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PROFILING CELLS AND BODY FLUIDS – CHROMATOGRAPHY AND TWO-DIMENSIONAL ELECTROPHORESIS AS COMPLEMENTARY TECHNIQUES

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SUMMARY

Recent results on the use of gas chromatography–mass spectrometry, high-performance liquid chromatography with diode array detector, amino acid analysis and high-resolution two-dimensional (2-D) electrophoresis to study body fluids and cells in health and disease are surveyed. The chromatography profiling techniques are particularly suitable for the diagnosis of inborn errors of metabolism, with DNA technology as a complementary tool for prenatal diagnosis. Both chromatography and electrophoresis were utilized to study pre-diagnostic sera from the JANUS serum bank and to classify certain bacteria. Protein profiling by 2-D electrophoresis was employed to identify marker proteins associated with the metastatic properties of cloned cancer cells. The electrophoretic technique is also appropriate as a preparative tool for isolating sufficient amounts of marker proteins for microsequencing of amino acids. Chromatography and protein profiling were complementary tools for evaluating the toxicity and mutagenicity of environmental samples in a new test utilizing living human leukocytes and fibroblasts.

INTRODUCTION

The body contains a large number of organic and inorganic compounds of low, intermediate and high molecular mass. The concentrations of all these compounds in the cells and body fluids are normally well regulated. Many diseases or other disturbances may lead to changes in the chemical milieu of cells, blood, urine, spinal fluid, etc., and information on these changes is of diagnostic importance. In modern clinical chemistry laboratories, variations in, e.g., certain enzymes, proteins, lipids, carbohydrates, electrolytes, hormones, blood cells and acid–base parameters are determined. These laboratories are usually highly automated and certain 'key' compounds or parameters on a large number of specimens can be handled daily. The output of such a laboratory often exceeds a million analyses per year.

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There is, however, also a demand for a different approach, namely the determination of a large number of compounds (200–3000) in fewer, selected samples. This 'profiling' approach or 'multi-component analysis' is used for both research and routine analyses, and a variety of specimens, including body fluids, biopsies, cells in culture and microorganisms, may be examined by multi-component analytical techniques.

Our Institute has been engaged in profiling of body fluids and cells for two decades. A variety of chromatographic and electrophoretic methods are in use, including gas chromatography (GC), gas chromatography–mass spectrometry (GC–MS), GC with radioactivity detection, high-performance liquid chromatography (HPLC), HPLC with diode-array detection, automated amino acid analysers, fast protein liquid chromatography (FPLC), sodium dodecyl sulphate (SDS) electrophoresis, isoelectric focusing, high-resolution two-dimensional electrophoresis and DNA electrophoresis (Southern blotting). In this paper we shall consider these methods as complementary techniques, overview recent examples from our studies on metabolic disorders, on tumour markers in pre-diagnostic serum samples from cancer patients, on cancer cells in culture, on the use of profiling methods for bacterial classification and on uses in environmental studies and describe the development of new tests for toxicity and mutagenicity using protein and DNA electrophoresis.

EXPERIMENTAL

GC–MS profiling

Two GC–MS instruments are in daily use with a third as back-up. The first is a Hewlett-Packard 5970 mass-selective detector (1987 model) coupled to a gas chromatograph with an automatic sample injection system (HP 5890 gas chromatograph with HP 7673A 100 sample autoinjector) and an HP 300 data system. The second instrument is a Finnigan Model 4021 C GC–MS instrument with an Incos/Nova 4 data system (1981 model) (Finnigan, Sunnyvale, CA, U.S.A.). The back-up instrument is a Varian-MAT 112 (1975 model), which is operated when required and used in a manual manner. The GC–MS instruments are fitted with fused-silica capillary columns (30 m) and the coatings are usually SP-1000 or SPB-5 (Supelco, Bellefonte, PA, U.S.A.). Nearly all GC peaks are automatically identified using mass spectral library searching. The libraries now in use contain the mass spectra of about 40 000 different compounds, including a number of drug metabolites, metabolites of biological interest and the collection of 230 mass spectra of urinary organic acids published by Spiteller and Spiteller [1]. In addition to the commercial computer search routines (Finnigan and Hewlett-Packard) we have developed a simple program that automatically identifies about 200 organic acids known to carry specific diagnostic information.

Samples for GC–MS profiling are usually urine, serum, cerebrospinal fluid (CSF) and occasionally tissue biopsies. Urine and CSF are used directly and the other specimens are deproteinized with ethanol before extraction. The protein-free samples are acidified, extracted with diethyl ether and the organic acids are subsequently methylated with diazomethane before injection into the GC–MS

system. Other laboratories prefer to use, e.g., ion exchange, for isolation of the acids and silylation to make them volatile (for reviews and books, see refs. 2–6).

HPLC profiling

The standard HPLC instrument is an LDC liquid chromatograph with two ConstaMetric pumps (Laboratory Data Control, Riviera Beach, FL, U.S.A.), equipped for variable-wavelength detection and rapid scanning detection. The diode-array detector is a Model 2140 rapid spectral detector (LKB, Bromma, Sweden) with an IBM-XT personal computer (IBM, New York, NY, U.S.A.). The LC-18 reversed-phase column (Supelcosil) is from Supelco and the Asahipak GS 320 H porous polymer column from Gasukuro Kogyo (Tokyo, Japan). The Bond Elut strong cation exchanger is from Analytichem International (Harbor City, CA, U.S.A.). An Aminex HPX-87H ion-exchange column (Bio-Rad Labs., Richmond, CA, U.S.A.) is used for the HPLC analysis of lactate and pyruvate in cerebrospinal fluid in cases of suspected cerebral lactic acidosis [7]. The instrument is also equipped with valves making column switching possible.

HPLC analyses are first carried out directly on urine (diluted about ten-fold depending on the creatinine concentration). When pathological HPLC isograms and chromatograms are seen, Bond Elut cartridges are used for sample clean-up before HPLC analyses, which may be carried out with or without column switching [8].

Amino acid profiling

A Biotronic automatic amino acid analyser (Biotronic, Maintal, F.R.G.) with an ion-exchange column and traditional ninhydrin detection is used. All samples, including urine, are deproteinized with sulphosalicylic acid prior to the amino acid analysis.

Two-dimensional gel electrophoresis

An ISO-DALT system (Electro-Nucleonics, Oak Ridge, TN, U.S.A.), as described by Anderson and Anderson [9], is used for high-resolution two-dimensional polyacrylamide gel electrophoresis (2-D PAGE). The ISO apparatus allows isoelectric focusing of twenty samples at a time, and the DALT tank for SDS electrophoresis in the second dimension holds twenty slab gels.

The analytical procedure is basically as described by Anderson's group at Argonne National Laboratory [9,10]. Serum is mixed with SDS–mercaptoethanol dissociation buffer [10] and tissue is mixed with urea–NP-40–mercaptoethanol–ampholyte dissociation buffer following pulverization to a fine powder in frozen conditions [11]. Cells (leukocytes and fibroblasts) are radiolabelled by culturing in media supplemented with [^{35}S]methionine, and are lysed in the urea dissociation buffer mentioned above [12].

RESULTS AND DISCUSSION

Metabolic disorders

This is a field where various chromatographic and mass spectrometric methods have proved to be very valuable diagnostic tools [2–5]. Many centres in different

parts of the world therefore use such methods to identify characteristic metabolites which occur in the body fluids as a consequence of an enzyme deficiency. The analytical system currently used in our laboratory [8] can diagnose about 120 different metabolic diseases. Selection of patients is based on a number of clinical symptoms or 'warning signals' typical of metabolic disease. These signals may be, e.g., progressive disease, metabolic acidosis, recurrent vomiting, peculiar smells from the body and urine, neurological symptoms of unknown etiology and indications of inheritance (similar cases in the family). Certain dip-stick tests and simple clinical chemistry analyses (e.g., creatinine concentration in urine) are carried out in addition to the following profiling steps:

(a) thin-layer and paper chromatography (one-dimensional) to determine glycosaminoglycans (mucopolysaccharides) and carbohydrates, respectively (urine and serum);

(b) quantitative, automated ion-exchange chromatography to determine the amino acid profile (urine, serum and CSF);

(c) capillary GC–MS with automated sample injection and peak identification by computerized mass spectral library searching to obtain the organic acid profile (urine only as a rule);

(d) HPLC with computerized diode-array detection to determine the urinary profile of, e.g., succinylpurines, orotic acid, uric acid and other non-volatile compounds. A special HPLC method for the quantitative determination of pyruvate or lactate in cerebrospinal fluid is used on certain clinical indications.

The outcome of this profiling may result in the correct diagnosis of a metabolic disease. A negative result, on the other hand, may exclude more than 100 known metabolic diseases. Follow-up studies, e.g., monitoring the effect of treatment and, in a few instances, prenatal diagnosis by direct analysis of cell-free amniotic fluid are also possible by means of GC–MS methods. In these instances the analytical procedures have to be modified to obtain quantitative data, e.g., by using special internal standards in HPLC and GC and by operating the mass spectrometer in the selected-ion monitoring mode (see also ref. 13).

In the following we report some typical recent results to illustrate how the various profiling techniques play important roles in diagnosis.

Triple H syndrome. A two-year-old boy was admitted to hospital and the clinical symptoms were frequent episodes with vomiting, lethargy and sopor/coma. The patient was psychomotoric retarded. Urine samples were analysed several times by our standard profiling procedure. The organic acid profile was normal, but both the amino acid profile (Fig. 1) and HPLC profile (Fig. 2) were pathological. The urinary excretion of the amino acids ornithine, homocitrulline and glutamine was highly elevated (Fig. 1). High glutamine often indicates hyperammonaemia, and special analyses of serum indeed showed elevated ammonia levels. The HPLC profile, as depicted at 194 nm in Fig. 2, contained an abnormal peak with the same retention time as orotic acid. The light absorption spectrum of the peak, as recorded with the diode-array detector (Fig. 2, insert) was identical with that of authentic orotic acid. Hence the patient had increased excretion of orotic acid, ammonia (and therefore also glutamine), ornithine and homocitrulline. This is typical of a rare metabolic disease, the triple H syndrome (hy-

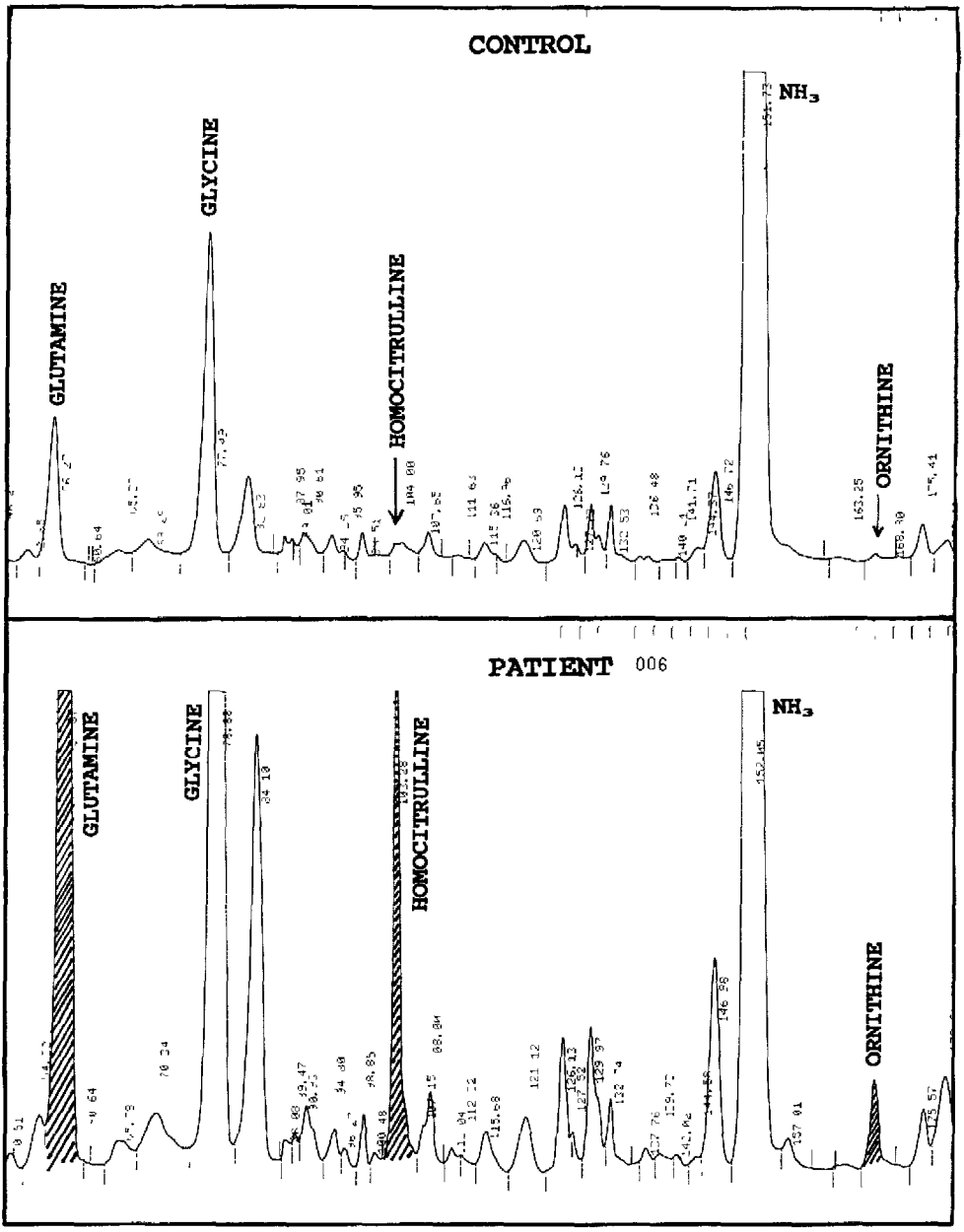


Fig. 1. Amino acid profile of urine from a control (top) and from a patient (bottom) with triple H syndrome. A Biotronic LC-5001 automatic amino acid analyser with an ion-exchange column and ninhydrin detection was used. Note the increased excretion of glutamine, homocitrulline and ornithine in the patient's sample.

perammonaemia, hyperornithinaemia, homocitrullinaemia), which appears to be due to a defect in the transport of ornithine into the mitochondria [14]. Reduced protein intake had a beneficial effect on the patient.

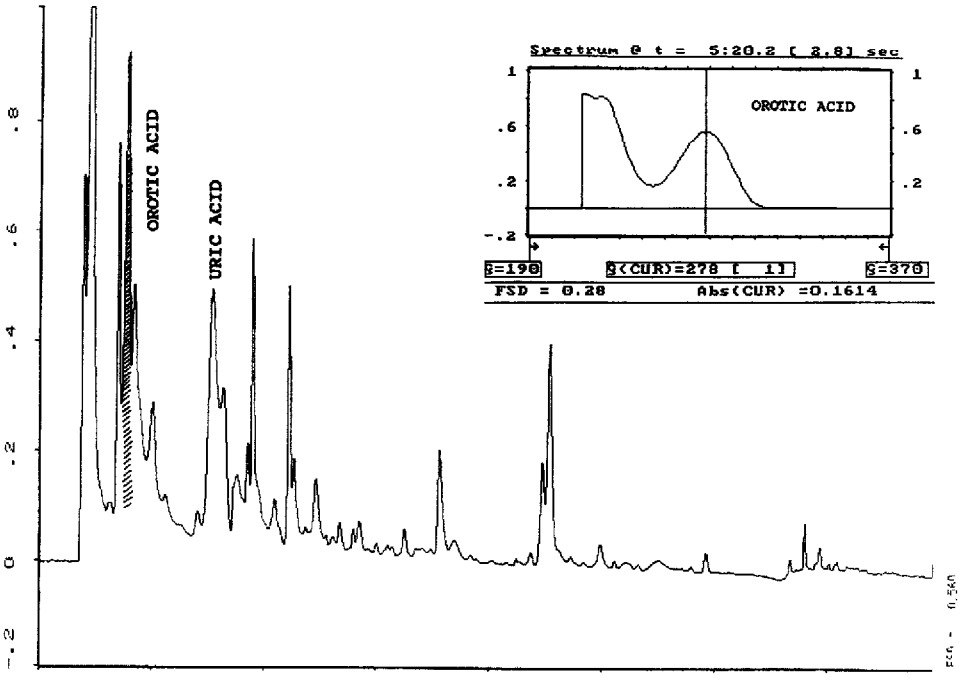
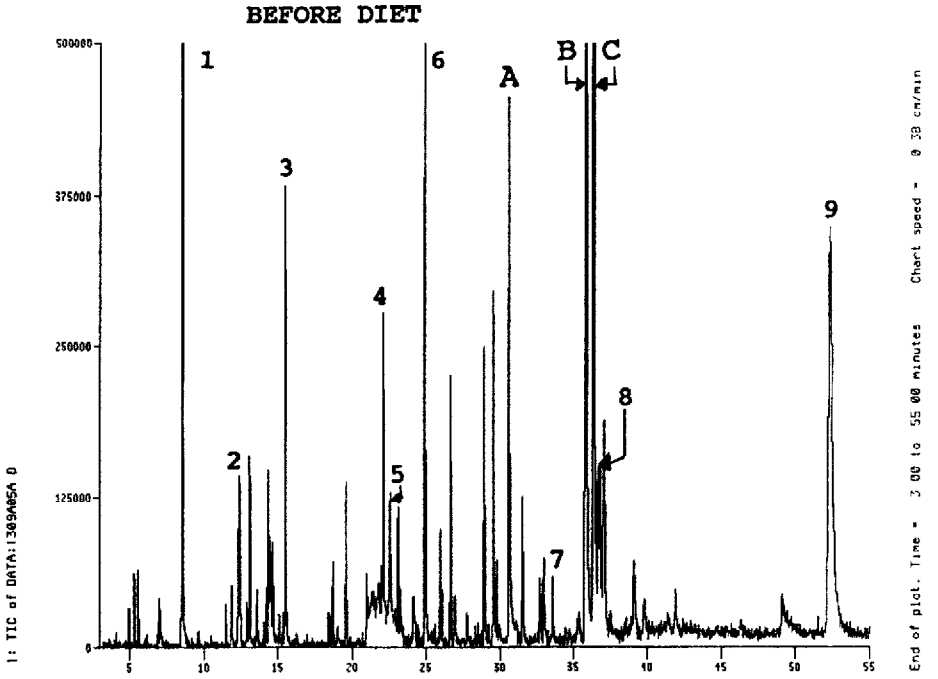


Fig. 2. HPLC trace (194 nm) of urine from a patient with triple H syndrome. A Supelcosil LC-18 column was eluted with a gradient system: starting solution, 5 mmol/l sulphuric acid; end solution, acetonitrile–water (4:6, v/v). Flow-rate, 1.0 ml/min. The diode-array detector was a Model 2140 rapid spectral detector with an IBM-XT personal computer. The peak designated orotic acid had a retention time and light absorption spectrum identical with those of authentic orotic acid (insert).

Propionic acidaemia. An eight-month-old girl was hospitalized with frequent vomiting and failure to thrive and to gain weight. She had an episode with convulsions. Laboratory findings included hyperglycinaemia. A urine sample was analysed with our profiling system. High glycine excretion was evident and the organic acid profile, as determined by GC–MS, showed several abnormal peaks (A,B and C in Fig. 3, top). The computerized mass spectral library search (Fig. 4) showed the identity of these peaks to be propionylglycine, tiglylglycine and methyl citrate. These metabolites are characteristic of the disease propionic acidaemia [15], which is due to a deficiency of the enzyme propionyl-CoA carbox-

Fig. 3. Organic acid profiles of urine from an eight-month-old girl suffering from propionic acidaemia. Top, before treatment; bottom, after instalment of a low-protein diet. The samples were acidified, extracted with diethyl ether and methylated with diazomethane before analysis. The separation was achieved with a Hewlett-Packard 5970 GC–MS system (see text) with an automated sample injection system. The SP-1000 fused-silica capillary column was programmed from 80 to 220°C at 4°C/min. Note the decrease in the metabolites A, B and C (propionylglycine, tiglylglycine and methyl citrate, respectively) after treatment with the low-protein diet. Some known metabolites are marked as follows: (1) 3-hydroxyisovalerate; (2) 2-methyl-3-ketovalerate; (3) succinate; (4) adipate; (5) 3-methyladipate; (6) 3-hydroxy-3-methylglutarate; (7) 3,6-epoxysuberate; (8) citrate; (9) hippurate. (A) Propionylglycine; (B) includes both tiglylglycine (leading edge) and 3-methyl citrate, peak 1; (C) 3-methyl citrate, peak 2.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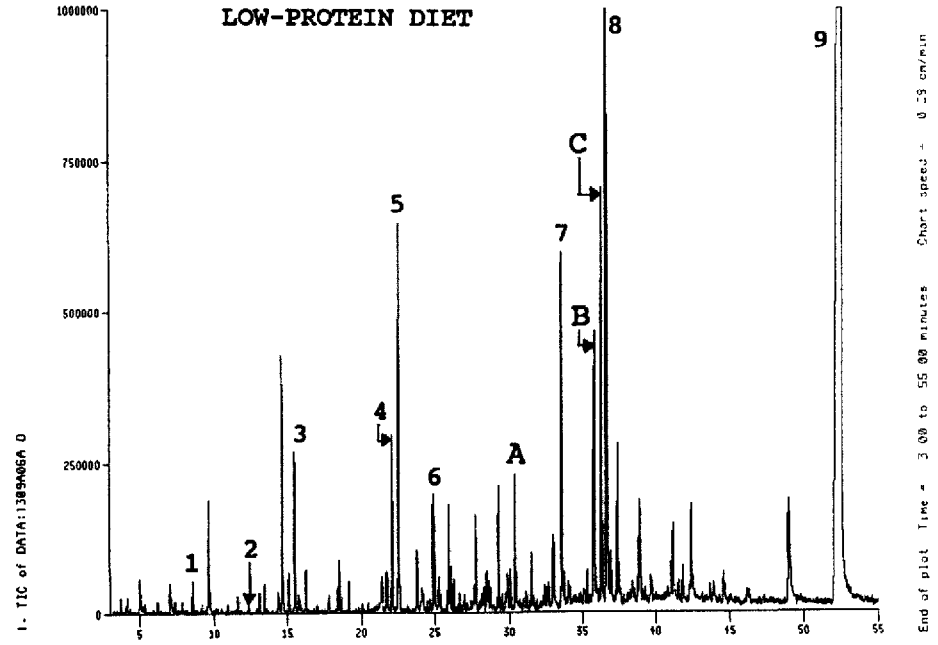
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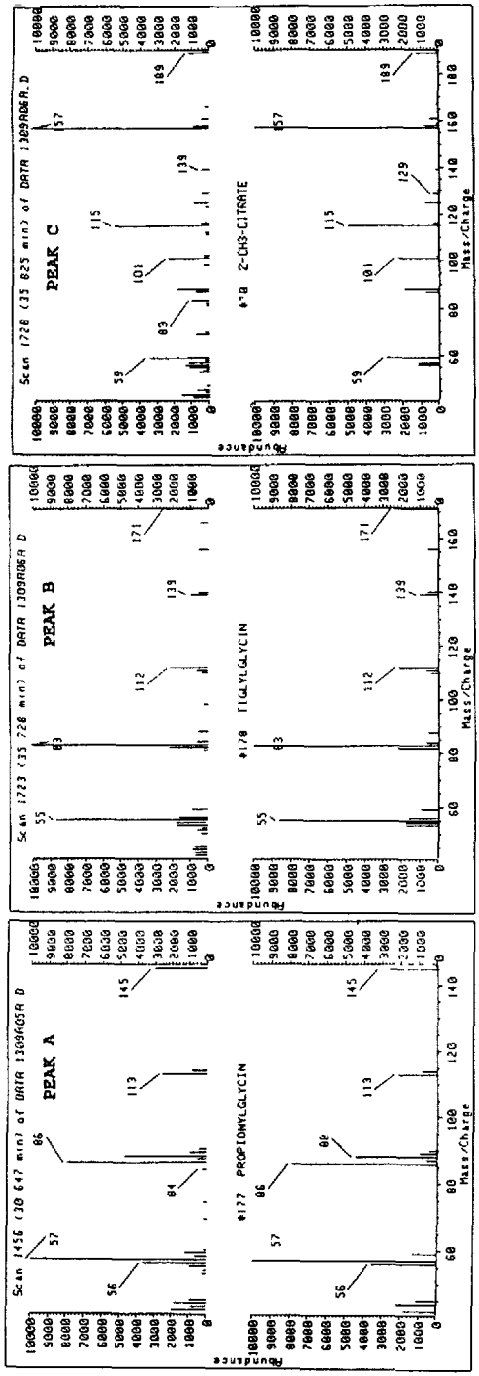

Fig. 4. Identification of peaks A, B and C in Fig. 3 by computerized mass spectral library search. The spectra of the unknown peaks are shown in the upper part of each section, and in the lower parts are shown the spectra of authentic propionylglycine, tiglylglycine and methyl citrate.

ylase [16]. Free propionate should also be present in this condition, but is not ordinarily seen in the organic acid profile, because the very volatile methyl ester of propionic acid is lost in the solvent front. A special low-temperature GC procedure proved the presence also of propionate.

Since instalment of a low protein diet, the excretion of the propionate metabolites has been at a satisfactory low level (Fig. 3, bottom), except in a few instances where the patient had a virus infection.

Biotin-responsive multiple carboxylase deficiency. A four-year-old patient had intermittant episodes with metabolic acidosis, ketosis and hypoglycaemia. The GC–MS profile (Fig. 5, top) showed the presence of large amounts of 3-hydroxyisovaleric acid and 3-methylcrotonylglycine. These metabolites are typical in several disorders of branched-chain amino acid metabolism, including multiple carboxylase deficiency. These disorders are known in several forms, one of which responds to large doses of biotin [17]. Fig. 5 (bottom) shows the complete normalization of the urinary organic acid profile in the patient after administration of biotin. The clinical symptoms soon disappeared and the child is now in good condition. Enzyme studies carried out later on cultured fibroblasts proved that the underlying defect in this patient was biotin-responsive multiple carboxylase deficiency [17].

Glyceroluria. A three-year-old boy was admitted to hospital with diarrhoea, dehydration and metabolic acidosis. Metabolic disorder was suspected. GC–MS analysis showed two pathological peaks in the organic acid profile (Fig. 6). These peaks were identified as glycerol and monomethylated glycerol (due to diazomethane treatment). Quantitative analyses showed a daily excretion of glycerol of about 20 g (the peak areas in Fig. 6 do not adequately demonstrate this large amount owing to the poor extractability of glycerol into diethyl ether; with our standard procedure glycerol is normally completely absent from the profile). Hyperglycerolaemia (glyceroluria) was first described in 1977 [18], and the underlying enzyme defect is glycerol kinase deficiency [18].

High-resolution two-dimensional electrophoresis (2-D PAGE) and metabolic disorders. The enzymatic deficiency in inborn errors of metabolism may have several different causes [19]. One underlying mechanism involves mutant enzyme–protein devoid of catalytic effect. Another cause is the failure of the cells to produce sufficient amounts of the enzyme in question. A mutant protein may have an altered charge and/or molecular mass, and can in principle be detected by 2-D PAGE. The absence of an enzyme–protein should also be detectable by the same method. Disease-associated alterations in 2-D gel patterns have been described in, e.g., Lesch-Nyhan syndrome [20], Duchenne muscular dystrophy [21] and Tangier disease (for a review, see ref. 22). Attempts in our laboratory to detect such changes in several other metabolic disorders, e.g., glutathione synthetase deficiency and hereditary tyrosinaemia, have failed. From the fact that apparently no laboratories, including ours, use 2-D PAGE for the routine diagnosis of metabolic disorders, it may be concluded that the technique is not well suited for this purpose. In a research context, however, 2-D PAGE has already made important contributions to human medical genetics [23].

DNA analyses and metabolic diseases. Recent progress in gene technology now

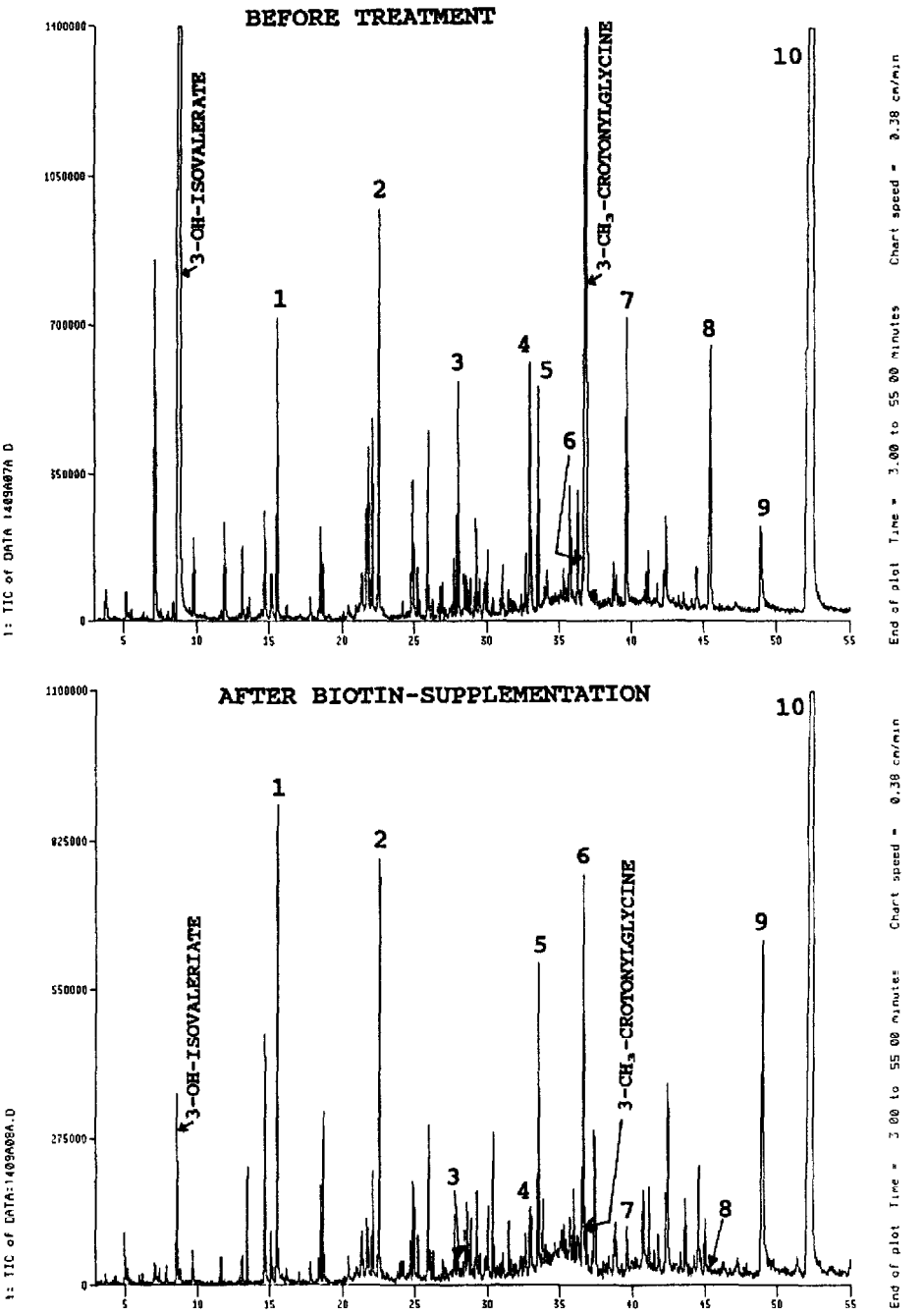

Fig. 5. Organic acid profile of urine from a patient suffering from biotin-responsive multiple carboxylase deficiency. Top, before treatment; bottom, after biotin supplementation. Experimental conditions as in Fig. 3. Note the normalization (decrease) in excretion of 3-hydroxyisovalerate and 3-methylcrotonylglycine after treatment. Some known metabolites are marked as follows: (1) suberate; (2) 3-methyladipate; (3) phenoxyacetate; (4) 2,5-furandicarboxylate; (5) 3,6-epoxysuberate; (6) citrate; (7) 5-hydroxymethyl-2-furoate; (8) N-methylsaccharine; (9) *p*-hydroxyphenylacetate; (10) hippurate.

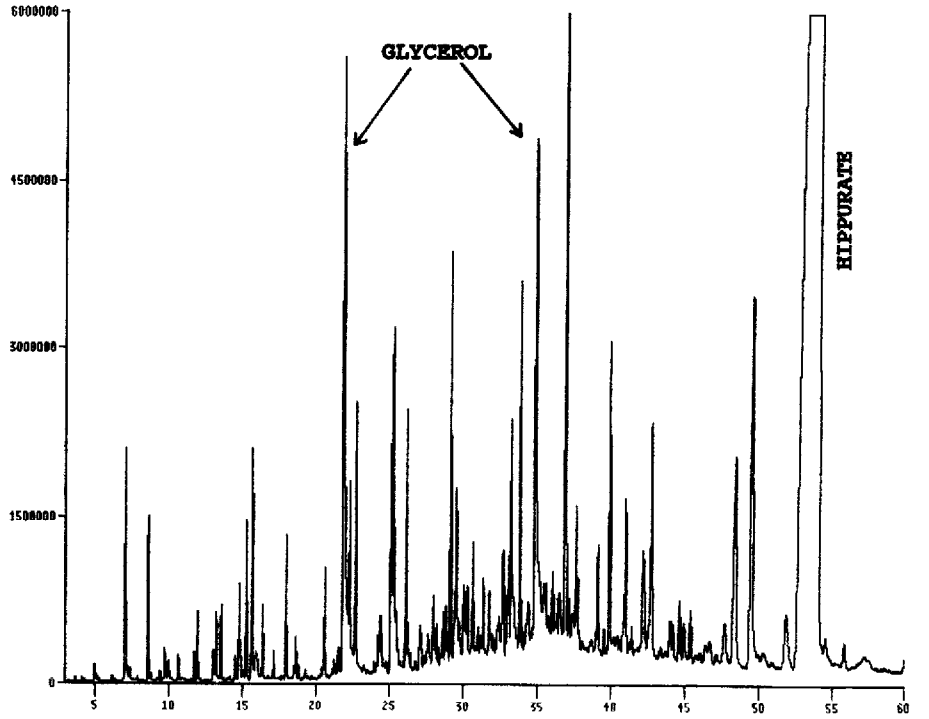

Fig. 6. Organic acid profile of urine from a three-year-old boy suffering from glyceroluria. Glycerol elutes in two peaks, the first corresponding to monomethylated glycerol due to the diazomethane treatment before analysis. Experimental conditions as in Fig. 3.

makes it possible to diagnose a number of metabolic disorders (currently about 25) at the gene level [24]. In principle the DNA is first isolated from cells of the patient (e.g., leucocytes, fibroblasts, amniotic cells or chorionic biopsies). After cutting the DNA by means of restriction enzymes, the resulting DNA fragments (more than 100 000) are separated by electrophoresis in agarose gel. The fragments are blotted on to a nylon membrane, converted into single strands and hybridized with a radiolabelled DNA probe complementary to the gene to be studied (Southern blotting). After autoradiography, a limited number of bands (often only one to three) show up. By comparison with similar patterns obtained by analysing DNA isolated from the mother, father and siblings, it is possible to determine whether or not the proband has a metabolic disease or is a carrier of the genetic defect. This method is in principle very simple and, as more and more genetic probes become available, diagnosis at the gene level is rapidly expanding.

The question may then be raised as to whether modern DNA technology will make chromatography obsolete for diagnostic purposes. One should realize, however, that DNA technology is suitable for diagnosis only in situations where the family already has had a child with a known disease. The DNA methods are therefore particularly appropriate for prenatal diagnosis. In contrast, the chromatographic methods can diagnose close to half of all the 250–300 metabolic diseases recognized today without knowing what to look for.

Conclusion. It can be postulated that chromatographic profiling techniques will continue to be most helpful tools for the diagnosis and studies of metabolic diseases for many years to come. These methods cannot be replaced by DNA technology, which, however, will become increasingly more important for prenatal diagnosis. High-resolution 2-D PAGE has little role as a routine diagnostic tool in the field of metabolic disease.

Profiling of pre-diagnostic sera from cancer patients: the JANUS project and early diagnosis of cancer

The on-going JANUS project was initiated by the Norwegian Cancer Society in 1973 [25]. The JANUS serum bank [26] now contains over 350 000 serum samples consolidated from several sources and is one of the largest serum collections currently available. The collection includes sera from cohort studies on cardiovascular disease conducted in four different counties in Norway. Up to three consecutive samples are available from each person. In addition, specimens from more than 26 000 Red Cross blood donors are continually being added to the collection and two to fourteen (average four) consecutive samples are available from each donor. The sera are stored at $-25\,^{\circ}C$ and a total of about 102 000 persons have made serum donations up to the present time [26].

Information concerning all cancer patients is collected in the Cancer Registry of Norway, where the registration of cancer cases has been compulsory since 1953. At regular intervals, usually once every year, the JANUS collection is matched against the files in the Cancer Registry. From 1973 up to March 1988 about 3500 of the donors have developed some form of cancer. Deep-frozen serum samples collected from a few months to fifteen years prior to clinical recognition of their disease are consequently available for research purposes. The aim of the JANUS project is to search in these premorbidity sera for chemical, biochemical, immunological or other changes that might be indicative of cancer development at early stages.

When samples from the bank are used in research projects, three age-, sex- and storage-matched control specimens (from JANUS donors who have not developed cancer) are retrieved for every patient sample. Samples from cases and controls are randomly numbered before distribution for assay purposes, and the code is not broken until all analytical results are available.

During the first ten years of the JANUS project the main task was serum collection and little was done with the deep-frozen samples. By 1983 the total number of cancer cases had reached 1800, with skin, breast, lung and intestinal cancer as the dominating types [26]. Preliminary research was then initiated according to two principally different analytical approaches, multi-component analysis using profiling techniques and single-component analysis. The latter type was aimed at the determination of specific antigens, enzymes, isoenzymes, hormones, viruses or other 'markers' assumed to be associated with cancer. In this paper we shall briefly describe some results obtained, including the use of GC–MS for profiling serum metabolites and 2-D PAGE for the determination of serum protein patterns.

Protein profiling of JANUS sera by two-dimensional electrophoresis. Samples

from various cancer types (lung, breast, intestine, etc.) were retrieved together with age-, sex- and storage-matched controls as indicated above. The sera were analysed by 2-D PAGE and silver-stained [27]. The patterns were carefully inspected and compared before breaking the code. Based on a number of experiments with over 200 samples, the conclusion could be drawn that no significant differences (apart from genetic polymorphisms) were found between control sera and pre-diagnostic cancer sera. This was the case even when using serum that had been collected only a few months prior to clinical diagnosis of disease.

The results may have several explanations. One is that possible cancer-associated proteins may be present in the pre-diagnostic sera only in minute amounts and thereby escape detection even with the highly sensitive silver staining. Another important factor is the masking effect of major serum proteins such as albumin and the immunoglobulins. Attempts have been made in our laboratory to remove albumin selectively by use of immobilized monoclonal human anti-albumin, but with poor results [28]. Finally, a 'marker' protein, if present, may not become stained with the present technique. It is known that certain proteins, e.g., carcinoembryonic antigen (CEA), metallothionein and the 'P-glycoprotein' [29] involved in multi-drug resistance, do not stain with silver.

The failure to detect tumour-related proteins in pre-cancer sera using 2-D profiling was at first considered negative. On the other hand, these studies clearly show that the 2-D protein patterns are remarkably unchanged (Fig. 7) even if the sera have been stored for many years in a deep-frozen condition. This again reflects the stability of the primary structure of the serum proteins and thereby of immunological properties associated with this structure. This knowledge has proved important in subsequent single-component analyses using monoclonal antibodies directed towards specific tumour-associated antigens (see below).

GC–MS profiling of JANUS sera. Both as an attempt to search for cancer-induced changes in metabolic profiles of sera and to obtain knowledge about the stability of serum metabolites, GC–MS profiling was carried out on selected pre-diagnostic sera and matched controls. A typical organic acid profile and a 'total' metabolic profile of serum are shown in Fig. 8. In the upper chromatogram the serum proteins had been removed by ethanol precipitation followed by extraction and methylation before separation. In the bottom chromatogram the serum had been evaporated to dryness, refluxed with methanol–hydrochloric acid and silylated before separation by GC. Although this metabolic profiling study has not yet been completed, it seems clear that most of the serum metabolites seen as peaks in Fig. 8 undergo little change with time in the deep-frozen sera. Other metabolites, including unsaturated lipids, fatty acids and certain vitamins, seem to be less stable. At this stage in the GC–MS profiling of the pre-cancer sera we cannot report on metabolite changes that are related to the development of cancer.

Single-component analyses of JANUS sera. It is outside the scope of this paper to discuss in detail the results of the many single-component analyses carried out on the JANUS sera, often as international collaborative studies. For the sake of completeness, however, it can be mentioned that the level of the tumour-associated antigen CA-125 is elevated several months prior to diagnosis of ovarian cancer [30], serum thyroglobulin may be a preclinical tumour marker in subgroups

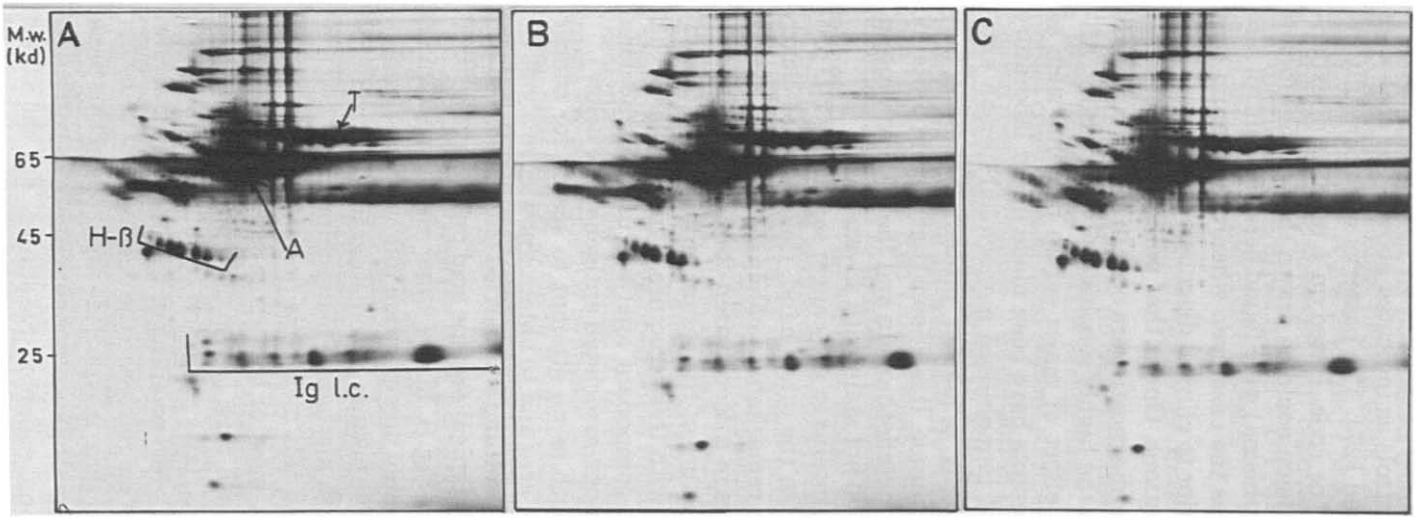

Fig. 7. Two-dimensional protein patterns of serum collected from the same person (A) in 1973, (B) in 1976 and (C) in 1981. The samples were stored at −25 °C and were analysed simultaneously (in 1988) as described in the text. The gels were stained with silver [27]. They are oriented with the acidic end to the left, and molecular masses are indicated on the left hand side. Very few changes in the protein pattern are noted, indicating good stability of the primary structure of the proteins. Marked proteins: A = albumin; T = transferrin; H-β = haptoglobin β-chain; Ig l.c. = immunoglobulin light chains.

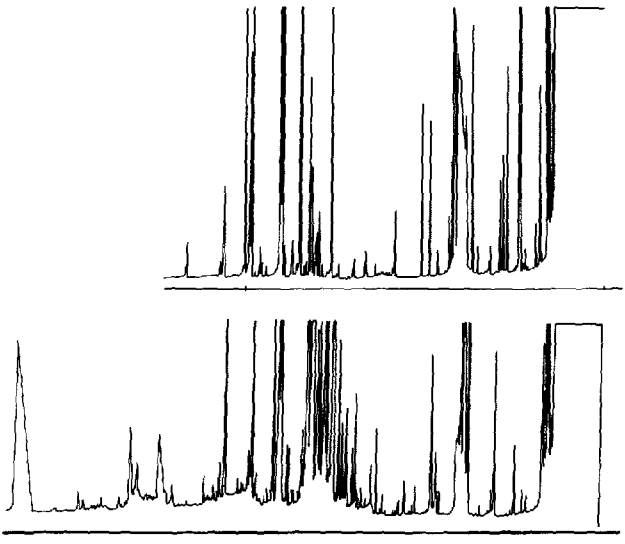

Fig. 8. Organic acid profile of serum. The proteins were removed by ethanol precipitation followed by extraction and methylation before separation. Bottom: 'total' metabolic profile of serum. The sample was evaporated to dryness, refluxed with methanol–hydrochloric acid and silylated before separation by GC. Column and GC conditions as in Fig. 3.

of thyroid cancer [31], a low level of selenium in serum reflects an increased risk of thyroid cancer [32] and raised antibodies in serum against Epstein–Barr virus is a risk factor for the development of Hodgkin's disease [33]. A number of other projects, particularly using new, specific monoclonal antibodies on the Janus material, are currently in progress.

Conclusion. Specific methods, particularly using monoclonal antibodies directed towards tumour-associated antigens, seem well suited to detect disease-associated changes in pre-diagnostic cancer sera. The JANUS serum bank has demonstrated its use as a resource in evaluating new serum tests for early detection of cancer. The profiling methods have so far not revealed changes in the pre-cancer sera that might indicate the development of cancer at early stages. GC–MS and 2-D PAGE profiling have, on the other hand, added valuable knowledge about the stability of stored, deep-frozen serum samples. This information is of importance in the maintenance and handling of the JANUS serum bank established for cancer research purposes.

Two-dimensional electrophoresis for profiling of cloned cancer cells: recognition of marker proteins associated with metastatic properties

Several laboratories have used 2-D PAGE to study qualitative and quantitative changes in the 2-D protein patterns of cancer cells and biopsies from e.g., renal carcinoma [34], breast cancer [35], leukaemia [36], hepatoma [37], sarcomas [38], colonic adenocarcinomas [39,40] and melanomas [28]. Further, Celis et al. [41] have used the technique extensively to investigate the process of malignant transformation. Strikingly few changes in the protein pattern following transformations were observed [41].

Our studies on polyps, colonic cancer and melanomas [28,40] made us realize the problems of applying 2-D profiling to biopsy materials. No matter how much care is exercized during biopsy collection and preparation, one is always faced with problems caused by tumour heterogeneity and artifacts due to contaminating normal cells and tissue. For this reason we prefer to work with cells in culture, preferably homogeneous cell lines, i.e., cloned cells.

In the following we shall briefly describe recent results obtained in a collaborative study (with I.A. Grimstad, Oslo, Norway) on metastatic properties. i.e., the spreading tendency of cancer cells. Two unrelated 3-methylcholanthene-induced murine fibrosarcomas were used as starting materials. Using a variety of cloning and selection procedures [42], seventeen cell clones, ten from tumour 1 and seven from tumour 2, were chosen for further experiments. Each of the seventeen clones was tumorigenic, i.e., formed tumors when injected into the footpad of female syngeneic mice. Seven clones formed local tumours, which in a number of repeated experiments never gave rise to metastases. These seven cell lines were therefore categorized as weakly malignant. The other ten cell clones formed tumours which in all experiments gave rise to metastases to the lungs of the animals. Hence these ten cell lines were highly malignant with a consistant tendency to spread. The seven weakly malignant and the ten highly malignant fibrosarcoma cell lines were allowed to grow in a nutrient medium containing radiolabelled methionine. After harvesting, lysis and separation of the proteins by 2-D PAGE, the spots were revealed by autoradiography.

Comparison of the 2-D profiles (Fig. 9) showed a close resemblance of the protein patterns of all seventeen cell lines. This was not surprising as all cell

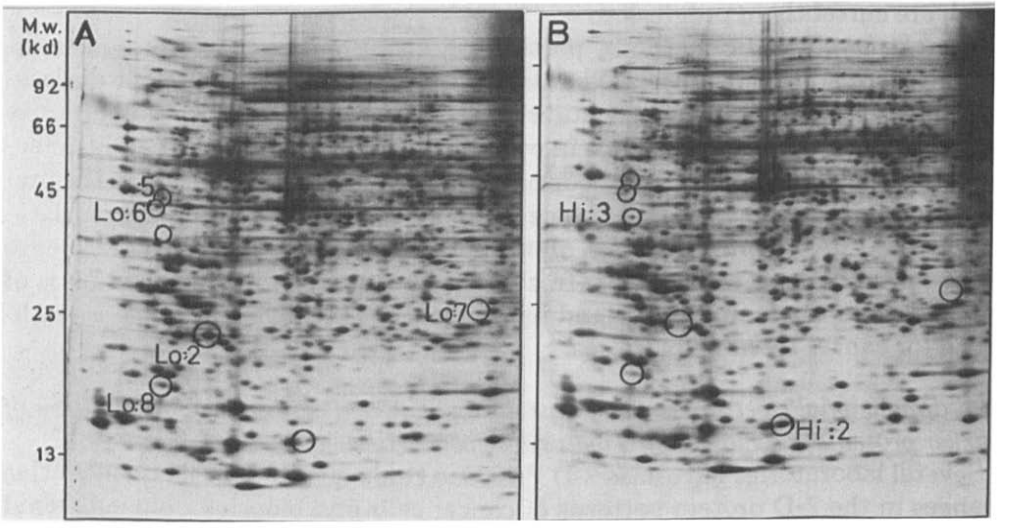

Fig. 9. Two-dimensional protein patterns of (A) a weakly metastatic cell clone and (B) a strongly metastatic cell clone from the same murine fibrosarcoma. Radiolabelling of the proteins, 2-D PAGE procedures and autoradiography are described in the text. The positions of proteins differing systematically between the weakly and the strongly metastatic clones are marked. The gels are oriented as in Fig. 7.

clones originated from the same two tumours. Careful inspection of the protein patterns, however, revealed consistent differences in the expression of seven specific proteins. One of these marker polypeptides, designated Hi:2, was very strongly expressed by all ten highly metastatic cell clones, but was absent from the weakly malignant clones. Another marker protein, Lo:6, was consistently most strongly expressed by the weakly metastatic clones. Five other polypeptides also distinguished between highly and weakly metastatic clones, but not as stringently [42]. These results add further evidence to the apparent fact that minor biochemical changes in the genes or gene products of a cell may lead to dramatic changes in its behaviour. In this particular case, a change perhaps in as few as two genes/gene products (corresponding to proteins Hi:2 and Lo:6) may be sufficient for a cancer cell to acquire high or low metastatic properties.

An obvious continuation of this study is the attempt to characterize further the marker proteins and their corresponding genes, and this is currently being done as follows. Twenty 2-D gels of, e.g., a highly metastatic cell clone are run in parallel. After staining with Coomassie Brilliant Blue, the Hi:2 spot is cored out in all gels and mixed. The isolated protein is blotted on to an Immobilon membrane [poly(vinylidene bifluoride)] (Millipore, Bedford, MA, U.S.A.). By use of modern microsequencing techniques it is possible to obtain the sequence of, e.g., the first ten amino acids of the protein molecule. The structure of the first part of the corresponding gene can then easily be deduced. A search in a genomic library may give clues to the structure of the gene. In parallel to these experiments attempts are also being made to raise poly- and monoclonal antibodies against the marker proteins, so that larger amounts of, e.g., Hi:2 may be isolated by immunological means.

Conclusion. Protein profiling using high-resolution 2-D PAGE is a suitable research tool for cancer studies. The method should be applied with great caution to biopsies, which are likely to be contaminated with normal cells and connective tissue. Application to cells in culture is greatly to be preferred. We have used 2-D PAGE to study closely related fibrosarcoma cell clones differing in metastatic properties. Certain marker proteins, particularly those designated Hi:2 and Lo:6, were only expressed in the highly and weakly metastatic cell clones, respectively. High-resolution 2-D PAGE is now used as a preparative technique to isolate sufficient amounts of these proteins to perform microsequencing of the first amino acids in the polypeptide, and thereby obtain information which may possibly lead to the characterization of the gene(s) responsible for the metastatic properties.

Chromatography and electrophoresis as complementary profiling techniques for the classification of bacteria

The first attempt to characterize bacteria by GC was carried out by Abel et al. in 1963 [43]. Since then a number of researchers have applied GC to the study of microorganisms.

Analytical procedures for profiling of fatty acids, sugars, etc., are well known (e.g., refs. 44 and 45). The GC technique is used in some microbiological laboratories to aid clinical diagnosis, although it is evident that the profiling methods are mainly of research interest. In collaboration with a microbiologist (I. Olsen,

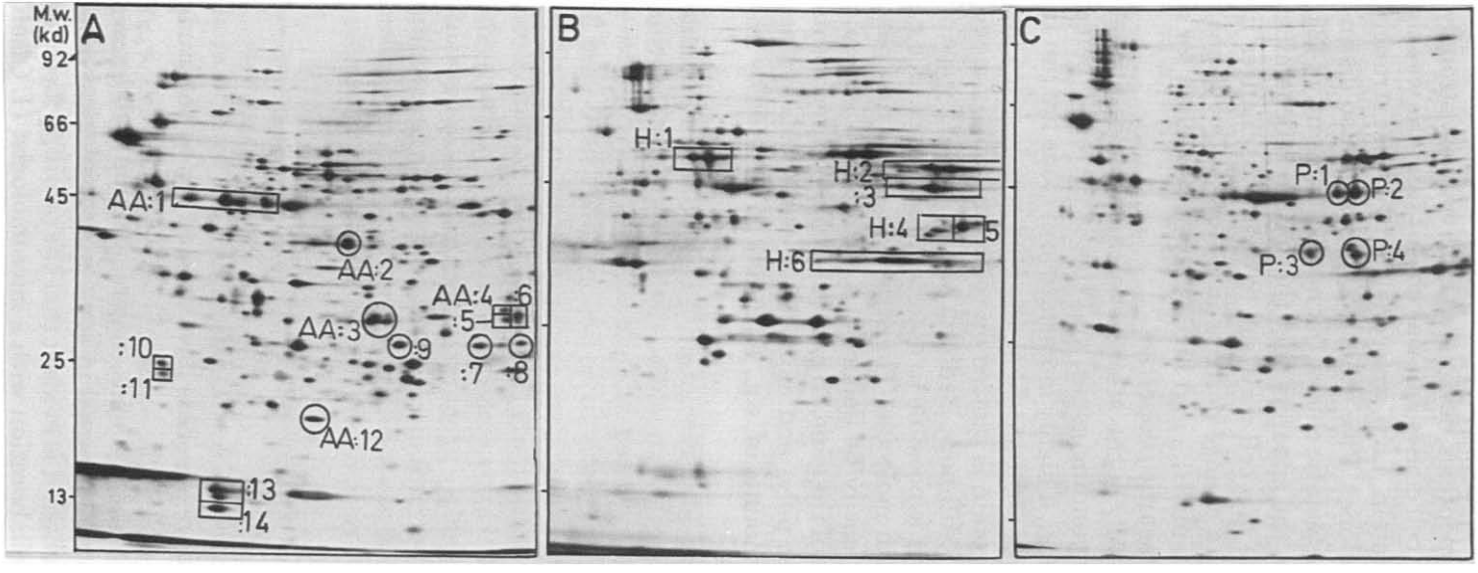

Fig. 10. Two-dimensional protein patterns of (A) *Actinobacillus*, (B) *Haemophilus* and (C) *Pasteurella*. The gels were silver-stained and are oriented as in Fig. 7.

Oslo, Norway) we have recently applied 2-D PAGE to distinguish between the closely related species *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* [46]. These species could also be differentiated by GC profiling [47]. The study has now been extended to include a number of other microorganisms of the *Haemophilus, Actinobacillus* and *Pasteurella* groups. Distinction of these clinically important organisms is often difficult, and doubt has been expressed as to their generic separation. Several strains of *H. paraphrophilus, H. aphrophilus, H. influenzae, A. actinomycetemcomitans, Pasteurella multocida, P. haemolytica* and *P. ureae* were cultured, isolated and subjected to 2-D PAGE [48]. After separation the gels were stained with silver.

The electrophoretic protein patterns of *Haemophilus, Actinobacillus* and *Pasteurella* were quite distinct (Fig. 10). Also, the patterns of *P. multocida, P. haemolytica* and *P. ureae* differed markedly. Except for certain strains of *H. paraphrophilus*, it was difficult to distinguish *H. paraphrophilus* from *H. aphrophilus* strains. *H. paraphrophilus* strains were similar, but *A. actinomycetemcomitans* could easily be distinguished from all species tested, including the related *H. aphrophilus, H. paraphrophilus, P. haemolytica* and *P. ureae*. The results suggest that *H. aphrophilus* and *H. paraphrophilus* seem to require revision of their current taxonomic positions [48].

Conclusion. It is the experience of our group and others (e.g., ref. 49) that high-resolution 2-D PAGE has considerable potential in the classification and identification of bacteria. Protein profiling seems to be a complementary technique to the well established GC and GC–MS profiling techniques currently used in microbiology.

GC–MS, HPLC, two-dimensional electrophoresis and DNA analyses for the evaluation of toxic and mutagenic compounds

A number of short-term tests to evaluate the genotoxic, carcinogenic and mutagenic properties of a chemical or an environmental mixture have been developed [50]. Most of these tests use plants or bacteria, followed by longer-term animal experiments. The most widely accepted test for the mutagenic behaviour of a chemical is the Ames test [51], utilizing mutants of *Salmonella typhimurium*. In collaboration with Canadian researchers (F.W. Karasek and co-workers) we have for the past few years taken a new approach to evaluate toxicity and mutagenicity utilizing human living cells and chromatographic and electrophoretic profiling techniques [52].

Environmental mixtures including organic extracts of incinerator fly-ash [53], diesel exhaust [54] and air-borne particulate matter [55], and also oil and petroleum products, have been extensively studied by the novel technique. After obtaining an environmental extract it may be separated by means of preparative HPLC, and several fractions can thus be obtained. Aliquots of each HPLC fraction may subsequently be analysed by GC–MS to characterize the organic compounds present. In this way over 300 organic substances, including polycyclic aromatics, chlorinated dibenzofurans and various dioxins, were identified in fly-ash extracts [53]. Each HPLC fraction was subjected to the new toxicity and mutagenicity test.

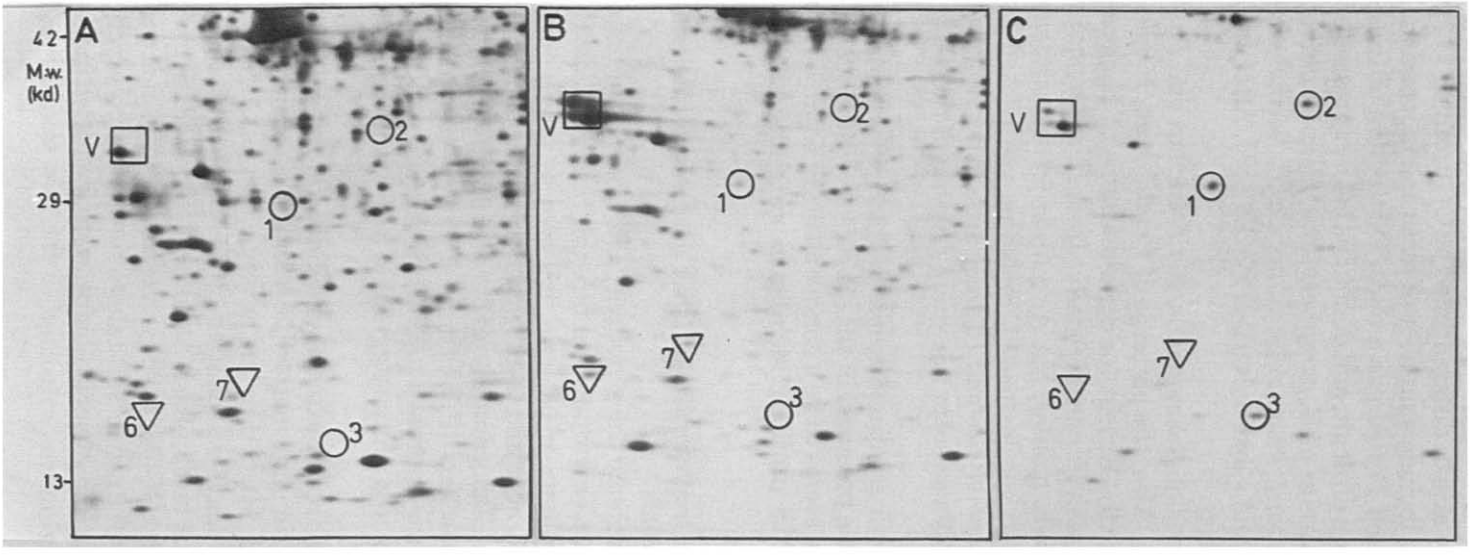

Fig. 11. Two-dimensional protein patterns of human peripheral blood leukocytes grown (A) in the absence and (B) and (C) in the presence of increasing amounts of extract of fly-ash. The proteins were revealed by autoradiography. The gels are oriented as in Fig. 7 and only sections of the total 2-D patterns are shown. Note the formation of new proteins, e.g., 1, 2 and 3.

In this test [52], human living cells, particularly fibroblasts or freshly isolated peripheral blood leukocytes in culture, are utilized. These cells are allowed to grow and synthesize proteins in the absence or presence of the added compound(s) to be tested. During the incubation period the proteins become radiolabelled as the incubation medium contains [^{35}S]methionine. After 2-D PAGE and autoradiography, changes in the 2-D protein profile are readily seen if the added chemicals have had toxic or mutagenic effects. In principle, the target of harmful chemicals may be the genes (DNA), the transcription process to mRNA, the various RNAs involved in protein synthesis, the ribosomes and the many enzymes involved in the making of new proteins. Attack at any one or more of these steps may lead to alteration of the cellular protein pattern. The effects of adding increased amounts of a toxic or mutagenic mixture usually are as follows: (1) the synthesis of most of the 2000 proteins seen on the 2-D gels are gradually and easily blocked; (2) the production of a limited number of proteins is virtually unchanged (resistant to the chemical); and (3) new proteins are being formed. The last observation is clearly shown in Fig. 11. The formation of new proteins on the 2-D pattern may be due to mutation, induction and/or post-translational changes.

The new test has been applied in several studies [52–55]. Most recently it was used to evaluate possible contamination of ground water by incinerator fly-ash deposited in the soil. In brief, it was found that water extracts of fly-ash had a marked toxicity towards human cells. The toxicity was the same whether or not the organic compounds had been removed (benzene extraction) prior to the water leaching. By means of inductively coupled plasma emision spectroscopy it was found that the water extract contained large amounts of inorganic compounds, particularly lead, cadmium, antimony and arsenic. These inorganics explain the toxicity of the water extract of the fly-ash. The conclusion of this study is therefore that the leaching of heavy metal compounds into the ground water probably represents a more important pollution problem than the leaching of dioxins and other organics. The latter compounds are firmly adsorbed to the fly-ash particles and are present only in trace amounts in the water leachates. (Details of this study will be reported elsewhere in collaboration with F.W. Karasek.)

Modern DNA technology opens up the possibility of developing a mutagenicity test closely related to that described above, except that damage directly on the genes themselves can be recognized. After incubating the living human cells in the presence and absence of the compound(s) to be tested, the cells are divided into two aliquots. One may be used for 2-D protein analyses as above. From the second aliquot DNA is isolated, treated with restriction enzymes and subjected to Southern blotting analysis as described above. Hybridization with probes that give many bands on the Southern blot, e.g., probes directed towards the HLA genes (human leukocyte antigens), is then carried out. If the chemical(s) have caused damage in one of the cutting sites for the restriction enzyme and this mutation is within the HLA region of the DNA molecules, then this will result in marked alterations in the band pattern as seen after Southern blotting and autoradiography. Preliminary results from our laboratory indicate that the mutagen nitropyrene does indeed cause such mutations of certain human genes in the HLA region.

Conclusion. We have described a new assay for toxicity utilizing living human cells and 2-D protein profiling. HPLC and GC–MS may be used as complementary tools to fractionate and identify the constituents of the chemical mixtures, e.g., environmental pollutants, to be tested. An outline is given of another new test which makes it possible directly to detect damage to certain vital genes.

CONCLUSION

We have demonstrated the complementary use of high-resolution chromatography and two-dimensional electrophoresis to solve a number of biomedical and environmental problems. For a laboratory involved in profiling it is of great advantage to be able to handle biochemical techniques such as cell culturing, in addition to the instrumental part of the approach. Inclusion of new technology into the profiling system should also be exploited. In addition to DNA techniques, new developments in the field of capillary electrophoresis [56] seem promising.

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