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Review

Keyhole limpet hemocyanin (KLH): a biomedical review

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Abstract

In this review we present a broad survey of fundamental scientific and medically applied studies on keyhole limpet hemocyanin (KLH). Commencing with the biochemistry of KLH, information on the biosynthesis and biological role of this copper-containing respiratory protein in the marine gastropod *Megathura crenulata* is provided. The established methods for the purification of the two isoforms of KLH (KLH1 and KLH2) are then covered, followed by detailed accounts of the molecular mass determination, functional unit (FU) structure, carbohydrate content, immunological analysis and recent aspects of the molecular genetics of KLH. The transmission electron microscope (TEM) has contributed significantly to the understanding of KLH structure, primarily from negatively stained images. We give a brief account of TEM studies on the native KLH oligomers, the experimental manipulation of the oligomeric states, together with immunolabelling data and studies on subunit reassociation. The field of cellular immunology has provided much relevant biomedical information on KLH and has led to the expansion of use of KLH in experimental immunology and clinically as an immunotherapeutic agent; this area is presented in some detail. The major clinical use of KLH is specifically for the treatment of bladder carcinoma, with efficacy probably due to a cross-reacting carbohydrate epitope. KLH also has considerable possibilities for the treatment of other carcinomas, in particular the epithelially derived adenocarciomas, when used as a carrier for carcinoma ganglioside and mucin-like epitopes. The widespread use of KLH as a hapten carrier and generalised vaccine component represent other major on-going aspects of KLH research, together with its use for the diagnosis of Schistosomiasis, drug assay and the treatment of drug addiction. Immune competence testing, assessment of stress and the understanding of inflammatory conditions are other areas where KLH is also making a useful contribution to medical research.

Keywords: Gastropod; Mollusc; Keyhole limpet hemocyanin; KLH; Didecamer; Electron microscopy; Biochemistry; Hapten carrier; Bladder carcinoma; Immunotherapy; Adjuvant; Vaccine

Contents

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1. Introduction and historical background

Biomedical interest in the high molecular mass hemocyanin of the marine mollusc, the giant keyhole limpet *Megathura crenulata* (commonly abbreviated as KLH) goes back more than 30 years, to the time when this copper-containing extracellular respiratory protein was first found to possess remarkable immunostimulatory properties in experimental animals and man (e.g. Curtis et al., 1970, 1971; Herscowitz et al., 1972; Weigle, 1964). KLH was even introduced as a test for human immunocompetence in 1967 (Swanson and Schwartz, 1967). Both the humoral and cellular immune responses are activated by KLH, and interestingly, in non-immunised people KLH-binding lymphocytes (Hersh and Dyre, 1974) and cross-reacting IgG antibody with avidity for KLH are already present (Burke et al., 1977; Moroz et al., 1973).

In addition, the use of KLH as a hapten carrier for small molecules, such as many chemicals (e.g. 2,4-dinitrophenol [DNP] and 2,4,6-trinitrophenol [TNP]), drugs, hormones, peptides, polysaccharides, lipids and oligonucleotides, against which it has often proved to be difficult or impossible to raise polyclonal antibodies, rapidly extended the applications of this protein within the broad field of immunobiology and immunochemistry. Perhaps not unexpectedly, tumour biologists and oncologists dealing with experimental tumour immunotherapy, via non-specific or active specific stimulation of the immune system, have also investigated the properties of KLH and KLH-conjugates. Thus, the possible benefits of KLH for the treatment of superficial bladder carcinoma in experimental animals and man emerged as early as 1974 (Olsson et al., 1974). It was also discovered that KLH has a cross-reacting carbohydrate epitope with one on *Schistosoma mansoni* (Dissous et al., 1986; Grzych et al., 1987), the trematode parasite causing bilharziosis.

The ready availability of *Megathura crenulata*, native to the Pacific coast of California and Mexico, the relatively large volume of hemocyanin-rich hemolymph that can be obtained from individual animals (compared to many other small marine and terrestrial molluscs) and the simple fact that in earlier studies this rather than any other hemocyanin was used, rapidly led to the inclusion of a research grade KLH product within the lists of a number of chemical suppliers. Subsequently, there followed the establishment of large-scale processing facilities for the production of a safe KLH-subunit clinical product, within Europe (biosyn Arzneimittel GmbH, *Immucothel*®/*Immunocyanin*) and later in the USA (Organon Technica, *KLH-ImmuneActivator*®). In addition, the biosyn company market the KLH-subunit pharmaceutical cGMP grade product VACMUN®, specifically as a carrier for a wide range of antigens.

Despite these advances, fundamental knowledge of the protein structure of KLH has lagged behind for many years. Two different subunit types were observed (Senozan et al., 1981), but the fundamental fact that there are indeed two independent oligomeric isoforms of KLH emerged much later (Markl et al., 1991b; Savel-Nienann et al., 1990); the currently used terminology “KLH1 and KLH2” was first introduced in 1994 (Gebauer et al., 1994).

For all the topics introduced briefly above, progress through the 1980s and early 1990s has been steady, if slow, with a marked acceleration in the quantity of both fundamental and clinically applied data published throughout the past five years. It is the intention of this review to expand upon these topics, firstly from a fundamental scientific point of view and then from a more applied/biomedical stance. The less than total success of the established immunotherapy for superficial bladder carcinoma with bacillus Calmette-Guérin (BCG), and cytotoxic/cytostatic chemotherapy with thioglucid and mitomycin C, usually following transurethral resection (TUR), and the more experimental use of other agents such as adriamycin, mitoxantron and thriotepa has given considerable credibility to clinical trials using KLH, from which it has emerged that immunotherapy with KLH is equal if not superior to BCG, and with far fewer side effects.

2. Biochemistry of KLH

Since the discovery of the two subunit isoforms of KLH (Markl et al., 1991b; Savel-Niemann et al., 1990; Senozan et al., 1981), then defined on the basis of the different

Table 1
Summary of biochemical and structural studies on KLH

Biosynthesis	In connective tissue "pore cells"
Purification of KLH1 and KLH2	Gel filtration and ion-exchange chromatography, electrophoresis + TEM of the two isoforms.
Proteolytic cleavage of KLH1 and KLH2	SDS-PAGE + TEM of reassociation products.
Protein biochemistry of KLH1 and KLH2	Functional Unit sequence, HPLC, immunoelectrophoresis, SDS-PAGE.
Carbohydrate biochemistry of KLH1 and KLH2	Monosaccharide components, N- and O-linkages to peptide chain. Oligosaccharide sequences.
Immunological analysis of KLH1 and KLH2	Definition of FU epitopes with mAbs. Immunoelectrophoresis and Immuno-EM.
Molecular biology of KLH1 and KLH2	Gene sequence and cloning of the two isoforms.
Oligomeric composition of KLH1 and KLH2. Control of oligomerisation and polymerisation	TEM of purified isoforms, and experimentally manipulated oligomeric and polymeric states.
Subunit reassociation studies with intact and proteolytically cleaved KLH1 and KLH2	TEM of reassociation products. Assessment of subunit–subunit interactions.
Quaternary (3D) structure of KLH1 and KLH2	High resolution TEM, single particle and 2D crystal digital image analysis.
Atomic structure of KLH1 and KLH2	X-ray crystallograpy of functional units and intact proteins.
Oxygen binding and release	Oxygen dissociation curve, oligomerisation and cooperativity.

electrophoretic migration rate of the two subunits in a non-SDS PAGE system following high pH dissociation, much biochemical work has been performed. The terms KLH1 and KLH2 (Gebauer et al., 1994) have become the standard nomenclature and will be used throughout this review, although the isoform terminology KLH-A/KLH-B has also been proposed (Swerdlow et al., 1996). Throughout the biochemical and electron microscopical sections it will be seen that considerable interplay between these techniques is emphasised, indeed the two approaches are mutually supportive, as indicated in Table 1.

2.1. Biosynthesis of KLH

In some pulmonate gastropods hemocyanin biosynthesis occurs in the connective tissue "pore cells", characteristically large cells with much endoplasmic reticulum and a distinctive fenestrated/pore surface, found widely dispersed throughout the tissues. Within these cells hemocyanin has been found to accumulate within the endoplasmic reticulum cisternae as intact cylindrical macromolecules and even forms intracellular crystalline arrays (Sminia, 1977). In tissues taken from the keyhole limpet, a prosobranch gastropod, pore cells have also been detected; however, it has not yet been possible to define intact KLH within these cells, but a diffuse accumulation of protein has been shown by electron microscopic study of thin sectioned tissue (Albrecht, 1996) and a positive signal for KLH was seen in these cells by immunolabelling at the light microscope level. The difficulty encountered here, with respect to the in situ visualisation of KLH, appears to be in accord with the difficulties faced during attempts to isolate hemocyanin-specific messenger RNA from the tissues of animals taken freshly from the Pacific ocean and from captive animals held for a period of weeks in a salt water aquarium (see below). Some evidence for the poor viability of keyhole limpet pore cells has been obtained, compared to pore cells from another marine mollusc, the abalone *Haliotis tuberculata* (Albrecht, 1996). Initially it appeared that hemocyanin biosynthesis in the keyhole limpet may be rapidly halted by subjecting the animals to the stress of removal from their natural environment. However, we now have reasons to believe that in this animal hemocyanin may be actively synthesised for very short periods, with long resting periods in between.

2.2. Biological role and the copper-containing functional units (FUs) of KLH

Oxygen uptake, transport and release is the function of the hemolymph extracellular hemocyanins, all of which are known to be oligomeric assemblies of one or more subunit types (Salvato and Beltramini, 1990). For the molluscan hemocyanins, each functional unit (FU) within the protein subunit contains a binuclear copper binding site that can bind molecular oxygen (O_2). The copper is in a cuprous state and oxygen binding generates light absorbance around 340 nm, responsible for the characteristic blue colour of oxygenated hemocyanins. The peptide sequence surrounding the two copper binding sites (termed A and B) is highly conserved, with three copper-liganding histidines at both sites. For *Octopus dofleini* hemocyanin, it has been possible to obtain by trypsin cleavage the FU *g*, followed by purification and crystallisation, from which the X-ray structure has been obtained (Cuff et al., 1998). For KLH, much N-terminal sequence analysis has been obtained on individual FUs and groups of FUs, but to-date it has proved to be difficult to isolate and purify the individual FUs in sufficient quantity for crystallisation. Fig. 1 shows the X-ray structure of the FU *g* from *Octopus dofleini* hemocyanin (Cuff et al., 1998), which has a high sequence homology to the FU *g* of both KLH1 and KLH2. Major differences have, however, been detected between arthropod and molluscan hemocyanins, which are currently considered to represent different multigene superfamilies, but both appear to be related in evolutionary terms to the tryosinases and the molluscan hemocyanins also exhibit a weak peroxidase activity.

Progress on the relationship between hemocyanin oligomer formation and reversible oxygen-binding behaviour and cooperativity (i.e. the Bohr effect) of KLH is still at an early

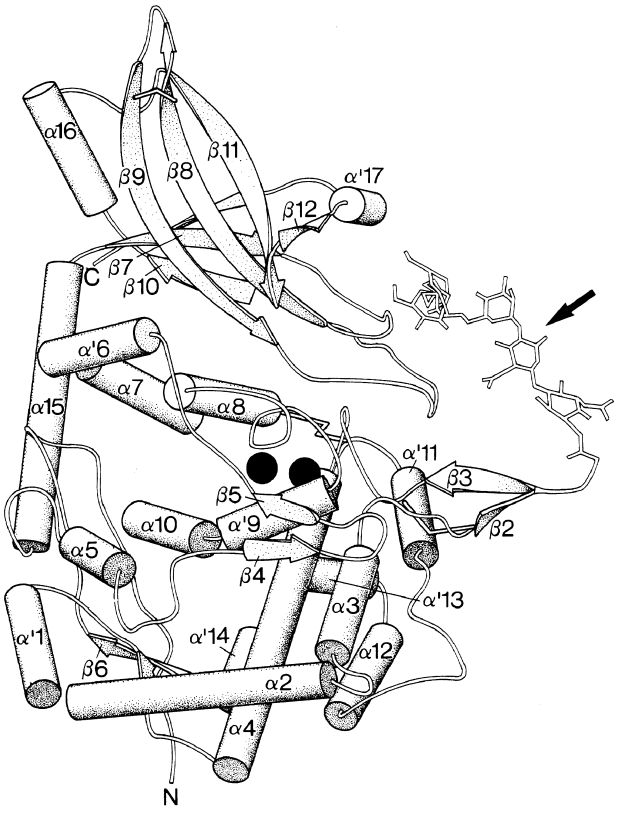

Fig. 1. The X-ray structure of *Octopus dofleini* hemocyanin functional unit *g*, the only currently available high resolution data from a molluscan hemocyanin. The location of the two copper atoms is indicated by the dark spheres. The attachment of a carbohydrate side-chain is indicated by the arrow. The carboxy- and amino-terminus of the polypeptide chain is indicated by C and N, respectively. Redrawn from Cuff et al. (1998).

stage. In view of the recent availability of the two KLH isoforms in a highly purified state and ultrastructurally defined oligomerisation states, it can be anticipated that an increasing number of physiological and biophysical studies using KLH will be undertaken in the near future.

2.3. Purification and oligomeric states of KLH1 and KLH2

Purification of KLH is routinely monitored by analytical non-denaturing native gel electrophoresis of the dissociated subunits of KLH1 and KLH2 (SDS-PAGE is not useful here, because the molecular mass of the two subunits is very close: ca. 390 kDa for KLH1 versus ca 360 kDa for KLH2 (Söhngen et al., 1997)). Preparative native electrophoresis has been used to purify the two subunits (Harris et al., 1997b; Savel-Niemann et al., 1990), but most efforts have been directed towards the purification of the intact molecules. The ability to assess the KLH oligomerisation state, molecular integrity and presence of contaminants by TEM of negatively stained specimens at the start and throughout any purification procedure adds considerably to the validity of the data. As a first step in the purification of KLH, centrifugal pelleting has become standard. This differential centrifugation enables the high molecular mass hemocyanin to be separated from the other lower molecular mass hemolymph proteins, most of which remain in the supernatant. Centrifugation of cell-free hemolymph provides quantitative recovery of total hemocyanin, which can be suspended as a very high concentration KLH solution and is extremely convenient for further biochemical work; at this stage it should not be considered to be a highly purified protein, but cf. Swerdlow et al. (1996). The ratio of KLH1 to KLH2 varies considerably in the pelleted hemocyanin from individual animals. An average of ca. 1:2 for KLH1:KLH2 has been obtained from 325 animals (Harris et al., 1997a). In practise it can be readily appreciated that the development of successful biochemical purification of intact KLH has been heavily dependent upon two techniques for monitoring the various separations: (i) transmission electron microscopy (TEM); and (ii) crossed immuno-electrophoresis (IE), be this achieved by ion exchange or gel filtration column chromatographic procedures. Early indications showed that animals held captive in saltwater aquaria for a period of months slowly lost most if not all of their KLH1. This enabled studies to be performed on a naturally occurring highly enriched population of KLH2 molecules, as in some animals KLH1 becomes undetectable. The health of such animals often appeared to be compromised, they do not feed well and no explanation is currently available as to why the KLH1 is selectively metabolised by the limpets. The possibility exists that these animals are in severe negative protein balance and that they are utilising the KLH1 to physiologically conserve amino acids, as well as the copper atoms.

Gel filtration chromatography of the mixed population of KLH1 and KLH2 oligomers yields a mass-dependent fractionation, but little separation of the two KLH isoforms (Harris et al., 1992; Markl et al., 1991b; Savel-Niemann et al., 1990). For both types of KLH the didecameric oligomer usually predominates. KLH1 also forms random didecamer clusters and KLH2 has a tendency to form elongated stacked-decamer oligomers/multimers, which are usually termed multidecamers. Gel filtration chromatography does not have the potential to discriminate between these higher oligmeric forms, and indeed under in vitro conditions inherent dynamic instability of the KLH2 didecamers leads to the formation of single decamers, in turn promoting a progressive increase of the number and length of the multidecamers during prolonged storage. When gel filtration chromatography is applied to the KLH2 taken from animals depleted with respect to KLH1, with the above caution in mind, it does nevertheless have the potential to produce a mass-dependent separation of the KLH2 multidecamers, didecamers and decamers (Harris et al., 1993).

A most important breakthrough occurred in 1995, when we came to some understanding of the confusing stability properties of total KLH in the presence of the ammonium molybdate solution used for the preparation of TEM specimens (Harris et al., 1995b). It was found that at pH

7.0 the KLH2 multidecamers had a tendency to dissociate into decamers (Harris et al., 1992; Markl et al., 1991b), and that when the pH was reduced further to the range 5.7–5.9 the KLH2 dissociated into subunits, whereas the KLH1 (individual didecamers and didecamer clusters) remained intact. This provided us with the possibility to achieve separation of the two types of KLH by gel filtration chromatography in the presence of ammonium molybdate, with the KLH1 eluting in the three leading peaks (as didecamer clusters, pairs of didecamers and didecamers) followed by a fourth peak containing the KLH2 subunit (Harris et al., 1995b). This purification procedure was also found to be successful with total KLH reassociated from the commercially available subunit product *Immucothel*®, following reassociation of both KLH isoforms in the presence of a low or high concentrations of calcium and magnesium ions (Harris et al., 1997a, 1998) (see Section 3.4).

Herscowitz and colleagues indicated that ion exchange chromatography on DEAE–cellulose has the potential to fractionate total KLH (Herscowitz et al., 1972), but at this early stage the two isoforms of KLH had not yet been defined. Indeed this alternative purification procedure, using ion exchange HPLC to separate KLH1 from KLH2 with both molecules in an intact oligomeric state, has proved to be difficult to control and establish as a reproducible method (Swerdlow et al., 1996), supported also by our own unpublished data. Whilst it is possible to achieve a good separation of the KLH2 by HPLC, there is some carry-over of the leading KLH2 peak into the following KLH1 peak. The adsorption of both KLHs, which are relatively fragile macromolecules, onto the ion exchange column matrix and release by elution with an increasing salt concentration could generate molecular instability. Re-chromatography of the KLH1 through the HPLC column can produce higher purity, but this then becomes an extremely long procedure. Nevertheless, for bulk separations (ie gram quantitites) both ion exchange and gel filtration chromatography have considerable potential for the purification of KLH.

2.4. Molecular mass determinations with KLH

Although analytical ultracentrifugation has been widely used for the determination of the sedimentation coefficient (from sedimentation velocity analysis) and molecular weight (from sedimentation equilibrium analysis) of molluscan hemocyanins (Van Holde and Miller, 1995), this technique does suffer from a lack of sensitivity, particularly in any system where a range of similar oligomeric/multimeric states is present, together with some dynamic instability and/or aggregation of the molecules. Early indications from KLH showed the presence of multimeric forms (Herscowitz et al., 1972), with predominant species sedimentating at 15S, 46S, 88S and 116S, but without any corresponding TEM images. Sedimentation coefficient values of 67S and 61S for the KLH1 and KLH2 decamer were established (Swerdlow et al., 1996), with 100S and 105S for the KLH1 and KLH2 didecamers. These values appear to be somewhat mutually contradictory, suggestive of the inherent limitation within the accuracy of this approach. For the KLH1 and KLH2 subunits, figures in the range 11S–13S were obtained (Swerdlow et al., 1996). After reassociation, the picture emerging from ultracentrifugation was even more confusing, further indicating the limitations of this approach, particularly when no parallel TEM data on the samples was presented. On the other hand, if purified and stable oligomers are subjected to analytical ultracentrifugation, accurate sedimentation coefficient and molecular weight data are likely to be obtained. Mass determination obtained from unstained dark-field images of KLH1 and KLH2 (subunits and didecamers) by scanning transmission electron microscopy (STEM) (Söhngen et al., 1997) provided quantitative data which correlates well with the sedimentation coefficient values, and at the same time confirms the presence of several oligomeric forms. Light scattering has also been used for the study of hemocyanins and their subunits (Van Holde et al., 1991), and in combination with STEM for assessment of the oligomerisation states (Hamilton et al., 1989). As with ultracentrifugation, light scattering is an indirect physical approach that has considerable limitations for the study of complex mixtures, but is very useful for the study of solutions of highly purified monomeric species. Mass spectrometry has recently been introduced for the determination of accurate molecular mass, for both low and high molecular mass proteins (e.g. Martin et al., 1996), but this technique also requires samples of high purity and stability; it is likely to be of considerable value for present and future studies on molluscan hemocyanins, subunit fragments and individual FUs (Stoeva et al., 1997). Molecular mass determination of the subunit, single FU (usually between 45 and 50 kDa) and FU multimers by SDS-PAGE has provided accurate values, which correlate well with values from STEM (Gebauer et al., 1994; Söhngen et al., 1997). From all this data it appears that the molecular mass of the KLH2 didecamer (7.5 MDa) is somewhat lower than that of KLH1 (8.3 MDa), in spite of the presence of eight FUs in each KLH subunit. This mass difference has been convincingly shown by independent methods (Swerdlow et al., 1996).

2.5. Functional unit (FU) structure of KLH1 and KLH2

The recent availability of purified KLH1 and KLH2 has enabled considerable analytical progress to be made into the subunit organisation of the two protein molecules. Gebauer and colleagues presented the first major contribution within which the molecular analysis of KLH2, obtained from KLH1-depleted animals held in aquaria, was described (Gebauer et al., 1994). This work drew significantly upon limited proteolytic cleavage of the 360 kDa subunit by bovine trypsin and *Staphylococcus aureus* V8 protease into multi-FU subunit fragments and into the individual

FUs by bovine elastase, followed by their immunochemical analysis (see Section 2.7). The molecular masses of the FUs and the multi-FUs were determined by SDS-PAGE together with their N-terminal amino acid sequences. Interpretation of this data at that time led to the definition of the seven FU sequence (*abcdefg*) within the subunit. Comparison of the KLH2 subunit and FU amino acid N-terminal sequence data with that available from other molluscan hemocyanins revealed considerable similarity (Gebauer et al., 1994). Independent supportive information has also been presented (Swerdlow et al., 1996). On the basis of copper analyses, these authors did, however, suggest that KLH2 contains eight FUs. A recent reassessment of our own data has now shown that under our isolation conditions, the FU *h* has been overlooked because of rapid degradation during the proteolysis with elastase (our currently unpublished data). Analysis of HPLC-purified proteolytic cleavage products from KLH1 (Söhngen et al., 1997) by SDS-PAGE and N-terminal sequence determination has now provided a complete data set for the molecular mass of the eight KLH1 FUs. Comparison of the N-terminal sequences of the individual FUs of KLH1 and KLH2 with those of other known molluscan hemocyanins (Söhngen, 1993) emphasises the presence of the common N-terminal sequence motif (–VRKDVXXL–), which is a structural characteristic for all these FUs.

2.6. Carbohydrates of KLH

Knowledge of the oligosaccharides of KLH is limited, but it is widely acknowledged that these sugar components are likely to be of prime significance for the antigenicity of the intact molecules, along with purified FUs or peptides that may be available from genetic engineering in the future. The carbohydrate content of total KLH was calculated to be ca. 4% of the molecular mass (van Kuik et al., 1990), and similar figures have been obtained for other molluscan hemocyanins (Stoeva et al., 1997). A significantly higher carbohydrate figure (9%) has been obtained for the Roman/vineyard snail *Helix pomatia* hemocyanin, which correlates with the higher molecular mass of ca. 55 kDa usually obtained by SDS-PAGE for the individual *Helix pomatia* hemocyanin FUs. By gas liquid chromatography, mannose, galactose, *N*-acetylglucosamine and *N*-acetylgalactosamine were shown to be present in KLH1 and in addition to these four carbohydrates, fucose was also detected in KLH2 (Stoeva et al., submitted). Three different lectins (concanavalin A [Con A] indicative of N-glycosides, peanut agglutinin [PNA] indicative of Gal(β1-3)GalNAC as the core complex of O-linked glycosides and *Amaranthus caudatus* agglutinin [ACA] indicative of the α-anomer of Gal(β1-3)GalNAc) were used to identify the saccharide components and carbohydrate–peptide linkages of KLH.

Using KLH1 multi-FU subunit fragments and single FUs, by immunoblotting Con A (which is specific for α-mannose, the main carbohydrate component of N-linked glycosides) was found to bind to FUs 1*ab*, 1*de*, 1*fg* and 1*h*, indicating the presence of N-linked glycosides. The lectin PNA did not bind to these KLH1 FUs, indicating the absence of O-linked glycosides. FU 1*c*, on the other hand, did not bind Con A, PNA or ACA, indicating the absence of carbohydrates.

For KLH2, by immunoblotting ConA was found to bind to all multi-FU subunit fragments and single FUs 2*a*, 2*b*, 2*d*, 2*e*, 2*f*, 2*g*, and 2*h*, but not to FU 2*c*, indicating that this FU does not contain any N-glycosylated carbohydrate chains. This is supported by its polypeptide primary structure (see Section 2.8). However, FU 2*c* was the only FU of KLH2 to bind PNA, and this FU did not bind lectin ACA, indicating that the core complex of this oligosaccharide must be the β-anomer of Gal(β1-3)-GalNAc. Lectins PNA and ACA did not bind to FUs 2*a*, 2*b*, 2*d*, 2*e*, 2*f*, 2*g* and 2*h*, indicating the lack of O-linked glycosides in all these FUs of KLH2.

The multiplicity of the monoantennary and diantennary molluscan hemocyanin N-linked oligosaccharide chains is indicated by the stuctural analysis of the α_D-hemocyanin of *Helix pomatia* (Lommerse et al., 1997). Although our molecular mass and sequence data indicate that the situation in KLH is less complex, it can be anticipated that it may be several years before the full structure of the KLH1 and KLH2 oligosaccarides will be available. Nevertheless, detection of the β-anomer of Gal(β1–3)GalNAc supports the data of Wirguin and colleagues who considered this to be the cross-reacting epitope of KLH essential for its efficacy as an immunotherapeutic agent for the treatment of bladder carcinoma (Wirguin et al., 1995).

2.7. Immunological analysis of KLH

In view of the importance of immunoelectrophoresis and immunoblotting for the structural analysis of hemocyanins, including KLH, some further comment on these techniques is appropriate. The long-established immunoelectrophoresis of hemocyanins and other water-soluble proteins in agarose gel (Herscowitz et al., 1972; Kroll, 1973; Weeke, 1973) using polyclonal antibodies has been an indispensable tool to provide the current understanding of the antigenic individuality of the two KLH isoforms, initially with respect to the subunit structure of KLH1 and KLH2 (Markl et al., 1991b; Savel-Niemann et al., 1990) and then for the identification and linear sequence determination of the eight FUs of the two subunits (Gebauer et al., 1994; Söhngen et al., 1997). Although formation of immunoprecipitation patterns requires much more polyclonal antibody than does immunoblotting, in the present context it is superior to the latter, because it allows direct estimation of the structural relationship of the applied proteins. To date, crossed immunoelectrophoresis is still the only method which allows visualisation of the full set of different FUs in a molluscan hemocyanin. From all these experimental approaches it is very clear that the eight FUs of both KLH isoforms are very different structurally, which means that total KLH represents a cocktail of 16 immunologically distinct FUs. Indeed,

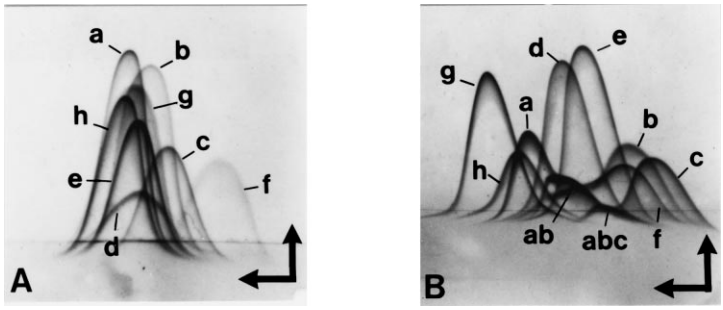

Fig. 2. The two-dimensional immunoelectrophoretic pattern of elastase-cleaved (A) KLH1 and (B) KLH2. For KLH1, the subunit has been completely split into the eight FUs (*abcdefgh*). For KLH2 the *ab* FU-dimer and *abc* FU-trimer are also present along with the eight individual FUs. For full interpretation of this data see Gebauer et al. (1994) and Söhngen et al. (1997).

sequence analysis of Octopus hemocyanin (Miller et al., 1998) has revealed differences of about 50% in the primary structure of the different FUs of this molluscan hemocyanin. Fig. 2 shows the immunoelectrophoretic analysis of elastase-cleaved KLH1 and KLH2 subunits, with definition of the eight FUs (*abcdefgh*) for both isoforms. Once characterised, the potential of monoclonal antibodies as reagents for the further immunological analysis within electrophoretic and blotting systems is considerable. They are also of value for the production of immune complexes in solution, from which epitope locational analysis of the native molecules can be performed by defining in TEM images the position on the molecules where the MAb is binding (Harris, 1996; Harris et al., 1993) (see Section 3.2).

2.8. Molecular genetics of KLH

In order to isolate cDNA clones coding for KLH1 and KLH2, cDNA expression libraries have been synthesised. For the construction of oligo(dT)- and randomly primed libraries, RNA from several different tissues from *Megathura crenulata* (e.g. mantle and mid-gut gland) and different developmental stages of freshly captive animals were used. The screening of several differently constructed cDNA libraries was performed with different monoclonal and polyclonal antibodies, mixed or alone, but no cDNA encoding KLH was isolated. However, parallel studies using the closely related mollusc *Haliotis tuberculata* yielded the complete coding regions for both hemocyanin isoforms (HtH1 and HtH2) (Keller et al., 1997). Furthermore, the analyses of the HtH genes elucidated the entire genomic organisation of these hemocyanins (Lieb et al., to be published). After having correlated all this information, genomic DNA from *Megathura crenulata* was isolated and investigation of the KLH genes by PCR is currently in progress. One future goal is to express single FUs or the whole subunit in bacteria or eukaryotic cell lines. From such cloned proteins or peptide fragments it should ultimately be possible to produce crystals suitable for X-ray crystallography and the elucidation of the high resolution 3D structure of the two isoforms of KLH. Another future aim of this work is to establish a cell lineage from *Megathura* expressing KLH, in order to exclude artefacts introduced by expression of a non-endogenous protein in cells from another organism. Currently, attempts are being made to characterise the factors responsible for the exclusive expression of KLH in specific tissues from *Megathura crenulata*.

3. Transmission electron microscopical study of KLH

Despite the ready availablility of KLH and the large quantity of early immunological data, study of this hemocyanin by transmission electron microscopy (TEM) lagged considerably behind the hemocyanins from a number of other molluscs (Fernandez-Moran et al., 1966; Van Bruggen et al., 1962a,b). Significantly, in 1972 this TEM work led to the production of a 3D reconstruction of the *Busycon canaliculatum* hemocyanin didecamer (Mellema and Klug, 1972) and to many studies on the hemocyanin from *Helix pomatia* and from other molluscs (Ghiretti-Magaldi et al., 1979; Van Bruggen, 1983). The first negatively stained TEM images of KLH appeared only in 1990 (Savel-Niemann et al., 1990). Subsequently, TEM studies have contributed significantly to the structural characterisation of the two isoforms of KLH, their oligomerisation states and polymerisation properties.

3.1. The native oligomers of KLH1 and KLH2

Whilst most samples of total KLH freshly isolated from individual animals contain a mixture of the two isoforms of KLH, the availability of some animals that were almost or totally depleted with respect to KLH1 has enabled an understanding of the complex mixture of oligomeric forms to be obtained. These KLH samples enriched with KLH2 were found to contain greater numbers of the elongated multidecamers. Size-dependent gel filtration chromatography (in the presence of stabilising buffer) of the intact oligomers present when there is a mixture of KLH1 and KLH2 (Harris et al., 1992; Markl et al., 1991b), showed that the leading fractions eluted contained a mixture of large multidecamers and didecamer clusters, with the main broad peak of protein

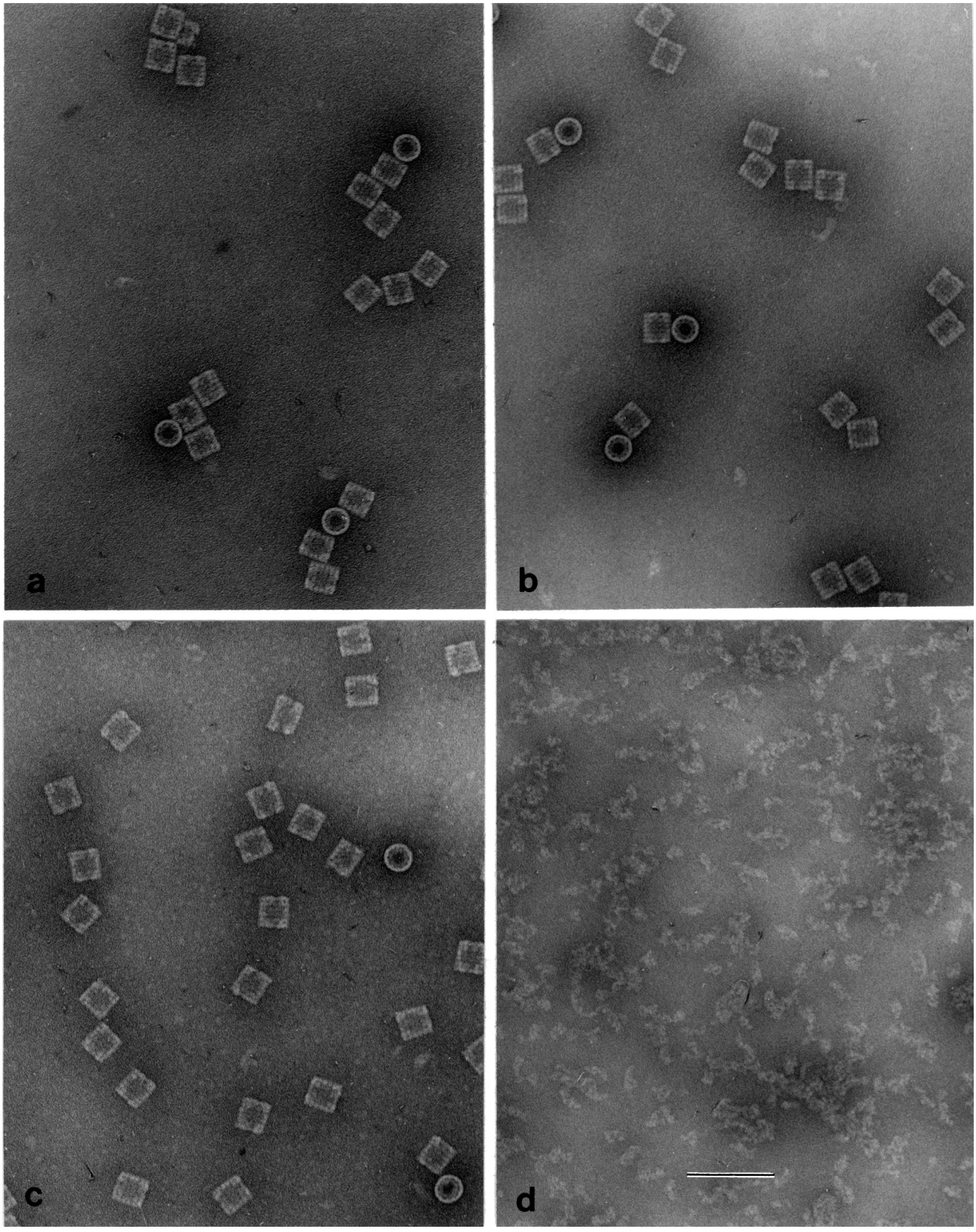

Fig. 3. The purification of KLH1. Separation of KLH1 didecamer clusters (a), doublets (b) and single didecamers (c) from dissociated KLH2 (d) was achieved by gel filtration chromatography, following selective dissociation of the KLH2 in ammonium molybdate at pH 5.7 (Harris et al., 1995b). Specimens were negatively stained with 5% ammonium molybdate (pH 7.0) containing 1% trehalose (Harris et al., 1995a). The scale bar indicates 100 nm.

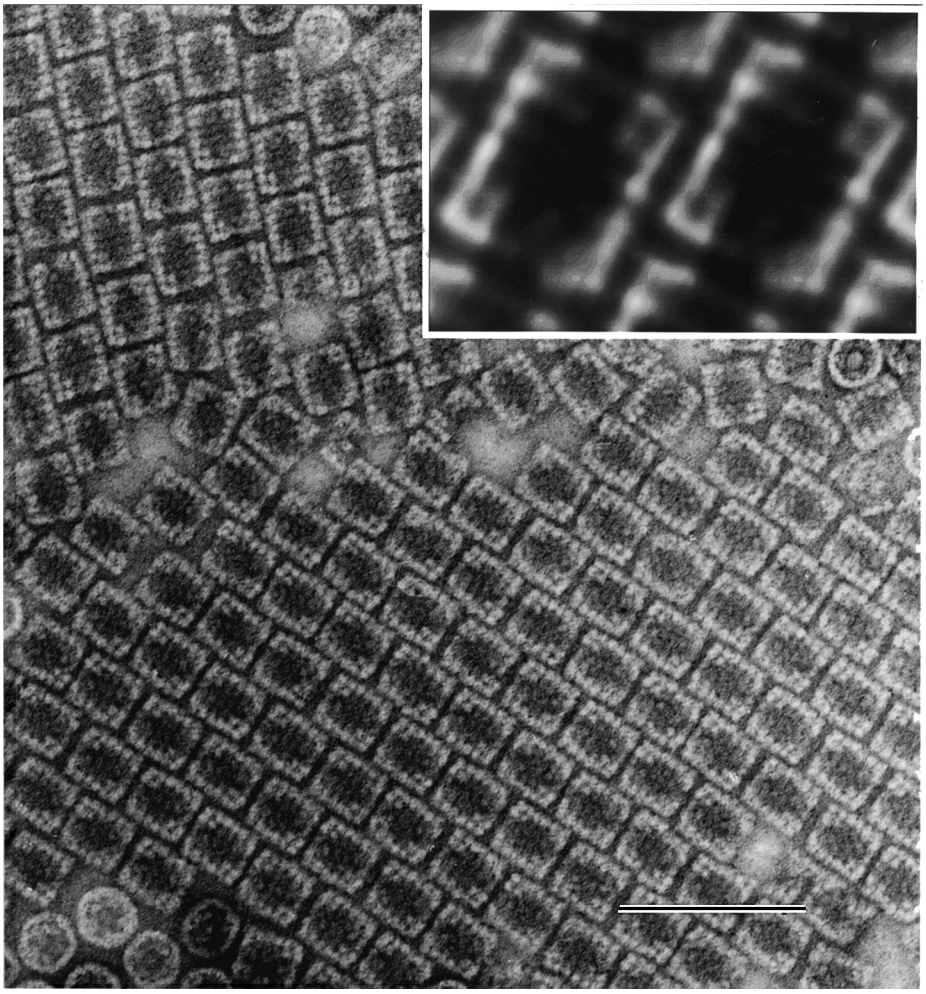

Fig. 4. Two-dimensional (2D) crystallisation of KLH1 didecamers, with production of the 2D projection average (inset) by crystallographic image processing (Harris et al., 1992). The specimen was negatively stained with 2% uranyl acetate. The scale bar indicates 100 nm.

containing didecamers. In all fractions, a mixture of the two types of KLH was present. On the other hand, when the KLH2-enriched starting material from older captive animals was subjected to gel filtration chromatography (Harris et al., 1993), the leading fractions contained only multidecamers, with a broad didecamer peak and decamers eluting last. Some dynamic instability of this fractionated KLH2 was observed, but not documented at the time, in that during in vitro storage the didecamer-rich fractions progressively tended to show the presence of some decamers and multidecamers, suggestive of instability of the didecameric oligomer, but stability of the multidecameric form. The importance of this characteristic feature of KLH2 stability will be expanded upon below.

Following dissociation of KLH2 in ammonium molybdate at pH 5.7 (see Section 2.3), gel filtration chromatography in the presence of this ammonium molybdate solution yields a size dependent separation of the KLH1 didecamer clusters from pairs or didecamers and single didecamers (Fig. 3). The purified subunit of KLH2 elutes as the final material from the column. This KLH2 subunit is suitable for many biochemical and immunological studies and can be reassociated into decamers, didecamers, multidecamers and a tubular polymer, if desired (see Section 3.4).

Using total KLH, we managed to produce a 2D projection average of the didecameric form in negative stain, following 2D crystallisation of the protein on mica in the presence of ammonium molybdate and PEG at pH 7.0 (Harris et al., 1992) (Fig. 4). In retrospect, we now believe this to be the crystallographic average of the KLH1 didecamer. Decamers of KLH2 produced by the pH 7.0 ammonium molybdate-induced splitting of didecamers and multidecamers, formed random 2D arrays (Harris et al., 1992). The first 3D average of the KLH didecamer was produced from total KLH in the unstained, frozen hydrated state (Dube et al., 1995), at a relatively low resolution level (ca. 40 Å), which is thought to represent an approximate mean of KLH1 and KLH2. Subsequently, from purified KLH1 didecamers, it was possible to obtain a 15 Å 3D reconstruction in air-dried negative stain + glucose at low temperature ($\backsim$4 K) (Orlova et al., 1997), from which many detailed features of the

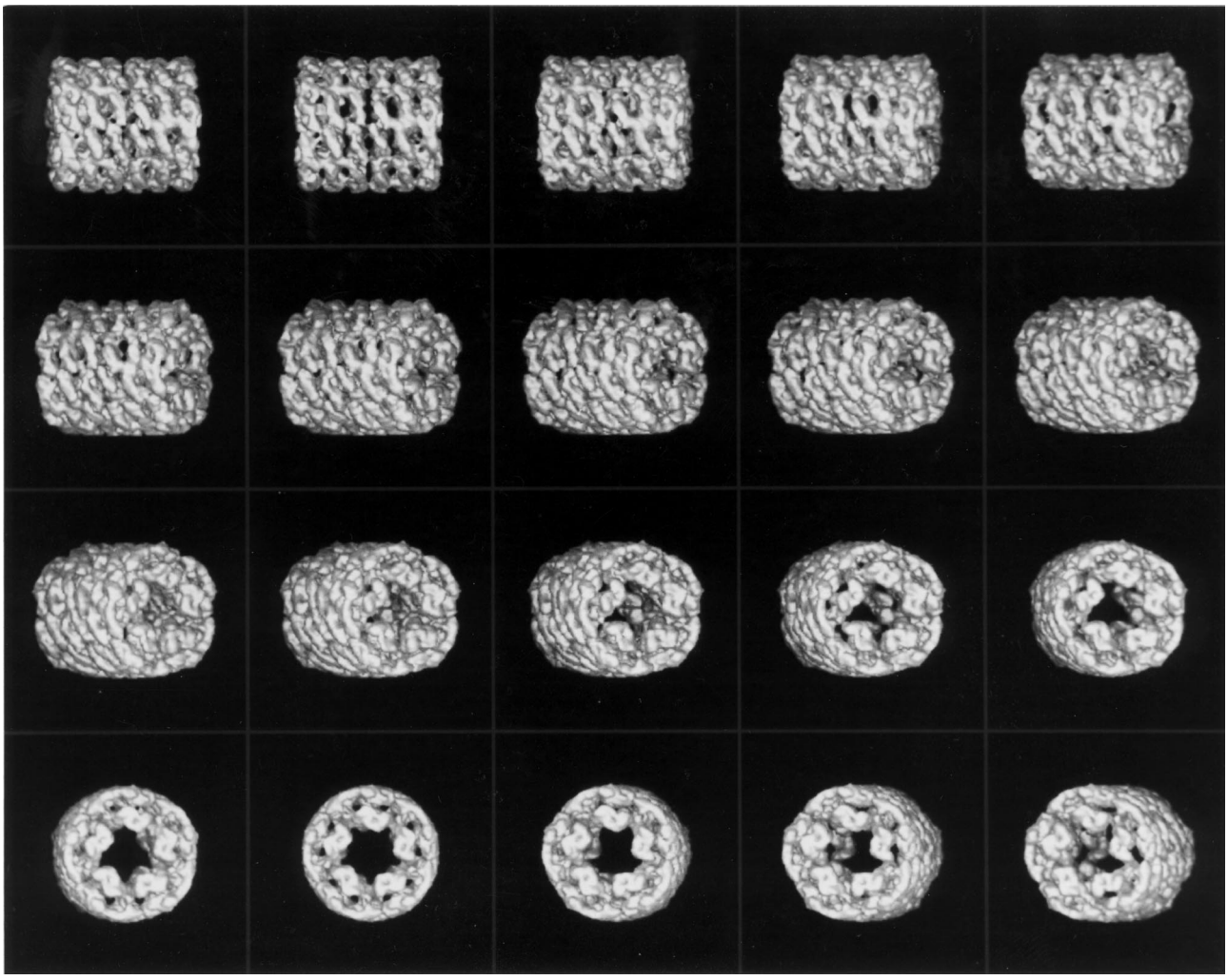

Fig. 5. A continuous stereo array of the 15 Å 3D reconstruction of the KLH1 didecamer, produced by low temperature transmission electron microscopy and angular reconstitution. Courtesy of Elena V. Orlova and see Orlova et al. (1997).

molecule emerge (Fig. 5). However, it is not possible even at this resolution to define the location of individual FUs, subunits or subunit pairs within the cylinder wall of each decamer, but it is clear that the ten masses forming each tier of the cylinder wall each represent a FU pair (often termed morphological units). Moreover, the FU components of the internalised arc and collar components (at each end of the KLH1 didecamer) have been located; yet it is not totally clear that each contains two *g* or two *h* FUs, and indeed if this is the case what it may imply for composition of the wall morphological units. Importantly, many symmetry features within the individual tiers of the didecamer wall are suggestive of a dyad symmetry within the FU pairs. These features are likely to be of importance for the future understanding of the manner in which subunit dimers are formed, which can in turn associate to form a closed pentameric ring (i.e. D5 symmetry) incorporating the ten subunits of the decamer. Whether or not the subunits within the subunit dimers are orientated in a parallel or anti-parallel manner remains to be firmly established. Our earlier immunoelectron microscopy of KLH2 (see Section 3.2) strongly implies that the subunit orientation within the decamer may be parallel, whereas the interpretation advanced by Orlova and colleagues for the KLH1 didecamer suggests that the subunit orientation may be anti-parallel, with complex divalent cation-dependent interactions between the intricately associated subunits (Orlova et al., 1997). Higher resolution TEM studies which permit the fitting of the known X-ray structure of FU *g* from *Octopus* hemocyanin (Cuff et al., 1998) into the TEM structure may help to provide answers to these questions.

3.2. Immunoelectron microscopy of KLH

Using monoclonal antibodies directed against epitopes on individual FUs (defined by immunoelectrophoresis), it has been possible to perform studies on the localisation of FUs within IgG-linked immune complexes of KLH2 (Harris et al., 1993), as shown in Fig. 6(a). This approach carries with it some inherent limitations, in particular when steric

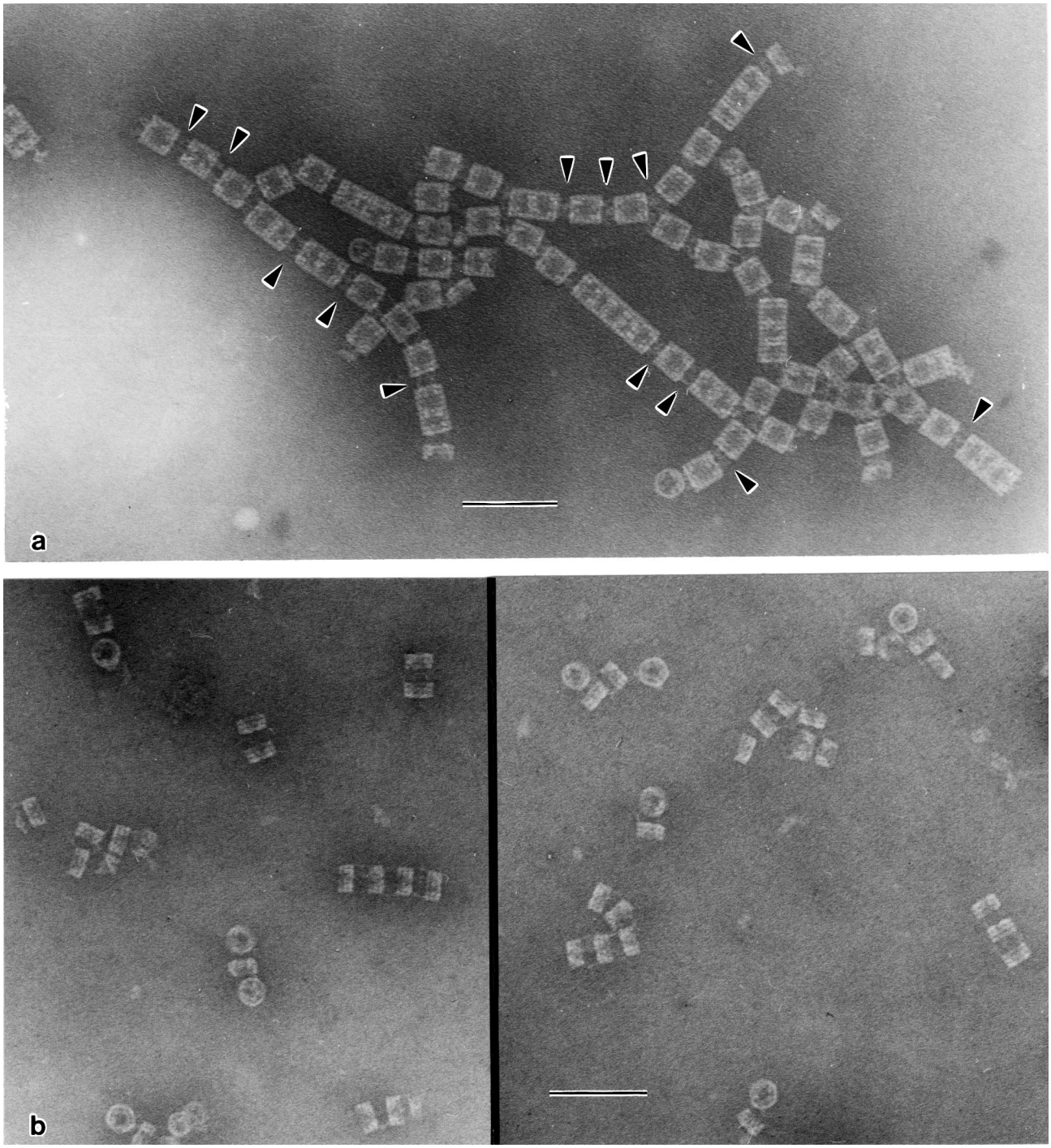

Fig. 6. (a) An immune complex of KLH2 didecamers linked with a monoclonal IgG directed against KLH2 FU-*h* (Harris et al., 1993). (b) Small immune complexes produced from KLH1 decamers following interaction with monoclonal IgG directed against KLH1 FU-*c*. Note that the KLH1 decamers are linked by IgG at *both* the "collar" and "non-collar" edges. Specimens were negatively stained with 5% ammonium molybdate (pH 7.0) containing 1% trehalose. The scale bars indicate 100 nm.

hinderance of an epitope occurs within the overall quaternary structure, and when there is insufficient antibody to occupy all available epitopes. Also, because of the structural similarities between the KLH FUs, some antibodies may recognise epitopes on more than one FU, but this is usually detectable in advance by immunochemistry. In theory, each decamer of KLH1 or KLH2 should have the possibility to bind a maximum of ten monoclonal IgG molecules. If, however, the position of the subunits within the molecule makes five of the ten epitopes inaccessible to the IgG, this fact may not be easily assessed. The use of purified decamers of KLH1 and KLH2 for immunonegative staining

studies could help to avoid anomalies due to this steric hindrance (Fig. 6(b)).

Whilst the use of *monovalent* Fab′ fragments provides the possibility for higher resolution epitope analysis than does use of *bivalent* IgG or (Fab′)2, the mass difference between a single Fab′ fragment (ca. 50 kDa) and a KLH decamer (ca. 4 MDa) is so great that in practice is difficult to detect a single bound Fab′, although one might expect that if the maximum of ten Fab′ fragments were attached per 4 MDa decamer that this would generate a characteristic change to the image of individual decamers, and didecamers. To date, Fab′ labelling has not been convincingly detectable for KLH, although considerable success has been achieved with other hemocyanins (Lamy et al., 1998), but using polyclonal Fab′ fragments.

3.3. Experimental manipulation of the oligomeric states of KLH in vitro

Exactly why the didecamer is the predominant oligomeric form of both KLH1 and KLH2, in vivo and following isolation from hemolymph, is not understood. In all probability this relates to the asymmetric nature of the decamer, with the collar region at one edge of the ring-like molecule. The hemocyanin of Cephalopoda (e.g. octopus and squid) exists as stable decamers, with a central (symmetrical) collar. In addition, the instability of purified KLH2 didecamers, with the formation of single decamers and multidecamers (containing a nucleating didecamer, with decamers added at one or both sides) are further aspects that require explanation. In an attempt to answer such questions, we have established procedures for the production of stable decamers from KLH1 and KLH2, which can then be used for a range of different experimental approaches.

For KLH1, we have found that by simply increasing the pH of the Tris-saline stabilising buffer to pH 8.5, the didecamers split into stable decamers (Söhngen et al., 1997) (Fig. 7(a)). This dissociation can be reversed by dialysis against pH 7.4 stabilising buffer (with 5 mM calcium and magnesium chloride), and dialysis against pH 7.4 stabilising buffer containing 100 mM calcium and magnesium chloride generates multidecamers. It should be noted that KLH1 multidecamers have not been detected in purified KLH1 samples obtained directly from animals; they can, however, also be produced by reassociation of the KLH1 subunit, under these high divalent cation conditions (see Section 3.4). No nucleating didecamer can be defined within these KLH1 multidecamers and there is a structural difference between these and the KLH2 multidecamers, expressed by the varying negative stain distribution along their hollow centres. This is surprising because to date, no convincingly defined structural difference has been detected between didecamers of KLH1 and KLH2, by comparison of computer-processed TEM images. On the other hand, both KLH isoforms consist of different subsets of FUs, and the two subunits are unable to co-assemble to form a hetero-oligomer (Söhngen et al., 1997). On dialysing the KLH1 multidecamers against the standard (5 mM $CaCl_2$ and $MgCl_2$) Tris-saline stabilising buffer, slow breakage occurred, yielding predominatly didecamers (Söhngen et al., 1997). No indication of the formation of randomly linked KLH1 didecamers has been obtained from these in vitro experiments; these didecamer aggregates have only been detected in KLH samples taken from living animals (Fig. 3(a)and (b)).

For KLH2, the production of decamers has proved to be slightly more difficult, as simple adjustment of the stabilising buffer pH had no effect. However, overnight dialysis against Tris buffer at pH 7.5 (i.e. in the absence of NaCl, $CaCl_2$ and $MgCl_2$) has been found to split purified samples of KLH2 (containing a mixture of didecamers and multidecamers) into a highly enriched population of decamers, but with instability, indicated by breakage of some decamers (Fig. 7(b)). Further dialysis of these KLH2 decamers against the usual KLH stabilising buffer causes didecamers to form again. Very significantly, when dialysis is performed against the stabilising buffer containing 100 mM calcium and magnesium, greatly elongated multidecamers were formed, often with more than one nucleating didecamer, but sometimes with no didecamer present (Fig. 8).

This experimental manipulation of the oligomeric states of KLH1 and KLH2 has provided data that correlates well with the known oligomeric forms obtained from total KLH formed in vivo, and extends our understanding of multimer formation by the two KLH decamers. It can be concluded that both isoforms of KLH exhibit characteristic oligomerisation features, be these produced naturally or experimentally.

3.4. Subunit reassociation studies

3.4.1. Reassociation of intact subunits

The production of molluscan hemocyanin subunits by dialysis of the intact oligomer(s) against a high pH buffer, with or without the presence of EDTA, is long established. Study of the hemocyanin dissociation intermediates has not been easily achieved. However, during the reverse process, subunit reassociation, this assembly process can be more readily monitored by electron microscopy and other physical techniques (Van Holde et al., 1991). Rather than the early stages of subunit reassociation of KLH1 and KLH2, we have been concerned with the study of the stable end-products. This work has been performed with purified subunit material produced from total KLH samples and also from commercially available Immucothel® (Biosyn GmbH, Fellbach, Germany). Initially, in our studies we concentrated upon rapid (two days) reassociation in the presence of 100 mM calcium and magnesium ions (Harris et al., 1997a,b) and more recently we have studied the slower (10 days) reassociation in the presence of 10 mM calcium and magnesium (Harris et al., 1998).

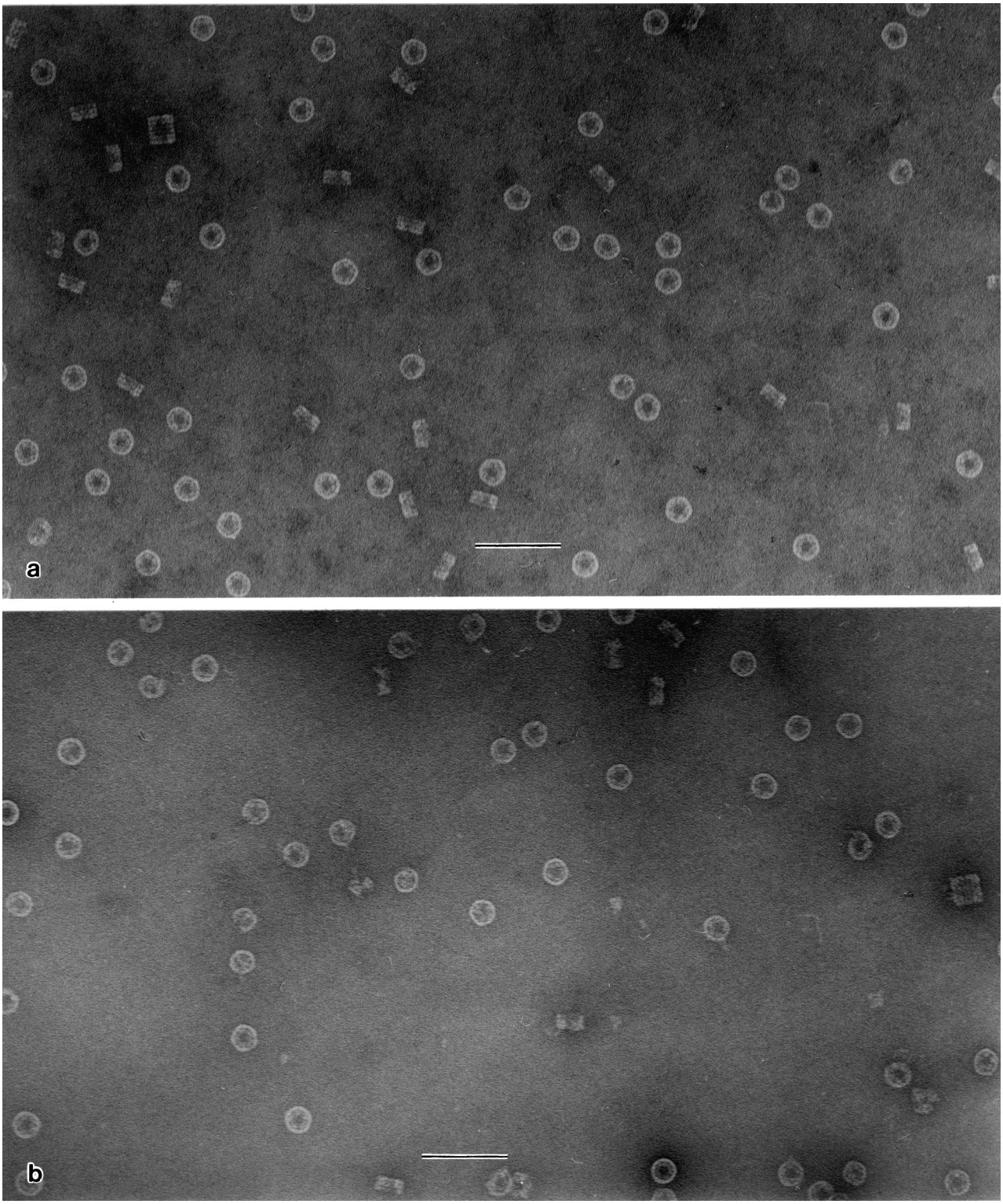

Fig. 7. Decamers of KLH1 (a), produced by increasing the pH of the Tris-saline stabilising buffer (which contains 5 mM $CaCl_2$ and 5 mM $MgCl_2$) to pH 8.5 to 9.5. These KLH1 decamers are relatively stable and can be used for oligomerisation studies and interaction with antibodies and Fab fragments for TEM immunonegative staining studies. Decamers of KLH1 are shown in (b), produced by dialysis against Tris buffer at pH 7.5 in the absence of calcium and magnesium ions. Under these condition the KLH2 didecamers and multidecamers split into decamers, but they have been found to be less stable than the KLH1 decamers. The scale bars indicate 100 nm.

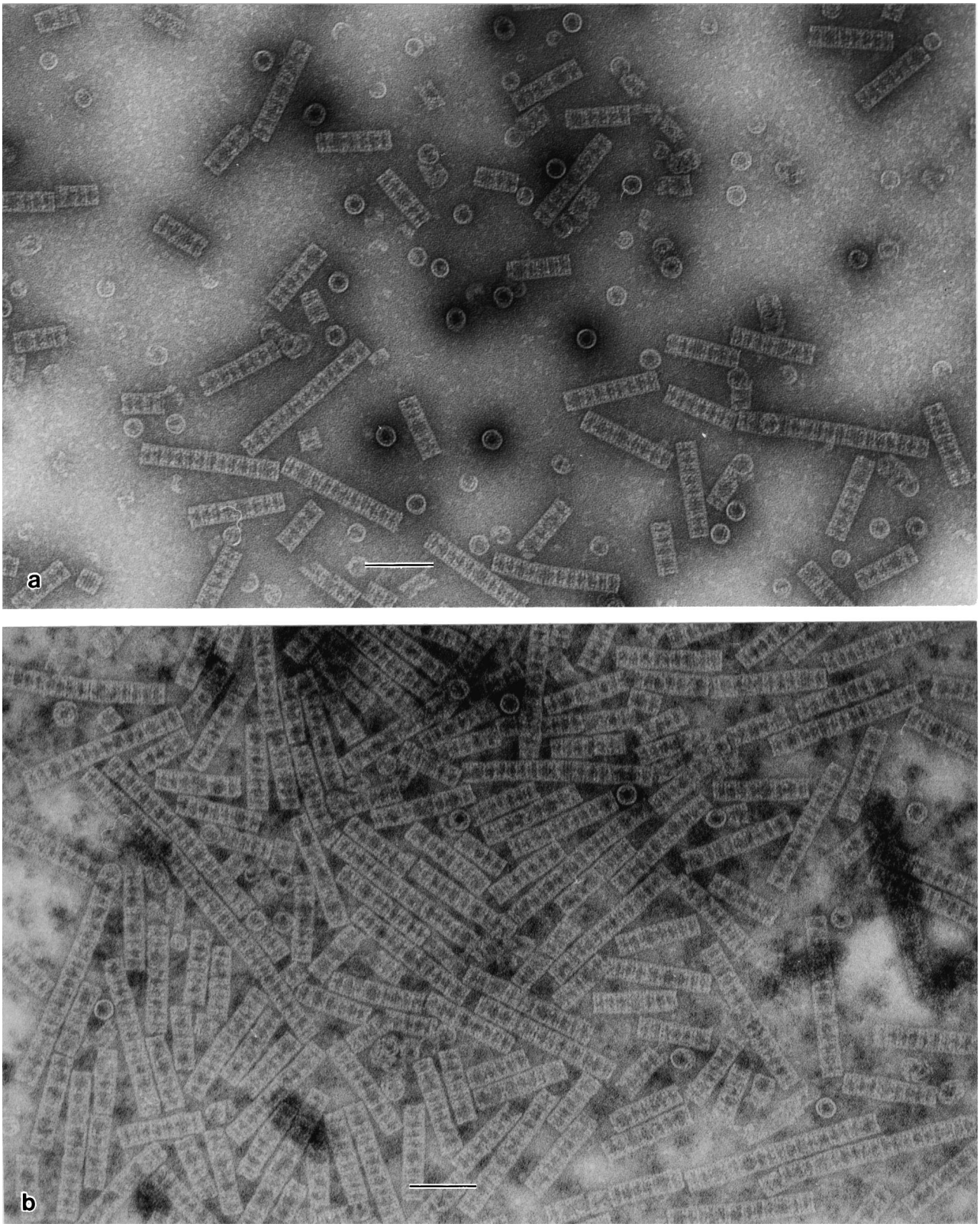

Fig. 8. KLH2 multidecamers formed by dialysis of a KLH2 decamer preparation (Fig. 7(b)) against a high calcium and magnesium concentration (100 mM) that potentiates linear-association of decamers. (a) Following 1 day, (b) following 2 weeks treatment. The scale bars indicate 100 nm.

At the high divalent cation concentration, it was found that KLH1 reassociates to form a mixture of oligomeric forms and a polymeric tubular form. Among the oligomers the didecamer predominates during the initial reassociation of the mixed KLH1 and KLH2 subunits in Immucothel® but following high pH dissociation and a repeated reassociation, multidecamers are also present. These KLH1 multidecamers have a tendency to form paracrystalline clusters and exhibit little indication of the stacked decamer collar/arc repeats characteristic of the KLH2 multidecamers (Harris et al., 1997b). The tubular polymer of KLH1 is of significantly smaller diameter than the multidecamers (ca. 25 nm versus 33 nm), and shows features characteristic of an incompletely annealed right handed helical ribbon of subunits. KLH1 tubules have a pronounced tendency to form disordered paracrystalline bundles. Under the 2D crystallisation conditions used during the negative staining-carbon film procedure, in the presence of ammonium molybdate and polyethylene glycol (PEG), individual KLH1 tubules have the potential to convert directly into multidecamers (Harris et al., 1997a), indicative of a helix–disc transformation.

The KLH2 subunit reassociates in the presence of stabilising buffer containing 100 mM calcium and magnesium to form a mixture of multidecamers (sometimes of considerable length) and a right-handed helical/tubular polymer that is more completely annealed than that produced from the KLH1 subunit under these ionic conditions. As with KLH1, the diameter of the KLH2 tubular polymer is less than that of the multidecamers. Electron optical images of the multidecamers and tubular polymers of KLH1 and KLH2 each exhibit a different and characteristic Fourier transforms (Harris et al., 1997b), from which it may in due course be possible to perform 3D helical image reconstruction, with assignment of the subunit location. It does appear, however, that the subunit–subunit interactions within the different oligomeric and polymeric structures varies, possibly due to some inherent flexibility or plasticity of the elongated multi-FU organization of the subunits, which can then associate in two different stable states. Biochemical SDS-PAGE analysis of such samples demonstrated that in both forms the subunit is intact; thus, the tubular polymers do not result from proteolytic digestion.

Reassociation of both KLH1 and KLH2 subunits in the presence of 10 mM calcium and magnesium is much slower and slightly less complete than with 100 mM concentrations of these cations. The oligomeric forms of both hemocyanins then predominate over the tubular polymers, indicative of association conditions that are closer to those encountered under physiological conditions (Harris et al., 1998). Although the TEM images of the reassociated didecamers of KLH1 and KLH2 appear to be identical to those obtained from untreated KLH samples taken directly from animals, some element of caution has to be applied, particularly in view of the stated possibility that ionically-induced variation in subunit shape and subunit-subunit interaction can be produced experimentally.

3.4.2. Reassociation of proteolytically cleaved subunits

Following limited proteolytic cleavage of KLH1 and KLH2 subunits with the V-8 protease from *Staphylococcus aureus*, the mixture of three FU (*abc*) and four/five FU (*defg/defgh*) fragments have been found to retain the ability to reassociate into tubular polymers in the presence of 100 mM calcium and magnesium ions (Gebauer and Harris, 1999). Of significance is the fact that no decameric oligomers have been detected under these conditions. Although some difficulty was encountered in establishing full control over the extent of proteolysis, reasonable reproducibility was established and purified multi-FU subunit fragments from the two KLH isoforms were obtained by HPLC.

The reassociated tubular polymers created from subunit fragments of protease V-8 cleaved KLH1 and KLH2 possess features in common with the tubular forms created from the intact subunits, but in both cases there are differences. KLH1 multi-FU peptide fragments *abc* + *def* + *defg* reassociate to form ca. 25 nm diameter tubules that anneal somewhat better than do those from the intact subunit, but they also aggregate to form parallel tubular bundles (Fig. 9). On reassociation, the KLH1 fragments *def* + *defg* possess the ability to form filamentous structures, which can create disorganised aggregates, indicating some inherent property within these FUs to generate higher assemblies. With KLH2, the multi-FU fragments *abc* + *defg* + *defgh* reassociate to form ca. 25 nm diameter tubules that are often revealed as poorly annealed helical/twisted ribbons in the EM images. Whilst extremely long tubules have been recovered following reassociation of the intact KLH2 subunit (Harris et al., 1997b), those formed from the multi-FU subunit fragments are generally somewhat shorter (Gebauer and Harris, 1999).

For both the KLH1 and KLH2 the tubules formed from the V-8 proteolytically cleaved fragments still have some of the internalised "collar and arc" FUs *g* and *h* present, and are therefore not strictly "hollow" tubules. They can nevertheless be distinguished structurally from the tubules created from the intact subunits. Experiments to generate a stochiometric mixture of the fragments *abc* + *def* and the complete sequence of the six KLH "wall" FUs *abcdef* are currently in progress.

4. Cellular immunology and KLH

It is clear that much of what is included in the following sections, dealing with tumour immunotherapy and the other uses of KLH, relates fundamentally to the immunological response. However, from the rather large, expanding and some might say confusing literature within the field of cellular immunology (from animal and human studies in vivo, and in vitro using isolated lymphoid cells) indicating the value of KLH as a non-specific immunostimulant/adjuvant and as a carrier for haptens conjugated to KLH, few articles will be discussed. It should be borne in mind that most of

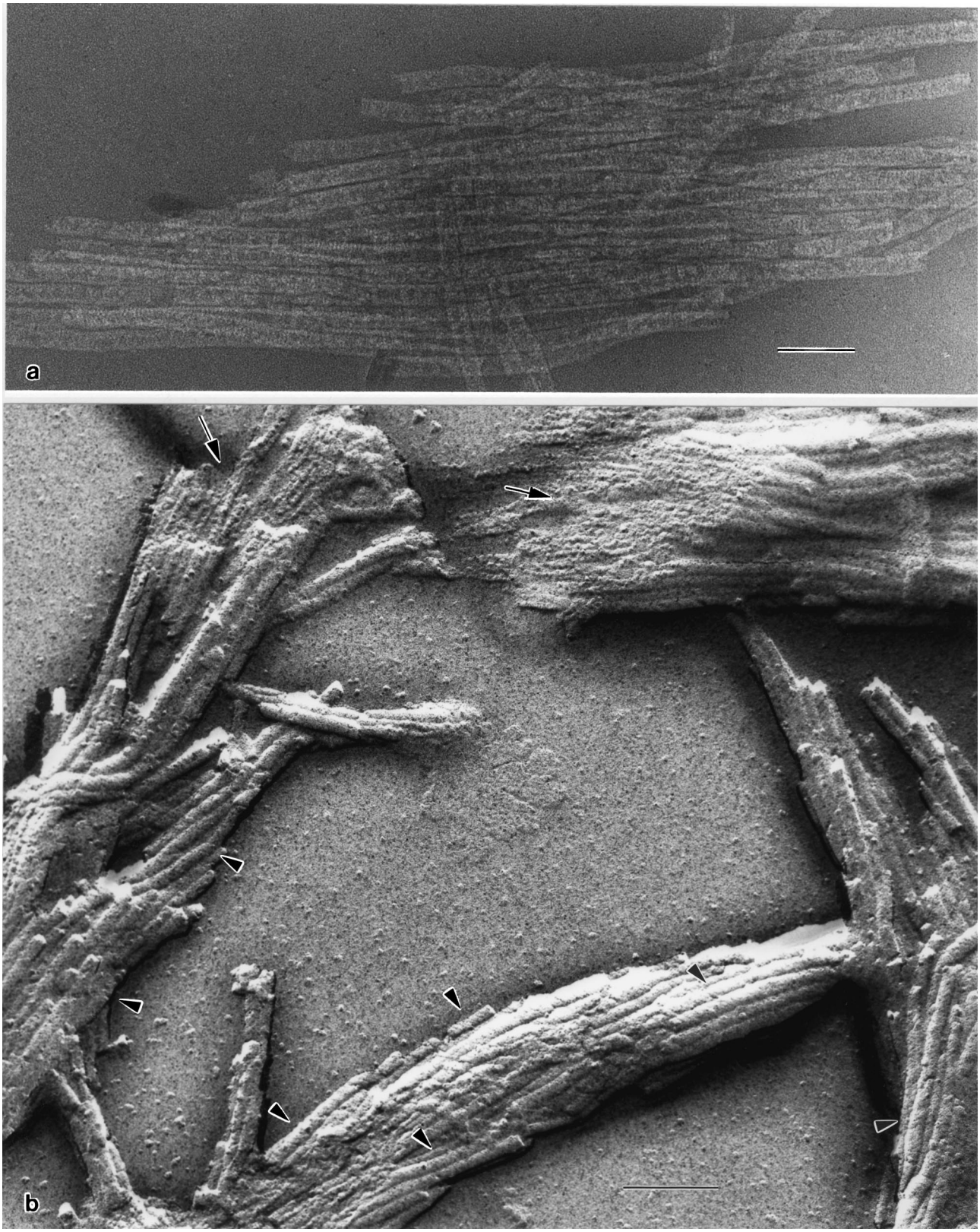

Fig. 9. Electron micrographs showing bundles of reassociated KLH1 tubular polymer, following cleavage of the polypeptide with the V8 protease from Staphylococcus aureus (Gebauer and Harris, 1999). In (a) the sample was negatively stained with 5% ammonium molybdate (pH 7.0) containing 1% trehalose and the specimen grid cooled to − 175°C before TEM study and in (b) the sample was freeze fractured and platinum-carbon shadowed (courtesy of Dr Milan V. Nermut, NIBSC, UK). Note the surface helical pattern on the tubules (arrowheads) and the places where cleavage of the bundles has occurred (arrows). The scale bars indicate 200 nm.

these studies relate to fundamental immunological problems rather than to the properties of KLH per se; they are therefore not directly relevant to the present discussion. Nevertheless, the popularity and frequent use of KLH as a highly immunogenic model protein antigen, alongside or as an alternative to a number of other commonly used proteins such as ovalbumin, has contributed greatly to the understanding of the $CD4^+$ T-cell response.

At first sight it might be considered that the low level non-specific blastogenic/lymphoproliferative action of KLH is somewhat more similar to phytohaemaglutinin (PHA), *Corynebacterium parvum* and bacterial lipopolysaccharide (LPS), than the usual polyclonal immunological response to a single protein. However, the mechanism of PHA action as a potent T-cell mitogen and LPS as a B-cell polyclonal stimulator, do appear to be fundamentally different. The intrinsic immunogenicity of KLH may relate to the existing population of memory B-cells, resulting from prior stimulation by a cross reacting protein or carbohydrate epitope, and to enhancement of T-cell cross-priming, or to some as yet unknown fundamental property of KLH.

Whilst the immunological response to KLH has often been attributed to the carbohydrate moiety, rather than protein alone, the polypeptide chain of eight globular functional units constituting the elongated KLH subunit and the highly organised quaternary structure of the native molecule, could create a scaffold upon which multiple carbohydrate epitopes can be initially made available to the immune system. Optimal steric spacing of sugar residues could potentiate the observed potent stimulatory response, both in vivo and in vitro. In general, however, essentially equivalent data has been obtained using the intact/native KLH1 + 2 oligomers and the KLH subunits, indicating that glycosylation of the ca. 400 kDa subunit could be the significant factor, in addition to protein mass and shape (Orlova et al., 1997). It should, nevertheless, be remembered that in general, immunologists do hold the opinion that high molecular mass proteins and protein aggregates are able to initially present antigens to the antigen processing cells (APCs)/dendritic cells (DCs) in more efficient manner than smaller mass proteins. Indeed Swerdlow et al. (1994) claimed that the oligomeric KLH was a more efficient immunogen in a mouse bladder carcinoma model, but the subunit of KLH has the ability to generate a greater range of polyclonal antibodies than the native oligomers (Weigle, 1964), indicative of sterically hidden epitopes within the intact molecule. Importantly, it can be predicted that carbohydrate side-chains will project from the surface of the intact KLH molecule, as well as from the subunits.

Following subcutaneous injection of KLH subunits or intra vesicle instillation for the treatment of bladder carcinoma, reassociation of the subunits will occur, and also in vitro in physiological cell culture medium. Whether or not KLH1 and KLH2 act in an identical manner is not currently known; present evidence indicates that there is a difference in glycosylation (see Section 2.6). Undoubtedly, major differences in the oligosaccharides of the two molecules exist, but both molecules are likely to carry multiple N-linked, branched carbohydrate chains, as indicated by the recent carbohydrate analysis performed using *Helix pomatia* haemocyanin (Lommerse et al., 1997). Also, the primary structure of both *Octopus* hemocyanin (Miller et al., 1998) and *Haliotis* hemocyanin (see Section 2.8) indicate that these molecules have putative glycosylation sites.

Of significance for the overall understanding of the direct immunotherapeutic action of KLH for the treatment of superficial bladder carcinomas is the study of Wirguin and colleagues (Wirguin et al., 1995), who showed that KLH carries oligosaccharides containing a terminal galactose (β1–3) *N*-acetyl galactosamine epitope (cross-reactive with the TF/Thomson–Friederich antigen) and that in vivo it induces a protective antibody against this carbohydrate sequence along with a cytotoxic T-cell response (expanded upon in Section 5.1). In other cases, the active specific immunotherapeutic potential of KLH-conjugates is thought to be due to the non-specific cellular immunostimulation derived from KLH, plus the generation of an antibody producing B-cell clone directed against the specific hapten.

5. Tumour immunotherapy using KLH

5.1. Bladder carcinoma

Based upon the extensive immunological background indicated above, it should not be surprising that the initially large volume of supportive data for KLH action in animal tumour models for bladder carcinoma has diminished somewhat in recent years e.g. (Lamm et al., 1993a; Recker and Rubben, 1989; Swerdlow et al., 1994). Perhaps of greater significance is the fact that this has been paralleled by an increasing number of clinical studies using KLH for the management of patients with superficial bladder carcinoma (superficial transitional cell carcinoma, TCC) extending through the 1980s (Ford et al., 1982; Jurincic et al., 1988; Lamm et al., 1981, 1982) to more recent phase II and phase III clinical trials, primarily using the KLH subunit product Immucothel® (Flamm et al., 1990, 1994; Jurincic Winkler et al., 1992, 1994, 1995a,b,c,d, 1996; Kleinknecht et al., 1992; Lamm et al., 1993b, 1996; Molto et al., 1991; Sargent and Williams, 1992). The clinical success of intravesicular administration of KLH to patients with bladder carcinoma (nearly always following surgical transurethral resection of the tumour) has, as already mentioned, been ascribed to the presence of the disaccharide epitope Gal(β1–3)GalNAc, from lectin and antibody binding studies in rats (Wirguin et al., 1995). This KLH epitope is believed to be cross-reactive with an equivalent epitope on the bladder tumour cell surface; the cumulative humoral and cellular immunological response to KLH can then be expressed as a cytolytic reduction of tumour growth. As indicated in Section 2.6, once available, the oligosaccharide analysis for KLH1 and

KLH2 is likely to confirm the multi-epitopic potential of these molecules.

Useful comparative overviews of the available treatments for bladder carcinoma have been presented (Crawford, 1996; Nseyo and Lamm, 1997) and a full account of the properties and use of *Immucothel*® appears in the scientific information on "Immunocyanin/Immucothel" available from the biosyn Arzneimittel GmbH, Fellbach, Germany. In patients with bladder tumours a beneficial antibody response to KLH is paralleled by an increase in natural killer (NK) cell activity, and a mean recurrence rate of 31%, has been obtained at a mean follow-up period of some 26 months. Overall, it can be concluded that the efficacy of KLH is comparable if not superior to non-specific immunotherapy with BCG and chemotherapy with mitomycin C, ethoglucin and thiotepa, with a marked reduction in side effects, such as febrile temperature and cystitis. The immunotherapeutic use of KLH does, however, represent only one of several possible lines of future treatment for bladder carcinoma (Nseyo and Lamm, 1997). Importantly, KLH is considered to be an extremely safe substance for in vivo use in man, as a direct antigenic stimulus and immunotherapeutic agent. This use of KLH has also been considerably extended following conjugation with tumour-specific peptides and oligosaccharides, and with anti-idiotypic antibodies, as will be discussed further immediately below.

5.2. Other carcinomas

It is apparent that the presence of weakly expressed tumour-specific antigens on the surface of animal and human carcinoma cells has not provided a ready access to successful and wide-ranging tumour immunotherapy, as once hoped. However, the mucin-like epitopes of the pancarcinoma antigens (T,TN,sTN), present on epithelially derived adenocarcinomas (e.g. colorectal, breast, ovarian) (Alduri et al., 1995, 1999; Agrawal et al., 1996, 1998; Longenecker et al., 1993, 1994; MacLean et al., 1996a,b; O'Boyle et al., 1996; Ragupathi et al., 1998, 1999; Reddish et al., 1995, 1996, 1997, 1998a,b; Sandmaier et al., 1999; Yacyshyn et al., 1995) together with the Lewis blood group epitope on other carcinomas (Ravindranath et al., 1998) and the expression of ganglioside epitopes on malignant melanomas (Helling and Livingston, 1994; Helling et al., 1995; Jennemann et al., 1994, 1996; Livingston et al., 1994, 1997; Livingston, 1995a,b), has revealed a number of potential targets for carbohydrate-based immunotherapy. In the main, synthetic oligosaccharides conjugated to KLH have been injected together with a range of different adjuvant materials such as aluminium hydroxide gel (alumina), BCG, DETOX (Schultz et al., 1995) (a monophosphoryl lipid A-containing adjuvant) and QS-21 (a saponin-based adjuvant), has enabled a specific cytotoxic antibody response to be raised in combination with a powerful non-specific stimulation of the immune system, sometimes in parallel with or following the administration of low-dose chemotherapy (MacLean et al., 1996a,b). Using a slightly different approach synthetic peptides (some 30 amino acids in length) have been produced, representing the protein epitopes present on the human mucin MUC1, which are also expressed on some cancers of epithelial origin (Zhang et al., 1996). Conjugates of these peptides to KLH injected into tumour-bearing mice, along with the adjuvant QS-21, indicated that the MUC1–KLH peptide vaccine is also suitable for clinical trials.

Agarwala and Kirkwood have reviewed the progress achieved during the treatment of melanomas. Gangliosides are a large group of ceramide oligosaccharides, carrying one or more sialic acid residues (Agarwala and Kirkwood, 1998). Although gangliosides were initially isolated from the brain, they are in fact widely distributed throughout vertebrate tissues. Fortunately, the gangliosides expressed on melanomas do show some structural specificity, so the use of this line of immunotherapy should not inadvertently be directed against normal tissues. It has usually been maintained that activation of cytotoxic T-cells and NK cells occurs in addition to the generation of melanoma-targeted neutralizing antibody. The Lewis(y) (Le[y]) blood group epitope, present on mucins and glycolipids [Fucα1–2Gal β1–4 Fucα1–3)GlycNAcβ1–3 Gal] and the sialyl Tn epitope [NANAα(2–6)GalNAcα-*O*-serine] widely distributed on tumour cells, likewise have sufficient uniqueness to lead clinical investigators to now use the phrase Active Specific Immunotherapy (ASI) (Kudryashov et al., 1998a,b; Miles et al., 1996).

The use of anti-idiotypic antibodies for immunotherapy, to mimic tumour-associated antigens, has been considered for several years. With a monoclonal antibody to the human high molecular weight-melanoma associated antigen (HMW-MAA), a mouse anti-idiotypic antibody MK2-23 conjugated to KLH was used to treat malignant melanoma in a small group of patients (Mittelman et al., 1995). Again, this specific immunotherapy was given along with non-specific BCG immuno/adjuvant therapy and cylophosphamide chemotherapy. The generation of anti-anti-id antibodies by the patients indicated their immune competence, in parallel with a beneficial effect on survival time. A similar approach, using an anti-idiotypic antibody to carcinoembryonic antigen (CEA) conjugated to KLH, has been investigated in a mouse colorectal carcinoma model (Pervin et al., 1997). Vaccination has also been tried with a syngeneic mouse lymphoma-derived cell surface Ig idiotype conjugated to KLH (Kwak et al., 1996). A markedly enhanced protective immunity was shown only when granulocyte macrophage colony stimulating factor (GM-CSF) was included, apparently by activation of the $CD4^+/CD8^+$ T-cell arm of the immune response rather than by anti-idiotypic antibody production. Similar work (Bohlen et al., 1996) has confirmed the potential of this immunization strategy for the improvement of long-term survival. In patients with non-Hodgkins lymphoma, Caspar and colleagues concluded that active immunization with

Table 2
VACMUN®: keyhole limpet hemocyanin (KLH) a pharmaceutical carrier for vaccines and antigens

Antigen (Ag) chemical identity	Antigen (Ag) functional identity	Physiological response to VACMUN®[a]–antigen conjugate
Peptides	Tumour-specific antigens	KLH as Adjuvant +
Proteins		Anti-tumour response
		Anti-idiotope response
Glycopeptides	Cytokines	
		Suppression of cytokine synthesis
Glycoproteins	Vaccines	
Carbohydrates (mono-oligosaccharides and polysaccharides	Allergens	Vaccination
		Anti-allergic effect
	Hormones	Production of monoclonal antibodies
Chemical compounds	Drugs and metabolites	Antidrug/hormone effect

[a] Modified from material supplied by BIOSYN® Life Science.

KLH-conjugated tumour idiotype can induce a polyclonal immune response that may be of value in the cytolysis of mutated tumour variants (Caspar et al., 1997). Cellular immunotherapy has been an approach used recently (Nestle et al., 1998). Autologous dendritic cells (DCs) from melanoma patients were cultivated in the presence of GM-CSF and IL-4, pulsed with a melanoma lysate or a cocktail of peptides recognised by cytotoxic T lymphocytes. DC vaccination was then performed, with KLH added in an unconjugated state, as a CD4 helper antigen. Reichardt et al. (1999) have provided further support for this approach. This cellular vaccine produced a delayed-type hypersensitivity to KLH and to the peptide-pulsed DCs, indicative of antigen-specific immunity, with regression of metastases. Some success has also been recently achieved for the treatment of renal cell carcinoma using DCs in combination with KLH (Holtl et al., 1998, 1999; Thurnher et al., 1998).

6. Other uses for KLH

6.1. As a generalised vaccine component

In this section we consider some of the non-oncogenic uses of KLH as a carrier for antigen presentation, alone and as a component of the adjuvant cocktail that could be useful for the immunotherapy of viral and other infections. The purified mixture of KLH1 and KLH2 subunits is commercially available (VACMUN®, biosyn Arzneimittel GmbH), for antigen/hapten conjugation without reassociation or after reassociation into the various oligomeric high molecular mass forms (Table 2).

From the literature it is clear that despite the large volume of evidence indicating the potential of KLH-conjugates for the generation of a specific immune response to small molecular mass haptens, it has not moved into widespread clinical or agricultural use as a component of viral and other vaccines. Early work with HIV proteins and peptides did indicate some success (Giard et al., 1992; Kahn et al., 1992; Naylor et al., 1991; Okuda et al., 1993), but the use of KLH has been limited and it has even been reported (Folks et al., 1997) that immune stimulation with KLH can cause enhanced progression of SIV-induced disease in rhesus macaques. Some success has recently been reported, using a synthetic 30-amino acid peptide (equivalent to amino acids 86 to 115 of HIV1 p17) when injected along with KLH and alumina (Zimmerman et al., 1998). Also, Meyer et al. (1998) have shown that a cocktail of synthetic peptides representing the hypervariable epitope construct of SIV, when conjugated to KLH and administered to rabbits and rhesus macaques, generated neutralising antibodies. These workers claimed that this approach could be an effective method for the generation of a broad cross-reactive humoral response, of value as a combination vaccine against HIV for the treatment of AIDS.

KLH–peptide vaccines have been produced from an N-terminal fragment of the L2 capsid protein of bovine papillomavirus-4, which in combination were highly protective, and one of which (peptide 14) contained the domain responsible for disease prevention (Campo et al., 1997). Using a similar approach, a number of peptide fragments from the envelope protein E of tick-borne encaphalitis virus were conjugated to KLH and administered to rats (Volkova, 1998). Two peptide antibodies were defined which were capable of neutralising the virus. Also using KLH, generation of a broad range of synthetic peptide conjugates, corresponding to sequences within defined epitopes of the major capsid protein of porcine parvovirus protein VP2 has proved to be useful (Kamstrup et al., 1998). The N-terminal region of VP2 was identified as the epitope of significance for viral neutralisation. Synthetic peptides of porcine zona pellucida ZP3 alpha conjugated to KLH have been successfully used to generate antibodies that inhibited boar sperm-pig zona attachment (Hall et al., 1995). This approach was considered to provide firm support for the continued investigation of synthetic ZP3 alpha peptides, which are cross-reactive with human zona pellucida, as potential target immunogens for anti-fertility vaccine development.

6.2. Diagnosis of schistosomiasis

Knowledge of the cross-reactivity of a carbohydrate epitope on the surface of larval schistosomes of *Schistosoma mansoni* and related species (defined as being on glycoprotein GP38) with an epitope on KLH (Dissous et al., 1986; Grzych et al., 1987; Ko and Harn, 1987) has led to continued interest in the biomedical significance of this observation. The use of immobilized anti-KLH antibodies or of KLH has been of value for the establishment of ELISA diagnosis and assessment of acute versus chronic infections of *S. mansoni* (Alves-Brito et al., 1992; Lambertucci, 1993; Mansour et al., 1989; Markl et al., 1991a), *S. japonicum* (Li et al., 1994, 1997; Taylor et al., 1998) and *S. haematobium* (Xue et al., 1993). In comparative studies of IgG and IgM antibodies raised against KLH and IgA antibody against soluble egg antigen (ovalbumin) (Liping et al., 1996; Xue et al., 1993), it was shown that both were useful for the detection of acute schistosomiasis. Recently, however, a note of caution has come from Thors and Linder who claimed that a cross-reacting anti-duct antibody, also detected with other worm infections, could interfere with the diagnosis of acute schistosomiasis by immunofluorescence microscopy (Thors and Linder, 1998).

Suppression of *S. bovis* egg production in cattle following injection of KLH and glutathione-S-transferase (Bushara et al., 1993) has indicated the possibilities for vaccination against this organism. Thus Bashir and colleagues were able to show that KLH vaccination of calves and sheep provided significant protection against *S. bovis* and *S. japonicum* (Bashir et al., 1994). No equivalent recent studies in man area vailable; although the earlier data (Ko and Harn, 1987) suggested that the Schistosoma epitope seen by non-protective antibodies cross-reacted with KLH, rather than with protective antibodies, there may be some potential for such treatment in man.

6.3. Drug assay and treatment of drug addiction

The use of drug–KLH conjugates to generate antibodies suitable for use in ELISA and other immunoassays ranges widely from well-known drugs such as cocaine (Ettinger et al., 1997) and morphine (Beike et al., 1997) to more exotic pharmacologically interesting and therapeutically useful, compounds such as triamcinolone-acetonide (Gabor et al., 1995), di- and trichloroacetylated groups (Pahler et al., 1998), capatopril (Narazaki et al., 1998), 2′-deoxycytidine (Darwish et al., 1998), thymidine glycol (Greferath and Neehls, 1997), lysergic acid diethylamide (LSD) (Kerrigan and Brooks, 1998), a thyrotropin releasing hormone analogue (Morikawa et al., 1998), mercury (Marx and Hock, 1998) and arsonate compounds (Vora et al., 1998), antitumour agent MKT 077 (Kawakami et al., 1998) and a range of peptides (Bergeron et al., 1998). In addition, the use of drug–KLH conjugates as active immunogens to reduce the free drug level in plasma may be beneficial for the provision of controlled withdrawal/drug abuse prophylaxis, for example from nicotine (Hieda et al., 1997) and cocaine (Bagasra et al., 1992; Ettinger et al., 1997).

6.4. Immune competence testing, stress and inflammation

The use of the varying immune response to KLH as an assessment of immune competence has been of interest in relation to both animal and human health. The immunological development of calves, and stresses of weaning and transport have been monitored by measurement of the immune response following injection of KLH (MacKenzie et al., 1997). On the other hand, the stress response of calves to overcrowding was not found to parallel behavioural changes or cortisol and anti-KLH antibody levels (Fisher et al., 1997). Differences in immune response of four breeds of pig to KLH were detected (Joling et al., 1993), from which it was proposed that selective breeding for a maximal response, and therefore immune competence, should be possible. The effect of chronic morphine treatment on the immune response of pigs to KLH has also been studied (Schoolov et al., 1995).

In man, it was concluded (Snyder et al., 1993) that under conditions of psychosocial pressures, the reduced primary immune response, measured as lymphocyte proliferation following KLH immunisation, was a more reliable indicator of such stress than biological factors such as weight gain. Postoperative stress, which also produces immunosuppression, has been assessed in rats using a paediatric model in which the cellular immune response was measured in animals sensitised to KLH (Mendoza Sagaon et al., 1998). From experiments with rats, Allendorf and colleagues showed that postoperative cell-mediated immune function was better preserved after laparoscopic-assisted bowel resection than after open resection (Allendorf et al., 1996). Similarly, classification of surgical patients on the basis of delayed-type hypersensitivity skin testing, following administration of KLH alone and together with a range of leukocyte mediators, indicated that a lack of response in patients with anergy could be countered by the inclusion of these mediators (Puyana et al., 1990). Patients with common variable immunodeficiency (CVID) were, following KLH injection found to be defective both at the cellular and humoral level (Kondratenko et al., 1997). Indeed, early work showed that cyclosporin inhibits the cellular and antibody response to KLH in normal humans (Amlot et al., 1986), whereas patients with chronic uveitis receiving cyclosporin responded with a normal blastogenic and antibody response, but with delayed cutaneous hypersensitivity (Palestine et al., 1985).

At the opposite end of the immunological spectrum, investigation of the pathological immune response associated with colonic inflammation (colitis) has also benefited from the use of KLH. Following TNP–KLH injection, the induction and prevention of colonic inflammation in mice was shown to be regulated by TGF-beta (Ehrhardt et al.,

1997; Ludviksson et al., 1997). Similarly, TNP–KLH lung immunisation was used in rats as an acute stressor, to assess the effects of inflammatory stress on pulmonary immune function (Persoons et al., 1997). Studies on asthma have also benefited from the use of KLH. Schuyler et al. found that atopic asthmatics responded to intrapulmonary KLH with more serum anti-KLH IgG4 than normal individuals, indicative of an increased Th2 response (Schuyler et al., 1997). Administration of bacterial cell wall and a synthetic muramyl peptide derivative (SDZ280.636) was also found to increase IL-6 levels in mice, while decreasing IFNγ, suggesting that this may be useful for the therapeutic and prophylactic management of allergic rhinitis, asthma and other atopic diseases in man (Auci et al., 1998). KLH administered along with heat-killed Listeria monocytogenes was shown to convert the established murine Th2-dominated response into a Th1-dominated response (Yeung et al., 1998). This was thought indicate the possibilities for KLH-vaccine therapies for conditions such as allergy and asthma.

Rats stressed by inescapable tail shock (IS) failed to respond with a normal Th1-like cellular response to KLH, an effect thought to be due to a diminished IFNγ level (Fleshner et al., 1995). These workers have continued to use KLH for their further investigations on stress (Fleshner et al., 1996, 1998). Immune injury in the inner ear, labyrinthitis, has been investigated by inoculation of KLH into the scala tympani of systemically sensitised guinea pigs (Ichimiya et al., 1998). Evidence for the invasion of circulatory inflammatory cells into the cochlea was presented, and claimed to be of significance for cochlea dysfunction.

7. Some conclusions and comments on the future potential of KLH

In this broad review we have tried to survey concisely various aspects of KLH biochemistry, structure and cellular immunology. We also integrate this basic information with the use of KLH as a therapeutic agent in oncology and other disciplines of medicine. Despite the fact that the subject is large, and rapidly expanding, it is appropriate to try and assess the future potential of KLH, directly as a potent adjuvant substance and as a hapten carrier for numerous small molecular mass substances against which it is desired to raise specific antibodies. Overall, our conclusions are extremely positive.

Nevertheless, some fundamentally important questions need to be addressed by the biochemical and pharmaceutical suppliers of KLH for experimental and clinical use. Firstly, can the current availability of *Megathura crenulata* from the Pacific ocean coast continue to satisfy the commercial demand, and if so can this supply be maintained for the foreseeable future without severely depleting a valuable natural resource. The alternative, to breed and maintain *Megathura crenulata* under small and large-scale aquaculture is under investigation. Second, the leading question has to be posed as to whether the hemocyanin from other marine, freshwater or terrestrial molluscs might act equally well as immunological and therapeutic agents. Limited information is available in this area, but there is evidence to indicate that the hemocyanin from the Roman/vineyard snail *Helix pomatia* may well have immunostimulatory properties, but it is almost certain that it does not carry identical oligosaccharide components to those of KLH. The hemocyanin from the marine abalone *Haliotis tuberculata*, which is already maintained successfully under aquaculture in France, for the domestic shellfish market, may also be a serious contender. Thirdly, it might be possible to make available a complete KLH1 or KLH2 subunit in quantity from gene cloning, but this is even more likely for single or small groups of functional units (FUs). However, the difficult question of appropriate glycosylation has to be addressed; it may be that chemical conjugation of structurally defined synthetic oligosaccharides to KLH produced from a bacterial source may be an easier option than KLH cloning in eukaryotic cells. Such considerations apart, in the short term (i.e. the next 10 years), it can be anticipated that KLH will continue to be readily available from it's current source in the natural environment.

From a strictly scientific view point, continuation and expansion of the basic scientific biochemical, structural and immunological work on KLH is fully justified, as it is only from this that a fuller understanding of the in vivo function of KLH as a therapeutic agent will be obtained. More specifically, future immunological studies should be carried out using purified KLH1 and KLH2, in a standardised molecular state (i.e. subunit and oligomeric forms), rather than simply with the commercially available total KLH. Indeed, it must be borne in mind that most of the early studies with KLH were performed with commercially available ammonium sulphate precipitate of total KLH, which probably contains low levels of other hemolymph proteins. For zoologists, the understanding of the structure and diversity of invertebrate hemocyanins, and of their evolution in parallel with the extracellular hemoglobins, is also a fascinating and worthwhile pursuit. From our own work, it is apparent that KLH has extremely interesting macromolecular properties relating to its varying oligomerisation states and the fact that it can be biochemically transformed into two different helical tubular/polymeric forms (characteristic of KLH1 and KLH2) is likely to be of considerable significance in protein/polymer chemistry. Indeed, the possibility of creating and controlling a reversible helix-disc transformation for KLH, somewhat akin to that established for tobacco mosaic virus protein, is an exciting prospect.

In the fields of bacterial and viral vaccines, and in oncology, it is apparent that KLH can be conjugated to multiple synthetic peptides, in order to create a potent antigenic cocktail, to generate a broad humoral and cellular response directed against bacterial, viral and tumour cell–surface antigens. In this way the continually changing antigenic

profile of some infectious agents may in the future be successfully combated, since the potential of synthetic chemistry to create defined peptide and oligosaccharide sequences and therefore epitopes of biological importance, is considerable. Similar considerations are likely to apply to tumours that rapidly mutate their cell surface glycoproteins. As a direct non-specific adjuvant, KLH must be classed as only one of the available useful materials, and it is likely that it will continue to be used alone and in combination with the other established powerful bacterial cell wall-derived adjuvants, most of which can potentiate the effects of KLH when it is used as an antigen carrier.

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