P. polycephalum*, it is an extremely unstable enzyme which is quantitatively inactivated during the nuclear isolation procedure. Preliminary experiments investigating the possibility of initiation of

transcription in isolated nucleoli demonstrated a slight inhibition of ^{3}H-UTP incorporation in the presence of the initiation inhibitor rifampicin AF/013 (Davies and Walker, 1978). This observation suggests the possibility that re-initiation of transcription may in fact occur in isolated nucleoli.

A potentially very interesting aspect of the work of Davies and Walker (1977) is their observation that nuclei, as well as nucleoli, synthesize full-length precursor ribosomal RNA molecules and, in addition, are capable of processing these large transcripts (44S) via the correct intermediate (34S) to the mature 26S and 19S ribosomal RNA. However, several features of these experiments require further clarification and a more detailed investigation would certainly be of great interest. Although the paper states explicitly that only about 40% of the transcription is resistant to α-amanitin, i.e. is ribosomal transcription, the gels presented indicate a virtually complete absence of non-ribosomal transcripts, if one is to follow the argument employed by the authors. Furthermore, gels displaying RNA synthesized either *in vivo* or *in vitro* for various lengths of time indicate a virtually complete absence of transcripts smaller than 19S, an observation so unusual that it would certainly warrant a closer investigation. The question of RNA processing is difficult to assess from the data presented since the resolution in the gel system used is rather poor, as the authors themselves acknowledge, and since the very likely possibility of aggregation artefacts has not been taken into account. If, however, the claims of the authors should be substantiated by more rigorous experimentation, the nucleolar transcription system from *P. polycephalum* would certainly be an even more attractive system for investigating the transcription of ribosomal genes in an eukaryotic organism.

Another system for *in vitro* transcription of ribosomal RNA has recently been established by the purification of a minichromosome containing the ribosomal genes and, among other chromosomal proteins, RNA polymerase I, but not II or III (Seebeck *et al.*, 1979; Kuehn *et al.*, 1979). In this system the endogenous polymerase transcribes the correct strands of the coding sequences but, in contrast to the observations discussed previously (Davies and Walker, 1977), the size of the transcripts is small. Since RNAase does not appear to be a major cause of this small size of the transcripts (G. Bindler and T. Seebeck, unpublished), the discrepancy between the sizes of the

transcripts obtained from the two systems remains unsolved. A speculative explanation for this discrepancy would be the loss, during isolation of the minichromosome, of factors which stabilize the transcriptional complex (e.g. Ernst and Sauer, 1977; see Section II.E). The transcriptional activity of the minichromosome is positively controlled by a non-histone phosphoprotein (see Section II.E); the mechanism of action of this control element still remains to be resolved.

In an attempt to map the initiation site for the transcription of the ribosomal precursor RNA, E. M. Johnson and his collaborators have worked with intact nuclei as their *in vitro* system. Using a technique which allows the separation of RNA synthesized *in vitro* from the pre-existing RNA (Reeve *et al.*, 1977), they were able to purify, by affinity chromatography, the newly initiated RNA chains which were synthesized in the nuclei in the presence of α-amanitin, i.e. by RNA polymerase I (I. Sun and E. M. Johnson, personal communication). Hybridization of these molecules, which carried a labelled 5′-terminal triphosphate group, i.e. which represent the 5′-termini of the newly initiated RNA, allowed the localization of the initiation site on the ribosomal DNA molecule (see Fig. 2). This location is compatible with a ribosomal RNA precursor molecule of the size of those actually observed *in vivo* (Jacobson and Holt, 1973; Melera and Rusch, 1973a). So, at present, the best estimate available for the localization of the initiation site of the ribosomal precursor RNA is the one indicated in Fig. 2. However, although three independent procedures (sizing of the ribosomal precursor RNA synthesized *in vivo*; electron microscopy of putative rDNP with nascent RNA chains; mapping of the initiation site *in vitro*) suggest a similar location, the possible pitfalls of all three approaches should be kept in mind and the site indicated in Fig. 2 should be regarded as tentative.

In vitro transcription by RNA polymerase II in isolated nuclei or chromatin is at present rather difficult to investigate in detail since no well defined hybridization probes for individual genes, which could be used for the analysis of *in vitro* transcripts, are yet available. However, specific genes have recently been identified from a library of cloned DNA from *Dictyostelium discoideum* (Kindle and Firtel, 1978), and at least some of these genes may be expected to give enough cross-hybridization with the corresponding *P. polycephalum* genes so that they could possibly be used as DNA probes until homologous clones are

available. Once such cloned DNA fragments carrying specific genes are available as hybridization probes for identifying *in vitro* transcripts, the analysis of *in vitro* transcription in *P. polycephalum* will enter a truly exciting stage.

Besides the lack of hybridization probes, transcription studies in *P. polycephalum* have also been hampered by the impossibility of preparing chromatin without introducing rather extensive damage to its structure. This problem is due to the extraordinary resistance of isolated nuclei to shear or lysis by EDTA, so that the preparation of chromatin always had to include one step of extensive sonication or vigorous vortexing in the presence of EDTA. This problem has now apparently been overcome by a significantly improved technique for the preparation of chromatin which has been reported by Schicker *et al.* (1979). The important step in this procedure is the lysis of the nuclear membrane with lysolecithin in the presence of 5 m*M* $MgCl_2$. Hereby the previously used sonication or vortexing can be avoided and the nuclei do not have to be exposed to EDTA which by itself disrupts the structure of *P. polycephalum* chromatin so that it can no longer be properly digested by micrococcal nuclease (J. Stalder, personal communication). Chromatin as prepared by Schicker *et al.* (1979) contains endogenous RNA polymerase activities of both types I and II. Salt optima for the two polymerases in chromatin correspond well with those found for purified enzymes. Analysis of the *in vitro* transcripts in denaturing formamide gels demonstrates a broad peak of RNA migrating with 20 to 30S, a homogeneous peak at 4 to 5S and a third pronounced peak of material migrating faster than 4S. Such a profile is in sharp contrast to those presented by Davies and Walker (1977) who could find no evidence for the *in vitro* synthesis of small molecular weight RNA molecules. Since these latter authors used non-denaturing gels of rather poor resolving power, the data presented by Schicker *et al.* (1979) may be more reliable. The addition of exogenous, purified RNA polymerase II to isolated chromatin resulted in a doubling of incorporation of ^{3}H-UTP and in a higher proportion of transcripts migrating in the 20 to 30S region during electrophoresis.

Such a stimulation of transcription is, however, only observed when the exogenous RNA polymerase is added under low salt conditions, and it is completely abolished when high salt (100 m*M* KCl) or the initiation inhibitor aurin tricarboxylic acid are present. Once initiation by the exogenous polymerase has taken place under low salt con-

ditions, a subsequent increase of the salt concentration no longer prevents transcription. Experiments using γ-^{32}P-labelled UTP as a precursor have given suggestive evidence of the occurrence of initiation of transcription by the endogenous polymerases, thus supporting the conclusions reached by previous workers (Davies and Walker, 1977) who investigated the effects of the initiation inhibitor rifampicin AF/013. However, both lines of evidence are rather indirect and none of the numerous pitfalls inherent in one or the other of the two methods has been rigorously excluded; hence the question of significant, site-specific initiation of transcription of mRNA in Physarum is still wide open, as it is in other eukaryotic systems as well (Grummt, 1978).

2. *Transcription in Isolated Mitochondria*

A single paper has appeared to date dealing with *in vitro* transcription in isolated mitochondria from *P. polycephalum* (Grant and Poulter, 1973). Here RNA synthesis is inhibited both by actinomycin D and by rifampicin (50% inhibition by both drugs is exhibited at around 5 μg/ml). The half-life of RNA synthesized *in vitro* is extremely short, namely around 2 to 3 min. This suggests, unless the rapid turnover is an experimental artefact, that the molecules made *in vitro* are not of ribosomal RNA, since ribosomal RNA would be expected to be much more stable.

Electrophoretic analysis indeed confirms that mitochondrial RNA synthesized *in vitro* migrates rather heterogeneously but exhibits a clear peak migrating more rapidly than the mitochondrial ribosomal RNA. No significant amount of RNA synthesized *in vitro* migrates in the positions of ribosomal or of 4 and 5S RNA. The nature of the mitochondrial RNA synthesized *in vitro* is unclear at present and certainly warrants closer investigation. The lack of interest in the transcriptional capabilities of the mitochondria from *P. polycephalum* is rather surprising since this organism offers the unique possibility of isolating rather large amounts of mitochondria from a homogeneous cytoplasm. Due to the natural synchrony of the cell cycle and the various life stages exhibited by this organism, the analysis of possible nuclear controls over mitochondrial transcription during the cell cycle or differentiation appears to be a promising approach and further information on this subject is certainly most eagerly awaited.

3. *Summary*

Transcription has been investigated *in vitro* both with isolated nuclei and nucleoli. During *in vitro* transcription with nucleoli, full-size ribosomal precursor RNA is apparently synthesized and processed into mature rRNA. Isolated chromatin from total nuclei still contains endogenous activity of both RNA polymerase I and II. Transcription of such chromatin can be strongly stimulated by the addition of purified homologous RNA polymerase. For lack of well defined hybridization probes, detailed investigation of *in vitro* transcription by bulk chromatin has not yet been possible.

III. Transcription in the Mitotic Cycle

A. *IN VIVO* TRANSCRIPTION

The question underlying much of the research done in this area is whether the well-known structural changes in the mitotic cycle are controlled at the level of transcription, or, to formulate a more descriptive question, whether there are quantitative and/or qualitative differences in the types of RNA molecules synthesized at different times of the mitotic cycle. In *P. polycephalum* some of the biochemical events of the mitotic cycle have been studied in considerable detail. In particular, it is well established that under standard growth conditions chromosomal DNA is replicated only in the first third of interphase (Nygaard *et al.*, 1960) and that each fraction of the genome replicates at a specific time during the S-phase (Braun *et al.*, 1965; Braun and Wili, 1969). Also, early replicating DNA contains fewer repeated sequences than does late replicating DNA (Fouquet *et al.*, 1974a; Fouquet and Sauer, 1975). In contrast to the main chromosomal DNA, however, r-DNA replicates throughout interphase with the exception of the first hour of the S-phase (Zellweger *et al.*, 1972). The second minor DNA fraction, mitochondrial DNA, replicates throughout the intermitotic period, as was found in several other eukaryotic cell types (Sonenshein and Holt, 1968; Braun and Evans, 1969).

1. *Differential Transcription in the Mitotic Cycle*

How does transcription relate to the mitotic cycle? A decrease of RNA synthesis during mitosis was already indicated by Nygaard *et al.*

(1960) and later autoradiographic observations of Kessler (1967) showed that very little, if any, RNA is made during the period of metaphase. This arrest of transcription only lasts about five min under standard growth conditions. These results, obtained by using pulse labelling techniques, have recently been confirmed for the two stable RNA types, rRNA and tRNA, by using an elegant isotope dilution procedure (Hall and Turnock, 1976; Fink and Turnock, 1977). In addition, these authors have shown that the rate of stable RNA synthesis clearly increases from early interphase to late interphase. For rRNA it was possible to compute the rate increase more accurately: it appears to rise continuously five- to six-fold from early interphase to late interphase (Hall and Turnock, 1976). The similarity in behaviour of both these stable RNA species is particularly interesting, since they are synthesized in a totally different manner. The genes for tRNA are chromosomal, whilst those for rRNA are extrachromosomal (Hall and Braun, 1977); in addition it is most likely that two different polymerases are required for their synthesis. It is not clear whether the mitotic arrest of synthesis and the gradual rate increase during the intermitotic period are regulated by the same mechanism. This does not seem very likely: the mitotic arrest may be caused by chromosome condensation and inability of the RNA polymerases to move further on their appropriate templates. The interphase rate, on the other hand, is probably controlled both by gene dosage as well as by some superimposed unknown mechanism. For rRNA the latter is postulated, since the rate of rRNA synthesis rises five- to six-fold over a mitotic cycle, while the template only doubles in amount. For the metaphase arrest of transcription other mechanisms than the one mentioned above can be put forward. One such possibility, which might be tested experimentally, is that RNA polymerase leaves the template during chromosome condensation.

Pulse labelling experiments with total RNA have shown up a biphasic pattern of uridine incorporation over the mitotic cycle with minima in mitosis and, unexpectedly, also in mid interphase (Mittermayer *et al.*, 1964; Braun *et al.*, 1966; Sauer *et al.*, 1969b). Many attempts have been made to see whether the two peaks of apparent transcriptional activity reflect the synthesis of different classes of RNA, and there is a great deal of evidence to suggest that differential transcription does occur as shown by sensitivity towards actinomycin D (Mittermayer *et al.*, 1964), base composition (Cummins *et al.*, 1966),

DNA-RNA hybridization under conditions wherein multiple copies reanneal (Fouquet and Braun, 1974), fractionation of polyA-RNA (Fouquet *et al.*, 1974b) and RNA redundancy (Fouquet and Sauer, 1975). In general, the G2-phase reflects predominantly rRNA synthesis, whilst in the S-phase peak other RNA species (presumably hnRNA) appear to be made in the largest amounts. Furthermore, studies of the transcription of unique sequence DNA strongly suggest that there are qualitative as well as quantitative differences between the RNA populations synthesized during S-phase and G2-phase (Fouquet and Braun, 1974). There may be about three times more different sequences transcribed in S-phase than in G2-phase. Taking all these results together it is clear that there is a control over both the type and the quantity of RNA transcribed during the mitotic cycle. This implies the existence of a transcriptional programme which gets repeated with every cell cycle. There is no compelling evidence to show at what stage of the mitotic cycle the transcription programme is reset, but metaphase itself would be a likely candidate for such an event. To investigate this problem further, it would be most helpful to have probes for individual mRNA species. As regards tRNA no differential transcription of 20 tRNA families could be detected over the mitotic cycle (Melera and Rusch, 1973b; Melera *et al.*, 1974). In contrast to most nuclear RNA synthesis, mitochondrial RNA seems to be made at a constant rate over the mitotic cycle (Grant and Poulter, 1973).

The intracellular pools of free ribonucleoside triphosphates, the immediate precursors of macromolecular RNA, have been the subject of several studies and appear to fluctuate considerably during the mitotic cycle (Chin and Bernstein, 1968; Sachsenmaier *et al.*, 1969; Bersier and Braun, 1974; Fink, 1975). This topic will not be dealt with any further here, since it is outside the scope of this review.

2. *Role of RNA Primers in DNA Replication*

The event by which the S-phase of the cell cycle is defined is the replication of chromosomal DNA. There appear to be two close connections between replication and certain functions of RNA. Firstly, Okazaki fragments contain RNA and, secondly, there may be some connection between replication of a particular DNA molecule and its transcription.

The first polydeoxyribonucleotides that are made in cells engaged in

DNA synthesis are single-stranded and only short. These so-called Okazaki fragments are about 200 nucleotides long in *P. polycephalum* and are subsequently joined to form much larger molecules (Funderud and Haugli, 1977). Interestingly, the transient Okazaki fragments carry a short polyribonucleotide sequence at the 5'-end of the nascent polydeoxyribonucleotide (Waqar and Huberman, 1975), as had previously been shown for the corresponding Okazaki fragments in *Escherichia coli* (Sugino *et al.*, 1972; for review see Sheinin *et al.*, 1978). These authors injected γ-^{32}P-labelled deoxyribonucleoside triphosphates into surface plasmodia (Waqar and Huberman, 1973), isolated Okazaki fragments containing a short RNA-fragment and showed that the RNA was joined to the 5'-end of the DNA. Any of the four ribonucleotides was found joined to any of the four deoxyribonucleotides thereby allowing all 16 possible RNA-DNA junctions, although the frequency of different junctions varied up to twenty-fold. As a starter, dGTP was used about ten times more frequently than the other three deoxyribonucleoside triphosphates. From these observations, and similar ones made in other organisms, it may be postulated that the first step in DNA replication is the synthesis of a short polyribonucleotide primer by an RNA polymerase and the subsequent joining onto this of deoxyribonucleotides to yield the nascent single stranded DNA chain. The RNA will obviously have to be removed at a later stage and replaced by DNA. It will be interesting to find out how transcription and subsequent removal of the RNA primers are regulated. Though the existence of an RNA primer for DNA replication has been unambiguously demonstrated, it is unclear at present if any of the RNA polymerases discussed in Section I are involved in the synthesis of the primer or if this type of transcription is performed by an altogether different enzyme or enzyme system.

3. *Coupling of Transcription with DNA Replication*

In *P. polycephalum* there may be a rather close connection between the replication of certain DNA segments and their transcription. This led Sauer (1978) to propose an interesting model of transcription control in the cell cycle. As will be shown, this model is still very hypothetical. Rao and Gontcharoff (1969) showed that during the S-phase inhibition by fluorodeoxyuridine of DNA replication leads to a considerable inhibition of uridine incorporation into RNA. Since fluorodeoxy-

uridine has this effect only during the S-phase and not during the G2-phase, it seems likely that previous replication is required for subsequent transcription of a specific DNA segment. It cannot be totally discounted, however, that the observed effect of fluorodeoxyuridine may be indirectly caused, e.g. by interference with the uptake or metabolism of the labelled uridine used as a precursor for RNA. More recently another inhibitor of DNA synthesis, hydroxyurea, has been used for similar experiments (Fouquet *et al.*, 1975b). In one hour pulses given at different times of the mitotic cycle, hydroxyurea inhibited uridine incorporation into RNA by up to 70%, but only in early S-phase. Again it cannot be completely excluded that this striking effect may arise indirectly, as suggested above for fluorodeoxyuridine. However, this objection is largely eliminated by the finding that isolated nuclei of cultures treated *in vivo* with hydroxyurea contain less RNA polymerase activity than nuclei from untreated cultures (A. Hildebrandt and H. W. Sauer, personal communication). RNA from both the S and G2 phases, labelled in the presence and absence of hydroxyurea, was extracted and partly characterized by RNA-DNA hybridization. The results tend to suggest that the RNA made in the presence of the inhibitor may in part be qualitatively different from that made in its absence, but to establish this firmly several different types of experiments would be required. It is perhaps surprising that different RNA precursors gave results that were somewhat at variance.

The replication-transcription coupling model makes the attractive suggestion that transcription may be re-aligned once per mitotic cycle, possibly during mitosis itself (Sauer, 1978). This would allow a simple control of events which occur periodically once per mitotic cycle or per cell cycle. Several observations with extracted RNA polymerase are in agreement with such a model, but they do not prove it (see Section III.B). In this context, studies on specific mRNA species, coded for by non-reiterated genes, would be welcome.

B. *IN VITRO* TRANSCRIPTION

1. *Isolated Nuclei*

Experiments on *in vitro* transcription in *P. polycephalum* led to the observation that the overall transcriptional activity of nuclei isolated during the cell cycle reflected the transcriptional activity of the intact plasmodia *in vivo* (Mittermayer *et al.*, 1966; Cummins and Rusch, 1967;

Grant, 1973). Basically, two peaks of transcriptional activity can be discerned when nuclei are isolated during the mitotic cycle. The first peak occurs during the S-phase, while a second peak becomes apparent during the G2-phase (Mittermayer *et al.*, 1966; Grant, 1972; Davies and Walker, 1978). When the RNA polymerases involved in these two phases of transcription were investigated, differential activities of polymerases I and II during the cell cycle could be recognized *in vitro*. In the first peak of activity, during the S-phase, maximal transcription occurs in the presence of Mg^{2+} ions and with low salt concentrations; the addition of Mn^{2+} and increase in the salt concentration to 100 m*M* KCl yield no further stimulation. However, during the second peak, when the overall transcriptional activity is lower than during the first peak, when assayed under low salt conditions, a considerable stimulation of transcription can be achieved by the addition of Mn^{2+} and by raising the salt concentration (Grant, 1972). This stimulation of transcription by Mn^{2+} and salt is completely inhibited by α-amanitin, a potent inhibitor of RNA polymerase II only. The basal activity, which is measured with Mg^{2+} and low salt, is resistant to the toxin. In contrast, the transcriptional activity during the first peak is sensitive to α-amanitin (Davies and Walker, 1977, 1978). These observations correlate well with the properties of purified RNA polymerases (see Section II.D), since polymerase I has a low salt optimum and is resistant to α-amanitin, whereas polymerase II has a higher salt optimum and is very sensitive to this inhibitor. Taken together, these observations suggest that during the first peak of activity (S-phase) it is chiefly RNA polymerase II which is active and, hence, it is messenger RNA which is predominantly synthesized; during the second peak (G2-phase), the predominant activity is that of RNA polymerase I synthesizing ribosomal RNA.

This contention was soon supported by the results of analysis of the base composition of the RNA synthesized both *in vitro* (Cummins and Rusch, 1967) and *in vivo* (see Section III.A). This demonstrated in both cases that RNA synthesized during the S-phase peak of activity has a rather DNA-like base composition (as would be expected for messenger RNA), while RNA synthesized during the G2-peak has a higher GC-content and hence bears more resemblance to ribosomal RNA. Although the validity of these data has been seriously questioned on technical grounds (Grant, 1973), the final conclusions of this work have gained support from the recent observations that, *in vivo*, polyA-

containing messenger RNA was mainly synthesized during S-phase (Fouquet and Braun, 1974; Fouquet *et al.*, 1975b), whereas the synthesis of ribosomal RNA was low during S-phase but predominated during G2-phase (Hall and Turnock, 1976).

Since RNA polymerase II can be quantitatively inhibited by low concentrations of α-amanitin, the specific transcription of ribosomal RNA, rather than that of the total RNA, can be conveniently studied *in vitro* (Davies and Walker, 1977, 1978). Experiments using isolated nuclei or nucleoli both showed that, again in accordance with the situation *in vivo*, no detectable transcription of ribosomal RNA takes place during metaphase. However, *in vitro* transcription reaches its maximal interphase level within ten min after mitosis and, quite in contrast to what has been observed *in vivo* (Hall and Turnock, 1976), no discernible differences in the rate of transcription of ribosomal RNA were observed *in vitro* during the cell cycle. Supportive evidence for this observation is provided by a preliminary experiment with the initiation inhibitor rifampicin AF/013 which showed that, *in vitro*, the rate of initiation of ribosomal RNA transcription did not apparently change during the cell cycle. The two sets of observations, *in vivo* and *in vitro*, need not necessarily be conflicting since all kinds of post-transcriptional processing schemes can be envisaged to account for the apparent contradiction that the rate of initiation seems to be constant during the cell cycle *in vitro*, while the rate of transcription of ribosomal RNA increases by a factor of five during the cell cycle *in vivo*. But before these findings should be considered in any depth, it would be desirable to obtain more reliable results *in vitro* in the initiation experiment.

The ability of chromatin or nuclei from different stages of the cell cycle to be transcribed by exogenous RNA polymerase II has recently been investigated (Schicker *et al.*, 1979). *In vitro* transcription by chromatin, or nuclei, isolated during the S-phase, where transcription by polymerase II predominates, can be strongly stimulated by the addition of exogenous, homologous, polymerase II. In contrast, the stimulation by exogenous enzyme is very much less with chromatin or nuclei from the G2-phase. This observation tentatively suggests that genes which are to be transcribed by polymerase II are inaccessible to the enzyme during the G2-phase, or else that inhibitors are produced during the cell cycle which selectively inactivate one or the other of the two polymerases and which would also inactivate, in G2-phase

chromatin, the exogenous polymerase. Several observations have provided hints that both types of transcriptional control might be operative. The presence of fluctuating concentrations of inhibitors is suggested by the observation that *P. polycephalum* contains approximately equal amounts (i.e. of units of enzyme activity) of polymerases I and II and that these amounts do not change appreciably during the cell cycle when semi-purified enzyme preparations are analysed (Hildebrandt and Sauer, 1976c). So the differential transcriptional activities observed both *in vivo* and *in vitro* cannot be caused by differential synthesis or rapid turnover of the polymerases, but rather through a modulation of their activity by controlling factors. A possible loss of such presumptive factors during the isolation of nucleoli could also account for the observation by Davies and Walker (1978) that no difference in transcription of ribosomal RNA during the cell cycle can be detected with isolated nucleoli (see Section II.F).

Other observations point to the possibility that the transcription of messenger RNA might be coupled to ongoing DNA replication (Fouquet *et al.*, 1975b; for a review see Sauer, 1978; see Section III.A), and thus that a particular conformation of a gene, or its surrounding chromatin, might be required to allow transcription by polymerase II. This suggestion is also supported by Schicker *et al.* (1979) who prepared chromatin from plasmodia, in the S- and G2-phases, which had been pretreated with 50 m*M* hydroxyurea to block DNA synthesis prior to the preparation of the chromatin.

When stimulation of transcription of such chromatin by exogenous polymerase II was tested, none could be achieved, either with S-phase or with G2-phase chromatin, though S-phase chromatin normally can be strongly stimulated (see above).

The evidence outlined so far indicates a general agreement that transcription observed *in vitro* with isolated nuclei or nucleoli reflects, at least to a large extent, the *in vivo* state of transcription. *In vitro* transcription systems therefore appear to be attractive instruments for analysing the various factors involved in the regulation of transcription during the cell cycle. A variety of concepts can be envisaged to explain how transcriptional regulation could be brought about, and most probably many different regulatory mechanisms are working in conjunction. An apparently obvious mechanism, namely that the fluctuation of the numbers of RNA polymerase molecules parallels the observed transcriptional activity, has been made unlikely by the

experiments which have determined the amounts of active RNA polymerase during the cell cycle (Hildebrandt and Sauer, 1976c; see above). The importance of a particular gene- or chromatin-structure for transcription, e.g. the need for concomitant DNA replication, has been suggested by different lines of experimentation (Fouquet *et al.*, 1975b; Sauer, 1978; Schicker *et al.*, 1979) but direct evidence has not yet been reported and might, in fact, be rather difficult to obtain at present.

2. *Regulatory Factors*

A third alternative, the presence of regulatory factors of various kinds, is amenable to analysis and evidence for several such factors has been reported. An organic polyphosphate of nucleolar origin, which is an inhibitor of RNA polymerase I, and to a much lesser extent of polymerase II, has been recently characterized and its function in the control of transcription of ribosomal RNA during differentiation has been suggested (Hildebrandt *et al.*, 1979; Hildebrandt and Sauer, 1977a; see also Sections II.E and IV.B). Preliminary experiments have indicated that this factor might be involved in the regulation of transcription during the cell cycle (Sauer *et al.*, 1969b), although this possibility still remains to be explored. A similar situation exists with a non-histone phosphoprotein which has been reported to act as a positive control element for the transcription of ribosomal RNA (Kuehn *et al.*, 1979). Here again, no information is yet available on its actual role in the regulation of transcription during the cell cycle.

In contrast, a recently isolated nuclear elongation factor for transcription (Ernst and Sauer, 1977; see Section II.E) has been correlated with transcriptional activity during the cell cycle *in vivo*. The concentration of this factor (measured *in vitro* as a stimulatory effect on RNA polymerase activity) fluctuates during the cell cycle. It reaches a sharp maximum during the peak of transcriptional activity of polymerase II during the S-phase, drops to background level (about 50% of peak activity) at the end of the S-phase and remains constant throughout the remainder of the mitotic cycle, including mitosis. It is interesting to note that the concentration of this elongation factor is covariant with the activity profile of polymerase II only, although, *in vivo*, the factor is capable of stimulating polymerase I as well as polymerase II, while it does not have any effect on *in vitro* transcription accomplished with *Escherichia coli* RNA polymerase. A second point of

interest is the observation that the activity of this factor, aside from the peak attained during the S-phase, remains constant throughout the cell cycle and does not change during mitosis, whereas transcription by both polymerase I and II drops to very low levels during mitosis (see Section II.E).

3. *RNA Polymerase*

Very little is known so far about the importance of subunit-modification for the modulation of transcriptional activity in Physarum. A recent analysis of RNA polymerase II from asynchronously growing cultures has shown that this enzyme, although homogeneous upon purification, exhibits a marked heterogeneity in its largest peptide subunit, the size of which can vary from 170,000 to 215,000 daltons (Smith and Braun, 1978; see Section II.D). This heterogeneity of the large subunit is reminiscent of the situation observed with RNA polymerases from other eukaryotic sources where polymerase IIA (the largest subunit has a molecular weight over 200,000) appears to be associated with resting, non-transcribing tissue whereas polymerase IIB (the largest subunit is smaller than 200,000 daltons) is predominantly found in actively growing tissue (Jendrisak and Guilfoyle, 1978). So, the variation in subunit structure of polymerase II from *P. polycephalum* could well reflect a mixture of enzyme populations from various stages of the cell cycle, and hence of different transcriptional activities.

A further possible regulatory mechanism has been suggested by the observation that in isolated nuclei various classes of polymerase II can be distinguished by the salt concentrations needed to extract them from the nuclei (Hildebrandt and Sauer, 1977b). The bulk of polymerase II is eluted during the preparation of nuclei in hypertonic sucrose solutions (Hildebrandt and Sauer, 1976b). Another 10% of enzyme activity can be solubilized from nuclei with 0.5 *M* NaCl. A small amount of additional activity can be eluted by 1.5 *M* NaCl; the proportion of this class of enzyme appears to increase during differentiation. The functional significance of the different solubility classes of RNA polymerase is not yet clear. The remainder of polymerase II activity is resistant to elution by 1.5 *M* NaCl and is considered by the authors to represent initiated enzyme. The relative distribution of total polymerase II activity between these "solubility classes" appears to fluctuate considerably during differentiation (see

Section IV.B); therefore it seems appropriate to speculate that differential binding of polymerase to template might constitute an important means of regulating transcriptional activity during the cell cycle.

IV. Transcription During Differentiation

A. *IN VIVO* TRANSCRIPTION

As mentioned in the Introduction, vegetative slime mould plasmodia can differentiate into resistant forms, usually under adverse environmental conditions. Although differentiation has been studied in much less detail with the acellular slime moulds than with the cellular slime moulds, several investigators have nevertheless studied the conditions under which sporulation or spherulation occurs and some of the biochemical changes that accompany these processes. The only acellular slime mould that has received any attention as regards transcription is again *P. polycephalum*. Since investigations at the molecular level have not advanced much in the last few years, and since the earlier observations have been succinctly reviewed by Chet (1973) for spherulation and by Sauer (1973) for sporulation, we will cover this subject only cursorily.

During the structural reorganization which occurs in differentiation, the RNA and protein contents of cultures, decrease, presumably through a breakdown of ribosomes. At the same time one could expect that new species of RNA have to be made, particularly mRNA for new structural proteins and new enzymes. Experiments with actinomycin D indicated that this might be the case for sporulation, though not for spherulation. The difference may however be due entirely to differential penetration of the inhibitor. More direct investigations were undertaken to visualise differences between newly made RNA and old RNA by sizing in either sucrose gradients or polyacrylamide gels. The results were negative, as there were no clear differences seen in RNA populations made at various stages of differentiation. Competition hybridization experiments on the other hand seemed to indicate that there were in fact different types of RNA molecules made at different times. Unfortunately these experiments gave no quantitative estimates of the number of molecular species concerned. Several reasons for this may be suggested: hybridization conditions only took account of RNA

species made on reiterated DNA sequences and, in addition, RNA extraction was not shown to be quantitative and to yield intact molecules. Particularly during differentiation, when RNA-degrading enzymes are known to be activated, such controls are essential to obtain biologically meaningful results. Undoubtedly *P. polycephalum* is a suitable and interesting organism in which to study the role of transcription and its regulation during various stages of differentiation. It will take a considerable effort to get some tangible information on the fate of the mixed population of mRNA molecules as well as on individual species coding for proteins with specific functions in differentiation.

B. *IN VITRO* TRANSCRIPTION

Differentiation in *P. polycephalum* can be induced by starvation and can proceed via two different pathways (see Section IV.A). Both types of differentiation seem to be accompanied by changes in the programme of RNA transcription. Again, as previously seen in cell cycle studies (see Section III.B), these changes in transcriptional activity are also reflected *in vitro* by isolated nuclei (Hildebrandt and Sauer, 1977b). When nuclei are isolated from microplasmodia after different periods of growth and starvation, their endogenous RNA polymerase II activity drops about five-fold from growing plasmodia to plasmodia which have been in culture for six days, i.e. which have initiated spherulation. A subsequent experiment indicated that this drop in activity does not reflect the absence of polymerase II in nuclei from stationary cultures, but that the enzyme becomes reversibly inhibited upon starvation. When nuclei from actively growing and from stationary cultures were treated with 2% Triton X-100 before RNA polymerase activity was measured, a reverse of the previous observation was obtained. Stimulation of transcription by Triton X-100 was very low in nuclei isolated from actively growing cultures (about 20% stimulation). In contrast, nuclei from old, stationary cultures were stimulated by Triton X-100 by about 400%. Transcriptional activity in nuclei isolated from cultures of different ages therefore reflects rather accurately the decline in transcriptional activity observed *in vivo*. Triton X-100 treatment of such nuclei restores polymerase II activity to the level found in nuclei from actively growing cultures. These findings can be interpreted to show that treatment of nuclei with the detergent reverses the inhibition blocking polymerase II to an increasing degree

during the stationary phase and the onset of spherulation. The authors of this study indicate that at least one of the regulatory factors involved in this inhibition had been identified tentatively. Here again, as previously discussed for the events of the cell cycle, regulation of transcription does not appear to operate on the level of turnover of RNA polymerase molecules, but rather via modification of the activity of a constant pool of enzyme protein.

A different picture emerges when the activity of RNA polymerase I is followed through the stationary phase and into spherulation (Hildebrandt and Sauer, 1976b). In these experiments, RNA polymerases were purified from whole plasmodia at different stages of starvation and the relative amounts of enzyme activity were measured after fractionation of the enzymes on DEAE-Sephadex columns. In agreement with the results obtained for RNA polymerase II activity described above, constant amounts of activity of this enzyme were detected in enzyme preparations from actively growing cultures, from starving cultures and from mature spherules. However, when the activity of RNA polymerase I was measured, a rather different picture was obtained. While during active growth approximately equal amounts of polymerase I and II (in terms of activity) were found, polymerase I dropped to near-background levels during starvation. In mature spherules, however, the level of polymerase I was again equal to that of polymerase II and quantitatively similar to the activity found during active growth. The drop in polymerase I activity during starvation again reflected the drop in transcription of ribosomal RNA which is observed *in vivo*. In contrast to the situation observed for polymerase II, the drop in enzyme activity seen *in vivo* was paralleled by the disappearance of soluble enzyme activity found *in vitro*. This drop in polymerase I activity could be achieved by a gradual degradation of the polymerase I population during starvation, followed by resynthesis of the enzyme during maturation of the spherule. Such a course of events would imply a completely different scheme for the regulation of the two RNA polymerases during differentiation. On the other hand, an apparent drop in enzyme activity could also be brought about by an inhibitor, which is formed during starvation and which binds reversibly to polymerase I, but not to polymerase II. This binding has to be tight enough so that it survives the purification procedure for polymerases. In such a situation, very little enzyme activity could be

recovered upon purification, although the overall amount of polymerase I protein in the cell had not changed. During spherule maturation, according to this hypothesis, the inhibitor is released from the enzyme and/or degraded so that full activity of the polymerase is restored.

Despite this point, the role of this polyphosphate inhibitor in the control of ribosomal RNA transcription during differentiation is of great interest (Goodman *et al.*, 1969; Sauer *et al.*, 1969b; Hildebrandt *et al.*, 1979; see also Section II). It is found in the nucleolus and its concentration increases continuously during starvation. While actively growing cells contain about 0.08 μg of inhibitor per 10^6 nuclei, this amount increases to a maximum of about 1 μg per 10^6 nuclei during prolonged starvation. This is sufficient to completely inhibit all RNA polymerase I found in this number of nuclei when assayed *in vitro*. During maturation of the spherules the inhibitor concentration decreases and in mature spherules its concentration has dropped to undetectable levels (Hildebrandt and Sauer, 1977a).

A further modulating element for transcription, which might be implied in the gradual shut-off of RNA synthesis during starvation, is a nuclear elongation factor (Ernst and Sauer, 1977) already discussed in Sections II.E and III.B. The activity of this factor (measured as stimulation of RNA polymerase II activity *in vitro*) gradually decreases during starvation to undetectable levels. From the evidence presently available it is difficult to judge if its activity, and hence its disappearance, is of consequence for one or other or both of the two RNA polymerases.

Taking into account all the observations outlined above, a picture of the regulation of transcription during differentiation emerges wherein the overall concentration of potentially active RNA polymerases I and II remains constant throughout starvation. Their respective activities are modulated according to the needs of the hour by reversible interaction of a variety of regulatory factors with one or the other of the enzymes. In the mature spherule, full transcriptional capacity of both polymerases is restored. A very interesting further possibility of modulating the activity of polymerases during differentiation, namely through chemical modification of subunit peptides, or through changes of the subunit composition of these enzymes, has not been investigated to date.

V. Concluding Remarks

A comparison of the title of this review with its actual content may leave some readers rather unsatisfied when they find that the acellular slime moulds, which are proudly announced in the title, are in fact represented almost exclusively by one single species, namely *Physarum polycephalum*. However, biochemical studies of transcription have been performed predominantly with this organism which has, over the last few years, undergone a remarkable development from a naive forest dweller into a pet organism of many laboratories in which transcription in eukaryotes is studied. This is not so surprising, since *P. polycephalum* offers the advantage of being a relatively simple unicellular organism which, however, resembles in many respects higher eukaryotes much more closely than does its more primitive cousin, the cellular slime mould *Dictyostelium discoideum*. An illustration of this point is the observation that in *P. polycephalum*, messenger RNA is probably transcribed as large precursor molecules, i.e. the heterogeneous nuclear RNA (see Section II.B), while in *D. discoideum* no such large precursors of messenger RNA have been detected. In addition, *P. polycephalum* has a greater complexity than that of *D. discoideum*. The recent observation that ribosomal genes of *P. polycephalum* contain insertion sequences which are not present in mature ribosomal RNA (Campbell *et al.*, 1979; Gubler *et al.*, 1979) furthermore suggests that *P. polycephalum*, in common with higher eukaryotes, has a "splicing system" to remove such sequences from the primary transcript.

When the structure of ribosomal genes or their *in vitro* transcription is considered, *P. polycephalum* offers the additional benefit that its nucleoli contain mainly ribosomal DNA, and very little or no other DNA. Thus nucleoli from *P. polycephalum* represent in many ways a rather simpler system than nucleoli from most higher eukaryotes where ribosomal DNA represents only a small fraction of the total nucleolar DNA.

Despite the attractiveness of *P. polycephalum* as an experimental system, the investigation of transcription has made rather slow progress and is still mostly descriptive. A relatively large amount of information is available on transcription during active growth. In contrast, virtually no recent data are available on transcription during differentiation and renewed emphasis should be put on the study of

these important regulatory pathways. Two major obstacles have considerably held up experimentation on transcription with *P. polycephalum*, but there is well founded hope that these will soon be overcome. Progress in the genetics of *P. polycephalum* has been much more sluggish and laborious than was initially envisaged and very few well defined mutants are yet available. But this field has been considerably revitalized in the last few years and the new developments clearly indicate that transcriptional mutants will eventually become available. Secondly, until now no defined genes, other than ribosomal DNA (Molgaard *et al.*, 1976; U. Gubler, personal communication), have been cloned from *P. polycephalum* and therefore no well-defined probes for transcriptional studies are yet available. Some progress has been made recently along these lines so that a more detailed analysis of transcription should soon be possible.

All in all, the dominating impression that one gains of the state of the studies on transcription with *P. polycephalum* is that a solid groundwork has been laid and that with the advent of transcriptional mutants and cloned genes this field now lies wide open: the most exciting stories are yet to be told.

VI. Acknowledgements

Supported by grant No. 3.312.78 of the Swiss National Science Foundation. We are very grateful to H. W. Sauer and L. Hall for carefully reviewing the manuscript and making many useful suggestions. We also thank U. Gubler for helpful discussions and D. Braun for a great deal of editorial help.

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Note added in proof

While the present review was in press, some progress has been made in the analysis of rRNA transcription and processing. *In vitro* transcription in isolated nuclei was used by Johnson and his collaborators in an attempt to map the site of initiation of rRNA transcription (Sun *et al.*, 1979). The use of -*S*-substituted nucleotides as substrates for the endogenous RNA polymerase allowed the isolation of newly initiated RNA chains by affinity chromatography on mercury-agarose columns. Hybridization of such *in vitro* initiated chains to restriction fragments of rDNA indicated that initiation pre-

sumably takes place in a region about 17.7 Kb from the end of the rDNA molecule, i.e. about 3.5 Kb from the start of the 19 S gene.

These results from transcription studies are in good agreement with data stemming from an analysis of ribosomal precursor RNA (Gubler *et al.*, 1980). Fractionation of total cellular RNA on denaturing agarose gels and subsequent identification of ribosomal RNA species by hybridization with appropriately cloned rDNA fragments revealed the presence of several ribosomal precursor RNA species. The largest of these, which contains 19 S, 5.8 S and 26 S ribosomal RNA sequences, was shown by R-loop mapping to be heterogenous in terms of the insertion sequences of the 26 S gene. Individual molecules of this precursor RNA class contain none, one or the other, or both introns, suggesting that, in *Physarum* sp. introns of ribosomal genes are transcribed and, in a later step, processed in a random fashion. Alignment of the precursor molecule containing both introns with the physical map of rDNA places the 5'-terminus of the precursor, and hence the presumptive start of transcription, within 1 Kb of the area proposed by *in vitro* transcription experiments (see above) to contain the site of initiation.

Whereas the above observations show considerable processing taking place at the 5'-end of the precursor molecule, R-loop analysis of the 3'- end showed no indication for occurrence of 3'-processing. These results suggest that termination of transcription coincides precisely with the end of the 26 S gene, as has also been observed in a variety of other systems (Sollner-Webb and Reeder, 1979).

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Nutrient Transport in Microalgae

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I. Introduction

The microalgae are of very great ecological importance as the major primary producers in large bodies of water (Parsons *et al.*, 1977) Microalgae are also found in benthic habitats, in soil, and in a number of symbioses, particularly corals and lichens (Bold and Wynne, 1977) A knowledge of their nutrient transport mechanisms is of great importance in interpreting their ecology, especially since a major limitation on phytoplankton productivity in the open sea is generally the availability of macronutrients supplying N, P (and, in diatoms, Si or micronutrients (Fe, B_{12}, biotin, thiamin). To this end, a large number of studies have been conducted on the transport properties of these planktonic algae, particularly with respect to long-term and short-term effects of fluctuations in external nutrient concentration. These studies have, however, been relatively isolated from current chemiosmotic interpretations of transport processes in microorganisms.

Less ecologically oriented studies have been carried out on "laboratory weeds", particularly such green algae as *Chlamydomonas*, *Chlorella* and *Scenedesmus*, mainly concerned with energy sources for transport, induction repression of transport systems, and effects on transport of the stage in the cell cycle (Raven, 1976a). Some of these studies have made very important contributions to our knowledge of solute transport in microorganisms (Tanner *et al.*, 1977).

The aim of this essay is to attempt a reconciliation of these two major approaches: how does chemiosmosis work in the real world? Microalgae may not seem to be the organisms of choice for investigating the mechanism of the transport of nutrients across the plasmalemma; they are usually photolithotrophs (often obligate), which poses problems both for the growth of the organisms and for unravelling the

energy sources for transport. In addition to the respiratory and fermentative energy sources of heterotrophic cells, there is also the possibility of direct intervention of photoproduced high-energy compounds in powering transport in the light (Raven, 1976a, b). Further, relatively few transport-associated mutations have thus far been detected in microalgae. Finally, it has not so far proved easy to separate the plasmalemma from other cell membranes in subcellular preparations, even in the structurally simple cyanobacteria. This is a prerequisite for detailed analysis of membranes and characterization of the transport systems found in them (Racker, 1975, 1976). However, their ecological importance and the possibility of testing current chemiosmotic views on the mechanism of membrane transport in organisms which live at extremes of pH and of osmotic pressure (Brock, 1969; R. H. Brown, 1978) make them very important experimental objects. Before considering specific transport aspects of the algae, we shall consider them as organisms.

II. The Microalgae

A. THE RANGE OF MICROALGAE

The organisms considered in this essay are the eukaryotic microalgae, together with the cyanobacteria. These organisms are considered together since they face similar environmental challenges with respect to nutrient transport, and it is of interest to see how these morphologically and chemically distinct groups (the O_2-evolving prokaryotes and micro-eukaryotes respectively) face their problems. The case for terming the blue-green algae (blue-green bacteria) cyanobacteria rather than cyanophyceae is vigorously upheld by Stanier and Cohen-Bazire (1977), and equally vigorously challenged by Bold and Wynne (1977). The eukaryotic microalgae are divided (on biochemical and ultrastructural characteristics) into a number of classes. The cyanobacteria, and the eukaryotic algal classes which have been most used in nutrient transport studies, are listed in Table 1, together with some of their relevant characteristics and some of the genera used in transport studies.

It should be noted that some of the algal classes shown in Table 1 contain algae which cannot be classified as microörganisms, and are thus outside the scope of this essay. Some of these organisms are very

TABLE 1. The major classes of algae and some of their attributes

Karyotic state	Class	Relevant biochemical and morphological characteristics	Representative genera
Prokaryote	Cyanobacteria (= Cyanophyceae)	Unicells, colonies, branched and unbranched filaments (differentiated into heterocysts, hairs) no flagella, all with cell walls (peptidoglycan)	*Anacystis* *Anabaena* *Plectonema*
Eukaryote	Chlorophyceae	Flagellate unicells and colonies (walled or wall-less); walled, non-motile unicells, colonies and filaments, and more complex macrophytes. Wall peptidoglycan or mainly polysaccharide	*Chlamydomonas* *Dunaliella* *Volvox* *Chlorella* *Scenedesmus* *Ankistrodesmus* *Selenastrum* *Polytoma* *Polytomella*
	Prasinophyceae/ Charophyceae	Similar range to Chlorophyceae	*Platymonas* *Mougeottia*
	Euglenophyceae	Wall-less flagellates	*Euglena*
	Cryptophyceae	Mainly wall-less flagellates	*Cryptomonas*
	Dinophyceae	Mainly flagellates with cellulosic armour resembling a cell wall but inside plasmalemma	*Gonyaulax* *Pyrocystis* *Amphidinium*
	Rhodophyceae	No flagellate forms or stages; a few unicells and simple filaments, mainly macrophytes	*Porphyridium* *Rhodella*
	Prymnesiophyceae (= Haptophyceae)	Mainly wall-less flagellates; many with scales (particularly coccolithophorids) containing $CaCO_3$	*Cricosphaera* (= *Hymenomonas*) *Gephyrocapsa* (= *Coccolithus*)
	Chrysophyceae	Mainly wall-less flagellates, many with scales containing SiO_2	*Ochromonas* *Monochrysis*
	Baccilariophyceae	All walled in vegetative stage; unicells or simple filaments (colonies); walls always contain SiO_2	*Cyclotella* *Cylindrotheca* *Navicula* *Phaeodactylum*
	Phaeophyceae	Simplest are differentiated branched filaments; mainly macrophytes	*Ectocarpus*

large: *Macrocystis pyrifera* (Phaeophyceae) can be 50 m or more long (Bold and Wynne, 1977; Lobban, 1978). *Macrocystis* and most of the large brown (Phaeophyceae) and red (Rhodophyceae) algae are multicellular, with a large number of differentiated, relatively small cells. Most of the macrophytic green algae are based on a coenocytic type of construction, i.e. are unicellular (or perhaps they may be better described as non-cellular). Despite this, they can attain a length of more than a metre and exhibit considerable thallus differentiation (Bold and Wynne, 1977). These coenocytic algae, and others composed

of a number of coenocytic cells, have proved of great value in studies of the mechanism of membrane transport (MacRobbie, 1974; Hope and Walker, 1975; Raven, 1975, 1976a).

Thus not all unicellular algae are microalgae. Conversely, some microalgae are multicellular; for the purposes of this article, we shall chiefly consider *unicellular* and *colonial* forms, rather than organisms with *differentiated* multicellular thalli, i.e. true multicellular organisms.

B. FINE STRUCTURE OF MICROALGAL CELLS RELATED TO TRANSPORT

Figure 1 shows a schematic cyanobacterial cell. Apart from the plasmalemma the only other membrane system which acts as a substantial barrier to transport of small molecules and ions is the detached and internalized photosynthetic thylakoids (Stanier and Cohen-Bazire, 1977). All other intracellular membrane systems in the cyanobacteria seem to be derived from the thylakoids (Andreis, 1975).

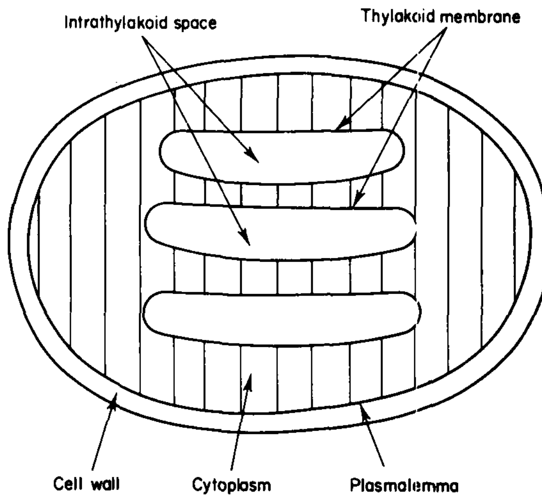

FIG. 1. A schematic cyanobacterial cell. The cyanobacterial cell wall is usually multilayered. The thylakoids only occupy about 7% of the intraplasmalemma volume of *Anacystis nidulans* (Falkner, Horner, Werdan and Heldt, 1976). The carboxysomes and the gas vacuoles are *not* membrane-bounded organelles in the sense of being enclosed by a "bimolecular lipid bilayer" membrane (Stanier and Cohen-Bazire, 1977; Shively, 1974). The comments about the influence of the cell envelope on measurements of plasmalemma transport properties in the caption to Fig. 2 are also relevant to cyanobacteria.

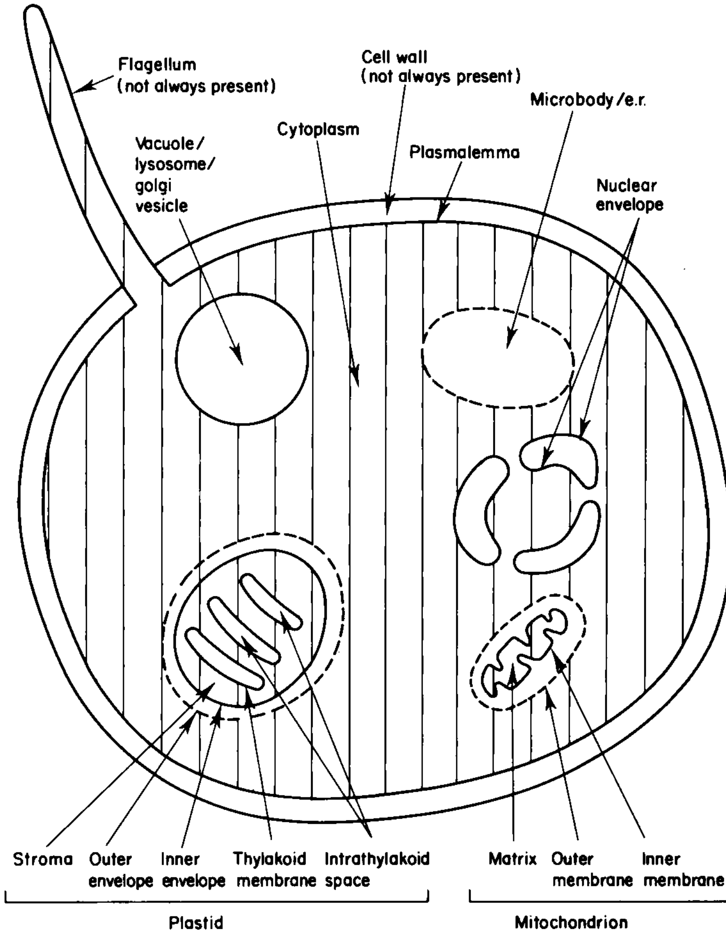

FIG. 2. A schematic eukaryotic microalgal cell. —— indicates membranes impermeable to small hydrophilic molecules (as well as to larger molecules); --- indicates membranes permeable to small hydrophilic molecules (but impermeable to larger molecules: see LeMasters, 1978); ▥ indicates intracytoplasmic space of a "cytoplasmic" nature, with DNA, RNA, a wide range of protein species (150–1000) and a total protein concentration of about 5 m*M*. This is the side of "tight" membranes (——) *from* which active transport of H^+, Na^+ and Ca^{2+} occur, and the side *from* which adenylates and pyridine nucleotides interact with ATPase and redox enzymes in the membrane; □ indicates intraplasmalemmar space of an "extracellular" nature, i.e. lacking functional DNA and RNA (lysosomes may be involved in the degradation of nucleic acids), a small range of protein species (less than 50) and a protein concentration up to 5 m*M* (in "leaky" compartments) or less (in "tight" compartments). It may be noted that the alternation (as one moves inwards from the plasmalemma) of "cytoplasmic" and "extracellular" spaces, and the sidedness of the membranes, are topologically explicable on *either* a "serial invagination" *or* an

Very few cyanobacteria lack these intracellular membranes: in those which do lack them (*Gloeobacter:* Stanier and Cohen-Bazire, 1977), membrane-associated photosynthetic and respiratory oxidation-reduction and H^+ transport reactions *must* be associated with the plasmalemma. In other cyanobacteria the evidence (largely indirect) is equivocal, or even contradictory, as to whether the membrane-associated redox reactions which are known to occur in the thylakoids are also found in the plasmalemma. This point is discussed later (Section IV.A).

The reason for our reliance on indirect evidence on this important point is that methods for separating the thylakoids from plasmalemma, which would enable rigorous physicochemical analysis of the two membranes such as has been done for photosynthetic bacteria (Oelze and Drews, 1972; Michels and Konings, 1978) have not so far proved feasible in cyanobacteria. Important considerations especially apposite to the cyanobacteria (Padan and Schuldiner, 1978) are that of the "sidedness" of the intracellular membranes (Mitchell, 1966, 1968, 1970) and the ontogenetic and/or phylogenetic derivation of the thylakoids as plasmalemma invaginations. This is discussed later in the context of eukaryotic microalgae.

Figure 2 shows the structure of a "composite" eukaryotic microalgal cell. In addition to the plasmalemma and the single-membrane bounded organelles (topologically homologous with the plasmalemma and thylakoids of the cyanobacteria), the eukaryotes have more complex organelles, the mitochondria, plastids and nucleus.

The caption to Figure 2 explains the topological relationships of the various spaces and membranes in the eukaryote cell. The number of compartments, and the diverse transport properties of the membranes enclosing them, means that transport studies with eukaryotic cells are

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"endosymbiotic" hypothesis for the derivation of energy-transducing organelles (see Raven, 1977d; Smith and Raven, 1978). The presence of extracellular fixed negative charge can have an important influence on the *chemical activity* of ionic species which are available for transport systems in the plasmalemma, but not on their *electrochemical activity*, in the cell envelope in comparison with the bulk medium (see Briggs *et al.*, 1961; Dainty, 1962; Stock *et al.*, 1977; LeMasters, 1978). Thus the presence of these fixed negative charges does not alter the energetics of transport as deduced from measurements in the bulk medium, but may have an influence on the *kinetic* properties of plasmalemma transport systems. Thus, with respect to the bulk medium, the membrane transport sites may "see" higher cation and lower anion activities than those in the bulk medium (but see Table 1 of Mettler and Leonard, 1979).

TABLE 2A. Quantitative structural data on eukaryotic microalgae: percentage of cell volume occupied by various organelles

Organism	Percentage of cell volume occupied by:				References
	Nucleus	Vacuoles	Mitochondria	Chloroplasts	
Chlorella fusca var *vacuolata* 211-8b (phototrophically grown)	8–12	8–13	2.5–3.0	33–40	Atkinson *et al.* (1974)
Chlamydomonas reinhardtii (phototrophically grown)	20	8	3	40	Schotz *et al.* (1972)
Polytomella agilis (obligate heterotroph)			19		Burton and Moore (1974)
Polytoma papillatum (obligate heterotroph)				12–33	Gaffal (1978)

Chlorella and *Chlamydomonas* are facultative photolithotrophs-heterotrophs; the cells grown for these measurements were photolithotrophs, and have a large fraction of the cell volume occupied by chloroplasts, and a small fraction occupied by mitochondria. The mitochondria are required for certain biosyntheses (e.g. those involving the Krebs cycle) in these photolithotrophic cells, and also for the supply of ATP in the dark phase of a light–dark cycle. Much of the ATP in the light can be supplied by the chloroplast (see Raven, 1976b). In the obligate heterotrophs *Polytoma* and *Polytomella* the mitochondrion is the sole location of membrane-associated ATP synthesis: the mitochondrion (mitochondria) occupy a larger fraction of the cell volume than in photolithotrophic algae. The plastid occupies a fairly large fraction of the cell volume, even though it is not active in photosynthetic energy conversion. In these chlorophyte algal cells the plastid is important as the sole site of starch storage and (probably) of nitrite reduction and ammonium assimilation within the cell (see Raven, 1976b; Miflin and Lea, 1976; Hewitt, 1975; Gayler and Morgan, 1976).

The algae in this table are generally classified as "non-vacuolate", although vacuoles of various kinds occupy up to 13% of the cell volume of *Chlorella*, and the very important (in volume regulation) contractile vacuoles of the three flagellate freshwater organisms (*Chlamydomonas*, *Polytoma* and *Polytomella*) are not accounted for in the table (see Raven, 1976a). In "vacuolate" algae, i.e. those in which the central vacuole can be readily seen by light microscopy, this organelle can occupy 85% or so of the volume inside the plasmalemma (e.g. in the charophyte (?) *Mougeotia* (Wagner, 1974) and the diatom *Ditylum* (Eppley and Rogers, 1970)). In these algae the fraction of the remainder of the volume inside the plasmalemma which is occupied by the chloroplast, mitochondria, nucleus, etc. is probably similar to the values quoted for the whole of the cell volume for the "non-vacuolate" algae in this table.

more practically and conceptually difficult than are those performed with cyanobacteria. As will be seen in Section III.C, the analysis of transport at the plasmalemma requires, ideally, that measurements be made of: (1) the concentration of the relevant solute in the ground cytoplasm in contact with the plasmalemma, (2) the electrical potential difference between the bulk medium outside the cell and the ground

TABLE 2B. Quantitative structural data on eukaryotic microalgae: area of other membranes as a fraction of the plasmalemma area

Organelle	Area of specified membrane as a percentage of the plasmalemma area in:	
	Chlorella fusca var *vacuolata* 2118b	A cell (e.g. *Mougeotia*, *Ditylum*) with the vacuole occupying 80% of the volume inside the plasmalemma
Vacuolar (tonoplast)	35	90
Inner mitochondrial	50–100	17–35
Outer mitochondrial	30–55	10–20
Nuclear envelope (inner and outer)	40–60	14–20
Thylakoids	1000	350
Inner chloroplast envelope	200	70
Outer chloroplast envelope	200	70

The first column gives data for *Chlorella* grown photolithotrophically. Vacuolar area is taken from Atkinson *et al.* (1972); inner and outer mitochondrial membrane area is taken from Forde *et al.* (1976). The area of the thylakoid membrane is taken from Raven (1978), who gives references on the chlorophyll content of *Chlorella* cells and the chlorophyll per unit area of thylakoid membrane in a variety of eukaryotes on which the estimate was based. The area of the envelope membranes of the perforate, cup-shaped chloroplast assumes that the area of each membrane is twice that of the plasmalemma.

The second column gives figures for a vacuolate cell in which the vacuole occupies 80% of the volume inside the plasmalemma. For a spherical cell of this type, the plasmalemma area is 2.9 times the plasmalemma area of a "non-vacuolate" cell with the same volume of other organelles (chloroplast, mitochondria, etc.) and of ground cytoplasm. The second column is based on the first column, with the same area of membranes (per cell) as for *Chlorella* except for a 2.9 times greater plasmalemma area and an area of tonoplast (central vacuole plus any smaller vacuoles) assumed equal to that of the plasmalemma.

cytoplasm, and (3) bidirectional tracer, and net, fluxes of the solute across the plasmalemma.

Table 2 gives some values for the areas of the membranes bounding various compartments, and the volumes of these compartments, in a number of microalgal cells. For hydrophilic small molecules and ions the fraction of the cell volume in which the concentration of the molecules is similar to that in the ground cytoplasm probably only represents some 50% of the cytoplasm (intracytoplasmic volume minus the volume of any large vacuoles). This volume corresponds to the "ground cytoplasm" plus the volume separated from it by leaky membranes (e.g. the microbodies) and the nuclear pores. The rest of the space within the plasmalemma is separated from the "ground cytoplasm" by "tight membranes"; in *Chlorella* and *Chlamydomonas* the

volume of these spaces (chloroplasts, mitochondria and vacuoles) is 43–56% of the total volume inside the plasmalemma (Table 2A); in more highly vacuolate cells this volume is a greater fraction of the volume inside the plasmalemma (see below). Since there is considerable evidence that these organelles can regulate their solute content at a value different from that of the ground cytoplasm (see Baker and Hall, 1975), it is likely that the concentration of specific solutes determined for the whole cell will not be identical with that in the ground cytoplasm. This has repercussions for the measurement of transmembrane tracer fluxes, since their determination by "flux analysis" (MacRobbie, 1971) is influenced by specific activities in, and exchange between, intracellular compartments. Finally, the electrical potential difference between the inside of the cell and the external medium, whether measured with microelectrodes whose tip position is difficult to measure and control, or with lipid-soluble strong electrolytes, may be influenced by electrical potential differences between the ground cytoplasm and other intracellular phases (see Raven and Smith, 1977).

For cells with large vacuoles the situation is even worse, in that the vacuole may occupy 70–90% of the total intraplasmalemma volume, and results obtained with giant-celled algae suggest that the vacuole may have a very different composition from the rest of the intraplasmalemma volume, and that there is usually an electrical potential difference (vacuole-positive) across the tonoplast (vacuolar membrane: see MacRobbie, 1974; Hope and Walker, 1975; Raven, 1975, 1976a). Thus whole-cell concentration and electrical potential difference measurements in vacuolate cells (e.g. most diatoms: Werner, 1977a) can give a very misleading idea of the values for the "ground cytoplasm". Further, tracer fluxes may largely reflect events at the tonoplast rather than at the plasmalemma. *Net* fluxes, determined by chemical analysis rather than as the difference between two fluxes measured with tracers, must reflect fluxes across the plasmalemma if they are based on whole-cell measurements or on analysis of the medium. However, the net flux may be limited by some intracellular flux, even though it represents a real transplasmalemma flux.

A further complication related to the structure of eukaryotic microalgal cells is the difficulty of isolating and recognizing the plasmalemma in the welter of cellular membranes. As will be seen from Table 2 the plasmalemma area is often a relatively small fraction (5–10%) of the total area of membranes within a cell of, for example, *Chlorella*.

C. ELEMENTAL COMPOSITION OF MICROALGAE AND THE NET FLUXES ASSOCIATED WITH ALGAL CELL GROWTH

Table 3 shows the elemental composition of two eukaryotic microalgae for which relatively complete data (albeit compiled for a number of strains from a number of sources) is available. These elemental contents represent, for *Chlorella* and *Euglena*, very largely elements which are present *within* the plasmalemma of the alga; the fraction of the total elements analysed which are present in the extraplasmalemma structures (cell wall and pellicle respectively for *Chlorella* and *Euglena*) is small. For other algae a very large fraction of particular elements may be present outside the plasmalemma; examples are sulphur (as sulphate ester in the cell envelope of unicellular red algae: Section IV.E), silicon (as scales and walls of chrysophytes and

TABLE 3. Elemental composition of *Chlorella* and *Euglena* (freshwater, non-vacuolate algae) and the concentration of available sources of the elements in freshwater (from Raven, 1981)

Element	Content in:				Freshwater μM
	Chlorella		*Euglena*		
	mol per kg dry wt	Relative to $C = 100$	mol per kg dry wt	Relative to $C = 100$	
C	40	100	40	100	10–2500 ($CO_2 + HCO_3^-$)
N	4–5	10–13	3.6–6.4	9–16	0.5–50 ($NH_4^+ + NO_3^-$)
S	0.12–0.24	0.3–0.6	0.12–0.6	0.3–1.5	100–1200 (SO_4^{2-})
P	0.12–0.77	0.3–1.9	0.20–0.74	0.5–1.8	0.5–1.8 ($H_2PO_4^- + HPO_4^{2-}$)
K	0.1–0.6	0.25–1.5	0.08–0.46	0.2–1.5	7–200 (K^+)
Na	0.008–0.2	0.02–0.5	0.008–0.01	0.02–0.025	5–500 (Na^+)
Ca	0.08	0.2	$8–120.10^{-4}$	0.002–0.03	11–3000 (Ca^{2+})
Mg	0.08–0.56	0.2–1.4	0.032–0.06	0.08–0.15	8–600 (Mg^{2+})
Cl	0.008	0.02	$4–8.10^{-3}$	0.01–0.02	4–300 (Cl^-)

Inorganic carbon is only available to *Chlorella* as CO_2 if it is grown at high (greater than 30–50 μM) CO_2 concentrations: HCO_3^- can also enter the cells if they are grown at atmospheric CO_2 levels (see Section IV.B, 1). The availability of HCO_3^- to *Euglena* is not established. Most strains of *Chlorella* and *Euglena* can also use organic compounds for growth (Raven, 1976b).

Inorganic nitrogen is only available to *Euglena* as NH_4^+; *Chlorella* can use NO_3^- if NH_4^+ is absent (see Section IV.C, 3). Many algae can use organic nitrogen as a nitrogen (and in some cases a carbon) source (see Sections IV.B, 2 and IV.C, 4).

Organic phosphorus is generally available to algae only after extracellular hydrolysis to produce inorganic phosphate (see Section IV.D).

diatoms: Section IV.H) and calcium (as $CaCO_3$ in the scales of prymnesiophytes, especially the coccolithophorids: Section IV.G). In all of these cases the element can properly be considered in mass balances of plasmalemma solute transport, since in all three cases the element which finally appears in the cell wall or scales has previously entered the cell, prior to excretion (generally by exocytosis after sequestration in a vesicle).

The two algae in Table 3 have very little of their intracellular volume occupied by vacuoles (this is shown by Table 2A for *Chlorella*). Freshwater cells with larger vacuoles (e.g. many diatoms) have higher inorganic ion contents (K^+, Na^+, SO_4^{2-}, Ca^{2+}, Mg^{2+}) relative to organic constituents, since these ions are the major solutes in the vacuole: the vacuole is one to two orders of magnitude less rich in organic compounds (per unit volume) than is the cytoplasm (Raven, 1975, 1976a). In marine algae, there is generally a greater content of inorganic ions than in comparable (non-vacuolate or vacuolate) freshwater algae, related to the greater osmotic potential of the contents of marine than of freshwater algae (Raven, 1975, 1976a, 1981; Kirst, 1977).

Also shown in Table 3 is the range of concentrations of the various solutes which supply the elements required in photolithotrophic growth. These typical concentrations show that, as suggested in the introduction, the elements for which the cell quota/external concentration is highest are nitrogen and phosphorus, suggesting that these may be the elements whose availability limits the *rate*, and ultimately the *extent*, of growth of natural phytoplankton populations. While the data shown in Table 4 are limited to freshwaters, similar considerations apply in the sea (Parsons and Takahashi, 1973).

Table 4 shows the net influx rate of the various solutes across the plasmalemma during photolithotrophic growth of *Chlorella*, granted the assumptions noted in the caption to the table. Even the highest flux calculated (that for carbon) is less than the maximum plasmalemma net flux for mediated transport noted in Section III.F); if the inorganic carbon were all to enter as HCO_3^- it would indeed be all mediated (Section IV.A). Even if the *total* net ion influx is considered, the mediated ion influx is still below the maximum value considered in Section III.F.

Some explanation is required for the presence of H^+ in a list of *nutrient* fluxes. Maintenance of cytoplasmic pH requires that excess H^+

TABLE 4. Net influx of nutrient elements, and net charge transfer across the plasmalemma, during growth of *Chlorella*

Element	Net influx during growth:	
	pmol $cm^{-2} s^{-1}$	Charge as pmol $cm^{-2} s^{-1}$
C	90	0 (all as CO_2) to −90 (all as HCO_3^-)
N	9	+9 (all as NH_4^+) to −9 (all as NO_3^-)
S	0.5	−1 (SO_4^{2-})
P	1	−1 ($H_2PO_4^-$)
K	1	+1 (K^+)
Na	0.05	+0.05 (Na^+)
Ca	0.05	+0.1 (Ca^{2+})
Mg	1	+2 (Mg^{2+})
Cl	0.01	−0.01 (Cl^-)
(H^+)		−10 (growth on CO_2 as C source, NH_4^+ as N-source) to +98 (growth on HCO_3^- as C source, NO_3^- as N-source)

Cell composition based on Table 3. Surface area/dry weight, and rate growth, from Raven (1978a, b). Photolithotrophic growth with CO_2 or HCO_3^- as carbon source, NH_4^+ or NO_3^- as nitrogen source. The influx quoted is based on the assumption that all of the net influx occurs during the light phase of a 12 hr light/12 hr dark growth cycle; for carbon the net influx in the light phase must exceed the value quoted in that there is a net loss of carbon from the cells in respiration during the dark period, while for the other elements there is often a slower net influx of nutrients during the light phase. Thus the values quoted are maximal values for the light phase for all elements but carbon, for which the quoted value is known to be an underestimate.

The charge transfer is quoted for influx of the various nutrient elements in their various common inorganic forms. Assimilation of HCO_3^-, NO_3^- and SO_4^{2-} involves a substantial net incorporation of H^+, while assimilation of NH_4^+ involves a substantial net H^+ production. The line for H^+ at the bottom gives the H^+ net influx or efflux required for pH regulation within the cell (and also for charge balance) during growth at the two extremes of intracellular pH stress.

or OH^- generated in essential biosyntheses be disposed of. In microalgae this generally involves transplasmalemma transport rather than biochemical neutralization (Raven and Smith, 1973, 1974; Smith and Raven, 1976, 1979; Davies, 1973a, b). Quantitatively, the required transplasmalemma flux can be determined from the charge difference between the form in which the element enters the cell and the form in which it is present after assimilation within the cell (Raven and Smith, 1973, 1974, 1976a; Brewer and Goldman, 1976; Raven and De Michelis, 1979). Taking the extremes of H^+ net fluxes across the plasmalemma which can be deduced from Table 4, *viz.* evidenced during growth with NH_4^+ as N-source and CO_2 as C-source, pH regulation in the cytoplasm requires net H^+ extrusion at up to 10 pmol

cm^{-2} s^{-1}, while growth with NO_3^- as N-source and HCO_3^- as C-source requires the net influx of about 100 pmol H^+ cm^{-2} s^{-1} (or a corresponding OH^- net efflux). Other combinations of nitrogen- and carbon-sources yield intermediate values of H^+ fluxes.

Thus $H^+(OH^-)$ can be regarded as a nutrient whose influx across the plasmalemma is essential for algal growth; large *net* H^+ fluxes can be involved in algal growth, aside from any H^+ cycling across the plasmalemma which may be involved in cotransport of other nutrients (Section III.D).

The maintenance of cytoplasmic pH is only one facet of the regulation of cytoplasmic composition which is essential for the existence of algal cells. Good growth rates, and even survival, of cells requires that their cytoplasmic composition be held within certain limits. In general the osmotic potential of the cytoplasm is at least as high as that in the medium: since most eukaryotic enzymes do not function well above about 0.5 *M* KCl or NaCl, in media of high external osmotic potential there must be additional "compatible solutes" in the cytoplasm (non-reducing sugars, polyols, quaternary ammonium compounds: Hellebust, 1976; A. D. Brown, 1978; Wyn Jones *et al.*, 1977a, b).

At the other end of the concentration scale, there must be a certain minimum concentration of enzymes, organic intermediates and inorganic cofactors present in functional cytoplasm (see Raven, 1977a, b). General constraints on cytoplasmic composition include very low free Ca^{2+} concentrations (less than 1 μM), K^+ generally at least 50 m*M*, Mg^{2+} about 5 m*M* and common inorganic anions (phosphate, nitrate, sulphate, chloride) limited to 50–100 m*M* *in toto*; the net charge difference of inorganic constituents (cations in excess of anions) is made up by a net organic negative charge (as phosphate esters and carboxylates). How this close regulation of solute concentration is achieved will be mentioned later; often (see Raven, 1981, and Section IV), a 1000-fold change in external concentration of a cytoplasmic solute (H^+, K^+, $H_2PO_4^-$, SO_4^{2-}) is associated with a change in cytoplasmic concentration of well under ten-fold.

III. Membranology and the Proticity Paradigm

A. INTRODUCTION

Having considered the structure of algal cells and their nutrient requirements, and before considering the transport of individual

nutrient solutes into microalgal cells, I propose to outline my interpretation of some of the hypotheses of membrane transport which may be relevant to studies of nutrient transport in algae. These hypotheses will be framed in a general way, drawing largely on data obtained with organisms other than microalgae: however, the problems of making the measurements by which the hypotheses may be tested will be discussed largely with respect to the microalgae.

B. NON-MEDIATED TRANSPORT THROUGH MEMBRANES

Algal plasma membranes consist of polar lipid bilayers (Davson and Danielli, 1943) modified by the presence of proteins. The peripheral proteins are hydrophilic and are associated with the membrane surface, while the integral proteins are *within* the membrane, displacing some of the lipid components (Singer and Nicolson, 1972; Singer, 1974). While much recent attention has been rightly focused on the transport functions of the integral membrane proteins which catalyse the "mediated", specific and often energy-coupled transport of hydrophilic solutes across the membrane (see C below), it is also important to consider the transport properties of the lipid part of the membrane (Davson and Danielli, 1943; Collander, 1954, 1959; Stein, 1967; Cole, 1976; Hill, 1977). Many important solutes (e.g. CO_2, NH_3, O_2, N_2, H_2O, urea and $B(OH)_3$: Raven, unpublished) can cross lipid bilayers by dissolving in the lipid portion of the membrane, diffusion to the other lipid-water interface, and dissolution in the aqueous phase on the other side of the membrane.

The equation describing the overall transport through the membrane is:

$$J_s = P_s(C_1 - C_2) \tag{1}$$

where J_s is the transmembrane flux of some neutral solute species s (mol cm^{-2} s^{-1}), P_s is the permeability coefficient for species s (cm s^{-1}) and C_1, C_2 are the concentrations of s (mol cm^{-3}) on the two sides of the membrane. When C_1 exceeds C_2, the net flux across the membrane is from side 1 to side 2; J_s is zero when $C_1 = C_2$.

P_s may be resolved into a term D_s/l, where D_s is the effective diffusivity of the solute species in the membrane material (cm^2 s^{-1}), and l is the length of the diffusion path (cm), i.e. the membrane thickness. D_s is termed the effective diffusivity because it is expressed in terms of the external concentrations of solute rather than the concentrations within the membrane lipid at the two sides of the membrane, i.e. it

involves the partition coefficient for the solute between the aqueous and membrane phases as well as the diffusivity within the membrane. For many solutes the value of P_s obtained for "model" bilayers can be quantitatively explained in terms of the diffusivity of the solute in the membrane lipid, the partition coefficient between membrane material and water, and the membrane thickness (Hill, 1977). Further, the P_s values are similar to those found with biological membranes of similar lipid composition, provided there is no mediated transport of the solute species.

The relevance of this value of P_s to the living microorganism requires consideration of both the rate of consumption (or production) of the solute (J_s) and the concentration difference ($C_1 - C_2$) which occurs in nature or during culture. If the known value of P_s can give a metabolically significant value of J_s in equation (1) when ($C_1 - C_2$) has a plausible value, then this mode of transport of the solute species s may be significant. It is certainly a major (if not the only) mechanism of transport of O_2 into respiring cells and out of photosynthesizing cells (Fischkoff and Vanderkooi, 1975; Samish, 1975; Longmuir, 1976; Raven, 1977c; Bakeeva, Chentsov and Skulachev, 1978). The transport of these low molecular weight, relatively lipid-soluble metabolites will be considered in detail in the relevant parts of Section IV.

Of experimental, if not ecological, interest is the transport of ions by "lipid solution". The driving force for ion movement involves an electrical term as well as a chemical potential term. Thus the free energy difference across a membrane for a neutral solute is given by

$$\Delta G_s = RT \ln \frac{C_1}{C_2} \tag{2}$$

where ΔG_s is the free energy difference of the solute between the solutions on the two sides of the membrane (kJ mol^{-1}), R is the gas constant (kJ mol^{-1} °C^{-1}), T is the absolute temperature, and C_1 and C_2 are as defined for equation (1).

It may be noted that, in the formalism of irreversible thermodynamics, the transmembrane flux J_s is related to ΔG_s by the equation:

$$J_s = L \Delta G_s \tag{3}$$

where L is a coupling coefficient between the driving force ΔG_s and the flux J_s; it has the units mol cm^{-2} s^{-1} kJ^{-1}. Equation (3) may be

simplified to Fick's equation when the term ln C_1/C_2 can be approximated to $(C_1 - C_2)$, and the RT term is subsumed in P_s. Both equations (1) and (3) agree in giving $C_1 = C_2$ as the equilibrium condition when J_s is zero.

For electrolytes, the equation corresponding to equation (2) (Briggs *et al.*, 1961; Dainty, 1962) is

$$\Delta G_e = RT \ln C_1/C_2 + zF\psi_{12} \tag{4}$$

where ΔG_e is the free energy difference of the electrolyte e between the two sides of the membrane (kJ mol^{-1}), z is the charge on the electrolyte (e.g. +1, −2), F is Faraday's equivalent (coulombs per mol), and ψ_{12} is the electrical potential difference across the membrane (volts). The equation relating this driving force to the transmembrane flux, J_e, of the electrolyte *via* a permeability coefficient with the same dimensions as that used in equation (1) is:

$$J_e = -P_e . \frac{zF\psi_{12}/RT}{1 - \exp(zF\psi_{12}/RT)} (C_1 - C_2 \exp(ZF\psi_{12}/RT)) \tag{5}$$

Values of P_e for most inorganic ions in lipid bilayers are very low (of the order of 10^{-10} cm s^{-1}); this is similar to the low value for many hydrophilic neutral solutes such as glucose (Hill, 1977). However, for a number of ions with "delocalized" charge and considerable lipid-solubility, the value of P_e in bilayers is much higher (Skulachev, 1971). These "lipid-soluble" cations, e.g. $TPMP^+$ (triphenylmethylphosphonium$^+$) and anions (e.g. SCN^-) can be used (see Section III.J) to measure the electrical potential difference between two phases; the condition for no net flux of the ionic species e between two phases (1) and (2) is given by setting $\Delta G_e = 0$ in equation (4), i.e.

$$\psi_{12} = \frac{RT}{zF} \ln C_1/C_2 \tag{6}$$

This is the familiar Nernst equation, and allows the measured equilibrium distribution of a strong, lipid-soluble electrolyte (C_1 and C_2) to be used to determine ψ_{12}.

The natural occurrence of these lipid-soluble strong electrolytes is very limited, so their importance is mainly as an experimental tool rather than as something which the organism has to deal with in nature. Similar considerations apply to the relevance of studies of lipid-soluble weak electrolytes, in which the transmembrane flux of both the

dissociated and the undissociated forms must be considered if the pH and pK values are such as to allow the two forms to coexist on one or both sides of the membrane. Here the flux of the un-ionized form is given by equation (1), while the flux of the ionized form is given by equation (5). At flux equilibrium, the net flux of the ionized and un-ionized forms is equal to zero, and the equilibrium distribution is given by:

$$P_u(C_{u1} - C_{u2}) = -P_d \cdot \frac{zF\psi_{12}/RT}{1 - \exp zF\psi_{12}/RT)} (C_{d1} - C_{d2} \exp(ZF\psi_{12}/RT)) \quad (7)$$

where the subscripts u and d refer to the un-ionized and the dissociated form of the solute, respectively.

There is no way of producing equations of the form of equation (6) for electrolytes, or $C_1 = C_2$ for non-electrolytes, which describe the equilibrium distribution without involving the permeability coefficients. As may be seen from equation (7) the calculation of passive flux equilibrium for a weak electrolyte with a finite value of P_u and of P_d, and with pH_1 and pH_2 sufficiently close to pK_a of the weak electrolyte for the ionized and un-ionized forms to coexist involves a knowledge of not only the concentration and electrical terms in equation (7), but also of P_u and P_d.

Equation (7) is important in three respects for transport in microorganisms: two are experimental and the other has ecological importance. The two experimental applications relate to the use of weak electrolytes to determine the pH in otherwise inaccessible compartments, and to the mechanism of uncoupling.

The use of weak electrolytes (such as 5,5-dimethyloxazolidine-2,4-dione) to measure the pH of inaccessible compartments is widespread: it is predicated on P_i being negligible with respect to P_u. Thus, at flux equilibrium, $C_{u1} = C_{u2}$. In the accessible phase 2, $C_{u2} + C_{d2}$ can be measured, as can pH_2; from the pK of the weak electrolyte, C_{u2} can be calculated, and equated to C_{u1}. From this estimate of C_{u1}, the measured value of $C_{u1} + C_{d1}$, and the pK of the weak electrolyte, pH_1 can be calculated. The assumption of $P_u \gg P_d$ may not be valid, particularly if ψ_{12} is very large; here even a small value of P_d might lead to a large value of J_d, and would thus cause an underestimate of the pH in phase 1 when the value of ψ_{12} was large and negative (see Raven and Smith, 1978a).

Many uncouplers appear to work by catalysing electrogenic uniport (see Section III.C) of H^+, by virtue of having high values of both P_u and P_d (equation 7): Mitchell, 1966, 1968; Skulachev *et al.*, 1968. These weak electrolytes may not have a P_u/P_d ratio much lower than that of the weak acids or bases used in pH measurements, but the uncouplers must be used at concentrations which permit P_d as well as P_u to be significant in allowing recycling across the membrane and hence net H^+ transport; the pH-indicators should not be used in the way described in the preceding paragraph when such net H^+ transport is significant. The use of uncouplers to determine the pH of inaccessible compartments will be discussed in Section III.J.

The physiologically and ecologically important aspect of equation (7) arises from the occurrence of mediated transport of the ionic forms of weak electrolytes such as NH_4^+–NH_3 and CO_2–HCO_3^- whose un-ionized form has a high intrinsic permeability (Sections IV.A and IV.B). Mediated transport of the ionized form and leakage "downhill" of the un-ionized form constitutes uncoupling (short-circuiting) of the transmembrane H^+ free energy difference, as well as constituting pump and leak for the solute (HCO_3^- or NH_4^+) which is subject to mediated, and usually energy-consuming, transport across the membrane. The energetic cost of such unavoidable "leaks" through the lipid part of the membrane of the undissociated form of transported ions will be considered in Sections IV.A and IV.B, together with the possible selective tradeoffs which make the energetic inefficiency worthwhile. A similar short-circuiting can occur for neutral solutes such as urea, where the finite passive permeability can cause a significant leak flux countering the active transport of the solute (Section IV.B).

C. FACILITATED TRANSPORT AND ACTIVE TRANSPORT

For a number of important nutrient solutes (glucose, amino acids, NO_3^-, $H_2PO_4^-$, HPO_4^{2-}, SO_4^{2-}, K^+, Mg^{2+}) the application of the criterion for the significance of the intrinsic permeability of lipid bilayers to the solute (Section III.B) shows that the bilayer intrinsic permeability is woefully inadequate. In terms of a physiologically significant value of J_s, and the possible magnitude of $(C_1 - C_2)$ (and, for electrolytes, ψ_{12}), equations (1) and (5) require P values many orders of magnitude greater than those found for unmodified lipid bilayers (Hill, 1977; Raven, 1977a, c). Here the lipid membrane constitutes an excellent

barrier to leakage of internal solute and to entry of external solute; for such solutes the unmodified bilayer puts membrane transport into a similar energetic position to the chemical interconversion of soluble metabolites in the absence of enzymes: the activation energy required for transport (or chemical reaction) is so high that negligible rates occur, even for reactions which are overall energetically favourable. The few important solutes with high intrinsic permeabilities in lipid bilayers (Section III.B) may be compared with the few important metabolites whose interconversion occurs rapidly without enzymic catalysis (see Morris, 1976; Raven, 1977a). In both cases the metabolites are mainly inorganic: in the case of the chemical reactions (e.g. hydration of CO_2, dismutation of O_2^-, breakdown of H_2O_2) even the rapid reaction in the absence of a catalyst may not be rapid enough, and enzymes (carbonic anhydrase, superoxide dismutase, catalase) which catalyse these reactions are found in many cells (see Morris, 1976; Raven, 1977a). Similarly, the effective rate of net transport of CO_2 and NH_3, with their high intrinsic permeability, may be considerably increased by specific transport systems for HCO_3^- and NH_4^+ respectively (Raven, 1977a; see Section III.B).

For the hydrophilic solutes mentioned above (sugars, amino acids, inorganic ions) the net flux (J_s) is enhanced by the presence of specific porters (intrinsic proteins) in the membrane (Mitchell, 1966, 1968; Stein, 1967; Singer, 1974). Porters analogous to enzymes which catalyse a unimolecular chemical conversion are uniporters, in that they move a single molecular species across the membrane. Such porters may be able to increase the net solute flux across the membrane by many orders of magnitude compared with the unmodified lipid membrane, but they do not alter the final position of equilibrium of the reaction. For a neutral solute a net flux through such a porter requires that the concentration of the solute on the sink side of the membrane is lower than that on the source side (equation (1)). For an anion, equation (5) shows that a net flux through a uniporter from a source at zero electrical potential to a sink at −58 mV (a not implausible transmembrane electrical potential difference) requires that the anion concentration at the sink side of the membrane shall be less than one-tenth of the concentration on the source side for a singly charged anion. For a singly charged cation, net flux under these conditions could occur provided the cation concentration on the sink side were less than 10 times that on the source side.

However, one cannot regard the transmembrane electrical potential difference as a long-term driving force for accumulation of cations in a cell whose cytoplasm is negatively charged relative to the bathing solution. The membrane capacitance of about 1 μF cm^{-2} means that a potential difference of 58 mV could be discharged by the transport of $Q = CV$ (Q = charge displacement in coulombs cm^{-2}, C = capacitance in F cm^{-2}, V = electrical potential difference in volts) = $5.8 \cdot 10^{-8}$ coulombs cm^{-2}, or 0.58 peq cm^{-2}. Reference to the fluxes involved in algal growth (Table 4) shows that the influx of NH_4^+ at 9 pmol cm^{-2} s^{-1} would completely discharge the −58 mV electrical potential difference in less than one-tenth of a second. Thus the use of the transmembrane potential difference to drive concentrative cation influx requires that the potential difference is maintained inside-negative by some other charge-transfer process operating vectorially across the membrane.

Returning to the neutral solutes and the anions, it is commonly found that a substantial net flux of such solutes (substantial relative to the fluxes required for growth: Table 4) occurs into cells, although the internal (sink) concentration of the solutes is higher than the values quoted above as allowing passive uniport, i.e. net transport occurs even when the internal concentration of a neutral solute exceeds the external concentration, and when the internal concentration of a singly charged anion exceeds one-tenth of the external concentration when the cytoplasm is 58 mV negative with respect to the medium. Clearly some additional driving force must be coupled to the transport process if net transport is to occur in a direction contrary to that predicted from the chemical and electrical driving forces acting on the solute. This process is *active transport*, and the additional driving force can be either an exergonic biochemical reaction (*primary active transport*) or an exergonic transmembrane solute flux (*secondary active transport*). In either case the overall coupled reaction must proceed with a *decrease* in free energy, just as is the case for passive solute uniport, or coupled biochemical reactions (e.g. the use of ATP to phosphorylate glucose).

Primary active transport of an ion, by coupling the uniport of an ion to a biochemical energy source (e.g. ATP) is an electrogenic process, i.e. it brings about charge transfer across the membrane; it can thus account for the origin of electrical potential differences across the membrane, and their maintenance at the expense of biochemical energy in the face of their dissipation by downhill ion uniport (see above). Electrical potential differences across membranes can also arise

from a mixture of downhill ion uniports, based on ion concentration differences across the membrane which may have been generated by neutral (charge-balanced) ion transport, and uniporters which give a variety of different effective P_{ion} values for the different electrolytes. Such a potential difference is termed a "diffusion potential", and may be clearly distinguished from an "electrogenic potential" in certain cases. The diffusion potential is dominated by the ion with the largest product of concentration difference and effective permeability; this ion is commonly K^+, since g_{K^+} greatly exceeds the g_{ions} for the sum of other ions (g being membrane conductance due to that particular ion, and is the effective product of the concentration and permeability terms mentioned above). The most negative possible diffusion potential approximates to the potassium equilibrium potential, ψ_{K^+}, obtained by inserting the C_1 and C_2 values for K^+ in equation (6) and solving for ψ_{12} which then equals ψ_{K^+}. Demonstration of electrical potential differences across the membrane which are more negative (high K^+ phase relative to low K^+ phase) than ψ_{K^+} provides good evidence for the occurrence of an electrogenic pump operating to transfer positive charge from the high K^+ to the low K^+ side of the membrane; in a microbial cell this means from the cytoplasm to the medium, suggesting either electrogenic cation efflux or electrogenic anion influx.

The magnitude of ψ_{co} (where c denotes the cytoplasm, and o the outside) in most microbial cells is such that the observed concentration of solutes in cytoplasm and bathing medium requires active influx of anions (Cl^-, NO_3^-, $H_2PO_4^-$–HPO_4^{2-}, SO_4^{2-}, glutamate$^-$, aspartate$^-$) to account for net anion influx during growth, active influx of many neutral solutes (sugars, neutral amino acids) is also required. For the cations, the net entry of many of them (Na^+, Ca^{2+}, Mg^{2+}, lysine$^+$, arginine$^+$) can be accounted for in terms of the observed value of ψ_{co} if equations (4)–(6) are applied. If the effective P_{cation} value in equation (5) is so high that the cation influx is higher than that required to account for the observed internal cation concentration as the cell grows, active (energy-coupled) efflux of the cation is required; this has been demonstrated for Na^+ and for Ca^{2+} (Harold, 1977a, b). Growth on NH_4^+ as the nitrogen source entering the cell requires a net H^+ efflux from the cell for pH-regulation (Table 4); this net H^+ efflux is active. In the case of K^+ there is sometimes an inwardly directed free energy difference for this ion, allowing accumulation by the same method as

for the other cations; in other cases the free energy difference is directed outwards, so energy-coupling to some exergonic reaction is indicated (Raven, 1976a; Jennings, 1976; Harold, 1977a, b; Hamilton, 1975).

It is important to note that only one of these active transport processes need be by a *primary* mechanism; granted that the active process involves *either* electrogenic efflux of a cation or electrogenic influx of some anion, the other transport systems could occur *either* by electrical coupling (cation influx) *or* by chemical coupling, i.e. secondary active transport (anion influx, neutral solute influx, cation efflux). This is an important tenet of the chemiosmotic hypothesis of energy conversion in solute transport.

D. CHEMIOSMOSIS 1: DIRECT USE OF BIOCHEMICAL ENERGY FOR ACTIVE TRANSPORT

A number of primary active transport systems have been identified in membranes from prokaryotes and eukaryotes; with the exception of the PEP-powered sugar transport and phosphorylation system of a few bacteria, these active transport systems all involve ions. H^+ pumps powered by light, by dark oxidation-reduction reactions and by ATP have been identified in prokaryotes and eukaryotes; an ATP-powered Ca^{2+} pump is found in metazoa, probably also in protozoa and in plants, and has recently been found in a prokaryote; while the ouabain-inhibited ATP-powered K^+, Na^+ pump seems to be restricted to metazoa (Mitchell, 1970, 1976; Racker, 1975, 1976; Junge, 1977; Grass and Marme, 1978; Kobayashi *et al.*, 1978; Harold, 1977a, b; Hamilton, 1975; Morris, 1978; Scarborough, 1977; Bowman and Slayman, 1977; Sachs, 1977a; Sachs *et al.*, 1978; Kagawa *et al.*, 1976, 1977).

Much of the definitive work on these various pumps has involved the use of subcellular systems; this is essential for work on the organelle-associated porters, and greatly facilitates the analysis of transport systems associated with the plasmalemma. Isolated organelles or plasmalemma vesicles represent a simplified system in which the solute and energy-source availability on the cytoplasmic side (see below) of the membrane can be much better specified than *in vivo*. Further clarification comes from purification of porter proteins from such preparations, and their subsequent reconstitution by incorporation into polar lipid vesicles and demonstration of active transport capacity.

This work has demonstrated the following features of these diverse transport systems.

Most of the ion pumps are electrogenic. This seems to be true of all the H^+ pumps so far identified, with the probable exception of the K^+, H^+ ATPase from vertebrate gastric mucosa (Sachs, 1977a; Sachs *et al.*, 1978). The Ca^{2+} ATPase is probably always electrogenic, while the K^+-Na^+ ATPase is at least facultatively electrogenic (Mitchell, 1976; Zimniak and Racker, 1978).

The polarity of pumping is in the same direction in all of the systems examined. Thus the direction of net H^+, Ca^{2+} and Na^+ active transport is *from* the cytoplasmic phase with high protein diversity *to* the phase of low protein diversity. This applies not only to the pumps in the plasmalemma: the topological fidelity is maintained in not only intracellular compartments readily identified as ontogenetic or phylogenetic derivatives of the plasmalemma, but also in more complicated organelles (plastids and mitochondria). Here the topology is consistent with *either* derivation of the organelles by endosymbiosis *or* their derivation by multiple invaginations of a single cell (Raven, 1977d; Smith and Raven, 1978). The distribution of these pumps in prokaryotic cells is shown in Fig. 3, while their distribution in eukaryotic cells of the plant (phototrophic) and fungal (saprotrophic) lines of eukaryote evolution is shown in Fig. 4.

In terms of energetics, there seems to be unanimity of opinion that the ATP/Ca^{2+} ratio is 1 : 2, and the ATP/Na^+ ratio is 1 : 3. This sets limits on the extent of the free energy gradient which can be produced by these pumps, granted an *in vivo* free energy of hydrolysis of ATP of 50 kJ mol^{-1}: at equilibrium the Ca^{2+} free energy gradient cannot exceed 25 kJ mol^{-1}, while Na^+ cannot exceed 17 kJ mol^{-1}. For H^+ the situation is much more complex, and values of 1 : 1, 1 : 2 and 1 : 3 have been suggested for the ATP : H^+ ratio for the plasmalemma of eukaryotes (1 : 1 to 1 : 2) and the plasmalemma of prokaryotes and the membranes of organelles (1 : 2 to 1 : 3); one distinction appears to be that ratios nearer 1 : 1 are found in membranes which are concerned with the conversion of ATP energy to transmembrane H^+ free energy difference, while the ratios nearer 1 : 3 are found in membranes which can interconvert redox and adenylate energy (Raven, 1977d; Smith and Raven, 1979; Morris, 1978). The 1 : 2 to 1 : 3 ratio found in the ATP-generating membranes probably relates to the value of ΔG_p *in vivo* and the size of the $\Delta\bar{\mu}_{H^+}$ "energy quantum" which can be conveniently

maintained across the membrane and/or generated by the redox processes driving phosphorylation. The maximum free energy difference for H^+ which can be generated by the various redox-driven H^+ pumps (either chemical or light) is generally about 22 kJ mol^{-1}. This could permit the observed powering of ATP synthesis by redox processes via a $\Delta\bar{\mu}_{H^+}$ (Nicholls, 1977; Moore *et al.*, 1978; Johnson and Hansford, 1977).

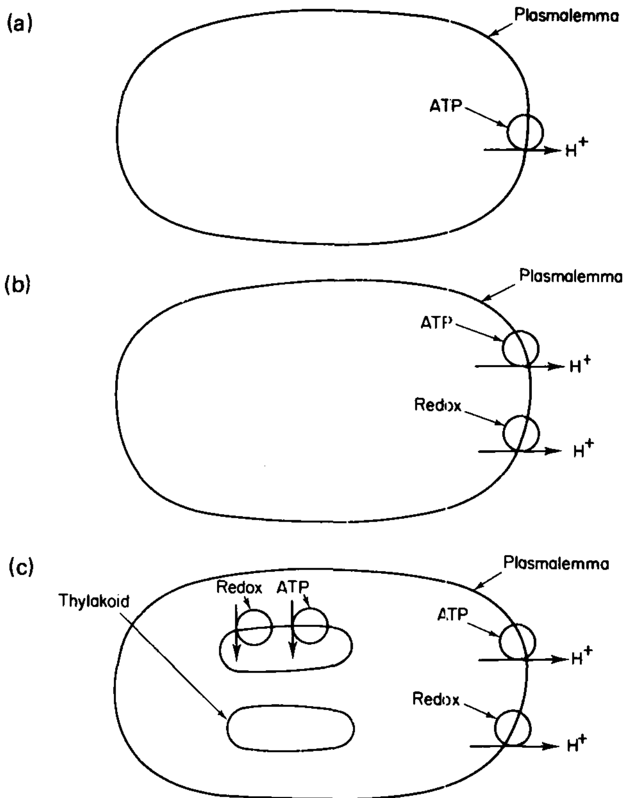


FIG. 3. Distribution of H^+ pumps in prokaryote cells. (a) Non-photosynthetic anaerobes lacking membrane-associated redox systems (see Thauer *et al.*, 1977). (b) Aerobic heterotrophs (and anaerobic heterotrophs with membrane-associated redox systems: see Haddock and Jones, 1977). (c) Photo- and chemo-lithotrophs (see Pfennig, 1977; Stanier and Cohen-Bazire, 1977). In addition to these primary H^+ pumps, at least group (a) have a primary active Ca^{2+} efflux pump at the plasmalemma (Kobayashi *et al.*, 1978).

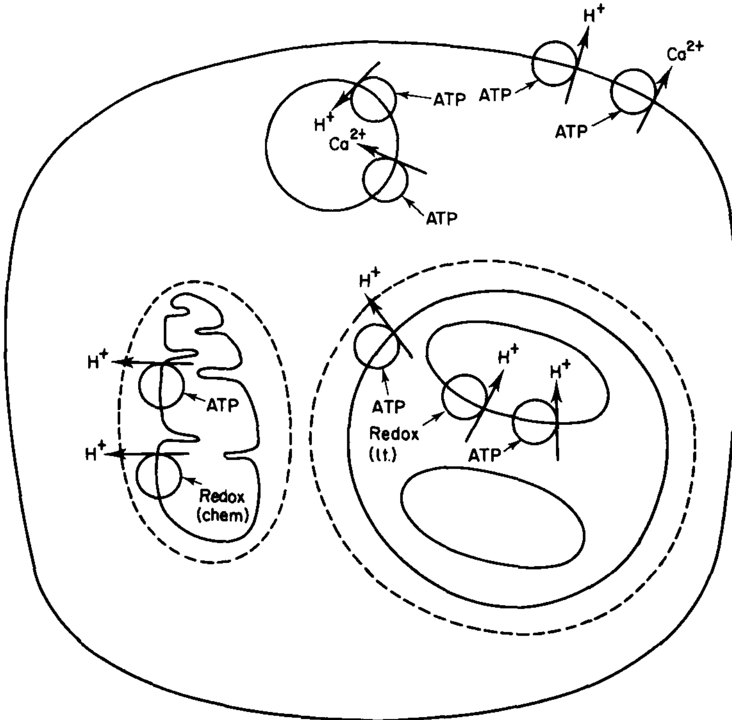


FIG. 4. H^+ pump distribution in photosynthetic eukaryotes (and in fungi) in the "tight" membranes shown in Fig. 2. Only an ATP-powered H^+ pump is shown as operating at the plasmalemma: evidence for redox-powered H^+ transport at this membrane is equivocal (Löw and Crane, 1978).

The other quantitative aspect of primary active transport relates to the power output per unit area of membrane. This is related to the energy transformed per ion transported *via* the H^+ flux (mol cm^{-2} s^{-1}), which in turn depends on the density of carrier sites and their rate of catalysis of transport:

$$\text{Solute flux} = \text{porter site density} \times \text{specific reaction rate}$$
$$(\text{mol}_s\,\text{cm}^{-2}\,\text{s}^{-1}) = (\text{mol}_p\,\text{cm}^{-2}) \times (\text{mol}_s\,\text{mol}_p^{-1}\,\text{s}^{-1})$$

where the subscript s refers to the transported solute and the subscript p refers to the porter. Harold (1977a) and Raven (1977d, 1978) have surveyed the literature, and it appears that the porter turnover for these active transport processes does not generally exceed 100 s^{-1}, while the density of porters does not exceed 2 $pmol_p$ cm^{-2}, i.e. the flux does not

generally exceed 200 pmol cm^{-2} s^{-1} (cf. Baker and Willis, 1972; Endo, 1977; Slayman and Gradmann, 1975).

The density of porters which can be accommodated in the membrane may well be limited by the requirement for some fluid, relatively unordered lipid in the membrane: perhaps 20 to 30 molecules of lipid per molecule of integral protein may be "ordered" by association with the protein. This upper limit probably applies to the sum of integral proteins, so the incorporation of more primary active transport sites into the membrane means that there must be fewer porters for passive uniport, symport and antiport (see E below).

There is evidence from some eukaryotic plant cells for primary active transport of anions at the plasmalemma. There is good evidence for ATP-powered electrogenic Cl^- influx at the plasmalemma of the giant, marine chlorophyte *Acetabularia* (Saddler, 1970; Gradmann, 1975); this possibly applies to the related algae *Bryopsis* and *Halicystis* (Raven, 1976a, 1981). ATP-powered Cl^- transport also occurs in salt glands of *Limonium*, a halophytic higher plant (Hill and Hill, 1976). Further, there may be primary active HCO_3^- influx at the plasmalemma of *Chara corallina* (Walker and Smith, 1977b). With the exception of the *Limonium* salt gland, these anion pumps are topologically analogous to the cation pumps discussed above, in that they transfer positive charge out of the cell. The net Cl^- flux across the plasmalemma of *Acetabularia* may exceed (by a factor of two or so) the "maximum" value of net primary active transport suggested above.

A final point relates to the limited number of different kinds of primary active transport processes which have so far been identified; this is especially the case if an individual membrane is considered. Individual membranes appear to be restricted to three kinds of active transport processes (e.g. the light-powered H^+, respiratory H^+ and ATP H^+ of *Halobacterium*). It may be that this restriction relates to an intrinsic difficulty in making primary active transporters, i.e. devices for crossing the scalar-vector boundary (Curie principle: Kleinzeller and Kotyk, 1961); at all events such porters seem to have much lower turnover numbers than is the case with uniporters and antiporters (see E below).

E. CHEMIOSMOSIS 2: COTRANSPORT PROCESSES

In Section D above we noted that there seem to be relatively few examples of primary active transport processes; however, the total of

active transport and electrically coupled transport processes (Section C above) is at least an order of magnitude greater. Ideas elaborated by Mitchell (1961, 1966, 1968, 1970, 1976), derived in part from earlier contributions by Lundegardh (1939), Davies (1951), Conway (1954) and Robertson (1960, 1968) show how the biochemical energy used by the primary active ion transport processes can power other transport processes. The basic scheme is shown in Fig. 5; in this scheme the

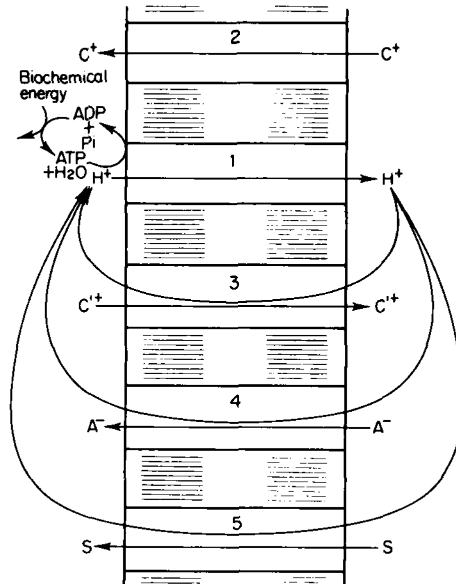


FIG. 5. Primary active H^+ extrusion and associated cotransport processes. (1) Primary, ATP-powered H^+ efflux. (2) Uniport of cation C^+. (3) Antiport of the cation C'^+. (4) Symport of the anion A^-. (5) Symport of the neutral solute (either undissociated, e.g. glucose, or bearing no net charge, e.g. alanine).

primarily transported ion is H^+ (process 1), although the scheme could be readily modified to accommodate any of the other ions known to be actively transported (see Section D above: Mitchell, 1970; Sachs *et al.*, 1978).

In its simplest form the coupling (as represented in Fig. 5) involves uniport for cation entry across the plasmalemma (process 2); electroneutral H^+-cation antiport (process 3) and H^+-anion symport (process 4) for the extrusion of cations and the entry of anions; and a 1 H^+:1

neutral solute symport stoichiometry for neutral solute influx (process 5). This imposes severe restrictions on the operation of such cotransport systems, particularly with respect to the influence of external pH on H^+-based cotransport. Over a range of external pH values the $\Delta\bar{\mu}_{H^+}$ in a number of organisms is maintained essentially constant as is cytoplasmic pH, with the ψ_{co} changing with external pH (Smith and Raven, 1979). Thus over a certain range of external pH values $\Delta\bar{\mu}_{H^+}$ is maintained constant, but with a variable contribution from the ψ_{co} and the ΔpH components; at extremes of pH the $\Delta\bar{\mu}_{H^+}$ itself declines (Smith and Raven, 1979). One of the major tests of the chemiosmotic model relates to its ability to cope with varying external pH, and the nutrient uptake by acidophiles and alkalophiles (Garland and Haddock, 1977; Krulovich *et al.*, 1978). In an elegant analysis, Rottenberg (1976) has suggested how the simple scheme shown in Fig. 5 could be modified to accommodate changes in the contribution of the two components of $\Delta\mu_{H^+}$ as external pH changes. At low external pH values, ΔpH is large while ψ_{co} is small. Thus electroneutral cotransport processes can still occur, while the efficacy of electrical coupled cation uniport is poor, so H^+-cation symport is invoked. A 1 H^+: singly charged cation symport allows a ten-fold cation concentration with a ψ_{co} of 29 mV rather than 58 mV. At higher external pH values the ΔpH component becomes small, or even negative, while the electrical component is large. Thus electrically coupled cation influx poses no problems, while electroneutral symport and antiport cannot occur. Rottenberg (1976) suggests that in this situation the $H^+:A^-$ (symport) or $H^+:C^+$ (antiport) ratio is more than 1 : 1, thus making the transport electrogenic (carrying positive charge inwards), and allowing the electrical component of $\Delta\bar{\mu}_{H^+}$ to be used to energize the transport. Evidence for some of these possibilities has been presented by Ramos and Kaback (1977a, b), Logarde (1977), Friedberg (1977), Schuldiner and Fischke (1978) and Borst-Pauwels and Peters (1977).

In order to achieve maximal energetic efficiency of cotransport, it would be expected that the H^+: solute stoichiometry would be a function of the external pH; thus substantial accumulation of A^- could occur at low external pH values with an $H^+:A^-$ ratio of 1, while a ratio of 2 or 3 might be needed to achieve the same accumulation ratio at higher external pH values. Conversely, while the accumulation of K^+ may need H^+ symport at low external pH values, this might not be needed at higher external pH values where the value of ψ_{co} is greater.

Evidence for such a variable stoichiometry is somewhat contradictory (Ramos and Kaback, 1977a, b; Friedberg, 1977; Schuldiner and Fischke, 1978). Energetic efficiency may also be improved if there is a variable stoichiometry of H^+:solute dependent on the external concentration of the transported solute: in order to maintain the influx from low external concentrations with an internal solute concentration largely independent of external concentration of the solute, more energy per mol of solute is required at the low external concentrations. For a cotransport mechanism for (e.g. phosphate) influxes, a higher H^+ : solute ratio might be expected at low external concentration of the solute. However, for phosphate uptake by *E. coli*, it is possible that a cotransport system at high external phosphate concentration is replaced by primary active transport at low external phosphate levels (Konings and Rosenberg, 1978).

A mechanism which does not really solve any of these problems is found in many marine and some freshwater bacteria: here most of the cotransport systems are driven by a $\Delta\bar{\mu}_{Na^+}$, which is in turn generated by H^+ : Na^+ antiport (MacDonald *et al.*, 1977; Belliveau and Lanyi, 1978).

Thus the scheme shown in Fig. 5 poses some problems for an organism living in the real world. As has been pointed out above, the organism attempts to limit the number of primary active transport systems which it has to make to only two or three per organism, possibly because they are less easy to contrive and to operate than vector-vector cotransport systems. Certainly the turnover of cotransport systems can be much higher than the rate of turnover of primary active transport systems. Instead of the specific reaction rate of 100 s^{-1} quoted for net primary active transport in Section D above, antiport (e.g. of Cl^- and HCO_3^- in red blood cells: Whittam, 1964; Ship *et al.*, 1977) can occur at specific reaction rates of 10^4 s^{-1}, while uniport (e.g. of Na^+ channels in nerve) can occur during an action potential with a turnover of $4 \cdot 10^8$ s^{-1} (Hille, 1970). However, these very high catalytic activities for the cotransport sites relative to the net primary active transport sites do not necessarily lead to a larger nutrient flux across the membrane than would primary active transport of all the solutes (with an assumed maximal turnover of 100 s^{-1}), since the rapidly turning over cotransport sites are limited by the slowly turning over primary active transport sites, whose density and turnover seem to be limited (see Section D above).

F. REGULATION OF TRANSPORT CAPACITY AT THE PLASMALEMMA

Constraints on the density of porters have been considered under Sections D and E above; it appears that the total porter density (mol_p cm^{-2}) rarely exceeds 3 mol_p cm^{-2}. Primary active transport porters seem to have specific reaction rates not exceeding 100 s^{-1}, so if there are 3 $pmol_p$ cm^{-2} of primary active transport sites, the primary net flux of (e.g.) H^+ will not exceed 300 pmol cm^{-2} s^{-1}. In terms of Fig. 5 (cf. Table 4) much of this H^+ will recycle across the membrane in cotransport; it is likely that a total density of 1 $pmol_p$ cm^{-2} of uniporters, symporters and antiporters could cope with a backflux of 300 pmol cm^{-2} s^{-1}, since the specific reaction rates of such porters can be as high as 10^4 s^{-1} (see Section E above). Such transmembrane H^+ fluxes may be required to account for the rates of ATP synthesis and nutrient transport in *E. coli* growing at its maximum relative growth rate, even allowing for some fermentative contribution to the ATP requirement at these rapid growth rates on complex media (cf. Stouthamer and Bettenhausen, 1975).

Similar computations may be carried out for the maximum catalytic capacity of soluble-phase metabolism, based on the turnover number of enzymes and their concentration in the cytoplasm (see Atkinson, 1977). Granted a knowledge of these area-based transport capacities of the plasmalemma, and the volume-based enzymic capacity of the cytoplasm, it should be possible to calculate the maximum relative growth rate which can be achieved for an organism of a given size and shape (and hence cellular surface/volume ratio), and whether the ultimate limitation is related to membrane transport or to soluble enzyme activity. However, it is likely that the limitation is more likely to be based on the "molecular biological" problem of the rate at which tRNA molecules can find rRNA molecules, which imposes a ceiling on relative growth rate for any given cell size which is lower than that imposed by the activity of soluble enzymes or of membrane transport (Maynard-Smith, 1969). As far as membrane transport capacity is concerned, it appears that the growth rate of microorganisms at saturating nutrient concentrations is not limited by the membrane transport capacity. At a constant carrier density and turnover, the important parameter is the cell surface area. In some elegant studies with haploid and diploid strains of *Saccharomyces cerevisiae* Adams and Hansche (1974) showed that the diploid and haploid strains had the

same relative growth rate on a medium in which all nutrients were present in excess, despite the diploid having a volume 1.7 times that of the haploid but a surface area only 1.45 times that of the haploid strain. However, when one nutrient was deficient the haploid grew faster than the diploid: it is not clear if this is related to a limitation in the transport capacity across the plasmalemma, or to greater problems with "unstirred layers" outside the cell in the larger diploid cells. The increase in mean cell size with increasing growth rate in *E. coli* (e.g. Helmstetter *et al.*, 1968; Donachie *et al.*, 1976), implying a decrease in membrane surface area relative to the cell's requirement for such membrane-associated processes as ATP synthesis and nutrient uptake, is also consistent with membrane transport rates not limiting the maximum relative growth rate which the organism can achieve on a complex medium. However, some evidence for *Saccharomyces cerevisiae* (Hennaut *et al.*, 1970) suggests that the addition of derepressed amino acid porters to the plasmalemma may require a decrease in the content of constitutive porters.

Data generally consistent with the suggestions made above come from studies of variation of cell size with growth temperature, the slower growth at lower temperatures (within the normal ecological range) is associated with an increased cell size (Finlay, 1977; Falkowski, 1977). In terms of the generally lower turnover of enzymes and porters at lower temperatures, and the occurrence of capacity adaptation to lower temperature by the synthesis of more catalysts per cell (see Raven and Smith, 1978b), it would again appear that the transport capacity of the plasmalemma is not rate-determining for growth, since the cell surface to volume ratio decreases with decreasing temperature.

Within the constraint of the overall density of porters in the membrane, an organism of a given size faced with the problem of taking up an essential nutrient from very low external concentrations can adopt one of three possible strategies to increase the influx (on a membrane area basis) from the low concentration of nutrient. The three strategies are:

(a) greater density of the porter concerned ($pmol_p\ cm^{-2}$), constant $K_{1/2}$* for the transported substrate, constant turnover ($pmol_s\ pmol_p^{-1}\ s^{-1}$)

* $K_{1/2}$ = substrate concentration at which the reaction rate is one-half of the rate at saturating substrate concentration.

(b) constant density of the porter, constant $K_{1/2}$ for the transported substrate, increased turnover, or
(c) constant density of porter, lower $K_{1/2}$, constant turnover at substrate saturation.

All of these strategies can (Fig. 6) lead to increased solute influx from a low (rate-determining) external concentration; they differ in the metabolic cost (capital and running costs), and the time scale over which they are effective. While strategy (a) can be adopted (in the presence of suitable regulatory mechanisms) within a generation time,

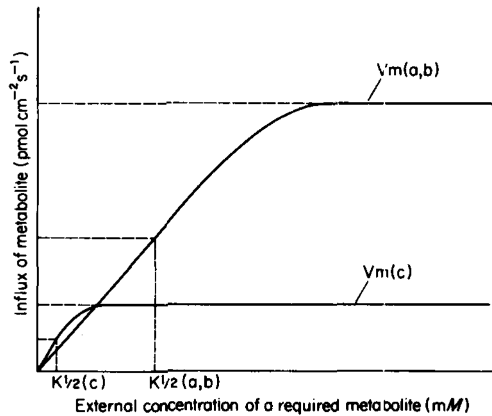


FIG. 6. Strategies for achieving a high influx capacity for a solute at low external concentrations of solute: (a) increased porter density at constant specific reaction rate and $K_{1/2}$; (b) increased specific reaction rate at constant porter density and $K_{1/2}$; (c) decreased $K_{1/2}$ at constant porter density and specific reaction rate.

(b) and (c) involve genetic change (selection of genotypes, if not mutation itself) and thus take longer than one generation. These various strategies have their counterparts in "classical" aqueous-solution enzymology (Albery and Knowles, 1976; Atkinson, 1977; Cole, 1976), and have as their end-point the limitation of the rate of catalysis of the chemical reaction or of transport by diffusion of the substrate from the bulk phase to the reaction site (Koch, 1971).

Regulation of porter density also occurs in the absence of changes in external conditions as a function of the stage in the cell cycle; this autoregulation must obviously result in the transport capacity per cell being twice as great just before binary fission as just after it, but the changes in capacity do not always parallel either cell mass or cell

surface area (Jeanjean, 1973a; Meszes *et al.*, 1967; Tischner and Lorenzen, 1978).

Related to changes in porter density in response to changes in nutrient concentration in the medium are changes in activity of porters for solutes which supply a nutrient element (or energy supply) for which alternative sources are available. The induction of glucose porters in photolithotrophic algae which can also grow heterotrophically upon exposure to glucose or to absence of light (Section IV.B) is a case in point.

G. NET AND TRACER FLUXES

With respect to net nutrient acquisition the microorganism requires efficient net uptake of nutrients during growth and the prevention of loss of nutrients when growth is not possible. When tracers are used it is frequently found that the measured net flux is the resultant of unidirectional tracer influx minus unidirectional tracer efflux. The nature of these component fluxes is of great importance in the energy economy of the cell (see B and E above).

There are two major possibilities for explaining the occurrence of these bidirectional fluxes. One possibility is that the tracer fluxes represent the "exchange reaction" of a single transport mechanism which brings about the net flux. The other possibility is that there are two porter systems: one case has already been discussed in Section D and E above (see Fig. 5), i.e. primary active transport and the cotransport systems powered by it. When H^+ is the circulating ion, however, tracers cannot be used to distinguish net from "exchange" fluxes due to exchange of the H isotopes with water. For other solutes (Na^+, Cl^-) which might be involved in recirculations related to cotransport, tracer fluxes can be measured. Another facet of the "two pathways" hypothesis is that there could be a "pump and leak" of the type described for K^+ and Na^+ in many vertebrate cells (Whittam, 1964). The pump would be a primary or secondary active transport, while the leak might be a uniporter. This has already been mentioned (C above) with respect to the regulation of cation content in cells; an influx of cations driven by the inside-negative ψ_{co} in excess of that required by the cell can be countered *via* extrusion by a cotransport mechanism. The two possibilities mentioned here are not mutually exclusive, in that a cotransport loop or pump and leak may themselves

be associated with tracer exchange fluxes in one or both of their components.

The first mechanism (large bidirectional fluxes relative to net transport in a single transport mechanism) *may* imply that, for active transport, the overall reaction is close to thermodynamic equilibrium. What we mean by "close" here is difficult to define, since there is no general prediction from irreversible thermodynamics of the relationship between energy dissipation in a reaction and the rate of forward and back reactions. While such a mechanism may be very efficient in terms of the fraction of the energy substrate used which is exploited to perform useful work, it also implies an excess of catalytic machinery to bring about a given net flux compared with what is needed when the overall reaction is further from equilibrium (Odum and Pinkerton, 1955). It may thus reflect a large investment of cell material and energy in the catalytic machinery.

A "pump and leak" system is also energetically wasteful, in that the "leak" flux dissipates part of the energy gradient established by the "pump". This has been emphasized (Barber, 1968b, d, e) as being particularly the case with non-vacuolate microorganisms such as bacteria, yeasts and *Chlorella*; however, it is probably equally important in vacuolate eukaryotic microorganisms, the important quantity being the ratio of plasmalemma area to cytoplasm (volume inside plasmalemma *minus* the vacuole) as far as cytoplasmic solutes are concerned (Raven, 1977a). The relative efficiency of pump and leak, with high running costs and with an unknown capital investment in the two separate channels for "pump" and "leak" fluxes, and the single porter species running close to equilibrium, is not known.

The requirement mentioned above for a separate leak channel is only applicable to hydrophilic solutes which have very low intrinsic permeabilities in lipid bilayers, e.g. inorganic ions, sugars and amino acids (Sections A and B above). For compounds such as CO_2, NH_3 and urea (Sections III.A and B, and Sections IV.A and B), mediated transport of HCO_3^-, NH_4^+ and urea respectively will be partially short-circuited by permeation of the undissociated species through the lipid portion of the bilayer. This is potentially more dangerous in terms of energy dissipation than is "pump" and "leak" where both fluxes are mediated, since the number and turnover of the porters is more readily regulated than is permeation through lipid; changes in permeability of the lipid portion of the membrane involve changes in the lipid

composition of the membrane which may have undesirable side-effects (e.g. on porter activity if the membrane becomes too solid).

A "pump" and "leak" system for hydrophilic solutes may have regulatory advantages over mediation by a single porter (Raven, 1977a), although what these advantages may be can only be discerned by detailed analysis (Stein and Blum, 1978). It is probable that "leak" is inhibited when growth is slow or absent, or when the solute concerned is absent from the medium. This regulation of the "leak" channel shows how difficult it is to establish the existence of a "pump" and "leak" system without extraction and purification of the relevant porters: the criterion of a diminished efflux of a solute which is actively transported into the cell when that solute is absent from the medium, is consistent with a single carrier mechanism "exchange diffusion"; however, if the "leak" channel in a pump and leak system becomes less active when the solute is absent from the medium, the evidence is also consistent with this mechanism. Similarly, decreased tracer fluxes under energy limitation or at low temperatures are as consistent with a regulated "pump" and "leak" as they are with a single channel mechanism.

By whatever mechanism the tracer fluxes in excess of net fluxes come about, it seems that when high free energy differences for the solute are being maintained across the plasmalemma (i.e. at low external solute concentrations in the case of metabolized anions), the efflux of the solute is much lower than when the external concentration is high, i.e. the net and tracer influxes are very similar. This may to some extent be an artifact of reaccumulation of any tracer which has been effluxed from the cell in the absence of unlabelled external solute to dilute it; however, it is very likely that there is a lower efflux at low external solute concentrations. In terms of the "one-carrier" hypothesis, the greater imbalance between influx and efflux at low external concentrations is embarrassing, as it implies that the influx process is further from equilibrium with the energy-supply reaction. This in turn requires that the energy available from the driving reaction increases at low external solute concentration to a greater extent than does the energy required for influx of the solute.

H. BIOCHEMICAL ASPECTS OF ENERGY SUPPLY

The major ultimate energy sources for transport at the microalgal cell plasmalemma are membrane-associated redox H^+ pumps; in those

cyanobacteria which have these processes in the plasmalemma (e.g. *Plectonema*: see Section IV.A), energy can be supplied to secondary active transport processes directly as the proton free energy gradient generated by these redox H^+ pumps (see Fig. 3); in cyanobacteria such as *Gleobacter* which lack internal membranes there must be redox coupled H^+ pumps at the plasmalemma: there is nowhere else for them to be located (Stanier and Cohen-Bazire, 1977). In other cyanobacteria (e.g. *Anacystis:* see Section IV.A), and in the eukaryotic algae, there are no redox H^+ pumps in the plasmalemma, and the plasmalemma transport processes are energized by ATP generated on internal cell membranes. In the cyanobacteria this ATP is produced by photosynthetic or respiratory reactions in the thylakoid membranes; in eukaryotes respiratory ATP comes from mitochondria and photosynthetic ATP comes from the chloroplasts (indirectly, *via* shuttles: Raven, 1976b; Heber, 1974; Simonis and Urbach, 1973; Gimmler, 1977).

The pathways involved in these H^+ pumps in the photosynthetic and respiratory membranes are shown in Fig. 7 together with the capacity of the pathways and the experimental means by which they may be temporally isolated from other redox H^+ sources (cf. Raven, 1974a, 1976b, 1981; Crofts and Wood, 1978). Application of these techniques to transport processes shows that all of the redox-coupled H^+ pumps shown in Fig. 6 can, *in the absence of the other pumps*, power active transport processes. In general the highest rates of active transport are found when all of the redox-coupled H^+ pumps are potentially able to operate, i.e. in the presence of oxygen for respiratory redox reactions and a range of wavelengths of light and of electron acceptors to permit both open-chain and cyclic photosynthetic redox reactions. The lowest rate of transport is found when all of the redox-coupled H^+ pumps are inhibited by the absence of light and of exogenous electron acceptors (i.e. anaerobically in the dark). Between these two extremes are the rates of transport found when particular redox H^+ pump systems are working in isolation. Generally, cyclic photosynthetic energy conversion supports at least as high a rate of transport as does the respiratory redox pump, while open-chain photosynthetic redox processes (often with incompletely suppressed respiratory and cyclic photosynthetic energy conversion going on at the same time) support a rate close to that found in control cells with all redox systems capable of operating.

A number of unsolved problems remain. In several cases the mediation of ATP between the redox H^+ pump at the internal

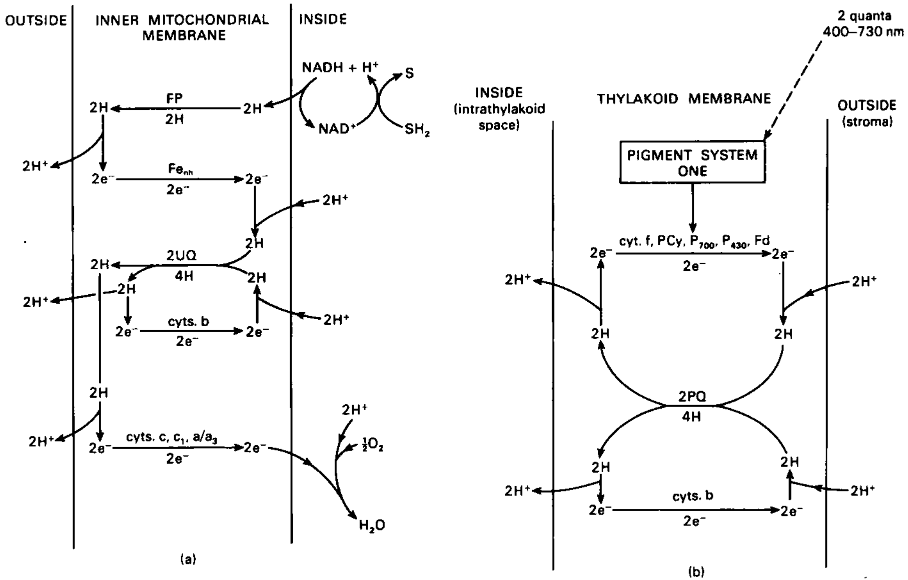
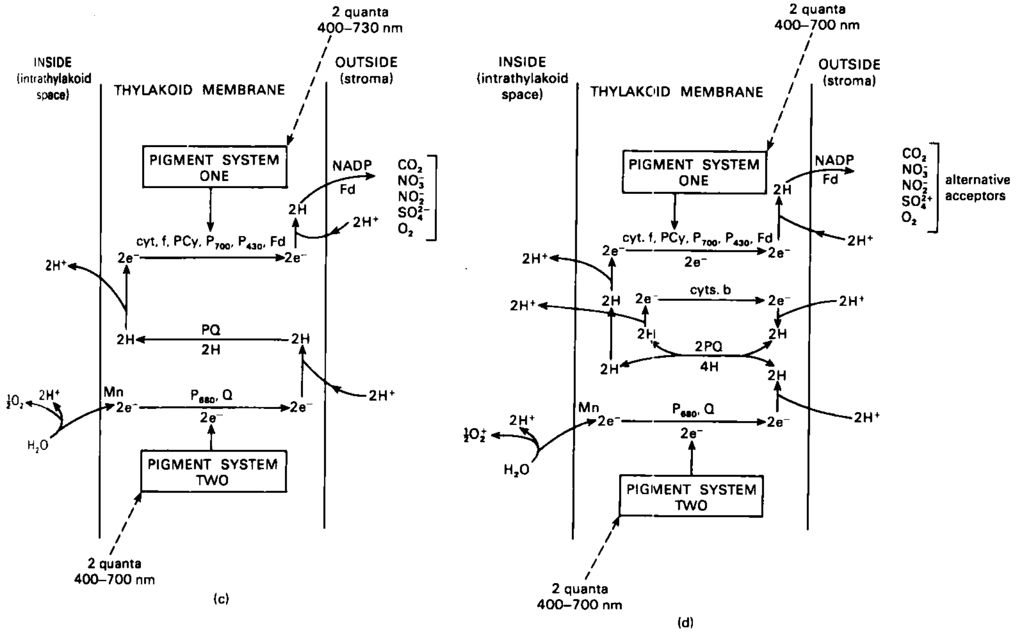


FIG. 7. ATP-generating processes in microalgal cells. (a) Oxidative phosphorylation; capacity of about 200 μmol ATP (mg chl)$^{-1}$ h^{-1}, P/e_2 of 3. With the redox H^+ stoichiometry shown, this implies an H^+/ATP of 2 for the ATP synthetase. Oxidative phosphorylation can be isolated from photophosphorylation by darkness in the presence of O_2. Under these conditions fermentation only supplies about 15 μmol ATP (mg chl)$^{-1}$ h^{-1} (cf. Raven, 1976b). (b) Cyclic photophosphorylation; capacity at least 200 μmol ATP (mg chl)$^{-1}$ h^{-1}, P/e_2 of 2. With the redox stoichiometry shown, this implies an H^+/ATP of 2 for the ATP synthetase (cf. MacCarty, 1978). Cyclic photophosphorylation can be isolated from oxidative and open-chain photophosphorylation by withholding O_2, CO_2 and light of wavelengths less than 700 nm, and by the addition of photosystem II inhibitor DCMU. Under these conditions fermentation only supplies about 15 μmol ATP (mg chl)$^{-1}$ h^{-1} of the (more than) 200 μmol ATP (mg chl)$^{-1}$ h^{-1} (cf. Raven, 1976b). (c), (d) Open-chain photophosphorylation; capacity of at least 2000 μmol ATP (mg chl)$^{-1}$ h^{-1}; P/e_2 up to 2 (Rosa, 1979). With the redox stoichiometry shown in (c), the only scheme here which does not involve a "proton-

membranes and primary active transport at the plasmalemma has been demonstrated by the use of energy transfer inhibitors such as DCCD which can inhibit both the ATP-generating and the ATP-consuming processes; however, in many of the examples quoted in Section IV such techniques have not been employed, and the role of ATP is rather less securely based. Attempts to demonstrate the role of ATP more directly by correlating the rate of transport with the intracellular ATP level, the



motive Q cycle", this requires an H^+/ATP for the ATP synthetase of 2, while the stoichiometry indicated in (d) can be accommodated with an H^+/ATP for the ATP synthetase of 3 (cf. Hauska and Trebst, 1977; MacCarty, 1978). Isolation from oxidative, and cyclic, phosphorylations is only achieved with difficulty, since the removal of O_2 to prevent all oxidative phosphorylation is difficult when O_2 is being continually produced, while the specificity of inhibitors (which are alleged to inhibit cyclic- and oxidative phosphorylation more than open-chain phosphorylation) is suspect (cf. Raven, 1976b). Alternative redox acceptors for this pathway are indicated in (c) and (d) (cf. Hauska and Trebst, 1977). Fermentation (isolated by withholding O_2 in darkness) has a capacity, in the absence of other pathways, of some 30 μmol ATP (mg chl)$^{-1}$ h^{-1}. Abbreviations: cyt cytochrome; Fe_{nh} non-haem iron; Fd ferredoxin; FP flavoprotein; P_{430} acting as primary acceptor in photoreaction one; P_{680} reaction centre chlorophyll of photosystem II; P_{700} reaction centre chlorophyll of photosystem I; PCy plastocyanin; PQ plastoquinone; Q primary acceptor of photoreaction II; UQ ubiquinone.

ATP free energy of hydrolysis, or the energy charge, have generally been unsuccessful in algae (e.g. Lilley and Hope, 1971; Raven, 1966, 1976a, b, c; Raven and Smith, 1978b; Sundberg and Nilshammar-Holmval, 1975; Jeanjean, 1976, 1977) in contrast to the excellent work of Slayman *et al.* (1973) on *Neurospora*. This suggests that the rate of primary active transport powered by ATP is not controlled directly by either kinetic or thermodynamic changes in the adenylate system.

Regulation of transport rate by "energy availability" appears to involve signals other than the adenylates; the response of a cell to a diminished energy supply is to decrease the rate of energy-requiring processes without a concomitant reduction in the free energy available from ATP or from the ion gradient generated by primary active transport and used for cotransport (Slayman, 1977). This permits the quality of the energy sources to be conserved even though the *quantity* available per unit time is reduced. The teleological function of this may be found in the requirement for a high-quality energy source to power essential maintenance processes even when energy availability precludes growth (Atkinson, 1968, 1977; Raven, 1976a).

This last point, in contrasting maintenance conditions and growth conditions, raises the final problem, that of determining which redox pumps supply energy to active transport processes under normal growth conditions. The only conditions employed in isolating the various energy sources which are normal for the photolithotrophic growth of the alga on a light-dark diurnal cycle are light in the presence of CO_2, and darkness in the presence of O_2. The other conditions which isolate various redox systems do not support photolithotrophic growth (e.g. the conditions required to isolate cyclic photosynthetic energy conversion). Net transport occurring under cyclic photoredox conditions probably reflects "luxury accumulation" of the solute (Raven, 1976a).

The approaches which may be used to attempt to delimit the energy sources for transport during growth are all indirect. One method is to follow the course of net nutrient transport during a synchronous light-dark cycle. In a number of cases (Raven, 1976a, b) the ATP demand for nutrient transport, and for subsequent processing of the nutrient taken up (excluding the basic reaction of the photosynthetic carbon reduction cycle requirement for ATP), is greater than the measured capacity for respiratory ATP synthesis. This is reasonable evidence for an involvement of photoredox processes in supplying the ATP required. The capacity for open-chain photophosphorylation is probably adequate to supply not only the CO_2 fixation reactions, but also the remaining ATP-requiring processes. The involvement of cyclic photophosphorylation in growing algal cells is still in dispute (Raven, 1976a, b, 1981; Simonis and Urbach, 1973; Gimmler, 1977).

Thus far the use of mutants has not been of great use in delimiting which energy sources may be used for transport in growing algal cells

(cf. Section IV.G). Thus the respiratory mutants of *Chlamydomonas reinhardtii* permit photolithotrophic but not heterotrophic growth, suggesting that little if any oxidative phosphorylation is essential for photolithotrophic growth (Alexander *et al.*, 1974). No mutants lacking cyclic photophosphorylation have so far been isolated. Even these approaches still do not tell us the sources of energy used when all of the energy sources are potentially available to power transport processes.

I. SHORT-TERM REGULATION OF NUTRIENT FLUXES

Long-term regulation involves changes in the number of porters present in the membrane as a function of the cell cycle and induction-repression by the transported substrate. Short-term regulation of the rate of transport involves a change in the achieved specific reaction rates of the porters. In general, this is thought to be a graded response, with all of the porters undergoing a quantitative change in activity, although "all or none" behaviour has been reported for passive OH^- porters in a giant-celled alga (Lucas, 1975, 1979; Lucas and Dainty, 1977).

This change in porter turnover may be regulated by the transported substrate(s), the energy substrate(s) or some other ligand. The transported substrate can cause changes in porter reaction rate by virtue of its concentration on either side of the membrane. On the "uptake" side the relation of activity to concentration is generally a rectangular hyperbola (or hyperbolae) although if more than one molecule of solute can be bound to one molecule of porter the kinetics at low solute concentrations may be sigmoidal. For active transport it is generally held that a kinetic model for transport involves a lower affinity of the porter for its substrate on that side towards which transport occurs (e.g. Komor and Tanner, 1974a, b, 1975). This relationship can probably account for much of the "trans-inhibition" of transport which cannot be accounted for thermodynamically, and which is often claimed to be allosteric in nature. The internal concentration on which "trans-inhibition" works relates to the rate of assimilation of the solute, either biochemically or by transport to another compartment.

The energy substrate, as has been already mentioned (H above) does not generally show large changes during considerable changes in the rate of transport which may reasonably be related to changes in energy

supply. In most cases the solute transport process is relatively far displaced from energetic equilibrium with the energy source (see Raven and Smith, 1978b). The nature of the signal which denotes a change in energy supply and which causes appropriate changes in energy consumption by (*inter alia*) transport processes is unknown: several cyanobacteria have been examined by Rogerson *et al.* (1978) to test for the involvement of guanosine 3′ diphosphate 5′ diphosphate ("magic spot") as the signal, with results which are relatively unfavourable to such an involvement.

Under the "miscellaneous" heading come ligands which are metabolic derivatives of the transported solute, and which can act as trans-inhibitors of active transport (e.g. some product of SO_4^{2-} metabolism causing inhibition of sulphate influx in *Chlorella*: Vallee and Jeanjean, 1968a). An analogous product of solute transport for a non-metabolized solute is, for a non-metabolized major osmotic solute, turgor pressure in a walled cell. Much evidence obtained with higher plants and giant-celled algae is consistent with an important role for turgor pressure (mediated by membrane compression) in altering the activity of porters for the turgor-generating solute (Gutknecht, 1968; Cram, 1976; Zimmermann, 1978).

J. METHODS OF DETERMINING INTRACELLULAR SOLUTE CONCENTRATIONS, TRANSPLASMALEMMA ELECTRICAL POTENTIAL DIFFERENCES AND SOLUTE FLUXES, IN MICROALGAE

Tests of the applicability of the hypotheses outlined in Sections III.B–III.I to microalgae require that ψ_{co}, internal and external concentrations (activities) of solutes, and transplasmalemma net and tracer fluxes, are measured under a variety of conditions.

The two methods of determining ψ_{co} in microalgal cells are (1) the use of an intracellular microelectrode (micro salt bridge), and (2) determination of the distribution of lipid-soluble strong electrolytes. The "microelectrode" technique involves direct measurement of the electrical potential difference across the membrane. A micropipette of tip diameter about 0.1μm, filled with 3 *M* KCl, is inserted through the plasmalemma into the cell interior; the micropipette is connected *via* a salt bridge to a reversible electrode (e.g. a calomel electrode) which interconverts electrolytic and electronic currents. A second micropipette in the bathing medium is connected *via* a salt bridge to a second reversible electrode. The potential difference between the two

electrodes is measured with a high input impedance electrometer (Barber, 1968b; Langmüller and Springer-Lederer, 1974, 1976; Zeldin, 1977). Aside from such problems as "tip potentials" of the intracellular pipette in contact with the cytoplasmic polyelectrolytes which cannot be corrected for by making "blank" measurements with the micropipette in the bathing solution, this method may be criticized when applied to small cells in that any current leakage round the point of micropipette insertion where the plasmalemma has not "resealed" properly will have a large effect on the measured potential difference, tending to lower it. This problem is proportionately greater with small cells (*cf.* Lassen *et al.*, 1971; Komor and Tanner, 1976). A further problem with eukaryotes relates to the positioning of the micropipette tip: it is very difficult to make sure that the tip is in the ground cytoplasm rather than the chloroplast.

Another method for determining ψ_{co} is to measure the distribution of a lipid-soluble strong electrolyte (measuring internal concentration as discussed below), and applying the Nernst equation (equation 6) to calculate the membrane potential causing the distribution. This is discussed in Section III.B; it is essential that the external concentration of the potential-measuring solute is kept low to avoid altering the potential difference due to permeation of the ion. Cations are preferred for measuring potentials with the membrane-bounded phase negative with respect to the bulk medium (i.e. cells with an external pH more alkaline than about pH 3: Raven, 1976a, 1981; Hsung and Haug, 1975, 1977; Krulovich *et al.*, 1978; *cf.* Lainsen and Field, 1976). While $TPMP^+$ has been successfully used with *Chlorella* (Komor and Tanner, 1976) and with *Anacystis* (Paschinger, 1976, 1977; Jeanjean and Broda, 1977), another cation (DDA^+, dibenzyldimethylammonium) has not yielded useful results with algae (see page 135 of Raven, 1976a).

An alternative to lipid-soluble cations is to use a lipid-insoluble cation such as K^+ or Rb^+ together with a suitable ionophore (e.g. valinomycin); however, unless it is certain that most of the potential difference to be measured is produced by an electrogenic ion pump rather than by a K^+ diffusion through natural K^+ uniporters this method can give misleading results if K^+ is not close to electrochemical equilibrium (Paschinger, 1977).

ψ_{co} has also been estimated with permeant fluorescent dyes whose fluorescent intensity is a function of the electrical potential difference

across the membrane of the system to which they are added; so far this technique has only yielded qualitative results for microalgae (Stavis, 1974; Adamich *et al.*, 1976).

In eukaryotes these methods based on solute distribution do not overcome the problem of compartments with different electrical potentials relative to the bathing medium which was mentioned earlier in connection with the use of micropipettes. In the case of solute distribution the value of $\Delta\psi$ obtained will be a weighted mean depending on the size of the various compartments and their potentials relative to the bathing medium.

Turning to the internal concentration of solutes, any determination based on whole cells will suffer from the problem of compartmentation mentioned above in connection with electrical potential measurements. For metabolized solutes there is the further complication that a precise measurement of internal concentration involves separation of the cells from the suspending medium which also contains the solute; during this period of separation the cells might metabolize an appreciable fraction of the internal solute (e.g. NO_3^-). Perhaps the best way to circumvent this problem is to use the silicone-oil-centrifugation technique used for isolated chloroplasts by (e.g.) Werdan *et al.* (1972). Here the cells are incubated with the solute whose distribution is to be determined in an aqueous phase above a silicone oil layer in a centrifuge tube. The incubation is terminated by centrifugation of the cells (plus a small quantity of incubation solution) through the silicone oil layer into a suitable "killing mixture" layered below the silicone oil layer. This prevents any further metabolism of the solute. In order to convert the quantity of the solute found in the killing mixture into a concentration within the cells, a correction must be made for any extracellular solute carried down during centrifugation. This can be done by incorporating another solute into the bathing medium (e.g. inulin) which does not penetrate into the cells; from the quantity of inulin appearing in the killing mixture the extracellular fluid entrainment can be quantified. A further measurement is required to determine intracellular volume; this can be done by the use of a permeant tracer (e.g. tritiated water) which measures the *total* water transferred from the bathing solution to the killing mixture, while the *extracellular* water can be estimated from the quantity of inulin in the killing mixture. The rapidity of quenching of this technique (a few seconds) is essential if the intracellular concentration of rapidly

metabolized solutes present at low concentrations is to be estimated with any accuracy (see page 140 of Raven 1976a).

An alternative approach for estimating the concentration of a metabolized solute in the compartment in which it is metabolized is to compare the *in vivo* rate of assimilation from a known external concentration of the solute with the *in vitro* V_{max} and $K_{1/2}$ of the assimilating enzyme. From the V_{max} of the enzyme *in vitro* on a cell basis, and its $K_{1/2}$ for the solute, it is possible to calculate what solute (substrate) concentration is needed to account for the measured *in vivo* assimilation rate. This probably gives a *minimum* value for the concentration of the solute, since the calculation assumes that the enzyme *in vivo* is working with all conditions optimal *except* the concentration of the solute whose concentration is to be determined (see Raven, 1970, 1976a).

From the point of view of the present analysis the determination of intracellular H^+ concentration (as intracellular pH) is very important in determining trans-membrane H^+ electrochemical potential differences, and in analysis of the distribution of weak electrolytes. In this latter regard the commonest method of determining intracellular pH is by setting a thief (the weak acid DMO) to catch a thief (weak electrolytes such as NH_3 and CO_2). The basis for the weak electrolyte method for measuring intracellular pH, and some of the pitfalls in its use, were discussed in Section III.B. Another method which could be used to determine intracellular pH, but which has not yet been used on algae, is ^{31}P nuclear magnetic resonance (e.g. Ogawa *et al.*, 1978). This powerful technique allows estimation of the concentrations of P_i and various organic phosphates as well as internal pH, and can also give some information about intracellular compartmentation (Salhaney *et al.*, 1975).

The use of intracellular pH-sensitive microelectrodes to measure intracellular pH (Spanswick and Miller, 1977a, have compared this method with the DMO distribution for giant-celled algae) demands the insertion of *two* microelectrodes (micropipettes) into a single cell; the pH-sensitive microelectrode measures the free energy difference for H^+, while a micropipette for measuring ψ_{co} is required to convert the $\Delta\bar{\mu}_{H^+}$ value into a ΔpH. This technique is thus not applicable to true microalgae; the same objection arises in the use of any ion-selective microelectrode to determine intracellular ion activities in microalgae (Lev and Armstrong, 1975). The other technique which has been used

to determine intracellular pH in algal cells is that of measuring the pH of a cell extract (Shieh and Barber, 1971). This method, like the weak electrolyte distribution method, gives an "average" value for intracellular pH, although the weighting of the different compartments will be different in the two techniques (Waddell and Bates, 1969).

The final measurement required is of the transplasmalemma flux. Net influxes can be measured as disappearance of the solute from the medium, or its appearance (or the appearance of an assimilated form) in the cells, and *vice versa* for a net efflux. The estimation of tracer fluxes requires that attention be paid to the possibilities of compartmentation, and hence may involve the technique of compartmental analysis to estimate fluxes at the various membranes. This technique can also be used to estimate the solute contents in the compartments (MacRobbie, 1971; Wagner, 1974; Schneider and Frischknecht, 1977a). However, it is not always easy to identify the compartments defined in this way with particular spatial (or, for metabolized solutes, chemical) entities.

IV. Transport of Specific Solutes across the Algal Plasmalemma

A. PRIMARY ACTIVE TRANSPORT AND ELECTROGENESIS

The operational definition of electrogenic transport, i.e. the occurrence of values of ψ_{co} which are outside the range of values which can be attributed to diffusion potentials, was discussed in Section III.C. It was pointed out that the most negative value of ψ_{co} which could be achieved by downhill ion transport is that which would be found if the membrane had a higher effective permeability to K^+ than to other ions, granted the ratio of internal to external ion concentrations. Thus if the measured ψ_{co} is more negative than the value of ψ_{co} calculated from equation (6) when K^+ concentrations are inserted, it is very likely that an electrogenic ion flux (cation efflux or anion influx) is occurring at the plasmalemma.

Table 5 shows some values of ψ_{co} which have been measured in microalgae, together with the method employed, the external medium in which the cells were bathed, and (where these could be calculated) values of ψ_{K^+}. All of the measurements show that the cytoplasm is negative with respect to the bathing medium, in agreement with the great majority of other cell types examined (see Section III.J and Raven, 1981). None of the values of ψ_{co} measured with microelectrode

(micropipette) techniques shows ψ_{co} to be more negative than ψ_{K^+}; it was noted in Section III.J that this technique is liable to underestimate the value of ψ_{co} in small cells. The only unequivocal case of ψ_{co} being more negative than ψ_{K^+} derives from the work of Komor and Tanner (1976) and Schwab and Komor (1978) on *Chlorella vulgaris* in which lipid-soluble cation distribution was employed as the method for measuring ψ_{co}. It is noteworthy (Table 5) that microelectrode experiments on other strains of *Chlorella* did *not* show ψ_{co} more negative than ψ_{K^+}.

The experiments on $TPMP^+$ distribution in *Chlorella vulgaris* may give an estimate of ψ_{co} which is too high (too negative) if $TPMP^+$ is metabolized and the labelled derivative is measured along with the unchanged $TPMP^+$, or if $TPMP^+$ is accumulated in intracellular compartments which are more negative than the cytoplasm. The first possibility ($TPMP^+$ metabolism) was examined and rejected by Komor and Tanner (1976). The second possibility cannot be refuted directly on the basis of the experiments of Komor and Tanner (1976), but it is rendered unlikely by consideration of possible volumes and potentials of these electrically negative compartments. The two most likely such compartments are the mitochondrial matrix and the chloroplast stroma. While the former may have a potential of some −150 mV with respect to the surrounding cytoplasm, its volume in *Chlorella* is very small (Table 2A; see also Fig. 1 of Raven and Smith, 1977), so an error in ψ_{co} of less than 10 mV is caused by assuming that the accumulation of $TPMP^+$ in the mitochondrial matrix is part of a higher average intracellular accumulation. Although the chloroplast stroma is a much larger fraction of the cell volume (Table 2A), it is likely to be only 10–20 mV negative with respect to the cytoplasm (Raven and Smith, 1977, Fig. 1), so again the assumption that $TPMP^+$ concentrated in the stroma is part of the average cell $TPMP^+$ concentration only alters the estimate of ψ_{co} by less than 10 mV. Less quantitatively important, and working in the opposite direction, is the exclusion of $TPMP^+$ from such positive phases (with respect to the ground cytoplasm) as vacuole and thylakoids. Thus the estimates of ψ_{co} for *Chlorella vulgaris* are unlikely to be more than about 10 mV too negative.

With respect to the estimates of ψ_{K^+}, it is unfortunate that there are no direct estimates of the intracellular K^+ concentration for the *Chlorella* strain used by Komor and Tanner (1976) and by Schwab and Komor (1978). However, these workers have measured ψ_{co} over a wide range of external K^+ concentrations; in the presence of 15 m*M* K^+ the value of

TABLE 5. Electrical potential difference between the cytoplasm and the bathing medium of microalgal cells

Alga	Method	Conditions	ψ_{co} (mV)	ψ_{K^+} (mV)	Reference
Anacystis nidulans	Distribution of $TPMP^+$	pH 7.7, 1 m*M* Tris/PIPES, light 50 W m^{-2}	−118		Paschinger (1976, 1977)
		pH 7.7, 1 m*M* Tris/PIPES, light 50 W m^{-2}, DCCD 0.15 m*M*	−77		
		pH 7.7, 1 m*M* Tris/PIPES, dark	−107		
	Distribution of $^{86}Rb^+$	pH 7.7, 1 m*M* Tris/PIPES, light 50 W m^{-2}, Valinomycin 2 μ*M*, Rb^+ 250 μ*M*	−107		
		pH 7.7, 1 m*M* Tris/PIPES, light 50 W m^{-2}, DCCD 0.15 m*M* Rb^+ 250 μ*M*	−77		
Chlamydomonas reinhardtii	Cyanine dye fluorescence		(inside negative)		Stavis (1974)
Chlorella pyrenoidosa	Microelectrode	5 m*M* KNO_3, 1 m*M* KCl, 2 m*M* $MgSO_4$, 0.25 m*M* $Ca(NO_3)_2$, 0.5 m*M* Na_2HPO_4, 0.5 m*M* NaH_2PO_4	−40	−71	Barber (1968b)
Chlorella fusca 2118b	Microelectrode	pH 7, 10 μ*M*, KCl, light, 30 °C	−55	−260	Langmüller and Springer-Lederer (1974); Paschinger and Vanicek (1974)
		pH 7, 10 m*M*, KCl, light, 30 °C	−30	−80	
		pH 9, 10 m*M*, KCl, light, 30 °C	−40	−80	
		pH 3, 10 m*M*, KCl, light, 30 °C	−20	−80	
		pH 7, 10 m*M*, NaCl, light, 5 °C	−12	−80	
		pH 7, 10 m*M*, NaCl, light, 30 °C	−25		Langmüller and Springer-Lederer (1976)
		pH 7, 10 m*M*, NaCl, light, 30 °C			
Chlorella vulgaris	Distribution of TPMP	25 m*M* NaP_i buffer:			
		pH 6, light–air	−135		Komor and Tanner (1976) (cf. Schaedle and Jacobsen, 1965)
		pH 6, dark–N_2	−87		

		pH 7, light–air	−150		1966, 1967; Barber, 1968a,b; Shieh and Barber, 1971)
		pH 5, light–air	−100		
		pH 6, light–air, 15 m*M* K^+ (citrate)	−125	−60	
		pH 6, light–air, 10 m*M* 6-deoxyglucose	−107		
		pH 6, light–air, 100 μM FCCP	−79		
		pH 6, light–air, 250 μM DNP	−63		
		pH 6, light–air, 10 m*M* NaN_3	−67		
Euglena gracilis strain *baccilaris* (dark-grown or apoplastidic mutant)	Microelectrode	0.1 m*M* $MgSO_4$, 0.1 m*M* KCl, 0.1 m*M* KH_2PO_4, 0.54 *M* mannitol, 5 m*M* MES, HEPES or cycloacid buffer			Zeldin (1977) (cf. Nicholls and Rikmenspoel, 1977); see Table 6 for internal K^+ concentration
		$pH_0 = 7–7.5$	−47		
		$pH_0 = 6$	−33	(−100)	
		$pH_0 = 5$	−20		
Gonyaulax polyedra	Cyanine dye fluorescence	0.5 *M* NaCl, 1 m*M* Tris, pH 8.1, ±KCl, valinomycin	(inside-negative)		Adamich *et al.* (1976)
Haematococcus pluvialis	Microelectrode	1 m*M* KNO_3, 0.4 m*M* K_2HPO_4, 0.3 m*M* $MgSO_4$	−20		Sineschekov *et al.* (1976)
Mougeotia	Microelectrode	0.65 m*M* NaCl, 0.675 m*M* Na_2SO_4, 0.05 m*M* K_2SO_4, 0.05 m*M* $CaSO_4$, 0.5 m*M* Na_2HPO_4/NaH_2PO_4, pH 7, light or dark	−40	−157	Wagner and Bentrup (1973)
Spirogyra	Microelectrode	0.1 m*M* KCl, light	−100		Fujii *et al.* (1978)
		0.1 m*M* NaCl			
		0.1 m*M*, $CaCl_2$, dark	−70		
		0.1 m*M* KCl, light	−146		
		0.1 m*M* NaCl, dark	−127		
		0.1 m*M* NaCl, light	−132		
		0.1 m*M* NaCl, dark	−122		

ψ_{co} was still −25 mV; if the observed ψ_{co} was a K^+ diffusion potential the value of $[K^+]_i$ would have to be more than 1.5 *M*. This is extremely unlikely, since work on two other freshwater *Chlorella* strains shows mean intracellular K^+ concentrations maintained at about 160–170 m*M* despite changes in the external K^+ concentration for growth in the range 0.4 to 100 m*M* (Schaedle and Jacobsen, 1965; Shieh and Barber, 1971), suggesting a ψ_{K^+} of about −60 mV when the external K^+ concentration was 15 m*M* (Table 5). In view of the dominant role of K^+ in the osmotic potential of *Chlorella* it is unlikely that the ground cytoplasm could remain isotonic with the organelles if its K^+ concentration were substantially different from that in the organelles, i.e. the ground cytoplasm K^+ concentration must be close to that in the whole cell.

Thus there is strong evidence for an electrogenic pump (cation efflux or anion influx) at the plasmalemma of *Chlorella*. Consistent with this, treatments which might be expected to decrease the rate of operation of a primary active transport process (Table 1; Komor and Tanner, 1976) diminish ψ_{co} to values which could be accounted for as diffusion potentials, although such treatments could also depolarize a membrane potential caused solely by passive ion diffusion by altering the relative permeabilities of the various contributing ions.

Granted the occurrence of a hyperpolarizing electrogenic pump, we are left with the problem of assigning its generation to a particular ion flux. A simple test of which ion is involved is to withhold that ion from the system; in this case we would need to withhold the suspected anions (for electrogenic anion influx) from the bathing medium, and the suspected cations (for electrogenic cation efflux) from the cytoplasm. Patently it is easier to test the anions in a whole-cell system. The only anion added deliberately to the bathing medium in the experiments of Komor and Tanner (1976) and of Schwab and Komor (1978) was phosphate in the form of 25 m*M* phosphate (neglecting the 25 μ*M* Cl^- added with the $TPMP^+$ as counter ion). Thus the electrogenesis could be due to active phosphate influx: certainly there are very large tracer fluxes of phosphate across the plasmalemma of *Chlorella* from external solutions containing 1 m*M* or more phosphate (West, 1967; Aitchison and Butt, 1973; Schneider and Frischknecht, 1977a). An indirect argument against the involvement of phosphate transport comes from experiments on sugar influx in *Chlorella* which appears to be driven by the electrogenic pump via secondary active transport: here the active

sugar influx can occur in the absence of external phosphate. HCO_3^- is present in the bathing medium in equilibrium with air, at least in the solutions at pH 6 and pH 7 where the electrogenesis is most pronounced (see Table 5). However, it is unlikely to be the ion causing electrogenesis, since the growth conditions of the cells (photolithotrophic, with 1–2% CO_2 in all; Kandler, 1955; Tanner, Dachsel and Kandler, 1965) would repress the synthesis of the active HCO_3^- pump at the plasmalemma (see Section IV.B).

With respect to internal cations, it is less easy to manipulate the composition of the cytoplasm than of the bathing medium. However, it is possible to change the internal pH of *Chlorella* (by adding weak acids or weak bases to the bathing medium: Tromballa, 1977), and it is also possible to increase the internal Na^+ (by growth on a low K^+, high Na^+ medium: Shieh and Barber, 1971). In both cases an increased intracellular level of the cation (H^+ and Na^+ respectively) can lead to an increased efflux of that cation in exchange for external K^+ when this is available; in the case of the Na^+-rich *Chlorella*, the internal pH is lower than that in control cells and some H^+ as well as Na^+ is excreted upon K^+ readdition (Tromballa, 1977; Shieh and Barber, 1971). Further, a net H^+ extrusion occurs when *Chlorella* grown on a low salt medium is supplied with an external K^+ salt, and net K^+ influx occurs: the K^+ in the cell is partnered by newly synthesized organic (carboxylate) anions whose synthesis generates the H^+ which is excreted (Schaedle and Hernandez, 1969; cf. Schaedle and Jacobsen, 1965). In none of these cases has the electrical potential difference been monitored during the net efflux of putatively electrogenic ion.

An obligate involvement of Na^+ in so fundamental a process seems unlikely since it is possible to grow *Chlorella* at near-control rates of growth in the complete absence of external or internal Na^+ (O'Kelley, 1974). Evidence consistent with a role for H^+ in electrogenesis comes from work already alluded to as being *against* a necessary role for phosphate influx in electrogenesis, i.e. the characteristics of sugar influx (Komor, 1973; Komor and Tanner, 1974a, b, 1976) in *Chlorella vulgaris*. The addition of sugar (glucose or an analogue) to cells with a derepressed glucose transport system causes an immediate net influx of H^+, which soon (in a few tens of seconds) ceases, with resumption of the previous small influx or efflux of H^+. However, the sugar influx continues at a steady rate. If the initial H^+-glucose stoichiometry of 1 : 1 for influx is maintained, there must be an H^+ efflux in the steady state

which just equals the sugar-coupled H^+ influx, and which must be through a different channel from the H^+-sugar influx. As will be shown below, a net H^+ flux through such a channel is against the H^+ free energy gradient by some 20 kJ mol^{-1}, i.e. must be active. Evidence that it is electrogenic comes from consideration of the time course of ψ_{co} after addition of sugar. After an initial depolarization (probably due to net positive charge entry by the H^+-sugar symporter) the membrane potential recovers to within some 20 mV of its previous value, i.e. still gives evidence of electrogenesis. If positive charge continues to enter, there must be some positive charge efflux which balances this and restores the potential difference to near its original value. Economy of hypothesis demands that the positive charge efflux be identified with the H^+ efflux. Thus the simplest explanation of the results of Komor and Tanner (1974a, b, 1976) is that there is an electrogenic H^+ extrusion pump in the plasmalemma of *Chlorella vulgaris*. (The absence of complete recovery of the electrical potential difference after addition of sugar can readily be explained by the occurrence of an additional leak channel which lets positive charge into the cell when the glucose-porter is working; the electrogenic pump is unable to maintain such a high H^+ free energy difference when transporting H^+ at the higher rate.) These results will be further analysed later in relation to pH regulation in the cytoplasm (Section IV.I). It may be noted that the fungal toxin fusicoccin, which has proved of great value in elucidating the electrogenic H^+ extrusion pump in higher plants does not seem to influence H^+ fluxes in microalgae including *Chlorella* (Marré, 1978). In higher plants this compound seems to increase the rate of pumping through an H^+-translocating ATPase; the absence of such an effect in the algae does not necessarily mean that an H^+-translocating ATPase is absent from these algae (there could be a fusicoccin-insensitive pump), but does not provide evidence *for* such a pump.

The balance of evidence thus seems to favour H^+ efflux as being the source of the electrogenic activity in *Chlorella*. Most of the properties of this which have been proposed to date have been deduced from studies of cotransport of H^+ with glucose and its analogues (Section IV.B). From experiments on the ultimate biochemical energy source for glucose influx (see Section III.H) it has been concluded that the energy source for active H^+ extrusion in *Chlorella* is ATP, or a very close metabolic relative of ATP (Komor and Tanner, 1974c). This ATP can be supplied by oxidative phosphorylation, cyclic photophosphoryl-

ation or (very slowly) by fermentation when these processes are the sole sources of ATP *in vivo* (Komor and Tanner, 1974c; Komor, Loos and Tanner, 1973).

A minimum estimate of the capacity for active H^+ efflux can be made from the maximum rate of glucose influx, the glucose/H^+ stoichiometry measured immediately after glucose addition, and the assumption (see above) that the absence of a net H^+ influx during steady-state glucose influx reflects active H^+ efflux balancing the H^+ influx during H^+-glucose symport. This minimum capacity estimate is 20 pmol cm^{-2} s^{-1} (Tanner *et al.*, 1974). Rather lower rates of H^+ efflux may be computed from the H^+ extrusion during restoration of normal intracellular K^+ and Na^+ levels in Na^+-rich *Chlorella* (Shieh and Barber, 1971) and during net K^+ influx and organic acid synthesis in "low-mineral" *Chlorella* (Schaedle and Jacobsen, 1965, 1966, 1967; Schaedle and Hernandez, 1969): these fluxes are 5 and 2 pmol cm^{-2} s^{-1} respectively. Large apparent net H^+ fluxes occur at light–dark (H^+ efflux) and dark–light (H^+ influx) transitions in *Chlorella* and its relatives, and indeed in most photosynthetic eukaryotic cells in an aqueous medium (Atkins and Graham, 1971; Neumann and Levine, 1971; Brinckmann and Lüttge, 1972; Raven, unpublished observations): however, the extent to which these apparent fluxes depend on photosynthetic and respiratory inorganic carbon fluxes is unclear. The initial light-dependent H^+ influx (and the corresponding dark efflux) occur, although at a diminished rate, when the bathing medium is sparged with CO_2-free air (Brinckmann and Lüttge, 1972; Raven, unpublished), although they seem to be essentially absent from *Chlamydomonas* mutants which lack phosphoribulokinase and hence are unable to carry out light-dependent CO_2 fixation (Neumann and Levine, 1971).

The H^+ free energy difference generated by the pump, and against which it works, is some 20 kJ mol^{-1}, based on measurements of the pH difference (Komor and Tanner, 1974a) and the electrical potential difference (Komor and Tanner, 1976) between the cytoplasm and the bathing medium in *Chlorella vulgaris*. This free energy difference may be rather greater at an external pH of 5 than at pH 7, since the increase in ΔpH as the external pH falls is energetically greater than the fall in ψ_{co}. For an ATP-powered pump, the usual free energy of hydrolysis of ATP *in vivo* of some 55 kJ mol^{-1} could power H^+ net efflux against a free energy difference of 20 kJ (mol $H^+)^{-1}$ with a stoichiometry of 2 H^+ per

ATP (cf. Walker and Smith, 1975, 1977a; Smith and Walker, 1976; Keifer and Spanswick, 1978).

The kinetics of transport of H^+ and sugar and their stoichiometry with metabolism argue for a $1H^+$/ATP stoichiometry. The argument (Raven, 1977d; Walker and Smith, 1977a; Tanner *et al.*, 1977) runs as follows. The influx of non-metabolized analogues of glucose in the dark is accompanied by a respiratory stimulation of more than 1/6 mol O_2 per glucose analogue transported; since the analogues are not metabolized, it is reasonable to attribute this stimulation to the increased energy demand for sugar influx (Decker and Tanner, 1972). With an ATP/O_2 of 6 (P/e_2 of 3), this suggests an ATP consumed/glucose transported ratio of 1. The respiratory stimulation is never as low as 1/12 O_2 per glucose which would be indicative of an ATP/glucose of 0.5; even on the unreasonable assumption that *all* of the basal ATP synthesis is diverted to sugar influx (leaving none for essential maintenance processes) this ratio is scarcely reached. Since the H^+/sugar ratio is 1 (Komor and Tanner, 1974a, b; Tanner *et al.*, 1974, 1977) and there is no net H^+ influx during steady sugar accumulation, there must be an equal active H^+ efflux, which accordingly must have an H^+/ATP of 1.

This conclusion has been challenged by Poole (1978). He argues from sugar influx under conditions (712 nm light, gas phase N_2, in the presence of the photosystem II inhibitor DCMU: see Fig. 7) when only cyclic photophosphorylation is available as an ATP source. Under these conditions the ratio einsteins of light (mol-quanta) absorbed by the cell to mol of glucose analogue taken up is 1.26 ± 0.51 under light-limiting conditions (Komor, Loos and Tanner, 1973). Poole (1978) further argued that the ratio of einsteins absorbed to ATP produced is 2 in cyclic photophosphorylation, and hence concluded that the ATP/glucose in the experiments of Komor, Loos and Tanner (1973) is 0.5, and that the H^+/ATP is 2 for the H^+ pump (the H^+/sugar ratio being 1). However, the evidence that the einsteins absorbed/ATP produced ratio is 2 for cyclic photophosphorylation *in vivo* is contradicted by a considerable body of data (see Raven, 1976b; Lindemann, 1977) which suggests that the ratio could be as low as 1 (*cf.* Fig. 7b). This in turn leads to the conclusion that the H^+/ATP ratio is 1, which is in agreement with the respiratory data of Decker and Tanner (1972): furthermore, it must be remembered that the cyclic photophosphorylation data do not allow a correction for "basal energy

metabolism" such as is possible for the respiratory data. The data of Tanner *et al.* (1968) on the quantum requirement of glucose uptake and metabolism in *Chlorella* (4.1 einsteins per glucose transported and assimilated under conditions in which only cyclic photophosphorylation can occur) can only with difficulty be accommodated in a scheme with an einsteins/ATP ratio of 2 in cyclic photophosphorylation and an H^+/ATP ratio of 2 at the plasmalemma. An ATP/glucose ratio of at least 1.5 is needed for the observed metabolism of glucose (Tanner *et al.*, 1968; Decker and Tanner, 1972), so even if only 0.5 mol ATP per mol glucose are needed for transport the overall einsteins per mol ATP is less than 2, even allowing for no other ATP-using processes in the cell. These data also fit more easily with an einstein/ATP ratio of 1 and a plasmalemma H^+/ATP ratio of 1, which also brings them into accord with the respiratory data.

If the evidence for primary H^+ extrusion at the plasmalemma is circumstantial in such freshwater eukaryotic algae as *Chlorella*, it is essentially non-existent in marine microalgae (or, indeed, in the giant-celled marine algae which have been much more intensively examined biophysically: Raven, 1976a, 1981). In marine algae it appears that cotransport systems are coupled largely to Na^+ (Ullrich-Eberius and Yingchol, 1974; Hellebust, 1978), although this need not be inconsistent with a primary H^+ extrusion which drives a (secondary) active Na^+ efflux and hence Na^+-coupled cotransport (cf. *Halobacterium:* Section III.E). The demonstration (Gimmler and Schirling, 1978; cf. Kaplan and Schreiber, 1977) of H^+–Na^+ antiport at the plasmalemma of *Dunaliella* begs the question, since it is likely that active H^+ efflux and active Na^+ efflux are necessary, so an Na^+–H^+ antiporter would be required regardless of which of these cations was transported by primary active transport and which by secondary active transport (Sachs, 1977b).

Recent work by Fujii *et al.* (1978) on the freshwater filamentous green alga *Spirogyra* has provided further evidence for the involvement of H^+ extrusion in electrogenesis in freshwater eukaryotic algae. The values of ψ_{co} obtained in these experiments (see Table 5) do not unequivocally show that the electrical potential difference is more negative than can be accounted for as a diffusion potential for K^+, since no values for the cytoplasmic K^+ concentration are available; comparison with the estimated cytoplasmic K^+ concentration of the related *Mougeotia* (Wagner and Bentrup, 1973) and the overall intracellular K^+ content of

two species of *Spirogyra* (Goldman *et al.*, 1972) suggest that the most negative potential measured by Fujii *et al.* (1978) may be close to ψ_{K^+}. The best evidence for an electrogenic pump in *Spirogyra* comes from the properties of the light-induced hyperpolarization of ψ_{co}: this hyperpolarization is independent of the presence of 0.1 m*M* of KCl or NaCl in the external medium (although it is abolished by 100 m*M* of KCl or NaCl), suggesting that it is not a result of light-induced changes in passive transport of these cations. Further, it is indifferent to the nature (Cl^- or SO_4^{2-}) of the external anion, suggesting that it is not caused by electrogenic anion influx. Thus, by the classic process of elimination, Fujii *et al.* (1978) suggested that the light-induced hyperpolarization is caused by an increase in the rate of active H^+ efflux.

In view of the fragmentary nature of the *in vivo* evidence as to the occurrence and nature of electrogenesis at the eukaryotic algal cell plasmalemma, we can turn to the study of plasmalemma-derived vesicles which have proved very useful in studies of transport in other microorganisms (Harold, 1977a, b). If these vesicles are "inside-out", it is possible to manipulate energy and transported substrate supply to transport systems in a way not possible in intact cells. In this way redox-dependent and ATP-dependent electrogenic H^+ transport, and ATP-dependent Ca^{2+} transport have been demonstrated (Section III.B), using suitable probes for measuring transmembrane free energy differences for the ions (Section III.J).

Thus far this powerful technique has not proved of much use with eukaryotic microalgae. It is not easy to identify and isolate the plasmalemma-derived vesicles in homogenates, and the preparations are generally leaky (Decker and Tanner, 1975; Tanner *et al.*, 1977). However, Grass and Marme (1978) have found evidence for ATP-powered Ca^{2+} transport in putative plasmalemma vesicles from the alga *Cryptomonas* as well as from a range of higher plants.

A related technique, which does not yield so much definitive data as does the isolation of transporting vesicles, is to identify ion-activated ATPases in the plasmalemma. Here the activation of the ATPase by ions which are known to be actively transported, and its inhibition by treatments which selectively inhibit active transport, can be used to associate an ATPase with a given transport process.

Meszes and Erdei (1969a, b) found a membrane-associated Mg^{2+}-ATPase in *Scenedesmus obtusiusculus* which was slightly stimulated by K^+

and Na^+ and slightly inhibited by ouabain (the classic inhibitor of animal K^+, Na^+-activated Mg^{2+}-ATPases). However, evidence that the enzyme was associated with the plasmalemma was not very convincing, and ouabain is not a potent inhibitor of K^+, Na^+ transport in algae (Raven, 1971, 1976a). Meszes and Erdei (1969a) showed that the activity of this enzyme in synchronous cultures did show some correlation with the rates of cation transport *in vivo*, which might indicate a transport function for the enzyme.

Sundberg *et al.* (1973) briefly reported on the ATPase activity of partially purified plasmalemma preparation from several green filamentous microalgae (*Mougeotia*, *Zygnema*, *Ulothrix* and *Oedogonium*). These ATPases were K^+ stimulated; however, this preliminary report gives very little data for the evaluation of this work.

Much more detail was provided by Sullivan and Volcani (1974a, b, 1975) on the ATPase activities associated with a variety of membrane fractions from the apochlorotic diatom *Nitzschia alba*. The plasmalemma had an Mg^{2+}-ATPase activity which was synergistically activated by K^+ ($K_{1/2}$ = 50 m*M*) and Na^+ ($K_{1/2}$ = 2 m*M*), and had a pH optimum of 8.5.

Falkowski (1975a, b) has detected a NO_3^-, Cl^--stimulated ATPase in a number of phytoplankters (*Skeletonema costatum, Ditylum brightwellii, Dunaliella tertiolecta, Eutreptia gymnastica, Isochrysis galbana* and *Chroomonas salina*), but not in *Amphidinium carterae*. The enzyme from *Skeletonema costatum* has a $K_{1/2}$ for NO_3^- of 0.9 μM, and for Cl^- 11 μM. The relation of this enzyme to the plasmalemma is unclear, although the $K_{1/2}$ for NO_3^- resembles the *in vivo* NO_3^- influx $K_{1/2}$ (Falkowski, 1975a, b). A relationship with electrogenesis is unlikely since NO_3^- is not an obligate nutrient for any of these algae.

This enzymic data does not give a clear picture of primary active transport at the algal plasmalemma. From the skeletal details given the enzyme of Sundberg *et al.* (1973) seems closest to the K^+-ATPase (which is thought to be the basis of an electrogenic or electroneutral active H^+ pump) from the plasmalemma of higher plants (Beffagna *et al.*, 1977; Balke and Hodges, 1977), *Neurospora* (Scarborough, 1977; Bowman and Slayman, 1977) and of gastric mucosa (Sachs, 1977a; Sachs *et al.*, 1978). However, many more details of the algal enzyme (particularly its pH optimum, which in the other K^+ ATPases is between 6 and 7) are required before a valid comparison can be made. The enzymes of Meszes and Erdei (1969a, b) and of Sullivan and Volcani (1974a, b,

1975) resemble the animal plasmalemma ATPase in being activated by both K^+ and Na^+ (particularly the diatom enzyme, which shows synergistic activation), although they are not very ouabain-sensitive. They differ from the K^+-ATPases mentioned above in having a more alkaline pH optimum, which implies some bizarre substrate kinetics for H^+ in the normal cytoplasmic pH range if they are to act as an H^+ pump: an *increase* in cytoplasmic H^+ would lead to a *decreased* activity of the enzyme. The anion ATPase of Falkowski (1975a, b) may be compared with the Cl^- ATPase of higher plants (Hill and Hill, 1976), although its major substrate is probably NO_3^- and its Cl^- affinity (if expressed at the outer surface of the plasmalemma) would be something of a problem in view of the NO_3^-/Cl^- ratio of seawater which can be as low as 10^{-6}.

For cyanobacteria the direct evidence for electrogenesis that a value of ψ_{co} more negative than ψ_{K^+} provides is lacking (Table 5). The only pertinent data here are those of Paschinger (1976, 1977), and while many of his values of ψ_{co} in *Anacystis nidulans* are much more negative than the ψ_{K^+} which can be calculated from the data of Dewar and Barber (1973) in their experiments with 10 to 40 m*M* K^+ in the medium, Paschinger does not provide data for ψ_{co} under these conditions; under the conditions in which he measured ψ_{co} (0.025 m*M* Rb^+ in the medium), the value of ψ_{K^+} (ψ_{Rb^+}) would be more negative than any of his values of ψ_{co}, based on the $[K^+]_i/[K^+]_o$ values at low $[K^+]_o$ of Fig. 1 of Dewar and Barber (1973). However, the general effects of experimental conditions (DCCD; presence of darkness) on the value of ψ_{co} are consistent with the presence of an electrogenic pump carrying positive charge out of the cell. Any such pump could not, in the experiments of Paschinger (1977), be attributed to anion influx (except possibly that of HCO_3^-), since the only external anion was that contributed by the buffers used (Tris/PIPES).

Further evidence for the occurrence of an electrogenic pump at the cyanobacterial plasmalemma, and which also provides evidence for the involvement of H^+ efflux, was supplied by Masamoto and Nishimura (1977a). These workers showed that there was a light-induced net efflux of H^+ when cells were illuminated (as had previously been shown for *Anabaena variabilis* by Scholes *et al.* (1969) and for *Anacystis nidulans* by Brinkmann and Simonis (1977), provided photosynthetic CO_2 fixation and putative solute-H^+ cotransport effects on the pH did not predominate) in 50 m*M* KCl at pH 6.14. This net efflux may be related

to an increased energy supply for pumping when photoredox as well as respiratory redox reactions can take place. The H^+ net efflux is rapidly slowed and stopped unless the cation permeability of the plasmalemma is increased (by adding valinomycin or gramicidin in the presence of the already high $[K^+]_o$ of 50 m*M*); such treatments increase the extent of the net H^+ efflux several-fold, and may also increase the apparent initial rate. Even the H^+ net efflux in the absence of membrane modifiers greatly exceeds that which could occur if there were no charge-balancing ion movements (cf. the discussion of the area-based H^+ flux below, and considerations of membrane capacitance: Section III.B). However, the finding that the effective addition of a high-conductance channel for cations in parallel with the H^+ efflux pumps allows it to pump faster and for longer is good circumstantial evidence for its electrogenicity, as is the finding that the net H^+ extrusion (in the presence or absence of valinomycin) is inhibited by CCCP or by nigericin. This electrogenic pump cannot immediately be associated with ATPase activity, since it is possible (see below) that *Plectonema* has a redox-associated H^+ pump in its plasmalemma.

There appears to be reasonable evidence, therefore, that active, electrogenic H^+ efflux occurs at the plasmalemma of the cyanobacteria *Anacystis nidulans* and *Plectonema boryanum*. The H^+ free energy difference between the cytoplasm of *Anacystis nidulans* and the bathing medium appears to be about 12 kJ mol^{-1} in the dark and 15 kJ mol^{-1} in the light when the external pH is 7.7, based on the values of ΔpH obtained by Falkner, Horner, Werdan and Heldt (1976) and the values of ψ_{co} of Paschinger (1977) using equation (4). No $\Delta\bar{\mu}_{H^+}$ values can be calculated on the basis of the available data for *Plectonema boryanum*, although the data of Masamoto and Nishimura (1977a) can be used to calculate estimates of minimum capacity for net H^+ extrusion, granted certain assumptions about the composition and dimensions of *Plectonema* filaments. The data (Fig. 3) of Masamoto and Nishimura (1977a) show that the maximum initial rate of net H^+ efflux upon illumination is about 140 μmol H^+ (mg chl)$^{-1}$ h^{-1} in the "control" treatment (with 50 m*M* KCl in the medium), 200 μmol H^+ (mg chl)$^{-1}$ h^{-1} with added valinomycin, and 225 μmol H^+ (mg chl)$^{-1}$ h^{-1} with added gramicidin. If chlorophyll is 1% of the dry weight (cf. Bottomley and Stewart, 1977), dry weight is 25% of wet weight, and the filament radius is 2 μm, these values correspond to area-based fluxes in the range 10–16 pmol cm^{-2} s^{-1}, i.e. of a similar order to those computed for *Chlorella vulgaris* above

(20 pmol cm^{-2} s^{-1}), and to the values of H^+ influx in symport with an H^+-sugar ratio of 1 in *Plectonema boryanum* (see Section IV.B) of some 22 pmol cm^{-2} s^{-1} calculated from the data of Raboy and Padan (1978) and of Ginzberg *et al.* (1976), using assumptions similar to those employed in analysing the data of Masamoto and Nishimura (1977a) above.

Granted the occurrence of active H^+ fluxes at the plasma-membranes of cyanobacteria which can transform energy at a rate of some 0.3 μW cm^{-2} (from the $\Delta\mu_{H^+}$ of $15 \cdot 10^3$ J mol^{-1}, and a flux of $20 \cdot 10^{-12}$ mol cm^{-2} s^{-1}) we may reasonably ask where the energy comes from. In the absence of active plasmalemma vesicle preparations from cyanobacteria, we must resort to indirect arguments. Paschinger (1976, 1977) has concluded from his studies on cotransport processes in *Anacystis nidulans* that there is only an ATP-driven H^+ pump at the plasmalemma of this alga, and that photosynthetic and respiratory energy is made available to this pump *via* ATP synthesis on the thylakoids and diffusion across the cytoplasm to the plasmalemma. Raboy and Padan (1978) have, by contrast, concluded from their studies on cotransport processes in *Plectonema boryanum* that there are both ATP-driven *and* redox-driven H^+ pumps at the plasmalemma of this alga.

The results obtained by these two groups of workers will be discussed in more detail later (Sections IV.F and IV.G, and Section IV.B, respectively), but they will be briefly mentioned here. Both groups' arguments hinge on the use of DCCD which inhibits (*inter alia*) H^+-ATPase activity. Paschinger found that this compound inhibited putatively H^+-coupled cotransport processes at the plasmalemma without inhibiting CO_2 fixation. He thus argued that ATP synthesis at the thylakoid membranes must still be able to occur in the presence of DCCD (since CO_2 fixation is the major ATP-using process in photosynthesizing cells: Raven, 1976b), and hence that the inhibition of cotransport requires that cotransport involves a DCCD-sensitive H^+-ATPase at the plasmalemma, this being the only other plausible locus of DCCD action. Since DCCD would not be expected to inhibit redox-driven H^+ pump activity at the plasmalemma, he regarded his results as confirming data in the literature which are consistent with the absence of photosynthetic and respiratory redox reactions in the cyanobacterial plasmalemma (except necessarily in *Gloeobacter violaceum* which lacks intracellular membranes: Section II.A).

Raboy and Padan (1978), by contrast, found that DCCD did not inhibit putatively H^+-coupled cotransport processes at the

plasmalemma, provided photosynthetic or respiratory redox processes could occur (i.e. in the light, or in the dark in the presence of O_2); by contrast, when only ATP generated by substrate-level phosphorylation was available as an energy source for transport (i.e. in the dark in the absence of O_2), DCCD did inhibit the very low rate of cotransport. The latter result shows that DCCD was indeed inhibiting the plasmalemma H^+-ATPase, while additional inhibition of the thylakoid ATPase was confirmed by the finding that DCCD in the light inhibited CO_2 fixation while leaving plasmalemma cotransport processes unaffected.

The location of membrane-associated H^+ pumps which is suggested by the work of Paschinger (1977) on *Anacystis*, and of Raboy and Padan (1977, 1978) on *Plectonema*, is shown in Fig. 8. It is to be emphasized that the apparent contradiction may be related to the different organisms used: each scheme may be correct for the organism employed. Indirect evidence as to the presence of redox systems in the plasmalemma of cyanobacteria (based on cytochemical location of redox activity, or electron microscopic evidence for the absence of phycobilisomes from the plasmalemma: Lang and Whitton, 1973) is unlikely to resolve this problem: the isolation of plasmalemma preparations is essential. The evidence available at presence of alleged "transport ATPases" of cyanobacteria (Mirisalikova, 1968; Batterton *et al.*, 1972) does not clearly establish that the activity measured is indeed associated with the plasmalemma rather than with the thylakoids.

It must be concluded that the evidence on the occurrence and characteristics of electrogenic ion pumps in microalgae is not as abundant or as direct as one might desire. In the three best-investigated algae (the eukaryotic *Chlorella vulgaris* and the prokaryotic *Anacystis nidulans* and *Plectonema boryanum*) the evidence favours H^+ efflux as being the main primary active transport process at the plasmalemma. Its role in powering cotransport processes, and in pH regulation, will be considered in the remainder of Section IV (B–J). It must be emphasized that, even if H^+ is not the *primarily* transported ion, it must be *secondarily* actively transported in order to account for the value of the cytoplasmic pH (cf. Sachs, 1977b).

B. TRANSPORT OF CARBON SUBSTRATES

1. *Inorganic Carbon Substrates: Photolithotrophy*

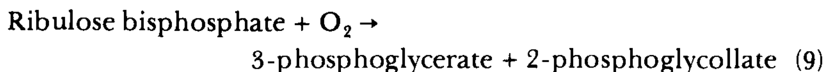
The fixation of inorganic carbon in microalgae requires the provision of CO_2 (not HCO_3^-: see Benedict, 1978) at the active site of

the enzyme ribulose bisphosphate carboxylase-oxygenase (RuBPc-o; 3-phospho-D-glycerate carboxylase (dimerizing), E.C. 4.1.1.39). This is the only carboxylase in O_2-evolving photolithotrophs which starts a reaction sequence leading to a net conversion of inorganic carbon to reduced organic compounds (see Walker, 1974; Raven, 1970, 1974b, 1977a, c; Benedict, 1978). The reaction (carboxylase activity, RuBPc) catalysed by this enzyme is:

$$\text{Ribulose bisphosphate} + CO_2 + H_2O \rightarrow 2 \times \text{3-phosphoglycerate} \quad (8)$$

This enzyme has three characteristics which may be construed as not overtly adaptive in an enzyme which is fixing CO_2 in an environment containing (at 15°C) some 10 μM CO_2 and 250 μM O_2. These are

(1) a high molecular weight of about 500,000; this, as we shall see, sets a limit on the quantity of enzyme per unit volume of photosynthetic organism, and thus has an important bearing on the length of the diffusion path for CO_2 from a source external to the organism (see Raven, 1977a, c, e, 1978);
(2) a relatively low turnover number at substrate saturation of about 10 s^{-1} (see Raven, 1978), thus compounding the problems introduced by (1); and
(3) the use of O_2 as an alternative substrate; this represents the so-called oxygenase activity (RuBPo), thus:



$$\text{Ribulose bisphosphate} + O_2 \rightarrow \text{3-phosphoglycerate} + \text{2-phosphoglycollate} \quad (9)$$

It has been suggested by Lorimer and Andrews (1973), and convincingly supported by data which has accrued in the intervening years (Andrews and Lorimer, 1978) that the oxygenase activity is an intrinsic and inescapable activity of this enzyme (but see Branden, 1978).

The relative affinities of the enzyme for CO_2 and O_2 mean that, with no diffusive limitation on CO_2 and O_2 supply to the active site of the enzyme (i.e. with the concentration at that site being 10 μM CO_2 and 250 μM O_2), the ratio of RuBPc to RuBPo (equations (8) and (9) respectively) is about 5 in air-equilibrated solution (see Raven, 1977a, c, 1978; cf. Laing *et al.*, 1974; Ku and Edwards, 1977a, b, 1978). This involves the production of the relatively unmerchandizable phosphoglycollate to the extent of 2C in phosphoglycollate per 11C

fixed: the phosphoglycollate is generally further metabolized by the photorespiratory carbon oxidation cycle (Lorimer *et al.*, 1978). The synthesis and metabolism of glycollate involves both O_2 uptake and CO_2 evolution, and thus runs counter to the reductive, carboxylative trend of photosynthesis; further, it consumes energy and demands the synthesis of additional enzymes.

The problem of RuBPo activity is exacerbated when the RuBPc-o is in a cell rather than in a well-mixed, dilute enzyme extract. Under these *in vivo* conditions, if CO_2 supply is by diffusion, then there must be a CO_2 diffusion gradient (concentration difference: equation 1) between the medium and the RuBPc-o molecules during net CO_2 fixation, i.e. $[CO_2]$ at the site of RuBPc-o activity must be less than that in the medium ($[CO_2]_o$). Conversely, the provision of reductant and ATP for the photosynthetic carbon reduction cycle requires O_2 evolution in the cell; disposal of this excess O_2 requires, in the steady state, that the O_2 concentration in the cell is greater than the O_2 concentration in the medium (Samish, 1975; Raven, 1977a, c, 1978; Berry and Farquhar, 1978). This has been analysed by Samish (1975), Raven (1977c) and Berry and Farquhar (1978). The concentration of CO_2 at the active site of RuBPc must be finite, i.e. the difference in CO_2 concentration between the medium and the cell is less than 10 μM. Since it is likely that D_{O_2} and D_{CO_2} in both the aqueous and membrane portions of the diffusion pathway are similar, and that the lengths of the diffusion paths are similar, then the difference in O_2 concentration between cell and medium is equal and opposite to the difference in CO_2 concentration. Since $\Delta [CO_2]$ is less than or equal to 10 μM, $\Delta [O_2]$ must also be less than 10 μM, so $[O_2]$ in the cell cannot be more than 260 μM when the external concentration is 250 μM. Thus the effect of diffusion gradients for the gases metabolized by RuBPc-o is much more important, relatively, in decreasing the CO_2 concentration at the site of enzyme activity than in increasing the O_2 concentration (Raven, 1977a, c, e).

This problem cannot be overcome by increasing the quantity of RuBPc-o per cell beyond a certain limit. The limited solubility and large size of the enzyme means that any additional enzyme molecule must be ever further from the source of CO_2 and the sink for O_2, and hence must operate at ever lower $[CO_2]/[O_2]$ ratios (see Raven, 1977c, 1978). Even in C_3 land plants, where CO_2 and O_2 transport outside the cell is by gaseous diffusion and convection, the $[CO_2]/[O_2]$ ratio in

the chloroplasts is sufficient to give a substantial C flux (more than one-fifth of the net C fixed) into phosphoglycollate and hence, by conversion to glycollate and operation of the "scavenging" photorespiratory carbon oxidation cycle, into compounds more useful to the plant than is glycollate. It is likely that terrestrial algae, and algae in lichens, conform to this pattern (see below). This pathway involves two sources of "inefficiency", the use of less than the full catalytic capacity of RuBPc-o for CO_2 fixation (some being used for O_2 metabolism), and the diversion of energy (see above) into the scavenging pathway.

In aquatic algae the situation is potentially much worse, in that the supply of CO_2 and the disposal of O_2 involves additional aqueous diffusion resistances, even when stirring (shearing) of the external medium is very effective (Raven, 1970; Samish, 1975; Lehman, 1978). This would result in a very low $[CO_2]/[O_2]$ at the site of RuBPc-o, and hence a very low ratio of RuBPc/RuBPo.

In a number of algae (enumerated by Raven and Glidewell, 1978) it appears that supply of CO_2 to RuBPc-o (and O_2 disposal from it) is only by diffusion (and extracellular convection) when the cells are grown at CO_2 concentrations in water substantially above the air-equilibrium values. This has been elegantly shown for *Chlamydomonas reinhardtii* by Berry and Bowes (1973): they showed that cells grown on "high CO_2" produced glycollate *in vivo* in a way which could be related quantitatively to the *in vitro* properties of the RuBPc-o extracted from the cells. Cells grown in this way have all of the "C_3 land plant" characteristics, i.e. inhibition of photosynthetic carbon fixation by O_2 (less photosynthesis with 10 μM CO_2 and 250 μM O_2 than with 10 μM CO_2 and low, near-zero, O_2), a finite CO_2 compensation concentration (the equilibrium concentration of CO_2 in a closed volume containing illuminated algal cells), a relatively high *in vivo* half-saturation constant for CO_2 fixation, and evolution of CO_2 in the light to CO_2-free air (and of tracer CO_2 from completely labelled cells to air): see Raven and Glidewell (1978). A further characteristic of such "high CO_2" cells is that the ratio of ^{13}C to ^{12}C in the organic C in the cells relative to that in the inorganic C supplied is characteristic of that expected from the operation of RuBPc not limited by diffusion of CO_2 (see Raven, 1970; Benedict, 1978; Estep, Tabita and van Baalen, 1978a; Estep *et al.*, 1978b). RuBPc fixes $^{12}CO_2$ faster than $^{13}CO_2$, provided the supply of ($^{12}CO_2$ + $^{13}CO_2$) is not limited by diffusion. If the enzyme

activity *is* diffusion-limited, then by definition the enzyme cannot pick and choose between the C isotopes, and the isotope ratio in the organic C reflects the (small) difference in diffusivity of the two isotopic forms of CO_2.

When these algae are grown at lower CO_2 levels, i.e. at or near those characteristic of air-equilibrated water, their photosynthetic properties change. Considering these properties in the order in which they were discussed for the algae grown at high CO_2 levels, they have a much smaller inhibition of CO_2 fixation from 10 μM CO_2 by 250 μM O_2 compared with near-zero O_2; their CO_2 compensation point is very near zero; their half-saturation constant for *in vivo* CO_2 fixation is much smaller; and evolution of CO_2 to CO_2-free air, or from completely ^{14}C-labelled cells to air, is very small and their carbon isotope composition relative to the inorganic carbon in the medium is consistent with transport limitation of inorganic C supply to the RuBPc (see Raven and Glidewell, 1977, 1978; Berry *et al.*, 1976; Lloyd *et al.*, 1977; Benedict, 1978; Barghoorn *et al.*, 1977). This "adaptation" to the lower CO_2 concentration is achieved without an increase in the activity of RuBPc-o per cell, or a change in the CO_2-saturated rate of photosynthesis *in vivo*; the half-saturation constant for CO_2 *in vivo* decreases while the *in vitro* half-saturation constant of RuBPc for CO_2 is unaltered (Berry *et al.*, 1976; Reed and Graham, 1977; Hogetsu and Miyachi, 1977; Findenegg, 1976a, b; Badger *et al.*, 1978b).

Granted the ability to extract and detect *in vitro* a very large fraction of the *in vivo* RuBPc-o activity (Lorimer, Badger and Andrews, 1977), it can readily be seen from a comparison of the *in vitro* properties of RuBPc-o and *in vivo* carbon fixation with respect to CO_2 fixed per cell per second and the $K_{1/2}$ with respect to CO_2 (Raven, 1970; Berry *et al.*, 1976; Raven and Glidewell, 1978) that CO_2 fixation *in vivo* by these algae at concentrations below 10 μM cannot be accounted for by a diffusive entry of CO_2. The *in vivo* rate of fixation and the *in vitro* properties of RuBPc can only be reconciled if the CO_2 concentration at the site of *in vivo* RuBPc-o activity is *higher* than the CO_2 concentration in the medium. This is particularly the case at high pH values (pH 9–10) where the internal CO_2 concentration would have to be more than ten times the external CO_2 concentration in order to account for the observed rate of *in vivo* photosynthesis with the measured *in vitro* properties of RuBPc-o (Raven, 1970; Raven and Glidewell, 1978; Berry *et al.*, 1976; Lehman, 1978).

The two most plausible explanations for this phenomenon both involve transport of carbon atoms at some part of the route from the bulk medium to the site of RuBPc-o in a form other than CO_2. By analogy with so-called "C_4" higher plants a preparatory carboxylation reaction could occur, with transport of the carboxylic acid anion produced to the site of RuBPc-o activity, where a decarboxylation step releases CO_2 for fixation by RuBPc. In the C_4 land plants the preparatory carboxylation is catalysed by phosphoenolpyruvate carboxylase (PEPc, E.C. 4.1.1.31), which brings about the reaction:

$$PEP + HCO_3^- \rightarrow \text{oxalacetate} + P_i \qquad (10)$$

This has the desirable attributes of a higher turnover number and a lower $K_{1/2}$ (expressed in terms of CO_2 in equilibrium with the true substrate, HCO_3^-, at cytoplasmic pH) than RuBPc, and furthermore is not inhibited by O_2. In order to make this "CO_2 pump" function efficiently, there must be some barrier to back-diffusion of CO_2 from the site of RuBPc action (where the decarboxylase regenerates it from the transported organic acid) to the medium. In the C_4 land plants this is achieved by a complex anatomical arrangement of cells with different enzymic complements, with substrate shuttling between them. Such an arrangement can quantitatively account for the characteristics of photosynthesis in such plants (see Raven, 1977c; Berry and Farquhar, 1978), including the conflicting requirements of preventing CO_2 leakage from the cells containing RuBPc-o without leading to the steady-state occurrence of very high O_2 levels which would tend to negate the CO_2 accumulation with respect to the ratio RuBPc/RuBPo.

Evidence for such a reaction in microalgae adapted to "natural" CO_2 levels is inconclusive: Döhler (Döhler, 1973, 1974a, b, c, 1976; Döhler and Wegmann, 1969; Döhler *et al.*, 1976) has long been an advocate of an important role for such a preparatory C_4 carboxylation, especially under transient conditions (Beardall *et al.*, 1976; Mukerji *et al.*, 1978). However, the evidence for an obligate role for such a reaction in steady-state photosynthesis (such as would come from $^{14}CO_2$ pulse-chase experiments) is lacking (cf. Döhler, 1974c; Kremer and Kuppers, 1977; Colman and Coleman, 1978). Further, it is not easy to see how such a mechanism could account for photosynthesis at high pH values in CO_2-depleted media, where the external CO_2 concentration is so low that the equilibrium HCO_3^- concentration in the cell (even assuming very high CO_2 permeability of the

plasmalemma—see below) would not support a sufficient rate of HCO_3^- fixation by PEPc.

The alternative is transport of inorganic C as HCO_3^-. It seems inescapable that photosynthesis at high pH values in media of low alkalinity and total inorganic C content, where the CO_2/HCO_3^- ratio is low, involves transport of HCO_3^- across the plasmalemma (Raven, 1970, 1974b; Raven and Glidewell, 1978; Lehman, 1978). The basis for this assertion is a consideration of likely cytoplasmic pH values, the RuBPc V_m and $K_{1/2}$, the observed rate of photosynthesis, and the external free CO_2 concentration. It is clear that the transport of inorganic C as HCO_3^- from the bulk medium to the outer surface of the plasmalemma, with conversion to CO_2 and CO_2 diffusion across the plasmalemma cannot account for the observed fluxes. Firstly, the calculations performed in support of transmembrane HCO_3^- transport employed the bulk CO_2 concentration rather than the lower CO_2 concentration close to the plasmalemma; and secondly the uncatalysed rate of conversion of HCO_3^- to CO_2 within the boundary layer is grossly inadequate to account for the observed carbon flux (cf. Burr, 1936; Briggs, 1959; Lucas, 1976; Lehman, 1978).

This transport of HCO_3^- must be facilitated: careful experiments on lipid bilayers (Kaethner and Bangham, 1977; Gutknecht *et al.*, 1977; cf. Raven, 1970) have shown that $P_{HCO_3^-}$ is very low (less than 10^{-10} cm s^{-1}; equation 5), while P_{CO_2} can be from 10^{-2} to more than 1 cm s^{-1}. Further, consideration of internal pH and the likely value of ψ_{co} require that the transport be active (Raven, 1968; 1970; Raven and Glidewell, 1978; Walker and Smith, 1977b). Further, entry of HCO_3^- cannot supply CO_2 sufficiently rapidly by the uncatalysed reaction $HCO_3^- \rightarrow CO_2 + OH^-$ to account for the observed rate of CO_2 fixation by RuBPc: this is consistent with the observed increase in the enzyme which catalyses this reaction (carbonic anhydrase) during adaptation to low external CO_2 concentrations (Reed and Graham, 1977; Findenegg, 1976a, b, 1977b; Berry *et al.*, 1976; Graham *et al.*, 1971; Badger *et al.*, 1978b). However, it should be noted that Findenegg (1976a, b, 1977b) found that inhibition of carbonic anhydrase did not greatly decrease the rate of photosynthesis by *Scenedesmus* cells at high pH, although photosynthesis by these low-CO_2-adapted cells was inhibited at lower external pH values.

HCO_3^- active influx may also be important at lower external pH values; the most elegant demonstration of this phenomenon comes

from the work of Findenegg (1976b) on *Scenedesmus*. He took advantage of the slow uncatalysed interconversion of CO_2 and HCO_3^- (these cells have no *extracellular* carbonic anhydrase) to follow the time course of C fixation when $^{14}CO_2$ *or* $H^{14}CO_3^-$ was added to an illuminated *Scenedesmus* suspension at a pH (pH 5.7) at which substantial concentrations of both CO_2 and HCO_3^- are present at equilibrium. After the tracer addition the equilibration reactions proceed slowly (with a half-time of 15-20 seconds at 20°C) to yield eventually an equilibrium mixture of HCO_3^- + CO_2 which was low enough to be rate-limiting for photosynthesis in Findenegg's experiments. If only CO_2 were entering the cells it would be predicted that the rate of carbon fixation would initially be higher than the final rate when the equilibrium CO_2/HCO_3^- ratio had been established, since the substrate (CO_2) concentration would *fall* as equilibration proceeded. Conversely, the carbon fixation rate immediately after addition of HCO_3^- should be negligible, and it would rise with time as equilibration proceeded and the external CO_2 concentration increases to the equilibrium value. In fact, Findenegg (1976b) found evidence for substantial HCO_3^- entry even at low external pH values (5.7). It should be noted that neither this result, nor the ability to photosynthesize at very high pH values, can be explained in terms of H^+ active efflux regulating internal pH followed by diffusive CO_2 entry (cf. Schilb, 1978).

Raven and Glidewell (1978) have argued that such an active HCO_3^- influx mechanism cannot account for *all* of the anomalous properties of microalgae grown at low CO_2 levels, since quantitatively similar active HCO_3^- influx mechanisms occur at the plasmalemma of organisms (e.g. *Chara corallina*) which do not seem to show the anomalous, C_4-type characteristics of photosynthesis. Thus, at least as far as eukaryotic algae are concerned, it is possible that an additional HCO_3^- transport ("CO_2-accumulation") step occurs at the inner membrane of the chloroplast envelope. This would account for the C_4-type characteristics of microalgae in very small volumes of external medium (Lloyd *et al.*, 1977; Bidwell, 1977). Here the CO_2 source is the atmosphere, and the volume of extracellular fluid is insufficient to permit conversion of CO_2 to HCO_3^- at a rate fast enough to account for the observed rate of carbon assimilation. However, a problem arises with the cyanobacteria, since they too show C_4-type characteristics even in the experiments with very small extracellular fluid

volumes ("artificial leaf experiments"), yet they have no membrane other than the plasmalemma between the medium and RuBPc-o (Lloyd *et al.*, 1977). Further, Schneider and Frischknecht (1977b) have shown that the carbonic anhydrase activity of air-grown *Anacystis nidulans* is inadequate to support the observed rate of C assimilation if an HCO_3^- to CO_2 conversion is obligatory, such as would be the case if HCO_3^- entered the cell but fixation was by RuBPc-o which uses CO_2. These observations also rule out CO_2 entry and an obligate "preparatory" fixation by PEPc, since this would require CO_2 to HCO_3^- conversion at a rate faster than the measured carbonic anhydrase activity could cope with. While HCO_3^- entry and initial PEPc fixation is possible (since no HCO_3^- to CO_2 conversion is required, and the decarboxylation which produces the inorganic carbon or RuBPc-o produces CO_2 rather than HCO_3^-: Raven, 1972a, b), this is not consistent with the experiments cited above (Lloyd *et al.*, 1977) which show CO_2 entry in *Anacystis nidulans*.

Raven and Glidewell (1978) have pointed out that the restriction of cyanobacteria in nature to pH values above pH 6 (Brock, 1973) is consistent with involvement of HCO_3^- entry in their normal photosynthesis. Many eukaryotic algae (see Raven and Glidewell, 1978) can grow at much lower external pH values when HCO_3^- is absent. However, it is not certain that these algae exhibit the "C_4-type photosynthesis": the acid waters in which they grow often have a CO_2 content greater than the air-equilibrium value (e.g. from ground-water enriched in metabolic CO_2), so they may not need a "CO_2-accumulating" mechanism. This may apply to subaerial algae and lichens mentioned earlier.

Thus far the discussion of "CO_2-accumulation" has been conducted in something of an experimental vacuum, with only indirect evidence that such accumulation occurs. Experiments have been carried out by Berry *et al.* (1978) and Badger *et al.* (1977, 1978a, b) on *Chlamydomonas* and *Anabaena*, and by Findenegg (1978) on *Scenedesmus*. These experiments involved measurement of the distribution of DMO and of inorganic C between the medium and the cells (see Sections III.B and III.J). The DMO distribution allowed the "mean" intracellular pH to be estimated; the inorganic C distribution could then be used to see if this could be accounted for in terms of passive processes (equations 2 and 7), or whether some active influx (presumably of HCO_3^-) is required. Berry *et al.* (1978) and Badger *et al.* (1978b) found that

inorganic carbon was present at a higher concentration in cells of both *Anabaena* and *Chlamydomonas* grown on low CO_2, but not in high-CO_2-grown cells of *Chlamydomonas* (Berry *et al.*, 1978; Badger *et al.*, 1977, 1978a, b), while Findenegg (1978) did *not* find any such accumulation in *Scenedesmus* grown at either high or low CO_2 levels. This latter finding is perhaps surprising, not only when compared with the work on *Anabaena* and *Chlamydomonas*, but also in relation to earlier work by Findenegg (1976b) which demonstrated HCO_3^- influx at the plasmalemma of *Scenedesmus* even at low external pH values. Clearly, more of these sort of experiments are required to resolve these seeming contradictions.

Thus the case for active HCO_3^- influx at the plasmalemma and/or chloroplast envelope membranes (in eukaryotes) has not been fully established. Even less evidence is available in favour of an obligate role for a preparatory carboxylation by PEPc which might also be expected to lead to a higher internal CO_2 concentration inside than outside. The observed low isotopic discrimination during algal photosynthesis at low external CO_2 concentrations (see above) could be accounted for by *either* transport limitation by a relatively non-discriminatory HCO_3^- pump (many porters can hardly distinguish K^+ from Rb^+ or Tl^+, so little discrimination between $H^{12}CO_3^-$ and $H^{13}CO_3^-$ would not be surprising), *or* by a major role for a preparatory PEPc reaction (Whelan *et al.*, 1973; Reibach and Benedict, 1977; Benedict, 1978). Alternatively, limitation by diffusion through the extracellular unstirred layers might be important (Lehman, 1978), in which case the isotopic fractionation cannot tell us much about the mechanism of C assimilation. Further investigations of isotopic discrimination in both organic and inorganic internal C, with different external pH values, external inorganic carbon concentrations and shearing rates might be useful in finding out what determines the discrimination in algae.

Aside from the question of the cause of the "C_4 characteristics" of algae, there is good evidence for HCO_3^- net influx at least at high external pH values and in "low-CO_2-adapted" algae. When such active HCO_3^- transport occurs, it is the dominant ion influx during steady-state growth (see Table 4); pH regulation in the cytoplasm (Section II.C and Section II.J) requires an effective net OH^- efflux (H^+ influx) almost equal to the HCO_3^- influx. The net active HCO_3^- influx (Table 4) may be quite close to the proposed upper limit for net active uniport (Section III.D); restrictions on passive H^+ uniport may be less

severe (Section III.E). However, it is by no means clear that the HCO_3^- influx is necessarily by primary HCO_3^- uniport; the relevant data from giant-celled algae is also consistent with H^+-HCO_3^- symport, with active H^+ efflux (primary active uniport) at sites on the membrane close to these symporters, and with passive H^+ uniport influx elsewhere on the plasmalemma. Whether this scheme of spatial separation of net HCO_3^- influx and OH^- extrusion which is derived from work on *Chara corallina* applies to microalgae is not certain. Further complications arise when the intracellular deposition of $CaCO_3$ is considered (Section IV.H).

A final point relates to the energetic inefficiency inherent in the operation of a pump for a charged species (HCO_3^-) into a compartment with a pH at which the uncharged species (CO_2) has a finite concentration after equilibration using carbonic anhydrase, and across a membrane which is permeable to the uncharged species (CO_2): cf. Section III.B. The maintenance of an internal CO_2 concentration of 40 μM with an external CO_2 concentration of 10 μM involves a transmembrane CO_2 concentration difference of 30 μM. A minimal estimate of P_{CO_2}, requiring a rather peculiar membrane composition (Gutknecht *et al.*, 1977), is 10^{-2} cm s^{-1}; inserting this value of P, and the concentration difference of 30 μM, into equation (1) shows that the net CO_2 leakage is 300 pmol cm^{-2} s^{-1}. This compares with a net C fixation rate in rapidly growing *Chlorella* (Table 4) of 90 pmol cm^{-2} s^{-1}. Thus a total influx of HCO_3^- of 390 pmol cm^{-2} s^{-1} is required to provide for a net fixation of 90 pmol cm^{-2} s^{-1}, and a leak of 300 pmol cm^{-2} s^{-1}. If each HCO_3^- taken up uses 1 ATP (e.g. H^+-HCO_3^- symport coupled to a 1 H^+/ATPase for H^+ extrusion), each net C fixed requires an additional 3.9 ATP in addition to the 3 ATP and 2 NADPH required by the biochemistry of the photosynthetic carbon reduction cycle.

However, there is an energetic offset to this extra ATP consumption: the maintenance of 40 μM CO_2 inside the cell largely suppresses RuBPo activity relative to RuBPc, even if the relatively low P_{CO_2} of 10^{-2} cm s^{-1} is also applicable to O_2 transport and the greater build-up of photosynthetic O_2 within the cell is considered (cf. Samish, 1975; Raven, 1977a, c). This suppression of RuBPo leads to an economy of capital expenditure and running costs of the photorespiratory carbon oxidation cycle (cf. Raven, 1981).

Whatever the mechanism, it is clear that microalgae can assimilate inorganic C from very low concentrations in the medium (this is not easy to explain in terms of any CO_2-concentrating hypothesis, since the

unavoidable CO_2 leakage cannot readily be recouped by active HCO_3^- influx in the absence of extracellular carbonic anhydrase). Whether this is the primary function of the mechanism is not clear: inorganic C is not usually the limiting nutrient for the growth of microalgae (Goldman *et al.*, 1972). R. H. Brown (1978) has argued convincingly that a role for the analogous "C_4 pathway" in higher plants is in economizing on the amount of N needed to support a given rate of C assimilation. The rationale here is that the additional N requirement to produce the enzymes of the preparatory CO_2 fixation pathway is more than balanced by the lower amount of RuBPc-o per unit C fixed per unit time due to the increased CO_2 concentration at the site of RuBPc activity, and the smaller quantity of enzymes of the photorespiratory carbon oxidation cycle. Whether this argument applies to algae is difficult to decide on the basis of currently available data: more selective advantage is likely to accrue from economizing in the use of a limiting nutrient (N) than in improving the efficiency of a less limiting nutrient (C). It also provides a rationale for the occurrence of the "CO_2-concentrating mechanism" in shade algae (Raven and Glidewell, 1975, 1978), where the major consideration may be that of economizing on the amount of energetically costly protein needed in the cell, since in a shade plant net energy acquisition is more likely to be limiting the growth rate than is net nutrient acquisition.

Finally in this consideration of inorganic carbon transport we return to microalgae which naturally obtain their inorganic carbon for photolithotrophic growth from the atmosphere. While it may be argued that the inorganic carbon for algal growth in a shallow pool at high pH is also supplied in part by the solution of atmospheric CO_2 into the water of the pool which has been depleted in CO_2 by net photosynthesis, the essential feature of terrestrial photosynthesis is that the aqueous phase diffusion path for CO_2 from the atmosphere to the carboxylase enzyme can be shorter than the minimal unstirred layer thickness for a cell submerged in a large volume of fluid. The diffusion path from the atmosphere-cell wall interface to the carboxylase can be about 1 μm, while the minimum unstirred layer thickness in microalgae in an aqueous medium is several micrometres at the minimum (Raven, 1970, 1977e; Raven and Glidewell, 1979). An important feature of cells carrying out terrestrial photosynthesis is some degree of water-repellency in the cell walls which maintains the air-water interface at the outer surface of the cell wall rather than at the surface of an external water film (Raven, 1977e).

The terrestrial plants relevant to this article are the free-living terrestrial algae (mainly chlorophytes/charophytes) and the lichenized chlorophyte/charophyte algae and cyanobacteria. The data on their photosynthetic physiology relevant to inorganic carbon transport is limited. It appears that the initial carboxylation step in free-living and in lichen algae is catalysed by ribulose bisphosphate carboxylase-oxygenase (Feige, 1974; Hill, 1976), and that the oxygenase activity of this enzyme is not suppressed as it is in many submerged algae. This contention is based on the high ^{14}C labelling in glycollate pathway compounds when the free-living alga *Trentepohlia* is supplied with $^{14}CO_2$ in the light, and an extrapolation of the net photosynthesis *versus* external CO_2 concentration relationship for illuminated lichens to zero CO_2 exchange which indicates a CO_2 compensation concentration of about 1 μM (Larson and Kershaw, 1975). These fragmentary observations suggest that terrestrial algae, like most of their terrestrial evolutionary derivatives (the macrophytic bryophytes, and tracheophytes or vascular plants) have C_3 metabolism and diffusive supply of CO_2.

As was indicated above, an important aspect of this mechanism of photosynthesis is a short liquid-phase diffusion path. Bertsch (1966) summarizes the effect of water content of terrestrial algae (free-living and lichenized) on their rates of net photosynthesis. The ability to carry out *some* photosynthesis at very low cell water contents is important in organisms which cannot regulate the loss of water to desiccating atmospheres (see Hoffman and Gates, 1970; Raven, 1977e); from our point of view the most interesting observation is that net CO_2 fixation is maximal in algae and lichens which are less than fully saturated with water. Saturation involves the presence of a surface film of water, and it is likely that the resistance to CO_2 transport is diminished when this surface film is absent in sub-saturated organisms (Bertsch, 1966; Collins and Farrar, 1978). This phenomenon cannot account for the results of Shomer-Ilan *et al.* (1979), who investigated the $^{12}C/^{13}C$ ratio in lichens growing in Israel in either the wet or the drier season. Shomer-Ilan *et al.* (1979) found that lichens which had been growing in the wet season had a greater ^{13}C content than those collected in the drier season, and suggested that this resulted from the greater fraction of photosynthate lost in respiration in the lichens, under dry as compared to wet conditions, rather than to differences in the carboxylase used in CO_2 fixation. Clearly the results are inconsistent with the hypothesis that under wet conditions the liquid-phase transport

resistance for CO_2 is increased, and that transport of CO_2 limits the rate of carboxylation and obliges the ribulose bisphosphate carboxylase to fix the ratio of $^{12}CO_2$ to $^{13}CO_2$ which diffuses to it, rather than being able to exhibit its intrinsic ability to discriminate between ^{12}C and ^{13}C. More work is needed to elucidate CO_2 transport in terrestrial algae.

2. *Organic Carbon Substrates: Heterotrophy*

Some algae are obligate heterotrophs, and rely on exogenous organic carbon as their energy and carbon sources. Only the obligate photolithotrophs cannot grow in this way, and even they can often take up organic compounds (often at a low rate). The nutritional middle ground of algae can grow photolithotrophically in light with CO_2, or heterotrophically in the dark with an organic carbon source. The organic carbon sources which can support the growth of algae include sugars, organic acids and amino acids (Droop, 1974). Of these, only the sugars will be considered here; amino acids will be considered under N sources in Section IV.C. Sugars can be used by the majority of algae, the major exceptions being the so-called "acid flagellates" which can grow heterotrophically only on organic (and sometimes amino) acids.

As has been discussed by Raven (1976a), algae capable of both photolithotrophic and heterotrophic growth show varying degrees of repression of the photosynthetic apparatus during growth in the presence of an organic carbon source; this repression should, at a naive level of analysis, allow the heterotrophic cell to be smaller than the photolithotrophic cell and/or to grow faster, as the photosynthetic apparatus can comprise 40% of the volume and weight of a phototrophic algal cell (Raven, 1976b). However, the precise selective advantages which such repressions may confer are not easy to analyse (Dykhuizen, 1978). At a simplistic level the uptake system for the organic carbon source, and the assimilatory enzyme(s) which convert the compound into a metabolite which is identical with a photosynthetic intermediate, are the heterotrophic equivalent of the photosynthetic apparatus during photolithotrophic growth. Despite the much smaller saving of materials and energy per cell associated with the repression of the specifically heterotrophic uptake and enzymic processes during photolithotrophic growth than accrues from repression of the photosynthetic apparatus during heterotrophic growth, such repression does occur to varying degrees.

In the Chlorococcales (*Chlorella, Ankistrodesmus, Scenedesmus, Bracteacoccus*, etc.) there are varying degrees of repression of glucose transport capacity during photosynthetic growth (Tanner and Kandler, 1967; Tanner, 1969; Tanner *et al.*, 1970; Sheath and Hellebust, 1974). Sugar specificity for derepression of the transport system is not the same as that for transport by the derepressed systems (Tanner *et al.*, 1970). The increased capacity for sugar transport when an "inducing" sugar was present was energy-dependent; the ATP required could be supplied by oxidative phosphorylation or by cyclic photophosphorylation or (at a much lower rate) by fermentation (Tanner and Kandler, 1967). The inhibitory effect of protein synthesis inhibitors (e.g. cycloheximide) on this sugar-dependent increase in sugar transport capacity suggested that protein synthesis was involved (Tanner and Kandler, 1967). This conclusion was substantially strengthened by the results of Tanner *et al.* (1974, 1977), who showed that induction of transport capacity led to an increased labelling (from tracer amino acid) of a membrane protein in *Chlorella vulgaris*.

A different regulatory mechanism has been described by Hellebust (1971a, b) for glucose transport in the diatom *Cyclotella cryptica*. Here the absence of light for 24 h from the photolithotrophically grown cells leads to the appearance of the sugar uptake system, even in the absence of the sugar substrate for transport from the medium. This increased capacity appears to involve protein synthesis (on the basis of inhibitor experiments); loss of transport capacity when light is restored is faster than mere dilution of stable porter during photosynthetic growth could account for. In another diatom (*Nitzschia laevis*) the glucose transport system is almost completely derepressed even in the light, as it is in some of the Chlorococcales (Lewin and Hellebust, 1978; Tanner *et al.*, 1970).

The sugar transport systems, regardless of their constitutive, substrate (sugar) induced or alternate (light, CO_2) substrate repressed nature, all seem to be capable of bringing about active sugar transport. The difficulties lie with determining the intracellular concentration of metabolized solutes (Section III.J) and hence of demonstrating active transport (equations 1 and 2). Despite this, Hellebust (1971a) has demonstrated a greater intra- than extracellular concentration of glucose during glucose uptake in the diatom *Cyclotella cryptica* although it is not entirely certain that the higher average intracellular concentration is not attributable to the accumulation of glucose in the vacuole rather than in the cytoplasm.

Generally, active sugar transport has been demonstrated predominantly using non-metabolized (or little metabolized) analogues. In this way, using for example 2-deoxyglucose, 3-*O*-methyl glucose, or 6-deoxyglucose, the accumulation of the sugar analogues inside *Chlorella vulgaris* cells to 1600 times the external concentration (equivalent to some 18.5 kJ mol^{-1}: equation 2) has been shown (Komor and Tanner, 1976). The evidence that these analogues are transported by the same mechanism as the metabolizable sugars comes from the mutual competition between the sugars and the analogues during uptake, and the parallel derepression of the capacity for transport of sugars and their non-metabolized analogues (Tanner, 1969; Komor and Tanner, 1971; Tanner *et al.*, 1970).

However, there must be some difference between the regulation of the porter activity by metabolized and non-metabolized sugars. The metabolized sugars generally only enter the cells as fast as the rate at which they are metabolized, with little accumulation of the free sugar, especially in the Chlorococcales (Decker and Tanner, 1972) with their very small relative vacuolar volume (see Table 2A), while the non-metabolized sugars can (as mentioned earlier) be accumulated up to 2000-fold (Sheath and Hellebust, 1974). Two possibilities for this difference are (1) that the metabolized sugars exert a much more effective trans-inhibition of influx than do the non-metabolized sugars, or (2) that some metabolic derivative of the metabolizable sugars is the regulatory agent. At all events, the transport of metabolized sugars is under kinetic control, while transport of the non-metabolized analogues proceeds to near thermodynamic equilibrium with the immediate energy source (Komor and Tanner, 1976).

The biochemical energy source for transport in the eukaryotes is probably ATP (see Section IV.A, and Komor and Tanner, 1974c). Most of the evidence suggests an indirect application of the energy from ATP, and that the immediate energy source is a trans-plasmalemma ionic free energy difference. In the freshwater green algae such as *Chlorella* there is very good evidence for H^+-sugar cotransport, while for the marine diatom *Cyclotella cryptica* the ion involved seems to be Na^+ (Komor, 1973; Komor and Tanner, 1974a, b, 1976; Hellebust, 1978).

The best-investigated case (by Komor and Tanner) is *Chlorella vulgaris*, where the addition of a transportable sugar or sugar analogue to transport-competent cells (i.e. those with both an induced porter system and an adequate energy source, i.e. oxidative or photosynthetic

phosphorylation) leads to both a net H^+ influx and a depolarization of ψ_{co} (Komor, 1973; Komor and Tanner, 1974a, b, 1976). After a few tens of seconds the net H^+ influx ceases, and even earlier the electrical potential difference begins a partial repolarization. Thus, during steady-state sugar influx, the H^+ *net* flux across the membrane is the same as it was prior to sugar addition, while the potential difference across the plasmalemma is less negative by some 20 mV (Komor and Tanner, 1976; see Table 6). This is consistent with H^+-sugar symport, which initially brings about a net H^+ influx which reduces internal pH and depolarizes the membrane. However, it seems that control mechanisms rapidly come into play which regulate ψ_{co} and pH_{cyt}. A major aspect of this regulation is an increase in the rate of active H^+ extrusion, although considerations of membrane capacitance mean that movement of some charge-balancing ion must occur very rapidly (see Sections III.A, IV.A and IV.J). This regulation is considered in more detail in relation to pH regulation (Section IV.J).

The H^+-sugar ratio deduced from the initial H^+ and sugar influx rates is 1 : 1. This kinetic value is consistent with the thermodynamic observation (Komor and Tanner, 1976) that the maximum free energy difference across the membrane for sugar analogues (18.5 kJ mol^{-1}) is very close to the measured $\Delta\bar{\mu}_{H^+}$ of 19.3 kJ mol^{-1}. The problem of the mechanism whereby the electrical component of $\Delta\bar{\mu}_{H^+}$ is used to bring about sugar transport is discussed by Schwab and Komor (1978).

Further kinetic analyses show that while initially upon addition of a transported sugar net and tracer influxes are equal, after considerable accumulation of a non-metabolized sugar analogue has taken place the net influx is less than the tracer influx, i.e. a tracer efflux is occurring. It seems that the tracer efflux is inversely related to the "energy availability" to the transport mechanism, i.e. the efflux is a greater fraction of the tracer influx in darkness in the absence of oxygen than in conditions in which oxidative phosphorylation and/or cyclic photophosphorylation can occur (Komor, Loos and Tanner, 1973). This "exchange diffusion" operation of the carrier appears to involve an energetic coupling between efflux and influx. The energy released during sugar efflux is only available for powering sugar influx: it cannot be used for "biochemical" energy-requiring processes (Komor and Tanner, 1974d; Tanner *et al.*, 1974). A facilitated diffusion operation of the active transport system does not occur under normal conditions at low external pH values (Komor *et al.*, 1972). This can be

induced by the addition of the polyene antibiotic nystatin (Komor *et al.*, 1974). In the presence of this agent (which interacts with sterol components of membranes) efflux of sugar analogues from pre-loaded cells can take place. This efflux is *via* the normal sugar carrier (Komor *et al.*, 1978).

Considerable progress has been made in modelling the sugar transport in *Chlorella vulgaris* in relation to the various equilibrium constants involved in glucose binding to, and release of glucose from, the protonated and unprotonated carrier, and the rate constants for transmembrane transfer of the various complexes (Komor *et al.*, 1973b), and also in accounting for the conversion of the high glucose affinity active transport system at low external pH values to a low affinity facilitated diffusion system at high external pH values (Komor and Tanner, 1975). At intermediate pH values the two systems coexist. This transition of porter properties with external pH leads to an efflux of previously accumulated sugar analogue when the external pH value is increased (Komor and Tanner, 1975).

A final point about the work on *Chlorella vulgaris* relates to the rate of transport as a function of the magnitude of the immediate driving force for transport, i.e. $\Delta\bar{\mu}_{H^+}$. It is likely that a substantial $\Delta\bar{\mu}_{H^+}$ remains under dark-anaerobic conditions in which the net active influx rate is greatly reduced (Komor, Loos and Tanner, 1973a). The evidence for maintenance of $\Delta\bar{\mu}_{H^+}$ under these "low-energy" conditions comes from the measurements of ψ_{co} under anaerobic, dark conditions (Komor and Tanner, 1976), and from the relatively small decrease in internal pH which is required to account for the observed decarboxylation of glutamate to γ-aminobutyrate (Lane and Stiller, 1970; Warburg *et al.*, 1957) in terms of the known *in vitro* pH dependence of the enzymic reaction. While it is not possible to predict precisely the rate-driving force relationship for cotransport from either rate theory or irreversible thermodynamics, it seems highly likely that purely thermodynamic control is unlikely (cf. Slayman, 1977), i.e. the rate of $\Delta\bar{\mu}_{H^+}$ consumption is regulated by a kinetic rapier rather than a thermodynamic bludgeon (cf. Section III.H).

In the marine diatom *Cyclotella cryptica* (Hellebust, 1978) the immediate energy source for sugar active influx appears to be a $\Delta\bar{\mu}_{Na^+}$. This conclusion is based on a dependence of transport on external Na^+ and inhibition of sugar transport by agents (e.g. the Na^+-selective cation uniporter monensin) which collapse $\Delta\bar{\mu}_{Na^+}$.

In cyanobacteria there is also evidence for active sugar influx, again involving the use of glucose analogues (Beauclerk and Smith, 1976, 1978; Raboy and Padan, 1977, 1978). In these prokaryotes the complications of intracellular compartmentation are much less significant than in eukaryotes, and the average intracellular sugar concentrations can be reasonably assumed to refer to the concentration in the ground cytoplasm. The evidence of Raboy and Padan (1977, 1978) on the active transport of 3-*O*-methyl glucose in *Plectonema boryanum* is generally consistent with H^+-coupled cotransport, e.g. the inhibition of influx under anaerobic, dark conditions by proton ionophores. Under these conditions the ionophores could not be interfering with ATP generation at the thylakoid membranes, and must be influencing the plasmalemma (aside from the remote possibility that they are causing massive hydrolysis of fermentative ATP *via* activation and short-circuiting of the thylakoid ATPases under these conditions). As was discussed in Section IV.A the results of Raboy and Padan (1977, 1978) are consistent with the occurrence of both redox-coupled and ATP-coupled H^+ pumps at the plasmalemma of *Plectonema boryanum*.

It remains to mention the ecological significance of these active sugar transport mechanisms. This is probably to be sought in the generally low sugar concentrations found in natural environments, and the *in vivo* kinetics of glucokinase (e.g. Hellebust, 1971a). The competitive heterotrophic ability of algae in comparison with more specialized microscopic heterotrophs is not clear.

C. TRANSPORT OF NITROGEN SOURCES

1. N_2: *Nitrogen Autotrophy*

Among the organisms discussed in this article only certain cyanobacteria are able to use N_2 as their N-source (Stewart, 1966, 1973). It might be expected that transport of N_2 from the medium to the site of nitrogenase activity would pose few problems, in view of the abundance of N_2 relative to other metabolized gases, and the high permeability of biological membranes to atmospheric gases (N_2 concentration in air-equilibrated water at 15°C is about 700 μM, compared with 250 μM O_2 and 10 μM CO_2; P_{CO_2} in a phospholipid-cholesterol bilayer is 0.35 cm s^{-1} (Gutknecht *et al.*, 1977). However, problems arise due to the universal O_2 inactivation of the purified N_2-fixing enzyme systems (nitrogenase) *in vitro* (Stewart, 1973). As will be

seen below, part of the ability of this enzyme to function *in vivo* in the presence of exogenous O_2 may be related to the presence of a permeability barrier to O_2 round the nitrogenase. This may pose problems for access of N_2 to nitrogenase, since the production of barriers to O_2 diffusion which are not quantitatively similar barriers to N_2 diffusion is unlikely (Crisp, 1963).

Stewart *et al.* (1978) list the cyanobacteria which have been tested for the ability to fix N_2. The general conclusions which can be drawn from this compilation are (1) not all cyanobacteria can fix N_2, (2) unicellular, and filamentous but non-heterocystous, cyanobacteria can only fix N_2 at low external O_2 levels, and (3) heterocystous algae can fix N_2 in the presence of atmospheric O_2 levels, although the optimal O_2 concentration is generally less than 250 μM (21 kPa of O_2). In the heterocystous cyanobacteria there is overwhelming evidence that the site of aerobic N_2 fixation is the heterocyst (a specialized cell in the filament: see Haselkorn (1978) for a recent review). Among non-heterocystous cyanobacteria aerobic N_2 fixation has been reported for pure cultures of *Gloeocapsa*, and for the marine *Trichodesmium* (probably an *Oscillatoria*), which is currently not available in pure culture (see Yates, 1978). The various mechanisms proposed for protection of nitrogenase from O_2 *in vivo* will be considered in relation to the involvement of transport of O_2 and of N_2.

Heterocysts of cyanobacteria lack photosystem II of photosynthesis and thus lack photosynthetic O_2 evolution; they have a high respiratory rate, and are surrounded by a cell wall with a composition different from that surrounding the adjacent, photosynthetically competent cells (which are termed vegetative cells). Organic carbon (as maltose?) for N_2 fixation enters the heterocyst, and organic nitrogen products of N_2 fixation are returned to the adjacent vegetative cells, *via* a cytoplasmic (symplastic) intercellular pathway which involves micro-plasmodesmata running through the intervening wall. On the heterocyst side of this path there is a "polar granule" of the arginine-aspartate copolymer cyanophycin. It has been suggested that these structural and metabolic characteristics provide protection of nitrogenase from O_2 by (1) not producing O_2, (2) having a high rate of O_2 consumption, and (3) having a cell wall which has a low permeability to O_2. Respiratory protection (alternative 2) is probably important in the aerobic heterotroph *Azotobacter* (Postgate, 1971; Yates, 1978), while Granhall (1976) and Haury and Wolk (1978) have shown

that aerobic N_2 fixation in the cyanobacterium *Anabaena variabilis* requires an intact, glycolipid-containing, heterocyst wall. The arguments used in Section IV.B suggest that the O_2 concentration in the neighbouring vegetative cells during photosynthesis are unlikely to be more than 10–20 μM higher than that in the surrounding water (250 μM or so).

To see if the possible rates of O_2 consumption in the heterocyst (possibility 2) are compatible with a value of P_{O_2} (= P_{N_2}) of the heterocyst which would allow N_2 entry at a fast enough rate to account for the observed rate of N_2 fixation with the observed *in vitro* nitrogenase characteristics, we can use the data obtained on the much-investigated *Anabaena cylindrica.* A low permeability to gases of the heterocyst investments reduces the amount of O_2 consumption needed in the heterocyst for respiratory protection but is difficult to achieve in terms of the permeability characteristics of biological materials, and may unduly restrict N_2 access to nitrogenase. For *Anabaena cylindrica* in continuous culture (continuously illuminated) with a generation time of 24 h, and nitrogen as 11% of the dry weight (7.8 μmol N per mg dry weight), the mean N fixation rate is 234 nmol (mg dry weight)$^{-1}$ h^{-1}, i.e. 117 nmol N_2 fixed (mg dry weight)$^{-1}$ h^{-1}. If the heterocysts occupy one-tenth of the filaments, the rate on a heterocyst basis is 1.17 μmol N_2 (mg dry weight heterocyst)$^{-1}$ h^{-1} (Bottomley and Stewart, 1976, 1977). This agrees closely with rates computed from acetylene reduction rates in these cells in the light, assuming that N_2 fixation is one third of the C_2H_4 assimilation. If the filament radius is 2 μm (inside the plasmalemma), the N_2 fixation rate calculated above corresponds to a flux *across the heterocyst plasmalemma exposed to the medium* of 8 pmol cm^{-2} s^{-1}. On the rather optimistic assumption that the capacity of nitrogenase exceeds the rate measured *in vivo* by a factor of 5 (cf. Shanmugam *et al.*, 1978), then the observed rate *in vivo* could be accounted for with one-fifth of the 700 μM N_2 used in *in vitro* assay present in the heterocyst, i.e. 140 μM. This involves a concentration gradient of 560 μM, which from equation (1) gives a P_{N_2} value of $1.4 . 10^{-5}$ cm s^{-1}.

Assuming that $P_{O_2} = P_{N_2}$, it is then possible to compute the rate of respiration in the heterocyst which would reduce the O_2 concentration there to some low value which would be consistent with the observed activity of nitrogenase (not more than 10 μM O_2 compared with the 250 μM outside). With an O_2 concentration difference of (250 − 10) or 240

μM, and $P_{O_2} = 1.4 \cdot 10^{-5}$ cm s^{-1}, the O_2 influx would be 3.4 pmol O_2 (cm^2 heterocyst exposed to the medium)$^{-1}$ s^{-1}. Reversing the computation by which the N_2 flux on an area basis was derived from the nitrogen assimilation per unit of heterocyst dry weight, this corresponds to an O_2 uptake of 500 μmol O_2 (mg heterocyst dry weight)$^{-1}$ h^{-1}. This is not excessive in relation to the rate of net carbon assimilation by the whole filament during growth with a generation time of 24 h, which is (from Bottomley and Stewart, 1977) 1 μmol C (mg dry weight)$^{-1}$ h^{-1}, i.e. 10 μmol C (mg heterocyst dry weight)$^{-1}$ h^{-1}. Thus "respiratory protection" would only require an increment of 5% of the net photosynthetic rate to account for the carbon respired in the heterocyst; this is well within the measured photosynthetic capacity of the organism (150 μmol O_2 (mg chl)$^{-1}$ h^{-1} or 1.5 μmol O_2 (mg total dry weight)$^{-1}$ h^{-1}) (Bottomley and Stewart, 1977). The measured respiratory capacity of heterocysts (using detached heterocysts: Fay and Walsby, 1966) is only some 100 μmol O_2 (mg heterocyst dry weight)$^{-1}$ h^{-1}, although the respiratory rate of the whole filaments from which heterocysts were derived in the experiments of Fay and Walsby (1966) was only some 50% of the rate measured by Bottomley (1975) for filaments grown under the conditions used in the experiments of Bottomley and Stewart (1976, 1977). Further, the high respiration-supported rates of N_2 fixation seen immediately after darkening (Bottomley and Stewart, 1977) requires an O_2 uptake to generate ATP (probably in the heterocyst) of at least 0.5 O_2 per N_2 fixed (cf. Table 3 of Shanmugam *et al.*, 1978) or 0.6 μmol O_2 (mg heterocyst dry weight)$^{-1}$ h^{-1}. While this, on a total dry weight basis, is a rather large fraction of the measured respiration rate (60 μmol O_2 (mg dry weight)$^{-1}$ h^{-1} out of 150 μmol O_2 (mg dry weight)$^{-1}$ h^{-1}: Bottomley, 1975), it is not impossible.

Thus respiratory protection is feasible, *provided* the low P_{O_2} (P_{N_2}) of $1.4 \cdot 10^{-5}$ cm s^{-1} can be achieved. As far as the heterocyst walls are concerned, the inner laminate zone may have a relatively low P_{O_2}. This zone appears to be constructed of at least 10 monolayers of a long-chain aliphatic glycolipid with a melting point of 113°C. Such "solid" monolayers may have a permeability to gases of as little as $2 \cdot 10^{-3}$ cm s^{-1} (Blank and Roughton, 1960), so ten such monolayers could have a permeability of only $2 \cdot 10^{-4}$ cm s^{-1}. This is an order of magnitude higher than the P_{O_2} (P_{N_2}) estimated earlier as being required for the operation of the "low O_2 permeability plus respiratory scavenging"

mechanism for keeping the O_2 concentration at the site of nitrogenase at a suitably low level. While the laminate zone of the cell wall may be more than 10 monolayers thick in the apical pore region, it must be remembered that this apical pore constitutes a high-permeability "leak" (a region of the cell wall pierced by microplasmodesmata, and apparently lacking the laminate zone) in parallel with the low-permeability wall in the laminate zone (cf. Haselkorn, 1978).

Thus the properties of the heterocyst envelope seem to be unable to account for the required barrier to O_2 entry, granted the assumptions made earlier as to nitrogenase activity *in vitro* and the respiratory capacity of the heterocyst (cf. Granhall, 1976; Haury and Wolk, 1978). If the *in vitro* capacity of nitrogenase is lower than that assumed earlier, a higher P_{N_2} must be assumed to supply N_2 at a sufficient rate to the lower catalytic activity in the heterocyst. While this is in better accord with the computed values of P_{N_2} for the heterocyst envelope, it implies a greater respiratory rate in the heterocyst to overcome the greater O_2 influx implied by the higher P_{O_2}. An upper limit on the extent of respiratory protection in a photolithotroph can be calculated as the difference between the maximum photosynthetic capacity of the organism and the observed net rate of carbon assimilation during growth; for *Anabaena cylindrica* the apparent surplus capacity is some 0.5 μmol C (mg total dry weight)$^{-1}$ h^{-1} (calculated from Bottomley, 1975; Bottomley and Stewart, 1976, 1977). It is not certain how much of this excess capacity is in fact used for respiratory protection during steady-state growth. Another aspect of N_2 fixation related to the permeability of heterocysts to gases is the recycling of H_2 (produced by nitrogenase reductase-nitrogenase) by the "uptake hydrogenase" (see Shanmugam *et al.*, 1978). Maximum recycling of H_2 requires a low permeability of the heterocyst envelope to gases, although the permeability to H_2 is likely to be higher than that to N_2 or O_2.

Another line of attack on the problem of permeability of the heterocyst to gases is to attempt to determine the extent to which N_2 fixation *in vivo* is limited by the rate of N_2 transport into the heterocyst. A possible test of diffusion limitation (see Section IV.B, 1) is the study of isotopic discrimination. If the nitrogenase enzyme *in vitro* can significantly discriminate between ^{15}N and ^{14}N, then the absence of discrimination *in vivo* would be consistent with diffusive limitation, since the nitrogenase would be forced to assimilate the two isotopes in the same ratio as they are supplied to the enzyme by diffusion (a

process which exerts very little discrimination). What little evidence is available suggests that N_2 fixation *in vivo* shows very little isotopic discrimination (Troughton *et al.*, 1974); it is not known whether this is due to an intrinsic lack of discrimination by the enzyme, or to diffusive limitation of an enzyme which does show significant discrimination. Measurements of isotopic discrimination by the enzyme *in vitro*, and measurements of *in vivo* isotopic discrimination in N_2 fixation by cyanobacteria, are required.

Turning to non-heterocystous cyanobacteria, Carpenter and Price (1976) have provided evidence for a spatial separation of photosynthetic O_2 evolution and N_2 fixation in *Trichodesmium*. Here it is suggested that the separation is based on a colonial aggregation of filaments, with the portions of the filaments in the centre of the faggot (meaning 1 of the "Concise Oxford English Dictionary"!) having little chlorophyll or capacity for light-dependent CO_2 fixation (and hence presumably having negligible O_2 evolution). Thus it is possible that the centre of the faggot may act as a "supracellular heterocyst" as long as the filament is intact, and indeed Carpenter and Price (1976) have shown that aerobic N_2 fixation by *Trichodesmium* is inhibited when colonies are disrupted. It is difficult to evaluate this hypothesis quantitatively on the basis of the data in the report of Carpenter and Price (1976): an analysis using the methods of Gerard (1931) could be attempted if data on colony size and on rates of O_2 metabolism were available. It must be borne in mind, however, that pure, N_2-fixing cultures of *Trichodesmium* are not yet available, and Knowles (quoted by Yates, 1978) has found aerobic heterotrophic N_2-fixers associated with *Trichodesmium*. If the N_2 fixation capacity of *Trichodesmium* were indeed associated with these heterotrophs, it is still possible that the non-photosynthetic centre of the faggots might be associated with respiratory protection of nitrogenase.

In the unicellular *Gloeocapsa*, where pure cultures have been shown to fix N_2 aerobically, it is possible that N_2 assimilation and photosynthetic O_2 evolution are temporally rather than spatially separated, with an inverse relation between the two activities in the growth cycle (Stanier and Cohen-Bazire, 1977).

Data currently available do not allow us to come to definite conclusions concerning the significance of transport processes in permitting aerobic N_2 assimilation in cyanobacteria: more critical data could, however, be obtained using extant techniques.

2. *Ammonia*

Ammonia can serve as a nitrogen source for all of the organisms considered in this article (Syrett, 1962; Morris, 1974). The term "ammonia" will be used for the large number of cases when there is no good evidence as to which chemical species (NH_3 or NH_4^+) is involved in a given transport or enzymic reaction; use of "NH_3" and "NH_4^+" indicates some certainty as to the species involved (see Cole, 1976; C. M. Brown, 1978). The evidence on this point is much less easily obtained than in the case of inorganic carbon. The reason lies in the rates at which the effective protonation-deprotonation reactions take place. While the ionic reaction $H_2CO_3 = H^+ + HCO_3^-$ is very rapid, the hydration reaction $H_2O + CO_2 = H_2CO_3$ is slow enough (when uncatalysed) to allow disequilibrium experiments (as discussed in Section IV.B.1) to be conducted, and thus for the identity of reactive species (CO_2 or HCO_3^-) to be established. For the reaction $NH_3 + H^+ = NH_4^+$ there is no such slow step, and less direct methods, such as the pH response of the reactions involving ammonia, must be used.

With the pK_a of NH_3/NH_4^+ of about 9.4, the dominant form (by a factor of about 100) at the cytoplasmic pH of 7.0–7.5 (Section IV.I) is NH_4^+. The great range of external pH values at which algae can grow means that the NH_3/NH_4^+ ratio varies widely. In the sea 5–10% of the total ($NH_3 + NH_4^+$) is present as NH_3. The entry of N into cells from media containing ($NH_3 + NH_4^+$) could (cf. Section III.B) be either as NH_3 (equations 1 and 2), or as NH_4^+ (equations 4, 5 and 6), or both NH_3 and NH_4^+ might enter (equation 7). When NH_3 equilibrates across the membrane, the equilibrium condition is that $[NH_3]_i = [NH_3]_o$, and net entry occurs when $[NH_3]_i < [NH_3]_o$. The total internal concentration of ($NH_3 + NH_4^+$) depends on the cytoplasmic pH. When NH_4^+ alone crosses the membrane, the equilibrium condition is that $\psi_{co} = RT/F \cdot \ln[NH_4^+]_o/[NH_4^+]_i$, and a net influx occurs when ψ_{co} is more negative than $RT/F \cdot \ln[NH_4^+]_o/[NH_4^+]$. Again, the total internal ($NH_3 + NH_4^+$) is a function of the cytoplasmic pH. More likely than either of these two extremes is that *both* the intrinsic membrane permeability to NH_3 (Section III.B) and the mediated transport of NH_4^+ (Section III.C) are involved in the determination of intracellular $[NH_3] + [NH_4^+]$.

Algae can grow at very low external ammonia concentrations when reduced inorganic N is their sole N source. The results of Eppley, Rogers and McCarthy (1969b), Eppley and Rogers (1970) and Eppley

and Renger (1974) (cf. Eppley, 1977) for marine microalgae, and of Healey (1977) and Steeman-Nielsen (1978) for freshwater microalgae, show that the relationship between short-term uptake and long-term N-limited growth is a rectangular hyperbolic function of the external $[NH_3 + NH_4^+]$. The apparent half-saturation constant is frequently well below 1 μM $[NH_3 + NH_4^+]$; this is similar to the values found for other "fixed" N-sources (NO_3^-, urea and amino acids: Sections IV.C, 3, 4 and 5), but is lower than for N_2 (Section IV.C, 1: $K_{1/2}$ of about 18 μM: Burris, 1966).

Further analysis of the mechanism of uptake (equations 1–7) is aided by measurements of the cytoplasmic $[NH_3 + NH_4^+]$. The determination of this during ammonia assimilation is made difficult by the problem of preventing metabolism during separation of cells from the bathing medium, and the possibility of compartmentation (Section III.J). A further problem arises from the possibility of ammonia release from (probably) amides during the killing and extraction procedures (Ohmori and Hattori, 1974). Many estimates of $[NH_3 + NH_4^+]$ levels in microalgae give very small values: some which give higher values (e.g. a few m*M* in *Platymonas* (Wheeler, 1977), 10 m*M* in *Ditylum* (Eppley and Rogers, 1970) and 6 m*M* in *Pyrocystis* (Kahn and Swift, 1978)) can reasonably be attributed to compartmentation. Certainly there is good evidence for a "non-metabolic" arginine pool in *Platymonas* (Wheeler and Stephens, 1977; cf. Matile, 1978). This putative accumulation of ammonia in vacuoles is supported by more direct data from giant-celled algae (Barr *et al.*, 1974; Smith *et al.*, 1977, 1978; Smith and Walker, 1978; Walker *et al.*, 1979a, b). Low cytoplasmic-phase (i.e. ground cytoplasm, mitochondrial matrix and chloroplast stroma) levels of ammonia are also consistent with the avoidance of uncoupling of photophosphorylation in photolithotrophs (cf. Tillberg *et al.*, 1977), and with the measured levels of ammonia in vertebrate cells (Abrahams and Younathon, 1971). Further, some regulated enzymes respond to m*M* concentrations of ammonia independently of the effect of NH_4^+ acting as an analogue of K^+ (Abrahams and Younathon, 1971).

This internally consistent, if circumstantial, evidence for a low level of ammonia in algal cytoplasm (less than 5 m*M*) is difficult to square with the data of Kempner and Miller (1974: see Table 6). These workers grew *Euglena gracilis* on four different growth media, and in all cases found large amounts of ammonia in the cells on a dry weight basis. Even with the high wet/dry weight ratio reported by these authors the

TABLE 6. Concentration of solutes in cells of *Euglena gracilis* grown in light–dark cycles on the minimal medium of Kempner and Miller (1972) with glutamate as carbon and nitrogen source

Solute	concentration mmol (kg wet wt)$^{-1}$	mmol +ve charge (kg dry wt)$^{-1}$	mmol −ve charge (kg dry wt)$^{-1}$
K^+	10.8	102	
Na^+	1.0	9	
Ca^{2+}	0.01	0.2	
Mg^{2+}	6.2	116	
NH_4^+	18.0	172	
Arginine$^+$ (free)	3.4	32	
Arginine$^+$ (protein)		77	
Lysine$^+$ (free)	1.0	9	
Lysine$^+$ (protein)		87	
Aspartate$^-$ (free)	0.75		7
Aspartate$^-$ (protein)			107
Glutamate$^-$ (free)	6.1		57
Glutamate$^-$ (protein)			158
Cl^-	0.1		1
Total P			565
		604	895

Data of Kempner and Miller (1965, 1972, 1974) and Miller and Kempner (1976). The dry weight of the cells grown on the Kempner and Miller (1972) medium is a very small fraction of the wet weight (10.6%); this accounts for the low concentration of (e.g.) K^+ on a wet weight basis. Higher fractional values of dry weight in *Euglena gracilis* have often been reported (e.g. Cramer and Myers, 1952; Cook, 1963). Organic and inorganic phosphates are all treated as having one negative charge per molecule (which is reasonable in view of the internal pH values shown in Table 7). SO_4^{2-} is not accounted for; neither are organic acids. Even with this known underestimate or negative charge, there is a considerable excess of negative over positive charge accounted for. The nitrogen balance sheet would allow the missing positive charge to be accounted for as membrane-associated choline and ethanolamine, and as polyamines (cf. Rolle *et al.*, 1971). Even if the high NH_4^+ level reported in this work is to some extent an artifact of hydrolysis of (e.g.) amides, this would not alter the overall charge balance situation, since —$CONH_2$ is giving rise to —COO^- and NH_4^+.

"average" ammonia concentration within the cell water is 18 m*M* (Table 6). This value is not easy to explain as being due to compartmentation since NH^{4+} is the single most abundant cation in the cells of those analysed. However, comparison of the positive and negative charges accounted for in the very complete analyses of the composition of *Euglena* undertaken by Kempner and Miller (1974; Table 6) shows that a lot of positive charge is unaccounted for. It is possible that whichever cation (or cations) balances the excess anions is cytoplasmic, and the NH_4^+ is largely vacuolar; there is sufficient nitrogen unaccounted for in these analyses to permit a substantial cytoplasmic

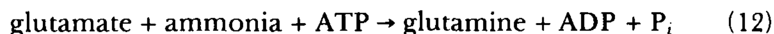
concentration of (e.g.) polyamines. The occurrence of 18 m*M* NH_4^+ in the cytoplasmic phases of *Euglena* would certainly be a problem for photophosphorylation, at least during growth on the photolithotrophic medium of Lyman and Siegelman (1967) which was one of the growth media used by Kempner and Miller (1974). However, evidence for a high cytoplasmic phase level of ammonia seems even more difficult to escape on the basis of the non-algal work of Conway and Breen (1945), who showed that yeast could be grown (albeit at a reduced rate) with essentially all of the intracellular K^+ replaced by NH_4^+.

On the basis of this discussion it is to be hoped that the reader will not feel that prejudice has triumphed over evidence in my conclusion that cytoplasmic phases of algae do not generally have *more* than 1 to 2 m*M* ammonia concentration in them. As to the *minimum* concentration of reduced inorganic nitrogen, we can turn to the known kinetics and activities of the ammonia assimilating enzymes in algae. The ammonia concentration needed to account for *in vivo* assimilation rates (per cell) on the basis of *in vitro* $K_{1/2}$ and V_{max} (per cell) characteristics of the assimilatory enzymes can be taken as the lowest level of ammonia which must be present at the site of assimilation.

Two ammonia assimilation pathways may be present in algae. The "historic" assimilatory enzyme producing amino compounds is glutamic dehydrogenase (E.C. 1.4.1.3), which catalyses the reaction:

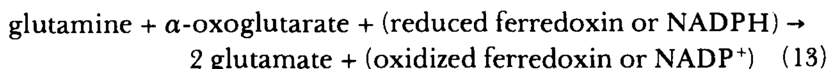
$$\alpha\text{-oxoglutarate} + \text{ammonia} + \text{NAD(P)H} = \text{glutamate} + \text{NAD(P)}^+ \quad (11)$$

The more recently discovered pathway involves the long established enzyme glutamine synthetase (E.C. 6.3.1.2) which catalyses the reaction:



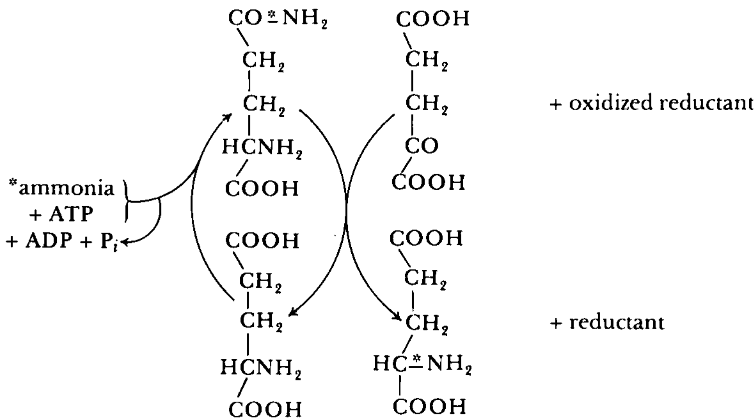
$$\text{glutamate} + \text{ammonia} + \text{ATP} \rightarrow \text{glutamine} + \text{ADP} + P_i \quad (12)$$

and the recently discovered (Brown *et al.*, 1974) glutamate synthetase (E.C. 2.6.1.53) which transfers an amino moiety from the amide group of glutamine to aminate alpha-oxoglutarate, thus:



$$\text{glutamine} + \alpha\text{-oxoglutarate} + (\text{reduced ferredoxin or NADPH}) \rightarrow 2\ \text{glutamate} + (\text{oxidized ferredoxin or NADP}^+) \quad (13)$$

Reactions (12) and (13) bring about the reaction sequence:



It has been suggested (Brown *et al.*, 1974) that glutamine synthetase-glutamate synthetase, which involves extra running costs (1 ATP per ammonia assimilated) and two enzymes rather than one, permits rapid ammonia assimilation at lower cytoplasmic ammonia concentrations than does glutamate hydrogenase (cf. Raven, 1976a).

Evidence as to which pathway of ammonia assimilation is followed *in vivo* comes from studies of enzyme activities *in vitro*, and of the *in vivo* kinetics of the assimilation of ammonia labelled with ^{13}N or ^{15}N (Brown *et al.*, 1974; Brown, C. M., 1978; Miflin and Lea, 1976; Falkowski and Rivkin, 1976; Edge and Ricketts, 1978; Meeks *et al.*, 1977, 1978); most evidence favours the glutamine synthetase-glutamate synthetase pathway, although in *Chlorella* spp. a specific NADPH-linked glutamate dehydrogenase is derepressed in media containing ammonia (Kretovitch *et al.*, 1970; Schmidt, 1974; Gronostajski *et al.*, 1978).

The required ammonia concentration needed to account for the observed *in vitro* rates of nitrogen assimilation *via* the glutamate dehydrogenase pathway in terms of the V_{max} and $K_{1/2}$ of the extracted enzymes is about 10 m*M* for the marine diatom *Ditylum brightwellii* (Eppley and Rogers, 1970; Eppley, Rogers and McCarthy, 1969b), and about 1 m*M* for *Chlorella* (Table 5; Kretovich *et al.*, 1970; Gronostajski *et al.*, 1978). It must be emphasized that the required *in vivo* concentration of ammonia depends not only on the amount of enzyme present and its $K_{1/2}$ for ammonia, but also on the degree of saturation with its other substrates. Particularly important in this regard is alpha-

oxoglutarate; if (as is likely) the alpha-oxoglutarate concentration in the cytoplasm (stroma?) is inadequate to saturate the enzyme (cf. Gronostajski *et al.*, 1978), relatively more ammonia is needed than if all the other substrates are present in excess (cf. Sims, 1976).

Despite the much lower $K_{1/2}$ for ammonia for glutamate synthetase in the presence of saturating amounts of the other substrates, and the generally high V_{max} of the enzyme *in vitro* relative to the V_{max} of ammonia assimilation *in vivo* (Brown *et al.*, 1974; Brown, C. M., 1978; Miflin and Lea, 1976; Falkowski and Rivkin, 1976; Edge and Ricketts, 1978), various factors conspire to increase the ammonia/ammonium concentration at the site of enzyme action which are required to achieve the *in vivo* rate of N assimilation. The chief among these is the high $K_{1/2}$ for glutamate. Generally the glutamate concentration in algal cytoplasm (as judged from the average intracellular concentration of glutamate in algae with relatively small fractional vacuolar volume, such as *Chlorella* and *Euglena*) is between 17 and 26 m*M* (Warburg *et al.*, 1957; Kanazawa, 1964; Lane and Stiller, 1970; Kempner and Miller, 1974), i.e. less than the $K_{1/2}$ for glutamate of algal glutamine synthetase (Miflin and Lea, 1976; Falkowski and Rivkin, 1976). In some freshwater algae (Guillard, 1960; Kauss, 1974) the total osmotic potential of the cells (37–75 mOs*M*) is not compatible with even this glutamate concentration, in view of the requirement for other solutes for cytoplasmic functioning (Raven, 1977a).

These considerations suggest that the ammonia concentration at the site of algal glutamine synthetase activity cannot be less than 10 μM to achieve the half-maximal rate of nitrogen assimilation (based largely on the results of Falkowski and Rivkin (1976) on the diatom *Skeletonema costatum*). The site of glutamine synthetase and glutamate synthetase (and of NADP-linked glutamate dehydrogenase, which has a much lower $K_{1/2}$ for ammonia than does the NAD-linked enzyme: Gronostajski *et al.*, 1978) is probably in the chloroplast stroma of eukaryotes (Haystead, 1973; Givan, 1975; Mitchell and Stocking, 1975; Anderson and Done, 1977; Gayler and Morgan, 1976). One cannot therefore argue directly from the minimum ammonia concentration needed to account for the *in vivo* rate of assimilation (in the chloroplast stroma of a eukaryote) to the concentration in the ground cytoplasm in contact with the plasmalemma. However, the alkalinity and electronegativity of the stroma relative to the cytoplasm (see Fig. 1 of Raven and Smith, 1977) are unlikely to effect a major concentration factor for ammonia

in terms of the driving forces implicit in equation (7), so we may approximate this minimal "assimilatory" concentration of ammonia within the cell to the required concentration in the ground cytoplasm.

For the marine *Skeletonema costatum*, then, the 10 μM ammonia concentration needed for half-maximal nitrogen assimilation rates is achieved at external ammonia concentration of about 1 μM (Eppley, Rogers and McCarthy, 1969b). We can see how plausible is the "lipid solution" hypothesis (Section III.B, equation 1) for NH_3 influx across the plasmalemma, by considering the P_{NH_3} required to account for the observed influx, making the limiting assumption of zero internal NH_3. From the data of Parsons *et al.* (1961) it is possible to calculate that the ammonia influx in *Skeletonema costatum* at 1 μM ammonia outside is 5 pmol cm^{-2} s^{-1}. At an external pH of 8 the NH_3 concentration is some 0.06 μM, so P_{NH_3} for the plasmalemma must be at least 0.1 cm s^{-1}. This is probably plausible for a molecule like NH_3 (Gutknecht *et al.*, 1977; Walker *et al.*, 1979b). However, if we look at the concentration gradient which is compatible with maintaining an internal [NH_3/NH_4^+] of 10 μM with a cytoplasmic pH of 7 (cf. Section IV.I), we find that the internal and external concentrations of NH_3 are equal, thus providing *no* net driving force for NH_3 entry. Granted the assumptions involved in the computations, the required internal concentration could be an order of magnitude lower for this rate of assimilation, thus providing a sufficient NH_3 driving force, granted a reasonable P_{NH_3} of about 10^{-1} cm s^{-1}. However, it is clear that the safety margin for NH_3 entry alone in supplying nitrogen assimilation in *Skeletonema costatum* is small. The situation becomes even more precarious for this hypothesis when we consider freshwater algae which can live at much lower pH values. A *Chlorella* cell growing at the rate shown in Table 4, with an N influx of about 5 pmol cm^{-2} s^{-1} at half-saturation concentration of the N source, and the *in vivo* $K_{1/2}$ for ammonia assimilation for freshwater microalgae of Healey (1977) of 1 μM at an external pH of 7, requires an internal [NH_3 + NH_4^+] of 1 μM at a cytoplasmic pH of 7. Since $pH_i = pH_o$, the internal and external NH_3 concentrations are equal, thus providing no net driving force for NH_3 influx even while providing an inadequate concentration of ammonia in the cytoplasm. The situation gets worse as the external pH is decreased.

These considerations, while not conclusive, do suggest that NH_3 entry by a lipid solution mechanism is only marginally acceptable as an explanation of ammonia entry in microalgae under ecological con-

ditions. The problem is, of course, compounded by not knowing what the cell's "objective" is in accumulating ammonia prior to assimilation: is the important parameter the NH_3 concentration or the NH_4^+ concentration? If the latter is important, a more flexible accumulation mechanism which does not depend on the external pH being higher than the internal pH is the electrogenic passive uniport of NH_4^+; here (equations 4–7) both NH_4^+ and NH_3 concentrations in the cytoplasm can be maintained at higher concentrations in the cell than outside over a range of external pH values as long as the inside-negative potential difference is maintained. In a range of non-algal microorganisms (heterotrophic bacteria: Strenkoski and DeCiccio, 1971; Kleiner, 1975; Stevenson and Silver, 1977; Kleiner and Fitzke, 1979; and fungi: Pateman *et al.*, 1973, 1974; Slayman, 1977; Roon, Levy and Larimore, 1977a; Roon, Meyer and Larimore, 1977b; Roon *et al.*, 1978) and in giant-celled algae (Smith *et al.*, 1977, 1978; Smith and Walker, 1978; Walker *et al.*, 1979a, b) there is evidence for a higher ammonia (methylammonia) concentration in the cells than can be explained in terms of NH_3 (CH_3NH_2) permeation and the internal pH value which has been measured or which seems plausible. The ammonia analogue methylamine has been very useful in these studies, since it is transported (with varying efficiency in different organisms) by the ammonia transport mechanism, yet is relatively slowly metabolized (Smith *et al.*, 1978). The most powerful and direct method of measuring the entry of the cationic form of these solutes involves "voltage clamping": since this involves placing two micropipettes in a single cell it is not appropriate to microalgae (Smith *et al.*, 1977; Smith and Walker, 1978; Slayman, 1977). The data of Abeliovich and Azov (1976) can be interpreted (Smith *et al.*, 1978) in terms of $CH_3NH_3^+$ influx in *Scenedesmus* cells. Other work on *Scenedesmus* (Pribil and Kotyk, 1970) used very high ammonia concentrations (up to 40 m*M*) and relatively unspecified external pH values, and the findings are therefore difficult to interpret, particularly when the internal ammonia concentration reaches 200 m*M*. Passive entry of CH_3NH_2 was assumed by Falkner, Horner, Werdan and Heldt (1976) in their investigations of internal pH in *Anacystis nidulans*.

Such an NH_4^+ ($CH_3NH_3^+$) uniport constitutes, in the presence of the apparently unavoidable passive fluxes of NH_3 (CH_3NH_2), an uncoupling mechanism, i.e. can bring about a net (downhill) H^+ influx across a membrane which maintains a large, inwardly directed H^+ free energy

difference. Thus (cf. equation 7) in a cell with an internal concentration (maintained by NH_4^+ uniport) of NH_3 + NH_4^+ of 10 μM at a pH of 7.3, i.e. with about 0.1 μM NH_3, and with negligible $[NH_3]$ outside (e.g. 1 μM NH_3 of pH 6), a P_{NH_3} of 10^{-1} cm s^{-1} gives a 1 pmol cm^{-2} s^{-1} flux. This would, for the cases quoted above when net influx is 5 pmol cm^{-2} s^{-1}, thus give a "short-circuit" flux of 20% of the total net flux. Thus the total NH_4^+ influx must be 6 pmol cm^{-2} s^{-1} and both the assimilated NH_4^+ and the leaking NH_3 leaves excess H^+ in the cell. NH_4^+ assimilation leaves an excess of about 1.2 H^+ in the cell, while each leaking NH_3 leaves 1 excess H^+ in the cell (cf. Raven and Smith, 1974, 1976a). Considerations of charge balance and the maintenance of an inside-negative potential difference, and of pH regulation (Sections IV.A and IV.I) require that all of this H^+ is actively extruded from the cell. The portion of this H^+ efflux which is equivalent to the NH_3 leakage represents the energetic cost of the leakage (uncoupling).

It is important to note that this leakage of NH_3 occurs even when the nitrogen source is N_2, NO_3^- or organic nitrogen, provided that the assimilation of these nitrogen sources requires the same $[NH_3 + NH_4^+]$ to be maintained in the cell to supply the assimilatory enzyme. This may well provide a powerful selective force to limit the internal $[NH_3 + NH_4^+]$ (cf. Rowell *et al.*, 1977) to little above that needed to supply the assimilatory enzyme, and hence limit the "uncoupling" leakage of NH_3. This leakage may be as significant as the action of ammonia on photophosphorylation in thylakoids. Granted that a finite leakage is unavoidable, it may be argued that some NH_4^+ porter activity is required even in the absence of exogenous NH_3 + NH_4^+ to recover the leaked NH_3 before it has escaped from the unstirred boundary layer; the rapidity of the $NH_3 + H^+ = NH_4^+$ equilibration means that this does not present the same problems as are posed by the scavenging of leaked CO_2 by HCO_3^- influx (see Section IV.B, 1). The ability to accumulate nitrogen from low external concentrations of (NH_3 + NH_4^+) (both when this is the sole nitrogen source and when it represents leakage from an intermediate pool in the assimilation of another nitrogen source), although it represents (energetically) uncoupling, may be energetically worthwhile when nutrient (nitrogen) availability rather than energy supply *per se* is growth-limiting.

This emphasis on countering ammonia net leakage should not obscure the ecological and experimental importance of such leakage. Under experimental conditions substantial excretion of ammonia can

occur, e.g. when nitrate uptake and reduction are dissociated from the formation of organic nitrogen (Eiselle and Ullrich, 1975, 1977; Ullrich and Eiselle, 1977), or when glutamine synthetase is inhibited by methionine sulphoxime (Stewart and Rowell, 1975; Rigano *et al.*, 1979). Ecologically, it may be significant in symbioses of N_2-fixing cyanobacteria and fungi or higher plants (Rowell *et al.*, 1977).

The mechanism of ammonia transport into storage compartments (vacuoles) in microalgae is not certain; neither is the mechanism (if any) which prevents leakage from this compartment (Eppley and Rogers, 1970; Kahn and Swift, 1978). It is likely (by comparison with giant-celled algae) that the ammonia concentration in the vacuole is greater than that in the cytoplasm, and that the vacuole is both more acid and more electrically positive with respect to the cytoplasm (Smith and Walker, 1978; Smith *et al.*, 1978). The simplest model for vacuolar storage of ammonia is that the acidity of the vacuole (maintained by an active H^+ pump: see Fig. 4) acts to provide an inwardly-directed diffusion gradient for NH_3 through the lipid part of the tonoplast, since the acidity (continuously maintained by the H^+ pump) displaces the equilibrium of $NH_3 + H^+ = NH_4^+$ to the right. This, in turn, could make the NH_4^+ electrochemical potential in the vacuole higher than that in the cytoplasm. The ammonia concentration in the vacuole would then be a function of the pH and ammonia concentration in the vacuole, the pH of the vacuole, the P_{NH_3} of the tonoplast, and the effective $P_{NH_4^+}$ of the tonoplast endowed with a (regulated) NH_4^+ uniport (see equation 7). A more acid vacuole increases the extent of ammonia accumulation in the vacuole; an increase in vacuolar pH, or an increase in the $P_{NH_4^+}$ leads to a decreased accumulation in the vacuole. The former mechanism is more economical, in that it does not (like the latter) constitute uncoupling at the tonoplast, but may be at variance with other metabolic demands for a constant vacuolar pH.

It is not possible to evaluate the mechanism on the basis of current data. The 10 m*M* ammonia in the vacuole of *Ditylum brightwellii* (Eppley and Rogers, 1970) would be at equilibrium with the cytoplasm in terms of NH_3 transport ($P_{NH_4^+} = 0$) if the vacuolar pH were 3, the cytoplasmic pH were 7, and the total cytoplasmic ammonia concentration were 10 μM (see the arguments earlier for *Skeletonema costatum*). The required vacuolar pH is higher if the cytoplasmic ammonia concentration is higher, but this (see above) leads to more leakage at the plasmalemma. The P_{NH_3} at the tonoplast need not be higher than 10^{-2} cm s^{-1} to permit

Skeletonema to store 0.1 of the total nitrogen taken up (using the assumptions used in the arguments about plasmalemma transport above). Further investigation is required.

The arguments and evidence deployed thus far have, I hope, provided a reasonable case for the distribution of ammonia being explicable in terms of equation (7) and the responses of NH_3 and NH_4^+ fluxes to their concentration differences and (for NH_4^+) the electrical potential difference. Clearly, a lot more data is required, since there is a large number of terms in equation (7) which are not yet available for any membrane in any algae. The model "fits where it touches", however; the ammonia concentrations in the cytoplasm of algae can generally be explained in terms of NH_4^+ uniport with a plausible value of the electrical potential difference across the plasmalemma. Indeed, the internal concentration probably does not approach the value permitted by ψ_{co} and $[NH_4^+]_o$, even taking leakage of NH_3 into account. This suggests that the internal concentration of ammonia is under kinetic control rather than proceeding to near thermodynamic equilibrium. This may not be the case for methylamine (Smith and Walker, 1978; Smith *et al.*, 1978), which is accumulated to higher levels: this may be another case (like sugar analogues) where the non-metabolized analogue is accumulated to higher levels than the metabolized solute (see Section IV.B, 2). Whether the vacuolar concentration is under thermodynamic or kinetic control is not clear.

The net NH_4^+ influx is generally light-stimulated, except in nitrogen-starved cells where any energetic advantage of assimilating nitrogen in the light (Raven, 1976b) is sacrificed to the need to assimilate nitrogen as fast as possible when it is growth limiting (Bongers, 1956; Syrett, 1962). The light-stimulation in *Ditylum brightwellii* is relatively insensitive to DCMU; the influx is inhibited by proton ionophores (Eppley and Rogers, 1970). This is consistent with the scheme outlined above for influx at the plasmalemma, but scarcely constitutes a rigorous test of it.

A final test which can be applied relatively easily is to check the occurrence of the bidirectional fluxes predicted from the leakage calculations above. While isotopic N(^{15}N: Baker and Thompson, 1961; Bassham and Kirk, 1964; Thang, 1961; Dugdale and Goering, 1967; Kretovitch *et al.*, 1970; and ^{13}N: Meeks *et al.*, 1977, 1978) has been used in studies of ammonia transport and metabolism in algae, the measurements have not been suitable for determining the trans-

plasmalemma tracer fluxes associated with labelling a small cytoplasmic pool (less than 1 m*M* concentration) in a small, non-vacuolate cell. Thus in a *Chlorella* cell with 0.25 $cm^2/\mu l$ of cell volume, and an influx of 10 pmol cm^{-2} s^{-1} (Table 4), the change in internal label is 36 μM s^{-1}: this is substantial, in view of the total concentration of 10–1000 μM (see above).

The ability to transport and metabolize ammonia in algae appears to be constitutive, although the pathway used may vary with conditions (Schmidt, 1974). However, it is possible that the capacity of the transport system varies with the external concentration of ammonia. Perhaps the best documented evidence for this possibility has been presented by Eppley and Renger (1974) for the diatom *Thalassiosira pseudonana*. These workers measured the relationship between specific growth rate and the ammonia concentration in the medium, and also determined the net influx/ammonium concentration relationship, in short-term experiments, for cells grown at the various concentrations of the nitrogen source. They found that the growth rate declined with increasing N-limitation of growth (decreasing nitrogen source concentration in the medium), and that the V_{max} for ammonia influx in short-term experiments increased. This, together with the observed constancy of the $K_{1/2}$ for ammonia influx in short-term experiments, suggests that the organism responds to nitrogen deficiency by increasing the capacity for ammonia uptake without altering the characteristics of the porters: presumably the porter density in the membrane is increased (Section III.F). This model can explain how, by increasing the capacity for transport to well above the rate at which ammonia can be used for growth, the apparent half-saturation concentration for growth can be well below the $K_{1/2}$ determined in short-term experiments with nitrogen-limited algae (Eppley and Renger, 1974; cf. McCarthy and Goldman, 1979).

3. *Nitrate*

Nitrate is probably the commonest nitrogen-source for algae in nature. Photolithotrophic growth of algae is largely restricted to habitats of relatively high (positive) E_h (Fergusson-Wood, 1965; his Fig. 2; cf. Cohen *et al.*, 1975), favourable to the activities of nitrifiers. However, under conditions of rapid grazing of algae by animals the ammonia produced by animal defaecation, excretion and death may be used by algae directly, prior to nitrification (Dugdale and Goering, 1967; Parsons and Takahashi, 1973).

Nitrate ion does not share the problems we encountered in studying the transport of the weak electrolytes CO_2–HCO_3^- and NH_3–NH_4^+ in that only the ionized form is encountered at physiological or ecologically important pH values. However, the analysis of its transport does share problems of measurement of cytoplasmic concentration. Nitrate reductase in prokaryotic O_2-evolvers may be associated with the (outside of the) thylakoid membrane (Hattori, 1970; Candeau *et al.*, 1976), while in eukaryotes it is probably cytoplasmic (Hewitt, 1975), so in each case the ground cytoplasm is the phase to which the NO_3^- for assimilation is delivered. This again is simpler than the situation for CO_2–HCO_3^-, or NH_4^+–NH_3, where the assimilation reactions in eukaryotes probably occur in the chloroplast stroma (Sections IV.B, 1 and IV.III, 2).

The meagre information on NO_3^- concentration in the cytoplasm which has been provided by direct analysis is, in the eukaryotes, subject to the constraint that there is always some fraction of the cell volume occupied by "storage" vacuoles which may have a higher NO_3^- concentration that the ground cytoplasm wherein the NO_3^- reductase is located. Thus the 10 m*M* NO_3^- in *Platymonas* cells which can be computed from the work of Wheeler (1977), using the cell volume data of Kirst (1977), may be partially in storage vacuoles (cf. Wheeler and Stephens, 1977). In *Chlorella*, no NO_3^- could be detected in NO_3^--assimilating cells (Ahmed and Morris, 1967; Pistorius *et al.*, 1978): similarly, no ClO_3^- could be detected in cells which were taking up ClO_3^- and reducing it (as an analogue of NO_3^-) to Cl^- (Tromballa and Broda, 1971). In *Chlamydomonas reinhardtii*, no NO_3^- was detectable in NO_3^--assimilating cells, but a finite (but unspecified) level was found in mutants lacking NO_3^- reductase activity (Syrett and Leftley, 1976).

In cells with a relatively large fraction of their intracellular volume occupied by vacuoles the NO_3^- concentration averaged over the entire cell water can be as high as 40 m*M* in diatoms (*Ditylum brightwellii*: Eppley and Rogers, 1970) and in some dinophytes (*Pyrocystis noctiluca*: Bhovichitra and Swift, 1977). In these organisms the 10,000 fold concentration difference between the NO_3^- storage compartment and that in the growth medium, together with the strong likelihood that ψ_{co} is inside-negative, means that active NO_3^- influx must occur between the medium and the vacuole (at the tonoplast, the plasmalemma, or at both of those membranes).

Further localization of the active NO_3^- influx process can be made from considerations of the *in vivo* and *in vitro* relations between NO_3^-

concentration and NO_3^- reduction rate, each on a per cell basis (cf. Sections IV.B, 1 and IV.C, 2). The external NO_3^- concentration needed to saturate NO_3^- influx (in short-term experiments) or growth on NO_3^- (in longer-term experiments) is very low (often less than 1 μM) in marine microalgae (Eppley, Rogers and McCarthy, 1969b; Falkowski, 1975a, b; Eppley, 1977; Serra *et al.*, 1978a, b). In freshwater microalgae the value can be equally low (Pickett, 1975; Steeman-Nielsen, 1978), or as high as 148 μM in *Chlamydomonas reinhardtii* (Wallen and Cartier, 1975).

Generally the V_{max} for NO_3^- reduction in algae extracts does not greatly exceed the V_{max} for *in vivo* NO_3^- assimilation (see Eppley and Rogers, 1970; Eppley, Coatsworth and Solarzaro, 1969a). This means that, for the *Ditylum brightwellii* used by Eppley and his colleagues, we can directly equate the *in vitro* NO_3^- reductase $K_{1/2}$ with the concentration of NO_3^- in the cytoplasm which is required to support half the V_{max} of *in vivo* NO_3^- assimilation. This value of 110 μM may be compared with the external NO_3^- concentration required to support half the V_{max}, i.e. some 0.6 μM (Eppley and Rogers, 1970; Eppley, Rogers and McCarthy, 1969b). This requires NO_3^- influx across the plasmalemma against a free energy gradient of 20 kJ mol^{-1} (assuming a 200-fold concentration ratio and ψ_{co} of −60 mV; equation 4).

For the freshwater microalgae with small vacuoles (Chlorococcales such as *Chlorella*, *Ankistrodesmus* and *Selenastrum*) the V_{max} for NO_3^- reduction *in vitro* is not more than five times the *in vivo* value on a cell basis (Pistorius *et al.*, 1976, 1978; Tischner, 1976) while the *in vitro* $K_{1/2}$ for NO_3^- reductase is 84–230 μM (Solomonson and Vennesland, 1972; Syrett, 1973). Thus the internal concentration of NO_3^- required for half-maximal rate of NO_3^- assimilation is at least 20 μM, compared with the *in vivo* $K_{1/2}$ in terms of exogenous NO_3^- of less than 0.5 μM (Pickett, 1975; Steeman-Nielsen, 1978). Thus the cytoplasmic NO_3^- concentration must be at least 40 times the external NO_3^- concentration, and net NO_3^- influx in *Chlorella* with ψ_{co} of −150 mV (Table 5) would need an energy input of 25 kJ mol^{-1}. Even with the high $K_{1/2}$ for influx (200 μM) shown by Tromballa and Broda (1971) an energy input of 5 kJ mol^{-1} is required.

For *Chlamydomonas* the evidence is less clear-cut, in that the $K_{1/2}$ for NO_3^- reduction *in vitro* (125 μM: Barea and Cardenas, 1975) and for NO_3^- assimilation *in vivo* in terms of external NO_3^- (148 μM: Wallen and Cartier, 1975) are similar, and the *in vitro* NO_3^- reductase capacity

is some five times that used *in vivo* (Thacker and Syrett, 1972a, b). Thus an internal NO_3^- of 25 μM could support half-maximal *in vivo* NO_3^- reduction rates when external NO_3^- is 148 μM; active influx would be needed if ψ_{co} were more negative than −50 mV (cf. Stavis, 1974; equation 4).

We may thus conclude that active influx of NO_3^- at the plasmalemma is ubiquitous in microalgae which are assimilating NO_3^-. Active influx from cytoplasm to storage compartment may also be needed: in *Ditylum brightwellii* where the vacuolar NO_3^- concentration is 40 mM, and cytoplasmic NO_3^- may be, say, 0.4 mM (higher than is needed for saturation of NO_3^- assimilation), transport of NO_3^- from cytoplasm to vacuole needs an energy input if ψ_{cv} is (as is almost certain) more negative than +120 mV.

The NO_3^- concentration in the cytoplasm of algal cells appears to be regulated at a fairly low level (less than 1 mM: cf. Raven, 1976a, p. 135) despite transmembrane fluxes (at the plasmalemma and tonoplast) and reduction which could individually alter the cytoplasmic NO_3^- concentration at a rate of 36 μM s^{-1} (assuming the NO_3^- influx in *Chlorella* in Table 4, and a surface/volume ratio for *Chlorella* of 0.25 cm^2 μl^{-1} if no regulation occurred). The regulatory hypothesis of Butz and Jackson (1977), which involves a membrane-associated NO_3^- reductase which also acts as a NO_3^- porter and reduces a fixed fraction of the NO_3^- it transports, seems too inflexible to account for much of the microalgal data. Certainly the regulation does not involve the complete sequence of NO_3^- entry, NO_3^- reduction and organic nitrogen formation, since NO_3^- entry and reduction "uncoupled" from nitrogen assimilation into organic compounds seem to show the same regulation of cytoplasmic NO_3^- concentration as does complete assimilation (Bongers, 1956; Eiselle and Ullrich, 1975, 1977; Ullrich and Eiselle, 1977).

This regulation greatly complicates the analysis of "biochemical" energy sources for NO_3^- entry at the plasmalemma; any treatment which inhibits NO_3^- assimilation will inhibit NO_3^- net influx (tracer fluxes have not been investigated) by feedback, and *vice versa* by substrate starvation (Pistorius *et al.*, 1978). Even when NO_3^- uptake into a storage compartment (e.g. cells with large vacuoles) is considered there is the problem of active transport at both plasmalemma and tonoplast, and of disentangling the effects of the experimental treatments on one transport process from that on the other. The

overall effects noted by Eppley and Rogers (1970) showed that NO_3^- uptake and storage were less light-stimulated than NO_3^- uptake and assimilation, and that the light-dependent component was less inhibited by DCMU in the case of uptake and storage than for uptake and assimilation. The biochemical energy supply is probably ATP (Ahmed and Morris, 1967, 1968; Falkowski and Stone, 1975). Further insights might be obtained by the use of mutants lacking NO_3^- reductase, although the high capacity for net NO_3^- influx relative to the intracellular storage capacity of a non-vacuolate unicell which can (see above) change the intracellular concentration by 36 μM s^{-1} in the face of a putative total storage capacity of 1000 μM means that short-term experiments with tracers are required. While $^{36}ClO_3^-$ may be useful as a tracer in this context, it is unlikely to be useful as a "non-metabolized analogue" of the 6-deoxyglucose or methylamine type, since $^{36}ClO_3^-$ is a good substrate for nitrate reductase in algae (Tromballa and Broda, 1971).

Turning to the coupling mechanism between ATP consumption and NO_3^- influx, the mechanism could involve primary or secondary active transport (Section III.D and E). Primary active transport is championed by Falkowski (1975a, b), who has found a NO_3^-, Cl^- activated ATPase in *Skeletonema costatum* and other marine phytoplankters. This is discussed in Section IV.A, and all that need be added here is that the evidence from giant-celled marine algae (*Halicystis*, *Bryopsis* and *Acetabularia*) suggests Cl^- rather than NO_3^- as the anion subjected to primary active transport at the plasmalemma. While the work of Falkowski falls short of proving primary active transport of NO_3^-, it should certainly be followed up using the membrane separation techniques of Sullivan and Volcani (1974a, b; 1975).

The other possibility is secondary active transport: for an organism with a primary H^+ extrusion pump at the plasmalemma this would involve an $H^+:NO_3^-$ symport. Since (see above) the ratio of NO_3^- concentration in the cytoplasm to that in the medium is 400 or more, while in sea water the external pH exceeds the cytoplasmic pH, a 1 : 1 $H^+:NO_3^-$ symport is not energetically competent to drive the influx. An $H^+:NO_3^-$ of 2 or more allows ψ_{co} to add to the driving force for NO_3^- influx. Evidence for such a mechanism has been presented for a giant-celled alga (Raven and Jayasuriya, 1977; Raven and De Michelis, 1979) and for the higher plant *Lemna* (Novacky *et al.*, 1978). No corresponding evidence for microalgae has thus far been presented,

although the techniques of Komor and Tanner (1976) should permit the detection of a transient depolarization of ψ_{co} upon addition of NO_3^- such as is required for NO_3^- influx in a positively charged complex with more than 1 H^+ per NO_3^-. Overall stoichiometries of H^+ net influx and NO_3^- net influx during growth generally reflect (when disentangled from complications due to CO_2/HCO_3^- assimilation) a pH-stat mechanism for maintaining cytoplasmic pH constant in the face of massive H^+ consumption during cytoplasmic NO_3^- assimilation (Raven, and Smith, 1974, 1976a; Smith and Raven, 1976; Raven and de Michelis, 1979); this involves less than 1 H^+ taken up per NO_3^- entering and being assimilated, so pH regulation during $2H^+ : 1NO_3^-$ symport involves net, active H^+ extrusion (Raven and Jayasuriya, 1977; Raven and De Michelis, 1979). This problem is further analysed in Section IV.I.

While the ability to transport and metabolize ammonia in microalgae seems to be constitutive (although the pathway used may vary with growth conditions), there is evidence for variation in the rate of transport and metabolism of nitrate depending on the growth conditions, although effects on uptake other than those related to feedback effects from assimilation are not generally observable directly (Pistorius *et al.*, 1978). Algae grown on a reduced N source generally have a low capacity to take up and metabolize NO_3^- (Morris, 1974). Growth on NO_3^- as nitrogen source, or the absence of a nitrogen source from the medium, leads to the induction of NO_3^- reductase and (probably) of the NO_3^- transport system; the induction of NO_3^- reductase and transport in the absence of an N source is *not* due to the production (by nitrification) of NO_3^- in the cells (Morris, 1974; Hipkin and Syrett, 1977; Hipkin *et al.*, 1978; Syrett and Hipkin, 1973). The apparent V_{max} for NO_3^- uptake and assimilation is increased in marine diatoms as the external NO_3^- availability decreases (Eppley and Thomas, 1969; Eppley and Renger, 1974); the increase in NO_3^- reductase capacity is less pronounced, so it is likely that this increased uptake represents an effect (derepression of synthesis?) on the transport system. Since the $K_{1/2}$ for NO_3^- (measured in short-term NO_3^- uptake experiments) is not changed as the growth NO_3^- level (and the relative growth rate) *decrease* and the short-term V_{max} for NO_3^- uptake *increase*, the apparent half-saturation value for NO_3^- when growth is measured as a function of external NO_3^- decreases (see Fig. 6, and Section III.D). Thus the adaptation to low NO_3^- availability

appears to involve an increase in the number of porters per unit of membrane area without a change in the characteristics of the porters, which increases the potential for uptake at low substrate concentrations.

4. *Urea*

Urea can be an important N-source for marine algae in coastal water (Antia *et al.*, 1975); it has long been a classic solute for studies of passive, "non-mediated" transport in biological membranes (Davson and Danielli, 1943; Collander, 1954; Stein, 1967; Wood *et al.*, 1968), although it is clearly transported by some "porter" mechanism in some animal cell plasma membranes (Hunter *et al.*, 1977). Recent evidence suggests some mechanism for urea (and the relatively non-metabolized analogue thiourea) transport at the algal cell plasmalemma other than "lipid-phase" transport.

The influx of urea into microalgae changes with increases in external concentration in the range 0–50 μM with saturation kinetics, although there is a further increase in influx at higher external concentrations which shows no evidence of saturation up to 1 m*M* or so urea (Williams and Hodson, 1976, 1977; Syrett and Bekheet, 1977; Rees and Syrett, 1978, 1979; Kirk and Kirk, 1978a, b; Kirk and Kirk, 1978; Bekheet and Syrett, 1979). This cannot be attributed solely to the maintenance of a low internal concentration of urea by the urea-assimilating enzymes (see later): there is good evidence for active urea (thiourea) influx in *Chlamydomonas*, *Volvox*, *Chlorella* and *Phaeodactylum*: Williams and Hodson (1976, 1977), Syrett and Bekheet (1977), Rees and Syrett (1978), Kirk and Kirk (1978a, b), Kirk and Kirk (1978); Bekheet and Syrett (1979); Rees and Syrett (1979).

For *Chlamydomonas*, *Volvox* and *Phaeodactylum* tracer (^{14}C) urea was added to the medium bathing the cells; with low external concentrations of urea it was found that very high ratios of internal to external concentration could be achieved (between 10 and $3 \cdot 10^3$-fold). In the case of the work of Williams and Hodson (1976, 1977) on *Chlamydomonas* the active nature of the influx was most readily demonstrated in the first ten minutes or so after the addition of urea to cells grown on an inorganic nitrogen source, during which time the activity of the urea-metabolizing enzyme was negligible. The rapid consumption of the internal urea when the assimilatory enzyme becomes active (Williams and Hodson, 1977) suggests that much of the

urea is in the ground cytoplasm rather than in a storage compartment; thus the concentration in the cell measured in *Chlamydomonas* (Williams and Hodson, 1977; Kirk and Kirk, 1978b) probably relates to the ground cytoplasm. The microalga with the highest accumulation ratio (*Phaeodactylum* with a ratio of up to 3000) has a large vacuole relative to the cytoplasm; again, we have no direct evidence for active transport at the plasmalemma rather than the tonoplast, but it seems very likely that such active transport does occur (Rees and Syrett, 1978, 1979).

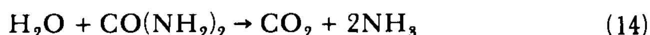
Further evidence for active urea transport at the plasmalemma comes from the work of Syrett and Bekheet (1977) on transport of the urea analogue thiourea: this compound is essentially non-metabolized in the *Chlorella fusca* used by these workers. Its utility as an analogue is confirmed by the competitive inhibition of its influx by urea (Syrett and Bekheet, 1977). In their experiments, Syrett and Bekheet (1977) demonstrated an internal concentration of 314 μM at flux equilibrium with 4 μM outside.

This evidence for active transport came from experiments at low (less than 60 μM) external concentrations of urea or thiourea, where the influx shows saturation kinetics. At higher concentrations (up to 3.5 mM: Williams and Hodson, 1977) a further increase in uptake with concentration is found: this component shows a linear increase with increasing concentration, with no evidence that internal concentration exceeds the external. This appears to be a passive influx; if the surface/volume ratio of *Chlamydomonas* is similar to that in *Chlorella*, i.e. some 0.25 $cm^2\ \mu l^{-1}$ (Barber, 1968a), then the influx of 50 pmol (μl cell volume) s^{-1} found at an external concentration of 3.5 mM (Williams and Hodson, 1977) corresponds to an influx of 18 pmol $cm^{-2}\ s^{-1}$. Less than 0.1 of this influx is by the active mechanism (judging from the influx found at 50 μM external urea, when the "active" component saturates). Treating the 18 pmol $cm^{-2}\ s^{-1}$ as being all passive, and assuming the internal concentration is zero (true in the initial stages of the experiment), P_{urea} is, according to equation (1), $5 \cdot 10^{-6}$ cm s^{-1}. This value of P_{urea} is very close to the value found for urea permeation through model lipid bilayers, i.e. $2\text{–}5 \cdot 10^{-6}$ cm s^{-1} (Vreeman, 1966; Lippe, 1969; Poznansky *et al.*, 1976).

The utility of *facilitated*, if not *active*, transport of urea is seen if the passive influx is computed for "natural" concentrations of urea. With a plausible but high estimate of natural urea concentration of 5 μM which is about the $K_{1/2}$ for the active transport mechanism in these

algae, the passive influx is only 0.0026 pmol cm^{-2} s^{-1} with P_{urea} of $5 \cdot 10^{-6}$ cm s^{-1}, while the observed active influx is 1.8 pmol cm^{-2} s^{-1} (Williams and Hodson, 1977). If the internal concentration is finite the net passive influx would be even lower. Before considering the requirement for active, rather than merely facilitated, transport in relation to the activity of the urea-metabolizing enzyme, it is worth enquiring as to the degree of "short-circuiting" of active transport by passive urea efflux with P_{urea} of $5 \cdot 10^{-6}$ cm s^{-1}. If urea is present in the cells at 50 *μM* (due to active influx from 5 *μM* outside, and metabolism inside the cell), the passive (*via* the lipid portion of the membrane) efflux is only some 0.023 pmol cm^{-2} s^{-1}, i.e. about 1% of the influx. Evidence for "pump and leak" of thiourea has been provided by Syrett and Bekheet (1977) for *Chlorella fusca.*

Turning to the requirement for the cytoplasmic urea concentrations needed to account for the observed rate of metabolism in terms of the extractable activities of the enzymes of urea metabolism, the enzymes involved are either urease (in the cyanobacteria, and the charophyceae, prasinophyceae, baccilariophyceae, xanthophyceae, chrysophyceae, cryptophyceae and euglenophyceae), or urea amidolyase (only in the chlorophyceae among the algae, and elsewhere in some fungi: Syrett and Leftley, 1976). Urease catalyses the simple hydrolytic reaction:

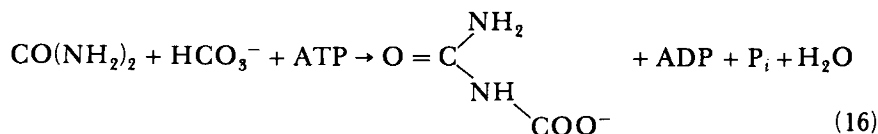


$$H_2O + CO(NH_2)_2 \rightarrow CO_2 + 2NH_3 \qquad (14)$$

while urea amidolyase catalyses the overall reacton:

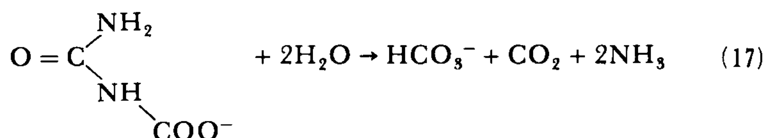
$$CO(NH_2)_2 + ATP + H_2O \rightarrow CO_2 + 2NH_3 + ADP + P_i \qquad (15)$$

which is comprised of two reactions catalysed by two separate enzymes: urea carboxylase catalyses the formation of allophanate



$$CO(NH_2)_2 + HCO_3^- + ATP \rightarrow O{=}C(NH_2)(NH{-}COO^-) + ADP + P_i + H_2O \qquad (16)$$

and allophanate lyase catalyses



$$O{=}C(NH_2)(NH{-}COO^-) + 2H_2O \rightarrow HCO_3^- + CO_2 + 2NH_3 \qquad (17)$$

As with the two alternative assimilatory systems for ammonia (glutamate dehydrogenase and glutamine synthetase/glutamate synthetase) the system which uses an extra molecule of ATP per mol substrate transformed, and requires two enzymes rather than one, has a lower $K_{1/2}$ value for its nitrogenous substrate (see Section IV.C, 2). Thus Syrett and Leftley (1976) found $K_{1/2}$ values of 460 μM for the urease from *Phaeodactylum tricornutum*, and 70 μM for the urea amidolyase of *Dunaliella primolecta*. Adams (1971) found a $K_{1/2}$ for the *Chlorella* urea amidolyase of 100 μM. Most ureases have higher $K_{1/2}$ values (Reith, 1971, quotes values of 3–100 mM) than the *Phaeodactylum* enzyme. The available data are not sufficiently detailed to allow an analysis of the cytoplasmic urea concentration of the type carried out in Sections IV.B, 1 and IV.C, 2 and 3, although it seems that the excess capacity of the assimilatory enzymes *in vivo* is such that the observed assimilation rate could not be sustained unless the internal urea concentration were *greater* than the external value (MacCarthy, 1972; Antia *et al.*, 1975; Syrett and Leftley, 1976). This is true for the higher-affinity, ATP-requiring urea amidolyase as well as the lower-affinity urease, so the use of the higher-affinity enzyme does not obviate the need for active transport of urea (cf. Syrett and Leftley, 1976). Again drawing an analogy with ammonia assimilation, the high-affinity glutamine synthetase/glutamate synthetase sequence does not appear to absolve the cell from the need for an energy-coupled influx of NH_4^+ from "ecological" ammonia concentrations.

The energy coupling of urea active influx is not well understood. It seems fairly certain (based on experiments on influx and/or final accumulation ratio at flux equilibrium) that in *Chlamydomonas*, *Chlorella* and *Phaeodactylum*, under various conditions, the biochemical source of energy is ATP. Transport is best supported in light-air when open-chain and cyclic photophosphorylation, and oxidative phosphorylation, can all occur, and is minimal in darkness in the absence of O_2 when only fermentative ATP is available. Intermediate rates are supported by oxidative (darkness in the presence of O_2) or cyclic photosynthetic (light with DCMU in the absence of O_2) ATP supply (Williams and Hodson, 1976, 1977; Syrett and Bekheet, 1977; Bekheet and Syrett, 1979; Rees and Syrett, 1978, 1979). Whether ATP energy is applied *via* primary or secondary active transport mechanisms for the influx of urea is not clear; while H^+ ionophores inhibit influx in light/air (Williams and Hodson, 1977; Rees and Syrett, 1978, 1979),

these effects could be on ATP supply from mitochondria or chloroplasts as well as on ATP use at the plasmalemma in generating a proton free energy difference.

5. *Amino Acids*

Although these compounds are classed here as N-sources, it is clear that many algae can also use amino acids as C sources for heterotrophic growth (Droop, 1974; Morris, 1974; Neilsen and Lewin, 1974; Hellebust, 1978). Not all algae can use all (or, indeed any) of the amino acids as their N-source; this may be due in part to the characteristics of the plasmalemma transport systems for these solutes. It is likely that the amino acid influxes which are significant for the nitrogen or carbon nutrition of algae from "ecological" concentrations of amino acids are due to the activity of specific porter systems; however, the permeability of lipid bilayers to at least *some* of the protein amino acids is rather higher than that to sugars and "lipid-insoluble" inorganic ions (Klein *et al.*, 1971; Wilson and Wheeler, 1973; Naoi *et al.*, 1977). The quantitation of the permeability coefficients has not yet been achieved, however, so the significance of this intrinsic permeability in allowing leakage of amino acids from the cell cannot be evaluated. It is clear that, as well as difference between species with respect to the occurrence of porter systems for specific amino acids, there are also effects related to the N-status of the cells (North and Stephens, 1972; Kirk and Kirk, 1978a, b; Kirk and Kirk, 1978).

At the risk of pre-judging the nature of energy coupling for amino acid transport, I propose to deal with the amino acids according to the net charge which they bear at "physiological" pH values, i.e. the negatively charged (acidic) amino acids such as glutamate and aspartate, the uncharged (neutral) amino acids such as phenylalanine and glycine, and the positively charged (basic) amino acids such as arginine and lysine. Their charge status has profound consequences for any cotransport system (Section III.E) for amino acids.

Of the anionic amino acids the best investigated example is glutamate. It seems that the influx of glutamate, at least in diatoms, is active according to the definition in Section III.B (Hellebust and Lewin, 1977). Thus the intracellular glutamate concentration can exceed that in the medium which, with the probably inside-negative ψ_{co}, means that the electrochemical potential for glutamate is higher in

the cells than in the medium. It is not, however, certain that the intracellular concentration measured refers to the ground cytoplasm in these vacuolate cells. In cells with a smaller fraction of the volume occupied by vacuoles (*Euglena*, *Chlorella*) the internal glutamate concentration is 6–30 m*M* (Table 6; Section IV.C, 2). In *Euglena*, where the internal glutamate concentration is at least 6 m*M* during growth on 20 m*M* glutamate (Kempner and Miller, 1974) and the value of ψ_{co} is perhaps −20 mV (Zeldin, 1977) in the acid growth medium, evidence for a thermodynamic requirement for an energy-coupled glutamate influx is not very clearcut. For *Chlorella* where the intracellular glutamate is up to 25–30 m*M* (Section IV.C, 2) the influx is low (Lynch and Gillmer, 1966) but (in view of the large inside-negative ψ_{co}: Table 5) it must be active. Kinetic evidence suggests that the acidic amino acids (glutamate and aspartate) share an uptake mechanism (Hellebust and Lewin, 1977).

The neutral amino acids, on kinetic evidence, may be transported into algae by more than one porter (Hellebust and Lewin, 1977). In a number of diatoms the net influx of neutral amino acids against a concentration gradient (Hellebust and Lewin, 1977) establishes the requirement for active transport. In *Chlorella*, where compartmentation of amino acids into vacuoles is less of a problem than in many diatoms, a thorough study of phenylalanine influx in *Chlorella fusca* has been undertaken by Petterson and Knutsen (1974), who showed influx of phenylalanine from a solution containing 2 μM phenylalanine into cells containing 2 m*M* phenylalanine.

The basic amino acids may also share a common carrier system (Hellebust and Lewin, 1977), although there is often a much more rapid influx of arginine than of lysine in ten green algae (including *Chlamydomonas* and *Volvox*: Kirk and Kirk, 1978a, b; Kirk and Kirk, 1978). In these algae, as well as in *Platymonas* (Wheeler, 1977) and a number of diatoms (Hellebust and Lewin, 1977) exogenous arginine can be accumulated to up to 225 times the external concentration. These average intracellular concentrations, together with the inside-negative value of ψ_{co} in algae (Table 5), suggests that the accumulation of these cationic amino acids could occur by ion uniport in response to the inside-negative electrical potential, although a 225-fold accumulation would require at flux equilibrium (equation 6) a ψ_{co} of −140 mV. There is good evidence for two metabolic compartments for arginine in *Platymonas* (Wheeler and Stephens, 1977; cf. Matile, 1978),

so the ground cytoplasm concentration of arginine may be less than the average intracellular concentration which would permit a less negative value of ψ_{co} to account for the observed accumulation.

How the observed intracellular concentrations of these amino acids relates to the regulation of their uptake, and to their ability to serve as sole nitrogen or carbon sources for the plant, is unclear. Certainly all of the protein amino acids are normal constituents of plant cytoplasm, as are enzymes for their biosynthesis. However, these enzymes may not be able to bring about mobilization of the amino acid for it to act as a sole nitrogen or carbon source even when its uptake can be fast relative to the alga's requirement for carbon and nitrogen (Kirk and Kirk, 1978a, b; Kirk and Kirk, 1978).

The energy coupling mechanism for amino acid influx in microalgae has been investigated by Hellebust and his coworkers (see Hellebust, 1978; Lewin and Hellebust, 1978). Energy appears to be provided ultimately from ATP, and Hellebust (1978) has evidence that the coupling mechanism is secondary in *Cyclotella cryptica* (as is the case for glucose influx in this organism: see Section IV.B, 2). Thus the influx of glutamate (anionic), alanine (no net charge) and arginine (cationic) is stimulated in the presence of Na^+ in the external medium of this marine diatom; the half-saturation concentration for Na^+ is about 50 m*M*. This is consistent with the coupling of amino acid influx to Na^+ influx down a $\Delta\bar{\mu}_{Na^+}$ generated by primary or secondary active Na^+ efflux (Section IV.A), although more evidence (e.g. amino acid stimulation of Na^+ influx, depolarization of ψ_{co} by the addition of a transported amino acid) is required before this mechanism can be considered proven.

Cotransport of anionic and neutral amino acids with Na^+ is to be expected if the energy for transport comes from $\Delta\bar{\mu}_{Na^+}$ (see Section III.E); however, it is perhaps unexpected for the cationic amino acid arginine. It was suggested above that the ψ_{co} may not be adequate to account for the observed accumulation ratio for arginine, and it is possible that Na^+:arginine$^+$ symport occurs, thereby increasing the energy available for producing an arginine concentration difference across the membrane (Rottenberg, 1976). The inhibitory effect of high concentrations of K^+ on influx of all three amino acids, even in the presence of 100 m*M* Na^+ in the medium (Hellebust, 1978) is consistent with a role for ψ_{co} in all three cases (if K^+ has its usual depolarizing effect on ψ_{co}), which is consistent with all three amino acids entering as

positively charged complexes (e.g. $2Na^+$ glutamate$^-$, Na^+ alanine, arginine$^+$ or Na^+ arginine$^+$).

In the freshwater *Chlorella fusca* the absence of Na^+ or K^+ effects on phenylalanine influx (Petterson and Knutsen, 1974) suggests that any cotransport does not involve these ions but rather (for example) H^+.

D. PHOSPHATE

The major form in which microalgal cells acquire phosphorus is as inorganic phosphate ($H_2PO_4^-$ + HPO_4^{2-}), which will be hereinafter denoted P_i. It appears that the ability of eukaryotic algae to take up phosphorus from a medium containing organic phosphorus or inorganic polyphosphate requires extracellular enzymic hydrolysis of the phosphorus compound with subsequent uptake of the resulting P_i (Kuhl, 1974). In cyanobacteria, however, sugar phosphates can be taken up intact (Rubin *et al.*, 1977). Despite its obvious role as a metabolized ion with a very rapid turnover (turnover times can be of the order of a second: Lilley and Hope, 1971) it is possible to determine the occurrence of P_i in actively metabolizing cells. This contrasts with NO_3^- (Section IV.C, 3), which is metabolized relatively more slowly than P_i. The possible difference here may relate to the unidirectional nature of NO_3^- metabolism (algal cells cannot nitrify reduced N to NO_3^-: Syrett and Hipkin, 1973: see Section IV.C, 3), while the rate of bidirectional P_i consumption (in phosphorylation) and release (in dephosphorylation) exceeds by perhaps 600 times the net rate of P_i incorporation (based on a C/P ratio of 100, Table 3, and a requirement for the hydrolysis of 6 ATP, per net C incorporated in photolithotrophic growth: see Raven, 1976b). Thus the killing procedure used inhibits both P_i incorporation and P_i release, and increases the chances of finding P_i in the cytoplasm. This is not the same as saying that the correct, *in vivo* cytoplasmic P_i level has been measured.

This reservation notwithstanding, there is considerable unanimity as to the P_i concentration in microalgal cells: the values are generally in the millimolar range for cells with relatively small vacuoles (Miyachi and Tamiya, 1961; Blum, 1966; Kanai and Simonis, 1968; Bassham and Krause, 1969; Domanski-Kaden and Simonis, 1972; Aitchison and Butt, 1973; Imafuku and Katoh, 1976; Schneider and Frischknecht, 1977a). The value is relatively constant with changes in external concentration of P_i; for *Ankistrodesmus braunii*, a change in P_i con-

centration in the medium from 0.1 μM to 5 mM for growth only increases the internal P_i level from 8 mM to 36 mM (Domanski-Kaden and Simonis, 1972; cf. Aitchison and Butt, 1973; Jeanjean and Ducet, 1974).

The extent to which the P_i level determined in these experiments represents the cytoplasmic P_i level is not clear. The most direct way of determining the cytoplasmic concentration would be to use ^{31}P-nuclear magnetic resonance (see Section III.J); here the P_i in a compartment which can be distinguished as cytoplasmic by its pH (using cytoplasmic to represent ground cytoplasm, stroma and matrix of eukaryotes) can be determined by use of this technique. Vacuolar P_i would appear distinctly as being in a more acid environment. Raven (1976a) has documented evidence for *Chlorella* that the chloroplast P_i level is similar to the average cell concentration. Arguments from the relative constancy of the free energy of hydrolysis of ATP in many cells may perhaps be used (in conjunction with measurements of ATP and ADP, and preferably Mg^{2+} and H^+, concentrations) to put limits on the P_i concentration in cytoplasmic phases (Wilson *et al.*, 1974; Erecinska *et al.*, 1978; cf. Meer *et al.*, 1978; Atkinson, 1977). Perhaps the 36 mM P_i in *Ankistrodesmus* grown at high P_i concentrations in the medium, and the even higher levels found in *Platymonas* (Kirst, 1977), may be attributed to some vacuolar storage (cf. Wheeler and Stephens, 1977).

The conclusion is inescapable, based on cytoplasmic negativity (Table 5) and even the minimum estimates of cytoplasmic P_i concentration, that P_i influx must be active. The cytoplasmic pH is probably higher than pK_{a2} of P_i, so HPO_4^{2-} is the dominant form of P_i in the ground cytoplasm. Over a wide range of external pH values the influx of *either* $H_2PO_4^-$ *or* HPO_4^{2-} requires an energy input (see Bieleski, 1973). As with NH_4^+ versus NH_3 uptake (Section IV.C, 2), experimental manipulation of the external pH does not give an unequivocal distinction between $H_2PO_4^-$ influx and HPO_4^{2-} influx. For most higher plants the pH optimum for P_i influx is on the acid side of neutrality, while for many algae the pH optimum is between pH 7 and pH 8 (Ullrich-Eberius and Yingchol, 1974).

The relation of P_i influx to external P_i concentration is complex and cannot be described by a single rectangular hyperbola. Ecologically the high affinity system is probably the most important in view of the generally low P_i levels available to microalgae in unpolluted waters, and the status of P as a potentially growth-limiting nutrient (Goldman

et al., 1972). The half-saturation constant for the high-affinity system is generally in the range 0.01–5 μM, determined from short-term uptake (giving the higher values) and from growth as a function of P_i concentration (see Jeanjean, 1969; Jeanjean *et al.*, 1970; Ullrich-Eberius, 1973; Simonis *et al.*, 1974; Lehman *et al.*, 1975; Falkner, Werdan, Horner and Heldt, 1974, 1976; Chisholm and Strauss, 1976a, b; Chisholm *et al.*, 1975; Eppley, 1977; cf. Blum, 1966). At this sort of concentration of P_i in the medium there appears to be only a very small tracer efflux of P_i to a solution containing the low concentration of unlabelled P_i (Jeanjean, 1969; Falkner, Werdan, Horner and Heldt, 1974), suggesting that net and tracer P_i influx are essentially equal (unless tracer recycling in the boundary layer round the cells is very important at these low concentrations: see Section III.G).

At higher external P_i concentrations (1 mM or more) there are very large bidirectional (tracer) fluxes relative to the net influx found during growth (West, 1967; Aitchison and Butt, 1973): tracer fluxes 260 times the net influx were found in *Chlorella* by Schneider and Frischknecht (1977a). It is not clear whether this reflects a tracer exchange through a single transport system, or "pump and leak" with distinct active and passive porters: pump and leak is unlikely on energetic grounds, since if 1 P_i transported into the cell involves expenditure of 1 ATP (or its equivalent), the tracer fluxes measured by Schneider and Frischknecht (1977a) involve the use of 260 ATP to accumulate 1 P, which with a C/P ratio of 100 (Table 3) involves 2.6 ATP per C assimilated in addition to the 6 ATP per C needed for normal photolithotrophic growth (Raven, 1976b). If there *is* a substantial leak at high external P_i concentrations, it must be subject to considerable regulation in that efflux is negligible at low external P_i values, yet the driving force for passive P_i efflux (equation 6) has greatly increased since cytoplasmic P_i content is little changed (Domanski-Kaden and Simonis, 1972).

Further ramifications of the dependence of P_i fluxes on external P_i concentration are seen if the apparent capacity for P_i influx by the high affinity system is followed when cells grown at high P_i concentrations (not rate-limiting for growth) are transferred to a medium with limiting P_i. This treatment increases the V_{max} of P_i influx by the high affinity system in *Chlorella* with no apparent change in $K_{1/2}$ (Jeanjean, 1969, 1973b). Increasing the external P_i concentration leads to a rapid decline in P_i influx capacity. The increased capacity of P_i influx when

the external P_i concentration is reduced is prevented if protein synthesis on 80 S ribosomes is inhibited with cycloheximide. This is consistent with the hypothesis that the increased V_{max} for P_i influx is a result of the synthesis of additional P_i porter protein (Jeanjean and Ducet, 1974). The cycloheximide does not appear to exert a direct inhibitory effect on the P_i porter (Jeanjean and Ducet, 1974). Jeanjean and Ducet (1974) also obtained evidence for turnover of P_i porter at constant external P_i concentration with a half-time of less than two hours (cf. Grensen *et al.*, 1968; Beever and Burns, 1978).

Reference to Section III.F suggests that the strategy which *Chlorella* adopts when faced with a diminished supply of phosphorus is that of increasing the number of P_i porters per unit area of plasmalemma without the need to invoke changes in the intrinsic turnover rate of the porter at P_i saturation, or its $K_{1/2}$ for P_i. Jeanjean and Ducet (1974) have suggested, without direct evidence, that the maximal capacity for P_i transport in *Chlorella* is limited by the capacity of the membrane to incorporate new porters. Be that as it may, an increase in carrier site density (Section III.F) can cause a decrease in the $K_{1/2}$ when viewed in terms of the cell's requirement for P_i in growth, which can impose a ceiling on net P_i influx in steady-state growth below that imposed by carrier site density and turnover; the expression of this capacity of the high-affinity system can be viewed ecologically as a recovery from phosphate deficiency.

Jeanjean (1973a) has further investigated changes in the apparent capacity for $^{32}P_i$ influx in *Chlorella* through a synchronous life cycle. Chisholm and Strauss (1976b) found a circadian rhythmicity of V_{max} for P_i influx, but not of $K_{1/2}$, in *non-dividing* cells of *Euglena gracilis*.

Much effort has been devoted to the investigation of the energy source (or sources) for P_i active influx in algae both in prokaryotes such as *Anacystis* and eukaryotes such as *Chlorella*, *Ankistrodesmus*, *Scenedesmus*, *Euglena* and *Porphyridium*.

In the eukaryotes most of the evidence, based on the effects of inhibitors of ATP synthesis on the influx, is consistent with ATP being the biochemical energy source for active P_i influx (Simonis and Urbach, 1973; Raven, 1976a, b; Ullrich-Eberius and Yingchol, 1974; Jeanjean, 1976, 1977), although the ATP concentration within the cell is not generally decreased as much as is P_i influx by treatments which reduce the rate of ATP synthesis (Jeanjean, 1976, 1977). The ATP can be made by fermentation, oxidative phosphorylation, and by photophosphorylation of the cyclic and open-chain types, the latter with CO_2, O_2 or

NO_3^- as the terminal redox agent (see Raven, 1976a,b, 1977f; Ullrich, 1971, 1973). In general the highest influxes are found with either open-chain or cyclic photophosphorylation as ATP source, with oxidative phosphorylation alone supporting a rather lower influx, and fermentation a much lower rate. The influx under these various conditions is not simply related to "energy availability" in terms of either the total capacity for ATP generation under the conditions applied, or the probable excess ATP after such major consumers as CO_2 fixation have been allowed for (Raven, 1976a, b, c; Section III.H).

From investigations with the prokaryote *Anacystis* (Bornefeld *et al.*, 1974; Simonis *et al.*, 1974) very similar results to those obtained with eukaryotes have been obtained with respect to biochemical energy sources, inasmuch as P_i influx in P-starved cells is inhibited when only fermentation is available. However, in prokaryotes there is the possibility that the biochemical energy source may be a redox-powered H^+ pump in the plasmalemma. The experiments of Simonis *et al.* (1974) with H^+ ionophores and various isolated photosynthetic and respiratory redox systems did not distinguish such a possibility from the involvement of ATP generated in the thylakoids as the energy source. Paschinger (1977) concluded that *Anacystis* lacks such plasmalemma-located redox chains, but investigation of the effects of such membrane ATPase inhibitors as DCCD on P_i influx in *Anacystis* are clearly required.

Granted that ATP is the *biochemical* energy source, it remains to be decided if primary or secondary active transport is involved in P_i transport at the plasmalemma. Considering first the prokaryote *Anacystis*, Brinckmann and Simonis (1977) have demonstrated some correlation between H^+ influx and $^{32}P_i$ influx, in that there is usually an increment of H^+ influx when P_i is being transported into P-starved cells. However, there is also some stimulation of H^+ influx due to P_i starvation *per se*, regardless of the presence of P_i in the medium, while in P-replete cells the withdrawal of P_i from the complete nutrient medium does not reduce the net H^+ influx. If the P_i influx does involve H^+ cotransport, the results of Falkner, Werdan, Horner and Heldt (1976) show that the $H^+ : H_2PO_4^-$ ratio must be greater than one if the $\Delta\bar{\mu}_{H^+}$ is the sole energy source, since P_i influx can occur up a P_i concentration gradient even when pH inside is less than that outside and hence the pH difference would make a negative contribution to powering $H_2PO_4^-$ entry (a similar argument could be applied to HPO_4^{2-} cotransport with an $H^+ : HPO_4^{2-}$ of 2). In order to involve the ψ_{co}

in energizing the influx, the $H^+:P_i$ complex must bear a positive charge. The significance for cotransport of the Mg^{2+} stimulation of P_i influx in *Anabaena* remains to be explored (Healey, 1973).

In eukaryotic algae, the best evidence for H^+–$H_2PO_4^-$ cotransport has been provided recently by Jeanjean (1979) working on *Chlorella*. However, most of the evidence on P_i cotransport in eukaryotic microalgae points to Na^+ as the cotransported ion (Baumeister and Conrad, 1966; Simonis and Urbach, 1963; Ullrich-Eberius and Simonis, 1970; Jeanjean *et al.*, 1973; Ullrich-Eberius and Yingchol, 1974). The influx of P_i is generally much greater in the presence of external Na^+ than when K^+ is the extracellular cation; however, a reciprocal effect of P_i on Na^+ influx has only been reported (Kylin, 1964c) as part of the response to re-addition of P_i to P-deficient cells. Furthermore, in freshwater green algae an absolute growth requirement for Na^+ has not been established, i.e. P_i influx can occur during growth in the absence of Na^+. Perhaps more P_i porters are made when the less efficient K^+ is used instead of Na^+ (i.e. Na^+ can lead to a faster turnover of the porters). Powering the P_i influx by a K^+ gradient presupposes an inwardly directed $\Delta\bar{\mu}_{K^+}$: this is a less regular occurrence than is the inwardly directed $\Delta\bar{\mu}_{Na^+}$. Even with Na^+ coupling, a high ratio of Na^+ to P_i would be required to account for the large P_i free energy differences against which P_i influx can occur. The energy required for $H_2PO_4^-$ influx with an external P_i of 0.1 μM, a cytoplasmic P_i of 1 mM, and ψ_{co} of −60 mV is 30 kJ mol^{-1} (equation 4), while the corresponding energy available from the influx of 1 mol of Na^+ is (with Na^+ inside and outside both 5 mM, and ψ_{co} of −60 mV) only 6 kJ mol^{-1} (see Section IV.F).

Thus the case for a complete reliance on cotransport as the energy source for P_i influx in microalgae remains to be made. In other microorganisms there may be primary active P_i transport at low external P_i concentrations (e.g. *Escherichia coli*: Konings and Rosenberg, 1978), while in *Saccharomyces* a change in the cotransport is seen upon transition from the low-affinity to the high-affinity P_i transport system (e.g. *Saccharomyces cerevisiae*: Borst-Pauwels and Peters, 1977; Roomans and Borst-Pauwels, 1977).

E. SULPHATE

Sulphate is the commonest form in which sulphur enters microalgal cells. Many algae can take up and metabolize organic sulphur at the

–SH level of reduction (e.g. as cysteine or methionine), although this form of sulphur cannot always fulfil the requirements of algae (e.g. *Chlorella*) for more oxidized sulphur in sulpholipid (O'Kelley, 1974; Sirensky, 1977). The magnitude of the sulphur influx into algal cells during growth can be seen from Tables 3 and 4; the sulphur influx here refers to intracellular S. Even in those marine algae (e.g. the Rhodophytes *Porphyridium* and *Rhodella*) in which a considerable fraction (0.5) of the total cell sulphur is in extracellular sulphated polysaccharides, all of the cell sulphur must have entered through the plasmalemma; the sulphate esters are subsequently secreted in the golgi vesicles (Ramus, 1972, 1974; Ramus and Graves, 1972, 1974; Ramus and Robins, 1975; Evans *et al.*, 1974).

Sulphate influx into microalgal cells is active. The cytoplasmic concentration of sulphate has been measured in *Chlorella* (Wedding and Black, 1960; Vallee and Jeanjean, 1968a), *Scenedesmus* (Kylin, 1964a), *Porphyridium* (Ramus and Graves, 1974), *Monochrysis* (Deare and O'Brien, 1975) and *Anacystis* (Utkilen *et al.*, 1976), with internal concentrations ranging from 38 to 2000 μM (see also Raven, 1976a). This internal concentration is regulated when the external sulphate concentration is changed (Wedding and Black, 1960; Utkilen *et al.*, 1976); in *Anacystis* the internal sulphate concentration only changes from 83 μM to 590 μM when the external concentration is increased from 0.1 μM to 10 μM. The regulation of sulphate transport which effects this regulation appears to use some organic sulphur assimilation product of sulphate, rather than sulphate itself, as the effector (Vallee and Jeanjean, 1968a; cf. Hodson *et al.*, 1971).

Consideration of the external concentration, and the inside-negative ψ_{co} in microalgal cells, in relation to the internal concentration of sulphate quoted above, shows that (equation 4) the influx of sulphate is an energy-requiring process. As with P_i, the relation of sulphate influx to the external sulphate concentration is complex, with a saturable high-affinity system and other, lower-affinity processes involved at higher external concentrations; the $K_{1/2}$ for the highest-affinity system ranges from 0.75 μM (*Anacystis:* Utkilen *et al.*, 1976), 1.2 μM (*Chlorella:* Vallee and Jeanjean, 1968a), 2.5 μM (*Porphyridium:* Ramus, 1974) up to 32 μM (*Monochrysis:* Deare and O'Brien, 1975). Thus the lowest $K_{1/2}$ for sulphate determined in these experiments is comparable with those for P_i, NH_4^+ or NO_3^- (Sections IV.C, 2 and 3; IV.D), despite the much smaller likelihood that sulphate would limit growth rate in nature than

would nitrogen or phosphorus (Parsons and Takahashi, 1973). As with P_i, there is evidence that net and tracer sulphate influxes are closely similar at low external sulphate concentrations (Jeanjean and Broda, 1977; Vallee and Jeanjean, 1968a; Ramus and Graves, 1974).

There is also evidence for an increased V_{max} for sulphate influx by the high-affinity system in S-deprived cells of *Chlorella* and *Anacystis* (Vallee and Jeanjean, 1968a, b; Vallee, 1968; Jeanjean and Broda, 1977). In *Chlorella* the apparent $K_{1/2}$ also seems to decrease in sulphur-deprived cells. However, this may not necessarily reflect a change in the kinetic properties of sulphate porters; it could also be explained in terms of fine control processes allowing a larger fraction of the low capacity for transport in sulphur-replete cells to be expressed than of the larger capacity found in sulphur-deficient cells (cf. Section III.F). However, it is not yet certain that the increase in measured rate of sulphate influx *via* the high-affinity system in sulphur-depleted cells is a function of protein synthesis (Vallee, 1968).

The biochemical energy source for active sulphate influx in eukaryotic microalgae seems to be ATP, although further experiments with energy-transfer inhibitors are required (Wedding and Black, 1960; Kylin, 1964a, b, 1966a, c, d, 1967; Vallee and Jeanjean, 1968a; Ullrich-Eberius, 1973; Ramus, 1974; Deare and O'Brien, 1975), with a similar set of effects of the conditions of energy supply to those previously noted for P_i (Section IV.D). In the prokaryotic *Anacystis*, Jeanjean and Broda (1977) showed that sulphate influx in light in the presence or absence of DCMU was greater than in the dark, again consistent with a primary dependence on photosynthetic (including cyclic) redox reactions. Accepting the views of Paschinger (1976, 1977) on the absence of redox reactions in the *Anacystis* plasmalemma suggests that these redox reactions are in the intracellular thylakoids, with energy transfer to the plasmalemma as ATP: this is consistent with the inhibition of sulphate influx by the energy transfer inhibitor DCCD (Jeanjean and Broda, 1977).

Little evidence is available as to the occurrence of cotransport reactions for sulphate in microalgae. Evidence consistent with H^+–SO_4^{2-} cotransport was obtained by Brinckmann and Simonis (1977), by experiments analogous to those carried out with phosphate, i.e. association of a component of H^+ influx with the presence of the anion in the bathing medium. Even less evidence is available for eukaryotes: in contrast to the Na^+ stimulation of P_i influx, sulphate influx in *Ankistrodesmus* is stimulated by K^+ (Ullrich-Eberius, 1973).

F. POTASSIUM AND SODIUM

1. *Introduction*

These two singly charged cations will be considered together; this does not imply that their distribution between the medium and microalgal cells is mechanistically linked in a K^+, Na^+ pump of the type common in metazoan cells (see Section IV.A). In general, the K^+/Na^+ ratio in microalgal cells is, like that in other organisms, well above one and is much higher than is the ratio in the medium. These concentrations are relatively easily measured in microalgal cells compared with the difficulties that are encountered in measuring those of metabolized solutes (Section IV.B–IV.E). Since K^+ and Na^+ are such major solutes within the cell, generally constituting perhaps 70% of the total positive charge on major osmotic solutes (see Raven, 1976a; cf. Table 6), there are strong osmotic arguments against gross differences in the $[K^+] + [Na^+]$ concentrations in the different compartments of eukaryotic cells. This argument holds with rather less force for the individual concentrations of K^+ and Na^+, although the case for K^+ is quite strong. Thus the total intracellular concentration of K^+ is probably close to the concentration in the ground cytoplasm (Section IV.A).

2. *Electrochemical State of K^+ and Na^+ in Eukaryotic Microalgae*

Despite our relative certainty concerning the K^+ concentration in the ground cytoplasm of microalgal cells, there is considerable disagreement in the literature as to the electrochemical state of K^+ within such cells. This was considered in Section IV.A in the context of detecting electrogenic pumps at the plasmalemma. In general it seems that K^+ is the intracellular ion which is closest to electrochemical equilibrium across the plasmalemma; however, the sign of the electrochemical potential difference for K^+ between ground cytoplasm and bathing medium can be either positive or negative. For *Chlorella*, the organism on which most of relevant experiments have been carried out, the sign of $\Delta\bar{\mu}_{K^+}$ varies with the method used to measure ψ_{co}. Microelectrode determinations suggest that K^+ is at a higher electrochemical potential in *Chlorella* than in the medium, while lipid-soluble cation distribution suggests the reverse. Since there seems to be no obvious reason to doubt the values of ψ_{co} obtained with $TPMP^+$ distribution, we may conclude that K^+ is, in some algae under some conditions, at a lower electrochemical potential in the ground cytoplasm than in the medium.

Na^+ is, by contrast, almost invariably at a lower electrochemical potential in microalgal cell ground cytoplasm than it is in the medium (see Raven, 1976a), even when the relatively low values of ψ_{co} measured with microelectrodes are used in equation (4). This means that, if there is sufficient Na^+ uniport (or symport: Sections IV.B, 2 and IV.D) to cause a larger Na^+ influx than is required to account for the net Na^+ influx into the cell which can be measured, this requires active Na^+ extrusion from the cell (a similar argument applies to K^+ when this ion is at a lower electrochemical potential within the cell than in the medium). However, in some cells the normal $[K^+]_i/[Na^+]_i$ is retained even in media with an extremely high K^+/Na^+ ratio, e.g. in the experiments of Kirst (1977) where the only Na^+ in the medium was from contaminants in the chemicals from which the nutrient solutions were prepared, and from glassware. Although ψ_{co} was not measured in these experiments, it seems very unlikely that it could have been negative enough to permit Na^+ in the ground cytoplasm to be at a lower electrochemical potential than in the medium. These observations are not in accord with the stimulating hypothesis of Skulachev (1978) on the possible energy-buffering role of the K^+ and Na^+ free energy differences across membranes.

Further evidence for homeostasis of the internal K^+ and Na^+ concentrations in algal cells comes from studies of the K^+ and Na^+ levels in the cells in response to a range of external K^+ and Na^+ concentrations. *Chlorella* can maintain its average internal K^+ concentration at 160–170 m*M* when the K^+ concentration in the growth medium is varied from 0.5 to 100 m*M* (Schaedle and Jacobsen, 1965; Shieh and Barber, 1971), but at lower $[K^+]_0$ the internal K^+ is progressively replaced by Na^+.

3. *K^+ Fluxes in Eukaryotic Microalgae*

The influx of K^+ (measured with $^{42}K^+$ or its "Doppelgangers" $^{86}Rb^+$ or $^{204}Tl^+$) shows a "dual isotherm", with the $K_{1/2}$ for the high-affinity system in the range 40–200 μM, and that for the low-affinity system being in the range 6–12 m*M* (Barber, 1968d; Kannan, 1971; Solt *et al.*, 1971; Paschinger and Vanicek, 1974). The $K_{1/2}$ for the high-affinity system in the related chlorococcalean alga *Scenedesmus* is 4 μM, i.e. considerably lower than in *Chlorella* (Meszes *et al.*, 1967). The variation of internal K^+ with the K^+ concentration in the growth medium in

Chlorella cited above suggests that net influx is effected by the high-affinity system alone, supporting the contention (Paschinger and Vanicek, 1974) that the low-affinity system deals in K^+–K^+ exchange rather than net K^+ transport. The high-affinity system can also bring about K^+–K^+ exchange (Barber, 1968d, e) under non-growing conditions, and may also bring about such an exchange at the same time as it catalyses net K^+ influx during growth (see computations by Raven, 1976a). Additionally this system can catalyse net K^+ influx with H^+ efflux as the charge-balancing process during K^+ organic anion-accumulation in *Chlorella* cells grown on low-salt media (Schaedle and Jacobsen, 1965; Schaedle and Hernandez, 1969), and also net K^+ influx with "charge balance" achieved by Na^+ efflux (and some H^+ efflux) in Na^+-rich *Chlorella* cells when exogenous K^+ is restored (Shieh and Barber, 1971; Barber and Shieh, 1972, 1973a), as well as tracer K^+–Na^+ exchange in normal, low Na^+ *Chlorella* cells (Barber, 1968c, d).

The fluxes of K^+ thus seem (at least in long-term "balance" experiments) to be cation exchanges independent of external anions (Schaedle and Jacobsen, 1965). The tracer $^{42}K^+$ ($^{86}Rb^+$) influx is generally larger than the net K^+ (Rb^+) influx during net K^+ (Rb^+) influx in exchange for H^+ (Schaedle and Jacobsen, 1965, 1966, 1967; Barber, 1968c). However, the net flux of K^+ in K^+–Na^+ exchange in "Na^+-rich" cells resupplied with K^+ is greater than the tracer K^+ in control, "low-Na^+" cells (Barber, 1968d; Shieh and Barber, 1971; Barber and Shieh, 1972, 1973a). The $K_{1/2}$ for external K^+ in this net K^+ accumulation in exchange for Na^+ (and H^+) is higher than that found for tracer K^+ influx in genetically similar cells with low internal Na^+ (Barber, 1968d; Shieh and Barber, 1971). The tracer K^+ influx, which is equal to the net K^+ influx during the rapid phase of recovery of the K^+/Na^+ ratio in high-Na^+ cells, returns to the lower level found in control cells grown at normal external K^+ levels very soon after recovery of the normal internal K^+ and Na^+ ratios (Barber, 1968d; Shieh and Barber, 1971; Barber and Shieh, 1972), although the influx in the dark is a larger fraction of the light value in cells which have recovered from K^+ deficiency than in cells grown at high K^+ levels (Barber and Shieh, 1972). For technical reasons related to the specific activity of $^{42}K^+$ the tracer K^+ influx could not be measured in the Na^+-rich cells at the very low external K^+ levels which were used to obtain these Na^+-rich cells (Barber and Shieh, 1972).Thus the data do not allow us to decide if there is an increased capacity for K^+ transport

by the high-affinity system in K^+-depleted cells, such as appears to be the case for the transport systems for solutes bearing C, N, P and S in cells deficient in these elements (Sections IV.B–E). The lower apparent affinity for K^+ during the high net K^+ influx associated with recovery of K^+ in the Na^+-rich cells is, however, more immediately consistent with relief of "fine-control" feedback inhibition of K^+ influx in the low-K^+ cells (see Sections III.F, III.H and IV.E). The extent of fine control involvement in the observed changes in tracer and net K^+ fluxes over the synchronous life cycle in *Scenedesmus* and *Chlorella* is not clear (Meszes and Cseh, 1973; Tischner and Lorenzen, 1978).

In terms of possible constraints on the magnitude of net fluxes across plasma-membranes (Section III.F), the maximum fluxes of K^+ reported at algal plasma-membranes are 20 pmol cm^{-2} s^{-1}; this has been reported for freshwater *Chlorella* during recovery of internal K^+ levels in Na^+-rich cells (Shieh and Barber, 1971; Barber and Shieh, 1972), and in the marine diatom *Cyclotella cryptica* during adaptation to an increased osmolarity of the medium (Brown and Hellebust, 1978). In the latter case K^+ influx is balanced in the long term by Cl^- influx rather than cation efflux.

K^+ efflux from *Chlorella*, except in exchange for K^+, Rb^+ or in cells with abnormally high internal pH, is very low (Schaedle and Jacobsen, 1965, 1966, 1967; Barber, 1968e; Muller *et al.*, 1970; Paschinger and Vanicek, 1974; Tromballa, 1977). This is consistent with either a low capacity for K^+ uniport when external K^+ is absent, or a very high negative value of ψ_{co} (cf. equation 5).

4. *Na^+ Fluxes in Eukaryotic Microalgae*

Tracer Na^+ fluxes in "normal", low internal Na^+ concentration cells of *Chlorella* are much lower than the K^+ fluxes (Schaedle and Jacobsen, 1965, 1966, 1967; Barber, 1968a, c, d, e). Efflux of Na^+ in these cells is stimulated by external K^+ with a concentration dependence similar to that for tracer K^+ influx (Barber, 1968c, d), although the fraction of the tracer K^+ influx which can be accounted for as a 1 : 1 K^+–Na^+ exchange is very small (Barber, 1968c, d). Tracer Na^+ fluxes (influx and efflux) are much higher in "Na^+-rich" cells, as is the net Na^+ efflux seen upon addition of external K^+, 5 and 15 pmol cm^{-2} s^{-1} respectively (Barber and Shieh, 1972, 1973a; Shieh and Barber, 1971). The net Na^+ influx into normal *Chlorella* cells is low compared with the tracer fluxes

(Schaedle and Jacobsen, 1965, 1966, 1967; Tromballa, 1974). A small net NaCl influx into *Chlorella* is seen upon illumination, with a corresponding efflux on darkening (Barber, 1969).

Na^+ influx (energetically downhill) shows saturation kinetics with respect to external Na^+ concentraton; the $K_{1/2}$ for Na^+ tracer influx in normal *Scenedesmus* (Meszes *et al.*, 1967) is about 100 μM, the $K_{1/2}$ for tracer Na^+ influx (exchange) in "Na^+-rich" *Chlorella* is some 800 μM (Barber and Shieh, 1973a), and the $K_{1/2}$ for Na^+ influx in cells of *Chlorella* loaded with Na^+ by exposure to high pH solutions is 2 mM (Tromballa, 1974). The (energetically uphill) spontaneous Na^+ efflux from cells transiently loaded with Na^+ by readdition of P_i to P-deficient *Scenedesmus* (Kylin, 1964c, 1966a, b), K^+-dependent efflux of Na^+ from *Chlorella* cells loaded with Na^+ by K^+ deprivation (Shieh and Barber, 1971), and the spontaneous (but K^+-stimulated) efflux of Na^+ from *Chlorella* cells loaded with Na^+ by exposure to high external pH upon return to a lower pH (Tromballa, 1974) all show a direct linear relationship to the internal Na^+ concentration.

5. *Biochemical Energetics of K^+ and Na^+ Transport in Eukaryotic Microalgae*

Detailed studies of the effects of varying energy-supply conditions on net and tracer K^+ (Rb^+) and Na^+ fluxes in *Chlorella* species have been undertaken by Springer-Lederer and Rosenfeld (1968), Barber (1968a, c, d, e), Kannan (1971), Shieh and Barber (1971), Barber and Shieh (1972, 1973a, b) and Muller *et al* (1970). Net and tracer influx and efflux of all of these ions is inhibited by the H^+ ionophores CCCP and DNP, and the ATPase inhibitor DCCD. This is consistent with ATP being the "biochemical" energy source for energy-dependent K^+ and Na^+ fluxes in *Chlorella* (i.e. active fluxes as defined in Section IV.B, together with uniport influx driven by metabolically maintained electrical potential differences). Further, it seems that the "exchange" role of the K^+ and Na^+ transport systems is a function of the energy state of the cell, in that ATP supply must be maintained even if there is no net consumption of ATP in the exchange of K^+ and Na^+ (Barber, 1968d, e; Muller *et al.*, 1970; Barber and Shieh, 1972, 1973a, b; cf. Glynn and Karlish, 1975).

Experiments by Springer-Lederer and Rosenfeld (1968), Barber (1968a, c, d, e), Shieh and Barber (1971), Barber and Shieh (1972, 1973a, b) and Kannan (1971) also involved measurement of the K^+ (Rb^+) and Na^+ fluxes when various ATP-generating systems were

present in isolation. These experiments showed that oxidative phosphorylation supported a higher rate of transport than did fermentation; cyclic photophosphorylation supported an even higher rate, while the highest rate was found when all of the photosynthetic and respiratory systems were able to operate. The extent of light-stimulation of K^+ fluxes (and, to a lesser extent, Na^+ fluxes) was greater than that found for sugar, phosphate and sulphate fluxes in *Chlorella* and its relatives (see Sections IV.B, 2, IV.D and IV.E). Ouabain, the classic inhibitor of the metazoan K^+–Na^+ ATPase, had no effect on the fluxes (Barber, 1968c, d; cf. Raven, 1971, 1976d).

This latter finding does not encourage the view that ATP use is coupled to K^+ and Na^+ transport *via* a metazoan-type ATPase; the evidence of Meszes and Erdei (1969a, b) on a K^+ and Na^+ stimulated Mg^{2+}-ATPase (see Section IV.A) again scarcely constitutes overwhelming evidence in favour of primary ATP-powered K^+, Na^+ transport in Chlorococcalean algae. In diatoms the work on plasmalemma ATPases by Sullivan and Volcani (1974a, b, 1975) is more favourable to such a link, but *in vivo* evidence appears to be lacking. The poorly documented ATPase from a plasmalemma fraction of various freshwater green algae (Sundberg *et al.*, 1973) seems to be more closely related to eukaryote plasmalemma H^+ pumps than to K^+, Na^+ pumps. The non-essentiality of Na^+ for the growth of many freshwater algae (O'Kelley, 1974) also constitutes an argument against the involvement of a strictly Na^+ coupled transport in K^+ influx in these algae.

6. *Indirect Use of Biochemical Energy in K^+ and Na^+ Transport in Eukaryotic Microalgae*

The alternative possibility of secondary active transport of K^+ and Na^+ is similarly poorly supported. The interesting work of Tromballa (1974, 1977, 1978) on the effects of changing external pH outside (by adding 5 m*M* NaOH to give a final pH of 11.4) and inside (by adding weak acids and bases to the medium) on K^+ and Na^+ fluxes in *Chlorella fusca* can be readily interpreted (as Tromballa indeed has) in a chemiosmotic framework. Thus the large net Na^+ influx observed in a solution containing 5 m*M* Na^+ at pH 11.4 could represent a reversal, at very high external pH which causes a large inside-acid pH difference across the plasmalemma, of an Na^+–H^+ antiporter which normally operates to bring about Na^+ efflux coupled to H^+ influx (Tromballa,

1974). This "normal" operation of the Na^+–H^+ antiporter may account for the net Na^+ efflux observed when the pH of the solution surrounding these "Na^+-loaded" cells is returned to pH 7 (Tromballa, 1974). This net Na^+ efflux is stimulated by, but is not absolutely dependent on, the presence of K^+ in the external solution (Tromballa, 1974). The net Na^+ efflux from Na^+-rich *Chlorella pyrenoidosa* cells (produced by withholding K^+) upon readdition of K^+ is similarly not a 1 : 1 K^+–Na^+ exchange, and again the balancing ion is H^+ (Shieh and Barber, 1971). However, in the K^+-deficient cells of Shieh and Barber (1971) there is a net H^+ efflux (active) during restoration of the normal K^+/Na^+ in the cells, which may be related to a disturbance of intracellular pH regulation during K^+ deficiency leading to cytoplasmic acidification when K^+–H^+ exchange is restricted (cf. Smith and Sinclair, 1967; Wolf and Deland, 1978). In the Na^+-rich *Chlorella fusca* cells of Tromballa (1974), produced by higher external pH treatment, the internal pH is probably *higher* than normal, thus allowing the H^+–Na^+ porter to manifest itself during recovery at lower external pH values; in the conditions used by Shieh and Barber (1971) any such H^+–Na^+ porter is masked by intracellular pH adjustment as described above. Other results which are consistent with H^+–Na^+ exchange in a green alga (*Dunaliella*) are cited by Latorella and Vadas (1973), Kaplan and Schreiber (1977) and Gimmler and Schirling (1978).

Tromballa (1977, 1978) has also investigated the effects of changing internal pH of *Chlorella fusca* (by the addition of weak acids or weak bases to the bathing medium; cf. Sections III.B, III.J and IV.A, and Lane and Stiller, 1970) on net K^+ fluxes. He found that decreasing internal pH (by the addition of a weak acid: cf. Spanswick and Miller, 1977b) caused a net K^+ influx, while increasing internal pH (by the addition of a weak base) led to net K^+ efflux. Findenegg (1978) has also found a net K^+ efflux associated with an increased internal pH in *Scenedesmus*: here the cytoplasmic pH increase was a result of the illumination of cells previously held in the dark, and the K^+ efflux appears to have been part of a "pH-stat" mechanism which brings about net H^+ influx and hence the observed restoration of the normal dark internal pH value after a few minutes in the light (Findenegg, 1978; cf. Brinckmann and Lüttge, 1972). The light-induced increase in cytoplasmic pH is probably of biochemical origin (see Section IV.A). These results of Tromballa (1977, 1978) and Findenegg (1978) can be interpreted most readily in terms of a coupling of H^+ and K^+ influxes

at the plasmalemma, with net K^+ influx by K^+ uniport (or K^+–H^+ symport if ψ_K is more negative than ψ_{co}: see Section IV.A) electrically coupled to H^+ active efflux, and net K^+ efflux by K^+ uniport (if the $\Delta\bar{\mu}_{K^+}$ favours net K^+ efflux when internal pH is high and H^+ extrusion is inhibited: see Section IV.J) or, if the $\Delta\bar{\mu}_{K^+}$ still favours net inward K^+ uniport even at high internal pH values, by K^+–H^+ antiport.

Evidence consistent with an inward transfer of positive charge during K^+ influx has been found for *Chlorella* in experiments in which ψ_{co} was measured as a function of $[K^+]_o$. Barber (1968b) and Langmüller and Springer-Lederer (1974, 1976) used micro-electrodes, and found (Section IV.A) that ψ_{K^+} was always more negative than ψ_{co}. Increasing $[K^+]_o$ decreased ψ_{co} (made it less negative). This effect was less marked with increases in $[Na^+]_o$, and the use of a Goldman-type equation with terms for only K^+ and Na^+ led to a ratio of P_{Na^+}/P_{K^+} of 0.1 (Barber, 1968b), although such an equation cannot quantitatively explain the magnitude of ψ_{co} (Langmüller and Springer-Lederer, 1974, 1976).

Komor and Tanner (1976) also used *Chlorella*, but determined ψ_{co} from the distribution of $TPMP^+$. Their basal medium (the choice of which may have been conditioned by fewer plant biophysical preconceptions than that of Barber (1968b) or of Langmüller and Springer-Lederer (1974, 1976)) contained 25 m*M* Na-phosphate buffer, and they found a much higher value of ψ_{co} than did the workers who used micro-electrodes, despite the much higher alkali cation content of this basal medium than of even the most concentrated media of Barber (1968b) and Langmüller and Springer-Lederer (1974, 1976). The addition of external K^+ had little effect on ψ_{co} until concentrations of 10–15 m*M* were reached, beyond which a decrease of ψ_{co} of some 50 mV more positive per ten-fold increase in external K^+ was found (Komor and Tanner, 1976; Schwab and Komor, 1978). An external concentration of K^+ of some 30 m*M* was required to effect a depolarization of ψ_{co} equal to that caused by the addition of a sugar analogue which caused an influx of positive charge (by H^+ symport) of some 20 pmol cm^{-2} s^{-1} (see Sections IV.A and IV.B, 2). The V_{max} of the low-affinity K^+ (Rb^+) transport system measured with tracers amounts to some 20 pmol cm^{-2} s^{-1} (Springer-Lederer and Rosenfeld, 1968; Kannan, 1971; Paschinger and Vanicek, 1974, assuming 0.25 cm^2 surface area per μl of cells) which would be attained at near 30 m*M*. However, this could not explain the continued fall in ψ_{co} as the K^+ concentration was increased to 300 m*M* (Komor and Tanner, 1976), and is not readily reconciled

with the evidence mentioned above for the "low affinity system" catalysing K^+ exchange unless the putative K^+–H^+ antiporter causes a parallel K^+ efflux (which must occur if the observed constancy of K^+ content of the cells is to be explained: Schaedle and Jacobsen, 1965; Shieh and Barber, 1971). However, such a large K^+ exchange *via* a "pump and leak" system would be expected to have very large effects on respiratory rates during dark K^+ exchange; the experiments of Kannan (1971) only show an increase of respiration after 2 h exposure to K^+ in the medium at more than 10 m*M* concentration (cf. Ried and Soeder, 1961). This contrasts with the effects noted by Decker and Tanner (1972) after sugar analogue addition, where the effect was immediate. It would appear that the K^+ effects on ψ_{co} in *Chlorella* cannot be used *quantitatively* as a measure of K^+ uniport, but that they *qualitatively* indicate its occurrence.

Thus we may conclude that there is positive-charge-carrying K^+ and Na^+ influx in *Chlorella*, with P_{K^+} greater than P_{Na^+}. There is also some evidence for H^+–Na^+ antiport, while H^+–K^+ antiport is not ruled out. How can these mechanisms be incorporated into a scheme which would allow K^+ net influx to occur when the net driving force (for passive uniport of K^+) were either directed inwards or outwards? Even if the ψ_{co} values found by Barber and by Langmüller and Springer-Lederer (*loc. cit.*) were too low due to leakage, the ability of *Chlorella* to grow at low external pH values suggests that K^+ accumulation by *Chlorella* can occur when the K^+ electrochemical potential in the cell is higher than that outside (cf. the depolarizing effects of low pH values in *Chlorella*: Table 5) as well as at high external pH values when K^+ is at a lower electrochemical potential inside the cell than outside.

Net K^+ influx when ψ_{K^+} exceeds ψ_{co} (i.e. is more negative) cannot be explained as K^+ uniport; a chemiosmotic mechanism would involve K^+–H^+ symport (Rottenberg, 1976), which would effectively make K^+ an ion with more than 1 positive charge. When ψ_{co} exceeds ψ_{K^+}, a K^+/H^+ antiport is required to remove any K^+ which has entered the cell (by K^+ uniport) in excess of that required to keep the internal K^+ concentration constant. There is a precedent in giant-celled algae for a decrease in the capacity of K^+ uniport at high values of ψ_{co} which would tend to limit the net passive K^+ influx when the driving force for this process is high (Kitasato, 1968, 1973). Mechanisms which might explain this lack of excessive K^+ accumulation when $\hat{\psi}_{co}$ is very negative relative to ψ_{K^+} involve temporal changes in the properties of K^+ porters

(Slayman *et al.*, 1976; cf. Barr and Ryan, 1978; Ryan *et al.*, 1978). All such mechanisms require a modulation of the properties of the K^+ transport system(s) as a function of $[K^+]_i$ and (probably) of ψ_{co}.

7. *K^+ and Na^+ Transport in Cyanobacteria*

Thus the distribution of K^+ and Na^+ in the best-investigated eukaryotic microalga (*Chlorella*) can be explained on the basis of experimental data and a number of unproven (but testable) assumptions within a chemiosmotic framework. The only cyanobacterium for which a similar analysis may be attempted is *Anacystis nidulans* (Dewar and Barber, 1973; Paschinger, 1976, 1977). Here the high $[K^+]_i$ and the low $[Na^+]_i$ is maintained over a wide range of external ratios of $[K^+]$ to $[Na^+]$ (Fig. 1 of Dewar and Barber, 1973), and it proved impossible to obtain cells with a higher internal Na^+ than K^+ concentration (such as were produced in *Chlorella*: Shieh and Barber, 1971) by manipulating the external concentrations of these ions (Dewar and Barber, 1973).

Internal $^{39}K^+$ exchanged with external $^{42}K^+$ in the absence of a net K^+ flux (Dewar and Barber, 1973). The finding that the K^+ ionophore valinomycin increased the tracer fluxes of K^+ across the plasmalemma without altering the internal K^+ concentration (even in the presence of the H^+ ionophore CCCP which should have allowed charge balance *via* net H^+ fluxes during any net K^+ flux induced by valinomycin) led Dewar and Barber (1973) to conclude that K^+ distribution was by passive K^+ uniport and that $\psi_{K^+} = \psi_{co}$, i.e. that K^+ was at passive electrochemical equilibrium. This is apparently consistent with the finding of Paschinger (1976, 1977) that ψ_{co} computed from $^{86}Rb^+$ distribution agrees closely with that calculated from $TPMP^+$ distribution. However, there are problems with comparison of the two sets of data. The $^{86}Rb^+$ distributions (Paschinger, 1976, 1977) were measured with 0.25 m*M* $^{86}Rb^+$ in the medium, and so the value of ψ_{Rb^+} of -107 mV (obtained in the presence of valinomycin: no control values are mentioned) corresponds to an internal Rb^+ concentration of about 25 m*M*. The measurements of Paschinger (1977) allow the computation of internal K^+ concentration as very similar to that reported by Dewar and Barber (1973), i.e. about 150 m*M*. The discrepancy between the original K^+ concentration in the cells and the final Rb^+ content is rather alarming. However, it must be pointed out

that the agreement between Rb^+ and $TPMP^+$ distribution as estimates of ψ_{co} extends to experiments carried out in the presence of the ATPase inhibitor DCCD (Paschinger, 1977), when the computed ψ_{co} was about −77 mV (decreased by inhibition of the electrogenic mechanism). This result implies a relative insensitivity of ψ_{co} to external Rb^+, since the $TPMP^+$ experiments were carried out in the absence of the 0.25 m*M* Rb^+ present for the Rb^+ experiments (assuming that the effects of the $TPMP^+$ on the value of ψ_{co} are negligible).

Thus while most of the data are consistent with a distribution of K^+ (Rb^+) by passive uniport in response to ψ_{co}, some data are difficult to accommodate in this sort of scheme. Such a passive distribution can also explain the effects of CCCP and DCCD in causing a net K^+ loss and Na^+ gain in *Anacystis* (Dewar and Barber, 1973; Paschinger, 1976, 1977), since DCCD has been shown to reduce the value of ψ_{co} and CCCP probably does so (Paschinger, 1977; Masamoto and Nishimura, 1977a, b).

With respect to Na^+ distribution the estimates of ψ_{co}, obtained by Paschinger (1977) suggest that active Na^+ efflux is needed if there is a finite permeability to Na^+ by a passive uniport mechanism. Dewar and Barber (1973) have shown that there is a tracer Na^+ influx in the absence of net Na^+ transport, although they did not show that this influx was by passive uniport. They found (Dewar and Barber, 1973) that the addition of CCCP increased the tracer Na^+ influx; this could not be explained in terms of a decreased ψ_{co} such as was invoked to explain the net K^+ loss under these conditions; an increased capacity for passive Na^+ uniport is required to explain the increased influx despite the reduced driving force.

Tracer Na^+ efflux was inhibited by CCCP (Dewar and Barber, 1973); this, together with the observed increased tracer influx, can explain the net Na^+ gain in the presence of CCCP. Paschinger (1977) showed that DCCD led to a net Na^+ influx in *Anacystis*; while this could be attributed to an inhibition of active Na^+ efflux when energy supply was blocked, with the observed Na^+ influx being by Na^+ uniport in exchange for K^+, Paschinger (1977) showed that this Na^+ influx was paralleled by an H^+ efflux. Since active H^+ efflux was blocked by DCCD, Paschinger (1977) attributes this to an $Na^+:H^+$ antiport. This is assumed to normally couple downhill H^+ influx to active Na^+ extrusion, but in the absence of generation of $\Delta\bar{\mu}_{H^+}$ it appears to run backwards.

Thus the overall conclusions for *Anacystis* are similar to those for

Chlorella (which also appears to lack redox pumps in the plasmalemma, and has its primary active transport processes there powered by ATP generated on intracellular membranes): Na^+ distribution is best viewed as passive Na^+ uniport influx down a $\Delta\bar{\mu}_{Na^+}$, with active Na^+ efflux *via* an $H^+:Na^+$ antiporter. K^+ distribution also involves passive K^+ uniport, although this is probably inadequate to explain K^+ distribution under all circumstances: $K^+:H^+$ symport and $K^+:H^+$ antiport may be required to explain K^+ distribution when ψ_{co} is less negative than ψ_{K^+} and more negative than ψ_{K^+} respectively.

G. CHLORIDE

As with transport of K^+ and Na^+, most of the data on Cl^- transport in microalgae are available for *Chlorella*, *Scenedesmus* and *Ankistrodesmus* among the chlorococcales, and for *Anacystis* among the cyanobacteria. These algae, when grown in their normal low-osmolarity medium, have low intracellular Cl^- concentrations (see Tables 3, 4 and 5 of Raven, 1976a; Kirst, 1977). This low Cl^- content is typical of the cytoplasm of freshwater plants: the volume occupied by the vacuole (which often has a higher Cl^- concentration than the cytoplasm: see Raven, 1976a) is only about 0.1 of the total in the chlorococcales, and is, of course, zero in *Anacystis*. In microalgae, including the halotolerant strains of *Chlorella*, which live in seawater or solutions of even higher osmolarity, the Cl^- concentration is higher even in relatively non-vacuolate cells (Tables 6.3, 6.4 and 6.5 of Raven, 1976a; Kirst, 1977; Chimiklis and Karlander, 1973; Gilles and Pequaux, 1977). The extent to which this Cl^- is localized in whatever vacuolar volume there is cannot be determined on the basis of currently available data. The vacuole presumably houses most of the Cl^- found in vacuolate freshwater and marine algae, with the vacuolar concentration probably being higher than in the cytoplasm.

On electrochemical criteria the influx of Cl^- into *Chlorella* (Barber, 1968a, b; cf. Komor and Tanner, 1976) and *Anacystis* (Dewar and Barber, 1974; cf. Paschinger, 1976, 1977) is active: this is probably also the case in the other microalgae as well (Raven, 1976a). The relation of Cl^- influx to external Cl^- concentration shows typical saturation kinetics: Meszes *et al.* (1967) found a value of 500 μM in *Scenedesmus*, using $^{82}Br^-$ as a tracer for Cl^-, while Neilsen (1965) found a value of 70 μM using $^{36}Cl^-$ in *Chlorella*.

The energetics of Cl^- transport have been extensively investigated (Raven, 1976a) in the freshwater alga *Chlorella* (Neilsen, 1963, 1965, 1966; Barber, 1968a, 1969) and *Scenedesmus* (Hope *et al.*, 1974; Findenegg, 1974, 1977a). Tracer Cl^- influx is light-stimulated, and the effects of inhibitors are consistent with ATP being the energy source, although most of the evidence was obtained with uncouplers rather than energy-transfer inhibitors. Treatments which isolate various ATP-generating processes, including (Hope *et al.*, 1974) the use of mutant strains of *Scenedesmus* deficient in the reactions of photosystem I or photosystem II (see Fig. 7), suggest that cyclic photophosphorylation can support most of the rate of $^{36}Cl^-$ influx in light; oxidative phosphorylation powers the considerably lower rate of influx found in the dark. In the cyanobacterium *Anacystis* light stimulation of Cl^- influx is less reproducible, and the photosystem II inhibitor CMU can *stimulate* Cl^- influx (Dewar and Barber, 1974). It has been argued that this may be related to a relief of competition for energy (ATP?) from photosynthetic CO_2 fixation which is blocked in the presence of CMU (which inhibits reductant generation: Dewar and Barber, 1974).

The primary or secondary nature of active Cl^- influx, and the relationship between Cl^- and other ion fluxes, is poorly understood. Paschinger (1976, 1977) suggested that Cl^- influx in *Anacystis* is by H^+ symport; this view seemed to be based on experiments with DCCD. In *Chlorella* Barber (1969) has shown that a light-induced net Cl^- influx (with a corresponding net Cl^- efflux upon darkening) is paralleled by changes in Na^+. However, Neilsen (1964) found a 15% lower $^{36}Cl^-$ influx in the steady-state in the light with Na^+ rather than K^+ as the counter ion. These experiments were carried out with cells grown at high external CO_2 levels; Findenegg (1974, 1977a) has shown that the nature of Cl^- transport in *Scenedesmus* is strongly influenced by whether the cells were grown in air or in air supplemented by extra CO_2. The equilibration of $^{36}Cl^-$ with initially (almost) Cl^--free cells is much more rapid in air-grown than in high-CO_2-grown cells in either light or dark. Only the air-adapted cells showed a substantial net Cl^- influx upon illumination with a corresponding net Cl^- release in the dark (cf. Barber, 1969). The negative charge entering the cell upon illumination is not balanced by influx of K^+ or Na^+; indeed, there is a net K^+ efflux from these cells upon illumination (cf. Findenegg, 1978). Thus there is even more net negative-charge influx to balance: the counter ion seems to be H^+ (Findenegg, 1974, 1977a, 1978). The H^+ influx is greater in the

presence than in the absence of external Cl^- (Findenegg, 1974). This constitutes the best evidence for the mechanism of Cl^- influx in microalgal cells, and points strongly at an H^+-Cl^- symport, with the H^+ influx associated with the Cl^- influx (and also that related to K^+ efflux) probably being part of a pH-stat for the cytoplasm. The generation of the pH-difference which is the driving force for this light-induced net Cl^- influx depends on photosystem II in that it is inhibited by DCMU; it is slowed by CCCP. The net fluxes of Cl^- involved in these light-dark changes in *Scenedesmus* may be 5-10 pmol cm^{-2} s^{-1} (Findenegg, 1974, 1977a, assuming 0.5 cm^2 per μl of cells). This is comparable with the flux of up to 20 pmol cm^{-2} s^{-1} required to explain the net loss of Cl^- (produced by ClO_3^- reduction) in *Chlorella* cells supplied with saturating external ClO_3^- (calculated from the data of Tromballa and Broda, 1971). The net influx of 20 pmol cm^{-2} s^{-1} of KCl during adjustment of cells of the marine diatom *Cyclotella cryptica* to higher external osmolarities occurs from a solution at least 200 times more concentrated in Cl^- than the external or external phases in the experiments on *Chlorella* and *Scenedesmus* (Brown and Hellebust, 1978).

H. CALCIUM AND MAGNESIUM

Relatively little is known of the transport of these two cations in microalgae. Mg^{2+} is well-established as an essential macronutrient in all microalgae; Ca^{2+} is only a micronutrient in a number of microalgae (O'Kelley, 1974). The two cations are considered here for similar reasons to those which lead to the consideration of K^+ and Na^+ together: they show interesting differences in biological behaviour (Williams, 1970, 1972, 1974).

Estimation of the chemical activity of Mg^{2+} and Ca^{2+} in algal cells is less simple than for K^+, Na^+ and Cl^-, in that while the activity coefficients of K^+ and Cl^- (and, to a lesser extent Na^+) in cytoplasm are close to those in free solution at the same concentration, Mg^{2+} is bound to a considerable extent (e.g. to phosphate compounds), while Ca^{2+} is almost completely bound, i.e. these ions have very low activity coefficients (Lev and Armstrong, 1975; Williams, 1970, 1972, 1974). It seems likely that the Ca^{2+} activity in the ground cytoplasm of healthy cells does not exceed 1 μM, and that of the mitochondrial matrix and chloroplast stroma does not exceed 10–100 μM (Duncan, 1976; Portis and Heldt, 1976). These considerations appear to be of general

biological validity, and have been invoked (e.g. by Blackwelder *et al.*, 1976) in a microalgal context. However, it is incumbent upon me to present algal evidence for this very low Ca^{2+} activity in the ground cytoplasm. Nicholls and Rikmenspoel (1977, 1978) have presented some evidence for *Euglena* and *Chlamydomonas* which is consistent with a very low Ca^{2+} activity in the ground cytoplasm. Their technique was to impale the cells on large (relative to the size of the cells) microelectrodes and investigate the response of flagella activity to the passage of electric current or the mechanical injection of solutions; free Ca^{2+} had to be at around 1 μM in the cells to allow flagella activity. Salisbury and Floyd (1978) have implicated the reversible sequestration of Ca^{2+} in golgi vesicles in the reaction sequence initiating flagella activity in *Platymonas*. Litvin *et al.* (1978) have shown that a transcellular ion flux associated with light perception in *Haematococcus* phototaxis appears to involve Ca^{2+}, and that the magnitude of this flux could only change the internal Ca^{2+} content by a very small amount, suggesting that the background level on which this triggering change is superimposed must be small..

Granted the occurrence of very low concentrations of free Ca^{2+} (relative to the total intracellular Ca^{2+}) in the ground cytoplasm, and a free Mg^{2+} level lower than the total Mg^{2+} concentration in the ground cytoplasm, it is clear that both Ca^{2+} and Mg^{2+} are at a lower free energy in the ground cytoplasm than in the medium. Hence, if the downhill influx of these ions exceeds the cell requirement, active efflux is required. Evidence from microalgae as to the occurrence of such active efflux is sparse. Grass and Marme (1978) have reported Ca^{2+} activated ATPase activity and ATP-dependent Ca^{2+} accumulation in a membrane fraction from *Cryptomonas* (cf. Section IV.A). The evidence that this "sarcoplasmic reticulum" type of Ca^{2+} transport is associated with the plasmalemma is not completely persuasive: from the point of view of regulating the Ca^{2+} activity in the ground cytoplasm uptake of excess Ca^{2+} into vesicles is as good as extrusion through the plasmalemma as long as the storage capacity of the vesicles is not exceeded. Unless exocytosis of the contents of the vesicles is possible the capacity problem is handed down to daughter cells in perpetuity (which has so far been 10^9 or so years for eukaryotic microalgae). In the filamentous alga *Mougeotia* (Wagner, 1974; Wagner and Bellini, 1976) there are tracer fluxes at the plasmalemma in excess of the net Ca^{2+} flux; this is consistent with active Ca^{2+} efflux in this alga which has a

large central vacuole which could act as an internal Ca^{2+} sink. In the prokaryotes, for which this internal storage cannot be in vesicles (Pautard, 1970), there is good evidence (none of it, alas, for cyanobacteria) for both primary and secondary active efflux of Ca^{2+} at the plasmalemma (Bhattacharya and Barnes, 1976; Tsuchiya and Rosen, 1976; Belliveau and Lanyi, 1978; Kobayashi *et al.*, 1978). Even less is known about Mg^{2+} transport in microalgae than about Ca^{2+} transport.

Perhaps the most widely investigated microalgal process involving Ca^{2+} transport, and the quantitatively most important, is coccolith formation in coccolithophoridaceae (Prymnesiophyceae). The coccoliths are extracellular plates of organic material (polysaccharide and protein) impregnated with $CaCO_3$. They are formed in the golgi apparatus prior to excretion from the cell: the Ca^{2+} which ends up in the coccoliths must have entered the cells across the plasmalemma (probably *via* uniport driven by the ψ_{co}), and then been translocated across the golgi membrane probably by active (ATP-powered) transport (see above, and Fig. 2); Paasche (1964, 1968), Pautard (1970), Darley (1974) and Borowitzka (1977). Thus all of the $CaCO_3$ which ends up in the coccoliths (and hence most of the $CaCO_3$ in cretaceous chalk deposits) has passed through two prymnesiophyte cell membranes.

Since the molar ratio of Ca^{2+} in coccoliths to total cell C (inorganic in coccoliths plus organic) can be as high as 0.4 (Paasche, 1962, 1968) the entry of Ca^{2+} in coccolith-producing algae can be a major net ion flux. For the two coccolithophorids for which most data are available (*Hymenomonas* and *Coccolithus*) considerations of mean cell size, cell generation time and Ca^{2+} content show that the net Ca^{2+} influx for coccolith formation is 2.5–3.5 pmol cm^{-2} s^{-1} (Paasche, 1962; Paasche and Klaveness, 1970; Blackwelder *et al.*, 1976; Weiss *et al.*, 1976). These fluxes seem low (relative to those quoted for *Chlorella* in Table 4) for an element which is of such quantitative significance. The reason is the relatively low maximum growth rate of these coccolithophorids (1.5–2.0 generations per day), combined with their small size (similar to that of *Chlorella*, i.e. a diameter of 2.5–5 μm): the net C influx during growth corresponding to the Ca^{2+} values mentioned above is up to 10 pmol cm^{-2} s^{-1}, and the maximum reported (Paasche and Klaveness, 1970) is about 20 pmol cm^{-2} s^{-1}. The fluxes of Ca^{2+} into the golgi vesicles is probably of a similar magnitude to the plasmalemma flux; neither of these flux magnitudes approach the values found for

possibly similar fluxes, i.e. Ca^{2+} influx into mitochondria of many animals and Ca^2 influx into sarcoplasmic reticulum for plasmalemma and golgi fluxes respectively (cf. Duncan, 1976).

It has been suggested by Blackwelder *et al.* (1976) that the low concentration of free Ca^{2+} in the ground cytoplasm may pose a problem for Ca^{2+} diffusion from the plasmalemma to the golgi vesicles. For *Coccolithus*, with coccolith formation in golgi vesicles near the centre of the cell between the two chloroplasts (Paasche, 1968), the diffusion path averages 2 μM, and with a net flux of 3.5 pmol cm^{-2} s^{-1} and a diffusion coefficient for Ca^{2+} assumed to be 10^{-6} cm^2 s^{-1} (cf. Raven, 1977b), the required concentration *difference* to explain the observed flux is (equation 1) 0.7 μM. This is perilously close to the upper limit on the *total* free Ca^{2+} concentration in ground cytoplasm, and diffusion of Ca^{2+} solely as free Ca^{2+} is impossible if the $D_{Ca^{2+}}$ is less than 10^{-6} cm^2 s^{-1} (Raven, 1977b). However, the ground cytoplasm contains several mM of chelated Ca^{2+} which exchanges rapidly with the free Ca^{2+}, and diffusion of these chelating agents can facilitate Ca^{2+} movement (Raven, 1977b). At the "source" end of the diffusion path the free Ca^{2+} concentration is higher than it is at the "sink" end, which implies that equilibration with the chelators gives a high ratio of "Ca^{2+}-charged" to "empty" molecules at the source end, and the reverse at the sink end. This creates diffusion gradients for "Ca^{2+}-charged" chelators from the source to the sink, with a reverse gradient for "empty" chelators with the highest concentration at the sink. The possible concentration gradients for the charged and uncharged forms of the chelators (such as citrate) could be each of the order of 1 mM even when the gradient for free Ca^{2+} was only from (say) 1.2 μM at the source end to 0.8 μM at the sink end, granted the intracellular total Ca^{2+} in coccolithophorids (Isenberg *et al.*, 1963, 1964), while the diffusion coefficient for the chelators is probably at least 10^{-6} cm^2 s^{-1} in the cytoplasm (Raven, 1977b); these values for P $(=D/1)$ and $(C_1 - C_2)$ inserted in equation (1) can readily account for the observed Ca^{2+} flux in the cytoplasm of coccolithophorids.

The overall process of coccolith formation requires not only the transport of Ca^{2+} into the vesicles, but it also requires a concentration of CO_3^{2-} in the vesicle sufficient to exceed the solubility product of $CaCO_3$, as well as the absence of any crystal poisons which would inhibit the precipitation. The mechanism for arranging the correct inorganic C content and pH to give this CO_3^{2-} concentration is not well

understood (Borowitzka, 1977). The "normal" direction of active H^+ transport (Fig. 4) is from the cytoplasm into such vesicles, and it is possible that a high pH in the vesicles is maintained by an inside-positive potential difference and H^+ passive uniport in the membrane or, even better, HCO_3^- uniporters: the potential difference is generated in this model by an electrogenic Ca^{2+} pump. It is possible (Borowitzka, 1977; cf. Paasche, 1964) that the inorganic carbon concentration in the ground cytoplasm is increased by active HCO_3^- transport at the plasmalemma (cf. Section IV.B, 1, IV.J).

The function of coccoliths has been considered at length; a recent suggestion (Bauman *et al.*, 1978) is that the main selective advantage bestowed by coccoliths is an increased ability to acquire nutrients. It has long been known that nitrogen deficiency induces otherwise recalcitrant strains of coccolithophorids to produce coccoliths, and Bauman *et al.* (1978) argue that the synthesis of coccoliths, by increasing overall cell density, favours sinking and thus may take the organism to a level at which N-sources are more abundant.

I. SILICON

Silicon is an essential element for diatoms, in which SiO_2 is an important constituent of the cell wall (Werner, 1977a, b), and also for some (perhaps many) chrysophytes with silicified scales (Klaveness and Guillard, 1975; Lee, 1978). It is also accumulated (not in a solid form) by the green flagellate *Platymonas*, for which it is *not* an essential element (Fuhrman *et al.*, 1978). As with the Ca^{2+} and CO_3^{2-} in coccoliths, and the $-O-SO_2-O^-$ in sulphated wall polysaccharides (Sections IV.G and IV.E respectively), the SiO_2 in the walls of diatoms and the scales of chrysophytes has entered the cytoplasm across the plasmalemma, and has been subsequently processed and packaged into a new cell wall or scale within a membrane-bounded vesicle, the "silicalemma" in diatoms and the chrysophyte golgi apparatus. Finally, the new cell wall portion or cell surface scale is excreted from the cell; in the case of the diatom cell wall it becomes a turgor-resisting structure (Werner, 1977b; Duke and Reimann, 1977; Darley, 1974). The silicon influx across the plasmalemma is substantial in terms of the other solute fluxes needed for growth of a diatom, since the silicon content of a diatom can be 0.25 or more of the carbon content (Werner, 1977b). For the large yet rapidly growing *Ditylum brightwellii* ($6 \cdot 10^4 \mu m^3$ volume, 3.2 divisions per day: Eppley, 1977), it is possible to compute silicon

influx values of up to 80 pmol cm^{-2} s^{-1}, compared to carbon net influx values of over 150 pmol cm^{-2} s^{-1} (data from Eppley (1977), Werner (1977b), Darley (1977); cf. Table 4). These values may be overestimates of the actual fluxes on a unit plasmalemma area rather than a unit cell surface area basis in view of the convoluted nature of the inner surface of the diatom cell wall in contact with the plasmalemma (Werner, 1977b).

Study of the energetics of silicon influx is complicated by the possibility of internal polymerization of silicic acid within the ground cytoplasm, and furthermore by some doubt as to the form in which silicic acid enters the cell. While $Si(OH)_4$ is the dominant form of silicic acid in natural waters at the pH values which diatoms tolerate, it is possible that $H_3SiO_4^-$ might be involved (Darley, 1974). In a number of cases the total silicic acid monomer concentration in the cytoplasm of microalgal cells taking up silicon exceeds that in the medium (Azam *et al.*, 1974; Fuhrman *et al.*, 1978). This can be used as evidence for active transport, regardless of the form of silicic acid ($Si(OH)_4$, $H_3SiO_4^-$ or $H_2SiO_4^{2-}$) taken up, since the influx is unaltered over the external pH range pH 7–pH 8, which includes the likely cytoplasmic pH (Werner, 1977b). Thus it is reasonable to assume that silicic acid uptake can occur when $pH_{cyt} = pH_{outside}$, and hence when the electrochemical potential of all three species of silicic acid is higher in the cytoplasm than in the medium.

The analogue germanic acid is easier to study, since it does not spontaneously polymerize and is not incorporated into cell envelope components. $Ge(OH)_4$ (or some ion thereof) is actively transported into diatoms (Azam and Volcani, 1974).

The $K_{1/2}$ for silicic acid uptake (expressed in terms of the total ionized and un-ionized monomeric silicate in the medium, corrected for an unusable residual concentration) has been measured at 0.8–3.4 μM in four species of diatom (Paasche, 1973a, b, c). Efflux of silicic acid monomer from diatoms is small relative to the net influx; it is rather larger for germanic acid, possibly as a result of the rather higher levels of this "non-metabolized" analogue accumulated within the diatom cell (Werner and Petersen, 1973).

The active influx of silicic acid appears to have ATP as its ultimate energy source (Lewin, 1955; Werner, 1977b). Light, probably *via* photosynthetic processes, stimulates the influx of silicate but not of germanate in *Cyclotella cryptica* (Werner and Petersen, 1973). The

stoichiometry of silicic acid influx with respiratory metabolism was determined by Werner (1965, quoted by Werner, 1977b). From the observed ratio of 36 μmol $Si(OH)_4$ taken up per μmol glucose (derived from stored chrysolaminarin) respired, the ATP used per $Si(OH)_4$ transported ratio cannot exceed one. Werner (1977b) further points out that this ratio refers to the whole reaction sequence (influx across the plasmalemma, transfer across the silicalemma, and polycondensation), so that if the initial uptake of $Si(OH)_4$ needs 1 ATP per $Si(OH)_4$ there can be no further energy requirement in the reaction sequence. No data seem to be available on the involvement of primary *versus* secondary transport of silicic acid.

J. PROTON TRANSPORT AND pH REGULATION

H^+ appears to be the ion responsible for electrogenesis in the microalgae *Chlorella*, *Anacystis* and *Plectonema* (Section IV.A), and many of the transport processes mentioned in Sections IV.B–IV.H involve transmembrane H^+ movements. In some cases there is good evidence for H^+-cotransport (particularly convincing for sugars in *Chlorella*: Section IV.B, 2); in others, in addition to any H^+ cotransport related to substrate transport into the cell, there are large productions (NH_4^+) or consumptions (NO_3^-, HCO_3^-) of H^+ within the cytoplasm during assimilation of these substrates (see Table 4).

The "metabolic" H^+ fluxes, associated with the disposal of excess H^+ or OH^- generated by metabolism of NH_4^+, NO_3^- and HCO_3^-, occasion net H^+ changes in the medium which can be measured in growth experiments as well as in short-term experiments (Brewer and Goldman, 1976; Raven and Smith, 1973, 1974, 1976a, b; Smith and Raven, 1976), although in the case of HCO_3^- some skill is needed in distinguishing between the effects due to CO_2 entry as opposed to HCO_3^- entry (Section IV.B, 1). The H^+ fluxes associated with solute cotransport *do not* appear as H^+ changes in the medium in growth experiments, or in uptake experiments conducted over times of the order of tens of minutes. These H^+ fluxes can generally only be detected in very short-term experiments (a few seconds) in which the putatively cotransported solute is added to the cell suspension and its uptake and any H^+ uptake are followed; while the cotransported solute generally shows a linear time-course, the net H^+ influx often occurs for only a few tens of seconds (Komor, 1973). From the stoichiometry of H^+ influx : solute influx for each solute required for growth, and the

net influx of each solute during growth (Tables 3 and 4), it would be possible to determine the total H^+ influx associated with cotransport processes during growth. Maintenance of intracellular pH requires an H^+ efflux equal to the H^+ influx which occurs in cotransport, with suitable additions or subtractions for internally generated (NH_4^+ assimilation) or consumed (NO_3^- or HCO_3^- assimilation) H^+. This requirement for intracellular pH regulation, and hence (in the presence of H^+-producing or H^+-consuming metabolic reactions) an imbalance of H^+ influx and H^+ efflux, must be made compatible with the maintenance of $\Delta\bar{\mu}_{H^+}$ across the plasmalemma.

The implicit assumptions in the foregoing are (1) that the only processes which are involved in regulating the acid-base balance of the cell are the transplasmalemma fluxes of H^+ ions and the metabolic production or consumption of H^+ in the primary metabolism of NH_4^+, NO_3^- and HCO_3^-, and (2) that the cytoplasmic pH is indeed maintained constant. As far as point (2) is concerned, Table 7 shows the measurements of microalgal cytoplasmic pH which have thus far been made, using a variety of techniques (Section III.I). The range of values is pH 6–pH 8, excluding those measurements (see Table 7) in which the method of measuring the cytoplasmic pH was itself likely to alter the pH value of the cytoplasm. The range of cytoplasmic pH values for any one alga is generally considerably smaller than the "overall" range of pH 6 to pH 8. The major experimental variables employed in these determinations are the external pH and the illumination conditions. While *Euglena* shows a considerable variation of intracellular pH with extracellular pH, the extent of change of intracellular pH with alteration of extracellular pH is between 0 and 0.2 units change in pH_{cyt} for a unit change in external pH. The effect of illumination is generally to cause a transient or prolonged increase in internal pH. The presence of an external solute (HCO_3^-) which can be taken up into the cells and metabolized with a large consumption of intracellular H^+ does not cause a large change in cytoplasmic pH in *Scenedesmus* or *Chlamydomonas*. This all betokens a fairly precise regulation of cytoplasmic pH in the face of the various assaults on its constancy due to H^+ transport or H^+-producing (or H^+-consuming) reactions, although further measurements of cytoplasmic pH during various transport (e.g. H^+-glucose cotransport) or metabolic (e.g. NH_4^+ or NO_3^- metabolism, or organic acid synthesis in fermentation) pH stresses would be helpful.

TABLE 7. Cytoplasmic pH values in microalgae

Alga	Method	External pH	Cytoplasmic pH	Reference
Anacystis nidulans	Distribution of DMO	6.5–8.0	7.5 (light) 6.9 (dark)	Falkner, Horner, Werdan and Heldt (1976)
Anacystis nidulans	Direction of net H^+ flux upon addition of H^+ ionophore; assuming ψ_{co} is zero immediately after addition of ionophore, pH at which net H^+ flux is zero $= pH_{cyt}$		8.5 (light) 7.5 (dark)	Masamoto and Nishimura (1977a)
Plectonema boryanum			8.5 (light) 7.4 (dark)	
Chlamydomonas reinhardtii (air-grown)	Distribution of DMO	7.15	7.05 to 7.08 (light, 10–250 μm HCO_3^-)	Badger *et al.* (1978) (cf. Badger *et al.*, 1977)
(high-CO_2 grown)		7.15	7.02 (light, 100 μm HCO_3^-)	
Chlorella	Distribution of 2,4-dichlorophenoxyacetic acid	5–7	5.3–5.8	Wedding and Erickson (1956)
Chlorella pyrenoidosa	pH of cell homogenates	6.8 (?)	6.50 (Na^+-rich, K^+-deficient cells 6.85 (Na^+-rich cells after recovery of normal K^+/Na ratio)	Shieh and Barber (1971)
Chlorella vulgaris	Distribution of DMO	5.2 5.8 6.7 7.7	6.8 (light) 7.0 (light) 7.2 (light) 7.4 (light)	Komor and Tanner (1974a)
Euglena gracilis (cultured in 12l flasks)	Distribution of DMO	6.60 7.40	6.0 7.5	Votta *et al.* (1971)
(cultured in 30 ml flasks)		7.25 8.4	6.0 7.5	

Euglena gracilis	Distribution of DNP	?	6.0–8.0 (1–2 mins after adding DNP) 5.5 (5–10 mins after adding DNP)	Evans (1975)
Scenedesmus obliquus	Distribution of DMO	5.0–7.0 5.0–7.0	7.0 (dark) 7.5 (light for 1 min) 7.0 (light for long periods)	Findenegg (1978)

Values obtained with the distribution of weak electrolytes are predicated *inter alia* on the assumption that insufficient of the weak acid accumulates in the cytoplasm to alter the cytoplasmic pH. The experiment of Evans (1975) with DNP as the weak electrolyte shows that this is not the case: cytoplasmic pH falls after a few minutes exposure to the weak acid. Similar results were found with the higher plant *Zea mays* by Humphreys (1975) and for the giant-celled alga *Nitella* (Denesh *et al.*, 1977); it is possible that the low estimate of cytoplasmic pH for *Chlorella* obtained by Wedding and Erickson (1956) for the weak acid 2,4 dichlorophenoxyacetic acid is subject to this error, since this compound had a similar uncoupling effect to DNP in these cells (Wedding and Black, 1961). However, it is likely that acidophilic algae have lower cytoplasmic pH values in their normal growth medium than do algae which favour higher external pH values, arguing by analogy with heterotrophic prokaryotes (Hsung and Haug, 1975; Krulovich *et al.*, 1978) and chemolithotrophic prokaryotes (Ingledew *et al.*, 1977).

With respect to the first point, i.e. the assumption that there are no pH-regulating processes other than transplasmalemma H^+ fluxes and primary metabolism of ionic C and N sources, the other major possibilities are (1) intracellular buffering by pre-existing buffer compounds with phosphate, carboxyl or amino or imino groups of the appropriate pK_a value, and (2) the synthesis of strong acids and bases from appropriate precursors (Raven and Smith, 1974; Smith and Raven, 1976, 1979). The use of pre-existing buffers is, in the long term, not a feasible means of regulating intracellular pH, since in the case of the carboxyl and amino (imino) groups it is the synthesis of these groups and their dissociation at the pH value of the cytoplasm which occasioned part of the pH stress in the first instance.

The synthesis of strong acids or bases is a stratagem widely employed in some microalgae, in fungi and in higher plants (see Raven and Smith, 1974, 1976a, b; Smith and Raven, 1976, 1979; Davies, 1973a, b) and to some extent in metazoa (Siesjo, 1973). The idea of the biochemical pH-stat, as outlined by Davies (1973a, b) involves the synthesis of malic acid (a strong organic acid) from neutral (sugar) and weakly acidic (CO_2) precursors by a mechanism which is stimulated by higher pH values within the physiological range. The pH-sensitive enzyme in this sequence is PEP carboxylase (see equation 10), and the overall result is that an increase in cytoplasmic pH within the physiological range (Table 7) leads to an increased synthesis of the strong acid. This sort of mechanism is particularly striking in the aerial shoots of land plants which are reducing NO_3^-, and in which there is no convenient sink for OH^- (Raven and Smith, 1976a; Raven, 1977b).

The reverse process, i.e. synthesis of a strong base to neutralize excess cytoplasmic acidity, is less readily accomplished by the biochemical pH-stat. As envisaged by Davies (1973a, b) the strong base was OH^- (HCO_3^-) produced by decarboxylation of the weakly basic malate anion by malic enzyme. Aside from problems related to the near-equilibrium status of the reaction catalysed by malic enzyme *in vivo* (Davies, 1977), this mechanism suffers from the further disability that the photolithotrophic cell only has a supply of malate anion insofar as it has synthesized malic acid from CO_2, and has disposed of the H^+ in excess of that required to produce the malate ion at the intracellular pH value. Thus, in the long term, the biochemical pH-stat is not a useful method of disposing of excess H^+ from cytoplasm (Raven and Smith, 1974, 1976a; Smith and Raven, 1976, 1979).

The operation of the biochemical pH-stat can be detected from studies of the composition of either growing cells or of their growth medium, or (preferably) of both. Taking NH_4^+-grown cells as the norm (since it is unlikely that their excess H^+ can be disposed of biochemically), the H^+ appearing in the medium generally amounts to about 1.2 H^+ per N assimilated, a value which can be confirmed from consideration of the titratable excess alkalinity of the ash produced from cell combustion (see Raven and De Michelis, 1980). If growth on NO_3^- produced cells of similar composition to those grown on NH_4^+, i.e. if there were no biochemical neutralization of excess OH^- within the cells, then there should be a disappearance of 0.8 H^+ per N assimilated from the medium, and the titratable excess alkalinity of the cell should be the same (on a cell N basis) as in the NH_4^+-grown cells (see Section III.C). If, however, the biochemical pH-stat is operative, then less than 0.8 H^+ should disappear from the medium per N assimilated, and the titratable alkalinity of the cell ash should correspondingly increase. Application of these tests to algae shows that there is no evidence for the operation of the biochemical pH-stat; although there patently are differences in composition between NH_4^+-grown and NO_3^--grown cells these are not related to pH-statting (Syrett, 1962; Morris, 1974; Brewer and Goldman, 1976; Raven and Smith, 1974; Smith and Raven, 1976; Raven and De Michelis, 1979, 1980). The energetic advantage of transmembrane transport of H^+ rather than a biochemical mechanism of pH regulation has been pointed out by Raven (1976a); a parallel advantage is the use of SiO_2 rather than organic polymers for cell wall synthesis in diatoms (Werner, 1977b).

Having shown that net transplasmalemma fluxes are indeed the major way of pH-regulation in microalgae, we might reasonably ask what evidence is available about the mechanism of this regulation, and its relationship to the maintenance of $\Delta\bar{\mu}_{H^+}$ at the plasmalemma. A reasonable starting point is the assumption that the increased active H^+ efflux when cotransport or H^+ production in NH_4^+ assimilation imposes an acidity stress in the cytoplasm is related to a decreased cytoplasmic pH, and vice versa for H^+ consumption in (e.g.) HCO_3^- assimilation (cf. Raven and Smith, 1977). Some idea of the extent of the pH change in the cytoplasm which is required to mediate this change in active H^+ efflux can be obtained by consideration of the data of Komor (1973) and Komor and Tanner (1974a, b) on H^+-glucose

cotransport in *Chlorella*. They showed that the glucose- (or glucose analogue-) induced net H^+ influx only lasted (with a declining rate) for some 60 seconds. In terms of the views expressed above, this decline in net H^+ influx may be interpreted as an increased active H^+ efflux which becomes equal to the cotransport H^+ influx after sufficient net H^+ entry has occurred to effect a small decrease in cytoplasmic pH. Since the sugar influx does not exceed 20 pmol cm^{-2} s^{-1}, and the H^+/sugar is 1, the net H^+ influx in the 60 seconds after sugar addition is unlikely to exceed 1 nmol cm^{-2}. The surface/volume ratio quoted by Tanner *et al.* (1974) suggests that this amounts to some 3 mmol H^+ per litre of intracellular volume. The buffer capacity of *Chlorella* cells seems to be about 20 mol H^+ l^{-1} pH $unit^{-1}$ (appendix of Raven and Smith, 1976b; Shieh and Barber, 1971), i.e. if the H^+ entering the cell had access to *all* of the intracellular buffer capacity, the pH decrease would only be about 0.15 pH units. If only the buffer capacity of the "ground cytoplasm" in contact with the plasmalemma, together with the volumes enclosed by "leaky" membranes (Section I.B) were available, then the pH decrease would be 0.3 units or so within the ground cytoplasm. However, in view of the large surface area of the "tight" membrane delimiting the other intracellular volume, and the large H^+ fluxes across them (Table 2A; Raven, 1978; cf. Gimmler *et al.*, 1974; Nobel, 1975), it seems likely that the initial assumption made above (access of the entering H^+ to all of the intracellular buffer capacity) is nearer the real state of affairs.

This suggests that an increase in the net active H^+ efflux across the plasmalemma of 20 pmol cm^{-2} s^{-1} can be induced by a change of pH at the inner face of the plasmalemma of less than 0.15 pH units. It is likely that most of this increase in net active efflux represents a stimulation of active H^+ efflux rather than a suppression of a "leak" H^+ uniport influx, based on the findings of Decker and Tanner (1972) on the effects of added sugars on dark respiration in *Chlorella*. Even if half of the basal respiration were associated with H^+ pump and leak at the plasmalemma (cf. Penning de Vries, 1975; Raven, 1976d) the H^+ leak (and balancing active efflux) would be less than 10 pmol cm^{-2} s^{-1}, based on the observed O_2 uptake and assuming an ATP/O_2 ratio of 6 and H^+/ATP ratio of 1 (Section IV.A).

Thus a decrease in the internal pH of 0.15 units (a 1.4-fold increase in H^+ activity) can cause an increase in active influx of some 20 pmol cm^{-2} s^{-1} from a basal level of less than 10 pmol cm^{-2} s^{-1}, i.e. at least a

three-fold increase in active H^+ efflux caused by a 1.4-fold change in cytoplasmic H^+ concentration. This is not necessarily in disagreement with kinetic regulation of a 1 H^+ ATPase, since there is no necessity for a stoichiometry of 1 H^+ transported per catalytic cycle to imply a direct linear relationship between the change in H^+ activity and the change in H^+ flux (cf. the apparent linear relationship between cytoplasmic Na^+ activity and active Na^+ efflux in *Chlorella* and *Scenedesmus* discussed in Section IV.F; and Portis and McCarty, 1976). The predicted decrease in internal pH should be measurable by the DMO technique with the precision achieved by Komor and Tanner (1974a), although this would not allow the extent of access of entering H^+ to intracellular buffers to be evaluated. Essentially complete access within 60 seconds is further supported by the fact that there are no subsequent changes in the H^+ net flux rate (Komor and Tanner, 1974a, b).

This analysis only considers one component of regulation of H^+ transport; the other consideration is charge balance. Walker (1976) and Walker and Smith (1977a), Harold (1977b) and Mitchell (1966, 1968) have considered the relative effects of net H^+ transport across a membrane on the pH difference across the membrane (a function of the volumes and buffer capacities of the phases separated by the membrane) and on the electrical potential difference across the membrane (a function, in the absence of any charge-balancing ion fluxes, of the membrane capacitance). Such considerations show that the sort of net H^+ flux considered above in relation to sugar-H^+ cotransport in *Chlorella* would, if not charge-balanced, lead to catastrophic changes in ψ_{co}. The 1 nmol cm^{-2} of net H^+ influx in *Chlorella* would, with a membrane capacitance of 1 $\mu F\ cm^{-2}$, give rise to a decrease in ψ_{co} of 10^5 mV compared with its initial value of about 135 mV! The observed depolarization of ψ_{co} does not exceed 75 mV (from an initial value of −135 mV) in considerably less than 60 seconds, and is restored to within 30 mV of this value within a further 60 seconds. The balancing net movement of positive charge out of the cell which occurs during the electrical potential changes and before the active H^+ efflux has become fully increased to its new value cannot be due to an anion influx since in many of the experiments of Komor and Tanner (1974a) the only external anions are OH^- and HCO_3^- whose influx would counter the observed net H^+ influx. The charge-balancing cation is probably K^+, whose net efflux would be increased as H^+ entry depolarized ψ_{co} (Section IV.E) (Tromballa, 1977; Findenegg, 1974,

1977a, 1978); the capacity for K^+ uniport is probably adequate for the peak flux required (20 pmol cm^{-2} s^{-1}; cf. Section IV.F). To the extent that, in the absence of K^+ downhill efflux, the net H^+ influx during the early stages of H^+-sugar symport would very rapidly (less than one second) take ψ_{co} down to below ψ_{H^+} and hence abolish the driving force for H^+ influx, the $\Delta\bar{\mu}_{K^+}$ could be considered as a short-term energy source for H^+-sugar cotransport (cf. Skulachev, 1978) before the active H^+ efflux rate has increased sufficiently to do so.

A possible example of *thermodynamic* regulation of active H^+ extrusion at the plasmalemma of microalgae is the light-induced net H^+ efflux found in *Plectonema boryanum* (Masamoto and Nishimura, 1977a, b). Here it is likely that light-driven redox H^+ pumps occur in the plasmalemma (Raboy and Padan, 1977, 1978); the maximum $\Delta\bar{\mu}_{H^+}$ which can be generated by such pumps probably does not exceed 20 kJ (mol $H^+)^{-1}$. An increase in the $\Delta\bar{\mu}_{H^+}$ at the plasmalemma upon illumination can be deduced from the results of Falkner, Horner, Werdan and Heldt (1976) and of Paschinger (1976, 1977) on *Anacystis nidulans*, although here it is likely that there is only an ATP-driven H^+ pump at the plasmalemma (Section IV.A).

With the assumptions as to the size of *Plectonema boryanum* used previously (Section IV.A and IV.B, 2), the net H^+ efflux in control cells (50 m*M* KCl) would cause an increase in internal pH of some 0.5 units, granted an internal buffer capacity of 20 mmol H^+ l^{-1} pH $unit^{-1}$; Masamoto and Nishimura (1977a) have detected a pH increase of this magnitude by an independent method (see Table 7), which in turn suggests that "biochemical pH-statting" is negligible at least on this time-scale. This charge transfer of about 0.2 nmol H^+ cm^{-2} out of the cell must patently be charge-balanced by some other ion movement (K^+ influx?) to avoid a change in ψ_{co} of about 17 V. The addition of valinomycin allows a much larger net H^+ efflux to occur (0.6 nmol cm^{-2}); this would give an increase in internal pH of well over one unit. The ability of valinomycin to catalyse K^+ uniport presumably permits a larger flux to occur, and hence depolarizes ψ_{co} and allows a pH difference to account for a larger fraction of a constant $\Delta\bar{\mu}_{H^+}$. In this instance it is clearly not the internal pH which prevents further net H^+ efflux when the normal (illuminated cell) pH_{cyt} has been reached, since further net H^+ efflux and rise in internal pH can occur when net charge transfer across the membrane by an additional, high-capacity uniporter (valinomycin) is possible. This is consistent with the normal

regulation of the fraction of $\Delta\bar{\mu}_{H^+}$ which is accounted for as a pH difference rather than a ψ_{co} being regulated primarily by a mechanism based on the transport of K^+ (or some other ion) through a regulated uniport channel. Valinomycin (with the 50 m*M* KCl present in all experiments of this type carried out by Masamoto and Nishimura, 1977a) bypasses this control mechanism.

The discussion of pH regulation up to here has been predicated on the occurrence of an electrogenic H^+ extrusion pump. This is used as a means of generating a $\Delta\bar{\mu}_{H^+}$, whose apportionment into a pH difference across the membrane and a ψ_{co} is a function of not only the cytoplasmic pH but also a number of other constraints, particularly charge transfer by K^+. This sort of model fits the growth of *Chlorella* on a variety of carbon and nitrogen sources such that the net generation of cytoplasmic acidity by H^+-symport processes exceeds any alkalinity generated by the metabolism of those solutes (Raven and De Michelis, 1979). Reference to Table 4 shows that, with an assumed stoichiometry of 1 H^+ cycled through the plasmalemma per singly charged ion pair or single neutral molecule accumulated, this constraint of net cytoplasmic acidification is met for glucose, CO_2 or HCO_3^- as the carbon source entering the cell, and NH_4^+, NH_3, urea, NO_3^- or amino acids as the nitrogen source entering the cell.

However, the possibility of primary, electrogenic influx of HCO_3^- was mentioned in Section IV.B, 1. This probably would not have been a significant factor in the experiments of Komor and Tanner (1974a, b, 1976) upon which so many of the arguments concerning active H^+ extrusion in microalgae in this review have been based, since the cells were grown at sufficiently high CO_2 levels to repress this transport system. Where it does occur (i.e. in cells grown at low levels of CO_2) the HCO_3^- pump, if it is primary and electrogenic rather than secondary and H^+-coupled (Walker and Smith, 1977b), could not only deliver inorganic C for photosynthesis but also generate a $\Delta\bar{\mu}_{H^+}$. In the absence of net CO_2 fixation (e.g. in the dark) the HCO_3^- pump could still function to drive cotransport, and as part of a pH regulation mechanism. Further experimentation is needed to test for the occurrence of active H^+ extrusion in cells which can assimilate HCO_3^- and conversely to investigate the possible electrogenicity of HCO_3^- influx. Such distinctions are not easy, and are still unresolved in the realm of animal physiology (Steinmitz, 1974; Schilb, 1978).

If electrogenesis in any microalga is due to primary active transport

of an ion other than H^+ or HCO_3^- then the electrogenic mechanism cannot of itself generate the $\Delta\bar{\mu}_{H^+}$ upon which the various other components of the pH regulation mechanism depends. As was pointed out in Section III.C, pH regulation in cells with some other primary active transport process (Cl^- or Na^+ are possibilities in marine algae) requires secondary active transport of H^+ as the $\Delta\bar{\mu}_{H^+}$-generating part of the mechanism (cf. Sachs *et al.*, 1978; Boron, 1977; Boron and Roos, 1978; Thomas, 1978).

V. Conclusions and Prospects

A. INTRODUCTION

The objective in this concluding statement is to consider the microalgal features discussed in Sections II and IV in relation to the generalized concepts of transport enunciated in Section III, and particularly in relation to the possible ecological adaptive significance of the various transport characteristics. This will emphasize the problems of life in the plankton, since this is the environment in which microalgae are dominant primary producers.

B. TRANSPORT THROUGH THE LIPID PORTION OF THE PLASMALEMMA

Under some ecological circumstances, the rate of net influx (Section III.B) of some nutrient solutes through the lipid phase of the plasmalemma can account for the rate at which the element borne by that solute is required by the cell (subject to constraints on growth rate imposed by availability of some other resource). This seems to be invariably the case for N_2 (Section IV.C, 1) for N_2 assimilation, and for the O_2 needed for respiration. A barrier in series with the plasmalemma (the heterocyst wall) may function to limit access of the deleterious O_2 to nitrogenase in cyanobacteria (Section IV.C, 1), acting as a kind of myelin sheath. CO_2 loss from respiring cells can be accounted for by passive diffusion through the lipid phase, as may inorganic C uptake as CO_2 during photosynthesis under many conditions (Section IV.B, 1). Under a number of conditions of low CO_2 concentration in the medium, however, there is evidence for active HCO_3^- influx, although O_2 efflux in photosynthesis seems always to be

passive (Raven, 1977c; Raven and Glidewell, 1979; cf. Steiger *et al.*, 1977). For ammonia, NH_3 entry may be adequate at high pH values, but facilitated NH_4^+ transport seems more generally to be required (Section IV.C, 2); for urea, active transport seems to be the rule (Section IV.C, 4).

In the cases in which facilitated transport of ions (or molecules) occurs with relatively high permeabilities through the lipid phase, there is the likelihood of short-circuiting of the facilitated transport by passive leakage. For natural selection, the energetic penalties associated with this "pump and leak" seem to be worth bearing in organisms in which the supply of nutrients is at least as likely to limit growth as is the supply of energy (Parsons and Takahashi, 1973; Guillard and Killham, 1977). Certainly the facilitated transport system is capable of reducing the external concentration of (CO_2 + HCO_3^-), (NH_3 + NH_4^+), or urea to very low levels despite the leak (Section IV.B, 1, C, 2, C, 4).

C. MEDIATED TRANSPORT

For other nutrient solutes the permeability of the unmodified lipid membrane is inadequate to support the required influx rate, and facilitated transport is the rule. The other requirement is that the cytoplasmic concentration of the nutrient solute shall be high enough to supply the assimilatory enzyme, and for most nutrient solutes concentrative transport is required when the external nutrient level is low. It can, of course, be argued that mere facilitation of transport would be adequate if the activity of the assimilatory enzymes were increased. This does *not* seem to be the general stratagem adopted (Raven, 1976a; Cole, 1976), and concentrative transport is the rule. This is even the case where there are two enzymic systems which can catalyse assimilation, of which one has a higher affinity for the substrate at the expense of the use of ATP and the production of two enzymes rather than one, i.e. the assimilation of ammonia (Section IV.C, 2) and urea (Section IV.C, 4). Here the cells which use the higher-affinity assimilatory enzyme still need concentrative influx mechanisms when ecological substrate concentrations are being used. Part of the explanation for this may be the extent to which the cytoplasm and the plasmalemma can, for a given surface/volume ratio, house additional protein molecules (Atkinson, 1977; Raven, 1977a, c, e; Section III.F). Concentrative transport is also clearly required for solutes used as

cytoplasmic enzyme activators (K^+) and vacuolar generators of osmotic potential (K^+, Na^+, Cl^-).

D. ACTIVE TRANSPORT

According to the definition of active transport as net flux in a direction contrary to that predicted from the solute electrochemical potential difference (Section III.A), concentrative influx of anions and neutral solutes is active, while concentrative influx of cations can often be explained as "passive" (but mediated) transport driven by ψ_{co}, which is itself maintained by active ion transport. With the exception of those cyanobacteria which have redox systems in their plasmalemmas, ATP appears to be the usual biochemical energy source for these concentrative transport processes. Algae appear to conform to the general pattern (Section IV.D, IV.E) whereby individual membranes have one or two (rarely three) distinct primary transport processes, and other concentrative and active transport processes are "cotransport" processes. For the best-investigated algae (the freshwater chlorophyte *Chlorella*, and the cyanobacteria *Anacystis* and *Plectonema*) the evidence favours primary active H^+ efflux (Section IV.A). For cotransport H^+ seems to be involved in many of the cases for which evidence is available; for P_i in many freshwater and marine algae, and for sugars and amino acids in a marine alga, the cotransported ion seems to be Na^+. In the freshwater algae the $\Delta\bar{\mu}_{Na^+}$ is probably itself generated by H^+-cotransport, while in the marine algae primary Na^+ active transport has not been ruled out. Other ions for which there are suggestions of primary active transport include Ca^{2+}, HCO_3^- and NO_3^-/Cl^-.

E. PORTER DENSITY AND FLUXES AT THE PLASMALEMMA

The density of occupation of the plasmalemma by porters is generally inferred in algae from the capacity for transport (pmol cm^{-2} s^{-1}) and assumptions about the turnover number of the porter (Section III.D, E and F). Net primary active transport probably has the lowest turnover number, and the most directly observed capacity for net primary H^+ efflux in algae (28 pmol cm^{-2} s^{-1}) is well within the range allowed by the considerations mentioned in Section III.D. However, the capacity for HCO_3^- transport (whether by H^+ cotransport or by primary HCO_3^- active transport) of up to 180 pmol cm^{-2} s^{-1} comes closer to the putative limit on capacity. In no case does the computed

total occupancy of the plasmalemma, taking all primary and cotransport systems required for growth into account, appear to involve an H^+ cycling much above 100 pmol cm^{-2} s^{-1}.

There is indirect evidence for increases in the transport capacity for a nutrient solute when the element supplied by that solute is growth-limiting; while in some cases the effect seems to be related to a relief of feedback inhibition of the flux, in others it is likely that synthesis of extra porter molecules can occur. This latter occurrence can, by increasing the V_{max} for transport on a cell basis (with a constant metabolic requirement per cell for the solute) decrease the $K_{1/2}$ for the solute in terms of growth rate. This seems to be the commonest way of increasing influx capacity at low solute concentrations in the medium. The intrinsic $K_{1/2}$ of the transport mechanism is less immediately changeable, and can be as low for solutes (K^+, SO_4^{2-}) which are rarely likely to be growth-limiting by being present at low external concentrations, as for solutes (P_i, NO_3^-, NH_4^+, urea, $Si(OH)_4$) which are often important limiting resources for algae. There is some evidence that the maximum rate of nutrient transport from low external concentrations in a given alga is related to the concentrations which that genotype normally encounters (Eppley *et al.*, 1969a, b; Guillard and Killham, 1977); these workers point out a number of other correlations, such as a high (or low) apparent $K_{1/2}$ for transport of one nutrient solute in a given alga being paralleled by high (or low) apparent $K_{1/2}$ values for other nutrient solutes, and a correlation may be related to the generally faster growth rate of smaller cells, and the higher apparent affinity of nutrient transport systems in faster growing cells (Guillard and Killham, 1977; D'Elia *et al.*, 1979; Nelson *et al.*, 1979). However, it is important to remember that the determination of $K_{1/2}$ values *in vivo* which involve rapid net transport from low concentrations are very likely to be subject to restrictions due to the rate at which the solute can diffuse from the bulk medium to the cell surface through the "boundary layer" (Lehman, 1978; cf. Gavis and Fergusson, 1975; Pasciak and Gavis, 1974, 1975; Neushl, 1972). It is teleologically sensible for the $K_{1/2}$ of the membrane transport system to be such that its value is slightly underestimated due to extracellular diffusion limitations even when it is determined with stirring of the medium as effective as is ever found in nature: diffusion limitation is the limit beyond which natural selection would not be expected to further increase the substrate-limited turnover rate of either soluble

enzymes or membrane transport systems (Albery and Knowles, 1976; Koch, 1971). Microalgae are not, of course, powerless to effect changes in this external diffusive limitation. Thus flagella may be as important in minimizing the thickness of the unstirred layer round the cell (Raven, 1976a) as in effecting net movement of the alga relative to the water body in which it is suspended, although such net movement can be useful in optimizing the relative supplies of light for photosynthesis and nutrients by vertical, diurnal migrations (e.g. Eppley *et al.*, 1968). Munk and Riley (1952), in an elegantly argued paper, pointed out the possible advantages of sinking as a way of not only getting to regions of potentially greater nutrient concentration, but also, by disturbing the unstirred layers, of increasing the instantaneous rate of nutrient solute supply to the plasmalemma (cf. Smayda, 1971; Bauman *et al.*, 1978; Walsby, 1975). Clearly cell size is important here in that it is related to both sinking rate and to the intrinsic thickness of the boundary layer in a given environment (Neushl, 1972), as well as to the intrinsic maximal growth rate of the organism (Maynard-Smith, 1969).

F. BIDIRECTIONAL FLUXES AND "PUMP AND LEAK"

In general the measurable extent of tracer efflux of nutrient solutes decreases with external concentration of the solute, although the precision of many of these measurements is inadequate to eliminate "tracer recycling" in the boundary layer as a source of the apparent lack of tracer efflux. These measurements have been largely confined to non-metabolized ions, sugars, P_i and SO_4^{2-}, with N-source providing solutes less thoroughly examined. The lack of convenient radio-tracers for N is a discouragement to such studies with N, as is the small steady-state pool size of ammonia and NO_3^- in the cytoplasm. However, such studies are essential if the mechanism of nutrient transport is to be understood. Most of the evidence is against a classical "pump and leak" for nutrients subject to active influx or, indeed, to concentrative influx by electrical coupling, although as was pointed out in Section V.A and B above it is difficult to see how some leakage of those solutes whose permeability coefficient in lipid bilayers is high can be avoided. Whatever the mechanistic significance of bidirectional fluxes they do not prevent the residual level of nutrient in the medium at which no further net influx can occur being low, particularly for the nutrients which provide the elements (N and P) which commonly limit growth in

nature. Frequently the organism is unable to grow at the lowest concentrations from which it can carry out net nutrient uptake, i.e. the rate of supply is lower than that which can support the lowest relative growth rate at which the organism is programmed to grow. Ecological data show (Guillard and Killham, 1977) that it is the large-celled diatoms with a low maximum relative growth rate which dominate in the later stages of a succession when nutrient levels are very low. Such organisms may be able to maintain growth at *lower* relative growth rates than smaller-celled species with a higher intrinsic maximum growth rate if the permitted range of growth rates for a given genotype only covers about a ten-fold range. This, however, leads to the conclusion that the ability to use very low nutrient concentrations *for growth* may be *negatively* correlated with the apparent affinity of the nutrient transport systems, since the smaller, faster-growing diatoms tend to have lower $K_{1/2}$ values for nutrient transport. Further investigations of *minimum* relative growth rates in relation to cell size and the characteristics of the transport systems are clearly desirable.

The occurrence of "leak" influxes for the solutes (mainly cations) which are subject to active efflux (i.e. H^+, Na^+, Ca^{2+}, Mg^{2+} and, in some cases, K^+) seems likely. In many cases the apparent "leak" influx of H^+ and Na^+ may be part of cotransport systems, while uniport of K^+ and Na^+ may have a function in adjusting the relative magnitude of the components (pH difference and electrical potential difference) of $\Delta\bar{\mu}_{H^+}$ (Skulacher, 1978; Section IV.J), and Ca^{2+} uniport may have importance in morphogenesis and sensory perception (Jaffe and Nucitelli, 1977; Litvin *et al.*, 1978). Active H^+ efflux is also required in some circumstances to rid the cell of excess H^+ produced in metabolism (Section IV.J; Table 4).

G. KINETIC AND THERMODYNAMIC REGULATION

In the cases in which there is a reasonable degree of confidence as to the energy per mol available from the energy source for transport, the energy involved in transporting one mol of solute, and the stoichiometry between the two processes, it is generally found that the steady-state free energy difference for the transported solute is *less* than the energy available from the driving reaction could maintain at thermodynamic equilibrium. This applies to the active extrusion of H^+ by *Chlorella* (granted the occurrence of a 1 H^+ ATPase; Section IV.A), to the

influx of metabolized sugars (such as glucose) in *Chlorella* (Section IV.B, 2), and (probably) to the influx of a number of cations if this is by electrically coupled uniport (Section IV.C, 2 and F), i.e. the flux is *kinetically* regulated. Redox-driven H^+ efflux at the plasmalemma of certain cyanobacteria may be close to thermodynamic equilibrium (Section IV.A and J), while it appears that the influx (by cotransport of a chemical or electrical variety) of non-metabolized analogues of "normal" solutes proceeds much closer to equilibrium than does transport of the "normal" solute. Perhaps the clearest example is that of glucose analogues in *Chlorella* where accumulation seems to occur to equilibrium with the immediate energy source ($\Delta\bar{\mu}_{H^+}$): Section IV.B, 2. Other examples may be the accumulation of methylamine to higher levels than ammonia (Section IV.C, 2), thiourea to higher levels than urea (Section IV.C, 4), and germanic acid to higher levels than silicic acid (Section IV.I). These effects are most readily observed in relatively non-vacuolate cells (e.g. *Chlorella*, *Chlamydomonas*) where vacuolar accumulation of the "normal" metabolite does not obscure the picture.

Since there is good evidence in all of the cases quoted that the analogue is transported by the same porter system as the "normal" metabolite, it would appear that the "normal" metabolite influx is subject to some kinetic regulatory mechanism which is absent for the non-metabolized analogue. The kinetic regulation of internal concentration is *not* generally *via* the rate of metabolism of the solute being limited by the transport rate; the alternatives seem to be *either* regulation by the internal concentration of the metabolized substrate by some mechanism which is inoperative with the non-metabolized analogue, *or* regulation by some metabolic derivative of metabolized solute which is not formed when the non-metabolized solute is transported (e.g. sulphate influx apparently regulated by an assimilation product of sulphate: Section IV.E).

Studies on algae have not shed great light on the problem of the possible relationship between exchange fluxes and the closeness of an energy-coupled transport process to thermodynamic equilibrium with the energy-supplying reaction (Section III.G). The exchange reaction catalysed by the H^+-sugar symporter in *Chlorella* (Section IV.C, 2) when there is a substantial accumulation of a non-metabolized sugar analogue in the cytoplasm does not behave in the way one might expect for increased back-flux as thermodynamic equilibrium is approached.

The large efflux relative to the influx anaerobically in the dark may be related to a regulatory mechanism which reduces net energy consumption when the rate of regeneration of energy sources is limited (Section IV.A and J).

H. TEMPORAL AND SPATIAL VARIATIONS IN FLUXES

1. *Temporal Variations in Fluxes*

Apparent changes in transport capacity (i.e. changes in the net rate of solute transport under otherwise optimal conditions for transport) occur in microalgae as a function of the stage in the cell cycle (studied with synchronous cultures), with a circadian rhythmicity in non-dividing cells, and in relation to the availability of the transported solute (Sections IV.B–J). The "luxury accumulation" of nutrients after a period of deprivation is apparently not attributable to an unregulated use of the increased transport capacity which develops under deficient conditions, but is part of a nutrient-storage strategy related to the likelihood of nutrient shortages (Cohen and Parnas, 1976; Parnas and Cohen, 1976; Sicko-Goad and Jensen, 1976).

The concentration of net nutrient uptake into the light portion of the diurnal light–dark cycle which is commonly seen in algae growing photolithotrophically on nutrient-sufficient media may be viewed as an energy-conserving mechanism in organisms whose growth rate is potentially limited by light supply (see Raven, 1976b for arguments suggesting that the direct use of photosynthetic cofactors for transport in unicells is the most energy-efficient stratagem). However, deficiency of one or more nutrients, when energy supply is not (by definition) limiting growth rate, causes many algae to turn to a less light-biased transport and assimilation of nutrients; here the acquisition of the nutrient at a high rate in both light and dark presumably takes precedence over the higher intrinsic energy efficiency of uptake in the light using directly photoproduced cofactors (Section IV.C). A "reverse" diurnal rhythm of nutrient uptake may occur in dinoflagellates which undergo diurnal vertical migrations from upper zones of the water column in the day (where illumination for photosynthesis is optimal) to the lower, nutrient-rich zones at night (Eppley *et al.*, 1968). Reliance on such diurnal (or longer-term) imbalances in the relative accumulation rates of various nutrients implies a storage capacity for those involved. Phosphorus can be stored in a reasonably benign form,

even in non-vacuolate cyanobacteria and in those eukaryotes with but small vacuoles as the metabolically and osmotically relatively innocuous inorganic polyphosphate (Harold, 1966; Kuhl, 1974). Cyanobacteria can store nitrogen as cyanophycin (an arginine-aspartate copolymer) and also as phycobilin pigment (Stanier and Cohen-Bazire, 1977). Eukaryotes store nitrogen mainly as inorganic nitrogen in vacuoles (Eppley and Rogers, 1970; Wheeler, 1977; Wheeler and Stephens, 1977; Bhovichitra and Swift, 1977; Kahn and Swift, 1978), although storage as organic nitrogen may also occur, e.g. as ribulose bisphosphate carboxylase-oxygenase, or as the various class-specific light-harvesting pigment-protein complexes.

2. *Spatial Variation in Fluxes: The Possible Role of Flagella*

The *indirect* involvement of flagella in nutrient transport in flagellate microalgae has been mentioned in Section V.D above in relation to both the movement of entire flagellate organisms to sites of high nutrient concentration, and the disruption of diffusion barriers around the cell which can limit nutrient transport from the bulk medium to the plasmalemma. The *direct* involvement of flagella in nutrient transport seems to have been little explored, despite the predisposing factors that (a) the plasmalemma of the flagella can comprise 10% of the total plasmalemma area in a *Chlamydomonas* cell (assuming two flagella each 11 μm long and 0.1 μm in radius: Randall, 1969; Bold and Wynne, 1977; see Tables 2A and B), (b) the flagella, by virtue of their small radius, have a very thin intrinsic unstirred layer, and are long enough to project beyond the unstirred layer of the cell body (cf. Neushl, 1972), and (c) the thickness of the flagellar unstirred layer is even further reduced by flagella movement (cf. Neushl, 1972).

These three considerations suggest that, if other factors are equal, the nutrient influx through the flagella plasmalemma could exceed that through the cell body plasmalemma *on a membrane area basis*. However, there may be constraints on energy-requiring nutrient uptake across the plasmalemma related to the supply of energy to the flagella plasmalemma. It is unlikely that algal flagella can synthesize ATP, and that ATP for primary active transport processes as well as for flagella movement is supplied by diffusion along the axoneme from the cell body. This situation has been quantitatively considered by Raff and Blum (1968) and by Nevo and Rickmenspoel (1970) for ATP supply to

dynein (the ATPase of the mechanochemical coupling system of eukaryote flagella). While their calculations involve a number of assumptions as to the steady-state ATP concentration in the cell body, the diffusion coefficient for ATP in the axoneme, and the *in vivo* kinetics of dynein, it is possible to conclude from their analyses that the 10–40 μm length of microalgal flagella is quite close to the limit for a functional flagellum supplied with ATP by diffusion. The situation may be even more critical in freshwater flagellates, where the higher internal osmotic pressure than that of the medium leads to a net water influx, balanced by water extrusion *via* contractile vacuoles (see Raven, 1976a). This involves a mass flow of water *down* the flagellum, opposing ATP diffusion *up* the flagellum, and halving the possible length of a functional flagellum compared with a similar flagellum in an isotonic medium (Raven, unpublished calculations).

It would thus appear that ATP supply *to dynein alone* may not have a large over-capacity in algal flagella, and that a substantial ATP demand for primary active transport processes across the flagella plasmalemma might lead to competition between dynein and active transport. The magnitude of the additional demand can be seen if primary active transport consuming 10 pmol ATP (cm^2 flagellar plasmalemma)$^{-1}$ s^{-1} (cf. Table 4) is assumed: this consumes as much ATP per μm flagellar length as does the dynein activity assumed by Raff and Blum (1968).

An alternative possibility for energizing nutrient uptake at the flagellar plasmalemma would be to restrict primary active transport to the cell body plasmalemma (where ATP supply would not be diffusion-limited), and to have *secondary* active transport of nutrients at the flagellar plasmalemma. Such a spatial displacement of chemiosmotic coupling (Skulachev, 1978) leads to difficulties in maintaining the intensive (kJ mol^{-1}) properties of the ionic "working substance" even if its extensive properties (mol (cm^2 flagellar membrane)$^{-1}$ s^{-1}) can be maintained at distances of tens of μm from the primary active transport reaction (Raven, unpublished calculations). Further investigations are clearly required in order to clarify the role of flagella in nutrient transport.

I. TOLERANCE OF PHYSICOCHEMICAL EXTREMES

Particular algal species, and higher taxa, are generally restricted to a particular range of environmental conditions. The ability to grow

under extreme conditions generally means that there is less competition for the available resources with other organisms at the same trophic level, since not many organisms can tolerate these conditions: indeed, Brock (1969) defines extreme conditions as those in which few microbial species can grow! This tolerance reflects the extreme influence of "Wallacian" (physicochemical) forces of natural selection rather than the predominance of "Darwinian" (biotic) influences seen in less physicochemically demanding habitats (cf. Harper, 1964, 1977).

The influence of transport mechanisms on the ability to withstand extremely high external osmotic potentials has been well documented elsewhere (Hellebust, 1976; A. D. Brown, 1978; Miller *et al.*, 1976) and will not be reiterated here. The chemical extreme of very low nutrient concentrations has been dealt with in Section V.E. and F above. A chemical extreme which is of great importance for the "proticity paradigm" is external pH. The extent to which cytoplasmic pH can be held constant, while at the same time maintaining $\Delta\bar{\mu}_{H^+}$ at a relatively constant level, is subject to constraints on the value of ψ_{co} (equation 4) which can occur. ψ_{co} in the cases measured in algae seems to be invariably inside-negative (see Section IV.A) which sets a lower limit on external pH at which $\Delta\bar{\mu}_{H^+}$ and pH_{cyt} can both be held constant (cf. the apparent inside-positive potential of acidophilic prokaryotes: Hsung and Haug, 1975, 1977; Krulovich *et al.*, 1978). The upper limit on ψ_{co} is probably set by the effects of the very strong electric field across the membrane on membrane structure: depending on the frequency of the applied electrical potential difference the "breakdown voltage" varies from 250 to 1000 mV (Coster 1969; Zimmermann *et al.*, 1974). The lower values are more appropriate for "steady-state" potential differences, and although these "punch-through" potential differences are higher at high external pH values than in more acidic media (breakdown at −250 mV at an external pH of 4.5 and −450 mV at an external pH of 9.0 in the giant-celled *Chara corallina*: Coster, 1969), the need for a safety margin suggests −300 mV as the most negative acceptable value of ψ_{co}. Such values are found in some eukaryotic plant cells (see Raven, 1977d; Smith and Raven, 1979). The maintenance of pH_{cyt} of 7.0 and $\Delta\bar{\mu}_{H^+}$ of 24 kJ mol^{-1} is thus only possible between external pH values of 4.0 and 8.0 if ψ_{co} is to lie between −60 and −300 mV. Since algae can grow at pH values well outside this range (*Cyanidium* at pH 1: Brock, 1969; *Scenedesmus* at pH 10, and *Coelastrum* at pH 11: Felfoldy, 1960), they must clearly be able to regulate their cytoplasmic pH to some

value near neutrality despite the extremes of external pH, even if they cannot maintain a large $\Delta\bar{\mu}_{H^+}$. Experiments on the pH regulation and possible cotransport processes in algae at these extremes of pH would be most valuable (Garland and Haddock, 1977; Krulovich *et al.*, 1978). It may be significant that only certain eukaryotic algae can live in these very acid media; cyanobacteria seem to be restricted to external pH values above 6 in nature (Brock, 1973; cf. Section IV.B, 1). Current evidence (Section IV.A) suggests that it is unlikely that this restriction is related to the occurrence of redox-coupled ATP synthesis at the plasmalemma of *all* cyanobacteria. The generally held view is that cytoplasmic pH regulation could well involve active H^+ efflux as the energy-requiring part of the mechanism at external pH values up to pH 10 or so; above this there may be a requirement for active H^+ *in*flux (Smith and Raven, 1979). Any given genotype of alga is unlikely to be found as a dominant in nature over a pH range of more than four units.

An important physicochemical variable is temperature. Algae as a group can grow over a range from below 0°C to above 50°C, although (as with external pH) a given genotype is only successful over a range of some 20°C within this range (Brock, 1969; Eppley, 1972). As has been pointed out in Section III.F, the generally observed decrease in surface/volume ratio of algae grown at lower temperatures suggests that the plasmalemma area available for porters to occupy is not limiting growth capacity, even at the lower temperatures where the area of membrane per unit volume of nutrient-requiring cytoplasm is smaller (cf. Raven and Smith, 1978b). Little seems to be known about any adaptation of algal nutrient transport to different temperatures; the different growth rates found in algae grown at the supply of the correct ratio of nutrients at the lower temperature in the face of possible (indeed, probable) differences in the intrinsic effects of temperature on the various transport systems (including, probably, the two components of "pump and leak systems") requires precise control of the number of porters and of their activity (cf. Raven and Smith, 1978b). A further point relates to temperature-induced changes in membrane fluidity with corresponding effects on both the activity of porters in the membrane (e.g. Raison, 1973) and on the permeability coefficient for lipid-soluble solutes (cf. Gutknecht *et al.*, 1977; Champigny and Moyse, 1975; Fischkoff and Vanderkooi, 1975; Kuiper, 1965). Further work on temperature adaptation of the capacity

and kinetics of mediated transport processes, and on membrane lipid composition and functional characteristics, should be valuable in interpreting data obtained in an ecological context of the apparent $K_{1/2}$ for nutrient transport in algae adapted to different temperatures (Eppley, 1972, 1977).

J. ECOLOGY AND EVOLUTION

Most of this essay has been concerned with the proximate (mechanistic) aspects of nutrient transport in algae, although occasionally the ultimate (evolutionary) aspect has been mentioned. Here we shall consider some general evolutionary aspects of plasmalemma nutrient transport in microalgae.

The basic pattern of nutrient transport (and, indeed, of membrane-associated energy conversion as a whole) appears to have arisen in relation to selective forces related to energy and nutrient availability in the early precambrian; this has been outlined by *inter alia* Mitchell (1970), Broda (1970, 1975), Morris (1978), Raven and Smith (1976c) and Smith and Raven (1978). It seems that the cyanobacteria and the eukaryotic microalgae received from their anaerobic chemoheterotrophic, photoheterotrophic and photolithotrophic ancestors (Olson, 1970; cf. Towe, 1978) a basic scheme of primary active H^+ transport, serving both to energize the membrane transport processes and as an essential aspect of intracellular pH regulation, and of various secondary (H^+-powered) transport processes involved in nutrient transport and pH regulation.

This primitive mechanism has been modified in a number of ways in the microalgae: there may be additional primary active ion transport processes, and "second tier" secondary active transport processes (Na^+-coupled cotransport, with the $\Delta\bar{\mu}_{Na^+}$ generated by cotransport based on primary active H^+ transport). Modifications are also needed to deal with the physicochemical extremes mentioned above (Section V.J), and with accumulation of nutrients from very low extracellular concentrations (Section V.D).

An ecologically very important aspect of plasmalemma transport relates to the size and shape of the organism (Paasche, 1960; Fogg, 1965; Barse, 1976; Bellinger, 1977; Kirk, 1976; Lewis, 1976; Taguchi, 1976; Guillard and Killham, 1977). One very significant point (which has already been mentioned in Section V.E) is that the *larger* cells

generally have a *lower* relative growth rate. However, it appears that the decreasing relative growth rate with increasing cell size is not sufficient to offset the decreasing plasmalemma area per unit of metabolizing cytoplasm, even in vacuolate cells (cf. Table 4; Section IV.I); thus the maximum flux across the plasmalemma during growth at the maximum relative growth rate may increase with increasing cell size. The extent to which this was an important selective force in the internalization of membrane-associated coupling (via H^+ fluxes) of redox and adenylate transformations in prokaryotes is not certain. The rationale here is that the nutrient transport *must* occur at plasmalemma, while redox-associated ATP synthesis (photosynthetic and oxidative phosphorylation) need not be at the plasmalemma. Calculations (Raven, 1977a 1978 and unpublished) show that the total plasmalemma ion fluxes associated with growth are much greater in a very fast-growing chemoheterotroph such as *Escherichia coli* (no necessary internalization, cf. the variable occurrence of mesosomes) than in the fastest-growing cyanobacteria (e.g. *Anacystis nidulans*, with perhaps four times the area of plasmalemma present as intracellular thylakoid membranes). The maximum relative growth rate of *Anacystis* could be achieved with H^+ fluxes at the plasmalemma lower than those in *Escherichia*, even if there was no internalization (this computation takes account of the additional H^+ fluxes in a photolithotroph associated with the ATP required for primary CO_2 fixation reactions). Further, there may be regulatory advantages in internalization (see Raven and Smith, 1976b; Smith and Raven, 1978). It seems certain (Raven, 1978) that *Chlorella* would have a grossly overladen plasmalemma if redox-coupled ATP synthesis were not internalized (cf. Table 4).

Further ecological aspects of surface/volume ratio on nutrient transport relate to the relationship of cell size (or, in the case of colonies, the size of the ecological unit: Lewis, 1976) to extracellular diffusion barriers to nutrient uptake. Other factors being held constant, there is an intrinsic increase in the thickness of the unstirred layers with cell size (cf. Section V.E), while the thickness of these layers can be diminished by flagella activity (cf. Koch, 1971; Raven, 1976a) and by movement relative to the surrounding medium (Munk and Riley, 1952: Section V.E). Cell size is also important in relation to light absorption (Kirk, 1976) and C transport (Section IV:B, 1), and to the biotic factor of grazing (see below).

However, a large chasm lies between our current understanding of the relationship between these various nutrient transport characteristics of different microalgae and their ecological characteristics. Attempts to fit algae and their nutrient transport into an "r-adapted"–"K-adapted" continuum have not been very successful (see Guillard and Killham, 1977). Perhaps more success would be found with a three-category scheme, which accounts not only for environmental stability but also for resource availability (e.g. nutrient concentration in the medium) such as has found some success in accounting for the ecology of terrestrial plants (Grime, 1974, 1977) and marine macroalgae (Dayton, 1975; Raven, 1981). Clearly the number of niches which must be invoked to account for the number of phytoplankton species co-existing requires that many biotic factors (competition at the primary producer level, and differential effects of grazers at the next trophic level) be invoked in addition to the measurable physicochemical heterogeneity of the environment. Darwinian factors must ultimately be used to account for the diversity of phytoplankters, but a lot of Wallacian work must be done on transport mechanisms and their interaction with the physicochemical environment before the various characteristics of individual algae can be accounted for in terms of both biotic and physicochemical factors (e.g. the relative influence of size on nutrient uptake and on grazing of phytoplankters): Guillard and Killham (1977).

VI. Acknowledgements

Discussions with Professor N. A. Walker, and Drs F. A. Smith, W. A. Hamilton and I. R. Booth have helped to clarify a number of points, particularly with respect to charge-balancing mechanisms during pH regulation by H^+ transport across the plasmalemma. Particular thanks are due to Dr S. M. Glidewell for her comments on the manuscript.

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Note added in proof

A number of significant papers on inorganic carbon sources for photosynthesis (IV.B) have recently appeared. Smith and Walker (*New Phytologist*, submitted for publication 1980) have considered the role of unstirred layers in inorganic carbon supply to microphytes and other aquatic plants, while Walker, Smith and Cathers (*Journal of Experimental Botany*, (submitted for publication) 1980) have suggested that the assimilation of inorganic carbon at high external pH values can proceed without the need for active HCO_3^- influx in certain freshwater macrophytes (cf. p. 73). The mechanism which they propose involves a spatial separation on the plant surface of sites of (active) H^+ efflux and (passive) H^+ influx: earlier interpretations (Lucas, 1975) involved active HCO_3^- influx at the "acid" sites now identified with H^+ efflux. The apparent use of HCO_3^- is then interpreted as uncatalysed conversion of HCO_3^- to CO_2 in the "acid" regions (cf. the calculations of Lucas, 1975, which were based on the assumption that the pH of the "acid" regions was higher than subsequent analysis has shown it to be) within the unstirred layer, with subsequent CO_2 diffusion into the cell. Such a mechanism is not necessarily more "leaky" than HCO_3^- active influx (see p. 117). However, the mechanism is absolutely dependent on the spatial separation of the "acid" and "alkaline" regions on a much larger scale than could be achieved in microalgae, so that the conclusions of Lehmann (1978) and of Birmingham and Colman (*Plant Physiology Lancaster* **64**, 892, 1979) as to the inadequacy of the uncatalysed conversion of HCO_3^- to CO_2 during microalgal photosynthesis at high external pH values are valid (see also, Pruder and Bolton, *Aquaculture* **17**, 1, 1979).

The confusion as to the nature of the inorganic carbon species entering the cells of microalgae under various experimental conditions has not been decreased by the findings of Miyachi and Shiraiwa (*Plant and Cell Physiology* **20**, 341, 1979) who showed, using techniques similar to those of Findenegg, 1976b, that *Chlorella vulgaris* 11 h cells showed no evidence of HCO_3^- influx at an external pH of 8 even when they had been cultured at low CO_2 levels. I have recently considered the mechanism of photosynthesis in terrestrial free-living and lichenized algae (Raven, in "Commentaries in Plant Science", Vol. 2, H. Smith ed., Pergamon Press, Oxford, 1980) in more detail than in the discussion on pages 119 and 120.

Findenegg (*Plant Science Letters* **17**, 101, 1979) has provided more data to support his earlier suggestions that HCO_3^- influx and OH^- efflux can be at very high rates in

Scenedesmus cells in experiments in which assays of intracellular carbonic anhydrase are performed with substrate (HCO_3^-) supplied externally. The fluxes at high external pH, when the HCO_3^- influx must be active, are up to 400 pmol cm^{-2} s^{-1}, with much higher values at lower external pH values when the exchange of HCO_3^- for OH^- may not need any energy input (cf. the HCO_3^-–Cl^- exchange in erythrocytes: see p. 76).

Craig and Budd (*Journal of Phycology* **15**, 300, 1979) have provided a substantial body of new data on Cl transport in *Anacystis* (see p. 174). The possibility of a substantial barrier to O_2 diffusion out of chloroplasts (Steiger *et al.*, 1977; see p. 193) has been disputed in a well-argued paper by Grodzinski, O'Connor and Vučinič in "Plant Membrane Transport", J. Dainty, W. J. Lucas and R. M. Spanswick eds, North-Holland, Amsterdam (1980).

The postulated low permeability of the glycolipid layers of the heterocyst wall (pp. 126–129) is supported by the finding of a low permeability of the glycolipid-rich outer membrane of coliform bacteria (Nikaido, *Angewandte Chemie, International Edition in English* **18**, 337, 1979).

The unpublished data on $B(OH)_3$ (see p. 61) is now in press (Raven, *New Phytologist* **84**, 1980).

Bacterial Production of Antibiotic Polypeptides by Thiol-Linked Synthesis on Protein Templates

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I. Introduction

Some years ago, I was searching for a peptide synthesis different from and less complex than the ribosomal one. Thus, I became aware of observations made in a number of laboratories that polypeptide antibiotics are produced in particle-free extracts from which RNAs have been removed by treatment with ribonuclease; also that their synthesis is resistant to protein-synthesis inhibitors such as puromycin (Mach *et al.*, 1963; Spaeren *et al.*, 1967; Yukioka *et al.*, 1965; Kurylo-Borowska and Tatum, 1966; Bhagavan *et al.*, 1966; Daniels, 1968). The background for our study to be reviewed here was laid by the earlier work of several groups: in Japan, those of Otani (Yukioka *et al.*, 1965) and particularly of Kurahashi (Tomino *et al.*, 1967), and in Norway that of Laland (Berg *et al.*, 1965; Spaeren *et al.*, 1967). These workers obtained extracts from the *Bacillus brevis* (A.T.C.C. 9999) that synthesizes the antibiotic polypeptide, gramicidin S. Their work and our own studies have shown that the basic mechanism of biosynthesis is essentially analogous for the three antibiotic polypeptides, gramicidin S, tyrocidine and linear gramicidin (still incomplete), and it has turned out that other polypeptides of bacterial origin are produced in the same manner.

The studies in this laboratory were begun after I had become interested in the origin of life (Lipmann, 1964, 1974) and hoped to find a possible precursor of ribosomal protein synthesis. The biosynthetic systems to be discussed in this review may be thought of as alternatives to the ribosomal system of polypeptide synthesis. They have proved to be polypeptide syntheses on protein templates which all use a protein-bound 4′-phosphopantetheine for collecting polypeptides from pre-bound amino acids. The latter are first thioester-linked to the polyenzyme subunits which initially activate the amino acids with ATP to form aminoacyl adenylates. This process has been compared in detail (Lipmann, 1971b) to a similar one in fatty-acid elongation from pantetheine-linked acetyl precursors (Vagelos *et al.*, 1966; Lynen *et al.*, 1968).

II. Synopsis of the Mechanism of Biosynthesis of Gramicidin S, Tyrocidine and Linear Gramicidin

Gramicidin S and tyrocidine are the two polypeptides for which the greatest detail has been obtained with regard to fractionation of the participating enzymes and identification of the pantetheine-linked protein (Lee and Lipmann, 1974) present in all polyenzymes; the latter are aggregates of subunits which each activate an amino acid. For their synthesis, the polyenzymes for gramicidin S and tyrocidine have to be combined with a mono-enzyme that activates and racemizes phenylalanine which, in the D-form, initiates the process in both cases. As will be seen later, initiation of biosynthesis for linear gramicidin is different (Akashi and Kurahashi, 1977). The structures of all three polypeptides are shown in Fig. 1.

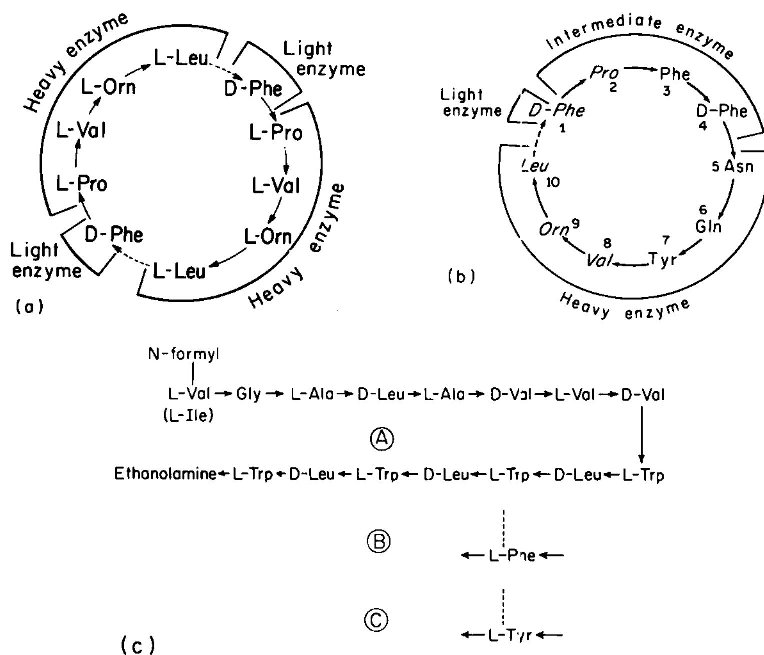


FIG. 1. (a) Amino-acid sequence in gramicidin S. The dotted arrows indicate cyclization of the two pentapeptides from the enzyme-bound Leu carboxyl group to the amino terminal group of phenylalanine. For enzyme identification, see Table 1. (b) Amino-acid sequence in tyrocidine. The ring closure between Leu and Phe is analogous to that in gramicidin S. For enzyme identification, see Table 1. (c) Amino-acid sequence in linear gramicidin (A, B and C). For preliminary enzyme identification, see Table 1.

In the process of characterizing the main features of the mechanism of amino-acid activation and polypeptide elongation, it was found in the early work on gramicidin S that the biosynthetic activity was due to multiple complementary fractions of different molecular weights (Gevers *et al.*, 1968; Kambe *et al.*, 1971; Lee *et al.*, 1973). As shown in Table 1, the number of units, i.e. mono- and polyenzyme, that participate in synthesis of gramicidin S is two and of tyrocidine three. For linear gramicidin, their number is as yet only partly defined. It may be noted that the molecular weights of the polyenzymes divided by the number of amino acids activated is regularly rather constant, indicating a subunit of 70,000 or slightly above for each amino acid. Every polyenzyme defined so far contains one mole of 4′-phosphopantetheine, independent of the number of amino acids activated.

From the amino-acid sequences in the three antibiotics shown in Fig. 1, analysis showed that the decapeptide in gramicidin S formed by *B. brevis* (A.T.C.C. 9999) is derived from two pentapeptides that cyclize head-to-tail to a decapeptide. Tyrocidine is also a cyclic decapeptide which, although formed by a different strain of *B. brevis* (A.T.C.C. 8185), contains the sequence found in gramicidin S but interrupted by five different amino acids. The start of biosynthesis of the chain in both antibiotics is the sequence: D-Phe-Pro, and the end is the sequence: Val-Orn-Leu. Linear gramicidin, also present in *B. brevis* (A.T.C.C. 8185), contains a sequence of 15 amino acids. Linear gramicidin and tyrocidine were originally isolated from this organism by Hotchkiss and Dubos (1940; cf. also Lipmann *et al.*, 1941).

TABLE 1. Enzymes participating in biosynthesis of gramicidin S, tyrocidine and linear gramicidin

Antibiotic	Number of enzyme	Mol. wt. ($\times 10^{-3}$)	Amino acids fixed in sequence	Approx. mol. wt. of each amino acid studied ($\times 10^{-3}$)	Pantetheine per molecule of enzyme
Half of gramicidin S	1	100	D-Phe	100	none
	2	280	Pro-Val-Orn-Leu	70	1
Tyrocidine	1	100	D-Phe	100	none
	2	230	Pro-Phe-D-Phe	76	1
	3	440	Asn-Gln-Phe-Val-Orn-Leu	74	1
Linear gramicidin (incomplete)	1	160; 180	Val-Gly	80	1
	2	350	Ala-D-Leu-Ala-D-Val-Val	70	1

A. ACCURACY OF AMINO-ACID INCORPORATION

One common feature of the aromatic parts of tyrocidine and linear gramicidin is that they are more or less interchangeable; the only aromatic amino-acid residue, D-phenylalanine, in gramicidin S is essentially non-changeable. The other D- and L-aromatic amino acids in tyrocidine (positions 3 and 4) are preferential for tryptophan, as are those in the final part of linear gramicidin, whereas that in position 7 of tyrocidine prefers tyrosine. Linear gramicidin is a linear pentadecapeptide that begins with formylvaline and terminates with four dipeptide sequences of aliphatic D-amino acids followed by tryptophan. At the tail end of the molecule, the last tryptophan carboxyl is blocked by ethanolamine.

TABLE 2. Substitutions in amino-acid activation in polymerization of gramicidin S

Amino acid replaced	Analogue	ATP-[^{32}P] PP_i exchange (%)	Antibiotic synthesis (%)
L-Phenylalanine	—	100	100
	D-Phenylalanine	100	105
	L-Tyrosine	35	1
	L-Tryptophan	43	0
	p-Fluoro-L-phenylalanine	95	94
	DL-Threophenylserine	69	0
	DL-Erythrophenylserine	1	0
	DL-β-Thienylalanine	89	25
L-Leucine	—	100	100 (6177 c.p.m.)
	D-Leucine	19	0
	L-Isoleucine	20	39
	D-Isoleucine	10	0
	L-Alloisoleucine	19	38
	L-Norleucine	23	22
	L-Valine	(100)	—
L-Valine	—	100	100
	D-Valine	1	0
	L-Threonine	1.5	1
	L-Isoleucine	18	48
	D-Isoleucine	9	0
	L-Alloisoleucine	17	58
	L-Norvaline	32	105
	L-Leucine	(100)	1.5

Experiments were carried out as described by Lipmann *et al.* (1971).

Altogether, the accuracy of incorporation is not as stringent as it is in ribosomal polypeptide synthesis. Thus, isoleucine will easily replace leucine and so will norleucine or alloisoleucine. Isoleucine will also very easily replace valine in linear gramicidin (Akers *et al.*, 1977). Other replacements, such as analogues, appear in Table 2. Recently it was shown by Altmann *et al.* (1978) that leucine, if added at high enough concentration to the gramicidin S-synthesizing system in the absence of valine, will replace it. Thus, they were able to synthesize gramicidin S with two leucines in each pentapeptide. In reverse, valine was found not to be inserted for leucine, perhaps because of the specificity of the terminal leucine residue for the cyclization reaction.

In view of the preferable uniformity of label in many of our biosynthetic experiments, we used phenylalanine as the exclusive precursor of the aromatic amino acids because Mach *et al.* (1963) had found that incorporation of the different aromatic amino acids is dependent upon their concentration in the medium. In confirmation, it has been our experience with tyrocidine and linear gramicidin that, if phenylalanine is the only aromatic amino acid present in the biosynthetic system, it is put into all the places occupied by the aromatic amino acids (Bauer *et al.*, 1972).

B. INDUCTION OF ANTIBIOTIC SYNTHESIS

It was observed early by Kurahashi and his group (Tomino *et al.*, 1977; Yamada and Kurahashi, 1968) that the enzymes that synthesize the antibiotics of the two strains of *B. brevis* (A.T.C.C. 9999 and 8185) which produce gramicidin S and tyrocidine, respectively, were induced at the turning point from the logarithmic to the stationary phases of growth, coincident with the onset of sporulation. For practical purposes, it was very important to collect the organism at the exact time when production of enzymes in soluble form was at its highest point. It was in order to spot the onset of production that we used different methods for determining formation of the antibiotic in samples taken at various times. The earliest assay was for the appearance of ATP-PP_i exchange with ornithine, an amino acid that is present in gramicidin S as well as tyrocidine but is absent from normal proteins.

This method is somewhat time-consuming, however, and, in order to get a quick assay, Dr. Lee used instead uptake of an amino acid present in the antibiotic, e.g. ornithine, or parts of the enzyme-bound

coenzyme 4′-phosphopantetheine, namely, radioactive β-alanine or pantothenic acid. These are very rapidly absorbed by the bacteria at the time when enzyme production is induced. It can be seen in Fig. 2 that, if one compares these curves with that for tyrocidine synthesis, the rise in uptake of β-alanine and pantothenic acid slightly precedes the onset of tyrocidine-synthesizing enzyme production. It was also observed during such experiments that addition of rifampicin very considerably delayed onset of enzyme formation, which indicated that new formation of protein was required for enzyme induction (Kurahashi, 1974; Lee *et al.*, 1975).

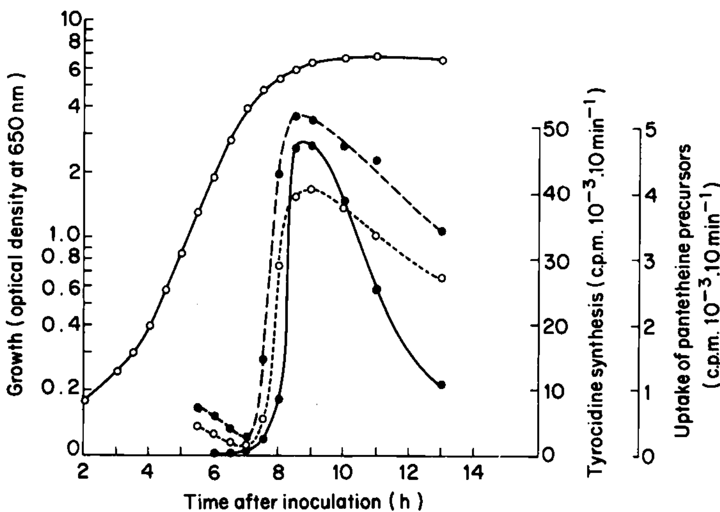


FIG. 2. Induction of tyrocidine synthesis (●—●) at the turn from the logarithmic to the stationary phase of growth (O—O), coincident with uptake of pantothenic acid (O---O), β-alanine (●---●) or constituent amino acids (not shown). From Lee *et al.* (1975).

Recently, we have always used as an assay the uptake of either [^{14}C]ornithine, [^{14}C]proline or [^{3}H]β-alanine to test onset of enzyme synthesis, and [^{3}H]β-alanine or [^{14}C]pantothenic acid for incorporation into the coenzyme, 4′-phosphopantetheine, in studies on isolation and function of the pantetheine-binding protein. This procedure takes very little time; a sample is incubated for a few minutes and collected on Millipore filters or sedimented by centrifugation without autolysis or enzymic determination.

C. COMMENTS ON THE RELATION BETWEEN SPORULATION AND ANTIBIOTIC FORMATION

It might be mentioned here that, in a paper published with Dr. Littau, an electron microscopist in Dr. Allfrey's laboratory at the Rockefeller University (Lee *et al.*, 1975), an effort was made to follow antibiotic formation and spore formation. It was found that, after the initial period of high yield of soluble enzyme, there was a prolonged period when antibiotic synthesis was very tightly bound to membranes and the enzymes were not easily obtained in soluble form suitable for analysis. The relationship between sporulation and antibiotic formation has been discussed by Kambe *et al.* (1974) in connection with the isolation of mutants that are deficient in enzyme production for antibiotic synthesis. Such deficient organisms sometimes develop a fragile spore, but a mutation was found in Kurahashi's laboratory that abolishes formation of antibiotic and might, to the contrary, cause an increased formation of spores. Paulus and his colleagues at Harvard (Mukherjee and Paulus, 1977) have also been concerned with this problem. They have reported that an organism deficient in synthesis of linear gramicidin developed rather fragile spores which could be rendered less fragile by addition of the missing antibiotic. It must be concluded from these studies that there is some relationship between spore formation and antibiotic formation. Because of our limited experience with this phenomenon, I do not intend to consider the relationship in detail.

III. Fractionation of the Biosynthetic Systems

The best method for surveying the contents of the different enzyme fractions is to separate the components in the supernatant of a crude bacterial extract chromatographically on Sephadex G-200. This aids in isolating the polyenzymes because they are involved in sequential synthesis. We chose for assay by ATP-P_i exchange those amino acids expected from their formulae to be present in the different parts of the molecule. As an example, Fig. 3 shows preliminary results taken from a 33–45% ammonium sulphate fraction of a crude supernatant of the tyrocidine-producing organism. The positions of the exchange peaks indicate the approximate molecular weights of the three polyenzyme fractions in sequential tyrocidine synthesis; they are generally called the light, intermediate and heavy tyrocidine polyenzymes, respectively.

Their molecular weights are more exactly identified in a sucrose density-gradient using the method of calculation of Martin and Ames (1961; Fig. 4). The molecular weights of the various enzyme fractions in the biosynthesis of gramicidin S and tyrocidine, and partially of linear gramicidin, were thus determined as listed in Table 1.

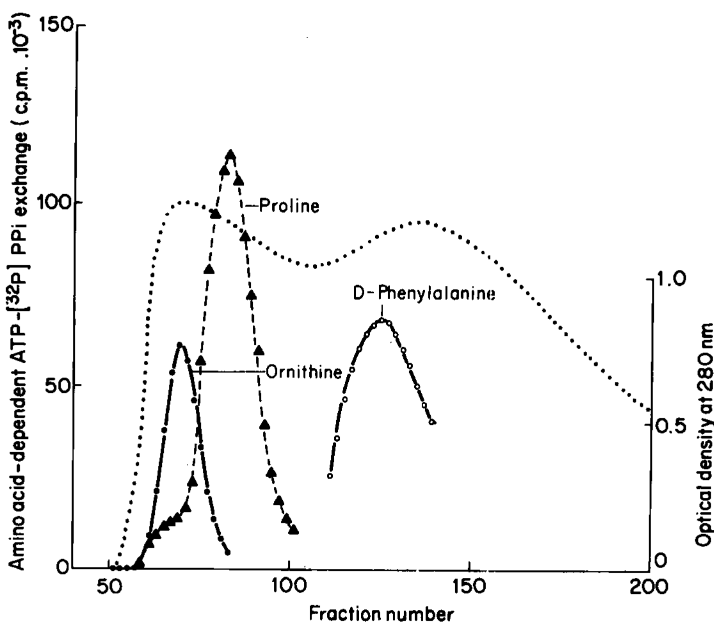


FIG. 3. Separation on Sephadex G-200 of an extract from *Bacillus brevis* A.T.C.C. 8185. ATP–PPi exchanges with amino acids indicate the position of the heavy enzyme (●—●), intermediate enzyme (▲---▲) and light enzyme (O—O) for tyrocidine synthesis. .... indicates optical density. From Lee *et al.* (1973).

A. ACTIVATION OF AMINO ACIDS BY ADENYLATION AND TRANSFER TO ENZYME-BOUND SULPHYDRYL GROUPS

This important feature of the biosynthesis of these polypeptides was observed early with gramicidin S. It was found that, in addition to an amino acid-dependent ATP-PP_i exchange, an ATP–AMP exchange, most pronounced with phenylalanine, also occurred (Table 3) (Gevers *et al.*, 1968, 1969). An overall reversibility was thereby indicated. As in the amino acid-tRNA transferase there was an initial activation to

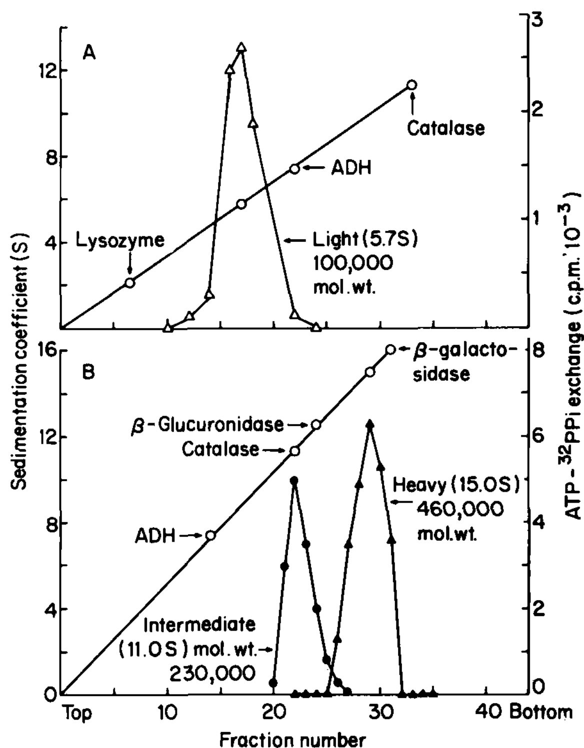
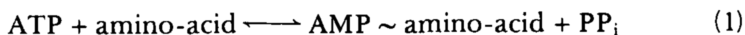


FIG. 4. Molecular weights for the light, intermediate and heavy enzymes for tyrocidine synthesis, as determined by sucrose gradient centrifugation. From Roskoski *et al.* (1970a).

aminoacyl adenylate from which the aminoacyl residue was reversibly transferred to a secondary attachment:



$$\text{ATP} + \text{amino-acid} \rightleftharpoons \text{AMP} \sim \text{amino-acid} + \text{PP}_i \quad (1)$$

$$\text{AMP} \sim \text{amino-acid} + \text{X} \rightleftharpoons \text{X} \sim \text{amino-acid} + \text{AMP} \quad (2)$$

This was confirmed using [^{3}H]ATP and ^{14}C-amino acids as labels (Table 4). The table shows that the equivalent of half of the reacting amino acid appeared as aminoacyl-[^{3}H]AMP, the other half as aminoacyl~X which remained in the trichloroacetic acid precipitate. In Table 5, the stability of the amino acid bound to the trichloroacetic acid precipitate is analysed by various tests; the instability to alkali, to mercuric acetate and, after exposure, to borohydride and performic acid, are features which indicate that fixed amino acid is thioesterified,

TABLE 3. Amino acid-dependent ATP-[^{14}C]AMP exchanges catalysed by heavy and light enzymes for gramicidin S synthesis

Activity	Amino acids added	Activated by	Heavy enzyme (25 μg)	Light enzyme (10 μg)
Exchange (c.p.m.)	L-Phe	Light	0	7015
	L-Orn	Heavy	370	0
	L-Val	Heavy	350	0
	L-Orn, L-Val	Heavy	720	0
	L-Orn, L-Val, L-Pro	Heavy	1080	0
	L-Orn, L-Val, L-Pro, L-Leu	Heavy	1450	0

For details, see Gevers *et al.* (1968, 1969).

TABLE 4. Isolation of protein-bound amino acids free of aminoacyl adenylates during gramicidin S biosynthesis

Materials	[^{14}C] Amino acid (pmoles)	[^{3}H]AMP (pmoles)
A. Sephadex eluate:	35.0	18.1
trichloroacetic acid-precipitable material, dissolved in dilute alkali	16.2	0
B. Sephadex eluate:	34.0	19.0
trichloroacetic acid-precipitable material, dissolved in dilute alkali	17.0	0

For experimental details, see Gevers *et al.* (1969).

TABLE 5. Stability of bonds between amino acids and denatured enzyme proteins during gramicidin S biosynthesis

Treatment	Percent cleavage of	
	leucine-protein	phenylalanine-protein
0.1 M Phthalate buffer, pH 2.5, 60°C	0	0
0.1 M Glycine-sodium hydroxide buffer, pH 10, 60°C	90	90
0.1 M Maleate buffer, pH 6.6, 37°C	5	8
0.1 M Maleate buffer, pH 6.6, 37°C together with 1% mercuric acetate	84	80
3 M Hydroxylamine, pH 6.1, 37°C	100	100
7.5 mM Sodium borohydride, pH 7.4, 25°C	100	100
Performic acid	95	95

With one exception, details of experiments are described by Kleinkauf and Gevers (1969). That for performic acid treatment was done by Kleinkauf *et al.* (1971).

presumably to an enzyme protein-sulphydryl group. Later, using the tyrocidine synthesis enzymes, the same separation of aminoacyl adenylate and thioester-linked amino acid was also obtained in the 80% ammonium sulphate precipitate where, however, the enzymic activity for polymerizing the amino acids was retained (Roskoski *et al.*, 1971).

B THE MECHANISM OF SEQUENTIAL ADDITION OF AMINO ACIDS IN ANTIBIOTIC POLYPEPTIDE SYNTHESIS

It was observed early by Ljones *et al.* (1968) and by Gevers *et al.* (1969) that, when incomplete sequences of amino acids were added to the mixture of complementary enzymes, polypeptide remained bound to protein. This is described by the data in Fig. 5, where the amino acids of gramicidin S were added in sequence and, after each new addition, the enzyme-bound polypeptides were precipitated with trichloroacetic acid and released by hydroxylamine. The peptidyl hydroxamates were determined by high-voltage electrophoresis which separates peptidyl hydroxamates according to length. Using equal samples, the hydroxamates were either labelled in all cases with [^{14}C]phenylalanine, the first amino acid, or with the ^{14}C label in the last added amino acid. After addition of the pentapeptide-terminal leucine, only 2 min were allowed for incubation instead of the 10 min after all other additions. This shorter exposure led to the formation of a bound pentapeptide, as indicated on the electropherogram. In a similar experiment (Table 6), using [^{14}C]phenylalanine as the marked amino acid, after 10 min incubation following the addition of each amino acid including leucine all [^{14}C]phenylalanine radioactivity present in the enzyme-linked incomplete chains was released and found in the cyclized gramicidin S.

In experiments with gramicidin S (Gevers *et al.*, 1969; Table 6) and tyrocidine (Roskoski *et al.*, 1970b), omission of an amino acid in the middle of the row showed that, in spite of the presence of the following amino acids, chain elongation broke off exactly at the position in which the missing amino acid would have been added. This is shown using many omissions in the experiments for tyrocidine synthesis in Table 7, which clearly indicate that polymerization is halted by a missing amino acid. It was found in the *in vitro* system for tyrocidine synthesis that cyclization to the final decapeptide as well as the sequential amino-acid fixation are much slower than they are in gramicidin S biosynthesis.

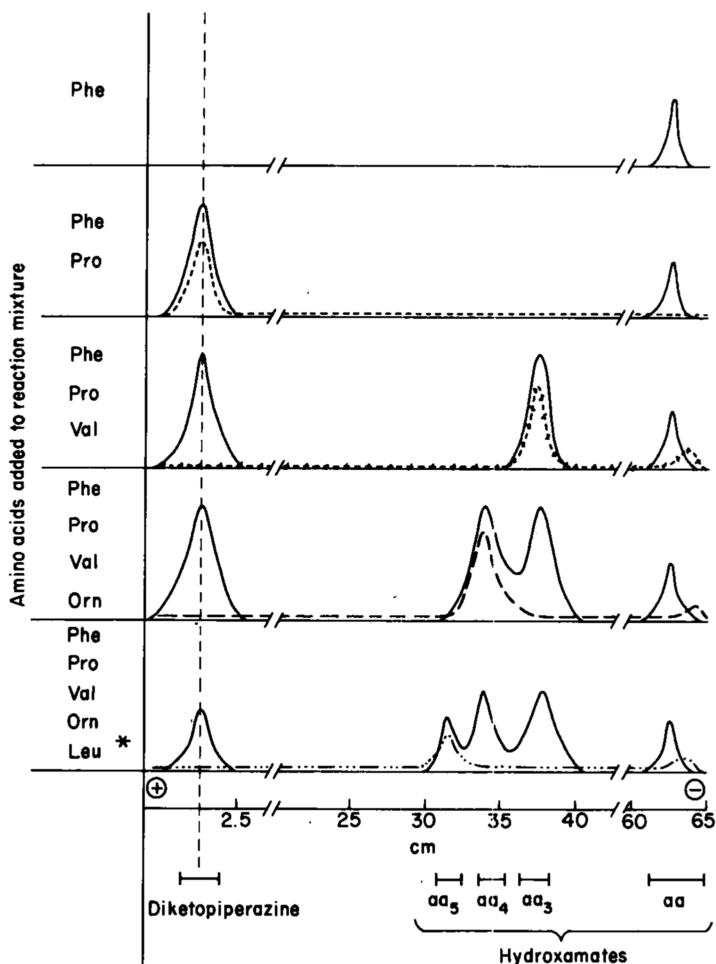


FIG. 5. Demonstration by electrophoretic separation of the formation of amino-acid and peptidyl hydroxamates on sequential amino-acid addition of the pentapeptide half of gramicidin. —— indicates [^{14}C]phenylalanine, ...[^{14}C]proline. + + + ^{14}C valine, – – – ^{3}H ornithine and —··—··— ^{14}C leucine. For details, see Gevers *et al.* (1969).

C. COMMENTS ON TYROCIDINE SYNTHESIS

As shown in Table 1, which summarizes the molecular weights of the polyenzymes of the different synthetases, a more or less constant subunit molecular weight of 70,000 is indicated for each amino acid activation. However, in the case of the intermediate enzyme of

TABLE 6. Biosynthesis of protein-bound nascent peptide chains and gramicidin S

	Radioactivity (c.p.m.) in			
	Sephadex eluate	Non-trichloro-acetic acid precipitate	Trichloro-acetic acid precipitate	Gramicidin S
[^{14}C]L-Phe	2,959	1,410	1,549	265
[^{14}C]L-Phe, L-Pro	7,671	1,670	6,001	375
[^{14}C]L-Phe, L-Pro, L-Val	11,460	1,455	10,005	1,531
[^{14}C]L-Phe, L-Pro, L-Val, L-Orn	16,160	1,835	14,325	1,005
[^{14}C]L-Phe, L-Pro, L-Val, L-Orn, L-Leu	3,531	1,502	2,029	25,409
[^{14}C]L-Phe, L-Val, L-Orn	3,101	1,401	1,610	376

Experiments carried out as described by Gevers *et al.* (1969).

TABLE 7. Formation of protein-bound nascent peptide chains in *in vitro* tyrocidine synthesis

Added amino acids	Bound radioactivity (pmoles)
[^{14}C]Phe	25.1
[^{14}C]Phe, Pro	49.4
[^{14}C]Phe, Pro, Asn	67.2
[^{14}C]Phe, Pro, Asn, Gln	101.0
[^{14}C]Phe, Pro, Asn, Gln, Val	132.0
[^{14}C]Phe, Pro, Asn, Gln, Val, Orn	148.0
[^{14}C]Phe, Pro, Asn, Gln, Val, Orn, Leu	182.0
[^{14}C]Phe, —, Asn, Gln, Val, Orn, Leu	25.1
[^{14}C]Phe, Pro, —, Gln, Val, Orn, Leu	50.5
[^{14}C]Phe, Pro, Asn, Gln, Val, —, Leu	120.0

Experiments carried out as described by Roskoski *et al.* (1970b). The horizontal lines indicate where addition of an amino acid was omitted. In the first seven reaction mixtures, with every addition of a new non-radioactive amino acid, radioactivity increased, indicating retention of every newly formed polypeptide on the enzyme system. When, as in all cases, 30 min were allowed for completion, also after leucine addition, the deca-peptide remained enzyme-bound.

tyrocidine, because we assayed an impure fraction, we were misled into believing that only proline was activated by this enzyme (Roskoski *et al.*, 1970b). This was puzzling because the molecular weight of 230,000 was too big for activation of one single amino acid, and 440,000 for the heavy enzyme was too small for activation of eight amino acids. We

were happy, therefore, when we were corrected by the work of Kambe *et al.* (1971) in Kurahashi's laboratory. They isolated the intermediate enzyme and found that it incorporated three amino acids, namely D- and L-phenylalanine and proline. Thus, as was more fitting, when divided by three and six, respectively, the molecular weights of both the intermediate (230,000) and the heavy enzyme (440,000) again showed a constant quotient of a little over 70,000 (Lee *et al.*, 1973). They were thus identified as polyenzymes and, accordingly, we also subsequently found one mole of 4′-phosphopantetheine in the intermediate enzyme (Table 1, p. 230).

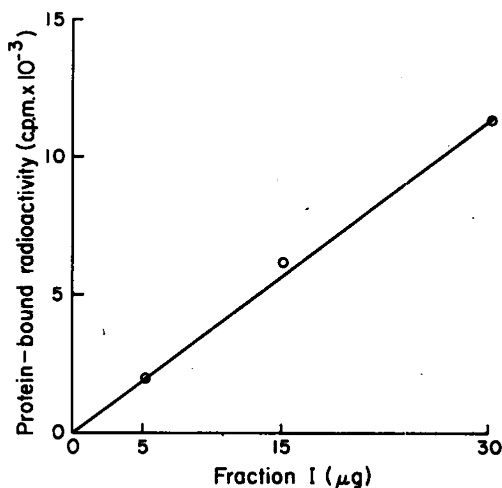


FIG. 6. Rate of enzyme-bound tetrapeptide: Phe-Pro-Val-Orn formation with a constant amount of inducing light enzyme (2 μg) and different amounts of heavy enzyme, fraction I, during gramicidin S synthesis. From Kleinkauf and Gevers (1969).

D. RACEMIZATION AND INITIATION BY THE LIGHT GRAMICIDIN S ENZYME

The light enzyme for phenylalanine activation contains a racemase (Yamada and Kurahashi, 1969; Takahashi *et al.*, 1971; Gevers *et al.*, 1969; Lee, 1976) and only D-phenylalanine is transferred. Racemization in this case occurs after the thioester linking of phenylalanine. An interesting experiment on the mechanism of initiation by the light gramicidin S enzyme in synthesis of the enzyme-bound tetrapeptide is shown in Fig. 6. Kleinkauf and Gevers (1969) observed that, with a constant input, i.e. 2 μg of phenylalanine-

activating enzyme, the rate of native peptide formation using [^{14}C]phenylalanine and the three following non-radioactive amino acids increased proportionately with addition of increased amounts of heavy enzyme, between 5 and 30 μg. It was concluded therefore that the phenylalanine-activating enzyme catalyses initiation of polymerization of the first four amino acids on the heavy gramicidin S enzyme.

An early, rather plausible, assumption by Gilhuus-Moe *et al.* (1970) is incorporated into their formulation (Frøshov *et al.*, 1978) of initiation of gramicidin S synthesis by transfer of phenylalanine from a mono-enzyme to a polyenzyme in the absence of proline. We tried to confirm this assumption (Kleinkauf *et al.*, 1971) by incubating a mixture of the two gramicidin S enzymes together with [^{14}C]phenylalanine and ATP, both alone and in the presence of proline. Each assay system was then chromatographed in Sephadex G-200 and the position of the enzymes identified by phenylalanine- and ornithine-dependent ATP-[^{32}P]PP_i exchange. In addition, the radioactivity of [^{14}C]phenylalanine was determined. We found (Fig. 7a) that phenylalanine is not transferred from the gramicidin S light enzyme to the heavy enzyme, except after addition of proline (Fig. 7b). This observation indicates that phenylalanine linked to the phenylalanine-activating enzyme cannot be accepted by the heavy enzyme before addition of proline.

E. SEPARATION OF 70,000 MOLECULAR-WEIGHT SUBUNITS FROM POLYENZYMES

With the gramicidin S synthesis system, Kleinkauf and Gevers (1969), using crude extracts of *B. brevis* (A.T.C.C. 9999), observed that prolonged incubation at 37°C abolished polymerization, except for production of the Phe-Pro-diketopiperazine, but not the ability to activate and thiol-link the individual amino acids. Their results were confirmed by Lee and Lipmann (1974) with crude extracts of the tyrocidine-synthesizing system (*B. brevis* A.T.C.C. 8185). They also observed that, on prolonged incubation at 37°C, subunit fractions decreased in size by discrete units of 70,000 daltons. These fractions were identified by Sephadex G-200 chromatography and eventually collected in a peak of about 70,000 molecular weight. The 70,000 molecular weight subunit fraction retained the ability to activate all of

the constituent amino acids and transfer them to a thiol in the fraction, while the ability to polymerize was lost except to form, if the light enzyme was present, the Phe-Pro-diketopiperazine (Lee and Lipmann, 1977). Formation of this diketopiperazine was observed earlier by Tomino *et al.* (1967) in *B. brevis* extracts that synthesize gramicidin S.

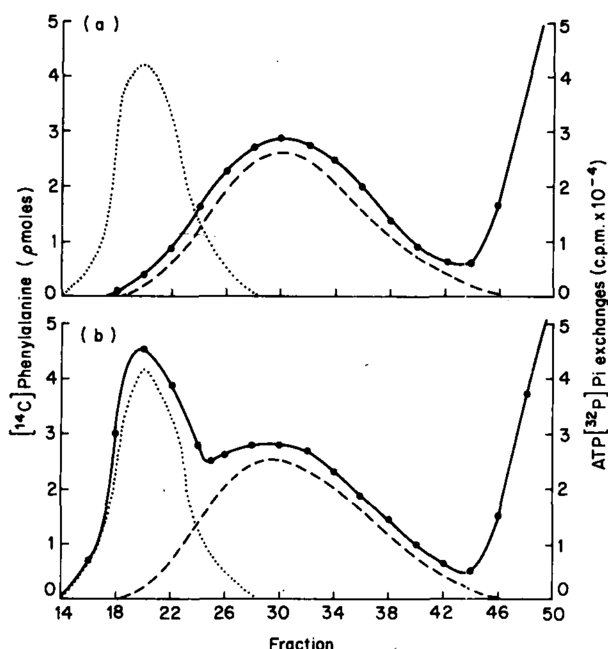


FIG. 7. Chromatographic separation of components of reaction mixtures showing binding of [^{14}C]phenylalanine (——) to the gramicidin S enzyme fractions. (a) describes binding of [^{14}C]phenylalanine in the absence of proline, and (b) in the presence of proline. The positions of enzymes were identified by D-phenylalanine (– – – –)- and L-ornithine (..........)-dependent ATP-[^{32}P]PPi exchange. From Kleinkauf *et al.* (1971).

Purified preparations of the tyrocidine intermediate and heavy enzymes were quite stable on prolonged exposure to 37°C. However, the clearest evidence of separation into subunits, including the small pantetheine-binding protein later isolated, was unexpectedly obtained by sodium dodecyl sulphate (SDS)-gel electrophoretic analysis of the two purified tyrocidine enzymes. This is shown in Fig. 8 (Lee *et al.*, 1973; Lipmann, 1973) where it appears that, although the largest amount of the enzymes remained at the bottom, a smaller part was decomposed to subunits which can be recognized on the electropherogram in distinct

fractions for the intermediate enzyme of three bands around 70,000 molecular weight. Similarly, the heavy enzyme was decomposed to five clearly visible fractions; of these, however, one is about twice as strong as the other four, indicating that it almost certainly contains overlapping subunits and, thus, the expected presence of six subunits. In addition to these approximately 70,000 molecular weight multiple fractions, in both electropherograms at around 18,000–20,000

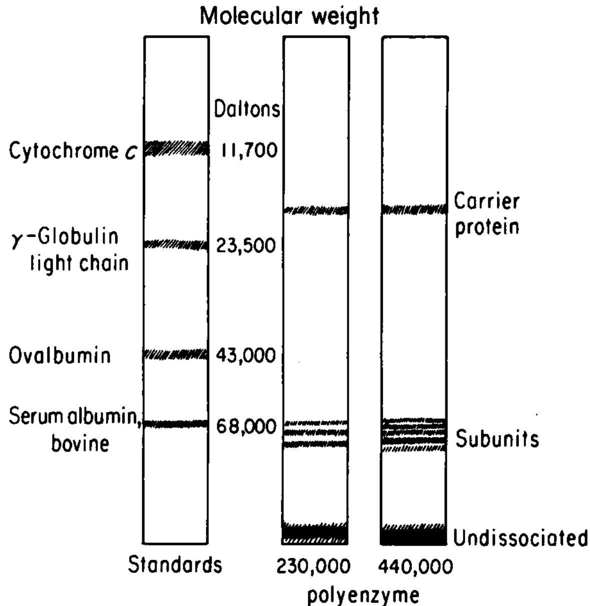


FIG. 8. Separation of subunits by sodium dodecyl sulphate-gel electrophoresis of the purified intermediate and heavy enzymes for tyrocidine synthesis. From Lee *et al.* (1973).

molecular weight, a single band appeared which we now know is the pantetheine-binding protein.

The more highly purified polyenzymes are generally quite stable to incubation at 37°C. However, they can be easily decomposed (Lee and Lipmann, 1974) by an extract of the residue of a crude homogenate that contains most of the membrane and detritus material. If this residue is extracted with Triton X-100, it yields on centrifugation a fraction which, when added to the purified polyenzymes, causes complete decomposition to 70,000 molecular weight subunits. This

process has the marks of an enzymic reaction, but we have been unable to define its nature. All experiments with known proteolytic enzymes have led to a random decomposition, and the enzymic effect of the residue extract may be due to a specific peptide split, or to the split of some other link between the subunits.

IV. Isolation of the Peptidyl Carrier 4′-Phosphopantethein Protein

The similarity between the mode of polymerization from the thio-ester of the amino-acid carboxyl moiety and that of fatty acid synthesis (Lynen *et al.*, 1968) suggested an involvement of 4′-phosphopantetheine, which we missed because we used unsuitable methodology. This was corrected, however, by Gilhuus-Moe *et al.* (1970) who used a microbiological pantothenic acid determination of Pugh and Wakil (1965) and reported on the presence of one mole of 4′-phosphopantetheine per mole of heavy enzyme, i.e. polyenzyme, in gramicidin S and its absence from the light mono-enzyme. We confirmed their results using the same method and found 4′-phosphopantetheine was present in the heavy enzyme of tyrocidine synthesis (Kleinkauf *et al.*, 1970) and later also in the intermediate enzyme (Lee *et al.*, 1973) as well as in the two polyenzymes that polymerize the first half of the amino-acid sequence of linear gramicidin (Akers *et al.*, 1977).

The earliest attempt to test for the expected function of the enzyme-bound 4′-phosphopantetheine in amino-acid polymerization was carried out in a rough manner using a combination of the three tyrocidine-synthesizing enzymes (Kleinkauf *et al.*, 1971). For this purpose, two sets of parallel experiments were done. The first involved incubating [^{14}C]asparagine alone, asparagine being the first amino acid bound to the large enzyme; the second entailed incubation of [^{14}C]asparagine within the sequence of the five initial amino acids: phenylalanine, proline, D-phenylalanine, phenylalanine and asparagine. After incubation with ATP, in the first experiment, we expected the single amino acid to be bound as a thio-ester directly to the enzyme while, in the second experiment, we expected the pentapeptide terminating in [^{14}C]asparagine to be enzyme-bound via pantetheine. To test this proposition, the incubated enzyme-substrate complexes were digested with pepsin for 18 h and chromatographed on thin-layer silica gel. As shown in Fig. 9, the position of the

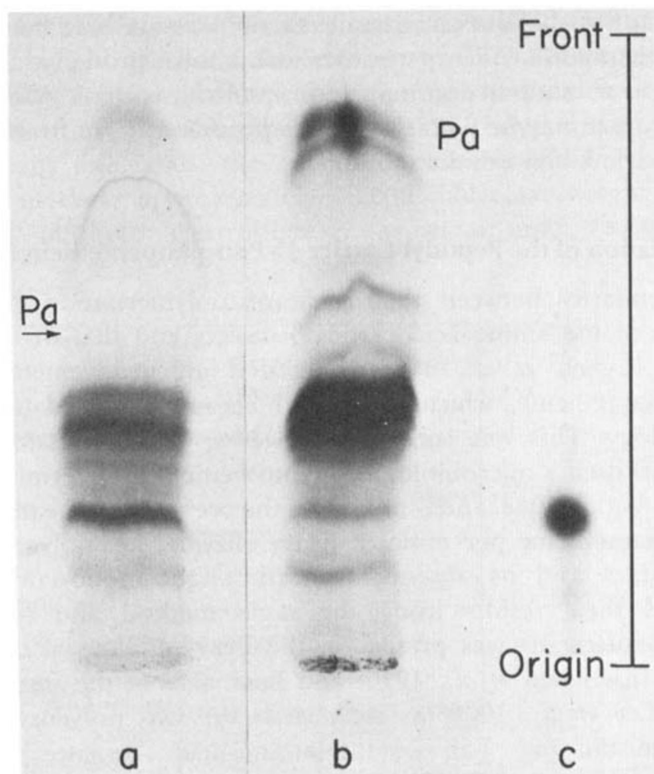


FIG. 9. Separation of the components by silica-gel chromatography followed by radio-autography of the components of a pepsin digest of the mixture of light, intermediate and heavy enzymes for tyrocidine synthesis, incubated with [^{14}C]asparagine and ATP alone, or with phenylalanine, proline, [^{14}C]asparagine and ATP to form the pentapeptide. For comparison, the position of pantetheine (Pa) was determined microbiologically (Pugh and Wakil, *1965) in zones of the silica gel (see text* for explanation) and found to overlap only with the peptide terminating in [^{14}C]asparagine. From Kleinkauf *et al.* (1971).

[^{14}C]asparagine on the chromatograms was detected by radio-autography and the position of the protein fragment bound to pantetheine was determined microbiologically as pantothenic acid by the method of Pugh and Wakil (1965) in silica-gel fractions taken from the chromatograms. In the first assay (Fig. 9a), [^{14}C]asparagine, singly bound to the enzyme, migrated far from the pantothenic acid band while, in the second assay (Fig. 9b), [^{14}C]asparagine in the pentapeptide migrated with pantothenic acid. It could also be shown that performic acid treatment would release the thio-ester of the peptide labelled with

[^{14}C]asparagine from pantetheine. At that juncture, we tentatively concluded that the peptidyl chain, but not the free amino acid, was linked to enzyme-bound 4′-phosphopantetheine.

When first observed, the appearance of a single 18,000–20,000 molecular weight fraction in the SDS-gel electrophoresis of both polyenzymes (Fig. 8, p. 244) was likely to indicate the position of the 4′-phosphopantetheine-containing protein, in accordance with the known presence of a single mole of 4′-phosphopantetheine per mole of polyenzyme. This proposition was confirmed (Lee and Lipmann, 1974) when we succeeded in incorporating either [^{14}C]pantothenic acid or [^{3}H]β-alanine into enzyme-bound pantetheine by adding them to the growth medium at the end of logarithmic growth. Crude biosynthetic extracts from bacteria thus labelled were prepared and incubated in two stages. First to produce polyenzyme-bound polypeptides, 7 g of [^{14}C]pantothenic acid-labelled bacteria were lysed with lysozyme and the lysate was incubated for 1 h at 37°C with [^{3}H]proline and all other tyrocidine amino acids except the terminal leucine. In the second stage, deoxyribonuclease was added and the mixture liquefied and incubated for a further 1.5 h to dissociate the polyenzyme system; after chilling and addition of ammonium sulphate to 33% saturation, the mixture was centrifuged and more ammonium sulphate was added to the supernatant to a concentration of 70% saturation. The precipitate was collected and dissolved in buffer. The chromatographic separation on Sephadex G-100 of this fraction is shown in Fig. 10. A large peak (C) at 35,000 molecular weight appeared in the chromatogram where the tritiated amino acid and [^{14}C]pantothenic acid labels overlapped. There was no peak at the level of the 70,000 molecular weight subunit, but there was a narrow peak (B) which overlapped in the region of 90,000 molecular weight, while peak (A) was ascribed to unresolved polyenzymes and peak (D) at 10,000 molecular weight presumably indicated ^{14}C-label in the acetyl-carrier protein. Peak B was assumed to be a high polymerization product; however, more recently, Lee and Lipmann (1977) found at the 90,000 molecular weight region in such a chromatogram a subunit which contained a complex of the 18,000 molecular weight pantetheine protein and the 70,000 molecular weight subunit (Lee and Lipmann, 1977). This might be double-labelled, carrying a tritiated peptide on [^{14}C]pantetheine.

The most important doubly-labelled peak C at 35,000 molecular weight indicated the position of the dimer of the pantetheine-linked

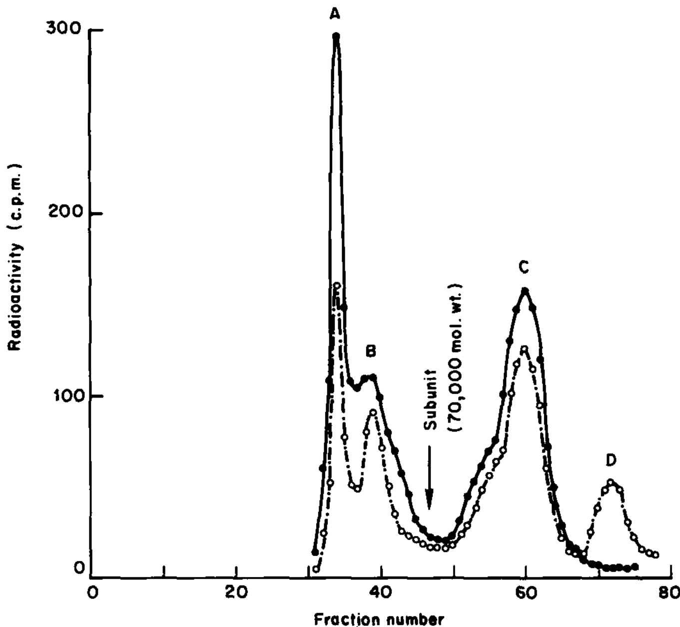


FIG. 10. Chromatography on Sephadex G-100 of crude homogenates from [^{14}C]pantothenate-labelled organisms. From Lee and Lipmann (1974). A indicates the appearance of multi-enzymes, B of the peptidyl-carrier protein polymer, C of the peptidyl-carrier protein dimer, and D of acetyl-carrier protein. O—O indicates activity of [^{14}C]pantothenate, and ●—● of ^{3}H-labelled peptide.

protein of 18,000 molecular weight that carries a polypeptide, and this fraction was further purified by DEAE-cellulose column chromatography and SDS-gel electrophoresis. A row of radioactive peaks appeared, the highest in the region of about 18,000 molecular weight, and smaller ones following in the regions of 35,000 molecular weight and larger polymers. Complete disaggregation to a single radioactive band at the level of about 17,000 molecular weight was obtained by first heating the sample to 100°C with 2% SDS for 1 h, followed by electrophoresis in 0.2% SDS at room temperature for nine hours (Fig. 11).

These experiments have been discussed in detail because they confirm the interpretation that polymerization is due to a swinging arm of the pantetheine-binding protein picking up the amino acids, lined up in the right order in the polyenzymes, as was assumed by Lynen *et al.* (1968) for the two carbon pieces in fatty-acid synthesis.

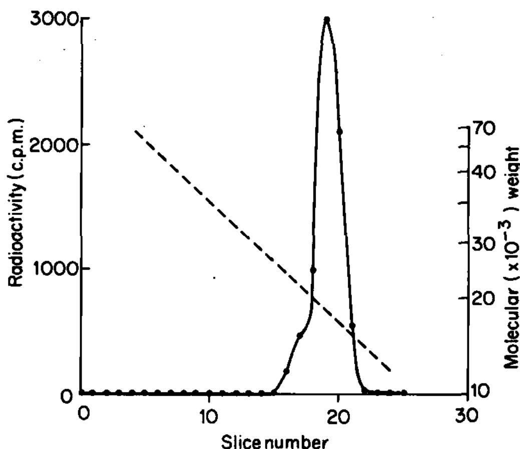


FIG. 11. Electrophoretic pattern of disaggregation of the carrier protein aggregates by high concentrations of sodium dodecyl sulphate. —— indicates radioactivity, --- molecular weight. From Lee and Lipmann (1974).

A. ISOLATION OF CARRIER PROTEIN COMBINED WITH 70,000 MOLECULAR-WEIGHT SUBUNIT

On hydrolysis at 37°C of the tyrocidine polyenzymes, the peak in the Sephadex G-200 chromatogram was relatively broad at 70,000 molecular weight. It could be subdivided by further chromatography on Sephadex G-100 into a smaller peak at 90,000 molecular weight and a larger one at 70,000 molecular weight (Lee and Lipmann, 1977). For further analysis, purified tyrocidine polyenzymes, prepared from *B. brevis* and labelled with [^{14}C]pantothenic acid, were decomposed by the described (Lee and Lipmann, 1974) Triton X-100 residue extract. For the tyrocidine intermediate enzyme with three subunits, the ratio between the 90,000 and 70,000 molecular weight peaks was 1 : 2 and, for the large tyrocidine enzyme with six subunits, it was 1 : 5. Only the 90,000 molecular weight peak was found to contain radioactive pantothenic acid and, on decomposition, it yielded the pantetheine-containing protein of 18,000–20,000 molecular weight.

In spite of its relatively small amount, the 90,000 molecular weight fraction contained all amino-acid activations, as did the large 70,000 molecular weight fraction. Thus, we assumed that, in the break-up of the polyenzymes into fractions, the presumably centrally positioned pantetheine-binding protein becomes linked to a small fraction of each

amino acid-activating subunit. Furthermore, as shown in Fig. 12, a single low-affinity constant for the fixation of amino acids in thio-ester linkage was found for the 70,000 molecular weight subunit, while for the 90,000 molecular weight peak the same low affinity appeared together with a high-affinity constant for thio-esterification that was specific for the pantetheine-thiol.

B. MECHANISM OF CHAIN ELONGATION IN ANTIBIOTIC POLYPEPTIDE SYNTHESIS

From the experiments described in the preceding sections, we concluded that, in every elongation step after transpeptidation, there is a rapid transthiolation from the growing polypeptide chain's initially formed carboxyl thioester to the enzyme protein on the carrier-bound pantetheine-thiol group because, on analysis, all polypeptides are found connected to the latter. The rapid transthiolation should be due to the higher affinity (see Fig. 12) of the carboxyl group of the freshly elongated polypeptide chain for the pantetheine-thiol group (Fig. 13). The pantetheine-thioesterified polypeptide then becomes transpeptidated to the amino group of the following amino acid, which is thioesterified to the activating protein. This sequence of transpeptidation-transthiolation continues until termination occurs, by cyclization in gramicidin S or tyrocidine, or in linear gramicidin by ethanolamination of the carboxyl group of the terminal thiol-linked tryptophan.

This transthiolation step is analogous to those involved in fatty-acid synthesis as well as ribosomal protein synthesis (Lynen *et al.*, 1968; Lipmann, 1971b), although the mechanism in protein synthesis is a translocation rather than a transthiolation because the newly elongated peptide bound to tRNA of the last added amino acid moves from the A-(amino acid) site to the P-(peptidyl) site on the ribosome to continue polymerization (Lucas-Lenard and Lipmann, 1971). The similarity is that, in a translocation, the shift of a newly elongated chain to the position from which it can then be further elongated is a general reaction that occurs in essentially homologous polymerization reactions, where a growing polymer has to remain linked to a macromolecule in order to complete the process. The uninterrupted one-by-one addition of amino acids to form a polypeptide that remains

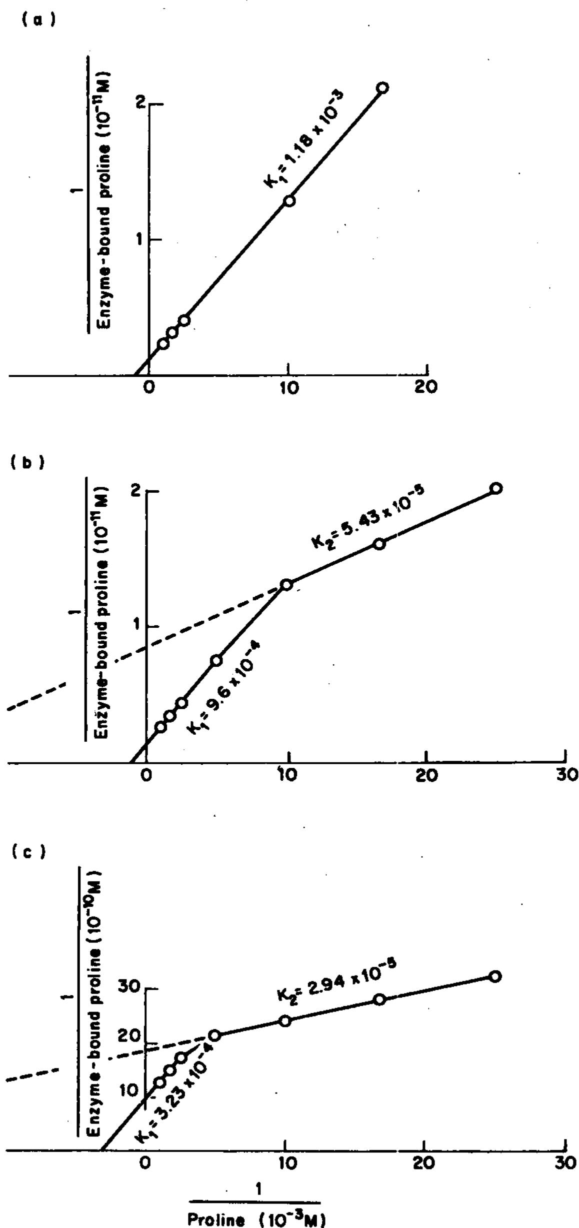


FIG. 12. Lineweaver-Burk plots for binding in thio-ester linkage of proline to the 70,000 molecular weight (a) and 90,000 molecular weight subunits (b) and to the undissociated intermediate enzyme (c). k_1 and k_2 indicate binding constants in gramicidin S synthesis. From Lee and Lipmann (1977).

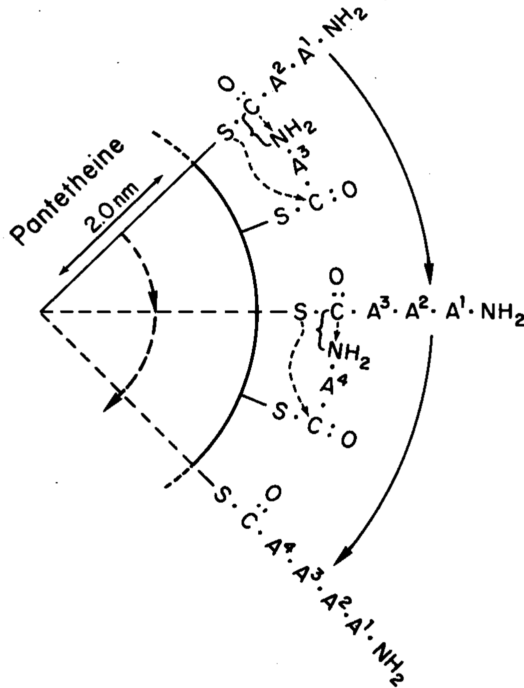


FIG. 13. Schematic representation of transpeptidation and transthiolation in biosynthesis of gramicidin S and tyrocidine, mediated by enzyme-bound pantetheine. Pantetheine is drawn as the central thiol-carrying arm charged with the nascent chain. It donates a peptidyl carboxyl group from pantetheine to the amino group of the succeeding amino acid to form the next longer peptide, which should have to remain only briefly on the peripheral thiol group. The now-liberated pantetheine thiol group then performs a translocation by transthiolation of the peptide from the peripheral acceptor thiol group to its own donor thiol group. Successive peptide transfers, each involving transpeptidation and transthiolation, are required to form the pentapeptidyl and decapeptidyl in gramicidin S and tyrocidine biosynthesis, respectively, before the decapeptides are released by cyclization.

linked to a solid phase has also been adopted in the non-enzymic system designed by Merrifield (1965).

C. USE OF A CHEMICALLY PREPARED AMINOACYL THIO-ESTER FOR TRANSFERRING AN AMINO ACID TO AN ACTIVATING ENZYME

We were able to show (Roskoski *et al.*, 1971) that, in a transthiolation from the D-phenylalanine thio-ester, chemically prepared with thiophenol, the amino acid will be accepted by the phenylalanine-specific

TABLE 8. Activity of the light enzyme in gramicidin S biosynthesis after pre-incubation with D-phenylalanyl-thiophenol

Composition of reaction mixture	Gramicidin S formed (pmol)
1. Heavy enzyme, [^{14}C]proline, [^{14}C]valine, [^{14}C]ornithine and [^{14}C]leucine	0
2. Light enzyme and D-phenylalanine, with mixture 1	0
3. Light enzyme and D-phenylalanyl-thiophenol with mixture 1	5.8

From Roskoski *et al.* (1971). Incubation of reaction mixture 1 does not lead to polymerization in the absence of the phenylalanine-charged light enzyme.

thiol group of the light gramicidin S enzyme in the absence of ATP. This thio-ester would have been very difficult to prepare with radioactively-labelled amino acid. Therefore, we chose to test for initiation by the light enzyme, pre-incubated with D-phenylalanyl-thiophenol. In the experiment, described in Table 8, the pre-incubated light enzyme was passed through Sephadex G-50, precipitated with 80% ammonium sulphate, and the precipitate dissolved in a small volume. It was then combined with the heavy enzyme, which had been precharged with ^{14}C-labelled amino acids and treated like the charged light enzyme. After incubation of the mixture for one hour at 37°C, gramicidin S was extracted with organic solvent, analysed by thin-layer chromatography and measured by radioscanning. The results indicate that, by this procedure, the light enzyme was charged with D-phenylalanine since it initiated synthesis of gramicidin S. It is concluded, therefore, that transthiolation occurred from the D-phenylalanyl-thioester to the active site on the light enzyme which then initiated biosynthesis of the antibiotic. When thio-esters of other constituent amino acids were tested, however, they were not accepted. This may be explained by the relatively high mobility of the thio-ester-linked D-phenylalanine for the light enzyme, which is indicated by the 20-fold faster ATP-[^{14}C]AMP exchange as compared to all other gramicidin S constituent amino acids (see Table 3, p. 237).

V. Biosynthesis of Linear Gramicidin

Early experiments on this problem were done by Bauer *et al.* (1972). Their analysis of this biosynthetic system remained rather preliminary because it soon appeared that the enzyme assembly needed for

synthesis of linear gramicidin is relatively unstable and they decided not to attempt an isolation of the constituent polyenzymes. In their experiments, they used extracts of *B. brevis* (A.T.C.C. 8185), which synthesizes both tyrocidine and linear gramicidin, and prepared a lysate from the organism in the usual manner by treating it with lysozyme and deoxyribonuclease and centrifuging it at 10,000 g. The supernatant fraction contained a crude but potent enzyme preparation that was used in experiments to be discussed later. First, however, I will describe the experiments done with a purified fraction obtained from the crude extract, which was prepared by precipitating inactive material with streptomycin and then taking the cut between 20% and 50% ammonium sulphate saturation. The precipitate obtained from the latter was dissolved in a 10% sucrose-containing buffer to give a concentration of approximately 25 mg protein/ml. This solution was applied to a Sephadex G-200 column and eluted with a triethanolamine buffer (pH 7.3) containing dithiothreitol. Aliquots were collected and tested for the ATP-PP_i exchange reaction of all the linear gramicidin constituent amino acids. As shown in Fig. 14, peaks, or double peaks depending on the presence of the same amino acids in different places, were obtained for all of them.

A. ISOLATION OF NON-FORMYLATED PENTADECAPEPTIDE

For the following experiments, fractions 60–87 of Fig. 14 were combined to isolate enzyme-bound polypeptides. It has to be remembered here that the extract contained both the tyrocidine- and the linear gramicidin-synthesis systems. However, the amino acids specific for tyrocidine were omitted in the attempt to isolate only the enzyme-bound polypeptides formed in linear gramicidin synthesis. Fortunately, by leaving out proline, the second amino acid in the tyrocidine sequence (eighth item in Table 7, p. 240), any synthesis of tyrocidine polypeptides was prevented because omission of one amino acid in the sequence prevents polymerization of the amino acids that follow the missing one. Therefore, in spite of the overlap with many amino acids in tyrocidine, by adding only the constituent amino acids of linear gramicidin, only enzyme-bound peptides suitable for comparison with those formed in linear gramicidin biosynthesis were obtained. It was one of the defects in our understanding of this biosynthesis that, in the *in vitro* experiments, we were unable to

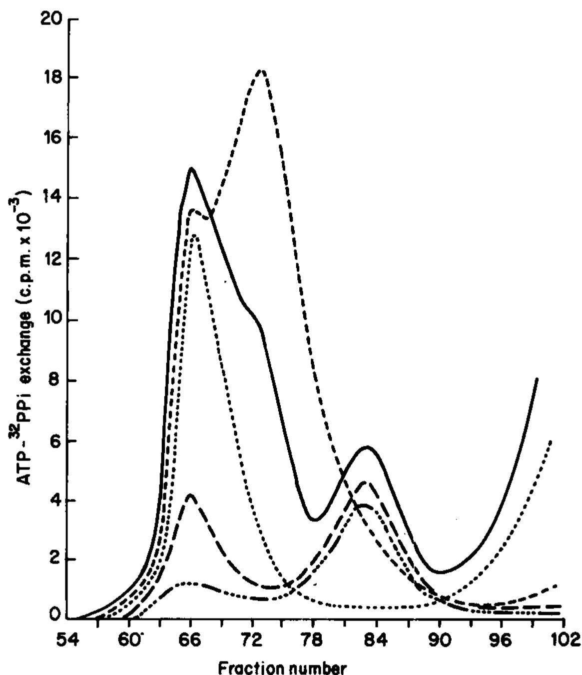


FIG. 14. Separation of linear gramicidin-synthesizing enzymes by Sephadex G-200 filtration. Adenosine triphosphate-[^{32}P]PP$_i$ exchanges are shown dependent on valine (——), leucine (..........), tryptophan (– – –), alanine (———)—all in the L configuration—and glycine (—··—), the constituent amino acids of linear gramicidin. From Bauer *et al.* (1972).

complete enzymic synthesis by ethanolamination which was needed for release.

For the moment, any discussion of the mechanism of initial formylation of the *N*-terminal valine will be postponed. The experiments to be described gave us reason to believe that, with the Sephadex G-200-purified system, it was possible to synthesize an unformylated pentadecapeptide. For this purpose, enzyme fractions 60–87 (Fig. 14) were combined and incubated with ATP and the linear gramicidin constituent amino acids. After incubation of these amino acids containing [^{14}C]glycine and ATP, the peptides formed remained enzyme-bound because the chain-terminating ethanolamine could not be supplied. The protein-bound peptides were precipitated with trichloroacetic acid, and the thiol link of the peptides to the protein was hydrolysed by treatment with potassium hydroxide (0.01 *N*). The

liberated peptide-containing solution was neutralized with perchloric acid, taken up with an excess of 90% ethanol, and the supernatant solution used for thin-layer silica-gel chromatography with butanol : acetic acid : water. With this eluant, the fraction moving fastest was expected to contain the longest peptide. It was visualized by radio-autography, eluted with 90% methanol, and rechromatographed on thin-layer silica gel with an acetate : pyridine : acetic acid : water mixture. The second chromatogram produced a fast-moving heavy band with an R_F value 0.45. This peptide was compared with the linear gramicidin pentadecapeptide, including use of acid hydrolysis. For this purpose, the precursor amino acids were labelled with ^{14}C, tryptophan being replaced by phenylalanine to permit exhaustive hydrolysis with hydrochloric acid. As shown in Fig. 15, the expected amino acids were present in the chromatogram of the hydrolysate.

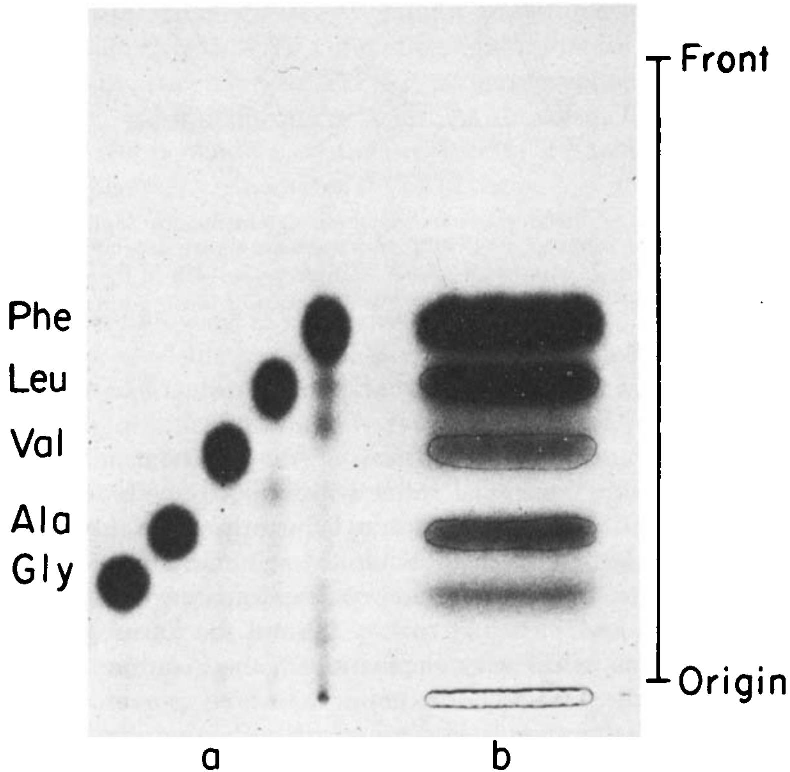


FIG. 15. Recovery of the precursor amino acids from the peptide (R_F value 0.45) after acid hydrolysis. From Bauer *et al.* (1972).

To verify quantitatively the identity of the R_F 0.45 peptide with the pentadecapeptide of gramicidin, the ratio of L- to D-amino acids was determined in the hydrolysate, as described by Gevers *et al.* (1969) by use of D- and L-amino acid oxidases. For this purpose, an acid hydrolysate of the peptide was prepared containing [^{14}C]leucine, or [^{14}C]valine or [^{14}C]alanine. The ratios are shown in Table 9 for alanine only in the L-configuration, for leucine only in the D-configuration, whereas for valine, with 55% in L- and 45% in D-configuration, the ratios closely approximated the value for D- and L-valine in the pentadecapeptide. Furthermore, in four separate experiments, the contents of [^{14}C]glycine, [^{14}C]alanine, [^{14}C]valine and [^{14}C]phenylalanine were each compared with the content of [^{3}H]leucine. As shown in Table 10, their ratios very closely approximated those to be expected, that is 1:2:4:4.

B. EXPERIMENTS WITH CRUDE EXTRACTS

When the crude extract was supplemented with constituent amino acids containing [^{3}H]leucine and [^{14}C]formate and incubated with ATP, the fastest moving peptide was again isolated as described. The peptide obtained here had an R_F value of 0.6 instead of 0.45. This penta-

TABLE 9. Optical configuration of amino acids obtained after hydrolysis of the R_F 0.45 peptide, a precursor in linear gramicidin S synthesis

	L-amino acid (%)	D-amino acid (%)
Leucine	0	100
Valine	55	45
Alanine	100	0

From Bauer *et al.* (1972).

TABLE 10. Stoicheiometry of amino acids in the R_F 0.45 peptide, an intermediate in linear gramicidin synthesis

[^{14}C]Glycine 1.45 pmoles	[^{3}H]Leucine 5.8 pmoles	Ratio 1:4
[^{14}C]Alanine 2.8 pmoles	[^{3}H]Leucine 5.4 pmoles	Ratio 1:2
[^{14}C]Valine 5.8 pmoles	[^{3}H]Leucine 5.4 pmoles	Ratio 1:1
[^{14}C]Phenylalanine 5.8 pmoles	[^{3}H]Leucine 5.4 pmoles	Ratio 1:1

From Bauer *et al.* (1972).

decapeptide, as shown in Fig. 16, contained [^{14}C]formate as well as [^{3}H]leucine. When the formyl group was removed chemically, chromatography revealed that the compound with an R_F value of 0.6 had been converted to one with an R_F value of 0.45, namely non-formylated pentadecapeptide. Thus, the crude extract contained the formylation system using formate as donor.

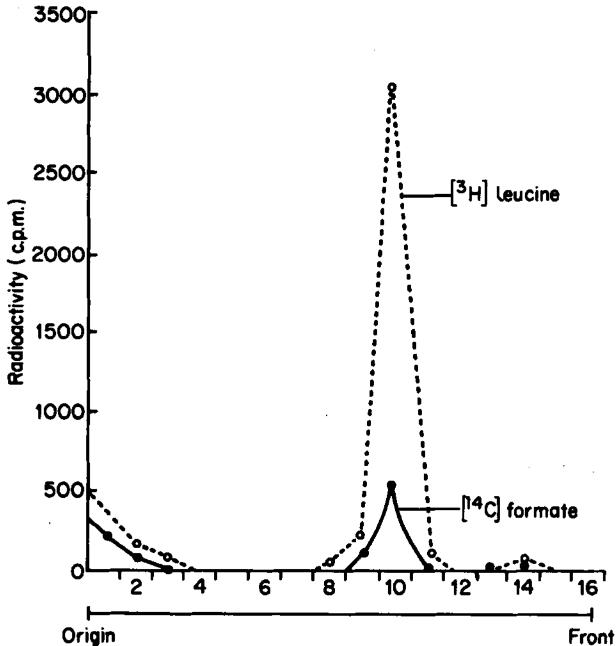


FIG. 16. Isolation of enzyme-bound peptide (R_F value 0.6) after liberation from the enzyme system for linear gramicidin biosynthesis. The incubation mixture contained [^{3}H]leucine, [^{14}C]formate and ATP. The chromatographic separation shows incorporation of both labelled compounds. From Bauer *et al.* (1972).

C. INITIATION BY FORMYLATION OF ENZYME-BOUND VALINE

Since we had obtained evidence with purified extracts for *in vitro* formation of a non-formylated pentadecapeptide, we erroneously concluded that formylation might be a secondary reaction after peptide formation. We should have been warned by the observation made in the same paper that, *in vivo*, aminopterin inhibited linear gramicidin synthesis to 95%, while at the same time it stimulated tyrocidine synthesis two-fold. This indicated that, when formylation

was prevented, linear gramicidin was not synthesized. Nevertheless, the identity of the non-formylated peptide (R_F value of 0.45) with the backbone of linear gramicidin was further confirmed by chemical formylation and release from the protein by ethanolamination of the bound thio-ester-linked formyl peptide using a high concentration of ethanolamine. This product of combined enzymic and chemical synthesis was found to be indistinguishable from authentic linear gramicidin.

Akashi and Kurahashi (1977) have now solved the formylation problem. They report on formylation of valine bound to an enzyme they called component I using formyltetrahydrofolic acid (THFA). This enzyme was isolated from *B. brevis* A.T.C.C. 8185, and contained the catalytic function of formyl transfer with formyl-THFA as the donor; it synthesized enzyme-bound formylvaline and formylvaline-glycine. They also reported identification in their *B. brevis* homogenate, by Sephadex G-200 chromatography, or multiple ATP-[^{32}P]PP_i peaks for all amino-acid components of linear gramicidin (gramicidin A). This chromatogram is similar to that described by Bauer *et al.* (1972) and Akers *et al.* (1977).

D. ISOLATION OF TWO POLYENZYMES FROM PURIFIED HOMOGENATES

Akers *et al.* (1977), using as a guide to isolation the high peaks of ATP-[^{32}P]PP_i exchange with glycine and alanine the two amino acids unique to linear gramicidin (Fig. 17), purified two enzymes they called the glycine and alanine enzymes. The former we judge to be analogous to component I of Akashi and Kurahashi (1977). We did not detect formylation of valine with free formate, but did not try formyl-THFA. The glycine enzyme readily fixed glycine as well as valine when added separately, but synthesized mostly Val-Gly-diketopiperazine when both were added together. On trichloroacetic acid precipitation, very little bound valine-glycine was found. When the purified enzyme was tested with ATP and valine or glycine separately, it formed equivalent amounts of trichloroacetic acid-precipitable valine and glycine, with traces only of alanine or ornithine. Using the approximately 75% pure glycine and alanine enzymes, each was found to contain one mole of 4′-phosphopantetheine as determined by the method of Pugh and Wakil (1965).

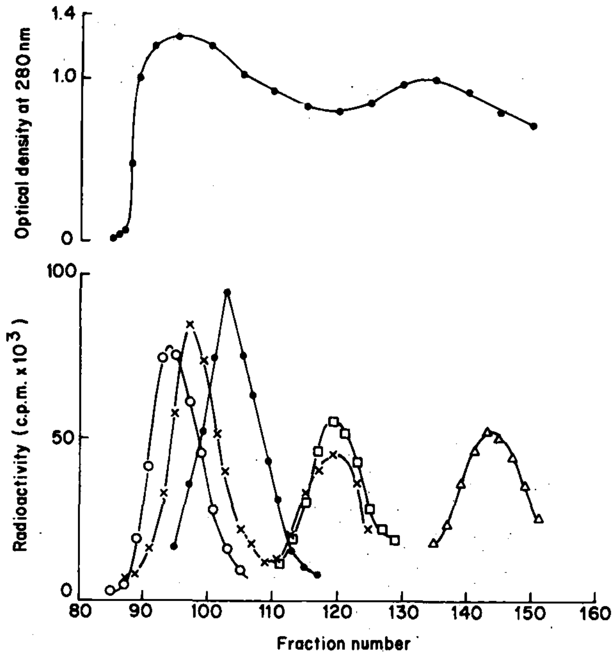


FIG. 17. Positioning of the alanine and glycine enzymes on a Sephadex G-200 chromatogram by ATP–PPi exchange with tyrocidine and linear gramicidin constituent amino acids. O—O indicates L-ornithine, x—x L-alanine, ●—● L-proline, □—□ glycine and Δ—Δ D-phenylalanine.

TABLE 11. Formation of protein-bound nascent peptide chains involved in synthesis of linear gramicidin

Experiment	Amino acid				Mol bound amino acid per mol alanine enzyme
	L-Val	Gly	L-Ala	L-Leu	
1	^{14}C	+	–	–	0.36
2	^{14}C	+	+	–	0.62
3	^{14}C	+	+	+	2.27
4	+	^{14}C	–	–	0.07
5	+	^{14}C	+	–	0.91
6	+	^{14}C	+	+	1.56
7	–	–	^{14}C	+	0.17
8	+	–	^{14}C	–	0.18
9	+	+	^{14}C	–	0.66
10	+	+	^{14}C	+	2.90

From Akers *et al.* (1977).

When the glycine enzyme was combined with the alanine enzyme and unlabelled alanine added, [^{14}C]glycine-valine binding was considerably increased (Table 11). This increase is comparable to the increase in incorporation of amino acids on addition of the next following amino acids in experiments with gramicidin S synthesis, where liberation of Phe-Pro-diketopiperazine was first observed in Kurahashi's laboratory (Kurahashi, 1961; Kurahashi *et al.*, 1969). We later found with the gramicidin S system (Fig. 5, p. 239) that, on addition of the third amino acid namely valine, an enzyme-bound tripeptide formed and was followed by appearance of a tetra- and pentapeptide after addition of ornithine and leucine. This is analogous to the present case where diketopiperazine formation between valine and glycine, as it occurs by simultaneous addition to their mixture, is minimized by addition of the alanine enzyme and the next following amino acid, namely alanine. This is further increased when leucine is added, as shown in Table 11 (experiments 3, 5 and 10) where, as indicated by the higher ^{14}C incorporation, longer enzyme-bound peptides are produced. We assume that *in vitro* production of the non-formylated pentadecapeptide (Bauer *et al.*, 1972) should be due to stabilization of the valine-glycine dipeptide after addition of the following amino acids, which permit longer chains to form. Nevertheless, Akashi and Kurahashi (1977) show that immediate stabilization of the enzyme-bound valine-glycine dipeptide is achieved by blocking the *N*-terminal group in valine, and they are quite right to assume that normal initiation of linear gramicidin synthesis is due to formylation of enzyme-bound valine. We consider, however, formation of enzyme-bound non-formylated pentadecapeptides under abnormal conditions to be well documented in the *in vitro* experiments of Bauer *et al.* (1972).

The approximate molecular weight of 160,000 found for the glycine enzyme compares reasonably well with that of 180,000 for component I of Akashi and Kurahashi (1977). It is consistent with activation of two amino acids, as is the molecular weight of 350,000 for the alanine enzyme with activation of five amino acids. Correspondingly, the purified alanine enzyme separately binds valine, alanine and leucine in the ratio of 2 : 2 : 1 (Akers *et al.*, 1977). We were somewhat uneasy about the possible binding of the D-valine that follows L-valine in the sequence, but felt that the molecular weight and ratio of valine-alanine-leucine binding appeared to indicate that the alanine enzyme activated the five amino acids that follow the valine-glycine sequence in

the glycine enzyme. For activation of the four following duplex sequences of a D-aliphatic-L-tryptophan, there is some indication that D-leucine-tryptophan sequences are activated by an enzyme in the low molecular-weight region of the Sephadex G-200 chromatogram (S. G. Lee, unpublished observation). This observation does not exclude the possibility that this activity may be due to a fragment of a larger enzyme.

Vi. Thio-ester-linked Amino or Hydroxy Acid Activation in some other Antibiotic Biosynthesis

Studies on the thio-ester-linked biosynthesis of both gramicidin S and tyrocidine in the laboratories of Kurahashi and Laland, and our own, were followed by observations that other antibiotics are synthesized in an analogous manner. In the following brief survey I have chosen only those compounds that have been more thoroughly investigated.

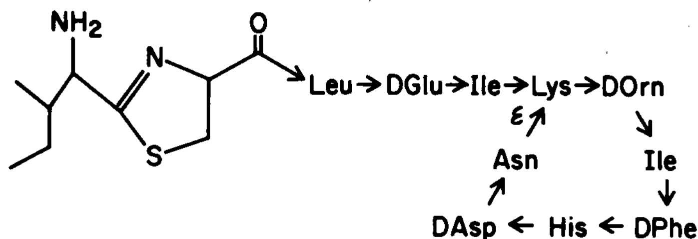


FIG. 18. Structure of bacitracin.

A. BACITRACIN

The structure of bacitracin is presented in Fig. 18. This rather complex antibiotic was first analysed by Ishihara *et al.* (1968) and further amplified by later work from their laboratory (Ishihara *et al.*, 1975). More recently, Laland's group has contributed to this analysis (Frøshov, 1977). Three enzymes, compounds 1, 2 and 3, with different molecular weights, were isolated by the Japanese group. They assume that the first polymerizes the first five amino acids, the second activates ornithine and lysine, and the third continues the chain by activation of the five following amino acids. They also found that each of the three

polyenzymes contained one mole of 4′-phosphopantetheine. Two cyclization reactions, in the first part of the molecule, between cysteine and isoleucine and in the second part from asparagine to the ε amino group in lysine, seem to be carried out after the initial chain has been formed.

B. EDEINE

The mechanism of biosynthesis of edeine has been well outlined by Kurylo-Borowska and her coworkers (Kurylo-Borowska and Sedkowska, 1974; Kurylo-Borowska, 1975). It is a hexapeptide of the following composition which contains many unusual amino acids:

β-Tyr → Ise → Dpr → Dha → Gly → Spermidine

(Ise indicates isoserine, Dpr diaminopropionic acid, and Dha 2,6-diamino-7-hydroxyazelaic acid). Two enzymes were isolated with molecular weights of 210,000 and 180,000 respectively and a monoenzyme of 100,000; the two higher molecular-weight enzymes were found to contain a covalently bound pantetheine. Edeine is blocked by spermidine on the *C*-terminal at the carboxyl group of glycine. At the *N*-terminal of the molecule, there is a β-tyrosine, the biosynthesis of which has been elaborated by Kurylo-Borowska and Abramsky (1972).

C. ENNIATIN B

Zocher and Kleinkauf (1978) described the mechanism of biosynthesis of this cyclodepsipeptide which is composed of a cycle of thrice-repeated D-2-hydroxyisovaleric acid-*N*-methyl-L-valine sequences. Using extracts of *Fusarium oxysporum*, they succeeded in synthesizing the cyclodepsipeptide and found that both components were activated by ATP apparently through amino-acyl and hydroxyacyl adenylates; this was followed by thio-esterification to protein as shown by a trichloroacetic acid precipitation with the respective compounds which can be liberated by performic acid. The didepsipeptide in thio-ester linkage is reported to be the intermediate in the cyclization of three didepsipeptides to form the cyclohexadepsipeptide.

D. MISCELLANEOUS

Kleinkauf and his collaborators have worked, furthermore, on the biosynthesis of a number of antibiotics which have been discussed in detail in a recent review (Kleinkauf and Koischwitz, 1978).

Biosynthesis of valinomycin, a cyclododecadepsipeptide which contains the thrice-repeated sequence of the tetradepsipeptide: L-lactyl-L-valyl-D-α-hydroxyvaleryl-D-valyl, has been explored by Anke and Lipmann (1977). Earlier attempts to identify the mechanism of valinomycin synthesis were made by Ristow *et al.* (1974).

It should be added that Mitnick and Reichlin (1971, 1972) published data which they considered to indicate that the hypothalamic thyrotropin releasing hormone (TRH), which is the tripeptide pyroGlu-His-Pro-NH_2, was synthesized in hypothalamic extracts by a non-ribosomal reaction similar to the bacterial antibiotic syntheses discussed in this review. Through helpful collaboration with Dr. Reichline's laboratory, Bauer and Lipmann (1976) could show, however, that the identification by Mitnick and Reichlin of formation of radioactively marked TRH, by comparison with authentic hormone, was due to the use of an unsuitable chromatographic system, and that, on chromatography in a second dimension with a different system, this coincidence disappeared. Dixon and Acres (1975) also reported their inability to obtain synthesis of TRH in hypothalamic extracts. To our knowledge, the type of polypeptide synthesis that emerged from analysis of the biosynthesis of bacterial antibiotics has not been found in animal tissues.

For an extensive general discussion of polypeptide synthesis, the reader is referred to the reviews by Kurahashi (1974), Kleinkauf and Koischwitz (1978) and Frøshof *et al.* (1978).

VII. Acknowledgements

The research reported here was supported by a grant to the author from the National Institutes of Health, United States Public Health Service, No. GM-13972. I should like to acknowledge the great help of my secretary, Chris Gillespie, in the preparation of the manuscript.

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Control of Metabolism in Yeast and other Lower Eukaryotes through Action of Proteinases

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I. Introduction

The involvement of proteinases in control of cellular metabolism has been neglected for many years. Over the past three decades, control of

metabolism through regulation of rates of enzyme synthesis (Cellular Regulatory Mechanisms, 1961) and through non-covalent (Stadtman, 1966) and covalent (Holzer and Duntze, 1971) binding of ligands on enzyme proteins regulating their activity has attracted much attention. More recently, the importance of proteolysis in cellular events has been realized. The recognition of intracellular proteolysis as a fundamental process in biology has greatly increased research in this field. The increase of knowledge is documented in several review articles (Schimke and Doyle, 1970; Pine, 1972; Goldberg and Dice, 1974; Miller, 1975; Holzer *et al.*, 1975; Goldberg and St. John, 1976; Switzer, 1977).

Most of these review articles are concerned with proteolytic events in bacterial and mammalian cells. Much work has also been done on lower eukaryotes; however, except for the review of Holzer *et al.* (1975), no writer has attempted to bring all of this work together.

In lower eukaryotes, the proteolytic system of yeast (*Saccharomyces cerevisiae*) is perhaps the most thoroughly studied. A variety of proteinases and specific inhibitor proteins are known and characterized, their subcellular compartmentalization is known in several cases, and culture conditions governing the activity levels of components of the proteolytic system have been studied. Furthermore, a variety of physiological events have been found which have been assumed to be governed by proteolytic action. The foregoing facts, together with the accessibility of yeast to biochemical studies under defined nutritional conditions and the possibility of using genetic studies to uncover links between *in vitro* activities of the proteolytic system and *in vivo* events, make yeast an ideal organism in which to study metabolic regulation by proteolysis in the eukaryote cell. Even though this article will concentrate on yeast, it will also include other lower eukaryotes to get a more general view of the involvement of proteolysis in the eukaryote cell.

Proteinases have been postulated or shown to be involved in the following types of processes: (1) So called "protein maturation events": (a) removal of *N*-formylmethionine or methionine from newly synthesized polypeptide chains (Stewart *et al.*, 1971); (b) removal of peptide extensions of proteins traversing membranes (Blobel and Dobberstein, 1975; Highfield and Ellis, 1978); (c) cleavage of the translation product of monocistronic mRNA coding for several distinct polypeptides (Fink, 1971); and (d) conversion of inactive

proproteins (zymogens) into the biologically active products (Neurath and Walsh, 1976). (2) Specific inactivation or modification of biologically active proteins (Rouget and Chapeville, 1971; Switzer, 1977). (3) Continuous turnover to regulate protein levels (Schimke and Doyle, 1970; Pine, 1972; Goldberg and St. John, 1976). (4) Degradation of unneeded cellular proteins in order to supply amino acids and energy under conditions of starvation or during differentiation (Mandelstam, 1960; Kornberg *et al.*, 1968). (5) Selective elimination of defective protein molecules (Goldschmidt, 1970; Platt *et al.*, 1970; Etlinger and Goldberg, 1977). (6) Degradation of intracellularly transported exogenous peptides to amino acids which ultimately satisfy nutritional requirements (Payne, 1976).

In the present article, a mosaic of the existing information on intracellular proteolysis of lower eukaryotic organisms will be summarized. This includes characterization of the intracellular proteinases and proteinase inhibitors, their regulation, as well as the processes known or postulated to be dependent on proteinase action. Even though knowledge of both proteinases and their inhibitors on the one hand and proteolytic processes on the other has increased considerably in the past few years, the reader will notice that large gaps still exist between parts of the mosaic, preventing us from seeing the picture as a whole. However, by assembling the parts of this mosaic as it has emerged until now, these gaps should become more clearly recognizable. Hopefully such a task will enable us to find missing pieces more rapidly and give us clearer notions as to where to insert them.

II. The Proteolytic System and its Cellular Localization

A. *SACCHAROMYCES CEREVISIAE*

1. *Proteinases*

Eighty years ago proteolytic enzymes had already been found in yeast (Hahn and Geret, 1898). A first characterization had been started by Dernby (1917) and by Willstätter and Grassmann (1926). Work of Lenney and Dalbec (1967), of Doi *et al.* (1967) and of Hata *et al.* (1967a, b) led to separation and characterization of three proteolytic enzymes in *Sacch. cerevisiae*, namely proteinase A, proteinase B, and proteinase C, an enzyme which because of its specificity was later on called

carboxypeptidase Y (Hayashi *et al.*, 1970, 1973). Detection of drastic effects of proteinase A and proteinase B on yeast proteins *in vitro*, resulting in activation or inactivation of the proteins tested, and the possible implications of these findings for *in vivo* processes, led to increased interest in these two proteinases (Cabib and Farkas, 1971; Katsunuma *et al.*, 1972; Cabib and Ulane, 1973a, b; Hasilik and Holzer, 1973; Saheki and Holzer, 1974; Schött and Holzer, 1974; Jusič *et al.*, 1976; Ulane and Cabib, 1976). It was found that proteinase A is an acid endoproteinase with a molecular weight of about 60,000 and an isoelectric point of 3.8 (Hata *et al.*, 1967b). The enzyme protein contains about 10% hexose (Hata *et al.*, 1967b). It hydrolyses casein and haemoglobin with high efficiency between pH 2 and 4 (Hata *et al.*, 1967a, b; Lenney and Dalbec, 1967), but it does not cleave any small peptide substrate that has been tested (Hata *et al.*, 1967a). Pepstatin is a highly effective inhibitor of proteinase A (Saheki *et al.*, 1974) indicating an aspartic-acid or glutamic-acid carboxyl group (Tang, 1976) at the active site of the enzyme. Inhibition of proteinase A is furthermore brought about by the intracellularly occuring inhibitor protein I^A (Saheki *et al.*, 1974; Holzer, 1975; Nuñez de Castro and Holzer, 1976; Bünning *et al.*, 1977; Holzer *et al.*, 1977) (see also Section II.A, 2, p. 274).

Using affinity chromatography, proteinase B was isolated (Fig. 1) as a single polypeptide chain of 32,000 (Bünning, 1977) to 44,000 (Ulane and Cabib, 1976) molecular weight, exhibiting an isoelectric point of 5.8 (Bünning, 1977). The discrepancy in the reported molecular weights might be due to different extraction procedures (Ulane and Cabib, 1976). As an endoproteinase, the enzyme cleaves acid-denatured haemoglobin and casein most readily between pH 6 and 10 (Hata *et al.*, 1967a). In addition, it attacks Azocoll (Cabib and Ulane, 1973b; Saheki and Holzer, 1974) and Hide Powder Azure in the neutral pH range (Ulane and Cabib, 1976). No small peptide substrate has been found to be cleaved as yet (Lenney and Dalbec, 1967). However, proteinase B exhibits some activity towards the ester substrates *N*-benzoyl-L-arginine ethyl ester (Hata *et al.*, 1967a; Ulane and Cabib, 1976), *N*-acetyl-L-tyrosine ethyl ester (Hata *et al.*, 1967a; Ulane and Cabib, 1976), *N*-acetyl-DL-phenylalanine-β-naphthyl ester (D. H. Wolf and C. Ehmann, unpublished observations) and several amino-acid *p*-nitrophenyl esters (Ulane and Cabib, 1976). Proteinase B activity is strongly inhibited by diisopropylfluorophosphate and

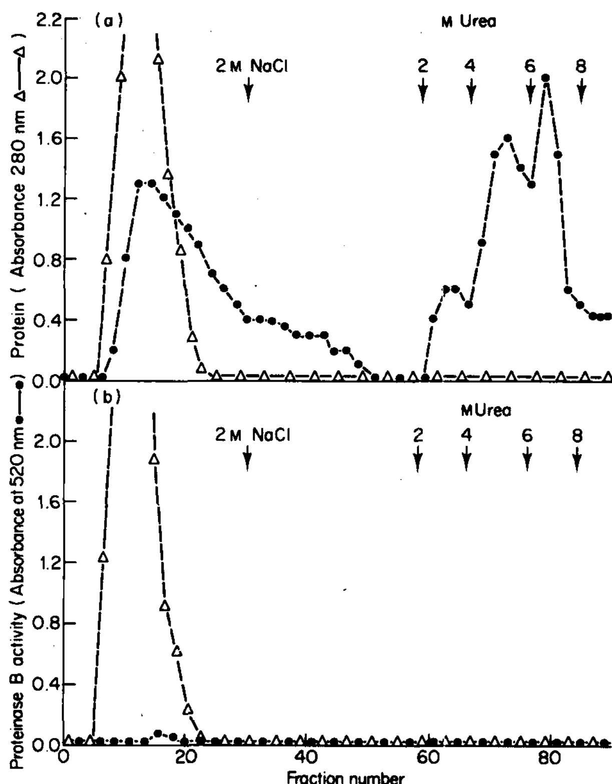

FIG. 1. Purification of proteinase B from *Saccharomyces cerevisiae*, showing elution patterns of proteinase B from immobilized nitrated proteinase B-inhibitor (a) and native proteinase B-inhibitor (b) sepharose-columns. Proteinase B-inhibitor (I^B2) or its nitrated derivative was coupled onto activated CH-Sepharose 4B. Nitrated proteinase B-inhibitor (3-nitrotyr[41] I^B2) was prepared as described by Bünning and Holzer (1977). Elution conditions and measurements of proteinase B activity (absorbance at 520 nm of the colour released from Azocoll by proteinase B cleaveage) are described by the same authors. Successful elution of proteinase B activity was achieved from the 3-nitrotyr[41] I^B2 affinity column by a stepwise gradient from 2 to 8 M urea in 0.1 M potassium phosphate buffer (pH 7) (part 1 of Fig. 8; Bünning and Holzer, 1977). In contrast, proteinase B activity was not eluted from immobilized native I^B2 under these conditions. These differences in elution behaviour are due to a lowered association constant of proteinase B to 3-nitrotyr[41] I^B2 of $3{\cdot}2 \times 10^8\ M^{-1}$ as compared with the association constant of proteinase B to unmodified I^B2 of 3.9×10^9. From Bünning and Holzer (1977).

phenylmethylsulphonylfluoride (Hata *et al.*, 1967a; Lenney and Dalbec, 1967) indicating a serine residue at the active site (Ulane and Cabib, 1976). Inhibition of the enzyme by *p*-chloromercuribenzoate or mercuric chloride (Hata *et al.*, 1967a; Lenney and Dalbec, 1967; Ulane

and Cabib, 1976; Bünning, 1977) points to the presence of sulphydryl residue(s) in the enzyme molecule which are necessary for protein-hydrolysing activity. Proteinase B activity is also strongly inhibited by the actinomycete metabolites chymostatin and antipain (Lenney, 1975; Ulane and Cabib, 1976). In addition, the intracellular inhibitor protein I^B acts strongly on the enzyme activity (Betz *et al.*, 1974; Ulane and Cabib, 1974; Bünning and Holzer, 1977; see Section II.A, 2, p. 274).

The broad amino-acid specificity of carboxypeptidase Y implicated its general role in intracellular peptide hydrolysis (Hayashi *et al.*, 1970) and its possible usefulness in studies on the amino-acid sequence of proteins was realized (Hayashi *et al.*, 1973). This led to extensive purification and characterization of the enzyme by different research groups (Doi *et al.*, 1967; Aibara *et al.*, 1971; Hayashi *et al.*, 1973; Kuhn *et al.*, 1974; Johansen *et al.*, 1976). The enzyme was found to be composed of one polypeptide chain of 61,000 molecular weight (Hayashi *et al.*, 1973; Kuhn *et al.*, 1974) exhibiting an isoelectric point of around 3.6 (Hata *et al.*, 1967b). Sixteen residues of glucosamine and about 15% of hexose are linked to the protein moiety (Hayashi *et al.*, 1973; Kuhn *et al.*, 1974). At pH 5.5–6.5, carboxypeptidase Y exhibits a prominent activity in hydrolysing most types of amino-acid residue, including proline, from C-termini of proteins and peptides (Hayashi *et al.*, 1970, 1973; Kuhn *et al.*, 1974; Hayashi, 1976). The enzyme also exhibits high activity against ester substrates, like *N*-acetyl-L-phenylalanine ethyl ester, *N*-acetyl-L-tyrosine ethyl ester (Doi *et al.*, 1967; Hayashi, 1976) and *N*-acetyl-DL-phenylalanine-β-naphthyl ester (Wolf and Fink, 1975). Also, the amide substrate *N*-benzoyl-L-tyrosine-*p*-nitroanilide is cleaved by carboxypeptidase Y (Aibara *et al.*, 1971). Diisopropylfluorophosphate and phenylmethylsulphonylfluoride are powerful inhibitors of carboxypeptidase Y activity (Doi *et al.*, 1967; Kuhn *et al.*, 1974). Quantitative analysis revealed one serine residue per enzyme molecule at the active site (Kuhn *et al.*, 1976). A similar inhibition of peptidase and esterase activity of carboxypeptidase Y can be brought about by benzyloxycarbonyl-L-phenylalanine chloromethyl ketone or tosyl-L-phenylalanine chloromethyl ketone. Quantitative studies with benzyloxycarbonyl-L-phenylalanine chloromethyl ketone uncovered one histidine residue that is necessary for activity (Hayashi *et al.*, 1975; Kuhn *et al.*, 1976). Inhibitory action of iodoacetamide and phenylglyoxal was restricted to peptidase activity of the enzyme, leaving the esterase activity unaffected (Kuhn *et al.*, 1976). Thus, the modified

single methionine residue and arginine residue are obviously located in the region of the active site, which is involved in peptide binding but not in binding of the ester substrates (Kuhn *et al.*, 1976). *Para*-Hydroxymercuribenzoate blocks a single cysteine residue, and concomitant inhibition of enzyme activity depends on the geometry of the tested substrate. This indicates that the modified cysteine is not functionally essential but is located at or near the substrate-binding site (Bai and Hayashi, 1975). Inhibition of carboxypeptidase Y can furthermore be brought about by its intracellularly occurring inhibitor protein I^C (Matern *et al.*, 1974b; see Section II.A, 2, p. 274).

In a *Sacch. cerevisiae* mutant lacking carboxypeptidase Y (Wolf and Fink, 1975), Wolf and Weiser (1977) found a second enzyme with carboxypeptidase activity, which they called carboxypeptidase S. In contrast to carboxypeptidase Y, the enzyme is metal-ion dependent and strongly inhibited by chelating agents like EDTA or *o*-phenanthroline (Wolf and Weiser, 1977; P. Bünning and D. H. Wolf, unpublished observations). Reactivation of enzyme activity is brought about by Co^{2+}, Mn^{2+} and most effectively by Zn^{2+} (P. Bünning and D. H. Wolf, unpublished observations).

Several aminopeptidases have been described in *Sacch. cerevisiae*. An enzyme called aminopeptidase I was purified and characterized by Masuda *et al.* (1975). A molecular weight of about 200,000 was estimated. The enzyme cleaves a variety of dipeptides and also amino-acid amides between pH 7.5–8.0. The enzyme is able to hydrolyse glucagon releasing only amino acids which are expected from the amino-terminal sequence. Aminopeptidase I is strongly inhibited by the chelating agents EDTA and *o*-phenanthroline indicating that its activity is dependent on divalent cations. In reactivation experiments, Zn^{2+} proved to be the most effective. Aminopeptidase I is claimed to be probably identical with an enzyme described by Frey and Röhm (Frey and Röhm, 1978) which they called aminopeptidase II. It cleaves a variety of *N*-terminal amino acids and cleaves *p*-nitroanilides (Frey and Röhm, 1978) as does aminopeptidase I from Masuda *et al.* (1975). The published molecular weight of 85,000 (Frey and Röhm, 1978) is different, however, from the molecular weight published for aminopeptidase I of Masuda *et al.* (1975).

Masuda *et al.* (1975) described also a second aminopeptidase, called aminopeptidase II. The enzyme has a molecular weight of around 34,000. The enzyme cleaves the same dipeptides tested as does

aminopeptidase I but, in addition, isoleucyl-L-phenylalanine, a peptide inhibitory to aminopeptidase I activity. In contrast to aminopeptidase I, aminopeptidase II does not cleave leucine *p*-nitroanilide and leucine amide. Ethylenediaminetetraacetic acid and *o*-phenanthroline inhibit the enzyme activity indicating its dependence on divalent cations (Masuda *et al.*, 1975). The aminopeptidase II of Masuda and coworkers is probably identical with aminopeptidase III described by Frey and Röhm, 1978. Matile *et al.* (1971) separated four aminopeptidase isozymes on starch-gel electrophoresis. Three of them are non-lysosomal but, as no further studies have been carried out, they cannot be correlated with the partially characterized aminopeptidases I, II and III. The fourth enzyme, however, was found to be an enzyme of high molecular weight, located in the vacuole and thus named "lysosomal aminopeptidase" (Matile *et al.*, 1971). Most likely it is this enzyme which was isolated from vacuoles by Frey and Röhm (1977). It has a molecular weight of 640,000, and is composed of 12 subunits (K. H. Röhm, personal communication). Enzyme activity can be inhibited by metal-chelating agents, and specifically depends on Zn^{2+}. Activation of the enzyme is brought about by Cl^{-}. The enzyme cleaves a broad variety of L-amino acid and peptide derivatives containing an amino acid with a free amino group, except *N*-terminal lysine (Frey and Röhm, 1978). A dipeptidase cleaving only dipeptides composed of L-amino acids has been found (Frey and Röhm, 1978). Glycyl-L-leucine, L-leucyl-L-leucine and L-leucine-L-valine were shown to be among the best substrates (Frey and Röhm, 1978).

2. *Proteinase Inhibitors*

In freshly prepared crude extracts of *Sacch. cerevisiae*, activities of proteinases A, B and carboxypeptidase Y are undetectable and their existence as inactive zymogens had been suspected (Hayashi *et al.*, 1967; Lenney and Dalbec, 1967). Further studies, however, uncovered the presence of inhibitory proteins rendering these proteinase activities inactive (Lenney and Dalbec, 1969; Lenney, 1973). Subsequently, three classes of proteins specifically inhibiting proteinase A, proteinase B and carboxypeptidase Y have been isolated and characterized (Betz *et al.*, 1974; Matern *et al.*, 1974b; Saheki *et al.*, 1974; Ulane and Cabib, 1974; Lenney, 1975; Nuñez de Castro and Holzer, 1976).

Saheki *et al.* (1974) isolated two proteinase A-inhibiting proteins, $I^{A}2$ and $I^{A}3$, from baker's yeast. The two proteins exhibit different

isoelectric points, 5.7 for I^A2 and 6.3 for I^A3, but show the same molecular weight on SDS-gel electrophoresis (Saheki *et al.*, 1974; Bünning *et al.*, 1977). Experiments using a haploid strain of *Sacch. cerevisiae* suggest that only I^A3 is present in this organism, whereas I^A2 occurs in *Sacch. carlsbergensis* (Bünning *et al.*, 1977). Inhibition by I^A3 of proteinase A was concluded to be of the "non-stoicheiometric-pseudo irreversible" type with an apparent K_i value of 5.5×10^{-8} *M* (Nuñez de Castro and Holzer, 1976). Protein I^A3 consists of 68 amino acids per molecule and contains a large number of polar amino acids. The amino acids proline, arginine, cysteine and tryptophan are absent (Nuñez de Castro and Holzer, 1976). The molecular weight of I^A3 was calculated to be 7676 (Nuñez de Castro and Holzer, 1976). The inhibitor protein is stable to heat and trichloroacetic acid (Saheki *et al.*, 1974). Of the known proteinases A, B and carboxypeptidase Y in *Sacch. cerevisiae*, it selectively blocks only proteinase A activity (Saheki *et al.*, 1974; Nuñez de Castro and Holzer, 1976).

Proteinase B-inhibiting activity was purified by Betz *et al.* (1974) and Ulane and Cabib (1974). While Ulane and Cabib (1974) found only one single proteinase B inhibitor protein in a haploid strain of *Sacch. cerevisiae*, Betz *et al.* (1974) starting from baker's yeast were able to purify two inhibitory proteins, I^B1 and I^B2. The discrepancy in these findings was resolved by Bünning and Holzer (1977) who showed that I^B1 occurs exclusively in *Sacch. carlsbergensis*, whereas I^B2 is the only proteinase B-inhibiting protein existing in *Sacch. cerevisiae*, and baker's yeast contains both inhibitors. Protein I^B2 only inhibits proteinase B; proteinase A and carboxypeptidase Y activity are not affected by its presence (Betz *et al.*, 1974). The enzyme-inhibitor association constant was calculated as to be 3.9×10^9 M^{-1} (Bünning and Holzer, 1977). Inhibition was found to be of the non-competitive type and was maximum between pH 6 and 9 (Betz *et al.*, 1974). A molecular weight of around 8500 for I^B2 was calculated from amino-acid analysis and SDS-gel electrophoresis (Ulane and Cabib, 1974; Maier, 1977). The inhibitor protein is stable to heat and acid (Betz *et al.*, 1974; Ulane and Cabib, 1974) and consists of 74 amino acids, an unusually high proportion of them being polar (Maier, 1977). The protein contains only one tyrosine and no arginine, tryptophan, methionine or cysteine residue (Ulane and Cabib, 1974; Maier, 1977). Amino-acid sequencing of the proteinase B inhibitor was performed by Maier (1977) (Fig. 2) uncovering threonine as an *N*-terminal amino acid and a sequence of

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
H_2N-THR	LYS	ASN	PHE	ILE	VAL	THR	LEU	LYS	LYS	ASN	THR	PRO	ASP	VAL	GLU	ALA	LYS	LYS	PHE
21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
LEU	ASP	SER	VAL	HIS	HIS	ALA	GLY	GLY	SER	ILE	LEU	HIS	GLU	PHE	ASP	ILE	ILE	LYS	GLY
41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
TYR	THR	ILE	LYS	VAL	PRO	ASP	VAL	LEU	HIS	LEU	ASN	LYS	LEU	LYS	GLU	LYS	HIS	ASN	ASP
61	62	63	64	65	66	67	68	69	70	71	72	73	74						
VAL	ILE	GLU	ASN	VAL	GLU	GLU	ASP	LYS	GLU	VAL	HIS	THR	ASN-OH						

FIG. 2. Primary structure of proteinase B inhibitor of *Saccharomyces cerevisiae* (I^B2). Taken from Maier (1977).

-Val-His-Thr-Asn-OH at the C-terminus of the protein. Proteinase B inhibition highly depends on the intact C-terminal sequence. Loss of the second amino acid, threonine, leads to a drastic decrease of inhibitory activity (Maier, 1977).

In contrast to the above-described inhibitors of proteinases A and B, the carboxypeptidase Y inhibitor (I^C), which was purified by Matern *et al.* (1974b), is not a heat-stable and acid-stable protein. It was shown to be one polypeptide chain with a molecular weight of about 25,000 consisting of 217 amino acids (Matern *et al.*, 1974b; Barth, 1977). An isoelectric point of 6.6 was determined (Matern, 1975). The inhibitor protein is specific for carboxypeptidase Y. Neither proteinase A nor B nor carboxypeptidase S is inhibited by I^C (Matern *et al.*, 1974b; D. H. Wolf, unpublished observations). Maximum inhibition of carboxypeptidase Y is brought about between pH 5 and 9, and was shown to be competitive, exhibiting a K_i value of 2.5×10^{-9} *M* (Matern, 1975).

3. *Cellular Localization of Proteinases and Inhibitors*

Studies of Matile and Wiemken (1967), Lenney *et al.* (1974) and Matern *et al.* (1974a) show proteinases A and B and carboxypeptidase Y

to be localized in the vacuole of the yeast cell, an organelle probably functionally related to the lysosome of the animal cell (Matile and Wiemken, 1967). Also the "lysosomal aminopeptidase" was found in the vacuole (Matile *et al.*, 1971). Preliminary experiments point also to a non-cytosolar localization for carboxypeptidase S, as its activity was found to be associated with a membranous fraction after centrifugation of metabolically lysed cells (P. Bünning and D. H. Wolf, unpublished observations). The localization of aminopeptidases I and II of Masuda *et al.* (1975) has not been reported as yet. Aminopeptidase II of Frey and Röhm (1978), which may be identical with aminopeptidase I of Masuda *et al.* (1975), has been reported to be partially localized outside the plasma membrane (Frey and Röhm, 1978). However, more data are necessary to elucidate the relationship of aminopeptidases I and II described by Masuda *et al.* (1975) and aminopeptidases II and III of Frey and Röhm (1978) and their localization. The dipeptidase has been found outside the vacuole (Frey and Röhm, 1978).

Two questions are not resolved as yet, namely: are the vacuolar-engulfed proteinases located together in one population of vacuoles or do there exist different populations of vacuoles bearing one proteinase activity or a certain set of proteinases? Secondly, are the vacuole localized proteinases always trapped in their compartment or do there exist *in vivo* conditions under which molecules of such trapped proteinases occur also in the cytoplasm? A cytosolar localization was found for the inhibitors of proteinases A and B and carboxypeptidase Y (Hasilik *et al.*, 1974; Lenney *et al.*, 1974; Matern *et al.*, 1974a).

B. *SACCHAROMYCES CARLSBERGENSIS*

1. *Proteinases*

The proteolytic system of *Sacch. carlsbergensis* is less well understood compared with that in *Sacch. cerevisiae*. Several apparent endopeptidases have been described by Maddox and Hough (1970). From the few data available, they do not show a high degree of similarity to the known endoproteinases from *Sacch. cerevisiae*.

Several peptidases have been purified from brewer's yeasts, the great majority of which consist of *Sacch. carlsbergensis* strains (Rainbow, 1970). Two peptidases—called α and β—have been characterized by Felix and Brouillet (1966). Peptidase α is sensitive to metal ion-chelating agents

like EDTA and *o*-phenanthroline. The enzyme is furthermore inhibited by *p*-chloromercuribenzoate and iodoacetate. Peptidase α has the properties of a carboxypeptidase as it cleaves a variety of *N*-carbobenzoxy dipeptides carrying a free carboxyl group.

Peptidase β also hydrolyses *N*-carbobenzoxy-dipeptides. In contrast to peptidase α, enzyme activity is sensitive against diisopropylfluorophosphate. The enzyme cleaves *N*-carbobenzoxy-di- or tripeptides. The carboxyl group of the C-terminal amino acid may be substituted by an amido group. The enzyme is assumed to be an endopeptidase (Felix and Brouillet, 1966).

A dipeptidase and two aminopeptidases were identified in brewer's yeast (Röhm, 1974; Metz and Röhm, 1976). The dipeptidase has a molecular weight of 130,000 and an isoelectric point of 6.3. Activity is strongly inhibited by metal-chelating agents like EDTA. The enzyme contains firmly bound Zn^{2+}. Only dipeptides containing uncharged side chains and free α-amino and carboxyl groups are hydrolysed by the dipeptidase (Röhm, 1974).

Aminopeptidase I is a glycoprotein of 640,000 molecular weight, exhibiting an isoelectric point of 4.7. The enzyme is composed of 12 identical subunits of 53,000 molecular weight (Metz and Röhm, 1976; Marx *et al.*, 1977a, b; Metz *et al.*, 1977). All types of L-amino-acid and peptide derivatives containing a free amino terminus are cleaved. Compounds containing leucine or another hydrophobic amino-acid residue at the amino terminus are hydrolysed best. The enzyme is strongly activated by Zn^{2+} and Cl^- and inactivated by metal-chelating agents (Metz and Röhm, 1976).

Little is known about the second aminopeptidase, except that it has a considerable lower molecular weight than aminopeptidase I and differs in substrate specificity from this enzyme (Metz and Röhm, 1976).

2. *Proteinase Inhibitors*

A heat- and acid-stable protein inhibiting proteinase A from *Sacch. cerevisiae* has been found in *Sacch. carlsbergensis* (Bünning *et al.*, 1977). This proteinase A-inhibitor (I^A2) has a similar molecular weight of 7700 compared with the *Sacch. cerevisiae* protein. The isoelectric point of 5.7 differs by about 0.6 pH units from the inhibitor from *Sacch. cerevisiae*.

Two heat- and acid-stable proteins inhibiting proteinase B from *Sacch. cerevisiae* have been isolated from *Sacch. carlsbergensis*. Protein I^B1 has the same molecular weight of 8500 (Bünning and Holzer, 1977) as

the *Sacch. cerevisiae* protein (Maier, 1977). A molecular weight of 14,000 was found for the second inhibitor protein, called I^B3 (Matern *et al.*, 1978; H. Matern, unpublished observations). Isoelectric points are 8.0 for I^B1 (Betz *et al.*, 1974) and 4.6 for I^B3 (Matern *et al.*, 1978). Proteinases in *Sacch. carlsbergensis*, which are specifically inhibited by I^A2, I^B1 and I^B3, have not been characterized as yet.

C. *NEUROSPORA CRASSA*

1. *Proteinases*

Five intracellular proteolytic enzymes have been isolated and partially characterized in *N. crassa*. These are an acidic and alkaline endopeptidase, one carboxypeptidase and two aminopeptidases (Siepen *et al.*, 1975). The acidic proteinase cleaves acid-denatured haemoglobin and casein with pH optima for activity at around 3.0 and 2.5, respectively. Also the B-chain of insulin serves as a substrate. The acidic proteinase is not inhibited by agents attacking serine residues or binding to sulphydryl groups, nor is the enzyme inhibited by metal ion-complexing agents. Ultracentrifuge sedimentation analysis revealed a molecular weight of 31,000 for the enzyme protein (Siepen *et al.*, 1975; Siepen and Kula, 1976).

The alkaline proteinase has a molecular weight of 24,000 and consists of a single polypeptide chain. Action of the enzyme on casein showed two distinct pH optima, at pH 6.2 and 9.1. The enzyme cleaves the B-chain of insulin into a number of small peptide fragments. The alkaline proteinase also exhibits esterolytic activity on *N*-acetyl-L-tyrosine ethyl ester and *N*-benzoyl-L-arginine ethyl ester. Caseinolytic activity is completely inhibited by diisopropylfluorophosphate, phenylmethylsulphonylfluoride and *p*-chloromercuribenzoate, indicating that at least one serine residue and a sulphydryl group are necessary for activity (Siepen and Kula, 1976; Siepen *et al.*, 1975).

The carboxypeptidase was found to be a protein composed of a single polypeptide chain of 60,000 molecular weight. The enzyme liberates free amino acids from casein with a pH optimum of 6.2, and a few amino acids from the C-terminal end of the B-chain of insulin. The enzyme hydrolyses *N*-carbobenzoxy-L-alanyl-DL-phenylalanine and exhibits esterolytic activity against *N*-acetyl-L-tyrosine ethyl ester but not against *N*-benzoyl-L-arginine ethyl ester. Also, *N*-benzoyl-L-tyrosine-*p*-nitroanilide is cleaved. Carboxypeptidase activity is strongly inhibited by serine-proteinase inhibitors diisopropylfluorophosphate

and phenylmethylsulphonylfluoride. Inhibition is also brought about by the sulphydryl-blocking reagent *p*-chloromercuribenzoate (Siepen *et al.*, 1975).

Aminopeptidase A1 has a molecular weight of 99,000. It does not hydrolyse casein, nor the B-chain of insulin. The enzyme cleaves a variety of peptides, and attacks several amino-acid *p*-nitroanilides exhibiting a pH optimum between 7.25 and 7.5. Inactivation of the enzyme is brought about by *p*-chloromercuribenzoate and EDTA indicating that the sulphydryl residue(s) and divalent metal ions are necessary for activity (Siepen and Kula, 1976; Siepen *et al.*, 1975).

Aminopeptidase A2 is composed of one single polypeptide chain with a molecular weight of 97,000. The enzyme shows a broad substrate specificity and releases the first *N*-terminal amino acids of the B-chain of insulin. The enzyme cleaves lysine-*p*-nitroanilide and leucine-*p*-nitroanilide, exhibiting a pH optimum of 8.6 on the latter compound. Inhibition of activity is brought about by *p*-chloromercuribenzoate and EDTA, pointing to sulphydryl residue(s) and metal ions being necessary for activity (Siepen *et al.*, 1975; Siepen and Kula, 1976).

2. *Proteinase Inhibitors*

Four inhibitory proteins have been found in *N. crassa* and have been analysed (Matile, 1965; Yu *et al.*, 1974, 1976). Two inhibitors, B and A2, have been found to be specific for the alkaline proteinase (Yu *et al.*, 1974, 1976). Molecular weights were estimated to be 24,000 for inhibitor B (Yu *et al.*, 1976) and 10,000 for inhibitor A2 (Yu *et al.*, 1974). Whereas inhibitor A1, a protein of molecular weight 10,000, had first been suspected to inhibit the acidic proteinase (Yu *et al.*, 1976), its specific action on both aminopeptidases A1 and A2 was uncovered in recent experiments (Kaehn and Kula, 1977). Inhibitor A3, a protein of 5000 molecular weight and formerly suspected to inhibit alkaline proteinase (Yu *et al.*, 1976), was later found to be a specific inhibitor of both aminopeptidases A1 and A2. The question is open as to whether inhibitor A3 is a cleavage product of A1 (Kaehn and Kula, 1977).

3. *Localization of Proteinases*

Studies of Heiniger and Matile (1974) indicate the location of intracellular endoproteinases to be vesicles, which probably represent precursor structures of vacuoles.

D. *PODOSPORA ANSERINA*

Five proteinases, called A, B, C, III and IV, have been found in *P. anserina* (Begueret and Bernet, 1973). Proteinases A and B were found to be serine proteinases with molecular weights of 22,000–25,000 and 28,000–30,000, respectively. Both proteinases hydrolyse casein at pH 6.7 and are inhibited by ovomucoid, phenylmethylsulphonylfluoride and *p*-chloromercuribenzoate. The two enzymes differ in the size of peptides they produce during casein hydrolysis. Whereas proteinase A produces peptides of molecular weight greater than 5000, proteinase B gives rise to smaller peptides and to amino acids. Proteinase C is an acid endopeptidase with a molecular weight of about 51,000–54,000. The enzyme hydrolyses casein with a pH optimum at 4.2. Proteinase III is only found when protoplasmic incompatibility is effective. The enzyme exhibits a molecular weight of 38,000–41,000, hydrolyses casein between pH 3 and 9, and is partially inhibited by ovomucoid, phenylmethylsulphonylfluoride and *p*-chloromercuribenzoate. Also, proteinase IV is only found during the incompatibility reaction. A molecular weight similar to that of proteinase C (51,000–54,000) was determined. In contrast to proteinase C, however, its activity against casein is partly inhibited by ovomucoid, *p*-chloromercuribenzoate and phenylmethylsulphonylfluoride. It is not clear whether there exists any relationship between the two enzymes (Begueret and Bernet, 1973).

E. *PHYCOMYCES BLAKESLEEANUS*

Three serine proteinases and one acid proteinase from *P. blakesleeanus* have been isolated and characterized (Fischer and Thomson, 1979). The acidic proteinase—by analogy to the *Sacch. cerevisiae* enzyme called proteinase A—was found to be a protein of 24,000 molecular weight. Proteinase A cleaves acid-denatured haemoglobin at pH 3. Its activity is strongly inhibited by pepstatin.

By analogy with proteinase B in *Sacch. cerevisiae*, the three serine proteinases were called B-type proteinases. According to their separation behaviour on ion-exchange columns, the three activities were named B-CM, B-DI and B-DII. All three B-type proteinases are able to cleave Azocoll. Optimum pH values for proteinases B-CM and proteinase B-DI were found to be 7.5; that for proteinase B-DII is 7.0. Of the three B-type proteinases, only B-DII is able to cleave the elastase

substrate *N*-succinyl-L-alanyl-*p*-nitroanilide. Isoelectric points were estimated to be 7.6 for proteinase B-CM, 5.1 for proteinase B-DI and 4.4 for proteinase B-DII. Molecular weights of 18,000–20,000 for proteinase B-CM, 22,000–23,500 for proteinase B-DI and 60,000 for proteinase B-DII were estimated by gel-filtration and sodium dodecylsulphate gel electrophoresis.

Activity of all three B-type proteinases is strongly inhibited by diisopropylfluorophosphate and phenylmethylsulphonylfluoride as well as by *p*-hydroxymercuribenzoate. This indicates that a serine residue and sulphydryl residue are critical for activity. Part of the proteinase B activity was membrane bound (Fischer and Thomson, 1979).

Two heat- and acid-stable inhibitory proteins specifically inhibiting the B-type proteinases have been found in *P. blakesleeanus* (Fischer and Thomson, 1979). They show a similar molecular weight of 9000–10,000, but differ however in charge. Inhibitor I exhibits an isoelectric point of 4.5 whereas a value of 4.95 was found for inhibitor II. The inhibitors were found in the soluble fraction of cell homogenates. Gel-filtration experiments indicate a 1 : 1 complex formation of proteinases and inhibitors (Fischer and Thomson, 1979).

F. *BLASTOCLADIELLA EMERSONII*

Two proteinases cleaving casein have been found in the water mould *B. emersonii* (Lodi and Sonneborn, 1974; Corrêa *et al.*, 1979). The alkaline proteinase was shown to exhibit a pH optimum of 7–10. Activity is strongly inhibited by antipain, phenylmethylsulphonylfluoride and a proteinase-inhibitor fraction from potatoes. The molecular weight was estimated to be 25,000–30,000.

The second caseinolytic enzyme has a pH optimum at around 5.5. Activity was shown to be partly inhibited by phenylmethylsulphonylfluoride and the proteinase-inhibitor fraction of potatoes. In contrast to the alkaline proteinase, activity of the second enzyme is insensitive to antipain, but can be inhibited by concanavalin A. Molecular weight of the enzyme protein was estimated to be around 55,000–60,000.

In addition to the proteinases already described, aminopeptidase activity against L-alanyl-*β*-naphthylamide has been detected in *B. emersonii*. Acrylamide-gel electrophoresis led to the separation of four activities (Corrêa *et al.*, 1979).

G. *PHYSARUM POLYCEPHALUM*

Several intracellular aminopeptidase activities have been found in the true slime mould *P. polycephalum* and partly characterized (Hoffmann and Hüttermann, 1975). Three clearly discernible aminopeptidases, designated 1, 2 and 3 according to their isoelectric-focusing banding pattern, were detected in growing plasmodia. Additionally, four aminopeptidase isoenzymes: 6, 7, 8 and 9 according to their isoelectric-focusing banding pattern, were found in starving plasmodia. All seven aminopeptidases exhibit considerable activity against L-leucine-4-nitroanilide, L-phenylalanine-4-nitroanilide, L-proline-4-nitroanilide and L-tyrosine-4-nitroanilide. Only aminopeptidase 1 shows some activity against L-cystine-bis(4-nitroanilide), glycine-4-nitroanilide and L-lysine-4-nitroanilide. Whereas aminopeptidase 1 exhibits very high activity against L-alanine-4-nitroanilide, activity of aminopeptidases 2, 3, 6, 7, 8 and 9 against this substrate is considerably lower. No overall activation of the aminopeptidases by divalent cations was observed, nor did EDTA influence enzymic activity. Nine intracellular acidic proteinase activities were found in growing plasmodia (Haars *et al.*, 1978). No further characterization has been reported yet.

H. *DICTYOSTELIUM DISCOIDEUM*

Intracellular acid proteinase activity in the slime mould *D. discoideum* had been reported by Sussman and Sussman (1969) and Wiener and Ashworth (1970). After gentle breakage of growing amoeboid cells (myxamoebae), about 70% of this activity was found in a sedimentable fraction possibly the lysosomal fraction (Wiener and Ashworth, 1970).

Fong and Rutherford (1978) identified two enzymes responsible for acid proteinase activity. Because of their similarity with cathepsins of higher organisms, they were called cathepsin B and cathepsin D. Cathepsin B activity is strongly inhibited by iodoacetate, iodoacetamide,. tosyl-lysyl-chloromethyl ketone, chloroquine and leupeptin. Cathepsin D activity is highly sensitive to inhibition by diazoacetylnorleucine methyl ester. At least a third acid proteinase has been suspected using haemoglobin-acrylamide gels incubated at pH 2.75 (Fong and Rutherford, 1978). Using electrophoresis in polyacrylamide gel containing denatured haemoglobin, North and Harwood (1979) separated

at least eight acid proteinase activities (named A, B, C, D, E, E′, G and H after their banding pattern) in extracts of axenically and bacterially growing myxamoebae. During fruiting body formation, an additional acid proteinase—called F—was detected (North and Harwood, 1979). The authors suggest their proteinase E activity to be identical with cathepsin D activity of Fong and Rutherford (1978), and proteinase C activity to be identical with cathepsin B activity of Fong and Rutherford (1978). Using dipeptides as substrates, three peptidase activities were discovered (Fong and Rutherford, 1978).

J. *TETRAHYMENA PYRIFORMIS*

An intracellular proteinase hydrolysing haemoglobin at pH 5.5 has been found in this ciliated protozoan and purified (Dickie and Liener, 1962a). The molecular weight of the enzyme was estimated to be 29,300. Enzyme activity is strongly activated by cysteine and EDTA (Dickie and Liener, 1962a). The enzyme hydrolyses *N*-benzoyl-L-arginine ethyl ester. Oxidized insulin chains A and B are cleaved at the following bonds: Chain A: -val_{10}-$cySO_3H_{11}$-, -$gluNH_2$ $_{15}$-leu_{16}-, -$aspNH_2$ $_{18}$-tyr_{19}-. The B chain was cleaved at -tyr_{16}-leu_{17}-, -tyr_{26}-thr_{27}- and -thr_{27}-pro_{28}- (Dickie and Liener, 1962b).

Levy *et al.* (1976) described intracellular proteinase activity cleaving haemoglobin, azocasein and α-*N*-benzoyl-D,L-arginine-2-naphthylamide, which copurify when gel filtration and affinity chromatography are used. However, α-*N*-benzoyl-D,L-arginine-2-naphthylamide-cleaving activity could be separated from the other two activities by DEAE-Sephadex chromatography. When azocasein is used as substrate, activity is substantially inhibited by leupeptin and chymostatin. Iodoacetamide, tosyl-L-lysylchloromethyl ketone, and tosyl-L-phenylalanylchloromethyl ketone were found to inhibit to a lesser extent. Mercuric ions completely block enzyme activity against azocasein. Thiol compounds and EDTA are required for optimal enzyme action. This fact, together with the roughly estimated molecular weight of 25,000, may indicate that at least part of the proteinase activity described by Levy *et al.* (1976) corresponds to the enzyme purified by Dickie and Liener (1962a, b).

K. *CHLAMYDOMONAS REINHARDTII*

An endoproteinase cleaving the presumptive precursor protein of the small subunit of ribulose 1,5-biphosphate carboxylase was found

in a post-ribosomal supernatant of this alga (Dobberstein *et al.*, 1977). The enzyme activity is insensitive against agents known to inhibit serine proteinases and metalloproteinases. The enzyme is completely inhibited by iodoacetamide and *N*-ethylmaleimide, and is therefore probably a sulphydryl proteinase.

Table 1 summarizes the intracellular proteinases of the organisms described above which are known at present. The intracellularly occurring proteinase inhibitors are summarized in Table 2.

III. Control of Proteolysis

One obvious tool to control intracellular proteolysis is the change of proteinase activity levels. In fact, as yet many cellular functions attributed to involvement of proteinases were, in part, based on the change in activity of a certain proteinase before or during the biological event in question. This point will be discussed in more detail in Section IV (p. 295). However, proteolytic events with high specificity absolutely necessary for sensitive control of intracellular metabolism require more than only activity changes of the proteinases.

There is the possibility of a proteinase detecting and binding a certain amino-acid sequence in proteins, which is limited to only a few species, all devoted to undergo a similar metabolic process. Proteolysis of the signal peptide of membrane-traversing proteins (Jackson and Blobel, 1977) might serve as such an example.

Alterations in the accessibility of a certain protein to proteinase digestion is another emerging possibility. Different tertiary structures of a protein may have very different affinities to proteolytic attack. Proof for the validity of such a model for *in vivo* control comes from the significantly enhanced susceptibility of proteins containing amino-acid analogues or puromycin and of mutant proteins to proteolytic hydrolysis as studied in bacteria (Goldberg and St. John, 1976). A change in the tertiary structure of functional proteins might be induced by newly appearing or disappearing metabolites, or removal of a coenzyme bound to the protein in its active state (Grisolia, 1964; Schimke and Doyle, 1970). Tryptophan synthase from *Sacch. cerevisiae* might serve as an example. *In vitro* studies show a two- to three-fold accelerated inactivation of the enzyme after removal of the coenzyme pyridoxal 5'phosphate by proteinases A and B (Manney, 1968a; Schött and Holzer, 1974). Additional possibilities for control of

TABLE 1. Intracellular proteinases from eukaryotic microorganisms

Organism	Enzyme	Characteristics	Molecular weight	Inhibitory agents	References
Saccharomyces cerevisiae	Proteinase A	Acid endopeptidase	60,000	Pepstatin	Lenney and Dalbec (1967), Hata *et al.* (1967a), Hata *et al.* (1967b), Doi *et al.* (1967)
Saccharomyces cerevisiae	Proteinase B	Serine endopeptidase	32,000–44,000	iPr_2PF, $PhCH_2SO_2F$, $ClHgBzO^-$, chymostatin, antipain	Lenney and Dalbec (1967), Hata *et al.* (1967a), Ulane and Cabib (1976), Bünning (1977)
Saccharomyces cerevisiae	Carboxypeptidase Y	Serine exopeptidase	61,000	iPr_2PF, $PhCH_2SO_2F$, $TosPheCH_2Cl$, $ClHgBzO^-$	Hata *et al.* (1967a), Doi *et al.* (1967), Hayashi *et al.* (1970), Hayashi *et al.* (1973), Kuhn *et al.* (1974), Kuhn *et al.* (1976)
Saccharomyces cerevisiae	Carboxypeptidase S	Exopeptidase (Zn^{2+})	not determined	EDTA	Wolf and Weiser (1977)
Saccharomyces cerevisiae	Aminopeptidase I (probably identical with aminopeptidase II described by Frey and Röhm, 1978)	Exopeptidase (Zn^{2+})	200,000	EDTA, $ClHgBzO^-$	Masuda *et al.* (1975)

Saccharomyces cerevisiae	Aminopeptidase II (probably identical with aminopeptidase III described by Frey and Röhm, 1978)	Exopeptidase (Mg^{2+})	approx. 34,000	not determined	Masuda *et al.* (1975)
Saccharomyces cerevisiae	Vacuolar aminopeptidase	Exopeptidase (Zn^{2+})	640,000	EDTA	Matile *et al.* (1971), Frey and Röhm (1977)
Saccharomyces cerevisiae	Dipeptidase	(Mg^{2+})	120,000	EDTA	Frey and Röhm (1978)
Saccharomyces carlsbergensis	Peptidase α	Exopeptidase (Zn^{2+}, Co^{2+})	not determined	EDTA, *o*-phenanthroline, $ClHgBzO^-$, Iodoacetate	Felix and Brouillet (1966)
Saccharomyces carlsbergensis	Peptidase β	Endopeptidase	not determined	iPr_2PF	Felix and Brouillet (1966)
Saccharomyces carlsbergensis	Aminopeptidase I	Exopeptidase (Zn^{2+}, Cl^-)	640,000	EDTA, *o*-phenanthroline	Metz and Röhm (1976), Marx *et al.* (1977a), Marx *et al.* (1977b), Metz *et al.* (1977)
Saccharomyces carlsbergensis	Aminopeptidase II	Exopeptidase	not determined	not determined	Metz and Röhm (1976)
Saccharomyces carlsbergensis	Dipeptidase	(Zn^{2+})	130,000	EDTA	Röhm (1974), Metz and Röhm (1976)
Neurospora crassa	Acidic proteinase	Acid endopeptidase	31,000	not found	Siepen *et al.* (1975), Siepen and Kula (1976)

TABLE 1 (continued)

Organism	Enzyme	Characteristics	Molecular weight	Inhibitory agents	References
Neurospora crassa	Alkaline proteinase	Serine endo-peptidase	24,000	iPr_2PF, $PhCH_2SO_2F$, $ClHgBzO^-$	Siepen *et al.* (1975), Siepen and Kula (1976)
Neurospora crassa	Carboxypeptidase	Exopeptidase	60,000	iPr_2PF, $PhCH_2SO_2F$, $ClHgBzO^-$	Siepen *et al.* (1975)
Neurospora crassa	Aminopeptidase A1	Exopeptidase	99,000	EDTA, $ClHgBzO^-$	Siepen *et al.* (1975), Siepen and Kula (1976)
Neurospora crassa	Aminopeptidase A2	Exopeptidase	97,000	EDTA, $ClHgBzO^-$	Siepen *et al.* (1975), Siepen and Kula (1976)
Podospora anserina	Proteinase A	Serine endo-peptidase	22,000–25,000	$PhCH_2SO_2F$, $ClHgBzO^-$, Ovomucoid	Begueret and Bernet (1973)
Podospora anserina	Proteinase B	Serine endo-peptidase	28,000–30,000	$PhCH_2SO_2F$, $ClHgBzO^-$, ovomucoid	Begueret and Bernet (1973)
Podospora anserina	Proteinase C	Acid endopeptidase	51,000–54,000	not found	Begueret and Bernet (1973)
Podospora anserina	Proteinase III	Acid endopeptidase	38,000–41,000	Partially inhibitory: $PhCH_2SO_2F$, $ClHgBzO^-$, ovomucoid	Begueret and Bernet (1973)
Podospora anserina	Proteinase IV	Acid endopeptidase	51,000–54,000	Partially inhibitory: $PhCH_2SO_2F$, $ClHgBzO^-$, ovomucoid	Begueret and Bernet (1973)

Phycomyces blakesleeanus	Proteinase A	Acid endopeptidase	24,000	Pepstatin	Fischer and Thomson (1979)
Phycomyces blakesleeanus	Proteinase B-CM	Serine endo-peptidase	18,000–20,000	iPr_2PF, $PhCH_2SO_2F$, $ClHgBzO^-$	Fischer and Thomson (1979)
Phycomyces blakesleeanus	Proteinase B-DI	Serine endo-peptidase	22,000–23,500	iPr_2PF, $PhCH_2SO_2F$, $ClHgBzO^-$	Fischer and Thomson (1979)
Phycomyces blakesleeanus	Proteinase B-DII	Serine endo-peptidase	60,000	iPr_2PF, $PhCH_2SO_2F$, $ClHgBzO^-$	Fischer and Thomson (1979)
Blastocladiella emersonii	Alkaline proteinase	Serine endo?-peptidase	25,000–30,000	Antipain, $PhCH_2SO_2F$	Lodi and Sonneborn (1974), Corrêa *et al.* (1978), Corrêa, *et al.* (1979)
Blastocladiella emersonii	"pH 5.5" Proteinase	Endopeptidase?	55,000–60,000	Concanavalin A	Lodi and Sonneborn (1974), Corrêa *et al.* (1978), Corrêa *et al.* (1979)
Blastocladiella emersonii	Four amino-peptidases		not yet characterized		Corrêa *et al.* (1979)
Physarum polycephalum	Nine acidic proteinases		not yet characterized		Haars *et al.* (1978)
Physarum polycephalum	Seven amino-peptidases	Exopeptidases	not determined	not found	Hoffmann and Hüttermann (1975)
Dictyostelium discoideum	Cathepsin B	Acid endopeptidase	not determined	Iodoacetate, iodoacetamide, $TosLysCH_2Cl$, leupeptin, chloroquine	Fong and Rutherford (1978)

TABLE 1 (continued)

Organism	Enzyme	Characteristics	Molecular weight	Inhibitory agents	References
Dictyostelium discoideum	Cathepsin D	Acid endopeptidase	not determined	N_2AcNle-*O*-Me	Fong and Rutherford (1978)
Dictyostelium discoideum	Seven additional acid proteinases		not yet characterized		North and Harwood (1979)
Dictyostelium discoideum	Three peptidases		not yet characterized		Fong and Rutherford (1978)
Tetrahymena pyriformis	"pH 5.5" Proteinase	Endopeptidase	29,300	Chymostatin, leupeptin, Hg^{2+}	Dickie and Liener (1962a), Levy *et al.* (1976)
Chlamydomonas reinhardtii	"Transport trigger" proteinase	Endopeptidase	not determined	Iodoacetamide, *N*-ethylmaleimide	Dobberstein *et al.* (1977)

[a] For a list of endogenous proteinase inhibitors see Table 2 (pp. 292–293).
[b] The following abbreviations are used:
EDTA, ethylene diaminetetraacetic acid;
N_2AcNle-*O*-Me, diazoacetyl norleucine methyl ester;
iPr_2PF, diisopropylfluorophosphate;
$PhCH_2SO_2F$, phenylmethylsulphonylfluoride;
TosPheCH_2Cl, *N*-tosyl-L-phenylalanine chloromethylketone;
TosLysCH_2Cl, *N*-tosyl-L-lysine chloromethylketone;
ClHgBzO$^-$, *p*-chloromercuribenzoate.

proteolysis must exist when proteinases and their substrates are located in different cellular compartments, as is for instance the case in *Sacch. cerevisiae*. At least four of the known proteinases are located in the vacuole of the cell (see Section II, p. 276). Control over degradation of cytosolar proteins might reside in a limited pinocytotic uptake of substrate into the vacuole, or in a selective release of proteolytic enzymes from the organelle into the cytosol. In general, compartmentalization of certain proteinases in subcellular structures results in high specificity of proteolytic processes, as substrate accessibility is limited to only a certain class of proteins. The cleavage of a signal peptide (Jackson and Blobel, 1977) or a "transport" peptide (Highfield and Ellis, 1978) from membrane-traversing proteins by proteinases located in the membrane is an example.

The presence of specific intracellular inhibitors against proteinases of the same organism, as were found in *Sacch. cerevisiae*, *Sacch. carlsbergensis*, *N. crassa* and *P. blakesleeanus* (see Section II, p. 269), might play a fundamental role in regulation of proteolytic events. Besides their hypothetical function as a safety guard against unwanted proteolytic cleavage upon leakage or breakdown of proteinase-bearing organelles, or during transport of newly synthesized proteinases from cytoplasmic polysomes to the organelles (Lenney *et al.*, 1974; Saheki and Holzer, 1974; Holzer and Saheki, 1976), they might be engaged in regulation of proteolysis by adjustment of a certain proteinase activity level. Furthermore, their regulatory power might be due to an enhancement of the "specificity" of a proteinase by lowering the apparent "sensitivity" of the proteinase against its protein substrates. More detailed studies concerning these mechanisms have been undertaken in *Sacch. cerevisiae*.

A several-fold parallel derepression of proteinase and inhibitor activities upon glucose exhaustion of *Sacch. cerevisiae* has been found by Saheki and Holzer (1975) and by Hansen *et al.* (1977). This finding might support the safety-guard theory.

In addition, studies using a conditional mutant with a defect in proteinase A-inhibitor synthesis showed that derepression of proteinase A can be dissociated from derepression of proteinase A-inhibitor (Beck *et al.*, 1976; Wolf *et al.*, 1979). This indicates that regulation of both proteins differs in at least one regulatory element. Thus, the cell has the tool necessary to regulate proteinase activity level by inhibitor level (Wolf *et al.*, 1979).

TABLE 2. Intracellular proteinase inhibitors from eukaryotic micro-organisms

Organism	Inhibitor	Specificity of inhibition	Molecular weight	Isoelectric point	Other characteristics	References
Saccharomyces cerevisiae	$I^{A}3$	Proteinase A	7,700	6.3	Heat and acid resistant	Saheki *et al.* (1974), Nuñez de Castro and Holzer (1976), Bünning *et al.* (1977)
Saccharomyces cerevisiae	$I^{B}2$	Proteinase B	8,500	7.0	Heat and acid resistant	Betz *et al.* (1974), Ulane and Cabib (1974), Bünning and Holzer (1977), Maier (1977)
Saccharomyces cerevisiae	I^{C}	Carboxypeptidase Y	25,000	6.6	Heat and acid labile	Matern *et al.* (1974), Matern (1975)
Saccharomyces carlsbergenesis	$I^{A}2$	Proteinase A from *Saccharomyces cerevisiae* — No proteinase from *Saccharomyces carlsbergensis* inhibited has been characterized as yet	7,700	5.7	Heat and acid resistant	Bünning *et al.* (1977)
Saccharomyces carlsbergensis	$I^{B}1$	Proteinase B from *Saccharomyces cerevisiae*	8,500	8.0	Heat and acid resistant	Bünning and Holzer (1977)
Saccharomyces carlsbergensis	$I^{B}3$	Proteinase B from *Saccharomyces cerevisiae*	14,000	4.6	Heat and acid resistant	Matern *et al.* (1978)

Neurospora crassa	A-1 (I1)	Aminopeptidase A1, A2	10,000	Not determined	Heat resistant	Yu *et al.* (1976), Kaehn and Kula (1977)
Neurospora crassa	A-2	Alkaline proteinase	10,000	Not determined	Heat and acid resistant	Yu *et al.* (1974), Yu *et al.* (1976)
Neurospora crassa	A-3 (I3)	Aminopeptidase A1, A2	5,000	Not determined	Heat resistant, cleavage product of A-1?	Yu *et al.* (1976), Kaehn and Kula (1977)
Neurospora crassa	B	Alkaline proteinase	24,000	Not determined	Heat resistant	Yu *et al.* (1974), Yu *et al.* (1976)
Phycomyces blakesleeanus	Inhibitor I	Proteinases B-CM, B-DI and B-DII	9,000–10,000	4.5	Heat and acid resistant	Fischer and Thomson (1979)
Phycomyces blakesleeanus	Inhibitor II	Proteinase B-CM, B-DI and B-DII	9,000–10,000	4.95	Heat and acid resistant	Fischer and Thomson (1979)

Studies on the carboxypeptidase Y-inhibitor-complex of *Sacch. cerevisiae* revealed a highly different degree of cleavage of a variety of peptide substrates by carboxypeptidase Y, despite the presence of the inhibitor protein (Barth *et al.*, 1978). The most likely explanation for this behaviour is a different capacity for the peptides to dissociate the inhibitor protein from the substrate-binding site of carboxypeptidase Y. This might lead to extension of the first-mentioned model of substrate structure as control element in proteolysis. Because of the rather broad substrate specificity of many proteinases, a variety of proteins would be cleaved erroneously. The inhibitor protein prevents this, allowing only cleavage of proteins, which by some metabolically triggered signal, expose amino-acid sequences and make them "good" substrates, able to dissociate the inhibitor protein from the proteinase. By lowering the apparent sensitivity of the proteinase against proteins in general, the inhibitor enhances proteinase specificity (Barth *et al.*, 1978). Thus, the substrate itself can control proteolysis at any time and at any locus of the cell where it appears. This model would also predict the reversibility of this process. After cleavage of the protein to be hydrolysed, the inhibitor protein could again bind and inhibit the proteinase.

An additional observation might extend the hypothetical view on regulation of proteinase activity by inhibitor proteins. It has been shown that proteinase activities in crude extracts of *Sacch. cerevisiae* can be activated in their inactive complexes with the respective inhibitor by proteolytic "cross hydrolysis" of inhibitor proteins (Lenney, 1973; Saheki *et al.*, 1974; Saheki and Holzer, 1975; Barth *et al.*, 1978). This autocatalytic cycle can be started by adjusting the pH value to around 5, which liberates small amounts of proteinase A from its inactive complex. This is followed by inactivation of proteinase B- and carboxypeptidase Y-inhibitor, leading to free proteinase B and carboxypeptidase Y. Proteinase B itself digests carboxypeptidase Y-inhibitor and proteinase A-inhibitor, leading to more free proteinase A activity (Saheki *et al.*, 1974; Holzer, 1975). Thus, metabolic changes like local alterations in pH value might, in addition, lead to proteinase-inhibitor dissociation *in vivo* and thus proteinase action at the site of metabolic shift. Studies on the purified carboxypeptidase Y-inhibitor complex have shown that proteolytic inactivation of the inhibitor protein is only possible after dissociation of the complex by a substrate (Barth *et al.*, 1978). Such conditions might well be present in a

crude extract, where inhibitor inactivation occurs. One may speculate that, *in vivo*, initial activation of one proteinase sensitive to a given metabolic signal is an additional regulatory device that leads to rapid and complete activation of another proteinase insensitive to the original signal, when its substrate to be hydrolysed is present.

IV. Metabolic Events and Proteinase Function

A. PROTEIN MATURATION

1. *Removal of Methionine from Nascent Polypeptide Chains*

Synthesis of cytoplasmic polypeptides in eukaryotes is initiated by methionine (Lucas-Lenard and Lipmann, 1971; Haselkorn and Rothman-Denes, 1973). The *N*-terminal methionine residue can be removed from the polypeptide chains. Analysis of iso-1-cytochrome *c* mutants of *Sacch. cerevisiae* supports the view that methionine is cleaved off by an aminopeptidase (Stewart *et al.*, 1971). The methionyl codon AUG was identified as the beginning of the message for iso-1-cytochrome *c*. Purified wild-type iso-1-cytochrome *c*, however, was shown to carry a threonine residue at the *N*-terminus and no microheterogeneity of the *N*-terminus could be detected (Stewart *et al.*, 1971). Sequence analysis of the iso-1-cytochrome *c* protein from revertants of chain-initiation mutants carrying new methionine codons at different positions revealed the following specificity for the proposed aminopeptidase. The enzyme cleaves methionine efficiently from threonyl and alanyl residues, less efficiently from valyl residues and not at all from leucyl, isoleucyl and arginyl residues (Stewart *et al.*, 1971). It is not known if one of the characterized aminopeptidases in *Sacch. cerevisiae* catalyses this reaction, or if the enzyme responsible for this maturation event has yet to be found.

2. *Removal of Prepeptide Sequences*

Recently, Blobel and Dobberstein (1975) proposed a model for transfer of proteins across membranes, and a variety of experiments have found this model most likely to hold true for secretory proteins in mammalian cell systems (Devillers-Thiery *et al.*, 1975; Dobberstein and Blobel, 1977; Jackson and Blobel, 1977; Lingappa *et al.*, 1977; Shields and Blobel, 1977). A unique sequence of codons, located immediately

to the right of the initiation codon, is present in those messenger RNAs whose translation products have to be transferred across a membrane. Translation of the signal codons on free ribosomes results in a signal peptide, which triggers attachment of the ribosome to the membrane, transferring the nascent chain through the membrane. A "signal peptidase" cleaves the signal peptide from the nascent polypeptide chain after transfer through the membrane (Jackson and Blobel, 1977). Signal peptides released from different secreted proteins were shown to consist of 16–30 amino-acid residues (Jackson and Blobel, 1977; Shields and Blobel, 1977).

This sequence of events was first also thought to occur during translocation of proteins synthesized in the cytoplasm of the cell but finally localized in an organelle (Blobel and Dobberstein, 1975). Ribulose 1,5-bisphosphate carboxylase, the key enzyme in photosynthesis, resides in the chloroplast stroma of green algae and higher plants (Kawashima and Wildman, 1970). The enzyme of several species has a molecular weight of about 550.000 and is composed of several copies of each of two non-identical subunits (Kawashima and Wildman, 1970; Iwanij *et al.*, 1974). The large subunit is synthesized in the chloroplast (Blair and Ellis, 1973), whereas the small subunit is synthesized on cytoplasmic ribosomes (Gray and Kekwick, 1974). Dobberstein *et al.* (1977) showed that translation of polyadenylated mRNA of *C. reinhardtii* in a cell-free wheat-germ system resulted in numerous polyeptides. One of them was a species of molecular weight 20,000 which was specifically precipitated by antibodies raised against the authentic 16,500 daltons small subunit of ribulose 1,5-bisphosphate carboxylase. Cleavage of the 3500 daltons larger precursor into two polypeptide chains, one exhibiting the size of the authentic small subunit and the other a small peptide, is brought about by a specific endoproteinase found in the postribosomal supernatant and also in association with polysomes of *Chlamydomonas* sp. (Dobberstein *et al.*, 1977). The endoproteinase requires sulphydryl groups for activity. In contrast to synthesis of secretory proteins in mammalian cells, where the signal peptide extension triggers binding of ribosomes to the microsomal membrane (Blobel and Dobberstein, 1975; Dobberstein and Blobel, 1977), synthesis of the precursor of the small subunit of ribulose 1,5-bisphosphate carboxylase was shown to proceed on free cytoplasmic ribosomes (Dobberstein *et al.*, 1977). This indicates that the peptide extension does not trigger ribosome attachment to the

chloroplast envelope (Dobberstein *et al.*, 1977). The presence of the processing proteinase in the supernatant is puzzling, and hard to understand in view of any vectorial transport of the polypeptide chain through the chloroplast membrane. The final model explaining transport of the small subunit of ribulose 1,5-bisphosphate carboxylase in *Chlamydomonas* sp. may be equivalent to the model elaborated for transport of the small subunit of ribulose 1,5-bisphosphate carboxylase in the pea plant. Highfield and Ellis (1978) found that the small subunit precursor is maximally processed in the presence of intact chloroplasts, suggesting that the processing enzyme is located in the chloroplast envelope. Those workers suggest that finding the processing proteinase in the soluble fraction of *Chlamydomonas* sp. (Dobberstein *et al.*, 1977) was an artefact due to the impossibility of isolating intact chloroplasts from this alga by the methods used.

As found in *Chlamydomonas* sp., there is in the pea plant no chloroplast envelope studded with ribosomes visible in the electron microscope; the small subunit precursor is synthesized on free ribosomes and can be processed after polypeptide completion in the absence of ribosomes (Highfield and Ellis, 1978). In the pea plant, this processing is highly stimulated by addition of intact chloroplasts. Furthermore, the precursor small subunit is resistant to hydrolysis by trypsin when incubated with intact chloroplasts, indicating its uptake into the organelle (Highfield and Ellis, 1978). From these results, Highfield and Ellis (1978) propose a model for uptake of the small subunit of ribulose 1,5-bisphosphate carboxylase into the chloroplast, which is different from the signal hypothesis for secretory proteins, and which they call "envelope carrier mechanism". A class of proteins exists on the chloroplast envelope which recognizes sites common to all of those proteins which are made on cytoplasmic ribosomes, but which are destined to function in the chloroplast. The recognition site of the entering protein must not be at the *N*-terminus. Cleavage of the peptide extension of about 50 amino acids by the proteinase located in the membrane triggers a conformational change in the remaining polypeptide chain leading to transport through the membrane.

One of the central problems of mitochondrial biogenesis, the question as to how cytoplasmically made proteins are transported into the mitochondria, has also not been answered as yet (Schatz and Mason, 1974). Recent experiments on the ATPase complex of *Sacch. cerevisiae* showed the involvement of a proteolytic processing step,

which might be one of the crucial events in the transport mechanism. Mitochondrial ATPase, the enzyme generating ATP from ADP and phosphate during respiration, consists of at least ten distinct polypeptides in *Sacch. cerevisiae* (Schatz and Mason, 1974). Five of these are associated with the cold-labile F_1-ATPase, one is the oligomycin sensitivity-conferring protein and four are hydrophobic polypeptides tightly associated with the mitochondrial inner membrane. The four hydrophobic proteins are synthesized within the mitochondria. In contrast, the F_1-ATPase and the oligomycin sensitivity-conferring protein are synthesized on cytoplasmic ribosomes (Schatz and Mason, 1974). Schatz and his coworkers (G. Schatz, personal communication) translated *Sacch. cerevisiae* mRNA *in vitro* and, by using specific antibodies against the authentic γ-subunit (32,000 molecular weight) of the cytoplasmically made F_1 portion of ATPase, they found precipitation of a precursor protein of the γ-subunit with a molecular weight about 6000 higher. Addition of yeast mitochondria led to the appearance of a processed γ-subunit which, in contrast to conditions when mitochondria are absent, is not accessible to chymotrypsin digestion. Protection against chymotryptic cleavage suggests that uptake of the processed precursor into the mitochondrion has occurred (G. Schatz, personal communication). Translocation of the cytoplasmically synthesized γ-subunit of F_1-ATPase into the mitochondrion might follow the same or a similar mechanism as found for passage of the small subunit of ribulose 1,5-bisphosphate carboxylase into the chloroplast, proteolysis playing a key role in the uptake step.

Carboxypeptidase Y from *Sacch. cerevisiae* has been shown to be localized in vacuoles (Matile and Wiemken, 1967; Lenney *et al.*, 1974; Matern *et al.*, 1974a). Using antisera against authentic carboxypeptidase Y, Hasilik and Tanner (1976, 1978) were able to precipitate material cross-reacting with the antibody but different from authentic carboxypeptidase Y. Sodium dodecylsulphate polyacrylamide-gel electrophoresis identified the cross-reacting material as a precursor protein of carboxypeptidase Y of about 6000 higher molecular weight compared with the authentic enzyme (Hasilik and Tanner, 1978). Electrophoretic studies eliminated the possibility that this precursor protein is the carboxypeptidase Y-inhibitor complex known to form in crude extracts. Trypsin and proteinase B from *Sacch. cerevisiae* are able to convert the precursor protein into a protein identical in size to carboxypeptidase Y (Hasilik and Tanner, 1978). Whether proteinase B

is responsible for this conversion *in vivo* is not clear as yet. Hasilik and Tanner (1978) believe the precursor is probably an inactive proenzyme. If this were true, then proteinase B is most likely not the activating proteinase, as mutants lacking proteinase B activity exhibit normal carboxypeptidase Y activity (Wolf *et al.*, 1979; Wolf and Ehmann, 1978b). However, the possibility that the precursor is a preform of the enzyme which is proteolytically cleaved upon transfer into the vacuole via some as yet unknown route is equally likely.

No precursors of the other vacuolar-trapped proteolytic enzymes of *Sacch. cerevisiae* have been found as yet. Studies of Beck *et al.* (1976) and Wolf *et al.* (1979) indicate that the cytoplasmically found inhibitor of proteinase A is not the cleaved part of a precursor molecule of the vacuolar-engulfed proteinase A. A mutant (*pai* 1) was isolated which appears to be conditionally defective in proteinase A inhibitor synthesis. In contrast, synthesis of proteinase A was shown to be unaffected. As synthesis of both molecules—inhibitor and proteinase—seem to be regulated differently, they are most likely not derived from a common single precursor polypeptide chain.

3. *Processing of Translation Products of Monocistronic Messenger RNA*

Cytochrome-*c* oxidase represents the terminal member of the mitochondrial electron-transport chain. The enzyme from *Sacch. cerevisiae* is composed of three hydrophobic subunits, which are synthesized on mitochondrial ribosomes, and four relatively hydrophilic polypeptide subunits which are synthesized outside the mitochondrion on cytoplasmic ribosomes (Schatz and Mason, 1974). Using antibodies specific against the holoenzyme or to either of two cytoplasmic subunits, Poyton and McKemmie (1979) were able to isolate a single polypeptide chain of molecular weight 55,000 from the cytoplasm. Peptide analysis identified the protein as a precursor of the four cytoplasmically synthesized subunits of cytochrome-*c* oxidase. The cumulative molecular weight of the four cytoplasmically synthesized subunits is 45,000, indicating that, in addition, a 10,000 molecular weight region exists, which is not converted into one of the subunits (Poyton and McKemmie (1979). Processing of the precursor protein into the individual subunits takes place after its incorporation into the inner mitochondrial membrane (Poyton and McKemmie, 1979). The processing proteinase has not been identified as yet.

The question as to whether proteolysis plays a functional role in maturation or regulation of gene products has still to be answered. From genetic data, the product of the *trp* 5 gene-region of *Sacch. cerevisiae*, tryptophan synthase, was proposed to be a dimer of a single bifunctional polypeptide chain catalysing conversion of indole-3-glycerol phosphate to indole and addition of indole and L-serine yielding tryptophan (Duntze and Manney, 1968). Purification of the enzyme and dissociation, however, yielded four monomers each of 35,000 molecular weight (Wolf and Hoffmann, 1974). From this finding, together with genetic data on strains habouring nonsense mutations near the middle of the *trp* 5 region yielding only a monofunctional active protein fragment of 35,000 molecular weight (Manney, 1968b), and taking the analogy to the bacterial enzyme (Yanofsky and Crawford, 1972), it might be tempting to conclude that tryptophan synthase is composed of two different monofunctional polypeptide chains. However, taking the analogy to the enzyme in a related organism, namely *N. crassa* (Matchett and DeMoss, 1975), would suggest a dimer of one bifunctional polypeptide chain for *Sacch. cerevisiae* tryptophan synthase. As *Sacch. cerevisiae* tryptophan synthase is known to be extremely sensitive to proteolytic attack (Katsunuma *et al.*, 1972; Wolf and Hoffmann, 1974), the two bifunctional chains may undergo cleavage into four monofunctional chains.

Until recently, genetic and biochemical data could not clearly answer the question as to whether the *his* 4 gene in *Sacch. cerevisiae* codes for three different polypeptide chains or one polypeptide chain with three discrete enzyme functions (Fink, 1971) responsible for catalysis of the third, second and tenth steps of histidine biosynthesis. Genetic data had indicated the *his* 4 gene coding for a monocistronic messenger, but purification of the gene product had led to detection of an enzyme complex of three different polypeptide chains (Fink, 1971). The idea was put forward that proteinase B might be responsible for maturation of a single polypeptide chain containing the three enzymes into three distinct active polypeptides which associate to a complex (Fink, 1971). Recently, however, Bigelis *et al.* (1977), using rapid purification techniques, were able to show that the *his* 4 region encodes a single 95,000 molecular weight protein containing the three enzyme activities.

The *arom* region of *N. crassa* has been shown to be organized in a tightly linked unit (Giles *et al.*, 1967). This gene region codes for five of the enzymes (dehydroquinate synthase, dehydroquinate, dehydro-

shikimate reductase, shikimate kinase and 5-enolpyruvylshikimate 3-phosphate synthase) which catalyse consecutive reactions in the central pathway leading to biosynthesis of the aromatic amino acids (Giles *et al.*, 1967). Until recently, the enzyme system was believed to be a multi-enzyme complex composed of at least four distinct subunits (Gaertner, 1972). Re-investigation concerning the nature of the "*arom* complex" led to the discovery that the five enzymes are organized as one polypeptide chain, which undergoes rapid proteolytic cleavage *in vitro* (Gaertner and Cole, 1976, 1977). The inhibition pattern of the proteinase responsible for cleavage of the pentafunctional polypeptide chain suggests that it is an alkaline proteinase (Gaertner and Cole, 1976).

Unlike cytoplasmic cytochrome *c*-oxidase maturation in *Sacch. cerevisiae*, where proteolytic cleavage of a precursor protein into different polypeptide chains plays a crucial role in assembly of the enzyme, there is growing evidence that proteolytic action on the other polyfunctional polypeptide chains referred to is not a maturation event. At first glance, one may view these proteolytic events as artefacts. However, in the case of the pentafunctional *arom* enzyme conjugate of *N. crassa*, Vitto and Gaertner (1978) were able to show that, *in vitro*, all five enzymes of the conjugate were coordinately protected from proteolytic inactivation in the presence of the first substrate, 3-deoxy-D-arabinoheptulosonate 7-phosphate. Use of the *pai* 1 mutant of *Sacch. cerevisiae*, which harbours a defect in proteinase A-inhibitor derepression leading to high levels of free proteinase A (Beck *et al.*, 1976; Wolf *et al.*, 1979), led to isolation of a *his* 4 protein fragment of 60,000 molecular weight instead of the 95,000 molecular weight protein (Bigelis *et al.*, 1977). In addition, it was shown that tryptophan synthase undergoes enhanced inactivation *in vivo* in stationary-phase cultures of the *pai* 1 mutant (Wolf *et al.*, 1979). Taken together, these observations might point to an involvement of proteinases in *in vivo* regulation of the polyfunctional enzyme conjugates under consideration.

4. *Transition of Inactive Proteins to the Active Form*

Proteolytic activation of an inactive pro-form of an enzyme produces an immediate response to a physiological signal and can initiate a new physiological function. From *in vitro* experiments, proteolytic action

has been proposed as a crucial event in budding of *Sacch. cerevisiae* (Cabib and Ulane, 1973b; Cabib, 1976; Ulane and Cabib, 1976). The primary septum that initially separates the cytoplasm of the mother and daughter cells appears to consist exclusively of chitin, a linear polysaccharide of 1-4-linked *N*-acetyl-β-glucosamine units (Cabib, 1976). The enzyme responsible for synthesis of chitin, chitin synthetase, has been found in an inactive "zymogen" form which is attached to the plasma membrane of *Sacch. cerevisiae* (Duran *et al.*, 1975; Cabib, 1976). Both findings fulfil part of the requirement necessary to explain the exact timing and location of chitin synthesis. According to the proposal of Cabib and his coworkers, *in vivo* transformation of inactive chain synthetase into the active form might occur through action of proteinase B (Cabib, 1976), as this enzyme has been found to activate chitin synthetase specifically *in vitro* (Cabib and Ulane, 1973b; Hasilik and Holzer, 1973; Cabib, 1976). Vesicles carrying proteinase B were thought to activate chitin synthetase at a precise location in the membrane (Cabib, 1976). Based on the findings of Cabib, a more detailed model for chitin synthetase activation was proposed, which takes proteinase and proteinase-inhibitor interaction into account (Holzer, 1975; Holzer and Saheki, 1976; Holzer, 1978).

An inactive form of chitin synthetase, which can be activated *in vitro* by proteolytic treatment, has also been found in *Mucor rouxii* (Ruiz-Herrera and Bartnicki-Garcia, 1976; Ruiz-Herrera *et al.*, 1977), *Candida albicans* (Cellini-Braun and Calderone, 1978) and *Phycomyces blakesleeanus* (Fischer, 1977; van Laere and Carlier, 1978).

Recent studies on *Sacch. cerevisiae* mutants lacking proteinase B activity (Zubenko *et al.*, 1979; Wolf and Ehmann, 1978b; Wolf *et al.*, 1979) suggest that involvement of proteinase B in activation of chitin synthetase of this organism is questionable. Mutant extracts have lost the activating activity for chitin synthetase, due to proteinase B absence (Wolf and Ehmann, 1979). However, the mutants are able to grow normally and do not show any aberrant budding behaviour (Wolf and Ehmann, 1978b; Wolf *et al.*, 1979). An abnormal phenotype, however, should be suspected if the budding event were affected due to lack of chitin synthetase activity (Cabib and Bowers, 1975). In addition, using the method to detect chitin by appearance of fluorescence when "brighteners" are added to the yeast cells (Cabib and Bowers, 1975), chitin insertion in mutant cells was shown to be normal (Zubenko *et al.*, 1979; Wolf and Ehmann, 1979). The lack of proteinase B involvement

in *in vivo* activation of chitin synthetase must not preclude a proteolytic activation mechanism. Other proteinases not able to cleave the synthetic protein substrates, but exhibiting overlapping specificity with proteinase B with respect to *in vivo* substrates, might exist (Wolf and Ehmann, 1978b). But other possible mechanisms for *in vivo* activation of chitin synthetase, like chemical modification of chitin synthetase or membrane alterations at the site of intended chitin synthesis, should also be evaluated.

Recently, activation of a hormone, a mediator of cellular signals, has been reported in *Sacch. cerevisiae*, in which participation of proteolysis seems to play a crucial role (Maness and Edelman, 1978). Transition from the haploid phase to the diploid phase in yeast is achieved by conjugation of two haploid cells of opposite mating type. This process is mediated by mating type-specific hormones excreted by haploid cells that synchronize cells prior to fusion. Cells of mating type α produce α-factor, an oligopeptide of 13 amino-acid residues, which is excreted into the medium and specifically arrests cells of mating type *a* early in the G1 period of the cell cycle (Bücking-Throm *et al.*, 1973; Stötzler and Duntze, 1976; Crandall *et al.*, 1977).

Maness and Edelman (1978) found that intact mating type *a*-cells were able to remove α-factor activity from the medium. This removal of activity was shown to be accompanied by limited proteolytic cleavage of α-factor. Interestingly, pre-incubation of *a*-cells with the proteinase inhibitors Trasylol or bovine pancreatic trypsin inhibitor was shown to inhibit α-factor-mediated G1 arrest. Inhibition could be overcome by high concentrations of α-factor. As removal of α-factor activity by limited proteolysis is not mediated by a diffusible proteinase, these findings led to the suggestion that proteinase inhibitors may render a membrane-bound proteinase inactive, which is necessary for α-factor activity. The hypothesis was put forward that α-factor may bind to *a*-cells and be degraded by a specific proteinase embedded in the membrane and, as a result, permit penetration of the cell by active α-factor fragments. However, other models for expression of α-factor activity cannot be completely excluded (Maness and Edelman, 1978).

B. INACTIVATION OF BIOLOGICALLY ACTIVE PROTEINS

Selective proteolytic inactivation of enzymes and other biologically active proteins, following appearance of a signal, enables the cell

immediately to stop physiological functions irreversibly, which have become unnecessary or are even harmful under the new environmental conditions. In contrast to enzyme inhibition by metabolites (Stadtman, 1966) or enzyme inactivation by reversible enzymic chemical modification (Holzer and Duntze, 1971), where reactivation of enzyme activity can occur rapidly, the cell may use degradative inactivation of enzymes as a regulatory tool in all those cases where a quick return to the original environmental conditions cannot be expected. Complete degradation prevents accumulation of unnecessary inactive proteins, and the cell may even use amino acids from inactivated protein as building blocks for protein synthesis.

1. *Enzymes Subject to Carbon Catabolite Inactivation*

In *Sacch. cerevisiae*, several enzymes have been found to undergo an irreversible glucose-induced inactivation or modification. These include galactose-uptake system (Spiegelman and Reiner, 1947; Matern and Holzer, 1977), α-glucoside (maltose) permease (Robertson and Halvorson, 1957; Görts, 1969), cytoplasmic malate dehydrogenase (Witt *et al.*, 1966; Neeff and Mecke, 1977), isocitrate lyase (C. von Kries and D. Mecke, unpublished observations), fructose-1,6-bisphosphatase (Gancedo, 1971), phosphoenolpyruvate carboxykinase (Gancedo *et al.*, 1974; Haarasilta and Oura, 1975), α-isopropylmalate synthase (Brown *et al.*, 1975), δ-aminolaevulinate synthetase (Labbe *et al.*, 1972; Labbe-Bois *et al.*, 1973), porphobilinogen deaminase-uroporphyrinogen III-cosynthetase (Labbe *et al.*, 1972; Labbe-Bois *et al.*, 1973) and uridine nucleosidase (Magni *et al.*, 1977). The term "catabolite inactivation" was invented for these processes and the possibility of a proteolytic mechanism was proposed (Holzer, 1976b).

It seems obvious that, in abundance of glucose, uptake of other sugars is superfluous. Matern and Holzer (1977) were able to show that the apparent K_m value of the galactose-uptake system increases from 3.6 to 11 m*M* galactose upon glucose addition to galactose-grown cells. "Reactivation" of the galactose-uptake system was shown to be dependent on *de novo* protein synthesis. Proteolysis of a modifying protein, which increases the affinity of the galactose-uptake system for galactose by protein-protein interaction was considered (Matern and Holzer, 1977). Also the decrease in activity of the maltose-uptake system upon shift of maltose-grown cells to glucose-containing medium was shown to be due to a decrease in the affinity of the uptake

system for its substrate. A rapid increase in the K_m value of the uptake system from 4 m*M* to about 50 m*M* maltose was observed. The modification of α-glucoside (maltose) permease affinity was also shown to be irreversible, reappearance of a functional protein being dependent on *de novo* protein synthesis (Görts, 1969).

The enzymes cytoplasmic malate dehydrogenase, isocitrate lyase, phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase are all involved in generation of glucose from C_2 and C_3 substrates via the glycoxylate cycle and/or reversal of glycolysis (Lehninger, 1975). Their action is unnecessary in the presence of glucose. Moreover, inactivation of phosphoenolpyruvate carboxykinase and fructose-1,6-bisphosphatase prevents the operation of harmful and futile ATP-splitting cycles (van de Poll *et al.*, 1974; Gancedo and Schwerzmann, 1976). Whereas very little data are available on inactivation of isocitrate lyase in *Sacch. cerevisiae*, inactivation of cytoplasmic malate dehydrogenase, another enzyme of the glyoxylate cycle, upon glucose addition to ethanol- or acetate-grown cells has undergone very detailed analysis in this organism. Duntze *et al.* (1968), using a tryptophan-requiring mutant, were able to show that cytoplasmic malate dehydrogenase is inactivated after glucose addition in the absence of tryptophan. This indicates that the "inactivating principle" is present and does not require *de novo* protein synthesis. This conclusion was confirmed by Neeff and Heer (1978) who, in a temperature-sensitive protein synthesis mutant, were able to show that inactivation of cytoplasmic malate dehydrogenase proceeds under restrictive conditions which do not allow protein synthesis in the mutant. Reappearance of cytoplasmic malate dehydrogenase activity following growth on ethanol or acetate is dependent on *de novo* protein synthesis; tryptophan-requiring mutants are not able to reactivate the enzyme when tryptophan is omitted from the growth medium (Duntze *et al.*, 1968). Further evidence that inactivation of the enzyme might be due to selective degradation comes from immunochemical experiments. Neeff *et al.* (1978a, b) were able to show that immunological cross-reaction with antiserum against cytoplasmic malate dehydrogenase is lost in cell extracts during the course of glucose-triggered inactivation. When inactivation proceeds in radioactively labelled cells suspended in buffer besides the disappearance of antigenic cytoplasmic malate dehydrogenase the appearance of peptides in the medium, which have been formerly protein bound, can be detected (Neeff *et al.*,

1978b). Excretion of about 10% of formerly protein-bound radioactivity during inactivation suggests degradation of not only the few known glucose-inactivated enzymes but also other unwanted proteins (Neeff *et al.*, 1978b).

Inactivation of phosphoenolpyruvate carboxykinase by glucose when added to ethanol- or acetate-grown cells was also shown to be irreversible. Recovery of the enzyme activity was dependent on *de novo* protein synthesis (Gancedo and Schwerzmann, 1976). Cycloheximide did not prevent inactivation, indicating the presence of the "inactivating principle" at the time of glucose addition (Gancedo and Schwerzmann, 1976).

The observed rapid inactivation of fructose 1,6-bisphosphatase upon addition of glucose to ethanol- or acetate-grown cells of *Sacch. cerevisiae* was also shown to be insensitive to cycloheximide, indicating a pre-existing inactivating principle (Gancedo, 1971). Furthermore, disappearance of enzyme activity was found to be insensitive to inhibitors of energy metabolism, like sodium azide, 2,4-dinitrophenol or iodoacetate. For reappearance of fructose 1,6,-bisphosphatase *de novo* synthesis was demonstrated to be necessary (Gancedo, 1971).

The enzyme α-isopropylmalate synthase, which catalyses the first step on the pathway from α-oxoisovalerate to leucine, was shown to be inactivated to about 50% of its initial activity after glucose addition to *Sacch. cerevisiae* growing on acetate (Brown *et al.*, 1975). Also, fructose or mannose, but not maltose, led to inactivation of the enzyme in lactate- or acetate-grown cells. The serine proteinase inhibitor phenylmethylsulphonylfluoride was shown to inhibit the glucose-triggered inactivation. Addition of leucine to the growth medium led to a decreased inactivation rate. The disappearance of only 50% of the enzyme activity was explained by the existence of two classes of the enzyme, of which only one is sensitive to catabolite inactivation. This interpretation was further based on the finding that the enzyme from glucose- and acetate-grown cells exhibited differences in leucine inhibition (Brown *et al.*, 1975). However, in recent experiments using the same *Sacch. cerevisiae* strain, no catabolite inactivation of α-isopropylmalate synthase after glucose addition to acetate-grown cells could be demonstrated (H. Matern and H. Holzer, unpublished observations).

Uridine nucleosidase is an enzyme necessary for utilization of internally and externally supplied uridine, catalysing its conversion

into uracil and ribose. Subsequently, the base can serve as a building block in UMP synthesis. The enzyme has been found to be inactivated upon glucose addition to ethanol-grown cells (Magni *et al.*, 1977). Reappearance of enzyme activity was shown to be dependent on *de novo* protein synthesis. Addition of phenylmethylsulphonylfluoride prevented the enzyme from glucose inactivation. Enzymes involved in haem biosynthesis—δ-aminolaevulinate synthetase and porphobilinogen deaminase-uroporphyrinogen III-cosynthetase—were shown to be irreversibly inactivated upon glucose addition to resting, respiratorily adapted *Sacch. cerevisiae* cells (Labbe *et al.*, 1972; Labbe-Bois *et al.*, 1973).

Glucose inactivation of α-glucoside (maltose) permease (Rijn and van Wijk, 1972), cytoplasmic malate dehydrogenase (Duntze *et al.*, 1968), phosphoenolpyruvate carboxykinase (Gancedo and Schwerzmann, 1976) and fructose 1,6-bisphosphatase (Gancedo, 1971) has been also found in *Sacch. carlsbergensis.*

Glucose inactivation is not restricted to yeast only. John *et al.* (1970) and Thurston *et al.* (1973) found that glucose triggered inactivation of isocitrate lyase in acetate-adapted cells of *Chlorella* sp. Inactivation of the enzyme was fastest and complete if glucose was added to cells suspended in nitrogen-free buffer. By following isocitrate lyase using polyacrylamide gel electrophoresis, John *et al.* (1970) were able to show disappearance of the authentic isocitrate lyase protein band during glucose-triggered inactivation. From isotope-labelling studies, it is concluded that the isocitrate lyase protein is selectively degraded. Studies with the uncoupling agent 2,4-dinitrophenol showed no inhibition of glucose-triggered inactivation of isocitrate lyase activity (Thurston *et al.*, 1973). However, disappearance of the inactivated protein was shown to be strongly inhibited, as deduced from polyacrylamide gel electrophoresis of cell crude extracts. These findings led to the conclusion that 2,4-dinitrophenol inhibits degradation of the inactivated enzyme (Thurston *et al.*, 1973). The authors suggest chemical modification or direct interaction of a metabolite with the enzyme as being the possible primary inactivation event, followed by an energy-dependent degradation of the enzyme (Thurston *et al.*, 1973). No proteinase possibly responsible for this degradative process has been found as yet in *Chlorella* sp.

For none of the enzymes found to undergo glucose-mediated inactivation is the exact *in vivo* inactivation mechanism known. Is the

mechanism the same or partly the same for all enzymes, or is it different for every single enzyme? An indication that there exists a common mechanism on an, as yet, unknown level for catabolite inactivation of some enzymes from the same organism comes from a mutant of *Sacch. carlsbergensis* described by van de Poll *et al.* (1974). The mutant, which has been shown to be defective in fructose 1,6-bisphosphatase inactivation, was shown to be also defective in inactivation of phosphoenolpyruvate carboxykinase and cytoplasmic malate dehydrogenase (Gancedo and Schwerzmann, 1976).

Some as yet unknown metabolite(s) of the sugars glucose, fructose, mannose and sucrose, or another molecule(s) appearing or disappearing after sugar addition, must be responsible for the final inactivation event. It is unknown on which cellular level they interact and finally lead to enzyme inactivation. The possibilities range from simply interacting with the enzyme to be inactivated or stimulating the inactivating enzyme(s) up to triggering a cascade of events with enzyme inactivation as the final step.

The irreversibility of the inactivation processes suggests involvement of proteolysis as the final step in glucose inactivation of enzymes. Strong support for this suggestion exists in the case of cytoplasmic malate dehydrogenase of *Sacch. cerevisiae* and isocitrate lyase of *Chlorella pyrenoidosa* where inactivation of enzyme activity was either found to be paralleled by loss of immunologically detectable enzyme protein (Neeff *et al.*, 1978b) or to be followed by loss of protein detectable on polyacrylamide-gel electrophoresis (John *et al.*, 1970). If proteolysis is part of the inactivation procedure, the following questions have to be put forward: (1) is proteolysis only involved in clean up of the already inactivated proteins (waste removal) and thus is preceded by the inactivation step; (2) does a proteolytic event itself render the proteins inactive?

In *Sacch. cerevisiae*, an unusually high *in vitro* sensitivity against proteolysis of the enzymes investigated in this aspect has been found. Cytoplasmic malate dehydrogenase is rapidly inactivated by proteinases A and B (Neeff, 1973; Jusić *et al.*, 1976), fructose 1,6-bisphosphatase is highly sensitive against proteinase B (Molano and Gancedo, 1974) and uridine nucleosidase is readily attacked by proteinase A (Magni *et al.*, 1978). This led to the working hypothesis that these proteinases might also be involved in *in vivo* inactivation of the enzymes (Neeff, 1973; Molano and Gancedo, 1974; Holzer, 1976b; Magni *et al.*, 1978).

Recent studies on mutants lacking proteinase B activity (Fig. 3), however, make this hypothesis in case of an involvement of proteinase B unlikely. Mutant extracts were shown to be devoid of cytoplasmic malate dehydrogenase- and fructose 1,6-bisphosphatase-inactivating activity, due to the absence of proteinase B (Wolf and Ehmann, 1979). In contrast, glucose triggered *in vivo* inactivation of cytoplasmic

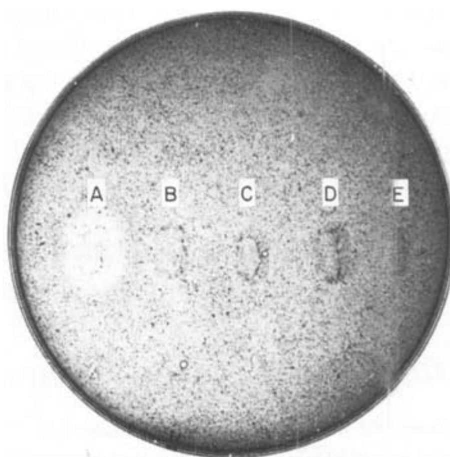

FIG. 3. Isolation of proteinase B mutants of *Saccharomyces cerevisiae*, using a petri-plate test for Hide Powder-clearing ability of temperature-sensitive cell-lysis strain 233 and four mutants derived from this strain lacking proteinase B activity. Cell-lysis strain 233 was mutated with ethylmethanesulphonate, plated on petri plates containing complete medium and grown for 4–6 days at 23°C. The colonies were replica-plated and incubated 1–3 days at 37°C on complete medium containing 10 mg of the proteinase B substrate Hide Powder azure and 0.05% sodium dodecylsulphate/ml. Incubation at 37°C leads to lysis of strain 233 and possible mutants derived from this strain. Cellular contents leaks into the medium. Complex formation of proteinase B with its inhibitor is prevented by sodium dodecyl sulphate. Mutants lacking proteinase B activity are detectable as colonies not able to cleave Hide Powder azure and this showing no halo on the petri plate. A shows cell-lysis strain 233; B, C, D, E represent proteinase B-mutant strains HP58, HP81, HP163 and HP232. Reproduced with permission from Wolf and Ehmann (1978b).

malate dehydrogenase, and fructose 1,6-bisphosphatase was unaffected by proteinase B absence (Zubenko *et al.*, 1979; Wolf and Ehmann, 1978b; Wolf and Ehmann, 1979). Also, two other catabolite inactivation-sensitive enzymes tested—phosphoenolpyruvate carboxykinase and isocitrate lyase—were found to be inactivated normally in a proteinase B mutant (Wolf and Ehmann, 1979). This suggests that

proteinase B is not involved in the immediate primary inactivation event of the enzymes tested. Alternatively, another proteinase with overlapping specificity with proteinase B *in vivo* might exist, and might be responsible for the inactivation events. It might equally be possible that a non-proteolytic inactivation event precedes a rapid final degradation of the inactivated proteins by proteinase B. Such a mechanism seems to operate in the case of isocitrate lyase inactivation in *Chlorella* sp., where inactivation can be separated from disappearance of the protein as shown by gel electrophoresis (Thurston *et al.*, 1973). However, the preceding inactivation step might also be due to a limited proteolytic cut, not one altering the electrophoretic behaviour of the isocitrate lyase protein, and this might remain electrophoretically undetectable. In the case of cytoplasmic malate dehydrogenase inactivation in *Sacch. cerevisiae*, simultaneous loss of enzyme activity and immunologically detectable enzyme protein (Neeff *et al.*, 1978b) seems to favour a mechanism where inactivation and protein alteration are tightly associated.

2. *NADP⁺-Dependent Glutamate Dehydrogenase*

Not only enzymes of carbon metabolism have been found to undergo irreversible inactivation which a proteolytic mechanism may account for. Inactivation also of enzymes of nitrogen metabolism has been attributed to proteolytic action.

Glutamate dehydrogenase is a key enzyme in nitrogen metabolism of many micro-organisms. The $NADP^+$-dependent enzyme synthesizes glutamate from ammonia and α-oxoglutarate. It is present in high levels in yeasts (Holzer and Schneider, 1957; Roon and Even, 1973; Folkes and Sims, 1974) growing in mineral medium containing glucose and ammonia.

Studies with *Sacch. cerevisiae* (Mazon, 1978) and *Candida utilis* (Hemmings, 1978) revealed that $NADP^+$-dependent glutamate dehydrogenase is rapidly inactivated upon glucose starvation. In *C. utilis*, this inactivation was found to be enhanced by addition of L-glutamate or L-alanine to a starvation medium lacking glucose and nitrogenous nutrients (Hemmings, 1978). Sodium azide, an inhibitor of the terminal oxidase, and 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation, were found to inhibit inactivation of $NADP^+$-dependent glutamate dehydrogenase in *Sacch. cerevisiae* and *C. utilis* (Hemmings, 1978; Mazon, 1978). This indicates an energy requirement

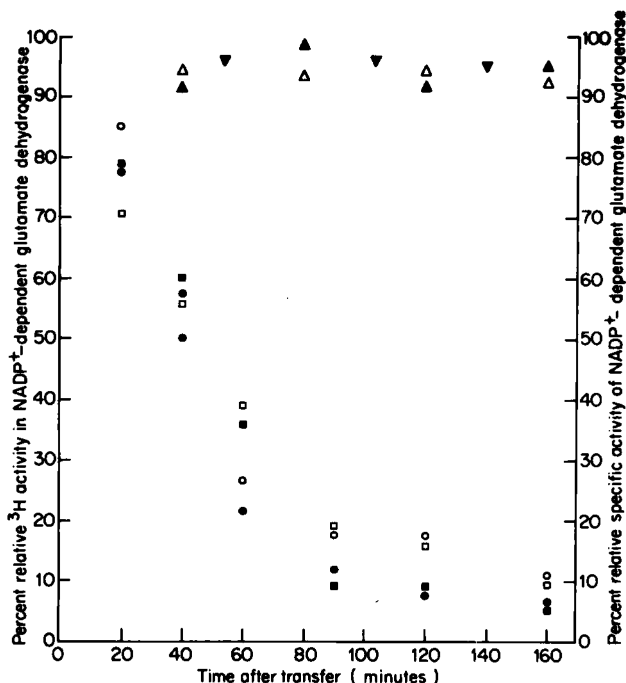

FIG. 4. Decrease in contents of NADP$^+$-dependent glutamate dehydrogenase (NADP$^+$-GDH) cross-reacting material and enzyme activity from *Candida utilis* following transfer of cells from ammonia-glucose medium containing 1.0 μCi of [^{3}H]leucine per ml to medium containing glutamate (24 m*M*), 0.5 m*M* leucine or fresh ammonia-glucose medium containing 0.5 m*M* leucine. Cell-free extracts, immunoprecipitation, and sodium dodecyl sulphate-polyacrylamide-gel electrophoresis were carried out as described by Hemmings (1978). Each point represents the number of counts per minute from 50 ml of culture in the three to four slices (2 mm) of a sodium dodecyl sulphate-polyacrylamide gel of antibody-purified material corrected for the local background in the gel. ●, ■ indicate percent relative NADP$^+$-GDH activity after transfer to glutamate-containing medium; ▲ percent relative NADP$^+$-GDH activity after transfer to ammonia-glucose medium; ○, □ percent relative [^{3}H]leucine in NADP$^+$-GDH after transfer to glutamate-containing medium; △, ▼, percent relative [^{3}H]leucine in NADP$^+$-GDH after transfer to ammonia-glucose medium. The data in the figure are from two separate experiments (●, ○, ▲, △, experiment I; ■, □, ▼, experiment II). From Hemmings (1978).

for some step in the inactivation process. Whereas in *Sacch. cerevisiae*, cycloheximide led to inhibition of the inactivation event (Mazon, 1978), the drug had no effect on the NADP$^+$-dependent glutamate dehydrogenase inactivation in *C. utilis* (Hemmings, 1978). Thus, in *C. utilis*, the "inactivating principle" seems to exist prior to inactivation and does not need *de novo* protein synthesis. Whether cycloheximide

inhibition of the inactivation process in *Sacch. cerevisiae* is due to a block in *de novo* synthesis of the inactivating system may need further investigation. Although cycloheximide inhibition of cytoplasmic malate dehydrogenase inactivation in *Sacch. cerevisiae* had been observed (Ferguson *et al.*, 1967), using an amino-acid auxotrophic mutant and a mutant conditionally defective in protein synthesis, it was shown later that the presence of the "inactivating principle" is independent from *de novo* protein synthesis (Duntze *et al.*, 1968; Neeff and Heer, 1978). Inactivation of $NADP^+$-dependent glutamate dehydrogenase was shown to be irreversible in *Sacch. cerevisiae* and *C. utilis* (Hemmings, 1978; Mazon, 1978). Strong evidence that disappearance of $NADP^+$-dependent glutamate dehydrogenase activity in *C. utilis* is due to proteolysis comes from immunochemical experiments (Hemmings, 1978). The rapid decrease in enzyme activity during starvation for a carbon compound and transition to glutamate-containing medium was shown to be paralleled by a decreasing amount of enzyme protein precipitable by a specific antibody raised against the enzyme (Fig. 4). No small molecular-weight protein could be found immunochemically as a result of inactivation, indicating a considerable alteration of the enzyme protein during the inactivation process. Azide and 2,4-dinitrophenol were found to inhibit loss of enzyme protein in parallel with enzyme activity. The enzyme was found to be very stable in cells under different non-inactivating conditions, indicating a selective increase in degradation of the protein when cells are subjected to inactivation conditions. Recently, the decrease in $NADP^+$-dependent glutamate dehydrogenase activity in *Sacch. cerevisiae* starved for glucose was shown to be paralleled by the disappearance of enzyme protein detectable by immunoprecipitation (M. Mazon and B. Hemmings, personal communication).

Only very little is known as yet about the sequence of events governing $NADP^+$-dependent glutamate dehydrogenase inactivation. The questions are similar to those raised already for carbon-catabolite inactivation. The simultaneous loss of enzyme activity and enzyme protein measured under different conditions seems to indicate that proteolytic cleavage is the inactivation step and is simultaneously responsible for further degradation of the enzyme protein. However, resolution of enzyme activity measurements and protein detection by immunochemical methods may be too low to preclude inactivation of the enzyme prior to rapid subsequent digestion.

In *Sacch. cerevisiae*, inactivation of the NADP$^+$-dependent glutamate dehydrogenase was shown to be partly inhibited by phenylmethylsulphonylfluoride (Mazon, 1978). Accordingly, involvement of a proteinase in the inactivation event was suggested (Mazon, 1978). As proteinase B is the only as yet known serine endoproteinase in *Sacch. cerevisiae*, participation of this enzyme in the inactivation process might be suspected. However, inactivation of the NADP$^+$-dependent glutamate dehydrogenase in a mutant absolutely devoid of proteinase B activity was shown to proceed as in the wild type, indicating that proteinase B might not be involved in the primary inactivation event (Wolf and Ehmann, 1979). Whether proteinase B is involved in removal of the inactive protein awaits further studies. A second proteinase in *Sacch. cerevisiae*, sensitive to phenylmethylsulphonylfluoride is carboxypeptidase Y. However, absence of this enzyme from a carboxypeptidase Y mutant (Wolf and Fink, 1975) does not alter the inactivation behaviour of NADP$^+$-dependent glutamate dehydrogenase (D. H. Wolf and C. Ehmann, unpublished observations).

Rapid loss of NADP$^+$-dependent glutamate dehydrogenase activity upon glucose starvation has also been found in *Aspergillus nidulans*, and a proteolytic mechanism was suspected (Hynes, 1974; Kinghorn and Pateman, 1974). Rapid inactivation of the ammonium-inducible NADP$^+$-dependent glutamate dehydrogenase upon removal of the inducer, i.e. ammonium ions, was observed in *Chlorella* sp. (Israel *et al.*, 1977). Inactivation was found to be irreversible, reappearance of enzyme activity being dependent on *de novo* protein synthesis following addition of ammonium ions. The possiblity of proteolytic degradation as being the inactivation mechanism is discussed (Israel *et al.*, 1977).

3. *Glutamine Synthetase*

Glutamine synthetase catalyses synthesis of L-glutamine from L-glutamate and ammonium ions. The enzyme occupies a strategic branch point in cellular metabolism as glutamine synthesis is the first step of the ultimately followed biosynthesis of a large number of different compounds (Stadtman, 1973). Thus, a finely controlled regulation of the level of enzyme seems crucial to meet cellular needs.

Rapid decrease in glutamine synthetase activity was observed in *C. utilis* following a sudden increase in ammonia supply or a decrease in glucose supply (Ferguson and Sims, 1974). This inactivation of glutamine synthetase was shown to be irreversible, and appearance of

enzyme activity was dependent on *de novo* protein synthesis. Inactivation of the enzyme was shown to be selective and not due to rapid turnover under normal physiological conditions. The irreversibility of the inactivation process indicates that proteolysis may be involved in glutamine synthetase inactivation.

4. *Nitrate Reductase*

Growth on nitrate as the sole nitrogen source requires the action of nitrate reductase for reduction of the nitrate ion as a first step on the pathway finally leading to production of ammonia necessary for biosynthetic metabolism. The enzyme is unnecessary when amino acids or ammonium ions are available for growth. In *N. crassa* (Sorger *et al.*, 1974) and in *Ustilago maydis* (Lewis and Fincham, 1970) disappearance of assimilatory nitrate reductase activity has been found upon addition of ammonium to nitrate-grown cells (Lewis and Fincham, 1970) or upon removal of cells from nitrate-containing medium followed by incubation under non-inducing conditions (Sorger *et al.*, 1974). In both organisms it was shown that inactivation of enzyme activity is irreversible and that *de novo* protein synthesis is necessary for reappearance of enzyme activity.

It is not clear as yet whether disappearance of nitrate reductase in *U. maydis* upon addition of ammonium ions to nitrate-grown cells is due to cessation of synthesis while degradation of the normal turnover process is continuing, or whether degradation of the enzyme is selectively enhanced (Lewis and Fincham, 1970). Using a tungstate method to examine synthesis and degradation of nitrate reductase activity, disappearance of enzyme activity in *N. crassa* was found to be selectively enhanced several fold upon removal of mycelia from nitrate-containing medium as compared to loss of activity in nitrate-grown cells. Immunoprecipitation experiments indicate that nitrate reductase protein is lost during inactivation of the enzyme (Sorger *et al.*, 1974).

5. *Phosphatase*

Phosphatase activity is believed to be, in part, responsible for the supply of phosphate from phosphate esters under conditions of phosphate deprivation (Liedtke and Ohmann, 1969). In this respect, the enzyme seems unnecessary when phosphate is present in the growth medium.

Studies on *Euglena gracilis* revealed that the high activity of derepressible phosphatase in cells grown under conditions of limited orthophosphate concentration disappeared rapidly after addition of phosphate to the growth medium. Cycloheximide inhibited loss in activity of the enzyme; however, an initial rapid loss of activity of about 30% also occurred in the presence of the drug. In addition, fluorophenylalanine did not prevent inactivation of the enzyme (Liedtke and Ohmann, 1969). It is therefore not clear if *de novo* synthesis of a protein is necessary for inactivation of the phosphatase. The inactivation was shown to be irreversible. Cycloheximide and fluorophenylalanine inhibited reappearance of enzyme activity, indicating that *de novo* synthesis is necessary. These findings may lead to the assumption that proteolytic degradation is involved in the inactivation process.

6. *Glycogen Phosphorylase*

Rapid irreversible inactivation of enzyme activity is expected to be not only a response of the cell to drastic changes in growth conditions but also a regulatory tool during cell differentiation. During this process, the cell has to rebuild its protein pattern to that characteristic for the differentiated cell. In this situation, proteolysis of unneeded proteins is advantageous. The cell has not to carry around unneeded proteins which, because of their unwanted catalytic action, have to be in an inactive state. It can use amino acids as building blocks for new protein synthesis. Proteolysis may proceed via normal turnover after a cessation of synthesis of unnecessary proteins (see Section IV.C, p. 318). Alternatively, in cases where quick disappearance of a certain protein is urgent for initiation of other processes, a selectively enhanced degradation may be operative.

A specific degradation of glycogen phosphorylase, the enzyme catalysing breakdown of glycogen, was found to occur at a certain developmental stage during differentiation in the cellular slime mould *Dictyostelium discoideum* (Thomas and Wright, 1976). Enzyme activity and, in parallel, the antibody cross-reacting enzyme protein were followed during different stages of development. It was shown that, while there is an increasing rate of synthesis of glycogen phosphorylase during the stages of amoebae aggregation, pseudoplasmodium and preculmination, degradation of the enzyme is not detectable in comparison with a considerable turnover rate of the other cell protein.

TABLE 3. *In vivo* inactivation or modification of enzymes in lower eukaryotes with proteolysis possibly involved

Enzyme inactivated or modified	Organism(s)	Culture conditions leading to inactivation	References
Fructose 1,6-*bis*-phosphatase	*Saccharomyces cerevisiae*		Gancedo (1971)
	Saccharomyces carlsbergensis		
Galactose-uptake system	*Saccharomyces cerevisiae*		Spiegelman and Reiner (1947), Matern and Holzer (1977)
α-Glucoside (maltose) permease	*Saccharomyces cerevisiae*		Robertson and Halvorson (1957), Görts (1969), Rijn and van Wijk (1972)
	Saccharomyces carlsbergensis		
Isocitrate lyase	*Saccharomyces cerevisiae*	Addition of glucose to cells growing on a non-fermentable carbon source; "carbon catabolite inactivation"	C. von Kries and D. Mecke (unpublished results),
	Chlorella pyrenoidosa		John *et al.* (1970), Thurston *et al.* (1973)
α-Isopropylmalate synthase	*Saccharomyces cerevisiae*		Brown *et al.* (1975)
Malate dehydrogenase (cytoplasmic)	*Saccharomyces cerevisiae*		Witt *et al.* (1966), Duntze *et al.* (1968), Neeff and Mecke (1977), Neeff *et al.* (1978a,b), Neeff and Heer (1978)
	Saccharomyces carlsbergensis		

Phosphoenolpyruvate carboxykinase	*Saccharomyces cerevisiae* *Saccharomyces carlsbergensis*	Addition of glucose to cells growing on a non-fermentable carbon source; "carbon catabolite inactivation"	Gancedo *et al.* (1974), Haarasilta and Oura (1975)
Uridine nucleosidase	*Saccharomyces cerevisiae*		Magni *et al.* (1977), Magni *et al.* (1978)
δ-Aminolaevulinate synthetase	*Saccharomyces cerevisiae*	Addition of glucose to resting; respiration-adapted cells	Labbe *et al.* (1972), Labbe-Bois *et al.* (1973)
Porphobilinogen deaminase-uroporphyrinogen III-cosynthetase	*Saccharomyces cerevisiae*		Labbe *et al.* (1972), Labbe-Bois *et al.* (1973)
Glutamate dehydrogenase ($NADP^+$-dependent)	*Candida utilis* *Saccharomyces cerevisiae* *Aspergillus nidulans*	Transfer of derepressed cells to media lacking glucose	Hemmings (1978), Mazon (1978), Hynes (1974), Kinghorn and Pateman (1974)
	Chlorella pyrenoidosa	Removal of ammonium ions from growth medium	Israel *et al.* (1977)
Glutamine synthetase	*Candida utilis*	Addition of ammonium ions or decrease in the glucose supply	Ferguson and Sims (1974)
Nitrate reductase	*Neurospora crassa*	Removal of cells from nitrate-containing medium	Sorger *et al.* (1974)
	Ustilago maydis	Addition of ammonium ions to cultures grown on nitrate	Lewis and Fincham (1970)
Phosphatase	*Euglena gracilis*	Addition of phosphate to cultures grown under limited orthophosphate supply	Liedtke and Ohmann (1969)
Glycogen phosphorylase	*Dictyostelium discoideum*	Nutrient starvation	Thomas and Wright (1976)

During the culmination stage, an additional increase in synthesis rate was observed but, at the same time, degradation of the enzyme protein is detectable at twice the rate of turnover of other cellular protein. During post-culmination degradation, the rate of glycogen phosphorylase is enhanced three-fold which, combined with a drop in rate of synthesis, leads to a rapid decrease in enzyme activity and protein of about 75% during this developmental stage. Enzyme activity and enzyme protein were found to be lost in parallel (Thomas and Wright, 1976).

As with catabolite inactivation, the exact mechanism of enzyme inactivation is not known. The irreversibility of the reactions and the need for *de novo* protein synthesis for enzyme reappearance point to proteolytic degradation of the enzymes. It is not known which cascade of events finally leads to this inactivation. It is furthermore not known whether a proteolytic step itself renders the enzymes inactive or whether inactivation by some other mechanism precedes proteolytic degradation of the already inactivated protein. Final solutions of these questions should come from experiments with mutants unable to inactivate the enzymes under consideration and with mutants lacking the proteinase activities probably involved in one of the events of the inactivation process. Promising steps in this direction have been taken (van der Poll *et al.*, 1974; Wolf and Fink, 1975; Jones, 1977; Zubenko *et al.*, 1979; Wolf and Ehmann, 1978b; Wolf *et al.*, 1979; Wolf and Ehmann, 1979). A summary of the enzymes irreversibly inactivated or modified and the conditions triggering enzyme alteration, are given in Table 3.

C. PROTEIN TURNOVER AND PROTEINASE ACTIVITY DURING GROWTH, STARVATION AND DIFFERENTIATION

Protein turnover implies continuous synthesis and degradation of intracellular protein constituents. Rate of synthesis and rate of degradation determine the concentration of a given protein at a certain time. Overall turnover must be regarded as the sum total of degradation and resynthesis of proteins with very different half lives, and different responses of their half lives to metabolic changes. Only following the fate of every single protein species will uncover whether its regulation following upon a signal is achieved by alteration of the rate of its synthesis, alteration of the rate of its degradation, or both alteration of synthesis and degradation rate. Thus, by measuring

general protein turnover, only a description of the overall regulatory pattern of the cell's protein is available leaving the intracellular fine regulation of single proteins undetectable.

1. Saccharomyces cerevisiae

During logarithmic growth of *Sacch. cerevisiae* in a glucose-containing rich medium, a rate of protein degradation of only 1% per hour was observed (Betz, 1976). Major membrane proteins have been found to be essentially stable in growing cells (Losson *et al.*, 1978). Concentration of proteinase A, proteinase B, carboxypeptidase Y (Saheki and Holzer, 1975) and the lysosomal aminopeptidase (Matile *et al.*, 1971) were found to be low during logarithmic growth on glucose-containing medium. Use of cycloheximide led to a two-fold enhanced protein degradation in logarithmically growing cells treated with the drug. A similar enhancement of protein degradation in growing cells was found when mutants conditionally defective in initiation of protein synthesis or chain elongation were shifted to restrictive conditions (Betz, 1976). From this observation, it was concluded that there may be stimulation of a pre-existing proteolytic apparatus in growing yeast cells upon cessation of protein synthesis (Betz, 1976).

A significant stimulation of protein turnover in late log-phase cells (Balkalkin *et al.*, 1976) and in resting cells (Halvorson, 1958a, b), as compared with growing cells, was observed. Dinitrophenol, an uncoupler of oxidative phosphorylation at a concentration of 4×10^{-4} *M*, was shown to inhibit protein breakdown by more than 90% (Halvorson, 1958a). The energy dependence of protein breakdown in resting cells was confirmed by other workers who found up to 95% inhibition of protein degradation when valinomycin or sodium cyanide, together with sodium fluoride, were used as energy blockers (Betz and Weiser, 1976a).

The energy dependence of protein breakdown is a puzzling question. As hydrolysis of peptide bonds is an exergonic reaction, there does not seem to be any need for energy requirement. Energy may be used, however, for events like activation of proteolytic enzymes, removing inhibitors or allowing proteins subject to degradation to enter proteinase-bearing organelles (Goldberg and St. John, 1976; Holzer, 1976a).

It has been shown that starvation of *Sacch. cerevisiae* for glucose and, to some extent, for nitrogenous nutrients leads to a several-fold

increase in activities of proteinases A, B, carboxypeptidase Y (Saheki and Holzer, 1975; Hansen *et al.*, 1977) and of vacuolar aminopeptidase (Matile *et al.*, 1971). Carboxypeptidase S activity was found to increase under conditions of nitrogen starvation but not under conditions of glucose deprivation (Wolf and Ehmann, 1978a). Thus, one might be tempted to conclude that these proteinases are at least part of a proteolytic system that is responsible for the enhanced degradation of proteins when cells are subjected to conditions of nutrient starvation. This view is strongly supported by studies on a proteinase B mutant. Protein degradation is lowered more than 40% in these mutant cells as compared to wild type (Wolf and Ehmann, 1979). A variety ot proteins may be unnecessary or even harmful under the new conditions; others are needed in lower concentrations. Amino acids resulting from proteolysis will be necessary for synthesis of new proteins and as an energy source, both necessary for adaption and survival under new environmental conditions. This concept also applies to sporulation in *Sacch. cerevisiae*. Development of this differentiated structure is accompanied by meiosis and subsequent ascospore formation. Subjecting cells to extremely poor nutritional conditions—only acetate is supplied as a nutrient—triggers this process (Croes, 1967a, b). Thus, adaptation of the cell's protein machinery to this differentiation event depends on the availability of building blocks which are already present intracellularly. Amino acids for protein synthesis must come from the internal amino-acid pool and from proteolysis of unnecessary vegetative protein.

Several investigators found intensive protein turnover in sporulating *Sacch. cerevisiae* (Hopper *et al.*, 1974; Magee and Hopper, 1974; Klar and Halvorson, 1975; Betz and Weiser, 1976a). Until the time that mature asci appeared, breakdown of 30% (Hopper *et al.*, 1974) to 70% (Betz and Weiser, 1976a) of pre-existing vegetative protein was measured. Treatment of cultures not able to sporulate (haploid cells or cell homozygous for mating type) led to protein breakdown ranging from 10% (Hopper *et al.*, 1974) to 50% (Betz and Weiser, 1976a). This indicates a specifically enhanced proteolysis in sporulating cells as compared with cells subjected to conditions of starvation but not able to sporulate. Disappearance of several enzymes (NAD^+-dependent glutamate dehydrogenase, isocitrate lyase, malate dehydrogenase and fructose 1,6-bisphosphatase) during sporulation had been investigated. However, none of the enzyme changes measured turned out to be

sporulation specific (Betz and Weiser, 1976b). They occurred also in haploid cells not able to sporulate. Protein turnover was also not found to be specific for vegetative-cell proteins. Proteins synthesized at different times during sporulation showed similar degradation rates (Betz and Weiser, 1976a). Uncouplers of oxidative phosphorylation and other inhibitors of energy metabolism inhibited protein degradation in sporulating cells, as had been found for degradation in starving cells (Betz and Weiser, 1976a). The possible role of this energy dependence of the degradative process has already been discussed.

During sporulation, a substantial increase in proteinases A and B, as compared with non-sporogenic cultures, was detected. Proteinase A activity increased between two- and 11-fold reaching its maximum 20 h after exposure of cells to the sporulation medium (Klar and Halvorson, 1975; Betz and Weiser, 1976a). Within the same time interval, the increase in proteinase B activity was found to range between six- and 12-fold (Klar and Halvorson, 1975; Betz and Weiser, 1976a). There is disagreement about the existence of a sporulation-specific increase of carboxypeptidase Y. Whereas Betz and Weiser (1976a) were not able to find a significant increase, Klar and Halvorson (1975) reported a five-fold enhancement of carboxypeptidase Y activity during sporulation. No sporulation-specific increase of activity was found for carboxypeptidase S (Wolf and Ehmann, 1978a). No other sporulation-specific proteinases have as yet been found (Klar and Halvorson, 1975; Betz and Weiser, 1976a).

In contrast to growing cells, cessation of protein synthesis by cycloheximide leads to a great decrease in protein degradation in sporulating cells (Magee and Hopper, 1974; Betz and Weiser, 1976a). This may be due to inhibition of synthesis of proteinases required for this process. It has been shown that increase in activities of proteinases A and B and carboxypeptidase Y during sporulation is dependent on *de novo* protein synthesis (Klar and Halvorson, 1975). Thus, one might speculate that these proteinases are part of the required proteolytic system that leads to acquisition of the protein pattern characteristic for the differentiated cell. Evidence for the validity of this hypothesis should come from studies of mutants lacking the proteinase activities. Diploid mutant cells homozygous for the absence of proteinase B exhibited a significantly decreased protein degradation rate of more than 40% as compared with wild-type diploids (Wolf and Ehmann, 1979). In addition, sporulation kinetics and sporulation frequency

of proteinase B-mutant diploids were found to be more than 50% decreased (Wolf and Ehmann, 1979). Zubenko *et al.* (1979) reported near cessation of sporulation in proteinase B-mutant diploids. As Zubenko *et al.* (1979) reported the mutation to be localized in the structural gene of proteinase B, the vital role for proteinase B in this differentiation process is obvious. Diploids homozygous for constitutively low proteinase A activity were also shown to have lost their sporulating ability (Betz, 1977). However, as the mutation does not seem to be in the structural gene for proteinase A (Betz, 1977), no convincing conclusion about a proteinase A requirement for sporulation can as yet be drawn. The mutation may be pleiotropic, affecting different unlinked functions.

Absence of carboxypeptidase Y was shown not to affect sporulation. Diploids homozygous for a mutation in the structural gene for carboxypeptidase Y leading to absence of activity were still able to sporulate (Wolf and Fink, 1975).

It is not known if there exist more proteinases than those already shown to be involved in turnover in growing or in differentiating *Sacch. cerevisiae*. Stimulation of protein turnover after cessation of protein synthesis by cycloheximide in growing cells might indicate a different proteolytic system (Betz, 1976); but, likewise, another as yet unknown mechanism might lead to stimulation of turnover by the pre-existing known proteinases.

One would furthermore like to know: (1) whether the known proteinases are involved in any specific process of maturation, activation or inactivation during differentiation; and (2) whether their action is restricted to rapid "unspecific" digestion of a large variety of proteins which the cell makes available by some mechanism to provide rapidly amino acids and energy for protein synthesis.

2. Blastocladiella emersonii

At any time during the growth phase of the water mould *Blastocladiella emersonii*, sporulation of the vegetative cell can be initiated by transfer to a starvation medium containing only calcium ions and a suitable buffer (Murphy and Lovett, 1966). Under appropriate conditions, the resulting zoospores are able to revert to vegetative cells (germlings), which are capable of prolific coenocytic growth (Truesdell

and Cantino, 1971). It has been shown that protein turnover is rather low during vegetative growth of the organism and in resting zoospores. Less than 1% per hour of the protein was found to be degraded during these phases (Lodi and Sonneborn, 1974). However, drastically increased turnover rates were observed during the differentiation phases of the organism. A degradation rate of 12% per hour was observed when cells were transferred to sporulation conditions. At least 35% of the pre-existing vegetative cellular proteins were hydrolysed during this differentiation process. During germination, a protein turnover rate of 5% per hour was detectable. Protein degradation during zoospore germination in growth medium was found to be unaffected by cycloheximide (Lodi and Sonneborn, 1974). Measurement of proteinase activities during the developmental stages revealed the following pattern. Four electrophoretically distinguishable aminopeptidase activities are present during all developmental stages, and the total aminopeptidase activity level does not change considerably. In contrast, alkaline proteinase and a caseinolytic activity with an optimum pH value of 5.5 were found to be absent during growth (Lodi and Sonneborn, 1974; Corrêa *et al.*, 1979). Upon onset of sporulation, alkaline proteinase and pH 5.5 caseinolytic activity appear. Alkaline proteinase reaches its highest activity level after 160 min and is released into the medium at the late phase of sporulation. No alkaline proteinase is detectable in free zoospores. In contrast, pH 5.5 caseinolytic activity reaches its maximum in free zoospores and remains at a high level during germination (Lodi and Sonneborn, 1974; Corrêa *et al.*, 1979). Appearance of both proteolytic activities during sporulation was found to be dependent upon *de novo* protein synthesis (Corrêa *et al.*, 1979). Antipain, a microbial peptide analogue which inhibits a variety of proteinases (Aoyagi and Umezawa, 1975), has been shown to inhibit sporulation of *B. emersonii* when applied at the onset of differentiation (Corrêa *et al.*, 1978). At the same time, protein degradation was decreased by 60%. Antipain was shown to exert no effect on vegetatively growing cells or during germination (Corrêa *et al.*, 1978). The findings that: (1) antipain selectively inhibits only the alkaline proteinase *in vitro* and; (2) antipain is effective only during the phase where alkaline proteinase is present, but not during all other periods of the life cycle of the organism, point to the alkaline proteinase as the target molecule for antipain action. This led to the conclusion that alkaline proteinase plays a crucial role

in sporulation (Corrêa *et al.*, 1978). Surprisingly, when added 90 min after initiation of sporulation, antipain showed no inhibition of differentiation but a strong inhibition of protein degradation was nevertheless observed (Corrêa *et al.*, 1978). This finding led to the suggestion that a considerable amount of protein degradation is not vital for sporulation (Corrêa *et al.*, 1978). The proteolytic steps critical for sporulation of *B. emersonii* are thought to be catalysed by alkaline proteinase in the first 90 min of sporulation (Corrêa *et al.*, 1978). This conclusion leaves open the question why alkaline proteinase activity peaks 70 min later in the sporulation process. The existence of another antipain-sensitive proteinase responsible for proteolytic steps critical for sporulation cannot completely be excluded.

3. Physarum polycephalum

Transfer of growing microplasmodia of *Physarum polycephalum* to a nutrient-free salts medium yields thick-walled diploid cysts, the so-called sclerotia or spherules (Jump, 1954). Turnover of protein was found to be very low in growing cells. In contrast, during starvation-induced formation of spherules, a very high turnover rate was detected, leading to degradation of at least 70% of the pre-existing protein (Wendelberger-Schieweg and Hüttermann, 1978).

Aminopeptidase activities have been examined during growth and differentiation of this organism (Hoffmann and Hüttermann, 1975). The seven enzyme activities found could be divided into three different classes. Two activities (called 2 and 3; numbers were given after the banding pattern during isoelectric focusing) were present only in growing cultures. They were shown to disappear during differentiation. One aminopeptidase activity (called 1) was found to be present in growing and differentiating cultures. Four aminopeptidase activities (6, 7, 8 and 9) appeared only in differentiating cells and were shown to be synthesized *de novo*. When the specificities of aminopeptidases in growing cells were compared with that of the newly appearing isoenzymes during differentiation, an increased preference for hydrophobic amino-terminal amino acids was observed in the differentiation-specific aminopeptidases. This altered specificity, together with very high aminopeptidase activity, is thought to be regulatory

resulting in differences in protein degradation during growth and differentiation (Hoffmann and Hüttermann, 1975).

4. Dictyostelium discoideum

During morphogenesis of the cellular slime mould *Dictyostelium discoideum*, vegetatively growing amoebae aggregate upon starvation to form a multicellular migrating pseudoplasmodium. This differentiates later into a sorocarp composed of stalk cells and spores (Bonner, 1967). During development a rapid decrease in cellular protein was detected (Hames and Ashworth, 1974). It has been shown that neither the rate nor the extent of decrease in cellular protein was changed during the developmental process, following provision of a metabolizable energy source such as glycogen. Furthermore, the major portion of the degraded protein was shown to be oxidized irrespective of the presence of an energy source (Hames and Ashworth, 1974). This led to the suspicion that massive net loss in protein during development of *D. discoideum* occurs for reasons other than energy provision (Hames and Ashworth, 1974). As a considerable part of the amino acids derived from protein degradation is oxidized, provision of amino acids for new protein synthesis cannot be the only aim of the degradative process.

As yet the fate of only one enzyme, namely glycogen phosphorylase, has been followed during development of *D. discoideum*. Its specific rapid inactivation during the postculmination stage, and in the young sorocarp, have already been reviewed in Section IV.B, 6, p. 315. Regulation of proteinase activities during differentiation of this organism is not clearly resolved as yet.

Cathepsin B and cathepsin D activities were followed during the life cycle by Fong and Rutherford (1978). Both activities were found to decrease throughout development. Whereas cathepsin D was shown to decrease gradually, a sharp decrease between the stages of aggregation and migration of pseudoplasmodia was detected for cathepsin B. In the differentiated sorocarp, no difference in the cathepsin D activity of stalk cells and spores could be observed. However, cathepsin B activity was found to be five-fold higher in stalk cells as compared to spores. From this finding, a possible role for cathepsin B in stalk-cell differentiation was proposed (Fong and Rutherford, 1978). North and Harwood (1979) described a decrease of their acid proteinases A, B, C and D during development. In addition, they reported the appearance

of a new proteinase, called F, during the culmination stage, when differentiation of stalk cells and spores occurs. Appearance of this enzyme could be prevented when post-aggregation development was blocked (North and Harwood, 1979).

5. Tetrahymena pyriformis

Shaking statically grown stationary-phase cultures of *Tetrahymena pyriformis* leads to a 60–80% decrease of three peroxisomal enzymes (lactate oxidase, isocitrate lyase and catalase) in the absence of cell growth. This decrease in enzyme activity was prevented by addition of cycloheximide or actinomycin D (Levy, 1973). Under these conditions, a two- to three-fold increase in activity of a neutral proteinase was detected. This increase was found to be blocked by cycloheximide and actinomycin D (Levy *et al.*, 1976). The coincidence in time of the decrease in activity of peroxisomal enzymes and the increase in proteolytic activity, both processes being inhibited by inhibitors of protein synthesis, led the authors to suggest that the proteinase is in part responsible for inactivation of the three peroxisomal enzymes (Levy *et al.*, 1976). Whether this inactivation event might be due to a specific proteolytic process, or part of turnover leading to disappearance of the enzymes after cessation of their synthesis, is not known.

6. Chlamydomonas reinhardtii

Synchronized vegetative cultures of *Chlamydomonas reinhardtii* can be induced to undergo gametic differentiation. Gametogenesis involves cell division in nitrogen-free medium producing four gametes from each vegetative cell (Kates *et al.*, 1968). Considerable protein turnover is detectable during this process (Jones *et al.*, 1968). The need for protein turnover during differentiation was shown by using a mutant (*arg* 2) defective in arginine biosynthesis. The mutant was able to continue protein synthesis and to undergo gametogenesis in the absence of a nitrogen supply and exogenous arginine, both of which are necessary for normal growth. However, starving the mutant of arginine in the presence of ammonia led to inhibition of protein turnover and also differentiation (Jones *et al.*, 1968).

D. REMOVAL OF DEFECTIVE PROTEINS

Accumulation of incorrectly synthesized proteins, which are inactive, would pose a serious problem for a cell's survival. Furthermore, in

bacteria, a growth-inhibiting role for a variety of di- and tripeptides has been proposed (Sussman and Gilvarg, 1971). Proteolysis of these deleterious compounds by proteinases and peptidases represents a vital mechanism enabling the cell to escape death. A proteolytic system serving this scavenger function has been found in bacteria (Goldberg and St. John, 1976). Very little, however, is known about such systems in eukaryotic cells. Studies of mutants defective in certain proteins suggest proteolytic removal of the aberrant proteins. In *Sacch. cerevisiae*, rapid degradation of subunits of the fatty-acid synthetase complex in a mutant not able to assemble the authentic multimeric enzyme was suspected (Dietlein and Schweizer, 1975; Schweizer, 1977). In a post-translational modification mutant of *D. discoideum*, low levels of β-glucosidase-1, α-mannosidase-1 and *N*-acetylglucosaminidase were found. These low levels of enzyme activity were thought to be most likely due to degradation of β-glucosidase-1 holoenzyme and degradation of α-mannosidase-1 and *N*-acetylglucosaminidase subunits before assembly, as a result of altered post-translationally modified polypeptide chains (Free and Schimke, 1978). It is not known whether degradation is brought about by established proteolytic enzymes, or whether additional proteolytic enzymes exist which specifically hydrolyse aberrant proteins.

E. METABOLISM AND PROTEINASE DISORDER

Many species of fungi fail to form heterokaryons between non-isogenic strains as a result of protoplasmic disintegration after hyphal fusion. This incompatibility reaction, followed by cell death, is thought to involve lytic processes (Begueret, 1972). In *Podospora anserina*, a considerable parallel increase in proteinase activity during the incompatibility reaction has been described (Begueret, 1972; Begueret and Bernet, 1973). Inhibition of protein synthesis by cycloheximide was found to stop the incompatibility reaction and, at the same time, increase proteinase activity. Mutations were found (*mod*$_1$) which suppress incompatibility and also show low proteinase activity (Begueret, 1972). A more quantitative determination revealed that, during release of the incompatibility reaction, two new reaction-specific proteinases (III and IV) appeared, which represent 90% of the proteolytic activity. The serine proteinase inhibitor, phenylmethylsulphonylfluoride, which is able partly to inhibit the two newly occurring proteinases, was shown partly to prevent cell disintegration

in vivo (Begueret and Bernet, 1973). The incompatibility reaction is regarded as a consequence of a disorder in the regulation of proteolytic enzymes (Begueret, 1972).

In *Sacch. cerevisiae*, a conditional mutant (*pai 1*) was found, which has a blocked derepression of proteinase A-inhibitor synthesis when cells undergo glucose starvation under restrictive conditions (Beck *et al.*, 1976; Wolf *et al.*, 1979). As proteinase A is normally derepressed in the mutant under these conditions, a surplus of proteinase A over proteinase A-inhibitor develops. One might suspect intracellular disorder by the big surplus of "free" proteinase A. In fact, the mutant cells are unable to reach wild-type cell density under restrictive conditions. The mutant does not show enhanced protein turnover; but those proteins known to be cleaved *in vitro* by proteinase A—tryptophan synthase and proteinase B-inhibitor—rapidly disappear upon proteinase A appearance. This altered mutant phenotype might be due to uncontrolled proteinase A action (Wolf *et al.*, 1979). Final proof would come from mutants defective in the structural gene for proteinase A-inhibitor.

F. FOOD SUPPLY

The role of extracellularly secreted proteinases and peptidases in supplying micro-organisms with amino acids from extracellularly present proteins and peptides is obvious. However, intracellular peptidases also play an important role in providing amino acids as source of nitrogen. Studies on *Sacch. cerevisiae* should serve as an example. Several studies showed that radioactively labelled peptides are taken up by the yeast. Intracellular cleavage of peptides could be demonstrated by their ability to promote growth of amino-acid auxotrophs. In addition, intracellular peptidase activity which cleaves peptides *in vitro* has been demonstrated (Becker *et al.*, 1973; Naider *et al.*, 1974; Marder *et al.*, 1977; Becker and Naider, 1977). It seems reasonable to believe that peptidases in *Sacch. cerevisiae* (see Section II.A, 1, p. 269) are involved in supplying the cell with the required amino acids from extracellularly supplied peptides. Direct evidence for this function has been demonstrated for carboxypeptidase Y and carboxypeptidase S. Both enzymes increase several-fold over levels found in amino acid- or ammonia-grown cells when they are grown on the dipeptide *N*-carbobenzoxy-glycyl-L-leucine as sole nitrogen source

(Wolf and Ehmann, 1978a). Furthermore, in contrast to wild-type, mutants lacking both carboxypeptidase activities are not able to grow on this dipeptide (Wolf *et al.*, 1979).

V. Concluding Remarks

As already mentioned in the introduction to this article, large gaps exist between our knowledge of the characterized proteinases and their possible involvement in cellular processes which are thought or found to depend on proteolysis. From the large number of facts that have accumulated, only in a very few cases are links between known proteolytic activities and metabolic events recognizable. Proteinase B from *Sacch. cerevisiae* is most likely involved in sporulation. A function in the same process may be attributable to alkaline proteinase in *B. emersonii.* Carboxypeptidases Y and S from *Sacch. cerevisiae* serve nutritional functions. In no other cases are connections between proteinase activity and definite metabolic events established as yet. In the case of proteinase B from *Sacch. cerevisiae*, hypothesized functions, such as chitin synthetase activation and participation in the primary inactivation event of several enzymes, will most likely have to be re-evaluated.

Predictions about the *in vivo* function of a proteinase by *in vitro* experiments are complicated or even impossible because the primary specificity of a proteinase lies in its ability to cleave a peptide bond which follows a certain amino-acid residue. However, an amino-acid sequence necessary for cleavage by a certain proteinase will reside in a large portion of proteins with different functions. A secondary specificity of a proteinase, namely its ability to bind certain proteins correctly, finally decides whether or not proteolysis occurs. However, depending on the "degeneration" of this secondary specificity, proteins may be bound and cleaved *in vitro* which, because of their inaccessibility to the proteinase, are not hydrolysed *in vivo*. As has been successfully demonstrated in *Sacch. cerevisiae*, mutants which lack one or more proteinases will provide the final link between proteinase activity and cellular function.

With only very few exceptions, such as the presumptive "transport trigger" proteinase of *Chlamydomonas* sp., known proteinases were detected by using non-physiological substrates. By searching for proteinases with these non-physiological substrates, it is possible that

researchers found only proteinases with a rather broad specificity. One might argue that most of these proteinases are involved only in *in vivo* processes which depend on proteinases exhibiting a broad substrate specificity, like general protein turnover supplying amino acids and energy, or waste protein removal. They might not be involved in very specific proteolytic processes like selective maturation, activation or inactivation of biologically active proteins.

It has been shown that eukaryotic proteinases can exhibit a very high degree of substrate specificity (Reich *et al.*, 1975; Jackson and Blobel, 1977). In the case of serine proteases from several eukaryotic organisms, a macromolecular recognition surface was identified formed by the tertiary structural folding of the enzymes. Only complementary protein structures can be bound to this surface. This recognition surface was found to define the macromolecular secondary specificity external from the active site, and variability in this region leads to different specificities which may be highly selective (Liebman and Fitch, 1978; M. N. Liebman, personal communication). Thus future work will show whether there exist other proteinases of higher specificity responsible for specific proteolytic events in the organisms described. Furthermore, the discovery of as yet unknown proteolytic processes in metabolism is quite likely.

VI. Acknowledgements

I am very grateful to Professor Dr H. Holzer for his encouragement, the many valuable suggestions he made and his critical reading of this review. I thank Professors G. Schatz and W. R. Lodi for providing research data prior to publication. Thanks are due to Dr W. Ghiorse and C. Ehmann for help during preparation of this manuscript. I am indebted to Mrs D. Montfort, Mrs L. Kötschau and Mrs R. Ch. Wilke for their expert assistance. The experimental work of the author cited in this article was supported by the Deutsche Forschungsgemeinschaft, Bonn–Bad Godesberg.

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Note added in proof

I. The Proteolytic System and its Cellular Localization

In a triple mutant of *Saccharomyces cerevisiae* lacking proteinase B, carboxypeptidase S and carboxypeptidase Y, four new intracellular proteolytic enzymes active at neutral pH values were detected with the aid of plasma proteinase substrates (Wolf and Ehmann, 1980). Enzymes, preliminarily called D and E, were found in the soluble fraction of disintegrated cells, and activities called M and P in the precipitable fraction. Two additional carboxypeptidases, preliminarily called carboxypeptidase γ and carboxypeptidase δ, were suspected from studies on the proteinase triple mutant (D. H. Wolf and C. Ehmann, unpublished observations).

In mycelia of *Neurospora crassa* separation, on polyacrylamide gel electrophoresis of eleven intracellular peptidases called I, II, IIIA, IIIB, IV, V, VI, VII, VIII, IX and X was reported by Tan and Marzluf (1979). Peptidase V may be identical with the imidodipeptidase, and peptidase IIIB may correspond to the methionyl dipeptidase of Johnson and Brown (1974). None of the three exopeptidases described by Siepen *et al.* (1975) and by Siepen and Kula (1976) (see Section II.C., p. 279) can be correlated with the II peptidases (Tan and Marzluf, 1979).

II. Metabolic Events and Proteinase Function

Proteolysis as a possible key step in membrane transfer of cytoplasmically synthesized proteins, which are functionally active in the mitochondria, has been further substantiated. Precursor proteins of 2000 to 6000 higher molecular weight of α-, β- and γ-subunits of adenosine triphosphatase, subunit V of cytochrome bc_1 complex, cytochrome c_1 and cytochrome c peroxidase of *Sacch. cerevisiae* have found to be synthesized in the cytoplasm. Transfer into the mitochondria is independent of translation, requires ATP and is accompanied by proteolytic processing (for reviews, see Schatz, 1979; Nelson *et al.*, 1979). In contrast to Poyton and McKemmie (1979a, b), who reported a polyprotein precursor of the four nuclearly coded cytochrome c oxidase subunits (see Section IV.A., p. 299), Lewin *et al.* (1980) found single individual precursor proteins of subunits IV, V and VI of the enzyme.

Experiments of Finkelstein and Strausberg (1979) and Ciejek and Thorner (1979) suggest proteolysis as an essential mechanism to release *Sacch. cerevisiae a* mating type

cells from G 1 cell-cycle arrest by the mating hormone α-factor. Mating type *a* cells degrade α-factor into discrete fragments, and initial endoproteolytic cleavage of the tridecapeptide between leu_6 and lys_7 was shown (Ciejek and Thorner, 1979). These authors discuss their findings in relation to the work of Maness and Edelman (1978), who suggested proteolysis necessary for α-factor activation (see Section IV.A., p. 303).

Glucose-induced inactivation of cytoplasmic malate dehydrogenase, fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase of *Sacch. cerevisiae*, which is proposed to be a proteolytic process, is not dependent on the presence of proteinase B-, carboxypeptidase Y- and carboxypeptidase S activities (D. H. Wolf and C. Ehmann, unpublished observations). Also, loss of antibody cross-reacting phosphoenolpyruvate carboxykinase protein, which parallels loss of enzyme activity during glucose-induced inactivation, is not impaired when proteinase B, carboxypeptidase Y and carboxypeptidase S are absent (Müller *et al.*, 1980). Thus, if the primary inactivation process is a proteolytic event, other proteinases must be responsible. This conclusion applies likewise to degradation of some inactivated phosphoenolpyruvate carboxykinase protein. Recently evidence accumulated, showing that, at least in some cases, a modification of the enzyme to be inactivated, with concomitant loss of activity preceeds final proteolytic degradation. Catabolite inactivation of fructose-1,6-bisphosphatase of *Sacch. cerevisiae* was shown to be initially reversible. The reversibility is independent of protein synthesis (Lenz and Holzer, 1980). Initial inactivation of NAD^+-dependent glutamate dehydrogenase from *Sacch. cerevisiae* upon glutamate starvation was found to be due to phosporylation of the enzyme. Antibody cross-reacting enzyme protein was found to disappear with a considerable lag period after onset of enzyme inactivation (Hemmings, 1980).

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