1. NMR IN BIOLOGY AND MEDICINE: OVERVIEW

G. K. Radda
University of Oxford

Professor Radda presented an overview of nuclear magnetic resonance as applied to biology and medicine. It has become a valuable tool for demonstrating pathological changes and determining their biochemical cause. It is a non-invasive and non-destructive method which can be used in vitro and in vivo.

Magnetic resonance is a property of nuclei with an odd number of protons. The simplest example of this is the hydrogen nucleus ('H), the single proton. This proton can possess two spin states, and the direction of spin in an applied magnetic field will determine that proton's 'energy'. Under normal conditions the vast majority of nuclei will be in the lowest energy state. However, by applying radiofrequency radiation of the correct energy, protons can be made to 'flip' into their higher, unstable energy state. When the radiation is removed and the protons are allowed to 'relax', they will re-emit the characteristic radiofrequency radiation.

The frequency emitted following relaxation is a function of the nucleus involved, the magnetic field strength and the chemical environment surrounding the nucleus. By applying a uniform magnetic field and 'sweeping' the radiofrequency range, a nucleus in different chemical environments will resonate at different frequencies. A spectrum is thus produced.

Not all nuclei are magnetic resonance active. Those of biological interest are 'H, 13C, 31P, 19F, 7Li, 23Na, 27Al, 31P and 87Rb.

The usefulness of these nuclei depends on their abundance and the sensitivity of their detection. 31P and 'H are the most widely used nuclei. An example of a 31P spectrum is shown in Fig. 1.

To produce an 'image' of the nuclei and therefore of molecules in three dimensions, it is necessary to apply magnetic field gradients in three dimensions and sweep the radiofrequency range in those defined directions. 'Slices' can be taken through tissues to trace the presence of these nuclei in various metabolites.

2. NMR INVESTIGATIONS IN CARDIAC METABOLISM

Anne-Marie L. Seymour
University of Oxford

Dr Seymour described the use of 31P magnetic resonance combined with biochemical measurements to determine the metabolic changes occurring in cardiac disease using the isolated rat heart as a model.

The normal, beating heart must replenish the ATP stores used for muscle contraction, for continuous working of ion pumps and for biosynthesis of large molecules. It does so by such processes as glycolysis, the citric acid cycle and oxidative phosphorylation. In exercise the heart beats faster and more strongly but still the supply of ATP must equal its demand.

In diseases of the heart there may be an energy imbalance. The problem can lie on either side of the equation. A mitochondrial myopathy may produce a deficit in energy production, whereas defects in the contractile mechanism of myosin will lead to a breakdown in energy utilisation. Alternatively, as a consequence of either process: (i) ion concentrations may change; (ii) the number of specific ion pumps may change; (iii) the rate of biosynthesis may change.

These changes in energetics may be followed using 31P magnetic resonance in the perfused isolated rat heart as a convenient experimental model. A force transducer is attached to the apex and the heart is artificially paced to give 300 isometric beats/min. The

Fig. 1. A 31P nuclear magnetic resonance spectrum of rat heart muscle: A, inorganic phosphate; B, phosphocreatine; C, ATP γ, α- and β-phosphates.

Intensity

Frequency

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heart is placed in a magnetic field and surrounded by a radiofrequency coil.

Figure 2 shows that ATP levels in the hypertrophied heart remain fairly constant but the ratio of phosphocreatine to inorganic phosphate shifts towards more inorganic phosphate and less phosphocreatine (and in fact reduced total creatine concentration). A broad phosphomonoester peak appears which, on amplification using tissue extract, shows a prevalence of the products of glycolytic enzymes such as glucose-6-phosphate and fructose-6-phosphate. Individual peaks can be identified using ‘titration’ and ‘spiking’ methods.

It is also possible to measure the flux of metabolites in biochemical systems using a method called magnetisation transfer. A selected peak (eg γ-phosphate of ATP) is magnetically ‘labelled’ and irradiated to saturation. The effects on the other molecular species are then studied.

In the hyperthyroid hypertrophied heart the flux of the glycolytic intermediates is increased. Biochemical assays support this finding. The heart usually relies on aerobic metabolism; the question therefore arises as to why there should be an increase in the flux of glycolytic metabolites. Dr Seymour put forward the hypothesis that in the hyperthyroid heart the Na⁺/K⁺ ATPase is working at a much greater rate than normal and that it is somehow selective for the ATP produced by glycolysis. It is possible to study Na⁺/K⁺ ATPase actively using nuclear magnetic resonance. Natural Na⁺ is visible to nuclear magnetic resonance but natural K⁺ is too insensitive. However, Rb⁺ is chemically similar to K⁺ and is magnetically visible. By measuring Rb⁺ flux, K⁺ flux is seen to be increased in the hyperthyroid heart, and this represents an increase in the action of the Na⁺/K⁺ ATPase.

3. USE OF 31P AND 1H NMR SPECTROSCOPY IN THE INVESTIGATION OF ANIMAL MODELS OF DISEASE

S. R. Williams
Royal College of Surgeons, London

Dr Williams began his talk with some questions about the possible roles of nuclear magnetic resonance. Can NMR spectra reveal pathology and, if so, which pathologies? Can the spectra be recorded quickly enough (20-40 min) and, if so, is the spatial resolution sufficient?

Dr Williams gave examples to illustrate the differences (also advantages and disadvantages; Table 1) in 1H and 31P spectra (Fig. 3); he used rat leg muscle as his model.

| Advantages | Disadvantages |
|------------|---------------|
| 1H | Low background | Ubiquitous | Magnetic field strength |
| 31P | Low sensitivity | Important metabolite | Simple spectra |

The problem with 1H spectroscopy is evident here; the water and fat signals saturate any useful information. However, it is possible to suppress the water peak by a number of methods (up to 10,000X), thereby enhancing the total spectrum, to obtain some more useful information. Peaks corresponding to such species as phosphocreatine and certain amino acids, such as taurine, can then be identified. The fat signal still masks important species such as lactate but, with a technique called spectral editing, the fat signal can largely be removed and the underlying lactate signal exposed.

The 31P spectrum is very clear and, although limited in the number of compounds that can be observed, gives important information on bioenergetics in vivo. In conjunction with the modified 1H spectrum, however, a wide range of molecules can be detected and useful physiology and biochemistry discerned.

A further example of the in vivo use of combined 31P and 1H magnetic resonance was illustrated by an investigation into gerbil brain ischaemia produced by unilateral occlusion due to an incomplete circle of Willis. By measuring, on each side of the brain, levels of P₃, PCr and ATP using 31P magnetic resonance, and lac-
tate levels using \(^1\)H, it was possible to demonstrate that
the ischaemic lesion was in the left hemisphere (due
to an inadequate blood supply).

\(^1\)H magnetic resonance is particularly useful in relation
to the brain, and can provide more information
than \(^3\)P. Two functionally important molecules in the
brain are glutamate and \(N\)-acetyl-aspartate (NAA), nei-
ther of which contains a phosphate group. In liver fail-
ure there is an increased circulation of ammonia in the
blood. This crosses the blood-brain barrier and reacts with glutamate to form glutamine. The change
in the glutamate/glutamine ratio can be measured
using \(^1\)H spectra. NAA is a very important ‘marker’
when viewing the brain. In infarction the level of NAA
in brain tissue drops; in astrocytoma it seems to be at a
very low level. In fact NAA is probably not present at all in astrocytoma and is just a neuronal marker.
Recent research has shown that it may be a marker of
maturation. By measuring levels of NAA, creatine and
choline it is possible to identify and ‘grade’ turnover
in pathological brain. This may become an important
diagnostic technique.

4. COMBINED USE OF MR IMAGING WITH MR
SPECTROSCOPY
L. D. Hall
University of Cambridge

Professor Hall explored some of the technical difficul-
ties associated with magnetic resonance spectroscopy
and imaging (MRS and MRI).

The devil at the heart of all spectroscopy is the low
signal/noise ratio. Amongst other approaches this can
be improved by maximising the number of scans, the
magnetic field strength and the probe filling factor.
This last factor is the percentage of probe filled by the
sample. The two techniques of detection used at the
present time are the surface-coil technique, in which
a flat coil is positioned over the surface of the sample,
and the volume-coil technique in which the sample is
placed within a coil volume. The former is best suited
for MRS of pathology located near the surface, but for
deep-seated pathology it is necessary to work in three
dimensions, which requires the use of three magnetic
field gradients. Signal detection can then be tailored
to the general position of the pathology, and much
information retrieved by computer processing. How-
ever, as the complexity of the ‘search’ increases, high-
er acquisition times are required, and kinetic data may
be lost.

In early studies it was necessary to use a rectilinear volume to study the pathology. What pathology is evi-
dent in this convenient form? The answer is none, and
by using rectilinear volumes non-essential information
may be recorded and the signal/noise ratio decreased.
Fortunately it is now becoming possible to define con-
vex and even spherical regions, using oscillating mag-
netic field gradients.

From the patient’s point of view, even more needs to
be done to reduce the claustrophobic nature of the MRI
examination, especially as the patient has to spend on average 45 minutes in the machine. Clearly a
more open source of magnetic field is required.

5. THE METABOLIC BASIS OF DISEASE:
OVERVIEW
K. G. M. M. Alberti
University of Newcastle upon Tyne

Professor Alberti outlined the importance of metabo-
lic regulation and integration in man. He used the reg-
ulation of glucose metabolism as the paradigm to illus-
trate how recent studies have begun to throw light on
the underlying molecular pathology of many metabo-
lic disorders. A large number of techniques have been
employed to study metabolism in man, varying from
measurements of blood, tissue and whole-body levels
to cross-tissue measurements, cell culture techniques
and recently DNA and gene analysis.

Glucose is an important fuel source in the human
and, not surprisingly, blood levels are tightly regulated
in health, with levels rarely straying outside the range
3.5-8.0 mM, despite large changes in supply (feeding)
and demand (fasting and exercise). Some organs,
notably the brain, have an obligatory requirement for
glucose as one of their fuel sources, so it is crucial that
blood levels be adequately maintained. The liver plays
a unique role in glucose homeostasis, removing glu-
cose from the blood when the concentration is above
normal and releasing it when the concentration is below normal. This function of storage and release of glucose is consistent with the liver’s anatomical location, draining the portal system and buffering the rest of the extracellular fluid from rapid changes in glucose concentration. For example, if 100 g of glucose were distributed instantaneously around the extracellular fluid, glucose concentration would rise by 37 mmol/litre with a large osmotic imbalance; instead the rise is only 2–3 mmol/litre in health.

Whilst the liver is the principal organ of glucose homeostasis, insulin is the major regulator of glucose metabolism. It is the primary anabolic hormone; it promotes fuel storage during feeding and has an anticatabolic role in fasting. Its action is antagonised by the major catabolic hormones, glucagon, cortisol and catecholamines. Growth factors serve tight regulation of enzymes and metabolic pathways. Thus the disorders with metabolic lesions in diabetes mellitus (a common disorder affecting some 80 million people worldwide and with a prevalence of 1–2% in the UK) the normally fine balance between anabolism and catabolism is disturbed. The tight regulation of glucose utilisation is deranged, resulting in the characteristic finding of hyperglycaemia.

Professor Alberti cautioned against looking for a single cause for the disturbance of metabolism in diabetes. Instead research has shown that diabetes is not a single disease but represents a heterogeneous group of disorders with hyperglycaemia as a common feature. Thus the complexity of glucose metabolism involving metabolic pathways and enzymes, which span more than one tissue and cell compartment, means that the types of metabolic defect in diabetes are likely to mirror the multi-enzyme nature of normal glucose metabolism. As an example of how studies of the regulation of enzymes and metabolic pathways can help in dissecting out metabolic lesions in disease, Professor Alberti described recent advances in the biology of the insulin receptor and glucose transporter found on target organ tissues.

In general terms, whereas type I (IDDM) diabetes is due to insulin deficiency the more common type II (NIDDM) diabetes is due to a combination of both impaired insulin release (insulin deficiency) and insulin insensitivity (insulin resistance). One of the many metabolic actions of insulin is to stimulate the transport of glucose across the cell membrane of target organs (e.g. adipocyte tissue and muscle).

Much is now known about the structure of the insulin receptor. It comprises two α subunits sited on the outer surface of the cell membrane and two β subunits which span the membrane. Both subunits are glycoproteins and are linked in a complex by disulphide bonds. The α subunits carry the insulin binding site; the β subunits are thought to be concerned with signal transmission across the cell membrane. The interaction between insulin and the α subunit causes phosphorylation of the β subunit. In insulin resistant states this interaction may be disturbed.

It is not known how the binding of insulin to its receptor is translated into the incorporation of one of the currently identified seven types of glucose transporters into the cell membrane, but it is now widely believed that the major causes of insulin resistance are not due to defective insulin receptors but are due to post-receptor defects. The exciting possibility exists that variants of type II (NIDDM) diabetes may be due to abnormalities of the glucose transporter and its translocation from intracellular pools to the cell membrane.

Professor Alberti ended by illustrating how single enzyme defects in the control of glucose metabolism can be inherited (e.g. hepatic glycogen synthase deficiency) or acquired either by external toxins and poisons or by mutations in mitochondrial DNA during life, giving rise to mitochondrial myopathies. Thus, by studying the various steps by which glucose metabolism is regulated, it may be possible to tease out the molecular basis of the individual lesions which together give rise to the metabolic and clinical features of diabetes.

6. FRUCTOSE: INTERACTIONS BETWEEN HEREDITY AND THE ENVIRONMENT

T. M. Cox

University of Cambridge

The metabolism of fructose is particularly important in man since this sugar is ubiquitous in the diet of industrialised societies (average consumption 50–75 g daily) and high dietary fructose intake has been implicated in the development of atherosclerosis, obesity and dental caries.

In his presentation, Professor Cox began by describing the case history of a family with hereditary fructose intolerance (HFI). This disease is an autosomal recessive disorder characterised by abdominal pain, vomiting and refractory hypoglycaemia following ingestion of fructose or related sugars. It is caused by a marked catalytic deficiency of the enzyme fructose-1-phosphate aldolase (aldolase B) which catalyses the cleavage of fructose-1-phosphate to dihydroxyacetone phosphate and glyceraldehyde in liver, intestine and kidney. The symptoms of the disease are first manifest at the time of introduction of sweetened artificial foods, fruit or vegetable-based solids at weaning. There is failure to thrive, with vomiting, diarrhoea and symptoms of a metabolic disturbance, most noticeably hypoglycaemia. Occasionally, the metabolic disturbance can be sufficiently severe to cause convulsions, coma and death. Survival to childhood is dependent on the reduction of fructose intake, and this is achieved mainly by the development of a marked aversion to most sweet-tasting foods, vegetables and fruits. The affected children are often branded as ‘food faddists’ and are occasionally referred for psychiatric help. Because of the reduction of dietary intake of sugar, there is a low frequency of dental caries in
patients with HFI. Often the condition escapes detection until adult life; Professor Cox described the remarkable instance of a medical student whose symptoms had eluded diagnosis by physicians and psychiatrists and who had made the diagnosis of HFI herself!

Although the incidence of the disease in different populations is not known with certainty, a survey in Switzerland has estimated a frequency of 1 in 20,000 live births, which would predict a carrier frequency of 1 in 70 of the population. The diagnosis can be made in affected homozygotes by an intravenous fructose tolerance test which induces hypophosphataemia and persistent hypoglycaemia. At present there is no simple test to detect asymptomatic carriers of the disease.

Naturally occurring mutations in aldolase B not only can interfere with protein function (as, for example, in sickle cell anaemia) but may also induce structural changes in the enzyme which can be recognised by impairment of antibody binding. Professor Cox then described how the characterisation of the first defective aldolase B gene was achieved in his laboratory, and how this had implications for clarifying the relationship between aldolase B structure and its catalytic function, and for carrier detection and diagnosis of HFI in affected families.

The aldolase B gene had been mapped to human chromosome 9 and sequenced. It consists of 9 exons, and the cognate mRNA encodes a protein of 364 amino acids. By comparing the sequence of the gene from an affected homozygote with the normal gene, the mutant was found to contain a single base-pair change (G→C) with respect to the wild type in exon 5. This mutation produced an amino acid substitution (alanine→proline) at position 149 of the protein, so it replaces a residue that is unique to aldolase B in the three species so far studied (Fig 4).

Professor Cox explained how this substitution would disrupt secondary polypeptide structure in the vicinity of residues that have been implicated in the binding of the substrate and have been shown, in the related isoenzyme aldolase A, to be localised to a binding pocket within the protein. Thus the mutation adversely affects the catalytic properties of the enzyme.

The G→C transversion creates a new recognition site for the restriction enzyme Ahal; by use of the polymerase chain reaction (PCR) to amplify exon 5 sequences specifically, it was shown that the patient from whom the defective gene was cloned was homozygous for the mutation. So far, 12 British patients with HFI have been studied; all of them were shown to have at least one copy of this mutation, and most were homozygous.

Professor Cox emphasised the power of the PCR technique by explaining that the genetic analysis can be carried out on DNA isolated from small samples of blood or even from epithelial cells obtained from mouth washings. Further studies using allele-specific oligonucleotides in conjunction with the PCR are in progress to ascertain the frequency of the A149P and other mutations in populations outside the UK, but Professor Cox indicated that this first genetic lesion accounts for the great majority of individuals affected by HFI. To support this he showed data from a study of 70 European patients; the majority of aldolase B sequences amplified from DNA by the PCR were shown to hybridise to a small panel of mutation-specific oligonucleotide probes. These probes had already been used to confirm the diagnosis of HFI in selected patients with symptoms suggestive of the disease.

Future advances in the population genetics and systematic characterisation of the pathology of the disease, including the application of site-directed mutagenesis to study the consequences of defined mutations on the expressed enzyme, should help to define critical regions of the enzyme and facilitate direct diagnosis. Given that data obtained so far suggest that a few missense mutations account for the majority of defective aldolase B genes, direct genetic diagnosis by molecular analysis of DNA is now a realistic possibility for this disease.

7. MONOCLONAL ANTIBODIES FOR IMMUNOSUPPRESSION AND TOLERANCE

H. Waldmann
University of Cambridge

One of the elusive goals of modern pharmacology has been to find a means of selectively abolishing an individual’s ability to mount an immune response to certain antigens while preserving responsiveness to others. Until recently the discovery of therapeutic agents able to induce immunosuppression or tolerance was due mainly to luck and empirical observations. However, advances in monoclonal antibody technology and immunology have allowed a more reasoned approach to the therapeutic modulation of the immune system. This has major applications in the fields of transplantation, the control of allergy, and in the treatment of autoimmune disease and various haematological malignancies.

The immune system is characterised by a high degree of cell surface activity. In his presentation Dr
Waldmann outlined some of the ways the cells of the immune system interact with each other. Such interactions are mediated by cell receptors and adhesion molecules (eg LFA-1) and are thought to be regulated by cytokines. From a therapeutic standpoint, one of the most important interactions is between T cells and antigen presenting cells. Dr Waldmann explained how therapeutic strategies using antibodies are aimed at removing T cells or antigen presenting cells or blocking the interaction between them.

The use of immunoglobulins as therapeutic agents is not new. However, there are a number of problems with using foreign immunoglobulins made in rodent species, the most important of which is the antoglobulin response which may interfere with therapy or cause allergic or immune-complex hypersensitivity. Since it has proved difficult to make human monoclonal antibodies of the desired specificity, alternative approaches to ‘humanise’ antibodies have been used. These include making ‘chimaeric’ antibodies consisting of mouse variable regions and human constant regions, and more recently making ‘reshaped’ antibodies by transplanting only the antigen binding sites, rather than the entire variable domain (Fig. 5).

Preliminary results using the ‘reshaped’ CAMPATH-1H antibody to treat two patients with non-Hodgkin lymphoma have been encouraging. In both patients lymphoma cells were cleared from blood and bone marrow without myelosuppression. No antoglobulin response was detected even after repeated administrations of the antibody.

Dr Waldmann also described the use of the CAMPATH series of monoclonal antibodies to purge bone marrow of T cells and so control graft-versus-host disease. Recently the use of a mixture of two CD45 monoclonal antibodies to perfuse cadaver kidney allografts has been shown to reduce the incidence of rejection episodes and improve allograft function.

Future developments using monoclonal antibodies to block other therapeutically important antigens such as T cell receptors, cell adhesion molecules, ‘homing’ receptors and cytokine receptors were discussed by Dr Waldmann. Such advances, and particularly the ability to produce new antigen specific reagents such as reshaped human antibodies and even more recently single domain antibodies, are likely to revolutionise therapeutic interventions for immunosuppression and tolerance.

8. APPLICATION OF GENETICS: OVERVIEW

M. Bobrow
Guy's Hospital Medical School, London

In recent years there have been enormous advances in our understanding of the molecular basis of human genetic disease. The information required to make all the proteins of our bodies is stored in the genetic code which consists of four deoxyribonucleotide bases: adenine, guanine, cytosine and thymine. Each set of three bases codes for a particular amino acid, so the coding regions of genes, called exons, are simply rows of base triplets carrying the information to build a polypeptide that has a particular amino acid sequence. Non-coding regions, called introns, contain some information relevant to the transcription and processing of the gene, although the bulk of DNA in the introns has no currently recognised function.

Sickle cell anaemia, a hereditary disease with 60 million carriers worldwide, was found to be caused by a single base substitution, adenosine to tyrosine, in codon 6 of the beta-globin gene. This results in an amino acid change from glutamic acid to valine, altering the polarity of the haemoglobin molecule and causing it to precipitate out of solution at low oxygen tension. Although there are millions of base variations throughout the human genome, very few result in genetic disease, since it is the position of the base mutations that determines whether they will exert phenotypic effects. Although 95% of the human genome does not code for proteins, it does not mean that this DNA is functionless, but most of these mutations have no phenotypic effect. Molecular genetic technology makes use of this normal genetic variation. Restriction enzymes isolated from bacteria cut DNA at specific

Fig. 5. Two types of 'humanised' antibody. Reprinted with permission from Nature, 332, 323-7. © 1988, Macmillan Magazines Limited.
base sequences; for example the enzyme HaeIII only recognises 5'-GGCC-3'. Digestion of DNA with a restriction enzyme, size fractionation of the fragments by gel electrophoresis and identification of a particular piece of DNA using a radioactively labelled complementary DNA probe allows the size of a piece of DNA to be visualised. In this way, base mutations that create or destroy a restriction enzyme recognition site adjacent to the region detected by the probe can be identified. When the enzyme recognition site is present the fragment detected is smaller than when the site is absent. These restriction fragment length polymorphisms (RFLPs) are used as markers to follow the inheritance of disease genes. When an RFLP and a genetic disease gene are linked (ie close together on the same chromosome) they tend to be coinherited. This means that, when an individual carrying a disease gene and RFLP allele A on one chromosome and a normal gene and RFLP allele B on the other chromosome has children, those children who inherit allele A will probably also inherit the disease gene, but those inheriting allele B will not. RFLPs are therefore useful to identify pre- or a-symptomatic carriers of disease genes and to identify affected offspring in utero. Currently there are efforts to produce a high resolution map of the human genome with RFLPs, so that the inheritance of all genetic diseases can be studied, to help locate the specific gene causing a hereditary defect, and eventually to sequence the whole human genome. One problem with these dimorphic RFLPs is that many individuals will be homozygous for one allele, and therefore the allele they pass on to each offspring cannot be identified. This problem has partly been overcome by the discovery of hypervariable markers, for which there are many alleles (described in the following paper), so only a few individuals are homozygotes.

One of the most exciting developments in molecular genetics is the use of the polymerase chain reaction (PCR) to amplify specific regions of DNA. The principle is to construct two primers, about twenty bases long, with sequences complementary to opposite strands of the 5' ends of DNA flanking the region of interest. The double stranded DNA is denatured by heating to about 94°C and the primers are then hybridised to the genomic DNA by cooling to about 55°C. A thermostable enzyme called Taq polymerase binds to the double stranded regions and, when the temperature is raised to about 70°C, it rapidly synthesises new complementary strands. These strands are then denatured and the cycle is repeated up to thirty times. Since the number of DNA strands synthesised during each cycle is doubled, after thirty cycles 10^10 copies of the region of interest can, in theory, be produced. After the first replication, all the synthesised strands are of equal length and can be visualised directly in the gel following electrophoresis and staining with ethidium bromide. The need to use radioactive DNA probes is eliminated and the experimental procedure is much simplified. The sensitivity of PCR makes it very useful for analysing extremely small amounts of DNA obtained from forensic specimens and chorionic villus samples for prenatal testing. Its ease of use has led to its application in many areas of molecular biology, including deletion screening for Duchenne muscular dystrophy and even as a highly specific test for HIV infection.

The present advances in the study of human genetics and the possibility of the sequencing of the whole of the human genome mean that we will very soon have a detailed understanding of the molecular pathology of most, if not all, monogenic diseases. We may then be able to apply this knowledge to investigate the role of specific genes in multifactorial diseases such as coronary heart disease and cancer.

9. GENETIC FINGERPRINTING: THE SCIENCE AND ITS APPLICATION
P. G. Debenham
Cellmark Diagnostics, Abingdon

DNA fingerprinting is a way of analysing many highly variable regions of the human genome simultaneously to produce a genetic 'bar code' which is specific to the individual from whom the sample has been obtained. Throughout human and animal DNA there are highly repetitive regions called mini-satellites. Variation in the number of copies of a short core sequence results in length polymorphism of each mini-satellite throughout the population. When human DNA is digested with a frequent cutting restriction enzyme, such as HinfI, it is generally broken into very small fragments. However, if the enzyme recognition site is not present in the mini-satellite core sequence, it will not be present anywhere in the repeated region. This results in the production of large fragments consisting of the mini-satellite region flanked by a short sequence of single-copy DNA. These fragments are fractionated according to size by agarose gel electrophoresis and transferred to a nylon membrane. As there is great similarity between the core sequences of mini-satellite regions, radioactively labelled probes based on the core sequence can be used to detect fragments from many regions simultaneously. In 1984, Jeffreys and co-workers discovered that the polycore probes 33.15 and 33.6 could each detect a highly complex band pattern in human DNA, resembling a supermarket 'bar code' (Fig. 6). By making pairwise comparisons between the DNA fingerprints of unrelated individuals they estimated that, on average, a fragment of a particular size is present in only about 25% of the population. The probability of two random unrelated individuals having identical DNA fingerprints is therefore less than 0.25 raised to the power of the average number of bands, ie <5x10^-11. DNA fingerprints can therefore be regarded as totally individual-specific and can be used as proof of identity.

In forensic biology, DNA fingerprints can be taken from a suspect and from a semen sample found at the scene of a rape or other violent crime and compared (Fig. 6). If they match, the sample must have come from the suspect. DNA fingerprinting is an extremely
sensitive technique and can be used on samples as small as 60 microlitres of blood or 5 microlitres of semen. It has been suggested that one day we may all be DNA fingerprinted and our genetic bar-codes kept on file so that the perpetrators of rape and violent crime will be immediately identifiable.

The mini-satellite regions detected by DNA fingerprinting are stably inherited in a Mendelian fashion. Half the bands present in a child’s DNA fingerprint will be present in the mother’s band pattern and half in that of the father. DNA fingerprinting can therefore be used in tests of relatedness such as in paternity testing and immigration cases. If the alleged father’s DNA fingerprint has few bands in common with that of the child, and the child’s band pattern contains several bands not present in that of either parent, the putative father is not the child’s true biological father. Conversely, if all the child’s bands are present in the DNA fingerprints of one or both of the parents, the putative father is the child’s true father (Fig. 7). The probability that a false match has occurred if the putative father and the true father are unrelated depends on the number of non-maternal bands in the child’s DNA fingerprint; on average it is $<1\times10^{-9}$, and if they are brothers it is $<1\times10^{-4}$. Immigration cases can be similarly resolved by comparing the DNA fingerprints of putative relatives, though the cases are often more complex if key individuals are unavailable for testing. This has revolutionised Britain’s immigration system; about half of applicants claiming to be closely related to a British citizen were rejected by the interview system, whereas about 9 out of 10 cases have been found to be genuine by DNA fingerprinting.

The polycore probes, 33.6 and 33.15, also detect highly complex genetic bar codes in animal DNA. This has proved useful in verifying the pedigree of prize dogs and it would be applicable to racehorses and in checking the authenticity of sperm samples for artificial insemination. It has also been used to study the demography and socio–biology of animal behaviour; for example, a recent study in Nottingham used the technique to estimate the rate of mate infidelity among house sparrows. DNA fingerprinting also has a variety of medical applications. It can be used to monitor graft rejection following bone marrow transplants, identify maternal cell contamination in chorionic villus samples, distinguish monozygotic from dizygotic twins and investigate allele loss in tumour development.

DNA fingerprinting is one of the most important new techniques to have been developed in molecular biology. Cellmark Diagnostics have already typed 19,000 individuals—mainly in forensic, paternity and immigration cases. As the technique comes into use in other laboratories, DNA fingerprinting will become a common tool in medical practice and scientific research.

**10. INFLUENCE OF GERMLINE-SPECIFIC IMPRINTING OF PARENTAL CHROMOSOMES**

**M. A. H. Surani**

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Classic Mendelian genetics was based on the assumption that the phenotypic effects exerted by a gene are independent of whether the gene was inherited from father or mother. However, in recent years it has been observed that the penetrance, severity (expressivity) and age of onset of certain inherited diseases are influ-
enced by parental transmission; for example, Huntington’s disease and spinocerebellar ataxia are of earlier onset if inherited from the father, and neurofibromatosis types I and II are of increased severity if inherited from the mother. This phenomenon of ‘genomic imprinting’ must be due to a germline-specific mechanism which alters gene expression during development, and this mechanism must be reversible in subsequent generations.

Recent work in which mouse eggs were reconstituted from newly fertilised eggs has provided some insight into the nature of genomic imprinting. If the haploid genomes inherited from mother and father are identical, a fertilised egg with two paternal pronuclei or two maternal pronuclei should develop normally. Androgenetic eggs (with two paternal components) and gynogenetic eggs (with two maternal components) can be constructed by removing a pronucleus from a fertilised egg and replacing it with a haploid embryonic genome from a parent of the opposite sex. Neither androgenetic nor gynogenetic eggs are able to develop normally and survive to term. The androgenetic eggs produce a proliferating trophoblast but a poorly developed embryo, and the gynogenetic eggs produce an underdeveloped trophoblast and initially a well developed embryo. This suggests that paternally derived genes are relatively more important for the development of trophoblast and maternally derived genes are necessary for the development of the embryo.

Foreign genes tend to integrate randomly in the mouse genome following injection of 200–500 copies of the gene into a pronucleus of a fertilised egg in vitro, the eggs then implant in the uterus of a pseudopregnant female following their transfer. Some of the resulting offspring express the foreign gene and are therefore called transgenic mice. These mice can transmit the transgene to their offspring because it has been permanently incorporated into the mouse genome. Breeding experiments with transgenic mice have shown that transgene expression is sometimes affected by genomic imprinting, and that genomic imprinting depends not on the transgene itself but on its site of insertion in the mouse genome. Where transgenes are influenced by imprinting, it is observed that maternally inherited transgenes are relatively hypermethylated and paternally inherited ones are undermethylated; such transgenes are only expressed after paternal transmission. The levels of transgene methylation and of gene expression have both been shown to be reversible when they are transmitted through paternal-maternal-paternal lines, fulfilling the criteria for imprinting. There is therefore good correlation between transgene methylation and its expression.

In one instance, Tk-lacZ transgenic mouse, transmission through the paternal line shows high expression in half of the offspring and low expression in the rest. Furthermore, the degree of transgene expression correlates well with DNA methylation. When mice with low expression are bred, transgene expression is lost within three generations of transmission, as the gene becomes increasingly methylated. Similarly, in the high expression subline, transgene methylation decreases and expression increases to a high level within three generations. This illustrates that hypermethylation and transgene inactivation are directly related and that grandparental transmission also affects the level of expression of a gene. The segregation of mice with the high and low expression is likely to be due to the action of modifier genes. Investigations of transgene expression in different inbred strains of mice have shown that their genetic background can affect transgene expression; in Balb/c mice there is a modifier repressor which affects the expression of this particular transgene. The modifier must be maternally derived if it is to exert any effect. This does not indicate that methylating enzymes provide the mechanism of modification in germline-specific imprinting, since methylation may be a consequence rather than the cause of gene inactivation.

In summary, the paternal and maternal components of the genome are not identical, and both are required for normal embryonic development. Germline-specific imprinting provides an explanation of why some single-gene disorders show different penetrance, expressivity and age of onset, depending on parental transmission.

11. PRODUCTION OF HUMAN PROTEINS BY TRANSGENIC ANIMALS

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Certain proteins are useful for the treatment of individuals with congenital or acquired deficiencies. Proteins obtained from donated blood are in limited supply and have the potential for the transmission of infectious agents, recently highlighted by HIV infection in haemophiliacs. Recombinant DNA technology may allow large-scale production of such proteins free from human pathogens, and in addition offers the possibility of production of proteins with altered properties. Bacteria are unsuitable for the production of proteins that require post-translational modification for activity (eg Factor IX) or stability (eg α-antitrypsin), and yields of proteins from eukaryotic cell culture are relatively low. The potential of transgenic animals for production of human therapeutic proteins is thus being investigated.

Milk contains large amounts of some proteins which are expressed in the mammary glands from a small number of genes, and it is particularly easy to harvest it in large amounts from animals such as cows and sheep. The aim was therefore to direct expression of human proteins to the mammary gland of transgenic sheep, using sequences from a gene expressed abundantly in this organ. To this end, the sheep β-lactoglobulin (BLG) gene was cloned. To investigate whether the BLG clone contained all the sequences
required to direct expression to the mammary glands, the gene was injected into one-cell mouse eggs to generate transgenic mice. Mice do not normally produce BLG, but the transgenic mice expressed BLG mRNA in their mammary glands, and BLG protein was secreted in large quantities into milk. There was no significant amount of BLG mRNA in other tissues, and in several other respects the transgene was expressed as expected. Mice expressing BLG or consuming milk containing BLG suffered no adverse effects.

Factor IX and α₁-antitrypsin were chosen as candidates for production in transgenic animals. Factor IX (FIX) is required by patients suffering from haemophilia B, and α₁-antitrypsin (AAT) may be useful for the management of patients with emphysema. These proteins are not usually synthesised in mammary glands. Two fusion genes (BLG-FIX and BLG-AAT) containing the entire BLG gene and cDNA encoding the human proteins were constructed to direct their expression to the mammary glands. The requisite techniques for the production of transgenic sheep were developed, and transgenic sheep carrying the BLG-FIX or BLG-AAT genes were produced. Both proteins were found in transgenic sheep milk, and the FIX was shown to be active in clotting assays. Both proteins were produced in extremely small quantities, insufficient for human needs; for example, FIX was present at only 1/200 of the concentration in human plasma.

To increase the levels of expression of the human proteins, the researchers returned to the mouse, a far more amenable experimental system than sheep. Several alternative designs of fusion gene were introduced into mice, and one of these designs was found to be particularly promising. This gene, a fusion between the BLG promoter and AAT genomic sequences, was expressed in the majority of transgenic mice in large amounts, and in one case AAT was present at extremely high concentration (~7 mg/ml). While mice produce too little milk for commercial production, this level of expression in sheep or cows would be sufficient. The fusion gene is being injected into sheep eggs for this purpose.

Before human proteins produced by transgenic animals can be used for therapeutic use, they will need to be purified from the milk to prevent immune responses against contaminating animal antigens. Once this can be achieved, yielding active proteins, transgenic animals may be extremely useful to the pharmaceutical industry.