



Institute of Biomedical Science

History of Biomedical Science

From Matula to Mass Spectrometry

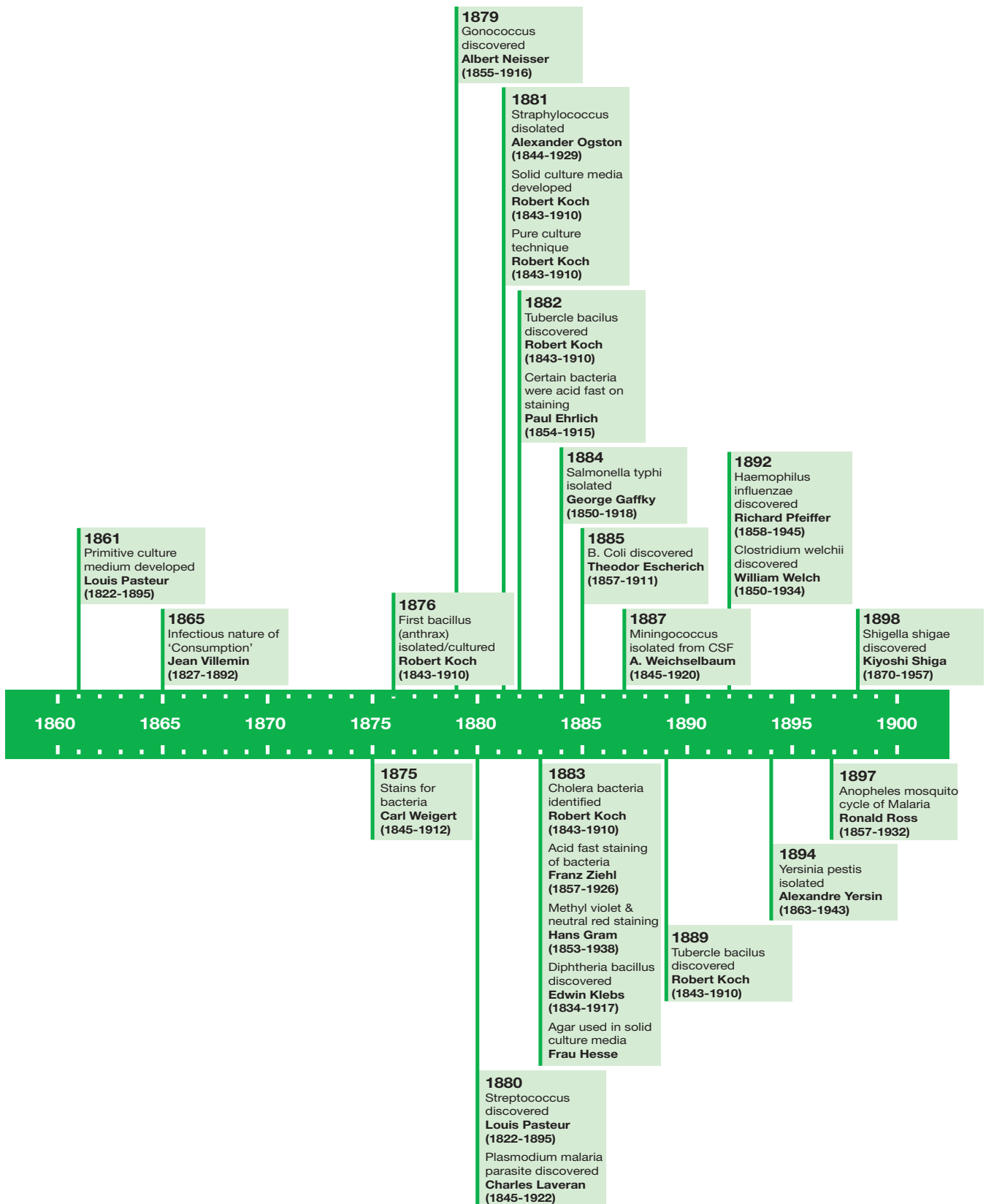
A history of urine tests in the investigation
of human disease

A booklet to accompany an exhibition presented by the
IBMS Historical Section Committee at the IBMS Biomedical
Science Congress

26th – 28th September 2005

Notable 19th Century discoveries in Microbiology

Developments in microscopy and microbiology were fundamental to the foundation of the first clinical laboratories in Britain during the 1880's.



History of Biomedical Science : ‘From Matula to Mass Spectrometry’

A history of urine tests in the investigation of human disease

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History of the Biomedical Sciences

This review has been compiled as a reference source of information in support of the display organised by the IBMS Historical Section Committee for the 2005 Congress

History of Biomedical Science : 'From Matula to Mass Spectrometry'

A history of urine tests in the investigation of human disease

Pre 16th Century

Introduction

This monograph describes the development of urine qualitative and quantitative tests from around 4000 BC to the present day. Urine has proved to be a most valuable sample source for a variety of disorders, notably diabetes mellitus, renal disease, inherited diseases, and infections and cancer of the genito-urinary tract. The organisation of the text is chronological and includes some brief biographical notes.

Urine was the first body fluid to be used in the investigation of disease due to its ready availability and distinctive appearance. This was limited to visual observation of urine in disease states and this 'matula' period remained dominant until the 17th century.



This shows a physician looking at a container of urine. In early days, the physician used his senses of sight, touch, hearing, smell, and taste, to make a diagnosis.

Further progress could not be made until advances were made in the anatomy and physiology of the kidney and developments in chemistry, microbiology and cell pathology to provide the technologies for more exacting investigation.

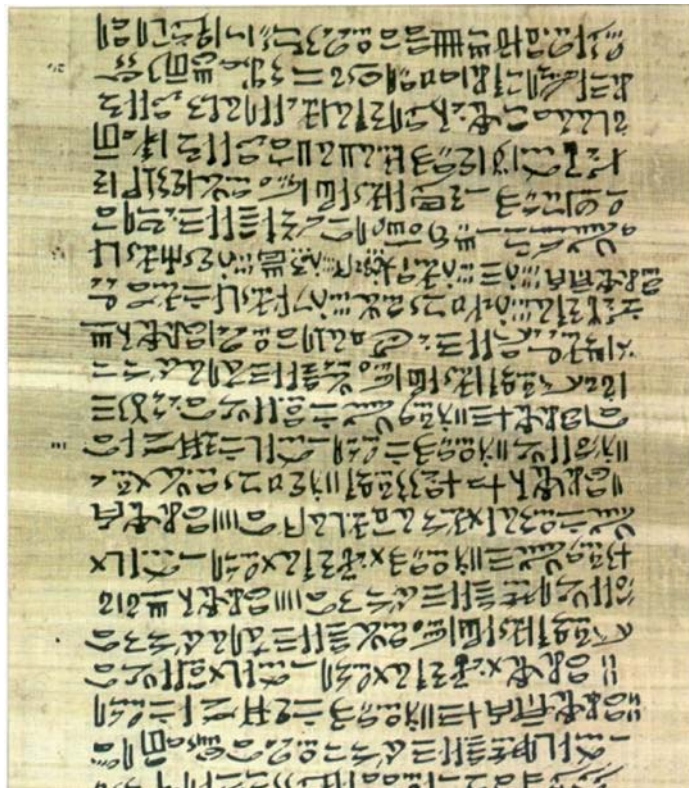
Consequently, prior to the details of urine tests, each section from the 16th century is preceded by a summary of relevant developments in these and related sciences. With this newly acquired knowledge and an improved understanding of pathophysiology a wide range of urine tests gradually evolved.

This monograph traces the origin and evolution of many of the urine tests used today in Clinical Chemistry, Microbiology and Cytology. It illustrates the progress achieved from visual observation to the complex analysis of urine by mass spectrometry. The interested reader is referred to the information sources quoted for more detail.

Magic and Mysticism Ancient medical practice

There is strong archaeological evidence that many of the diseases present today existed in the ancient civilisations from at least 3000 BC. Primitive surgery was performed and it is claimed that lithotomists were the earliest surgeons.

Egyptian documents such as the **Ebers papyrus** refer to **polyuria** in about 1500 BC and they were most probably referring to the increased level of urination in persons with diabetes. The document is based on previous papyri of about 5000 BC.



A representation of what the Ebers papyrus would have looked like.

Early forms of treatment included the administration of herbal and plant extracts. Illness was associated with evil spirits and incantations, charms and other forms of mysticism were used by physicians, philosophers and priests to expel 'demons' in conditions such as dropsy (oedema in renal, liver or heart disease). Records show that physicians in Babylon as early as 4000 BC made observations of the colour and consistency of urine. A more scientific approach to medicine was established in India and notably in ancient Greece from around 1000 BC.

Theories and Observation Hippocrates to Rhazes

Hippocrates of Cos (460-370 BC) Greek physician who is regarded as the founder of clinical medicine. He rejected the concept that sickness was sent by the gods and recognised that diseases had natural causes. He closely observed clinical symptoms and proposed the causes of disease but followed the Greek practice that placed an emphasis on prognosis rather than diagnosis. The ancient Greek physicians regarded the kidney as a simple filter and Hippocrates took into account the appearance, colour of urine and its sediment. The white 'clouds' he observed and reported may have been due to albuminuria in renal disease.

He realised that the volume of urine excreted was related to fluid intake. Greek physicians also recognised the difference in urine voided in pre puberty and adolescence now known to be due to the presence of sex hormones.

The distinguished Greek philosopher and scientist **Aristotle** (384-324 BC) around 336 BC illustrated and accurately described the gross anatomy of the genito-urinary system including the kidneys, bladder, ureters and blood supply. **Areataeus** (81-138) of Asia Minor in about 100 AD introduced the name 'diabetes' to a condition with wasting, thirst and polyuria. The Roman scholar **Varro** (116-26 BC) suggested that some diseases were caused by minute 'particles' entering the body and the Romans made important advances in the supply and purification of water, improving sanitation and promoting hygiene.

Claudius Galen (c131-201AD) born in Pergamum in Asia Minor was the most famous of the Roman physicians and is regarded as the founder of clinical pharmacology with his description of over 300 herbal remedies. He also studied anatomy and physiology by animal dissection and made significant advances in the anatomy of the kidney, bladder and nervous system. He proposed that blood did not flow through the kidney and that waste products were 'attracted' to the kidney.

Despite this error he advocated that the visual examination of the urine played an essential role in the assessment of disease.

'Sweet urine'!

The Ancient Brahmins ca 500 AD were skilled medical practitioners and made the observation that the urine of 'diabetics' attracted insects due to its high sugar content and it was described as mellita urina or 'honey urine'. The same observation was made later by the Chinese physician, **Chen Chuan** in 643 AD.

Persian medicine ca 630-1500 AD

This was mainly based on Greek medicine but adapted for their religion, climate and culture. This had a strong influence on European medicine from the Middle Ages to the 16th Century.

Rhazes (850-932) Persian physician who is regarded as the pioneer of genito-urinary medicine. He considered that haematuria was a symptom of bladder disease, he introduced specialised surgery to relieve urine retention and designed a number of surgical instruments including various forms of catheter.

The Matula – Uroscopy and Uromancy ca 476–17th Century

Naked eye examination of urine for diagnostic purposes was widely practised during the Middle Ages. Urine was collected and examined in a bulbous flask (matula), some of which were marked with a scale to estimate sediment and carried in a special wicker basket to the physician or 'prophet'. Haematuria and pyuria were recognised but uroscopy was based on a false assumption originated by the Egyptian physician **Isaac Judeus** (845-940) of the Salerno school of medicine that a characteristic appearance of urine existed for every known disease. Urine appearance was sometimes used in isolation without even seeing the patient! Uroscopy relates to the use of charts of various colours and physical appearance eg turbidity, of urine in disease correlated with written advice for physicians for diagnosis and treatment.

Avicenna (980-1037) an Arabian physician observed differences between urine passed in the morning and other times of the day. He also noted the effects of age, diet and drugs upon the colour of the urine voided. However, urines judged as 'abnormal' were often attributed by Avicenna and others to disorders of digestion!

A book published in England in 1541 'The Differences, Causes and Judgements of Urine' suggested that uroscopy and the pulse were the two most important procedures in clinical investigation



The urine color wheel was a chart used for the examination of urine from about 1430 AD.

The Urologist' A van Ostade 1665

The 16th Century

Significant advances were made in anatomy, notably at the University of Padua, by **Andreas Vesalius** (1514-1564) and **Gabriello Fallopius** (1523-1562). **Bartolommeo Eustachio** (1524-1574) a professor of anatomy in Rome produced one of the earliest illustrations of the structure of the kidney in 1564.

In 1588 the Spanish surgeon **Francisco Diaz** wrote the first treatises on diseases of the bladder, kidneys and urethra to pioneer the foundation of urology. Other relevant events were laws passed to permit the dissection of human corpses in England and most significantly towards the end of the century the Dutch spectacle makers, **Zacharias Jansen** and his son **Hans**, produced the first simple microscopes.

A chemical approach to medicine

Philippus Paracelsus (c1493-1541) Swiss physician and alchemist also known as **Theophrastus von Hohenheim**, challenged Galen's earlier 4 humour theory and Avicenna with a holistic approach to medicine in that diet, environment and the doctor-patient relationship were vital in curing disease. He adopted a more chemical approach to medicine, after studying in the mines of the Tyrol and used minerals, such as iron and lead, as well as tinctures and alcoholic extracts as medicines and drugs. It is reported that he also performed experiments on urine involving distillation and weighing but the results and applications of these studies are obscure. He pioneered the treatment of syphilis with mercuric compounds and the basic principles of homeopathy. He suggested alchemy should be used to cure disease and drugs were the province of the chemist. He was one of the first to use the term 'iatrochemistry'.

An early Germ theory

Infectious diseases, such as smallpox, scarlet fever, malaria, syphilis and bubonic plague were prevalent from ancient times and were major causes of mortality. Little was known of their causes, prevention and treatment.

Girolamo Fracastoro (c1483-1553) an Italian physician proposed a primitive germ theory describing syphilis and epidemic diseases. He wrote 'De Contagione et contagiosis morbis' in 1546 which included different modes of transmission. His work was undermined by the doctrines of Paracelsus until his concepts were proved correct more than 300 years later by **Robert Koch** and **Louis Pasteur**.

The 17th Century – the first scientific age

A more rational and logical approach to scientific study was evident and important advances were made during this century by many famous scientists. Progress in anatomy and physiology was initially led by **William Harvey** (1578-1657) in his discovery of the circulation of blood in 1628. The structure of the kidney was further elucidated by the Italian anatomists Lorenz Bellini (1643-1704) who discovered the renal excretory ducts and **Marcello Malpighi** (1628-1694) who used microscopy to identify the uriniferous tubules in 1666.

Antonie van Leeuwenhoek (1632-1723) Dutch draper and microscopist and the first to observe bacteria from teeth scrapings and protozoa from animal gut in 1674. He also gave accurate descriptions of spermatozoa (1677) and red blood cells (1684).

His discovery of 'animalcules' (bacteria) by microscopy and that they existed in a corn preparation (culture) led slowly to the development of microbiology in the 18th century.

The transition of alchemy to chemistry took place during this century. It is believed that alchemy originated in ancient Egypt, India and China. Primitive 'chemical' methods were used to obtain the 'essence' which would not only convert base metals into gold but act as a panacea for diseases and provide eternal youth. The Greek theories of just four elements and four humours had also restricted developments in chemistry. The first textbooks of chemistry appeared in 1606 and 1610 and **Robert Boyle** (1627-1691) is recognised as one of the founders of the scientific experimental method and introduced the term analysis which infers precise and exact investigation. He also performed pioneer studies on the chemical analysis of blood in 1684.

The rise in physics and chemistry led to attempts to introduce their application in medicine. Iatrophysics applied the laws of physics and mathematics to explain human physiology particularly relating to movement and respiration. It had little merit apart from the studies of **Santorio Santorius** (1561-1636) an Italian physician who performed the first scientific study of basal metabolism in 1614.

Iatrochemistry was based on the concept that physiology and disease could be investigated and explained by chemical changes in body 'juices'. Leading proponents included **Boyle** and **Jan Baptista van Helmont** (1579-1644) who was the first to make accurate calculations of urine specific gravity by comparing the weight of an equal volume of urine and rain water. **Thomas Willis** was also regarded as a respected iatrochemist.

Thomas Willis (1621-1675) English physician who was Oxford professor of natural philosophy. He also favoured urinalysis, recording colour and fluid intake and his 'Treatise of Urines', published in 1674, marked a more scientific approach over medieval uroscopy.

Chemical tests on urine

One of the earliest tests was devised in 1694 by **Fredericus Dekkers** (1648-1720) professor of medicine at the University of Leyden. Albumin in the urine was detected by boiling urine to denature and sediment the protein which did not redissolve when dilute acetic acid was added. However, he did not know the reason why the urine became milky on boiling or appreciate its clinical significance.

The 18th Century – the chemical revolution

Some of the major English hospitals eg Guys(1724), Addenbrooke(1762), John Radcliffe Infirmary(1766) and Great Ormond Street Hospital for Sick Children (1768) were founded during this century. The correlation of pathology with surgery and post mortem rose in prominence by the studies of the British surgeon John Hunter (1728-1793) and the Italian pathologist **Giovanni Morgagni** (1682-1771) respectively. The Swiss biologist **Albrecht von Haller** (1708-1777) was a noted pioneer of experimental physiology.

Advances in physics, chemistry and the biological sciences continued throughout Europe and also in the United States of America at the end of the century.

Developments in chemistry were led by the Dutch physician and medical tutor **Hermann Boerhaave** (1668-1738), the British chemist **Joseph Black** (1728-1799) and the French chemist



Herman Boerhaave

Antoine Lavoisier (1743-1794) who is regarded as the leader of the chemical revolution in the 18th century and the founder of modern chemistry.

Concepts in the use of photometry for analysis were developed by the French physicist **Pierre Bouguer** (1698-1758) and the Swiss born, German physicist **Johann Lambert** (1728-1777). This was to have major implications in the use of colorimetry for the quantitative analysis of urine (and blood) constituents in the early 20th century.

Chemical tests on urine

Analytical chemistry was becoming an established science and attempts were made to establish the various normal and pathological constituents of urine. Phosphate of lime was identified by **Marggraf** in 1757, uric acid and sodium phosphate by **Carl Scheele** in 1770 & 1776 respectively, urea was discovered independently by **Rouelle** and the English physician **William Cruikshank** in 1773 and ammonium magnesium phosphate by Fourcroy in 1790. In 1797 William Cruikshank also observed that coagulation occurred when certain urines were heated.

Urine sugars

In 1776 **Matthew Dobson** (1713-1784) physician at Liverpool Infirmary noted that diabetic urine underwent fermentation and the residue after evaporation had the taste of brown sugar. In 1780

Francis Home (1719-1813) a Scottish physician devised the yeast fermentation test to detect the presence of urine sugars. Glucose and fructose are fermented by yeast at 37°C to form alcohol and carbon dioxide. The evolution of carbon dioxide indicated a positive result. This method continued to be described in standard textbooks as late as 1962 but with a note of caution to avoid known sources of error.

At the end of the century and in the early 19th century the British physician and chemist **William Hyde Wollaston** (1766-1828) demonstrated the presence of uric acid, calcium, ammonium salts and cystic oxide(cystine) in renal calculi.

Origins of Microbiology

An Italian physiologist **Lazzaro Spallanzani** (1729-1799) performed investigations of microscopic life in nutrient culture media which became significant for the later studies of Louis Pasteur and in 1778 **Wilhelm von Gleichen** (1717-1783) devised stains for bacteria using carmine and indigo.

The 19th Century

Microscopes with higher magnification and resolution were developed by physicists, such as **Giovanni Amici** (1786-1863) and **Ernest Abbe** (1840-1905). The cell theory and the recognition that changes in cell structure were related to disease states were firmly established by around the midpoint of the 19th century. Clinical cytology was pioneered by **Johannes Muller** (1801-1858) professor of anatomy in Berlin who in 1838 described the microscopic structure of various types of cancers.



Rudolph Virchow

The extensive studies of the German pathologist **Rudolph Virchow** (1821-1902) laid the foundations of cellular pathology.

The origins of urine cytology

In 1853 **F Donaldson** published 'The Practical Application of the Microscope in Cancer' with methods to identify malignant cells in various body fluids. In the following year Lionel Beale of King's College, London published 'The Microscope and its Application in Clinical Medicine' which included illustrated detail for the cytological examination of the urinary sediment in the diagnosis of cancer.

Physiology and structure of the kidney

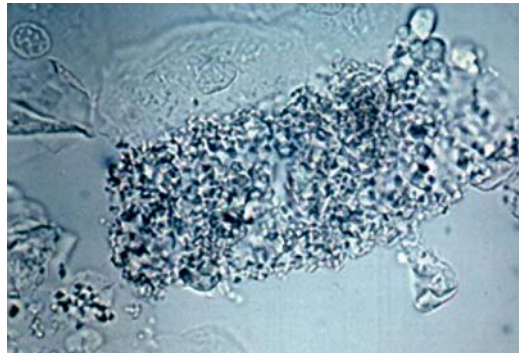
In 1842 the English physician **William Bowman** (1816-1892) concluded that urine formation occurred by glomerular filtration, via a capsule, and tubular secretion. In 1844 **Carl Ludwig** (1816-1895), a German physiologist, suggested that blood pressure forces waste fluids out of renal capillaries into the nephrons and that water was reabsorbed in the tubules to concentrate the urine. In 1899 the English physiologist **Ernest Starling** (1866-1927) proposed that an osmotic gradient was responsible for this concentration effect. This 'countercurrent' theory was confirmed by AN Richards during the 1920's. It was established later that waste products such as urea and uric acid were secreted into the tubules.

Urine Microscopy

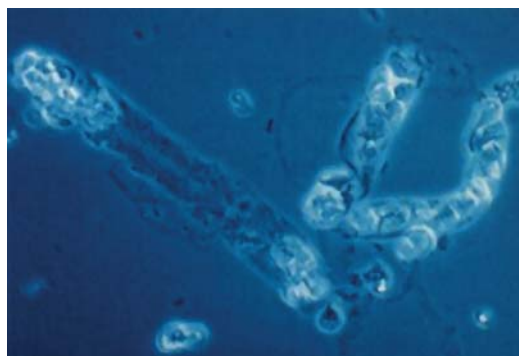
Microscopy was introduced into clinical practice during the 1840's and the study of urinary deposits in 'wet films' led to the recognition of red blood cells, pus and a variety of crystals. In 1844 Golding Bird, a physician at Guy's Hospital published 'Urinary Deposits : Their Diagnosis, Pathology and Therapeutical Indications'.

Casts

These were first reported by **Hermann Nasse** (1807-1892) in 1843 and in the following year **Gustav Henle** (1809-1885) established by postmortem that casts arose in the kidneys. Casts are formed as 'moulds' of the renal tubules and may reflect tubular damage. A number of different types may occur such as hyaline casts which are transparent, cylindrical structures and are associated with albuminuria. Other casts of pathological significance may contain epithelial cells, granules, red or white blood cells

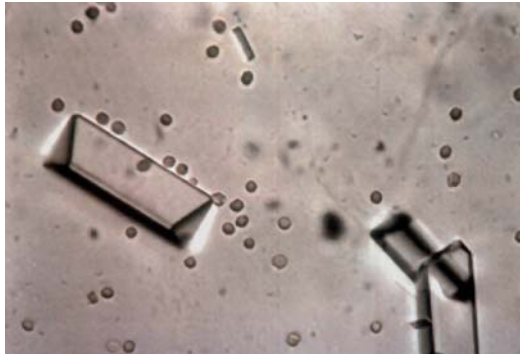


Trichomonas vaginalis – BF

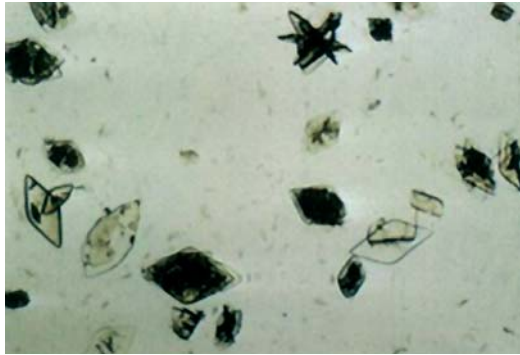


Hyaline/WBC cast – PC

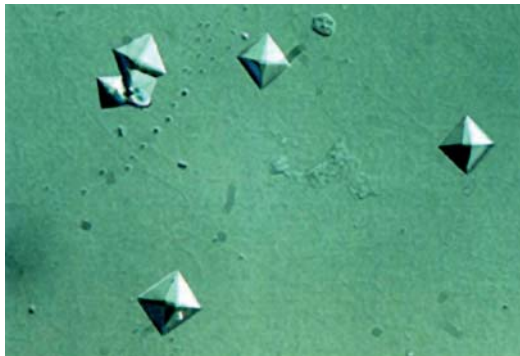
Crystals



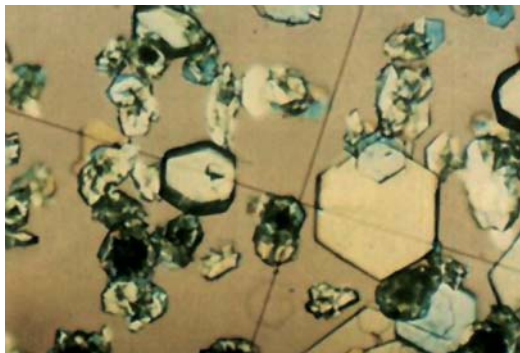
Triple phosphate – BF – Normal crystal of alkaline urine. Uncommon, especially in fresh urine specimens. Associated with struvite (infection) stones.



Uric acid – BF



Typical bipyramidal calcium oxalate crystals (ICM, x400)



Cystine – PL

A variety of crystals may occur in the urine and are identified by their characteristic morphology. Ammonium magnesium phosphate may appear as prisms or in fernlike form and are associated with bacterial infection, uric acid crystals may be rhombic and have little clinical significance. Calcium oxalate forms are octahedral or 'envelope' and are normal constituents of urine. Cystine crystals are colourless hexagonal plates and occur rarely notably in cystinuria. Other amino acids, such as tyrosine and leucine observed in urine deposits may be associated with liver disease. **H Reider** and **AS Delepine** published an 'Atlas of Urinary Sediments' in 1899 which helped to assist identification of casts and crystals.

Scientific Germ theory

The spontaneous generation theory of infectious disease was swept aside during this century and replaced by the germ theory. The earlier studies of **Agostino Bassi** (1773-1856) and **Casimir Davaine** (1812-1882) led **Louis Pasteur** (1822-1895) to confirm this important landmark in 1861. From this time the fundamental 'tools' of microbiology evolved. The coal gas/air burner was invented by **Robert Wilhelm von Bunsen** (1811-1899) and a prototype autoclave by **Charles Chamberland** (1851-1908). A number of culture media and stains were developed to isolate and identify specific bacteria and in 1887 **Julius Petri** (1852-1921) introduced Petri dishes for plating bacteria. These developments heralded the most intensive period for the discovery of pathogenic bacteria (See Table 1).

A Golden Age of Microbiology

Bacteria were also observed in urinary deposits in the 1840's but at that time their clinical significance was not appreciated. Once the germ theory was firmly established techniques were developed to identify the bacteria present in urine and their clinical significance. In 1881 rod shaped bacteria were observed in urine, associated with cystitis by William Roberts (1830-1899) a physician at Manchester Royal Infirmary.

In 1882 the German physician **Ernest Wagner** (1829-1888) described pyelonephritis and the clinical importance of chronic pyelonephritis. In the same year the German bacteriologist **Robert Koch** (1843-1910) discovered the tubercle bacillus as the causative organism of tuberculosis.

Koch proposed that each type of infection is caused by a specific bacterium and his 'postulates' were a significant landmark for the further development of microbiology. Koch devised a culture medium for growing the tubercle bacillus using blood solidified by heat at 65°C. He also developed a stain using acid fuchsin which was later much modified as an acid fast stain by **Paul Ehrlich, Franz Ziehl** (1857-1926) and **Friedrich Neelsen** (1854-1894). A stain of universal importance was developed in 1883 by the Danish physician **Hans Christian Gram** (1853-1938)

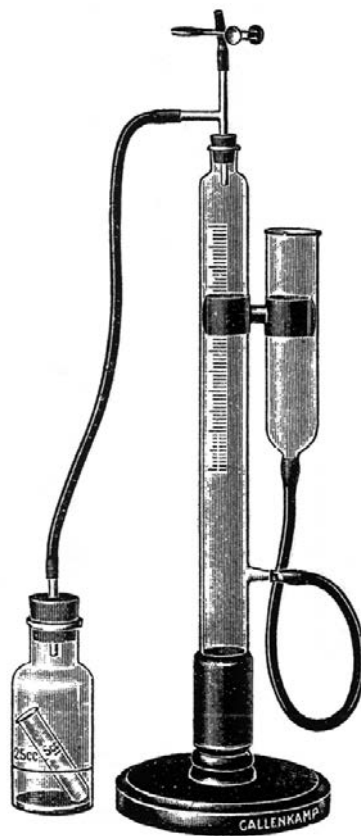
He observed that certain bacteria, after double staining were not decolourised by alcohol. A drop of urine or deposit is spread on a glass slide, allowed to dry and fixed by flaming. The film is stained with crystal violet, treated with iodine, rinsed, decolorised with alcohol and counterstained with safranin O and observed under the microscope. This famous discovery allowed a classification of bacteria into those staining purple-black (Gram positive) and those which stain pink (Gram negative). The Gram negative bacteria *B.coli* and *B Proteus* were first described in urine in 1885 and found to be a common cause of urine tract infections. Many other pathogens, such as gonococci, and the Gram positive staphylococci and streptococci, which may occur in urine were discovered in the latter part of the century. Biochemical techniques to aid identification of bacteria was pioneered by the Russian microbiologist **Sergei N Winogradsky** around 1900. **Emil von Behring** (1854-1917) introduced immunotherapy against bacterial infections with the discovery of antitoxins in 1890. The concept of a virus as an infectious agent, unlike a bacterium, was established by the studies of **Dmitri Ivanovski** (1892), **Martinus Beijerinck** (1898) and **Friedrich Loeffler** with **Paul Frosch** (1898).

Physiological chemistry

The English chemist **Humphry Davy** (1778-1829) discovered the electrolytes sodium and potassium at the turn of the century. In 1811 the Swedish chemist **Jons Berzelius** (1779-1848) introduced chemical symbols for the elements and tabulated their atomic weights. In the following year he performed a quantitative analysis of the chemical composition of urine.

The fusion of organic chemistry with physiology, as physiological chemistry, was mainly due to studies performed by the German scientists **Friedrich Wohler** (1800-1882), **Justus von Liebig** (1803-1873) and **Emil Fischer** (1852-1919). Wohler prepared urea, the first human biochemical substance to be synthesised, from potassium cyanate & ammonium sulphate.

Two books published by von Liebig, notably 'Organic Chemistry in its Application to Physiology and Pathology' (1842) were the basis of the early work on biochemistry. Towards the end of the century Fischer performed important studies on the structure of carbohydrates and proteins. In addition the French physiologist **Claude Bernard** (1813-1878) performed his classic experiments on



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carbohydrate metabolism. He recognised cells as the functional units of life and developed the concept of the internal environment in which cells carry out their activities that is regulated towards a 'steady state'. He used some of the urine tests, such as those for sugars and total solutes (by freezing point depression), that are described below.

The kidney plays a major role in homeostasis by excreting the waste products of metabolism, conserving useful substances and maintaining fluid and acid base balance.

Chemical tests developed at this time concentrated on renal disease, diabetes and liver disease supported by some significant clinical studies.

A number of textbooks devoted to urinalysis were published during the mid 19th century. These included 'A Treatise on the Pathology of Urine, including a Complete Guide to Its Analysis' by

J Thudichum (1858).

The first 'dipsticks' were developed in 1850 by the French chemist **Jules Maumene** (1818-1898) using a strip of wool impregnated with stannous chloride to detect urine sugar. A primitive colorimeter was devised by **Louis Jules Duboscq** (1817-1886) in 1854 and gravimetric and volumetric analysis and the necessary equipment were developed by the end of the century. During this period analytical chemistry laboratories were operating in industry and the industrial revolution in Europe helped to provide many of the chemicals that were used in the urine tests described.

Classical chemical tests on urine

Protein

Increased amounts of albumin and blood in urine were observed in patients with dropsy by the American physician **William Wells** (1757-1817) in 1812. This observation was given greater clinical significance by the work of Richard Bright.

Richard Bright (1789-1858) English physician who worked at Guy's Hospital from 1820-1843. A meticulous clinical researcher he correlated his observations with careful postmortem examinations. In his 'Reports of Medical Cases' of 1827 he described oedema and used a candle to heat urine on a spoon as a test for albuminuria found in nephritis ; he also recognised haematuria as a symptom of nephritis. He was greatly assisted in technical work by **George Owen Rees** and another Guy's colleague **John Lever** (1811-1858) reported in 1843 that albuminuria also occurred in eclampsia of pregnancy.

Further tests for urine albumin included the 'ring' test devised in 1844 by the Austrian physician **Johan Heller** (1813-1871). Concentrated nitric acid was carefully added down the side of a test tube of urine and if protein was present in significant amounts a white ring formed at the urine surface. The use of phenol and acetic acid to denature and precipitate albumin was devised by the English physician **Charles Tidy** (1843-1892). However, the heat test was more widely used and assumed greater importance by the observations of **Henry Bence Jones** (1814-1873), an English physician at St George's Hospital, London who in 1847 described the presence of an unusual protein in the urine of a patient, with fractured ribs. The protein precipitated when heated to 70°C but redissolved on further heating only to reprecipitate on cooling. He termed the condition myelopathic albuminuria and later the protein was named Bence Jones protein.

This finding was found to be most often associated with tumours arising in the bone marrow. Electrophoretic studies performed in the 20th century established that the protein was a gamma globulin, the light chain component of an immunoglobulin and the molecular structure was determined by the German molecular biologist **Robert Huber** in 1974.

In 1874 the French physician **Georges Esbach** (1843-1890) introduced his method for the semi-quantitative determination of urine protein. He designed a marked tube, an albuminometer, to which urine was added to mark U and his reagent of 1% picric acid and 2% citric acid was added to mark R. The tube was corked and mixed and left to stand for 24 hours. The height of the precipitate formed was read against graduations to calculate the percentage protein present.

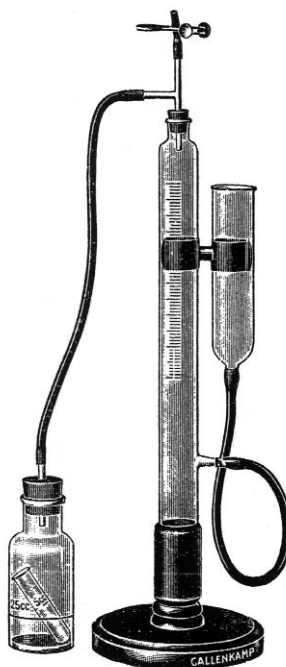


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Urine urea

Urea is the main end product of human protein metabolism and is formed by the deamination of amino acids in the liver. The British chemical physiologist **William Prout** (1785-1850) investigated the properties of urea in 1815, isolated urea from urine in 1818 and in 1848 investigated urea excretion in a number of disease states.

The origin of his methods to measure urine urea is obscure but it is likely that these involved the measurement of nitrogen liberated on addition of alkaline hypobromite using specially designed glass assemblies which were termed 'ureometers' eg Gerrard ureometer. In 1882 **Woldemar von Schoeder** (1850-1898) German physician devised his test for urea using bromine in chloroform.

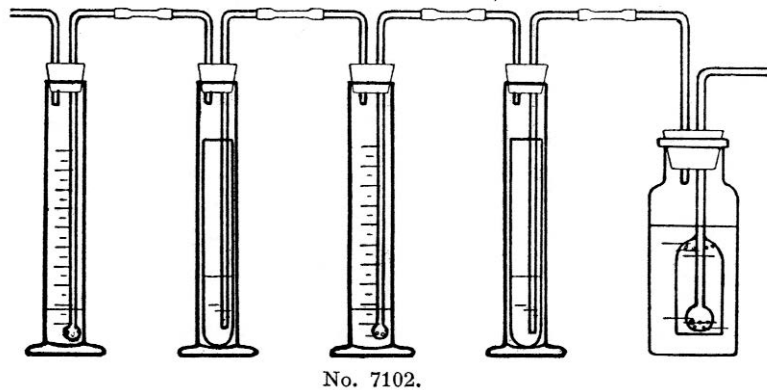


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Urine total nitrogen

Nitrogenous substances in urine are mainly urea but also include uric acid, creatinine, amino acids and ammonia. Urine total nitrogen was used mainly in metabolic balance studies.

A popular, but technically demanding, method was devised by the Danish chemist **Johan Kjeldahl** (1849-1900) in 1883. His apparatus was used in several modes as a micro or macro technique with different ways to measure the ammonia formed by aeration, distillation or using Nessler's reagent. Urine is treated with a digestion mixture of copper sulphate and sulphuric acid, heated strongly and ammonia is generated by the addition of sodium hydroxide. The ammonia is distilled and trapped in a boric acid-indicator solution and titrated with standard hydrochloric or sulphuric acid.



Creatinine

Creatinine is a waste product derived from creatine generated on muscle contraction. The daily amount excreted is related to muscle mass, exercise and creatine intake. It is probable that methods were developed at that time merely to explore the chemical composition of urine without known clinical applications. In 1865 **John Thudichum** devised a qualitative colorimetric method using ferric chloride.

John Thudichum (1829-1901) German physician who was a pioneer in biochemistry and the first lecturer in chemistry at St Thomas' Hospital, London. He also performed important studies on urine pigments and haematin.

Further methods were introduced by **Leopold Salkowski** in 1880 and most notably by **Max Jaffe** around 1886 using the red colour formed with alkaline picric acid.

Max Jaffe (1841-1911) Russian born German biochemist who developed a number of other qualitative urine tests such as those for the detection of urobilin (1868) and indican (1877). Most methods used today for the quantitative analysis of urine and serum creatinine are based on the Jaffe reaction.

Two other tests of renal function were devised by **Dresler** and **Albarran**. In 1892 **Heinrich Dresler** compared total solute in urine and blood and **Joaquin Albarran** (1860-1912) described an oral water loading test to detect tubular epithelial damage.

Urinary 'sugars'

In 1815 **Michel Chevreul** (1786-1889) a French chemist showed that the sugar present in diabetic urine was glucose. A simple test which utilised the reducing properties of sugars was developed in 1841 by the German chemist **Karl Trommer** (1806-1879). An alkaline cupric sulphate solution was added to urine and heated. Cuprous oxide was formed and caused the solution to change in colour from green to yellow and red cuprous oxide was precipitated. Another German chemist **Hermann**

von Fehling (1812-1885) introduced a modification of Trommer's method in 1848 by adding tartrate to hold cupric ions in solution.

Manfred Bial (1870-1908) a German physician devised a qualitative colour test for pentose sugars. Urine was heated with his reagent of orcinol, hydrochloric acid and ferric chloride to produce a green colour with pentoses. **Feodor Selivanoff** (b1859) a Russian chemist introduced a test for fructose in which fresh urine is treated with acidified resorcinol solution to produce a red precipitate with fructose which redissolved in ethanol.

Acetone 'bodies'

Three abnormal constituents, collectively known as 'acetone or ketone bodies', were discovered in diabetic urine during this century. These are products of the catabolism

of fatty acids which becomes the dominating metabolic pathway in uncontrolled diabetes mellitus and if untreated will lead to hyperglycaemic coma. In 1857 **Wilhelm Petters** observed that acetone may be excreted in the urine. In 1883 Ernest Stedelman discovered beta hydroxy butyric acid and its presence in diabetic ketoacidosis was demonstrated by (1865-1955) in 1899. **Carl Gerhardt** (1833-1902) a German professor of paediatrics devised a test for acetoacetic acid, derived from acetone, which gives a purple colour with 10% ferric chloride.

Bile pigments

Urine containing excessive amounts of bile pigments may be intensely yellow, reddish-brown or even black in colour.

Bilirubin

Two of the earliest tests used were based on the oxidation of bilirubin to the green and blue pigments, biliverdin and bilicyanin. **Alphonso Dumontpallier** (1826-1898) a French physician used a diluted tincture of iodine as oxidant which was added carefully to urine to form a layer at the surface. The test was regarded as positive if a green ring formed at the interface. **Leopold Gmelin** (1788-1853) a German chemist and professor of medicine and chemistry at Heidelberg used nitric acid to produce a green, blue or violet ring. In 1884 the eminent German bacteriologist **Paul Ehrlich** (1854-1915) used the diazo reaction with bilirubin to form red azobilirubin as a positive test.

Urobilinogen & Urobilin

Conjugated bilirubin is reduced by intestinal bacteria to urobilinogen, some of which is excreted in urine and may form urobilin on exposure to air. In the course of experiments on trypanosomes Paul Ehrlich observed that the addition of an acidic solution of paradimethylaminobenzaldehyde to certain urines produced a bright red colour. This was found to be due to the presence of urobilinogen and he introduced this test in 1883. The Viennese physician **Wilhelm Schlesinger** (b1869) introduced a test based on the green fluorescence produced with urobilin by zinc acetate in alcohol.

Bile salts

Studies on the composition of bile were performed in 1803 by the French organic chemist **Louis Thenard** (1777-1857). A greater understanding of the physiology of digestion was achieved through the work of **William Beaumont** (1785-1853) , **Theodor Schwann** (1810-1882) and **Claude Bernard**.

In 1844 **Max von Pettenkofer** (1818-1901) a German chemist devised a test for urine bile salts. A small volume of 3% sucrose was added to urine, the tube was sloped and concentrated sulphuric

acid carefully trickled down the wall of the tube. If bile salts are present in excess a red ring of furfural forms at the surface.

A simpler test was devised in 1886 by **Matthew Hay** (1855-1932) professor of forensic medicine at Aberdeen University. Bile salts lower the surface tension of urine and powdered sulphur added to the surface of urine sinks for a positive result.

Other urine pigments

Indican

Indican, indoxyl sulphate is derived from the bacterial decomposition of tryptophan in the large intestine. Indicanuria may occur if there is excessive intestinal putrefaction and the urine voided may have a blue tint and develop a greyish-blue colour on standing. Two tests were developed using acid to liberate indoxyl from indican followed by oxidation to produce indigo blue. In 1877 **Max Jaffe** acidified urine and used potassium chlorate as oxidant and extracted the indigo blue into chloroform. The Austrian physiological chemist **Friedrich Obermayer** (1861-1925) used acidified ferric chloride with chloroform extraction.

Homogentisic acid

Urine which starts to darken from the surface into a deep brown or black colour may occur in the rare metabolic disorder alkaptonuria. Cases were observed from at least the 17th century and the condition was first well described by the British physiological chemist **Alexander Marcet** (1770-1822) in 1822. The excretion of homogentisic acid in alkaptonuria was reported by **Carl Boedeker** (1815-1895) in 1859. The darkening of urine is due to oxidation products of homogentisic acid. Homogentisic acid has been identified by the reduction of silver nitrate or Fehlings reagent to produce black solutions whilst oxidation by 10 % ferric chloride gives a transient blue/green colour.

Melanin

Urine darkens on standing due to the oxidation of colourless melanogen to melanin. Tests for melanogen must be performed on freshly voided urine and include the deep brown or black colour with ferric chloride. **Johann Thormahlen** a German physician used sodium nitroprusside, alkali and acetic acid to produce a blue to blue-black colour.

Haemoglobin and related substances

The technique of spectroscopic analysis was developed by the German physicists **Gustav Kirchoff** (1824-1886) and **Robert Wilhelm von Bunsen** (1811-1899) in 1859.

They discovered that elements in the gaseous state emit characteristic spectra. This technique for the identification of pigments in the clinical laboratory was pioneered by **John Thudicrum** and **CA MacMunn**, Honorary Pathologist at Wolverhampton General Hospital.

The 20th Century

In a century of outstanding discoveries in all forms of science and technology, notably in biochemistry and genetics, the contribution of the clinical pathology laboratory to the practice of medicine was dramatically transformed. The origins of specialisation to form the main disciplines in the biomedical sciences can be found in the first three decades of the century. Prolific scientific research and innovation in medicine and pathology provided more opportunities to develop more clinically useful urine tests. This combined with advances in technology improved the range and quality of services available.

Developments in Microbiology

By around 1912 many of the bacteria responsible for serious human disease had been identified and concentrated efforts were made to develop other vaccines and agents to combat infection. Earlier in 1904 Paul Ehrlich had proposed a 'side chain' theory to correlate drug structure with biological effects and in 1910 he introduced Salvarsan for the effective treatment of syphilis.

Dyes had been long shown to have bactericidal properties and in 1932 **Gerhard Domagk** (1895-1964), director of research at Bayer established that the azo dye, prontosil red, could control coccal infections by virtue of a sulphonamide group. Four years previously **Alexander Fleming** (1881-1955) had made the landmark observation that the Penicillium mould killed staphylococci in culture. However, it was not until 1940 that penicillin was prepared for clinical purposes by **Howard Florey** (1898-1968) and **Ernst Boris Chain** (1906-1979). In 1944 **Selman Waksman** (1888-1973) isolated streptomycin from *Actinomyces griseus* and this was first used in the treatment of tuberculosis in 1946.

During the last fifty years a vast number of antimicrobial agents have been developed. Oral amoxicillin, nalidixic acid, trimethoprim can be used to treat urinary tract infection (UTI) whilst parenteral therapy with a cephalosporin or aztreonam may be effective in patients with pyelonephritis or recurrent UTI.

The increased availability of antibiotics added a new role for the microbiology laboratory and methods were developed to determine the sensitivity of organisms to appropriate antibiotics to select the most effective treatment. A disc diffusion method adapted by **Joan Stokes** of University College, London was adopted by many laboratories in the UK. Breakpoint/abbreviated minimum inhibitory concentration methods were introduced in the 1980's and more recently materials and methods conforming to national standards are employed. Automated systems are also now available for susceptibility testing.

Shortly before World War II the MRC established the Emergency Public Health Laboratory Service to combat possible bacteriological warfare and the threat of infectious diseases. Research and epidemiological studies continued post war and central reference laboratories were established. The PHLs were formally adopted in 1946 with the passage of the NHS Act. Recently the Health Protection Agency has been set up with a much wider remit.

In 1900 **Walter Reed** (1851-1902) and **James Carroll** showed that Yellow Fever was caused by the first human filterable virus and was transmitted by mosquitoes. Initially diagnostic virology was confined to serological tests mainly by complement fixation techniques. In 1949 **John Franklin Enders** (1897-1985) and his colleagues developed techniques for growing polio virus in non-neural cell culture.

By the mid century methods were also developed which used chick embryo for virus propagation and subsequently cell or tissue culture led to the recognition of many new groups of viruses. The use of electron microscopy, and the detection of viral antigens using immunological techniques including immunofluorescence were also significant developments.

The importance of addressing viral disease has been demonstrated by two major viral pandemics in the 20th century, an outbreak of a severe strain of influenza ('Spanish Flu') killed at least 25 million people in 1918-1919 and the appearance of AIDS from the 1980's.

During the last decade techniques in molecular biology, notably polymerase and ligase chain reactions, have been introduced for the investigation of infections due to mycobacteria, Chlamydia and viruses such as Norwalk, Hep B, C and HIV.

Drug resistance eg MRSA has proved problematic and those relevant to UTI include vancomycin resistant enterococci and extended spectrum beta lactamase found in some strains of E.coli and Klebsiella.

Other significant issues include the development of antiviral vaccines and the resurgence of sexually transmitted diseases. Despite the progress achieved it is estimated that infections still account for 70,000 deaths each year in the UK. Global figures are even more alarming with 13 million deaths a year, responsible for 1 in 2 deaths in developing countries and more than half are children under 5 years old.

Urine tests in Microbiology

By the mid century urine microbiology was used in the investigation of a wide range of clinical infections. These included renal abscess, cystitis, prostatitis, septicaemia, lobar pneumonia, gonorrhoea, syphilis, pyelonephritis, fevers and parasitic infections.

The workload was low and concentrated efforts were made to identify the bacteria responsible and select the most effective treatment with the limited number of antibiotics available. The main techniques employed for the identification of bacteria in urine were microscopy of untreated urine, a Gram (or ZN) stain of the urinary deposit, culture using a variety of media including that devised by **Alfred MacConkey** (1861-1931) in the early 1900's, and a range of biochemical and serological tests. Set strategies with a combination of tests, some of which, are described below were used for positive identification.

An increasing emphasis was placed on UTI and **L Rantz & CS Keefer** introduced the diagnostic criterion of >100,000 organisms/ml in 1940 and this was substantiated in 1957 by **EH Kass** who had also demonstrated the importance of 'clean catch' or mid stream urine collection in a comprehensive study performed two years previously.

Studies have consistently shown that E.coli is by far the most common bacterial cause of UTI with a greater occurrence in general practice eg ~80% but this may fall to around 50% in hospital patients. The next most common causes are Staph. saprophyticus, Proteus mirabilis and Klebsiella aerogenes. Pyuria without bacterial growth on conventional media may be due to tuberculosis of the urogenital tract.

From this period the workload has steadily increased and the medium sized microbiology department may now receive 300 urine samples daily. Urine tract infections are common, especially in females, and cause significant morbidity and mortality. Studies have shown that about 25% of septicaemias in hospital patients arise from UTI and that recurrent UTI in children with vesicoureteric reflux may lead to chronic pyelonephritis. UTI may account for at least 25% of hospital acquired infections and is particularly associated with invasive urological procedures eg catheterisation, cystoscopy, transrectal prostatic biopsy.

Screening for bacteriuria is now performed in pregnancy, those patients undergoing invasive urological investigations, patients with renal disease or structural abnormalities in addition to those patients with typical symptoms of frequency, dysuria and loin pain. It is now recognised that UTI may be considered to be uncomplicated or complicated and the latter will require more detailed investigation. These factors and the high workload has necessitated some changes in methodology to provide the relevant diagnostic information with economy of labour and resources.

Sample collection

The need to avoid contamination and to perform investigations as soon as possible has long been recognised. Catheterisation was often considered necessary for female patients but this is not generally advised as the process itself carries a risk of infection.

By 1940 it was also proposed that staged micturition, the 'two' or 'three' glass tests were useful in the location of gonococcal infections.

It is now recommended that urine should be received within two hours of collection and before antibiotic treatment has started. Dip-slides, boric acid preservation or sorbitol transport medium (for Cytomegalovirus) have been introduced if delays are inevitable. In the investigation of tuberculosis three successive early morning urines are now advocated to improve the detection rate.

Urine dipsticks

Two dipsticks are now available which are useful in the detection of urine tract infection and their simplicity has led to their use in point of care testing at GP surgeries and on hospital wards.

Nitrite test strip(Griess)

This involves the formation of an azo dye pioneered in the German dye industry by the chemist **Johann Griess** and developed into a test for nitrites by Ilosvay Lajos. This was first applied to urine by **J Cruikshank & J Moyes** in 1914 who showed that nitrate was reduced to nitrite by many of the bacteria associated with urinary tract infection (UTI). It is claimed that most of the common pathogenic organisms in an early morning sample can be detected with this technique.

Leucocyte esterase test strip

This is based on the esterase activity of granulocytes (and histiocytes) to hydrolyse an indoxyl ester to free indoxyl which reacts with a diazonium salt to form a violet dye.

It is claimed that the detection limit is 10 leucocytes/cmm and >25 leucocytes/cmm regarded as pathologically significant.

Automatic test strip reading equipment is now available eg Clinitek Status, Bayer to improve reliability of results and has been used to select only positive urines for culture.



Microscopy

By 1940 light microscopy of either the uncentrifuged urine or the urine deposit were performed to observe bacteria, cell types, casts and rarely fungal granules and parasitic ova. Dark ground illumination microscopy, often with staining, was used to detect spirochaetes and *Trichomonas vaginalis*.

In 1925 **T Addis** introduced a technique for the quantitation of epithelial cells, erythrocytes and leucocytes in the deposit. This was time consuming and later simplified by other workers.

A more practical approach was more commonly used with the quantitation of cells and casts using a Fuchs-Rosenthal counting chamber technique. An invasive procedure was described by JS Joly in 1935 to establish the site of infection when excessive numbers of 'pus cells' were detected. Today ultrasound and radiological investigations are more practical approaches.

Haematuria was considered to be of pathological significance notably in calculous disease, renal tuberculosis or neoplasm and infection with *Schistosoma haematobium*. Numerous granular, leucocyte or epithelial casts suggest renal involvement in an infected patient. Gram staining of the urine deposit became routine and the Gram reaction and morphology greatly aided identification.

In the succeeding years a number of specialised microscopy techniques were introduced which include phase contrast, polarised light and the use of selective colour filters. Following urine concentration, to improve sensitivity, an auramine phenol technique with fluorescence microscopy can be used for mycobacteria .

Leucocyte counting

The method of choice today for leucocyte counting for laboratories with a high workload is that devised by **JD Rant** and **W Shepherd** using an inverted binocular microscope and clear plastic microtitre plates with flat bottomed wells. A set volume of urine is pipetted into the well of a microtitre plate and after allowing time for sedimentation the number of polymorphs are counted and the number per ml can be estimated from reference tables. It is now well recognised that leucocyte counts exceeding 10/cmm are usually indicative of acute UTI, however, UTI may occur without pyuria.

Culture media

By the mid century both liquid and solid media prepared 'in house' were used based on the evidence provided by the wet film and staining of the urine deposit. Typically nutrient agar, blood agar and MacConkey agar were most often used. Heated blood agar ('Chocolate' agar) was used in the culture of streptococci whilst meat infusion with blood or **JW McLeod's** medium were preferred for gonococci. The selective, enriched sulphite medium developed by **WJ Wilson** and **E Blair** in 1926 was used in the culture of *S.typhi* and *S.paratyphi*.

Mycobacteria proved more difficult to culture and, if suspected from acid fast staining, the urine was pretreated with acid or alkali to remove contaminants before culture. A variety of media such as Petroff's egg-meat-juice medium and Lowenstein egg medium were used for the isolation of mycobacteria. Until around 1970 guinea pig inoculation was also used to confirm the presence of mycobacteria but this often required an incubation period of between 6-8 weeks. More recently modifications of the

Lowenstein-Jensen medium with the addition of the antibacterial malachite green have been used with incubation at various temperatures to improve differentiation of strains.

Today many laboratories have introduced rapid culture methods for mycobacteria using automated fluid culture with fluorimetric detection methods. Specific DNA techniques may be used by reference laboratories or in research to confirm positive culture results.

Manual and semi automated methods of media preparation inevitably led to wide variations in composition and quality of performance.

The introduction of national quality control schemes for microbiology in 1971 and the availability of commercial culture media have been important factors in the standardisation of culture techniques and antimicrobial susceptibility testing.

An important advance was made in 1966 when **JP Mackie & GH Sandys** developed the cystine lactose electrolyte deficient (CLED) medium which is widely used today for the culture of a wide range of pathogens found in UTI. It has significant advantages to MacConkeys medium. It is a suitable medium for more types of organism, with coloured lactose fermenting colonies and characteristically pigmented colonies for the most common organisms found in UTI eg E Coli, Klebsiella.

Bacterial counting

By the mid century qualitative comments were made of the urinary bacteria seen on microscopy or culture. Once again variations in technique and quality of sample gave rise to difficulties in interpretation. The scientific evidence for the diagnosis of UTI led to a number of methods to quantitate bacteria.

Today a surface viable count can be achieved by spreading set volumes of urine on a CLED agar plate. Following incubation each colony corresponds to at least one bacterium and the approximate number can be calculated. An alternative and simpler method is to apply a standard loop (2ul) of urine to the surface of a CLED agar plate incubate and count colonies. Single or double sided dip slides can also be used for semi quantitation.

In 1964 **DA Leigh & JD Williams** developed a reliable and inexpensive paper strip technique to apply urine to the surface of a CLED agar plate. After incubation the number of colonies in the application area is counted and if over 25 colonies this corresponds to at least 100,000 organisms/ml.

Urine culture results

The experienced and skilled microbiologist will often be able to identify the organism isolated from colony appearance alone. On CLED E.coli colonies are smooth, circular and opaque and coloured if lactose fermenting and on blood agar some strains are surrounded by zones of haemolysis. Klebsiella colonies are large, raised and often mucoid and Klebsiella aerogenes ferments lactose. Proteus organisms produce a 'fishy' odour and a swarming appearance on blood agar. Swarming is inhibited on CLED and compact pale blue, green or grey non lactose fermenting colonies are formed. Staph.aureus forms deep yellow opaque colonies whilst Staph.albus and Staph.saprophyticus are pale yellow or white. Candida albicans appears as pale colonies with a distinctive yeasty odour and Pseudomonas colonies on CLED are blue-greenish with a matt surface and rough periphery.

Following preliminary identification biochemical tests are required for more positive identification. Subcultures may be required with more selective media for mixed growth or if there is sufficient doubt in identification.

Biochemical tests

The majority of these simple tests were first devised towards the end of the 19th century and the early 20th century to aid identification. Since then there has been little variation in chemistry but other tests were added during the 1950's. Today a wide range of all these tests are available commercially in multi-test kits. The API 20E (Analytical Profile Index) strip contains 20 biochemical tests used to identify enterobacteria. After inoculation and incubation the results are scored to give a numerical profile compared to table or computer software systems for interpretation of results.

O.Voges-B.Proskauer

These two associates of **Robert Koch** first introduced this test in 1898. Alkali is added to a glucose phosphate peptone (GPP) broth culture to produce a pink colour if positive. It was designed originally to distinguish *E.coli* (negative) & *Ent.aerogenes* which give negative and positive results respectively. Modifications in procedures were described by **R O'Meara** (1931) **M Barritt** (1936).

It is now recognised that other enterobacteria also produce a positive reaction.

Methyl red

This involved the addition of this pH indicator to a GPP broth culture to form a red colour if acid is produced during the fermentation of glucose. Typical *E coli* gives a positive reaction.

Citrate

This was used to assess the ability of the organism to use citrate and an ammonium salt as sole sources of carbon and nitrogen for energy and growth using a synthetic chemical medium eg Koser. Typical strains of *E coli* are unable to grow on these limited medium.

Indole

This is used to assess if the organism decomposes tryptophan, added to peptone/saline medium, to indole which is detected by a pink colour formed with an acidic, alcoholic solution of paradimethylaminobenzaldehyde. *E.coli* and *Proteus vulgaris* are examples of indole positive organisms

Oxidase

Some bacteria, such as *Neisseria* and *P.aeruginosa*, contain oxidases which catalyse a redox reaction with di or trimethyl p-phenylene diamine to form a pink, then purple to black colour. A semi-transparent medium gives a more distinct colour definition with positive colonies. The dye solution may be flooded over the culture or applied to filter paper. All species of enterobacteria give negative reactions.

Catalase

This test is performed in a capillary tube containing the culture isolate and hydrogen peroxide solution. Oxygen released at the culture surface indicates a positive reaction.

Urease

Bacteria containing urease can hydrolyse urea to ammonia and carbon dioxide. Ammonia production can be assessed by the purple-pink colour change of phenol red or by the dark brown colour produced with Nessler's reagent. Typical strains of *E coli* do not hydrolyse urea unlike *Proteus spp.*

Coagulase

The culture is added to oxalate rabbit plasma and incubated for 3 hours at 37' and observed for coagulation. *S.saprophyticus* may be a cause of UTI in sexually active young women and is coagulase negative. *S.aureus* gives a positive reaction.

Gelatin

Organisms which contain proteolytic enzymes liquefy gelatin or coagulated serum. Nutrient gelatin media or gelatin agar are suitable media. The addition of an acid solution of mercuric chloride to the culture produces clear zones around gelatin liquefying colonies. J Kohn introduced gelatin charcoal discs in 1953 with carbon particle deposition for more rapid positive results. Typical strains of E coli do not liquefy gelatin.

Amino acid decarboxylase

This was devised by V Moller in 1955 and is based on the ability of some bacteria to decarboxylase basic amino acids eg lysine to produce an amine and liberate carbon dioxide. The pH of the culture medium becomes more alkaline detected by an indicator colour change. E coli & Ser. marcescens give positive reactions

Fermentation tests

In addition to MacConkey or CLED lactose fermentation it may be necessary to use other carbohydrate fermentation tests for greater discrimination. Lactose, glucose, inositol and mannitol are most often used in the investigation of the differentiation of enterobacterial infections. Each carbohydrate is added separately to nutrient media such as peptone water or sugar agar, containing a pH indicator. Following inoculation and incubation the culture is observed for the production of acid and gas or acid alone.

E. coli and Klebsiella produce acid and gas from glucose and lactose.

R Hugh and E Leifson test

This was described in 1953 to distinguish aerobic and anaerobic breakdown of carbohydrate. A semi-solid tube medium contains a pH indicator and the carbohydrate.

The production of acid at the surface (aerobic) infers oxidation of carbohydrate but if this occurs at lower layers (anaerobic) implies fermentation. Enterobacteria typically show fermentation whilst Pseudomonas strains are usually oxidative

Urine cytology

A number of stains had been developed in the previous century to differentiate cellular structures and specific types of tissue. The use of methylene blue and eosin as a counter stain for the cytoplasm was originally devised by the Russian physician **Dimitri Romanowsky** (1851-1921). Variations of the haematoxylin stain were introduced by **W Waldeyer** (1863) **J Delafield** (1885), **Paul Ehrlich** (1886), **HF Harris** (1900) and **P Mayer** (1903). Its combination with eosin was first introduced between 1875-1878 and later was adopted worldwide.

From 1917 onwards **George Papanicolaou** (1883-1962) a Greek born American anatomist and cytologist began his landmark studies into the use of cytology in the diagnosis of cancer. During these studies he devised his famous PAP stain which has become the dominating stain used in urine cytology today. Its primary role is to stain urothelial cells shed from the kidney and urinary tract to assess changes in nuclear morphology and staining characteristics associated with malignancy.

Other useful cytology stains include May Grunwald Giemsa, toluidine blue, haematoxylin and eosin, and Oil Red O. Autostaining equipment is now available to standardise the process as well as stain coated slides eg Roche Testsimplets.

Phase contrast microscopy (PCM) of wet preparations of the urinary sediment provides a rapid screening method in malignancy. PCM or differential interference microscopy may be used to

demonstrate the presence of parasitic infections eg *Trichomonas vaginalis*, or fungal infections such as *Candida albicans*. Electron microscopy can be used in the investigation of urine viral infections.

Despite the progress made many reviews emphasise the possible pitfalls in urine cytodiagnosis. These include sample quality, the presence of calculi, cancer treatment and bacterial or viral infection which may produce cellular distortion, atypical cells or cells which mimic malignant changes. Consequently during the last twenty years semi automated procedures have been described for the preparation and screening of the urine sediment for more rapid processing and reduction of human diagnostic errors. In addition greater diagnostic accuracy has been reported using computerised image analysis, and flow cytometry methods using the metachromatic fluorescent dye acridine orange to stain DNA and RNA may become an important technique in the future. Liquid based (thin prep) cytology methods can also be applied to urine.

A review published in 2004 recognises urine voided cytology as the current gold standard for bladder cancer but other methods eg proteomic profiling of urine are under evaluation.

Urine cytology investigations

The microscopic screening of urine for malignancy by an increased number of red blood cells was proposed in 1920. This was replaced in 1951 in the UK by more specific urine cytology at 6 monthly sampling intervals for workers in high risk manufacturing industries eg dyes, rubber.

Clinical requests may result directly from clinical symptoms eg haematuria, or when lesions are found during urological or radiological procedures. The main objective is

the early detection of urothelial carcinoma esp bladder and in the differentiation of transitional cell tumours from other tumour types. It can be used to evaluate the efficacy of cancer treatment and detection of recurrence and is regarded as complementary to cystoscopy.

Urine cytology has also been used to detect rejection episodes in renal allograft and indicators used include cell pattern changes (**EH Bossen**, 1970) and the increased number of renal tubular cells (**P O'Morchoe**, 1976). The successful use of fine needle aspiration cytology of the kidney to assess rejection was first described in 1968.

Samples

Many studies have shown that sample quality is highly significant for the reliable interpretation of results. It is now advocated that three voided urines should be collected and if a delay is anticipated urine should be stored at 4°C or preserved by fixation with alcohol. Catheter urine or bladder washings may be received and for location studies a catheter 'brush' technique was devised by **W Gill** in 1973

Preparation

For more reliable diagnostic interpretation it is important to concentrate the urine to provide an adequate number of cells whilst retaining their morphology and staining characteristics. Cellulose membrane filtration eg Gelman, Millipore or centrifugation eg Cytospin are most commonly used. A smear of the urine deposit can be stained with methylene blue/toluidine blue or PAP, haematoxylin and eosin or alternatively examined by PCM. Fixation is then required for a permanent preparation.

Microscopy

In keeping with their specialised function different types of epithelial cells are found within the kidney and urinary tract. Tubular, squamous, columnar and transitional epithelial cells may be exfoliated into the urine in various pathological conditions.

Voided normal urine contains few urothelials but a greater number in sheets or clusters may be seen in urine collected by catheterisation due to forcible detachment

Abnormal results

Cancer

Haematuria is common in malignancies of the bladder, ureter and the renal pelvis. Malignant changes of mainly transitional cells or less often metastatic squamous or adenocarcinoma may be observed with PAP staining. Adenocarcinoma of the kidney is tubular in origin and cells are only found in the urine at a late stage if there is invasion of the renal pelvis. Demonstration of cytoplasmic lipid using Oil Red O has improved diagnostic accuracy.

Primary carcinoma cells from other sites may be detected by PAP staining when there is invasion through the bladder wall from the primary site eg colon, prostate, cervix

Malakoplakia

This is a chronic inflammation of bladder wall and cytoplasmic inclusions (Michaelis-Gutmann, MG bodies) which are calcified lysosomes possibly associated with E coli infection may be observed.

Fungal infections

Candida albicans is the most common fungal infection and spores and pseudohyphae which stain pink with PAP may be seen especially in patients with Diabetes mellitus and immunosuppressed patients and the fungal ball may block the ureter.

Parasites

Schistosoma haematobium is a trematode worm found commonly along Nile delta. The ova are deposited in the submucosa of bladder/distal ureters causing inflammation and fibrosis of bladder wall and ova may be seen in the urine deposit. Affected patients have a tendency to develop squamous cell carcinoma of the bladder. Toxoplasma gondii produces cysts which are shed in urine and flagellated Trichomonas vaginalis may also be easily identified. Rarely, trophozoites of the parasitic amoeba, Entamoeba histolytica, may be found in the urine of patients with amoebic ulceration of the genitalia. Eggs of the giant kidney worm, Dioctophyma renale, and larvae of the nematode, Strongyloides, have been recovered from urine sediments.

Viral infection

This may occur in impaired immunity eg renal allograft, AIDS, chemotherapy

Cytomegalic virus (CMV)

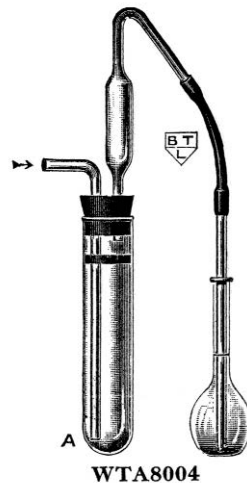
This is an encapsulated DNA virus, first isolated in 1956, and with haematoxylin and eosin staining the infected urothelials are enlarged with a nuclear 'birds eye' inclusion with a surrounding halo. It is relatively common but rarely causes disease except in renal allograft patients to cause pyrexia or pneumonia. It is serious in pregnancy by transplacental transmission and may cause foetal death or severe mental or physical retardation in surviving infants.

Human polyomavirus

DNA virus-many infected cells are shed with a cyanophilic nucleus with a basophilic inclusion with a variable halo. Infection occurs in childhood and remains latent until there is reduced immunity. Electron microscopy can be used to identify this virus and differentiate from CMV

Clinical Chemistry

By around 1950 the structure and function of proteins, amino acids, vitamins, enzymes and hormones had been discovered. This increased the range of urine tests which could be devised and the introduction of visual and photoelectric colorimetry permitted quantitative analysis. A large number of micromethods for blood chemistry were developed in the United States, notably by **Donald Van Slyke**, **Otto Folin** and **Ivar Bang** during the first two decades. Thus, for example creatinine or glucose could be measured in both urine and plasma to assess renal function as a 'clearance test' or as a 'tolerance test' to a glucose load respectively. The discovery of insulin in 1922 by the Canadian surgeon **Frederick Banting** (1871-1941) and the Canadian physician **Charles Best** (1899-1978) increased the importance of blood and urine glucose assays.



Hormones, notably aldosterone and anti-diuretic hormone, were identified that act on the renal tubules to regulate salt and water balance leading to a greater understanding of the composition of urine in health and disease. The kidney was shown to have synthetic functions in the secretion of the enzyme renin and the production of the active form of Vitamin D and erythropoietin. These discoveries led to a greater understanding of the occurrence of hypertension, bone changes and anaemia in renal impairment.

The autoimmune nature of glomerulonephritis was suggested by the American paediatrician **Bela Schick** (1877-1967) in 1903 and micronodular lesions in the glomeruli were observed in diabetic nephropathy by **Paul Kimmelsteil** and **Clifford Wilson** in 1936. The different forms of nephritis were classified by **Donald Van Slyke** (1930), **Arthur Fishberg** (1931) and **Arthur Ellis** (1942). Treatment prospects for patients with chronic renal failure were greatly improved by the introduction of haemodialysis in 1956 with a twin coil system designed by **Willem Kolff** and associates. This allowed a period of 'stabilisation' prior to renal transplantation which was first successfully performed in 1958. Developments in immunology for graft compatibility and pharmacology for immunosuppressive drugs reduced the risk of rejection to further improve the success rate. The lithotripter, an ultrasonic device to fragment kidney stones without bleeding was invented by **Eisenberger** and **Chaussey** in 1972.

A number of advances in analytical chemistry and immunology were applied to the separation, identification and quantitation of individual urine components. In 1941 the London biochemist **Archer Martin** (1910-2002) with **Richard Syngé** (1914-1994) developed partition paper chromatography which could be used for amino acids, sugars, hormones and drugs. During the

following 30 years more quantitative techniques were developed such as gas liquid chromatography (GC), high performance liquid chromatography (HPLC) and GC-Mass spectrometry (GCMS).

Dry chemistry systems, strips impregnated with chemicals for colour reactions eg as dipsticks and multi-film layers, were also introduced in this period.

Paper electrophoresis was developed by **Emmett Durrum** in 1950 and cellulose acetate introduced as a more efficient support medium by **J Kohn** in 1957 which could be used for urine amino acids and proteins.

The introduction of immunoassay, pioneered by **Yalow** and **Berson** in 1959, and the use of monoclonal antibodies by **Milstein** and **Kohler** in 1975 greatly improved the sensitivity and specificity for the quantitative analysis of urine hormones and drugs.

Many variations of immunoassay, notably non-isotopic labelling techniques have been developed in the last twenty years with significant practical advantages.

Urine chemical tests

Galactose tolerance test

This was used by Bauer in 1906 to assess the impaired synthesis of glycogen in liver disease. Galactose was measured in urine collected in the five hour after oral loading and is greatly increased in liver diseases, such as hepatitis and cirrhosis. More sensitive methods using the estimation of blood galactose were introduced during 1940-1948 notably by **NF Maclagan**. In 1940 **E King** proposed an intravenous loading and an evaluation performed in 1978 showed it could detect early liver damage and may be useful in assessing prognosis.

Clearance tests

The importance of glomerular filtration in renal function led to attempts to develop methods to estimate glomerular filtration rate (GFR). In 1904 **Otto Folin** (1876-1934) published quantitative methods for urine creatinine & creatine by the Jaffe reaction. This is claimed to be the first recorded use of a colorimeter for chemical analysis related to medicine.

In 1919 **Folin and Hsien Wu** (1893-1959) applied the same principles to the measurement of plasma creatinine. In 1926 the Danish physician **Paul Rehberg** (1895-1985) used urine and plasma creatinine to measure creatinine clearance. It is recognised now this is only an approximate estimate of GFR, however, it is adequate for most clinical requirements and is low or decreased in glomerulonephritis, ureteric obstruction and pyelonephritis. In 1954 **JA Owen** and **co-workers** published their 'true' creatinine methods using bacterial enzymes and adsorption on aluminium silicate (Lloyd's reagent) prior to the alkaline picrate assay to improve specificity. Kinetic mode assays and other enzymatic assays were described between 1979-1992. The urea clearance test was first proposed as a renal function test by the French physician **Leo Ambard** (b1876). Urea clearance was introduced in 1928 by **Eggert Moller, JF McIntosh & Donald Van Slyke** (1883-1971) but was limited by dependence on urine flow rates and diet. However, it remained a popular test for many years until superseded by creatinine clearance. More accurate estimations of GFR were described during the 1940's using the clearance of inulin, thiosulphate and mannitol. Currently it is accepted that once GFR has fallen significantly radioisotope markers clearances are more appropriate.

Acetone and acetoacetic acid

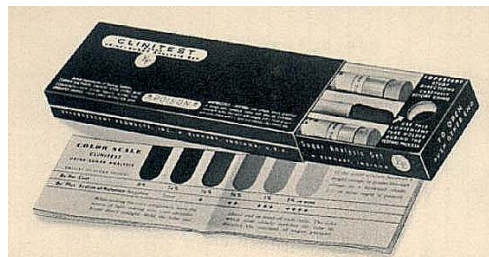
Cecil Hamil Rothera (1880-1915) devised the nitroprusside test for urine acetone and acetoacetic acid in 1908. **Donald Van Slyke** developed selective methods for each ketone 'body' in 1917 using gravimetric or titrimetric techniques but these were laborious and technically demanding. In 1946

RM Dumm & RA Shipley used a dry powdered reagent containing sodium nitroprusside, ammonium sulphate and sodium carbonate on a filter paper to which a drop of fresh urine is added. This was adapted during the 1950's by the Miles-Ames company (Indiana) to produce a tablet form of this test (Acetest) and later a single 'dipstick' strip (Ketostix).

Urine reducing substances

In 1909 **Stanley Rossiter Benedict** (1884-1936) produced a modified copper sulphate reagent for the detection of reducing substances which was more sensitive than previous tests described. In 1911 he also introduced a quantitative test for urine reducing substances. Other reducing sugars, certain drug metabolites, uric acid and creatinine also reduce copper sulphate. In 1934 **Joseph Hiram Roe** devised a specific colorimetric test for urine and plasma fructose. Confirmation of the presence of other reducing sugars used tests developed in the last century (eg Bials test) until paper chromatography was applied to carbohydrates by **SM Partridge** in 1948.

Benedict's quantitative test was adapted during the 1950's by Miles-Ames in tablet form as Clinitest and a more specific dipstick, Clinistix, that contains glucose oxidase, o-tolidine and a vegetable peroxidase. The coupled hexokinase method developed in the 1970's can also be used for urine glucose but quantitative assays are rarely required.



CLINITEST® Reagent Tablets, was the first in vitro diagnostic test kit (1941).

Amylase

Amylase, originally named diastase, was the first enzyme to be used in clinical diagnosis. In 1910 **Julius Wohlegemuth** (1874-1948) detected an increase in urine amylase in pancreatitis. He used an iodometric method to follow the course of starch digestion by urine under standard conditions. In 1938 **Michael Somogyi** (b 1883) an American biochemist introduced a saccharogenic method with urine reducing substances estimated before and after incubation with starch substrate. Chromogenic methods employ a number of dye linked starch solid substrates, such as Phadebas (Pharmacia) and were introduced during the 1960's with colorimetric end point measurements of amylase activity. This was adapted to automated dry chemistry analysers (eg Kodak Ektachem) and a coupled enzyme assay was developed by **RA Kaufman** and **N Tietz** in 1980. Amylase isoenzyme methods were described by G Skude in 1976.

Phenolsulphonephthalein (PSP) excretion

This test is based on the ability of the kidneys to excrete foreign substances from blood. It was first described in 1910 by the American physicians **Leonard Rowntree** (1883-1959) and **John Geraghty** (1876-1924) and **Eli Marshall** (1889-1966) demonstrated that PSP excretion was related to the secretory efficiency of the renal convoluting tubules. Following the administration of one ml of 0.6% PSP i.m. it is measured colorimetrically in urine voided at one and two hours. Normally 60-80% of the dye is excreted in two hours with much lower rates in most chronic renal diseases. Dye excretion is affected by pre-renal factors and although still described in current textbooks it is not as reliable or sensitive as the concentration tests described later.

Uric acid

This is the end product of the metabolism of purines derived from the diet and the breakdown of nucleoproteins. **Otto Folin** and **Willey Denis** reported a quantitative colorimetric assay in 1912 using the reduction of phosphotungstic acid in alkaline solution. In 1915 Stanley Benedict used arsenophosphotungstic acid with the addition of sodium cyanide to improve colour yield and sensitivity. More specific methods were developed by Otto Folin in 1933 with initial separation as the silver salt and colorimetry. A UV technique without and with uricase was described by

TV Feichtmeir and **HT Wrenn** in 1955. Studies performed in 1969 by **GR Boss** have proposed that urine uric acid concentrations may be used to select the type of drug used in the treatment of asymptomatic hyperuricaemia. Although various forms of HPLC were described in the late 1970's coupled uricase methods are most often used today.

Haemoglobin and related substances

The pocket direct vision spectroscope was first used successfully for this purpose and in 1914 **Hamilton Hartridge** (1886-1976) a British physiologist developed his reversion spectroscope to investigate haemoglobin pigments and porphyrins in urine and blood. Oxyhaemoglobin, methaemoglobin and myohaemoglobin may occur in the urine with characteristic spectra. During the 1950's spectrophotometers with improved optical systems replaced visual spectroscopes. It is now common practice to use dipsticks eg Haemastix (Miles-Ames) with the haem proteins acting as peroxidases with oxidation of a chromogen eg o-tolidine, to a blue chromogen. This is a very sensitive test and can detect 0.02 mg haemoglobin/ml urine. Haemoglobinuria, in the absence of haematuria, usually infers severe intravascular haemolysis. Microscopic urine examination is the appropriate and most sensitive test for haematuria to detect intact red blood cells. Methods specific to myoglobin include separation by ultrafiltration, nephelometry and a radial immunodiffusion technique (in 1977).

Between 1910-1940 **Hans Fischer** (1881-1945) professor of organic chemistry in Munich performed extensive research into porphyrins and their structure and in 1913 he identified uroporphyrin in urine. In porphyrias the urine is typically port wine in colour but may be reddish-brown. In acute idiopathic porphyria the urine contains excessive porphobilinogen (PBG) and in 1941 CJ Watson and S Schwartz devised a test for PBG using Ehrlich's reagent and chloroform extraction. This test was adapted in 1974 as a single stage 'point of care test' with a modified Ehrlich's reagent. Between

1949-1958 **C Rimington** and associates developed a number of methods for the detection of the many subtypes of porphyrins including extraction with fluorescence and chromatographic separation techniques.

Screening for porphyrins using solvent extraction and their native fluorescence was described in 1969. A range of reverse phase HPLC methods have been developed notably by **RE Ford** (1981) and **CK Lim** (1988).

Bilirubin

A simple and popular qualitative test was devised in 1917 by the French chemist and physician **Andre Fouchet** (b1894). 10% barium chloride is added to an equal volume of urine and filtered. The addition of Fouchet's reagent, 25% TCA and 0.9% Ferric chloride, to the dried precipitate produces a blue-green colour with bilirubin.

During the 1950's Miles Ames company developed Ictotest, a dry chemistry test for bilirubin. The tablet contains a coupled diazonium compound and yields a blue purple colour with bilirubin.

Ehrlich's test for urobilinogen was modified by Richard Henry and associates as a semi quantitative technique in 1964. Ascorbic acid is added to prevent reformation of urobilin, sodium acetate to reduce interference and enhance the colour intensity, which is measured spectrophotometrically.

Water dilution test

The ability of the kidney to produce dilute urine on water loading was proposed by **Franz Volhard** (1872-1950) a German physician in 1918. Following overnight fluid restriction 1500 ml of water is given to drink. The volume and specific gravity was measured of urines collected each hour over 4 hours the next morning. The normal response is to excrete almost 1500 mls of urine within 4 hours with a specific gravity between 1.001-1.004, neither of which is achieved in renal disease.

Modifications of this test were introduced in 1952 and 1961 in the investigation of Addison's disease and severe hypopituitarism which may show a delayed diuresis. However, there is a significant risk of water overload and the test is rarely performed..

Sodium and Potassium

A colorimetric technique for potassium was first described in 1918 and a gravimetric methods for sodium in 1920. Both were refined during the 1930-1940's but remained most laborious. The flame emission spectrophotometer was first produced in the United States in 1945 and developed for the clinical laboratory notably by **RB Barnes**,

JW Berry and **AG Spencer** and their associates. In 1945 **Barnes** introduced the concept of an internal standard, using lithium, to improve specificity and sensitivity.

Flame photometry was an important landmark in clinical chemistry allowing rapid and accurate measurements of these two clinically important cations. This was used extensively for over twenty years until ion selective electrodes were developed for use in automated equipment from around the mid 1980's. Urine sodium measurements are clinically useful in the investigation of adrenal disease, salt losing nephritis and diuretic therapy. Studies performed in the late 1970's demonstrated that a low urine sodium with high osmolality may distinguish pre-renal, post-ischaemic and nephrotoxic forms of acute renal failure. Urine potassium can be helpful in adrenal and primary renal diseases, renal tubular necrosis and monitoring steroid therapy.

Urea concentration test

This was devised in 1920 by **Hugh Maclean** (1879-1957) professor of medicine at London University and director of the Medical Clinic at St Thomas's Hospital. No food or drink is taken 12 hours before the test and the patient is given 100 ml of flavoured 15% urea to drink. Urine is collected for next three hours and the volume and urea measured. A urea concentration of at least 2.5% is achieved normally with lower concentrations in renal failure.

Hippuric acid synthesis test

This was used to assess the detoxification function of the liver in removing benzoic acid by combination with glycine to form hippuric acid which is excreted in the urine.

It was first described in 1920 by **Emile Achard** (1860-1944) and modified in 1933 by **Armand Quick** (1894-1977) an American physician and physiologist who was professor of biochemistry at Milwaukee University. Oral and intravenous forms of the test were developed and hippuric acid determined by titrimetry. Low excretion of hippuric acid indicates liver damage.

Protein

More quantitative methods for urine total protein were developed by **Folin** and **Denis** in 1914 using nephelometry. **William Exton** (1876-1943) an American physician introduced a turbidimetric assay using a reagent containing salicylsulphonic acid and sodium sulphate. This is a simple and sensitive test which is still used today. In 1951 **OH Lowry** and colleagues devised a method for the analysis of serum and urine protein using the Folin phenol reagent and measurement of absorbance at 280nm

The advent of electrophoresis allowed separation and identification of different urine proteins preceded by membrane or column urine concentration. This can be used to identify Bence Jones protein or other low molecular weight proteins of tubular proteinuria. Comparison with serum protein electrophoresis may indicate selectivity in glomerular proteinuria. Total protein excretion rates may be measured from 24 hour collections but studies reported in 1983 showed that protein/creatinine ratios in random urines are more convenient. In heavy proteinuria eg nephrotic syndrome, IgG/albumin ratios are more appropriate and may be used to select and monitor treatment.

Studies reported by **GC Viberti** and colleagues in 1982 showed that increased albumin excretion rates can be predictive of nephropathy, end stage renal disease and retinopathy in type 1 Diabetes. Albustix (Miles Ames Co) can be used as a primary screen and contains bromophenol blue buffered to pH 3 which is converted from a yellow protonated form to the anionic blue-green form by protein. A latex agglutination test (Albu Sure, Cambridge Life Sciences) is semi-quantitative but immunochemical methods developed in the last 15 years are more sensitive and specific and include radioimmunoassay, radioimmunoassay and enzyme linked immunosorbent assays.

Glucose tolerance test (GTT)

The physiological response to a glucose load was first studied in 1916 by the Chicago physician **William Sansum** (b1880). **Hugh Maclean** (1879-1957) described a glucose tolerance test in 1921 for the investigation of diabetes and glycosuria. A number of different forms of this test have been described with variations in load and duration. Typically the test is performed after an overnight fast, a fasting blood sample collected and the patient given a solution containing 50 or 75g glucose to drink ie oral GTT Blood and urine is collected at 1 and 2 hours. Blood glucose is measured and urines tested for glucose Variations include a one hour GTT with two oral loads by **Sylvester Gould** (b1900) in 1937 and an iv glucose loading procedure by **Samuel Soskin** (b1904) in 1943. The OGTT was used for about the next 30 years mainly in the investigation of asymptomatic glycosuria and glycosuria during pregnancy. The interpretation of the response was often difficult and following studies performed in 1975 and 1979 WHO set new criteria with a revised classification based on the fasting blood glucose. It is used selectively now and urine tests are not performed. Glycosuria will occur when the renal threshold for glucose is exceeded and because this may vary urine glucose does not provide additional or clinically useful information.

Acidity of urine

In 1904 **Otto Folin** introduced the term 'titratable acidity' which when combined with urine ammonia gives an estimate of the ability of the body to conserve base. This involved titrating urine with 0.1M sodium hydroxide using phenolphthalein as indicator. In 1957 **K Jorgensen** devised a titrimetric method to measure net acid excretion.

pH of urine

In the early 20th century urine pH measurements were made using the Lovibond Comparator. Methyl red was added to urine and compared to standard glass discs calibrated to set pH values. In 1925 **AB Hastings** developed a colour comparison method to measure urine pH by mixing equal volumes of urine and indicator in capillary tubes. In 1934 **Arnold Beckman** developed an improved pH meter.

The pH of urine may vary between pH 4.8-7.8 depending on the acid base status, diet and drug therapy. Alkaline urine may be due to infection with urea fermenting bacteria notably B.Proteus. **H Davies** and **O Wrong** described an acid excretion test using oral loading with ammonium chloride in 1957. Urine is collected for 6 hours and pH and ammonia estimated. Normally urine is at least pH 5.3 or lower. In renal tubular acidosis urine pH is typically between 5.7-7.0 and ammonia excretion reduced.

Urine Ammonia

Acids are removed by the kidneys by the formation of ammonium salts. A number of different methods were developed to measure ammonium salts. In 1914 **Van Slyke** and **GE Cullen** used an aeration-titration method and in 1917 **Folin** and **RD Bell** used ion exchange resin- nesslerisation. The ingenious and versatile double chambered glass unit based on gas diffusion was introduced by EJ Conway for urine ammonia in 1933 and was also applied to urine urea and later for blood carboxyhaemoglobin.

Urine concentration

1903 **George de Santos** (1876-1911) a New York physician described a micromethod for urine SG using his hydrometer. The urine concentration test was adapted as a clinical procedure in 1930 by another New York physician **Arthur Fishberg** (b1898). It was designed to test the ability of the kidneys to concentrate urine. Fluids were deprived in the evening and during the night and the specific gravity of urine voided the next morning measured. A number of methods were introduced to measure specific gravity including the urinometer, a calibrated hydrometer, the pycnometer, a microvolumetric flask which was weighed, the flotation of glass beads or drops of organic solvent mixtures. With normal renal function the urine specific gravity should exceed 1.022 which is not achieved in renal impairment. Refractive index measurements can be correlated with specific gravity with portable meters (eg American Optical Corp, New York) available. During the 1950's freezing point osmometers were introduced and it now common practice to measure urine osmolality in the assessment of urine concentration function tests.

Clearance concepts have been applied using osmolality as osmolal clearance and free water clearance to define the limits of water conservation and excretion in health and disease.

Urine Vitamins

Studies performed at the turn of the century by **Eijkman** and **Hopkins** confirmed the existence and importance of 'accessory food factors'. The first 'vitamine', thiamine(B1) was identified by the Polish chemist **Casimir Funk** (1884-1967) in 1911. Many others were subsequently discovered and urine and blood assay methods introduced into the clinical laboratory from around 1935. In 1945 **RE Johnson** et al developed fluorimetric assays for urine B1(thiamine), B2 (riboflavin) and niacin which have now been superseded by HPLC methods introduced in the 1970's. Loading tests and the measurement of metabolites, which use specific vitamins as coenzymes, have also utilised urine tests. Today the measurement of vitamins by specialised laboratories has assumed greater importance in deficiency states eg 'dietary cultures', malabsorption, haemodialysis and total parenteral nutrition ; or with excessive intake.

Urine Vitamin C (Ascorbic acid)

Scurvy was first described in the 13th century and its treatment with citrus fruits was recorded during the 16-18th centuries. The role of ascorbic acid in scurvy was identified by **Albert Szent-Gryogyi** in 1928. In 1935 **LJ Harris & SN Ray** devised a urine test, using the reducing properties of the vitamin to convert the dye dichlorophenolindophenol to its colourless leucobase. Harris with **MA Abbasy** developed an oral saturation test in 1937 using the dye test for urine collected between 4-6 hours after a body weight corrected dose. This test is notable as a rare example of a test which is not only analytical, diagnostic but also has a therapeutic outcome. Fluorometric and HPLC methods were developed during the 1970's mainly for quantitative measurement of plasma ascorbic acid.

Urine Vitamin B12

In 1953 the American haematologist **Robert Schilling** (b1919) devised a urine test for Vitamin B12 absorption which allowed differentiation of pernicious anaemia and intestinal malabsorption.

Following i.m B12, oral doses of Cobalt57 and Cobalt 58 are given and measured in a 24 hour urine collection. Isotope excretion rate and their ratios allow differential diagnosis of pernicious anaemia and ileal malabsorption..

B12 is required for the conversion of methylmalonic acid (MMA) to succinic acid and urine MMA is increased in B12 deficiency. This can be detected by a variety of chromatography techniques such as gas chromatography -mass spectrometry

eg **K Rasmussen** in 1989.

Urine hormones

Sex hormones

In 1928 **Bernhard Zondek** (1891-1966) & **Selmar Aschheim** (1878-1965) developed the first reliable pregnancy test using a bioassay to detect the induction of ovulation in mice. In 1931 **Maurice Friedman** (1903-1991) with **Maxwell Lapham** (1899-1983) developed a similar but more rapid test with mature, female virgin rabbits. In 1937 **RK Callow & NH Callow** devised a bioassay for urine androgens using measurements of comb growth in castrated capons.

Chemical methods for total oestrogens were based on their colour with phenol and sulphuric acid first described by **S Kober** in 1931. Numerous variations were developed for pregnancy urines in the next 20 years. In 1955 **JB Brown** introduced modifications for the measurement of individual oestrogens in non pregnancy and this was applied to studies of the menstrual cycle and infertility treatment. The Kober reaction combined with a reagent developed by **G Ittrich** in 1958 led later to the automation of urine total oestrogens eg **Lever** et al 1973 used extensively to monitor risk pregnancies until replaced by ultrasonic methods during the 1980's. The development of serum oestradiol & progesterone radioimmunoassay and more recently by non isotopic automated immunoassay is now used in infertility investigations and treatment including IVF.

However, urine continues to be used in pregnancy testing and the in vivo types of tests described above were replaced during the next 20 years by haemagglutination or latex particle agglutination inhibition assays slide (eg Gravindex, Ortho) or tube form (eg Pregnosticon, Organon). Self test kit tests became available in the next decade (eg Advance, Ortho) based on enzyme immunometric principles.

Adrenocorticosteroid hormones

By 1940 over twenty steroids secreted by the adrenal cortex had been identified with a characteristic 4 ring structure. Many of the urine assay methods were based on the colour reaction of 17 ketosteroids with alkaline metadinitrobenzene first described in 1935 by the German physician **Wilhelm Zimmermann** (b1910). This was ingeniously adapted notably by **JK Norymberski** and associates (in 1952,1956) to differentiate the different steroid groups eg 17 ketogenic,17 hydroxycorticosteroids. Alternative colorimetric techniques were devised by **G Pincus** (1943) and **CC Porter** and **RH Silber** in 1950. These techniques were used extensively for the next twenty years in the investigation of disorders of the adrenal and pituitary glands and were also used with dynamic function tests of suppression or stimulation.

Today comprehensive urine steroid profiles can be obtained by HPLC or Capillary GC and immunoassays for plasma cortisol are used in dynamic function tests.

Adrenal medullary hormones

The catecholamines, adrenaline and noradrenaline, are physiologically active pressor amines and increased urine excretion in phaeochromocytoma was reported by **A Engel** and **Ulf von Euler** in 1950. Methods were described by **A Lund** (1949) and Ulf von Euler (1955) for total catecholamines using oxidation to produce a fluorescent product. Preliminary separation with alumina or ion

exchange resins improved sensitivity and specificity. Methods to determine their metabolites, metanephrines and hydroxymethoxy mandelic acid using oxidation to vanillin were developed by **JJ Pisano** in 1960 and 1962 respectively. HPLC techniques developed in the 1980's are now considered the method of choice.

Homovanillic acid is the major metabolite of dopa and dopamine, and urine measurement is important in the diagnosis of neuroblastoma. The early TLC screening methods have now been replaced by HPLC.

5 Hydroxyindoleacetic acid

This is the main metabolite of serotonin and increased excretion occurs in carcinoid tumours. In 1955 **S Udenfriend** and associates introduced qualitative and quantitative colorimetric tests using the purple colour generated with nitroso-naphthol and nitrous acid. Fluorimetry, GC, and immunoassay methods are available but HPLC techniques developed in the 1980's are now preferred.

Urine drugs

An exponential increase of drugs in medicine and in society has taken place during the 20th century. Increasing concern about pollution and occupational exposure has also led to the need to monitor trace metals and gases. Toxicology gradually evolved as a speciality within the larger Clinical Biochemistry laboratories and urine tests continue to play a significant role in the services provided. Generally drug screening and quantitation has attempted to keep pace with pharmacological developments, clinical and forensic toxicology requirements.

By the 1930's simple colorimetric screening tests only were used to detect drugs such as morphine, bromide and salicylates all discovered in the 19th century. However, a quantitative assay for urine salicylates by steam distillation and colorimetry was devised in 1915 by **TW Thoburn**. Tests for barbiturates involved cumbersome extractions and colorimetry with Millons reagent or the blue colour with alkaline cobalt nitrate. Sulphonamides, discovered during the late 1930's were quantitated in urine and blood by **AC Bratton & EK Marshall** in 1939 using diazo methods.

The introduction of more 'toxic' drugs, such as phenytoin, led the American pharmacologist **Eli Marshall** (1899-1966) and others to develop the concept of therapeutic drug monitoring in 1937.

Improved methods were introduced for urine and serum salicylates by **BB Brodie** (1944) and notably by **Paul Trinder** (1954) using the colour reaction of the phenolic group with ferric salts.

In 1951 EJ Conway adapted his microdiffusion unit method with titrimetry for urine and blood ethanol. **Peter Broughton** published his popular method for the quantitation of barbiturates in 1956 using chloroform extraction and UV absorption spectrophotometry. During the 1960's extraction schemes were devised, notably by **EGC Clarke**, to obtain acidic, basic and neutral fractions from a single urine sample. Chemical tests, such as FPN reagent (**IS & FM Forrest**, 1960) for phenothiazines, could be applied to the relevant fraction. The extract could be used for silica thin layer chromatography developed extensively by **AS Curry** using the same chemical tests as location reagents. This provided the basis for establishing the identity of an unknown or suspected drug. During the last 20 years drug dipsticks and complete TLC/location systems (Toxilab, Varian) can be used for selective or multidrug screening. Confirmation techniques include GC, HPLC with GC-MS regarded as the definitive technique. The increased demand for screening for drugs of abuse has been met by the application of enzyme immunoassays particularly by EMIT and more recently using CEDIA (Cloned Enzyme Donor Immunoassay) both of which can be automated.

Urine metals

Early tests for the detection of metals include a test devised by the German physician **Adolf Reinsch** (1862-1916). Urine was heated with acid in the presence of copper foil to form coloured

deposits eg arsenic (black), mercury (purple). More specific tests were developed for lead (J E Kench, 1940) copper (**MR Mattice**, 1936) and mercury (**DM Hubbard**, 1940). These methods were laborious with wet oxidation to destroy organic matter followed by colorimetry, particularly with dithizone.

The major landmark in metal analysis was the introduction of atomic absorption spectroscopy (AAS) and notably the invention of the hollow cathode lamp as the radiation source by **Alan Walsh** in 1955. Improvements in equipment and sampling technique are more recent with graphite furnace AAS and matrix modifiers currently used to quantitate urine aluminium, copper, lead and mercury.

Urine amino acids

Amino acids were shown to be important in digestive processes and 'inborn errors of metabolism' by **Otto Folin** and **Archibald Garrod** (1857-1936) respectively in the early 20th century. The urine output of nitrogen due to amino acids was estimated by titrimetric methods by **E Kirk** and **Van Slyke** (1936) and **AA Albanese & V Irby** in 1944. The use of electrophoresis and most notably paper chromatography was developed between 1942-44 by **John P A Martin** (1910-2002) & **Richard L M Syngé** (b1914) allowed the separation and identification of urine amino acids. This was used extensively and many solvent systems were devised to improve resolution. In 1948 **CE Dent** using this technique showed that around 60 different amino acids could be excreted in urine. Ion exchange chromatography for the quantitative analysis of urine and plasma amino acids was introduced by **Stanford Moore** (1913-1982) & **William Howard Stein** (1911-1980) of the Rockefeller Institute in 1950. This method with modern micro-columns and computerised integration of results is still recognised as the reference technique today and combined with the more recent use of thin layer chromatography, in one or two dimensional mode, has led to the discovery of many 'inborn errors' of amino acid metabolism. Other alternative forms of chromatography, such as GC and HPLC, have been introduced in the last 20 years.

Urine screening tests developed much earlier such as the cyanide/ nitroprusside test for cystine (**E Brand**, 1935) continue to be used.

Urine organic acids

Urine is the preferred sample for the investigation of disorders of organic acid metabolism. Diagnostic accuracy is important in these disorders due to their complexity and severity. A range of quantitative chromatography methods have been developed in the last twenty years for this purpose. This includes capillary GC and HPLC, and most significantly GC-MS. GC-MS techniques have been described by **E Jellum** (1972), **K Tanaka** (1980) and in the UK many applications were developed by **RA Chalmers & AM Lawson**.

Mass Spectrometry (MS)

Mass spectrometry is a high power analytical technique which has become a definitive method for the positive identification of a wide range of biomolecules, notably proteins, drugs and complex metabolic products. Ions generated are characteristic of the test compound and are identified by their mass/charge ratios. The instrumentation required was first developed by **JJ Thomson** (1856-1940) and his research assistant **Francis Aston** (1877-1945) at Cambridge University in the first two decades of the 20th century. During the mid 1950's commercial instruments became available and **R Gohlke** and **F McLafferty** introduced improved performance by combining gas chromatography with MS. As previously described this has become critically important in the identification of urine drugs and metabolites in toxicology and inherited diseases of metabolism respectively.

Concluding reflections

'History, in illuminating the past, illuminates the present and in so doing can give light to the future'

This monograph has attempted to reconstruct the past history of urine tests and shed some light on the evolution of the tests used in the pathology laboratory today. It demonstrates that a vast amount of research and development of methods has been performed worldwide by those cited, and many others, on a fluid which has proved to be more complex than first anticipated. Progress made particularly in technology has rendered some of the early, simple manual chemical tests obsolete with a trend towards more quantitative and definitive methods. Paradoxically 'dipsticks' have retained their popularity mainly due to their simplicity and economic benefits in selecting out 'positive' tests for confirmatory but more expensive laboratory tests. Current trends in urine cytology and microbiology are also moving to screening and automation to cope with increasing workload demands. The way forward in these disciplines may well depend on advances in molecular genetic techniques.

Future historical updates may well be entitled 'From matula to molecular genetics' ???

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