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Cytogenetics, A Diagnostic Service

H. E. Hutchings, FNZIMLT

*Pathology Department, Palmerston North Hospital

From a paper presented at the 27th Annual Conference of the NZIMLT, Wellington, August, 1971

Introduction

The term Cytogenetics Unit or Department is a readily recognised term today and there are a number of such departments throughout New Zealand. Many are primarily research departments and when the phenomenal rate of development in this field over its apparent short history is considered this is hardly surprising.

Human chromosomes have been a source of study however for many years. At the end of last century and at the beginning of this, the number of chromosomes present in the human cell was thought to be 48. The fact was so firmly established that even Painter (1921)¹ who found 46, in the face of previous work and some surprisingly clear bivalent XY pictures from a meiotic metaphase declared the number to be 48.

A significant advance was made with the work of Barr and Bertram in (1949)². Their astute observation that in a high percentage of cells from females only, there could be found a dense body lying along the nuclear membrane, introduced for the clinician a simple diagnostic test in cytogenetics. The fact that there was one more sex chromatin or Barr body than there were X chromosomes in the cell introduced the probable answer to a number of previously unexplained sex anomalies. It also introduced a decisiveness to the sexing of the indeterminate newborn or even should it be required, the unborn (1956)³.

The use of colchicine to arrest metaphase and the effect of hypotonic solution to disperse the chromosomes, techniques already used in plant biology, were utilised by Tijo and Levan (1956)⁴ to establish that there were indeed 46 chromosomes in the human cell. They used a tissue culture from lung explants of aborted fetuses and their finding was soon confirmed by Ford and Hamerton (1956)⁵ on testicular material.

The classic paper of Le Jeune *et al.* (1959)⁶ and Jacobs *et al.* (1959)⁷, pointing out that the mongoloid had 47 and not 46 chromo-

somes began a virtual revolution in Cytogenetics.

One of the more important factors in this revolution was the accidental discovery by Nowell (1960)⁸ that an extract from a bean, phytohaemagglutinin, acted as an antigenic stimulus to lymphocytes causing a wave of mitotic activity in these cells during a 48 to 72 hour period when cultured under artificial conditions.

The use of radioisotopes during this period had been applied to a number of aspects in plant and human biology. By introducing minute quantities of tritiated thymidine into the culture media approximately two to five hours before harvest of the cells, whether they be lymphocytes or fibroblasts, allows the uptake of the isotope labelled thymidine by the chromosomes during the S period of mitoses. Chromosomes do not all replicate together and some, particularly the sex chromosomes replicate late in the mitotic phase. The technique has therefore been particularly valuable in establishing anomalies of the X chromosome.

It was declared previously that the use of phytohaemagglutinin was one of the more important factors in the revolution of Cytogenetics but Laura Zech (1969)⁹ using a well-known fluorescent dye of the acroflavin family made the astute observation that a bright fluorescent area at the distal end of the Y chromosome was easily recognisable. Caspersson *et al.* (1970)¹⁰ and Pearson *et al.* (1970)¹¹ supported this observation with many others. This gave rise to a most exciting technical breakthrough in Cytogenetics which was quickly followed by yet another technique simply called Giemsa banding (1971)¹² or now, because of the variations in techniques, DNA banding.

Methods

The many procedures collected to date while arising as research tools are undoubtedly of diagnostic value and therefore form the basis of a diagnostic service within the precincts of a Pathology Department. The following is a brief review of the techniques commonly used in such a department.

* Current address: Health Science Department Central Institute of Technology, Petone.

Standard metaphase preparations are made from peripheral blood lymphocytes cultured in Glaxo T.C. 199 medium using phytohaemagglutinin as a mitotic stimulant. The culture period is 72 hours. Mitoses are arrested by the use of Acqua-Colchin (Park, Davis and Co. Ltd.) diluted to a concentration of .0002 micrograms per ml of media.

Cultures when required are treated with tritiated thymidine (H_3 TDR. 1 microcurie per ml of media) five hours before harvest and two hours before the addition of colchicine.

Where tissue culture was necessary a fast-growing confluent growth of fibroblasts was obtained from a small skin biopsy cultured after the method of Hyman (1968)¹³ and was lifted with 0.025 percent trypsin and harvested as for lymphocytes. Buccal smear cells were collected onto microscope slides previously treated with a very light coating of egg white, were wet fixed in ether alcohol 50/50 for two hours and air dried. Hair follicle sheath cells were examined for sex chromatin using the method of Schmidt as described by Culbertson *et al.* (1969)¹⁴. While thionin gave the most clear-cut preparations, Cresyl violet has now been substituted as it has been found that one can overstain fluorescent preparations with it and demonstrate the sex chromatin bodies of both X and Y in the same preparation.

Fluorescent staining was performed following the method described by Pearson *et al.* (1970)¹¹ except that the pharmaceutical product 'Atabrin' hydrochloride (Winthrop) was used at a concentration of 0.6 g percent with a staining time of eight minutes.

The above procedure was used for the fluorescent preparation of metaphase plates from lymphocyte and fibroblast cultures. These were examined microscopically using a Leitz Ortholux microscope, transmitted light from a HBO 200W Mercury vapour lamp and a 1.5 mm or 2 mm BG 12 exciting filter. A well-oiled dark field condenser, a 54x fluoride objective and a K530 or K510 barrier filter were used. Selected spreads were recorded and photographed using a Canon camera and Kodak high contrast copy film.

Slides from Tritium labelled cultures were coated with Kodak AR 10, exposed for seven days in the dark at 5°C and developed with Ilford ID 11 developer.

The most successful DNA banding was achieved following the method of Seabright (1971)¹⁵, using trypsin plus immersion in salt solution for two hours at 56°C.

Case Studies

The following cases are used to demonstrate the usefulness of the procedures mentioned.

Case 1

A teenage girl presented with amenorrhoea. She was 4 feet 7 inches in height, had a shield chest, poorly developed sex characteristics, deficient pubic and axillary hair, a small uterus and streak ovaries.

The buccal smear preparation showed a sex chromatin body in 35-40 percent of the cells. It was noticed however that the sex chromatin body was larger than normal.

On karyotype the lymphocyte culture showed the 2N number of chromosomes to be 46 but on size and centromere position there was an apparent extra number three chromosome with a deficit in number, of one chromosome, in group 'C'. Careful measurement demonstrated that one number 3 seemed exactly metacentric.

Autoradiography demonstrated that this chromosome was late replicating and was taken as extra evidence that it was an isochromosome X. (Fig. 1.)

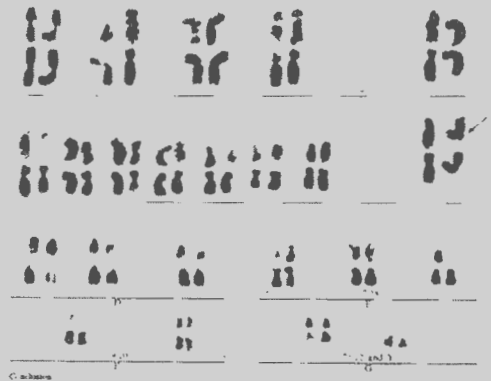


FIG. 1.—A karyogram with an isochromosome X labelled with tritiated thymidine from case 1.

Fluorescent staining confirmed the finding in that this chromosome had a fluorescent banding pattern resembling that of the q arms of an X chromosome on either side of the centromere.

Case 2

A male aged 31 was 16 stone in weight and 6 feet in height. At examination he had an acromegaloid facial appearance, short neck, broad pelvis, scanty body hair and female distribution of pubic hair. He had a small penis, with small and atrophic testicles. His prostate and seminal vessels were barely palpable.

A buccal smear demonstrated a sex chromatin body in 28 percent of the cells. Fluorochrome staining of the buccal cells demonstrated a small fluorescent spot due to the Y chromosome.

A karyogram from routine lymphocyte culture demonstrated a chromosome complement of $2N+1$. The extra chromosome (Fig. 2) morphologically fitted into group 'C' and was presumed to be an extra X. This was confirmed by autoradiography which demonstrated two late replicating chromosomes.



FIG. 2.—A karyogram demonstrating a sex chromosome complement of XXY—case 2.

Fluorochrome staining of cells in metaphase from the lymphocyte culture showed the Y chromosome with the typical fluorescence at the distal end of the q arms and after careful pairing also demonstrated that in the C group there were two chromosomes with the X pattern of fluorescent banding.

Case 3

A male infant born at full term with a birth weight of 6lb 6oz made normal progress until it was noticed that he was jaundiced. The father was of mid European descent and the mother, a radiotherapy technician, was New Zealand born. There was no family

history of possible blood dyscrasia. The only laboratory investigation of note was an increasing bilirubin. Histology on a limited liver biopsy indicated a macronodular cirrhosis of the liver.

A report from another centre, after referral, indicated an abnormality of the Y chromosome in the infant. Chromosome analysis was therefore carried out on both parents and repeated on the infant. No abnormality was demonstrated in the karyogram from the mother but lymphocyte and tissue cultures from the father and the son gave karyograms in which a small metacentric chromosome was found in place of the normal Y chromosome.

Fluorochrome staining with quinacrine hydrochloride indicated that the most ready explanation was that of a pericentric inversion. (Fig. 3.)

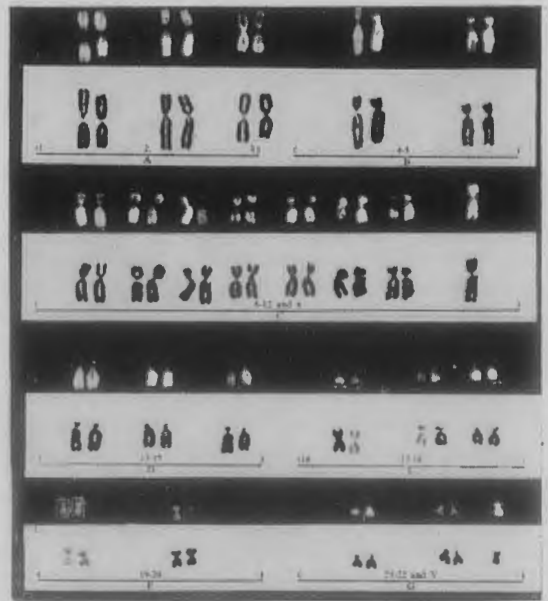


FIG. 3.—A karyogram demonstrating fluorochrome staining and aceto-orcein staining of the chromosomes of case 3. The metacentric Y is shown in group 'G'.

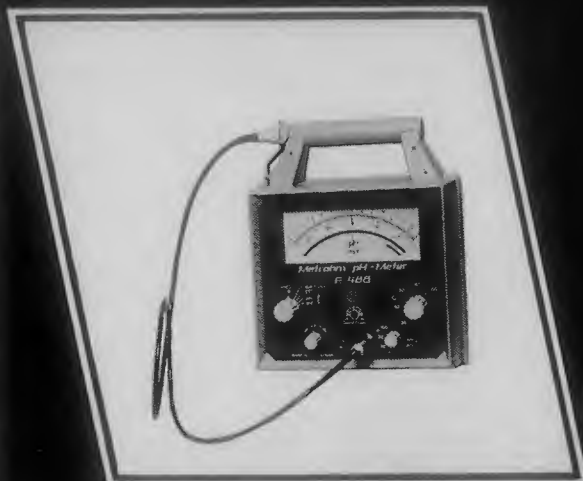
In view of the absence of any history of liver dysfunction in the father it seemed improbable that the clinical finding in the son was related to the chromosome anomaly.

Case 4⁽¹⁶⁾

In 1965 Woolf *et al.* (1965)¹⁷ described a condition which phenotypically closely re-

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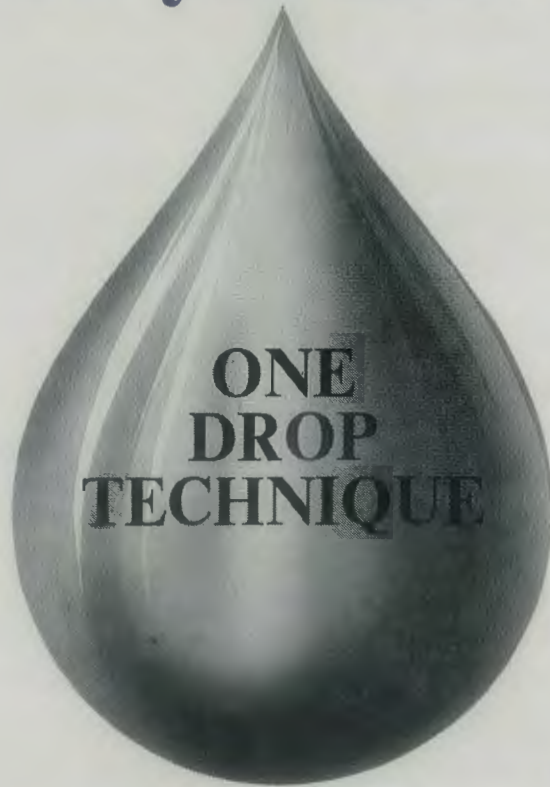
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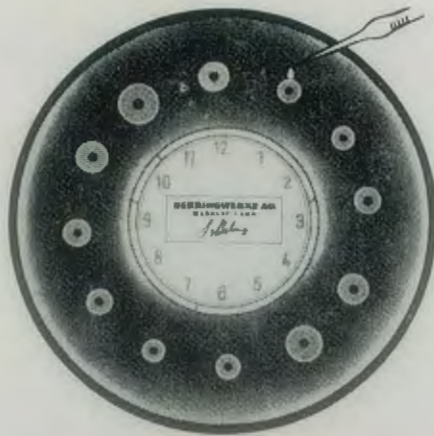
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sembled 'Cri du Chat.' Apart from various additional abnormalities, the most noticeable feature was the absence of the typical cry. Woolf demonstrated, using autoradiography that this deletion was of the short arms of one of the number 4 chromosomes rather than that of number 5.

This patient showed the typical facial features including the hooked nose with a very broad bridge and large, simple, low-set ears. Chromosome analysis carried out on peripheral blood lymphocytes gave a chromosome number of 46 and it was noticed microscopically and confirmed by karyotype that one of the 'B' group chromosomes could not be paired.

Fluorochrome staining confirmed that the number 5 pair was intact and that the deletion was indeed from the p arms of a number 4 chromosome.

DNA banding even more clearly confirmed that the deletion had occurred in the p arms of a number 4 chromosome. (Fig. 4.)

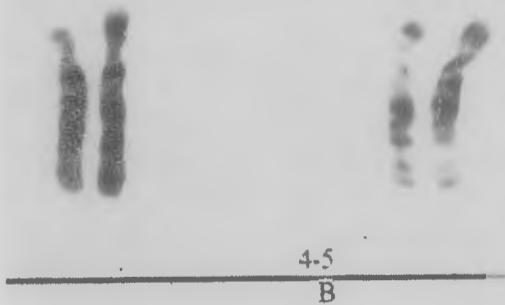


FIG. 4.—The B group chromosomes of case 4. The DNA banding in this karyogram demonstrates 5 bands in the q arms of number 4 chromosomes and 7 bands in the q arms of number 5. One number 4 chromosome has a deletion in the p arms.

Case 5

The following case is included because by attempting to confirm a diagnosis an error was in fact discovered.

The child, a male mental retardate, slightly above normal percentile in growth, was confidently diagnosed XYY on karyotype from the culture of peripheral blood lymphocytes. There were two acrocentric chromosomes slightly larger than the remaining 'G' group chromosomes and slightly smaller than those of group 'E'. (Fig. 5.)

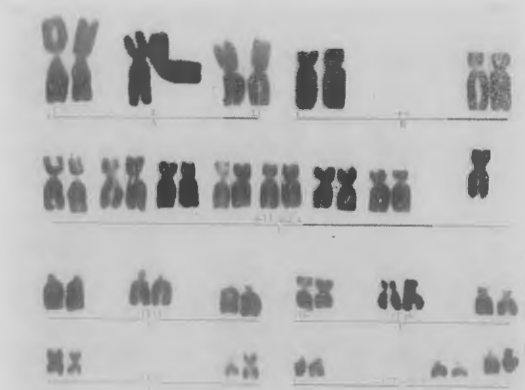


FIG. 5.—The karyogram from case 5. The chromosomes are stained with Giemsa stain. Two acrocentric chromosomes are seen in group G which are similar, larger than 21 and 22 but smaller than group E. Only one is a Y chromosome.

Fluorochrome staining of the hair follicle cells showed however that instead of the expected two fluorescent spots due to the Y chromosomes, only one could be found. (Fig. 6.)

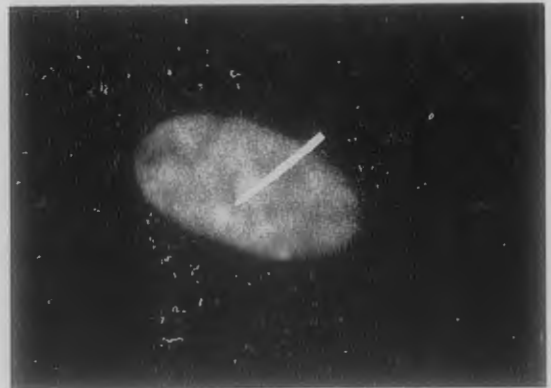


FIG. 6.—Fluorochrome staining of a cell in interphase from the hair follicle sheath of case 5. The fluorescence of the Y chromosome is indicated by an arrow.

Fluorochrome staining was carried out on the metaphase plates from the lymphocyte cultures and after karyotype it was demonstrated that the typical fluorescent pattern of the Y chromosome was present in one of the small acrocentric chromosomes but not in the other. The fluorescent pattern of the latter, if anything more nearly paralleled that of chromosome number 18 than any other, but the consistently smaller size is still unexplained.

Summary

The techniques routinely used in a diagnostic cytogenetic unit are outlined and a series of five cases are described to demonstrate the usefulness of the varying techniques available today.

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A Simple Routine Method for the Culture and Identification of Human Strains of *Mycoplasmataceae*

Barbara J. Blanchard, ANZIST

Drs Alexander and McCafferty's Laboratory, P.O. Box 30044, Lower Hutt

Adapted from a thesis submitted for the Diploma in Applied Science of the Wellington Polytechnic and received for publication, September, 1972

Introduction

The aim of this study was to devise a method to enable the recovery of human species of mycoplasmas as part of the daily routine of the bacteriology department of a diagnostic clinical microbiology laboratory. Since any new addition to a laboratory's routine must disrupt the normal procedures as little as possible, the mycoplasma method of choice should be one for which materials are readily available with the minimum of manipulations with regard to the preparation of the media, culture and identification.

Any steps which would reduce the period between initial culture and report were desirable, as some mycoplasma species may require as many as six weeks to form identifiable colonies on agar. Although identification of a mycoplasma may take so long, treatment of a patient with an illness suspected to be due to a mycoplasma infection would not be withheld. Since the patient is treated on an assumption that a mycoplasma is present it would appear to reduce the positive identification of such an organism to a point of mere academic interest but it provides information useful for the patient's medical history as well as indications of the distribution of mycoplasmas in the population and their involvement with disease.

Historical Background

Mycoplasmas were first recognised by Nocard and Roux in 1898 and the generic name *Mycoplasma* was introduced by Novak in 1929 with the Family *Mycoplasmataceae* and Order *Mycoplasmales* being adopted by Edward and Freund¹⁰ in 1956. Over the years, a number of names were given to this organism, e.g. *Asteromyces*, *Micromyces*, *Coccobacillus*, *Asterococcus*, *Borrellomyces*, *Bovimyces* and the term 'pleuropneumonia-like organisms' (PPLO) was later applied to the group (Marmion²⁵).

The type species is *Mycoplasma mycoides* which, as the causative agent of Bovine Pleuropneumonia, was the first mycoplasma to be connected with a disease process. A variant found in goats, *M. mycoides* var. *capri*, differs in complement-fixing antigens.

There are also saprophytic strains which have been isolated from soil, manure and sewage as described by Marmion²⁵. *M. laidlawii* is normally regarded as a saprophytic species but it has been isolated from animate sources such as tissue cultures (Marmion²⁵).

The first indication that there might be a mycoplasma human pathogen was in 1961 when several workers (Marmion *et al.*²⁶ Clyde,⁴ Chanock, *et al.*²) discovered that Eaton's agent, recognised for about twenty

Table 1 (after Marmion²⁵)

SPECIES	REFERENCE	AREA	STATUS
<i>M. pneumoniae</i>	Eaton <i>et al.</i> ⁶	Respiratory tract	pathogen
<i>M. hominis</i> Type 1*	Nicol <i>et al.</i> ²¹	Genitourinary tract, Respiratory tract	commensal and pathogen
<i>M. fermentans</i>	Ruiter <i>et al.</i> ^{28, 29, 30} Ford <i>et al.</i>	Genitourinary tract	commensal
<i>M. pharyngis</i> (orale)	Herderschee <i>et al.</i> ¹⁰ Lemcke ^{20, 21, 22} Taylor-Robinson <i>et al.</i> ³⁷	Respiratory tract	commensal
<i>M. salivarium</i>	Dienes <i>et al.</i> ⁸ Nicol <i>et al.</i> ²⁷ Huijsmans-Evers <i>et al.</i> ³⁷	Respiratory tract	commensal
strain 'umbilicus'	Ruiter <i>et al.</i> ³¹	Little is known of the aetiology of this strain	

* Strains grouped in the species *M. hominis* Type 2 have been found to possess similarities with a strain of *M. arthritis* (rat polyarthritis) which

indicated that *M. hominis* Type 2 may not be a human species only, therefore it is permissible to omit the 'Type 1' parenthesis.

years previously as the cause of cold-agglutinin-positive Primary Atypical Pneumonia, was a mycoplasma (Marmion²⁵). Eaton had recovered a filterable agent (now known as *M. pneumoniae*) from patients with Primary Atypical Pneumonia (PAP) which he was able to maintain in tissue culture in chick embryos.

Mycoplasmas in Humans

Possible pathogenicity of *M. hominis* for man has been seen in studies of patients with non-specific urethritis (NSU), puerperal sepsis, salpingitis and ovarian and pelvic abscesses. *M. hominis* has also been found in healthy subjects.

Shepard^{33, 34} and Shepard *et al.*³⁵ isolated 'T' strains (T = tiny — 50 μm to 30 μm diameter) from NSU. These organisms, though atypical in colonial form, bear many serological and structural similarities to classic large colony mycoplasmas. Some workers hesitate to include them with mycoplasmas but they are more strongly implicated as a cause of NSU than other species found in the genito-urinary tract.

Tissue cultures even before inoculation have been found to be contaminated with mycoplasmas. Marmion²⁵ indicated that it is possible that the mere introduction of pathogenic material onto a tissue culture may

provide some particular combination of conditions which enables previously latent mycoplasmas to begin to multiply on an apparently sterile culture medium.

The most common contaminating mycoplasmas are *M. hominis* and *M. pharyngis* but others have also been identified (*M. gallisepticum*, *M. laidlawii* and *M. hyorhinae*).

The discovery that mycoplasmas can cause diseases in animals accompanied by arthritis, conjunctivitis, synovitis, polyserositis and keratitis fostered interest in the possibility that mycoplasmas might cause similar diseases in humans.

The variable and often unreproducible circumstances in which the mycoplasmas have been grown, and the fact that suggested pathogens can be isolated from an ostensibly healthy subject has led to uncertainty in defining a specific pathogenic role for the organism.

Biological Background

By definition, mycoplasmas are the smallest organisms to grow on cell-free medium, solid or fluid. The minimal reproductive units or elementary bodies are 125 nm to 250 nm in diameter, and colonies on agar may range from 10 μm to 100 μm in diameter (Sabin³², Edward⁹). Each unit is bounded by a 8 nm

to 15 nm lipoprotein membrane (Marmion²⁵) as there is no cell wall, and the nucleus has no membrane. The nucleus is an electron-dense area of fibrillar or granular material, about 3 nm thick (Maniloff, *et al.*^{23,24}). The total nucleic acid content varies with the species and the stage in the growth cycle.

It is difficult to produce any constant description of the ultra-structure of mycoplasmas using electron-microscopy because the units are so plastic that preparation methods for examination may change their final appearance, (Clark, *et al.*³). Mycoplasmas are poorly stained by Gram's method, but are gram-negative. The general characteristics of mycoplasmas include globular growth in fluid media and 'fried-egg' colonies on solid media (seen microscopically) (Sabin³², Edward⁸). Figure 1. shows colonial forms typical of those usually encountered. They require, for growth, basic broth or agar supplemented with serum or ascitic fluid, and, for some species, yeast extract. Some will grow on serum-free medium. These are, understandably, saprophytes, e.g. *M. laidlawii*.

Mycoplasmas are resistant to polymyxin B and sulphonamides, and since they have no cell wall, are not susceptible to penicillin and bacitracin. They are sensitive, in varying degrees, to tetracycline, tylosin, kanamycin, erythromycin, chloramphenicol, streptomycin, ethidium bromide, organic gold salts, arsenical salts and also nystatin and amphotericin B. (Lampen *et al.*¹⁹, Marmion²⁵, Harwick *et al.*¹³), although Blazevic¹ suggests amphotericin B as a fungicide to be included in mycoplasma media.

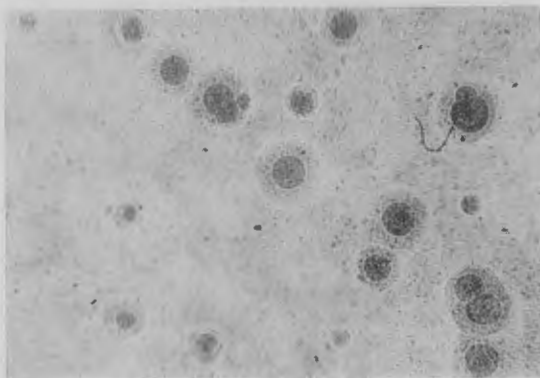


FIG. 1.—Shows typical mycoplasma colonies. There are some 'double-yolked' colonies.

The organisms are moderately insusceptible to osmotic shock because they are plastic and have a low internal osmotic pressure. Units may be lysed by freezing and then thawing in water. They are heat-labile at 45°C to 60°C and are inactivated by lipid solvents and surface active agents (Marmion²⁵). Atmospheric growth conditions vary with species but the family includes aerobes, anaerobes and micro-aerophils. Mycoplasmas are classified partly on their biological properties and partly on antigenic analysis. Biologically, a species may reveal its identity through a distinctive host range in laboratory animals, also its colonial form, gaseous requirements for growth, temperature and pH, carbohydrate fermentation, aerobic reduction of tetrazolium, haemolysis of various species of erythrocytes, haemadsorption of erythrocytes onto colonies, the need for yeast extract and growth in the presence of methylene blue. The fermentative species tend to attack the same range of carbohydrates; glucose, fructose, maltose, starch and glycogen, with the production of acid.

Antigenic analysis may be by such serological methods as haemagglutination, complement-fixation, fluorescent antibody methods, gel diffusion, and variants of growth inhibition techniques.

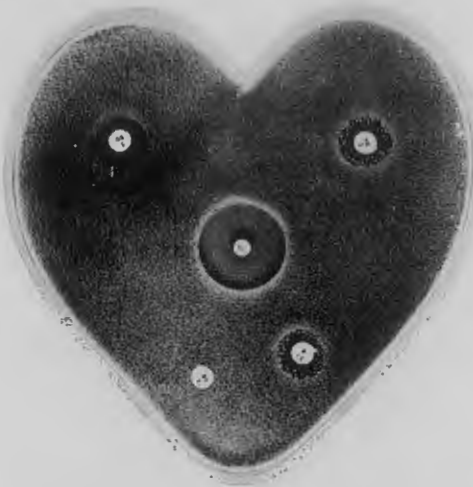
Materials and Methods

Specimens

In April 1970, interested local doctors were encouraged to duplicate their swabs from oral, nasopharyngeal and genital sites, placing one in Stuart's transport medium for routine bacteriology and the other in mycoplasma diphasic medium for mycoplasma culture. Seventy-three specimens were received over a 2 month period. From August 1969 to January 1971 a total number of 672 specimens was examined, from all sources, 66 of which gave cultures of mycoplasma species. Principally, the 672 specimens were derived from patients referred to the laboratory by local medical practitioners.

Those specimens in transport medium (usually Stuart's Transport Medium) were used in preference to dry swabs, since mycoplasmas require moisture for survival away from the host. Some swabs were placed in mycoplasma diphasic medium as a transport medium before they reached the laboratory.

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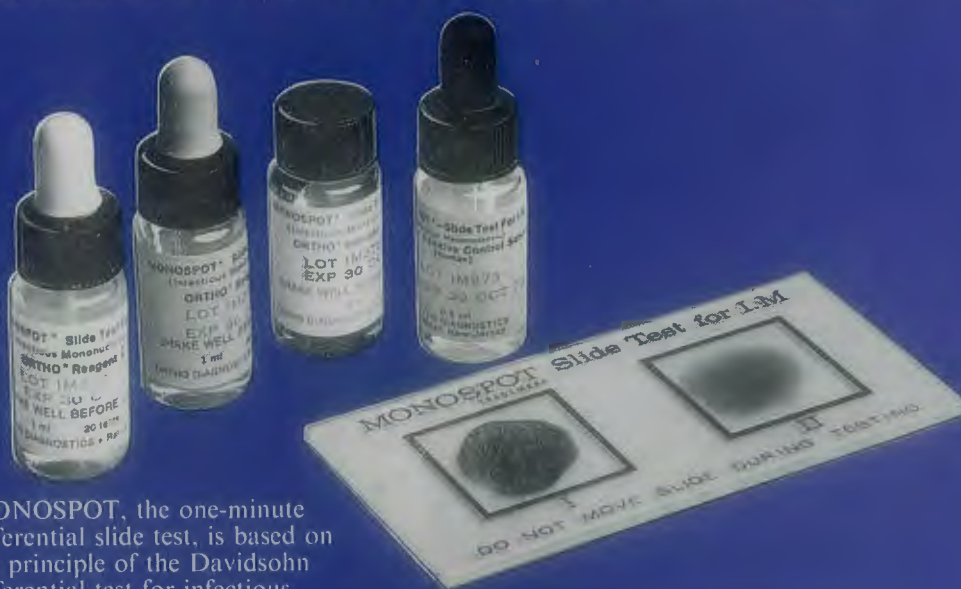
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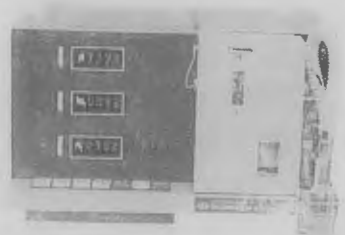
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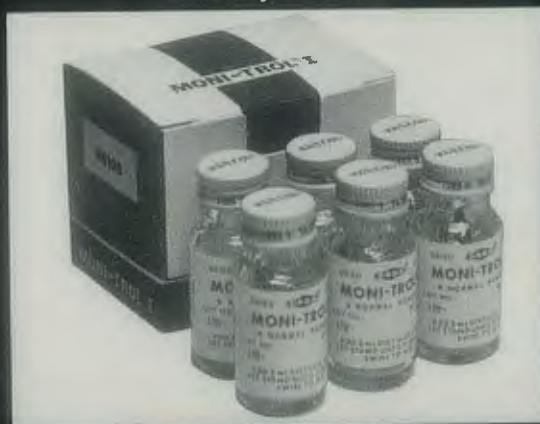
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Media

The medium chosen was that described by Marmion²⁵ which is a modification of Hayflick's¹⁴ medium.

To 70 ml of Mycoplasma agar or broth base is added 10 ml bakers' yeast extract, 20 ml horse serum or ascitic fluid, 1 ml sodium deoxyribonucleate, 2 ml 1.0M di-potassium hydrogen ortho-phosphate, and 0.2 ml of a 50,000 units per ml solution of penicillin. The final pH is 7.8 to 8.0. Ideally, the serum used should be sterile, uninactivated agamma horse serum which may be obtained from Difco. Agamma serum was preferred by Hayflick¹⁴ as it appeared to eliminate pseudocolonies from cultures. (Pseudocolonies are artifacts resembling mycoplasma colonies and are caused for example by soap crystals forming on the agar surface.)

Human ascitic fluid can be used, also human serum, both sterile and uninactivated. Human serum may contain antibodies active against mycoplasmas, but in this study it was used continually with no obvious adverse effects on the stock culture mycoplasmas.

Yeast extracts may be commercial products e.g. 'Oxoid' yeast extract, BBL yeast extract dialysate; or prepared from bakers' yeast. All those mentioned were used with success but the most suitable were the BBL yeast extract dialysate and the bakers' yeast extract.

A 0.2 percent solution of the sodium salt of deoxyribonucleate prepared from calf thymus was added to the complete medium; also 1.0M di-potassium hydrogen ortho-phosphate.

Bacterial inhibitors: Thallous acetate was used as an inhibitor of many gram-negative organisms and water soluble penicillin (50,000 units/ml) to inhibit any gram-positive contaminants.

Ampicillin (1 mg/ml) can be substituted for both of these as the thallous acetate affects 'T' strains adversely. It is effective against nearly all gram-positive organisms and many gram-negative bacteria.

Optional additives: A 0.4 percent solution of phenol red indicator can be incorporated into the broth phase of the diphasic medium.

When the red colour changes to yellow, the presence of a viable culture is indicated and subcultures may then be carried out. The red to yellow colour change does not indicate

specifically that the organism which has grown is a mycoplasma. It may be a contaminant which is able to grow in the presence of penicillin e.g. a penicillinase-producing *Staphylococcus* or a species of *Proteus*. A colour change from red to purple indicates alkalinity and may be due to the growth of a contaminant such as a *Candida* species. In both cases of contamination mycoplasmas may be included in the micro-organism population, but this can be confirmed only after the broth has been subcultured onto agar and the organism is allowed to grow into identifiable colonies. Approximately 2 percent glucose may be added to the broth phase of the diphasic medium also to encourage stronger growth (Marmion²⁵). Omission of this from the broth had no noticeable disadvantages.

A diphasic medium was used for primary cultures, which consisted of 2 ml of complete mycoplasma agar in a sterile Bijou bottle, overlaid with 2 to 3 ml of complete mycoplasma broth.

Agar plates of complete mycoplasma agar were used for subculturing from diphasic and agar plate cultures.

The basic medium, when sterilised and kept at 4°C to 10°C, keeps indefinitely but the complete medium (with enrichments and antibacterial substances added) will retain optimal growth-promoting properties for about three weeks only, although plates 4 or 5 weeks old were used successfully in the subculture of laboratory-acclimatised strains of mycoplasma. Conditions of culture used were a BBL Gas Pak anaerobe jar and aerobic culture (using normal atmosphere). In the latter case, plates were incubated in a sealed polythene bag to retain moisture. The air was changed every 2 or 3 days.

Cultures

Fresh specimens were placed in mycoplasma medium and incubated for one week at 37°C before being subcultured onto agar plates using a swab or a sterile Pasteur pipette. The mass inoculum was streaked over the agar surface with a sterile wire loop. Usually, cultures were undisturbed for 7 to 10 days before examination. The presence of mycoplasma colonies was determined by examining the underside of each plate without removing the lid, at a magnification of X 100 on the micro-

Subcultures from agar to agar were made by block transfer. A square of agar showing colonies was cut from the parent plate using a sterile scalpel or wire loop and transferred face down onto a fresh agar plate. The block was pushed over the agar to wipe off the colonies from the block onto the fresh medium. Blind passages were not made at any time.

Culture Contamination

At all stages in this study, a constant problem was contamination of culture plates by penicillinase-producing *Staphylococci*, yeasts, some gram-negative bacilli and airborne moulds.

In cases of bacterial contamination a compound antibiotic ring was laid upon the evenly inoculated agar surface and the plate incubated for 7 to 10 days. Mycoplasmas growing in the clear area of sensitivity around an antibiotic disc to which the contaminant was susceptible were then transferred to fresh medium to give a pure growth of mycoplasmas. Millipore filtration of the broth phase of a diphasic culture made from the affected plate yielded a pure growth of mycoplasmas, with a low failure rate.

The occasional failure with this method does not reduce its value. Progressively thin streaking of a contaminated mycoplasma inoculum over an agar plate may dilute both mycoplasma and contaminants sufficiently to allow a single colony to be cut out on an agar block and transferred to fresh medium to obtain a pure culture.

Staining

The staining procedure chosen was the modification of Dienes' method as described by Klieneberger-Nobel¹⁸. This was the simplest and avoided any distortion of the colonies because they were stained *in situ*. If a permanent record is required, the Intensified Giemsa method from Marmion²⁵ or Klieneberger-Nobel's Agar Fixation method are more suitable.

Staining colonies of elementary bodies has no real significance and is merely a location procedure.

Biochemical Tests

Since the mycoplasma species grown were able to be identified by the Growth Inhibition Technique (GIT) as Clyde⁵ described, bio-

chemical tests as an aid to identification were not used.

Growth Inhibition Test (GIT): This employs a growth inhibiting antibody specific for mycoplasma species which, when applied to a culture of a homologous species, will inhibit the growth of the organism; Stanbridge and Hayflick³⁶. The antiserum is prepared, using rabbits, and tested for specificity.

A known and constant volume of suitable antiserum is soaked into small absorbent paper discs, dried, and stored at -20°C at which temperature the antiserum is stable for 6 months. When a disc is placed upon the agar surface of a freshly-inoculated plate the moisture present (from condensation or from the broth from which the subculture may have been made) elutes the antiserum which diffuses through the agar. The plate is examined after 7 to 10 days for any ring of inhibition of growth of the mycoplasma. If no colonies are visible, the plate is re-incubated and examined again at weekly intervals.

The narrow range of sensitivity of the GIT is the principal disadvantage i.e. the antiserum cannot be diluted to any large extent and still induce inhibition of the culture. Obviously, the high degree of specificity and simplicity of performance are the cardinal advantages the GIT has over the other methods of identification of mycoplasmas.

Antiserum discs can also be used selectively in a culture of mixed serotypes of mycoplasmas. For example, a culture of *M. hominis* and *M. fermentans* with an anti-*M. hominis* disc will inhibit the former, allowing *M. fermentans* to grow within the margin of inhibition. This uninhibited mycoplasma can then be subcultured for a pure growth. The antiserum-impregnated discs used in this study were commercially prepared BBL "Taxo" discs, which were numbered to correspond with 6 species of mycoplasmas.

Fluorescent Antibody Technique (F.A.)

The direct F.A. method is the simplest of the F.A. methods available and in principle involves mixing a fluorescent dye-labelled antibody with its specific antigen to give a fluorescent product (i.e. antigen-antibody complex).

The method chosen was that of del Giudice *et al*¹² as being potentially the most rapid.

Since no mycoplasma antiserum was available already conjugated with fluorescent dye, an attempt to conjugate commercially prepared antiserum with fluorescein isothiocyanate was made. This was unsuccessful, but the F.A. technique would be the most suitable for the prompt and specific identification of mycoplasmas if a fluorescent conjugate could be developed.

Results

Sixty-six of the 672 specimens tested provided cultures of mycoplasma species. Table II shows the species identified.

Table II

Species	Number
<i>M. salivarium</i>	12
<i>M. pharyngis</i>	12
<i>M. hominis</i>	8
<i>M. fermentans</i>	4
unidentified	1
died on subculture	29

The unidentified mycoplasma which was isolated was from an aural swab taken from a

3-year-old child with otitis media. It is a common problem with mycoplasmas to be unable to subculture some species from the primary culture. This tendency may be due partly or wholly to the retention by the mycoplasma in primary culture some growth-promoting material from the host which cannot be transferred to fresh media or supplied by it. The benefit is, therefore, lost when the mycoplasma is subcultured.

No. *M. pneumoniae* was isolated.

Clinical Interpretation

Of 66 mycoplasmas isolated 19 were from patients exhibiting signs and symptoms which could be considered consistent with a primary mycoplasma infection. In all of these routine bacteriological investigation had revealed nothing significant.

In eight of these patients antibiotic treatment might have suppressed bacterial activity thus the assumption that a mycoplasma was the pathogen might be incorrect. Table III shows these separately for this reason. The other 47 isolates were regarded as of doubtful pathogenicity for various reasons. Some were not identified due to loss on subculture, in others a pathogenic micro-organism was recovered which was felt to be a more likely pathogen than the mycoplasma.

Table III

Species	Source	Significant pathologically	Patients on antibiotics
<i>M. hominis</i>	2 vagina	2	1 panmycin
	1 urethra (male)		2 no record
<i>M. pharyngis</i>	Throat	1	1 penicillin
<i>M. fermentans</i>	Vagina	1	0 no record
<i>M. salivarium</i>	Throat	7	1 ampicillin
			1 panmycin
			1 penicillin, but no effect on sore throat
unidentified		0	1 penicillin
	TOTAL:	11	8

Discussion

1. Isolation rate: The culture medium chosen fulfilled every requirement as far as could be ascertained, although the mycoplasma isolation rate was low at approximately 10 percent (Table III). This low figure could

have been due to several factors. The chief factor may have been the length of time between taking the swab from the patient, and its incubation in mycoplasma diphasic medium (initial or primary culture). A series of swabs taken from the throats of staff volunteers and

incubated promptly showed 90 percent cultures positive for mycoplasmas, although these were a strain fairly easily grown, *M. pharyngis*.

One group of specimens which may have been left at room temperature for times in excess of three hours because of transport difficulties, failed to grow any mycoplasmas.

The fact that patients could have been taking antibiotics effective when the samples were taken would have reduced the mycoplasma recoveries. Most specimens taken from the bacteriology department were received in Stuart's transport medium.

Of the 585 specimens received, 9.5 percent produced positive cultures; 87 specimens received in diphasic transport medium produced 11.5 percent positive cultures.

Other factors influencing the isolation rate included the contamination of cultures by various organisms.

Despite the statement of Blazevic¹ that Amphotericin B does not affect the growth of mycoplasmas we found that it did inhibit growth as did Marmion²⁵.

The failure to isolate *M. pneumoniae* was due, we think, to lack of suitable clinical material.

Conclusion

I. The following method for the isolation and identification of mycoplasmas has proved applicable to a routine clinical microbiology laboratory.

1. Placing of freshly-taken swabs in diphasic medium and incubation at 37°C in an appropriate atmosphere for one week.
2. Subculturing onto mycoplasma agar with further incubation.
3. Identifying mycoplasmas on the agar plates using the growth inhibition technique and BBL 'Taxo' discs.

The time from initial culture to identification and reporting may range from three to six weeks.

II. 672 specimens yielded 66 positive cultures of mycoplasmas of which 19 were clinically significant and 47 were thought to be commensals. An attempt to develop fluorescent antibody conjugates was unsuccessful but it is clear that given appropriate materials this would give speedier identification and more accurate recognition of mycoplasmas.

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Serological Studies in Pigeon Fanciers

M. J. Gratten, ANZIMLT

Pathology Services, Department of Microbiology,
Christchurch Hospital

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Introduction

In a previous communication⁴ it was reported that two individuals with allergic interstitial alveolitis due to inhaled organic pigeon components possessed strong reactivity in a complement fixation (CF) test using pooled hydatid cyst fluid as antigen. This finding does not appear to have been recorded elsewhere. Both patients also demonstrated multiple precipitating serum antibodies to a variety of pigeon antigens including blood, faeces and feathers. Cross reactivity of such antibodies with components of the fluid antigen were excluded by absorption studies.

It was subsequently decided to determine the incidence of antibodies to various pigeon and hydatid antigens in the blood of symptomless pigeon fanciers actively engaged in their hobby.

Material and Methods

Blood from 42 healthy pigeon fanciers was removed by venepuncture, the serum separated aseptically and stored at -25°C until tested. The age of the donors ranged from 15 to 68 and the length of exposure to pigeons varied from $1\frac{1}{2}$ to 60 years.

The collection and preparation of pigeon dropping extract (PDE), pigeon serum (PS) and hydatid cyst fluid antigen have been described earlier as has the crossed electrophoresis method on cellulose acetate which was used to demonstrate serum precipitating antibodies to pigeon antigens⁴.

Three procedures were used to test sera for antibodies to hydatid components:

(a) Hydatid CF test (HCF). A one-fifth volume Kolmer technique² using pooled sheep liver cyst fluid at a 1 in 30 dilution was employed. All positive results were quantitated.

(b) Latex Hydatid test (LHT). A commercially available sensitised latex reagent (Italdiagnostic Echinococcosis Agglutinotest) was used following the manufacturer's instructions. Sera were tested undiluted and at 1 in 5.

(c) Fluorescent-antibody test (FA scolex) using a commercially acquired lyophilised hydatid scolex reagent (Burroughs Wellcome and Co. (N.Z.) Ltd.). This material was reconstituted as instructed with distilled water, a small amount spread over an encircled area on a 3 x 1 inch glass slide, air dried and fixed for 5 minutes in AR acetone. The preparations were layered with the test sera both at zero and 5 dilutions, incubated in a moist atmosphere at room temperature for 30 minutes, excess seum removed and washed for 10 minutes in phosphate buffered saline (PBS) pH 7.5. Fluorescein-labelled antihuman globulin serum (Burroughs Wellcome (N.Z.) Ltd.) was then applied and the slides incubated as above for 30 minutes. Following a further brief wash with PBS the preparations were mounted with glycine-saline glycerol pH 8.2, coverslipped and immediately examined using a Leitz SM binocular microscope fitted with a Philips CS 150W ultraviolet lamp with 1.5 mm UG I primary filter, a darkfield substage condenser, x 10 objective n.a. 0.25 and x 16 eyepieces. Reactive sera produced a marked peripheral fluorescence of the scolices which in

strong results tended to involve the entire organism. Negative sera occasionally produced a weak discontinuous peripheral staining pattern.

Results

Of 42 sera tested 15 or 35.7 percent were found to possess antibodies to PDE and 3 (7 percent) to components in PS. Eight of the reactive sera showed a multiple response by producing two or more precipitin lines to the antigens used. No relationship appeared to exist between the age of an individual or the period of contact with pigeons and the intensity or multiple nature of the antibody response (Table I).

The results of HCF, LHT and FA scolex tests can also be seen in Table I. With the exception of the LHT which was negative in all sera tested the results of the HCF and FA scolex procedures closely parallel those findings in sera which reacted with components in PDE. Only 3 of 15 sera with antibodies to PDE failed to react with hydatid scolices while 6 were negative in the HCF test. In positive HCF tests titres varied from 5 to 40. Additionally, two sera which had not demonstrated precipitins to either PDE or PS were both reactive in the FA scolex test while one also gave a positive HCF to a dilution of 1 to 10.

Discussion and Conclusion

A significant incidence of precipitating antibodies in healthy fanciers to various pigeon antigens has been noted by other investigators. Barboriak *et al.*¹ found antibodies to PS and PDE in sera from 7 of 43 healthy members of a pigeon racing club. Fink *et al.*³ detected a similar response in 41 percent of 80 asymptomatic breeders.

These results emphasise the need to carefully evaluate similar findings in those suspected of suffering from Bird Fanciers disease. Here, in addition to demonstrating specific serum antibodies the induction of symptoms by inhalation tests, either by aerosols or exposure to the birds themselves has been used. The demonstration of an Arthus-like response following intradermal challenge has also been widely employed.

The frequency of antibodies to hydatid material in the blood of healthy individuals actively engaged in a common hobby was not anticipated. Earlier work has indicated a lack of cross reactivity of serum precipitating anti-

Table 1: Antibodies in Pigeon Fanciers

Sample Number	Age (Years)	Contact Period (Years)	Precipitin Lines to				
			PDE	PS	HCF	FA Scolex	LHT
2	44	3	multiple	double	+(40)	+	0
9	63	17	single	0	0	0	0
10	41	11	double	0	+(5)	+	0
12	51	33	single	0	+(5)	+	0
15	17	2½	single	0	0	0	0
17	65	45	double	0	0	0	0
19	18	5	double	0	+(40)	+	0
20	68	60	single	single	+(5)	+	0
24	40	7	single	0	+(10)	+	0
32	32	15	multiple	0	+(10)	+	0
34	15	5	multiple	0	0	+	0
36	16	2	single	0	0	+	0
38	15	3	single	0	+(10)	+	0
40	45	11	double	0	0	+	0
41	41	7	multiple	double	+(40)	+	0

0 = Negative

+

() = Titre

bodies to PDE with components in hydatid cyst fluid. Further work is required to characterise the factor or factors in hydatid cyst fluid and scolices which are implicated in such a surprising response in many individuals in contact with pigeons. The unrefined nature of the antigens used in this study has probably contributed substantially to the results gained and suggests that components in common exist. It seems at least that a reactive HCF or FA scolex test in any person unsupported by other evidence of hydatid disease should be viewed cautiously until contact with pigeons and possibly other avian antigens can be excluded. It is of course well known that hydatid serology tests, especially the HCF, can give false positive results due to a variety of causes.

The consistent lack of activity of the LHT is of real interest but it is not known whether this is related to the kinetics of this type of serological reaction or to the purity of the reagent used to coat the latex particles.

Summary

Fifteen of 42 healthy pigeon fanciers were found to possess antibodies to one or more

organic pigeon components. Of these 80 percent reacted in an immuno-fluorescent procedure using hydatid scolices and 60 percent in a CF test using hydatid cyst fluid as antigen.

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The Use of Fluorescent Chelates for the Determination of Antibiotics and other Biologically Important Substances

M. Lever

Pathology Department, Green Lane Hospital, Auckland, 3

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Introduction

A number of assays for antibiotics and other substances have recently been introduced into this laboratory, and a general approach for the development of new procedures has been found to have broad applicability. This is to form a fluorescent chelate of the compound of interest, or of a derivative of it, and measure the fluorescence in non-aqueous solution. The formation of chelates is familiar as a means of determining cations such as calcium and magnesium, but use as a means of determining ligands has been much less frequently exploited. It is hoped that the principles described here will help others recognise cases where this type of approach can be used, and provide some guidance to workers in the development of new and useful methods.

Effect of Chelate Formation on Fluorescence

When energy is absorbed by a molecule an excited state is formed and this can return to the ground state by either emission of light (fluorescence), or by radiationless decay which involves a sequence of vibrational and rotational transitions. The latter course is the more common, and the energy is dissipated as heat. Fluorescent substances are characterised by having rather rigid molecular structures that do not allow much scope for rotations and vibrations. For example, fluorescein and rhodamine have the structures shown in fig 1. These are highly fluorescent, although similar compounds without the oxygen bridge between two aromatic rings have only the weak U.V. fluorescence associated with aromatic com-

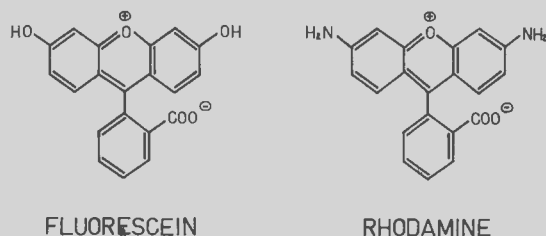


FIG. 1.—Structure of two highly fluorescent compounds.

pounds in general. These two compounds also have another property required before a compound can have analytically useful fluorescence: they absorb light in an accessible part of the spectrum, thus making it possible to form the excited state with readily available instruments.

Now consider a molecule such as glyoxal bis-(4 hydroxybenzoylhydrazone) (fig. 2A). This has relatively weak, blue fluorescence, characteristic of substituted phenols. The molecule is not rigid, and no intense, visible fluorescence is observed. However, in alkaline solution and with a suitable cation present a chelate is formed, probably with the structure shown in figure 2B (its absorption spectrum shows it to be a 1 : 1 chelate), although not necessarily planar. It can be seen that a rigid molecule has been formed, and it is one with an extended conjugated system that leads to intense absorbance in the 400-500 nm region of the spectrum. The chelate is intensely fluorescent, with a wavelength (depending on cation) between 520 and 550 nm, giving a green

colour. Thus the chelate has a fluorescence mode which is not found in either the cation or ligand, and in principle this could be used to determine either.

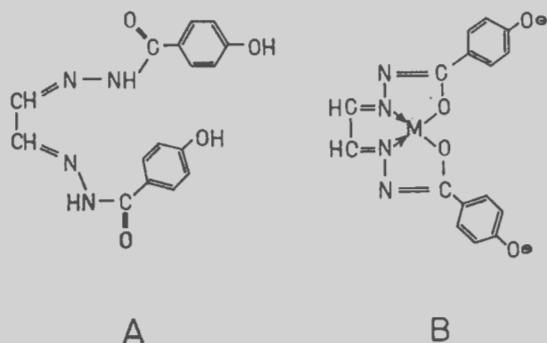


FIG. 2.—(A) Glyoxal bis-(4-hydroxybenzoylhydrazone), neutral form. (B) Postulated structure of chelate of glyoxal bis-(4 hydroxybenzoylhydrazone), in its anion form, with a divalent cation.

Since a chelate is more rigid than the ligand, the chelate will be more fluorescent than a weakly fluorescent ligand (e.g. tetracycline). Usually the fluorescence wavelength is shifted, because the chelate is a distinct molecular species with different excited states. A fluorescence assay depending on chelate formation with a particular cation therefore usually has a high degree of specificity as well as sensitivity.

Effect of Solvent on Fluorescence

In clinical chemistry, fluorescence assays other than those for steroids are usually carried out in water or occasionally in a lower alcohol. Since hydrogen bonding in the excited state aids radiationless decay to the ground state, such hydrogen bonding solvents could be expected to lead to low fluorescence yields⁸, and in many cases this is true. However, most biologically active substances are not readily soluble in non-polar solvents, and more polar solvents often lead to higher fluorescence yields when the hydrogen bonding effect is not paramount⁹. The type of compound the clinical chemist commonly meets presents a dilemma, since highly polar solvents which do not favour hydrogen bonding are rarely met in routine work. Yet there is little doubt that many analytical systems could be improved by exploring the possibilities of solvents other than water, methanol and ethanol.

Effect of Solvent on a Fluorescent Chelate

The salicylaldehyde anion (fig. 3) forms a fluorescent chelate with magnesium. This chelate is not soluble in the more non-polar hydrocarbon solvents, but it is in a wide range of alcohols, esters and other solvents. It will be seen from fig. 3 that while there is the possibility of hydrogen bonding between solvent hydrogens and the solute, the solute has no suitable hydrogen atoms to be involved in such bonds.

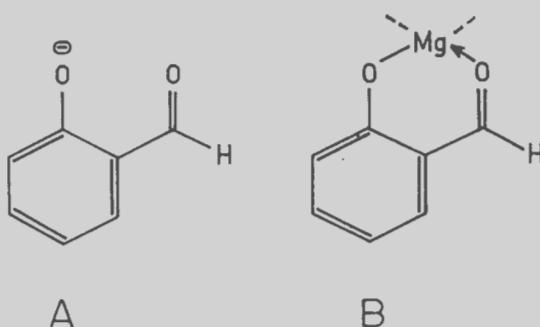
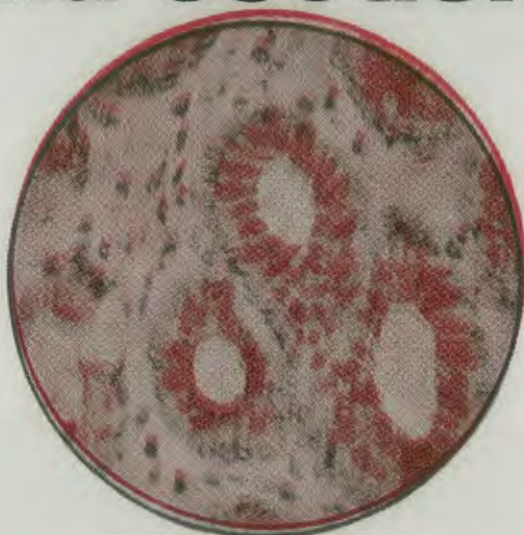


FIG. 3.—Salicylaldehyde anion (A) and fluorescent salicylaldehyde anion-magnesium chelate (B).

Figure 4 shows how the fluorescence of this chelate varies in solution in the series of normal alcohols. The base used to form the anion of the ligand can be sodium hydroxide, a quaternary ammonium hydroxide, or diethylamine, with little change in the results. In formamide there is little, if any, fluorescence and similarly in chloroform. Dimethylsulphoxide is a polar solvent which cannot form hydrogen bonds with the fluorescent species in this particular case, and in this solvent the fluorescence is very intense (46 on the scale of fig. 4), with a peak at 464 nm.

This pattern of results is very much what would be expected if hydrogen bonding adversely affects fluorescence, and a polar solvent is favourable, as discussed in the previous section. However, in ethanediol, propane 1, 2 diol and butane 1, 3 diol much higher fluorescences are obtained than expected on this model. The fluorescence in ethanediol is at 479 nm and between ethanol and methanol in intensity (12 on scale of fig. 4), and in butanediol it is at 472 nm and at about the plateau level (32 on scale of fig. 4). This effect could be interpreted in several ways, but a likely explanation is weak chelating

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between the diols and the cation, and this is consistent with the fact that magnesium hydroxide does not precipitate from the diol solutions under conditions which will precipitate it from simple alcohols. It will be seen from fig. 3 that one or two molecules could possibly chelate with the magnesium as well as the salicylaldehyde, and the resultant molecule would have a rigid structure.

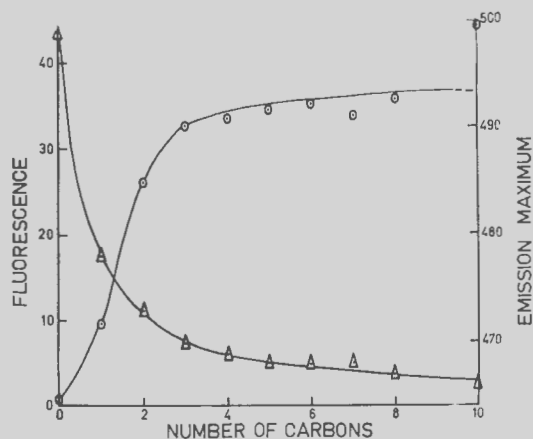


FIG. 4.—Fluorescence of magnesium-salicylaldehyde chelate in normal alcohols, from C 0 (water) to C 10 (decanol). Salicylaldehyde 0.1 mM, base 0.25 mM and Mg^{2+} 10 mM. Excitation wavelengths 380 nm. Triangles : emission maximum. Circles : maximum fluorescence intensity.

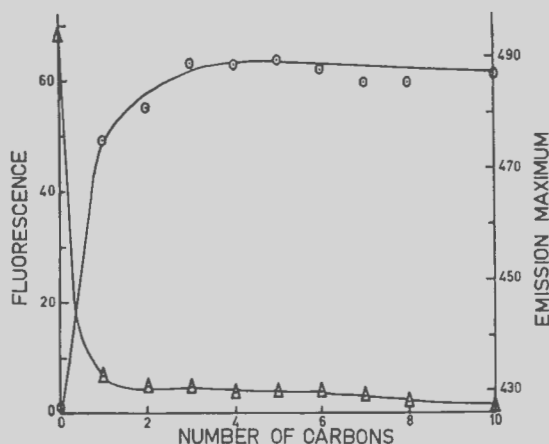


FIG. 5.—Fluorescence of magnesium-salicylaldehyde chelate in normal alcohols with diethanolamine present. As for fig. 4, except that 0.25 M diethanolamine added as base.

Figure 5 shows the corresponding results obtained with diethanolamine as base. In this case there are higher fluorescences at shorter wavelengths. Dimethylsulphoxide behaves similarly to methanol in this case (in this solvent the fluorescence is 46 at 434 nm), and low but significant fluorescences are observed with formamide (0.7 at 437 nm) and chloroform (4.4 at 430 nm). The diols behave as expected, ethanediol being less favourable than methanol (22 at 434 nm), though still 20-fold better than water. These results are consistent with chelate formation between diethanolamine and the cation.

One thing that is quite clear is that water is not a good solvent for fluorescence reactions of this type. The fluorescence in water is only about 2 percent of that in organic solvents. This is possibly because water is an ionising solvent as well as a hydrogen bonding solvent, and since the maximum fluorescence is developed under basic conditions water may cause the charge on chelate or ligand to be unfavourable.

Tetracyclines

The discussion of the magnesium-salicylaldehyde chelate probably provides a basis for understanding the analytical procedure for tetracycline antibiotics which are used here¹. Figure 6 shows that tetracycline contains an arrangement of functional groups closely similar to those found in salicylaldehyde, a phenol with an α -carbonyl substituent *ortho* to the phenolic hydroxyl. The magnesium chelates of tetracyclines, in the presence of excess base and in ethanediol, fluoresce at 475-495 nm emission (excitation 380-405 nm) which is close to the magnesium-salicylaldehyde chelate (excitation 380 nm, emission 479 nm). Earlier workers have used the fluorescence of these magnesium chelates in aqueous solution for the determination of tetracyclines, a rather insensitive procedure, and previously a β -diketone system has been supposed to be the functional group involved in chelate formation.

An alternative method for tetracyclines was a calcium chelate, claimed¹ to have the structure shown in figure 7. This gives a good fluorescence in a number of organic solvents, ethyl acetate being originally recommended¹. However, barbiturates do not appear to have

any more effect on the fluorescence of either calcium or magnesium chelates of tetracyclines than do a number of other weak bases. Barbiturates do not appear to be essential for fluorescence in this assay system¹⁰ and it can be questioned whether the species shown in figure 7 does, in fact, exist.

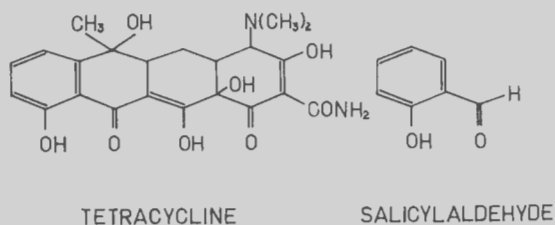


FIG. 6.—Structure of tetracycline (free base) compared with salicylaldehyde.

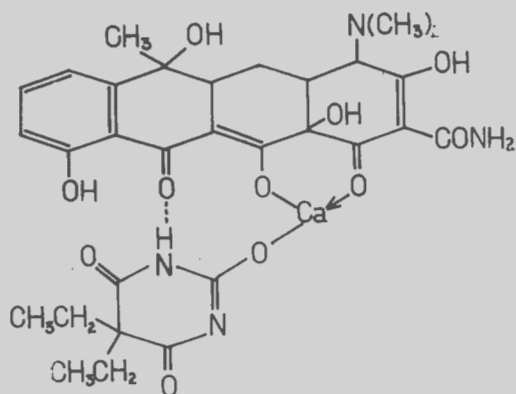


FIG. 7.—Postulated structure of tetracycline-calcium-barbitone chelate, after Kohn¹.

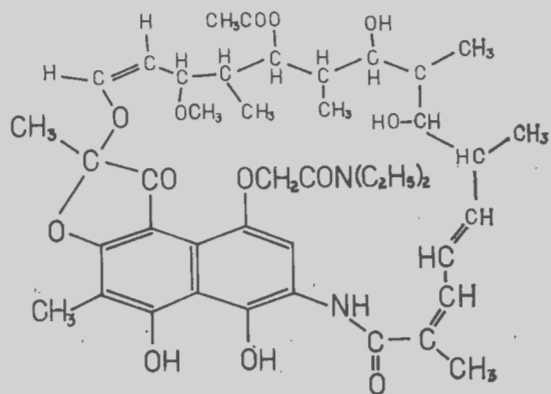


FIG. 8.—Structure of rifamide.

Rifomycins

Rifamide (figure 8) is an example of the rifomycin antibiotics, which all possess a 1, 8 dihydroxynaphthalene ring system. This is not unlike the systems found in salicylaldehyde and tetracyclines, and rifamide in fact forms a highly fluorescent chelate with magnesium in ethanediol-diethanolamine mixtures. Rifampicin forms only a weakly fluorescent chelate, but is readily converted by acid to a fluorescent form (Lever and Johnson, unpublished data).

Salicylate

Salicylate is often determined by its native fluorescence. However, the excitation maximum is about 300 nm in water or ethanediol. With magnesium and base in ethanediol the excitation maximum is shifted sufficiently to permit the use of glass cuvettes, and the fluorescence is intensified very considerably. This provides the basis of a very simple and rapid microassay for salicylate⁶.

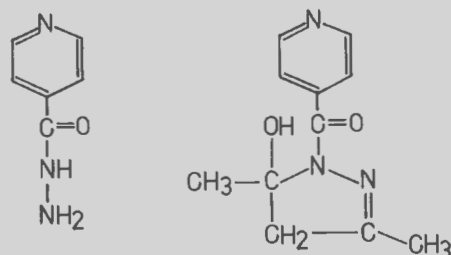
Isoniazid

The assay for isoniazid is an example of a method where a derivative of the substance being determined is the ligand of a chelate. Isoniazid forms a hydrazone with pentanedione which probably has the structure shown in figure 9, in neutral or weakly acidic solution. In the presence of base this derivative forms an anion, possibly with the structure shown in figure 10. This forms fluorescent chelates with a number of cations, but especially with zinc. This fluorescence reaction is highly specific and provides the basis of a convenient and sensitive assay system³.

Carbohydrates

Carbohydrates react with 4-hydroxybenzoic acid hydrazide in alkali to give bis-hydrazones of glyoxal and methylglyoxal, the former having the structure shown in figure 2. As observed earlier, these derivatives form fluorescent chelates, in the presence of base, with a number of cations, the calcium chelate giving the most intensive fluorescence. However, in attempting to measure (for example) glucose by measuring the fluorescence of this calcium chelate in the presence of excess calcium ion, as soon as the prepared sample is placed into the intense light source of a

fluorometer, rapid photo-decomposition takes place. As a result it is impossible to make a reliable estimate of the fluorescence. The type of rigid molecule that gives a good fluorescence



Isoniazid

Probable structure of derivative

FIG. 9.—Structure of isoniazid and the probable structure of its condensation product with pentane 2, 4 dione (acetylacetone). Condensation product in neutral form.

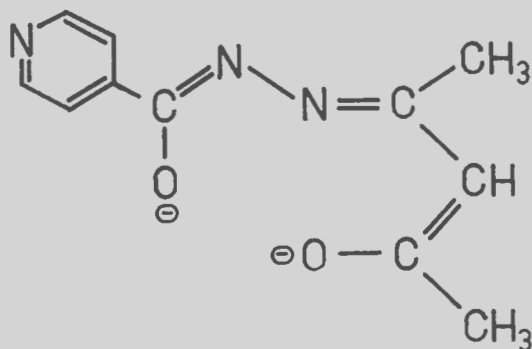


FIG. 10. —Probable structure of yellow anion formed by the isoniazid derivative in the presence of strong base.

often proves to be photolabile, and in fact this problem has presented itself during development of several of the analytical methods described here.

The reason for this can be readily understood. Since the molecule fluoresces, there are few paths other than the emission of radiation by which an excited state can readily return to the ground state. When a solution of the fluorescent substance is put in an intense light source, such as that used in a fluorometer, excited species are formed rapidly and there will result an unusually high equilibrium concentration of excited species. This means that

there is a relatively high chance of molecular interactions involving excited species, and hence a relatively rapid rate of decomposition reactions.

Usually this effect can be minimised by altering the reagents in the final solution; such factors as cation concentration, strength and concentration of base, and presence of water, are important. In the cases of the calcium chelates of the aroylhydrazones, however, the problem has not been solved. Fortunately lanthanum forms a reasonably stable chelate with a good fluorescence and convenient fluorescence spectra, and this has been used in an analytical method for nanogram quantities of glucose⁵.

Extraction from Sample

In most of the assays described here, the substance being determined (or a derivative of it) is extracted from serum into amyl acetate. This solvent is readily available in a pure form, it is non-toxic and not readily flammable. It is also immiscible with ethanediol and diethanolamine. An alternative solvent, with similar properties, is 4-methylpentan-2-one. (*iso*-butyl methyl ketone).

Bilirubin and its breakdown products are among the worst interfering substances in these assays, because they absorb light in the 400-500 nm region of the spectrum, which causes quenching of the fluorescence. Fortunately amyl acetate is not an efficient extraction solvent for these. A number of ways of further reducing the extraction of bilirubin and its derivatives have been developed; thus a buffer containing sodium sulphite is used at low pH in the tetracycline assay⁴. The isoniazid derivative extracts from carbonate solution, whereas bilirubin is too acidic to do this³. Another method is to add cations such as zinc to the sample; these form non-extractable complexes with many acidic interfering substances.

High concentrations of salts are used to facilitate extraction by salting out. Thus 3M NaH₂PO₄ and 1 M Na₂SO₃ are used in the tetracycline assay, and 5 M potassium carbonate is used in the isoniazid assay.

Extraction into the Fluorescence Reagent

The solvent for the fluorescence reagent in these assay systems is ethanediol, diethanolamine, or a mixture of these. The reagent contains the cation (usually as the acetate,

since acetates are frequently available in pure form and are readily soluble) and if necessary a base such as sodium barbitone. Because of chelate formation, the ligand is extracted with good recovery into the fluorescence reagent.

In an earlier article² the use of Brown cuvettes for the last step was described. This technique has been further aided by the introduction of a vertical shaker⁷ which enables reproducible results to be obtained even with viscous fluorescence reagents.

Summary

Some studies of the fluorescence of chelates in non-aqueous solution have been described. Diols are unexpectedly favourable solvents, possibly because they take part in chelate formation. Some fluorometric assays for clinically important substances have been described which utilise this property. In some cases the substance is extracted and then back-extracted into an ethanediol-based reagent containing a cation with which the substance forms a chelate. In other cases the substance itself either cannot be extracted or does not form fluorescent chelates. A simple derivative may be formed if the substance contains functional groups such as carbonyl or amino

groups capable of undergoing condensation reactions. The derivative may be extracted and then back-extracted to give an ethanediol solution of a suitable chelate. In either case the measurement of the fluorescence of the chelate can provide the basis of a sensitive, specific, rapid and technically simple analytical procedure. It is hoped that the work described here will assist others in the development of analytical methods for substances of concern to them.

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A Preliminary Evaluation of a Latex Test for Hepatitis-Associated Antigen

M. J. Gratten, ANZIMLT and D. S. McConnell, ANZIMLT

Pathology Services, Christchurch Hospital

J. A. Walker, FNZIMLT

Pathology Laboratory, Christchurch

S. W. Entwistle, ANZIMLT

Pearson Laboratory, Christchurch

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Introduction

With the recent availability of a simple screening procedure for Hepatitis-Associated Antigen (HAA) using coated latex particles an opportunity for rapid and sensitive testing appeared imminent. At least one report (Fritz and Rivers, 1972)¹ has suggested that latex particles sensitised with anti-HAA is somewhat more sensitive than complement fixation and that such a method could be adapted to large scale screening. Leach and Ruck² have also recorded the suitability of the latex agglutination test for the detection of HAA. A certain lack of specificity in such a proce-

dure appears to have been eliminated in a variety of ways, none of which are readily explicable. Some investigators have heated sera in a 56°C water bath for 30 minutes while others have mixed test sera with diluted guinea pig serum prior to reacting with the coated latex reagent. Specificity is also said to be enhanced by "aging" sera before testing at refrigerator or deep freeze temperatures.

Materials and Methods

The present study compared a HAA Latex Test (LX) distributed by Hoechst (N.Z.) Ltd. with three widely used techniques. The commercial reagent was employed as instructed by the manufacturer except that following the

five minute mixing period slides were examined for macroscopic agglutination only. No attempt was made to evaluate results microscopically. With the exception of an ancillary study on a number of rheumatoid factor-containing sera latex reagent of the same batch number was used throughout.

The method was used in conjunction with a complement fixation (CF) procedure referenced previously², Ouchterlony gel diffusion (OGD)⁴ and immunoelectro-osmophoresis (IEOP). The latter method used barbitone buffered (pH 8.6) 1.5 percent Oxoid ionagar No. 2 layered onto glass plates 90 x 50 mm. Prior to testing wells 1.5 mm in diameter (to contain anti-HAA) and 3 mm in diameter (to contain test sera) were punched in the gel, each pair 3 to 4 mm apart. After inoculation plates were run at 140 V for 60 minutes at 24 mA per plate and then examined for precipitin lines. In the CF procedure marked prozoning is common in strongly positive sera. Test samples were therefore assayed at dilutions of 5, 50 and 100.

The source of anti-HAA used in the IEOP test differed from that used in the other two methods although both antibody-containing sera were gathered from haemophiliacs. Neither serum had undergone any purification.

Ninety-six sera were tested by each of the four methods described above. The samples derived from out-patients who had produced biochemical results suggestive of viral hepatitis, individuals with chronic renal failure on maintenance dialysis and those known or suspected of involvement in drug abuse. Hospital in-patients contributed about one-fifth of the total samples. All sera were held at -25°C for a minimum of 24 hours prior to testing.

In addition, IgG and IgM levels were determined on 12 sera considered to be giving falsely positive latex results. These same samples were also examined for rheumatoid factors. A further 20 sera known to contain rheumatoid factors were tested using the HAA latex procedure.

Results

Of 96 sera tested 20 (21 percent) were positive by LX. Only six of these could be confirmed by the other procedures each of which were reactive in all cases. None of the remaining 14 LX positive sera reacted by OGD, IEOP and CF. A total of 12 HAA-containing sera were detected by CF, 11 by

IEOP and nine by OGD. Those positive by OGD were confirmed by IEOP and CF; two sera positive by IEOP and negative by OGD were confirmed by CF. The sample positive only by CF was drawn from an individual who had been previously positive with all tests including LX.

On five occasions a LX-negative serum reacted with one or more of the other three procedures. In three instances OGD, IEOP and CF were all reactive, in one both IEOP and CF reacted and in one only CF was positive. Prozone anomalies with the latex reagent were excluded by diluting the 5 sera 1 in 5 with 0.85 percent NaCl and retesting without change in results previously gained. It is of some interest that three of the above specimens were removed from two individuals who had previously reacted in all four tests. These results are compared in Table 1. It will be seen that on each occasion the LX method was one of the first to become negative.

Table 1: Summary of HAA test results on 2 patients over a 4 week period.

Date Specimen Removed	LX	OGD	IEOP	CF		
				1/5	1/50	1/100
Patient A 1. 7.7.72	+	+	+	+	+	+
2. 21.7.72	0	+	+	0	+	0
3. 3.8.72	0	0	0	+	0	0
Patient B 1. 9.6.72	+	+	+	0	+	+
2. 7.7.72	0	0	+	+	0	0

0 = Negative

+

Twelve of 14 sera giving positive LX results unconfirmed by other procedures were assayed for IgG and IgM levels by radial immunodiffusion and for rheumatoid factors using latex agglutination⁵ and a modified Rose Waaler procedure (Denver Laboratories Pty. Ltd.) These results appear in Table 2.

It will be noted that only one of the 12 samples above demonstrated normal amounts of both IgG and IgM while seven gave abnormally high values of both immunoglobulins. Nine of the 12 sera showed substantial increases in IgM. Four revealed some evidence of rheumatoid factor activity although in none of these were results of diagnostic significance; only

one of these four sera showed any increase in IgM.

Table 2: Results of tests for IgG and IgM levels and rheumatoid factors on sera giving false positive HAA latex reactions.

SAMPLE NUMBER	IgG (mg%) (normal: 770-1150)	IgM (mg%) (normal: 90-170)	Rheumatoid Latex	Factors Rose Waaler
1	1600	570	0	0
2	1450	540	0	0
3	860	140	+(1/128)	+(weak)
4	1680	890	0	0
5	1080	400	0	0
6	1330	130	+(1/32)	+(weak)
7	1450	750	0	0
8	1000	850	0	0
9	1500	225	0	0
10	1680	510	0	0
11	1370	324	+(1/64)	+(weak)
12	1500	128	+(1/32)	+(weak)

0 = Negative
+ = Positive

Finally 20 randomly selected sera all known to contain significant levels of rheumatoid factors were subjected to LX testing. Sixteen (80 percent) gave a positive reaction and of these nine demonstrated immediate and complete clumping of the latex particles. The batch number of the latex reagent used in this section of the study differed from that used throughout the remainder of the investigation.

Discussion

The results of this study indicate that the HAA latex procedure is deficient both in specificity and sensitivity. Positive reactions gained with the latex reagent that were not able to be confirmed by other methods have been classified as false. Here it has been suggested that such non-specific values may be related to qualitative and quantitative IgM abnormalities in test sera. In this respect 16 of 20 sera containing rheumatoid factors—antibodies to altered gamma globulin generally occurring in the IgM class—agglutinated the HAA latex reagent. Although advised of

this possibility by the manufacturer the frequency of such results in sera containing this auto antibody was not anticipated. This finding suggests that antibodies of the same class present at least in other autoimmune processes may also contribute to this lack of specificity.

Immunoglobulin levels were investigated in 12 of 14 falsely positive samples and quantitative abnormalities were common. A number of sera showed substantial increases in IgM while elevations in the IgG class were of a more moderate nature. It seems likely that elevated IgM values, directly or indirectly, play a significant role in the reactivity of this latex reagent.

The incidence of falsely negative results is disturbing and would seem to preclude use of the HAA latex method in a screening context. An essential deficiency in sensitivity is indicated by the findings in those two patients presented in Table 1. It seems that the hepatitis-associated antigen of a serum must be quantitatively high before specific aggregation of the latex particles occurs. The latex reagent appears to produce more false negative results than the gel diffusion procedure which is generally regarded as the least sensitive of all commonly used methods.

Summary and Conclusion

A commercially available latex reagent for the detection of HAA in blood has been compared with three commonly employed techniques and found to produce significant numbers of both false positive and false negative results. Its use in a screening or definitive context cannot be recommended at this time.

Acknowledgments

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Use and Abuse of Antibiotics

Dr S. M. Bell

Prince of Wales Hospital, Sydney

From an address to the 28th Annual Conference of the NZIMLT August 1972

Introduction

Last year the Commonwealth Government of Australia spent \$34,000,000 on antibiotics prescribed in practice under the pharmaceutical benefits scheme. The Health Department spends a considerable amount of money ensuring that their regulations are complied with in the writing of the prescriptions and safeguarding against possible fraud under this scheme. However, there is no information regarding the actual use of the antibiotic once it has been dispensed and in the hands of the patient. Under these circumstances we cannot say with any certainty to what extent antibiotics are misused in practice; in fact, we cannot estimate what the true extent of the use of these drugs is in the community.

On the other hand we can say with certainty that practitioners receive misleading information concerning antibiotics which must influence their prescribing for patients with infection and would result in the misuse of antibiotics. I wish to refer to the two major sources of misleading information concerning antibiotics and examine how these affect the prescribing of these drugs.

A major source of misleading information given to practitioners is the pharmaceutical industry. It is possible upon request to pharmaceutical companies to receive a tremendous service with regard to information concerning the antibiotic produced by that company. This information will include reprints of various publications and details of the pharmacology and toxicology of the antibiotic. Unfortunately this is not the service which is generally given to practitioners: unsolicited literature which practitioners receive from pharmaceutical companies emanates from the sales division of the company and the information it contains is restricted to that which is most likely to convince the practitioner to use the antibiotic it markets.

One popular technique used in the promotion of an antibiotic is to emphasise an isolated *in vitro* property of the drug which may not

have anything to do with the way it acts in a patient. If a drug happens to be bactericidal, this is often quoted as giving the drug an advantage over a bacteriostatic antibiotic. Antibiotics are simply bacterial inoculum reducers and most of the work in ridding the body of infection is done by such bodily defence mechanisms as phagocytosis; therefore in the majority of infections it doesn't matter how you reduce the inoculum, either by cidal or static activity, the end result is the same—the patient gets better. There are some exceptions to this of course, and these are mainly where phagocytosis is deficient or because of local conditions phagocytosis is not effective, such as in subacute bacterial endocarditis or in patients treated with drugs which depress these defence mechanisms, but these examples are pretty rare. The therapy of typhoid fever demonstrates how little the cidal versus static properties matter. Chloramphenicol, a purely bacteriostatic antibiotic, consistently beats ampicillin, a bactericidal drug, in any comparative trial of these drugs in this disease. On the other hand, ampicillin will clear a typhoid carrier of *Salmonella typhi* but chloramphenicol will not. Incidentally, the synergistic bactericidal combination of sulphonamide and trimethoprim has failed miserably in typhoid fever in two trials reported in the recent literature.

Another example of misleading information from pharmaceutical firms is the promotion of an antibiotic for diseases caused by an organism sensitive to the antibiotic but where antibiotics play no part in the resolution of the disease. This is most commonly seen with staphylococcal infection. Invariably in any promotional literature considerable emphasis is placed on the value of the antibiotic in the treatment of superficial staphylococcal infection, and indeed many reports of a new antibiotic's appraisal will often contain a number of patients successfully treated with that antibiotic for staphylococcal skin infection. This practice continues despite conclusive evidence that antibiotics play no part in the resolution

of this disease. In controlled trials of antibiotic treatment of staphylococcal skin sepsis the untreated group of patients gets better just as quickly as the group treated with antibiotics.

In the same way antibiotics are promoted for upper respiratory tract infection without further qualification, but only something like a third or less of cases of upper respiratory tract infection, such as pharyngitis, are due to a Beta-haemolytic streptococcus and it is only these which would respond to antibiotic therapy. The remainder are viral and antibiotics are of no value.

Erythromycin stearate for example is indeed effective against upper respiratory tract infection and skin sepsis, provided the infecting organism is *Streptococcus pyogenes* and provided, furthermore, that the antibiotic is taken before meals. Unfortunately it is the use of these incomplete statements which seem to be part and parcel of most promotional literature and I would like to emphasise not confined to this example which I happened to pick.

More serious than the use of the incomplete statement is the use of false information in the promotion of an antibiotic. Fortunately this is not as common but it does occur and is demonstrated in the claims made in one brochure relating to cephaloridine.

One could probably take issue with each one of the statements but let us look at some in particular. The claim for lack of toxicity is not supported by fact; published literature frequently makes reference to the nephrotoxicity of cephaloridine and even in patients with normal renal function a dose of 6 grams a day or more can be toxic. The resistance of cephaloridine to staphylococcal penicillinase is only marginally better than that of benzylpenicillin. Cephaloridine is approximately 40,000 times less resistant to staphylococcal penicillinase than is methicillin and the failure of cephaloridine in the treatment of serious staphylococcal disease is well documented in the literature.

In regard to the last two statements, the cross allergenicity of the penicillins and the cephalosporins is well established and the danger of the use of cephaloridine in a penicillin-allergic subject is emphasised thus.

In penicillin-sensitive patients, cephalosporin and derivatives should be used with

great caution. There is clinical and laboratory evidence of partial cross-allergenicity of the penicillins and the cephalosporins. Instances of patients who have had severe reactions to both drugs, including death from anaphylaxis, have been reported.

This is the warning notice which must appear on all promotional and prescribing literature concerning the cephalosporins published in the United States. It warns of the danger of serious and even fatal reactions which have occurred in patients with a history of allergy to penicillin who have been treated with cephalosporin derivatives.

In Australia we have no equivalent authority to the American FDA which regulates what can be said in promotional literature and there are many people in my country who see a need for such an authority. On the other hand there is a danger in this that if bureaucratic restrictions are too repressive they could impede the development of the pharmaceutical industry. This industry depends on an active sales force for its survival and we, in turn, depend on this industry for the development and supply of life-saving antibiotics. Rather than attempt to restrict it by regulation it seems to me preferable that this industry which claims the title "ethical" should formulate a code of ethics governing the promotion of drugs. In addition, I see it as our function in the laboratory to provide the practitioner with accurate and unbiased information concerning the diagnosis and antibiotic treatment of the individual patient.

This brings us to the second source of information concerning antibiotics which is available to the practitioners, that is, the information from pathology laboratories. Over the past three years we have had an opportunity of studying the accuracy of information arising from pathology laboratories in Australia and New Zealand by means of the microbiology survey which we conduct for the College of Pathologists each year. These surveys have demonstrated that the information emanating from the pathology laboratories can be as inaccurate as that which the practitioner receives from the pharmaceutical companies.

The first example examines the laboratories' ability to isolate and identify a common pathogen and is from the 1970 survey. The participants were supplied with a specimen

TABLE I
Specimen 1: Isolation and Identification of Organisms Present

Organism I		Organism II
B. haemolytic streptococci Group A	48	Proteus sp. 73
B. haemolytic streptococci	9	
Anaerobic streptococci	5	
<i>Streptococcus faecalis</i>	2	
Streptococcus	2	
Not isolated	7	
TOTAL	73	73

simulating a vaginal swab of a patient with a septic abortion. Despite the fact that in this situation *Streptococcus pyogenes* is a lethal pathogen, only 48 out of the 73 labs, that is less than 65 percent, were able to isolate and correctly identify the pathogen.

TABLE II

Specimen 3: Assessment of Significant Pathogen	Number of Reports
None of the organisms significant	14
Klebsiella significant	40
Klebsiella and Clostridium significant	11
Klebsiella and Staphylococcus significant	2
Combinations of other organisms	7

In the same year the laboratories' ability to interpret the results of culture was tested by requesting them to nominate what they considered to be the significant pathogen and to report the antibiotic sensitivity of this organism.

In this example the swab simulated a specimen from a varicose ulcer of many years' duration. The specimen contained three organisms, Klebsiella, *Staphylococcus albus* and *Clostridium welchii*, all commonly found as normal flora in varicose ulcers and which have no effect on the course of the disease. Only 14 laboratories considered that none of these organisms was significant and more than half considered that the Klebsiella was significant and reported the sensitivity of this organism to kanamycin, which implied that this case

should be treated with a potentially toxic antibiotic.

The surveys have also examined the accuracy of antibiotic sensitivity testing and the results do not justify the faith with which clinicians accept this laboratory test.

In the 1970 survey the laboratories carried out antibiotic sensitivity testing on a member of Proteus species and there was about a 50 percent error in the results with sulphonamides and a 10 percent error with ampicillin. These are the two most commonly used agents against gram-negative infections in clinical practice.

TABLE III

Specimen 1: Antibiotic Sensitivity Testing of Proteus Vulgaris

Antibiotic	Number of Reports		Minimum inhibitory Concentration $\mu\text{g/ml}$ *
	Sensitive	Resistant	
Sulphonamide	28	22	12.5 (Sensitive)
Ampicillin	5	46	>40 (Resistant)
Tetracycline	35	16	5.0 (Intermediate)
Kanamycin	50	0	2.5 (Sensitive)

* Inoculum: 2.5×10^4 organisms per ml.

In 1971, when six strains of staphylococci were tested by laboratories using their own method of sensitivity testing—shown here as Part 1—there were errors of 11 percent in penicillin sensitivity testing, 12 percent in ery-

thromycin testing and 19 percent when testing against methicillin. All of these errors are potentially lethal, particularly with penicillin where the error is invariably the failure to detect resistance to that antibiotic. A clinician treating a patient suffering from a penicillin-resistant staphylococcal septicaemia could be misguided by the laboratory to use benzylpenicillin with almost certainly disastrous results.

TABLE IV

Percentage of incorrect reports received in parts 1 and 2 of the survey of antibiotic sensitivity of all six strains of staphylococci to each of the five antibiotics tested

Antibiotic	Part 1	Part 2
Benzylpenicillin	11%	0.9%
Tetracycline	4%	0.2%
Chloramphenicol	5%	0.5%
Erythromycin	12%	0.6%
Methicillin	19%	6%

The results shown here as Part 2 are where the laboratories followed a standard method of sensitivity testing and the dramatic improvement in results demonstrates the need in this area of laboratory medicine for standardisation of the methods used.

TABLE V

Comparison of the percentage of incorrect reports of methicillin sensitivity of the two strains of methicillin-resistant staphylococci in parts 1 and 2 of the survey

Strain Number	Percentage of Incorrect Reports	
	Part 1	Part 2
2	30%	1%
6	64%	25%

With methicillin the failure to detect resistance was even more common: 64 percent of laboratories failed to identify one of the strains as methicillin-resistant and there was a 30 percent error with the other methicillin-resistant strain. Again this is a potentially catastrophic error.

The results from the 1972 survey of antibiotic sensitivity testing which examined sensitivity testing of gram-negative organisms in more detail are not yet available but I was

able to extract one piece of information which is relevant to the discussion and I thought would be of interest to you today.

TABLE VI

Glossary of abbreviations and terms used in reporting antibiotic sensitivity by participants in Microbiology Survey 1972

Abbreviation Used	Laboratory's Interpretation
S	= Sensitive
R	= Resistant
DS	= Diminished sensitivity
NS	= Not sensitive
+	= Sensitive
O	= Not sensitive
I	= Inhibited
N	= Not inhibited
I	= Intermediate
I	= Moderately resistant
M	= Moderately sensitive
VS	= Very sensitive
MR	= Moderately resistant
M	= Moderately resistant
—	= Resistant
—	= Insensitive
—	= No zone
PS	= Partially sensitive
WS	= Weakly sensitive
(Blank)	= Insensitive
‡	= Fully sensitive
†	= Zone reduced—antibiotic likely to be effective
+	= Small zone—antibiotic unlikely to be effective
?	= Doubtful
++	= Fully sensitive
+	= Moderately sensitive
±	= Doubtful sensitivity
D	= Doubtful
=	= Doubtful
MS	= Resistant
SW	= Moderately sensitive
S?	= Sensitivity weak
	= A few resistant colonies within the zone of no-growth
R!	= A very small zone of inhibition of growth

This slide is a glossary of thirty different ways the participants said "sensitive" or "resistant". The participants were asked to report sensitivity of six strains of *E. coli* using the same terms of sensitivity report that they normally issue from their laboratories. As you

can see the same abbreviation can have completely different meanings to different laboratories. The information issued by the laboratories in this situation would not only be misleading but must result in confusion to the practitioner in his choice of an antibiotic.

In conclusion, I suggest that the scarcity of unbiased and accurate information concerning antibiotics which is available to prac-

tioners and the lack of support from us in the laboratory can lead to a misuse of antibiotics in clinical practice. We cannot expect the clinician to be selective in his choice of antibiotics unless he is supported by a reliable laboratory service, and, it is unreasonable to criticise the practitioner in those situations where he finds, because of lack of support, a need to use empirical or shotgun antibiotic therapy.

Technical Communications

An Unusual Member of the Genus *Bacillus* Isolated from a Blood Culture

Identification of organisms based upon morphological characteristics and a limited range of metabolic activities, if interpreted without critical evaluation, can lead to the wrong identification and misreporting of isolates. Recent experience in this laboratory with an isolate from a blood culture demonstrates this point. Blood cultures in the Auckland Hospital are normally collected by the resident medical staff, and are placed into two types of media. Medium I consists of a 3oz standard panel glass bottle, containing a slope of Columbia agar (BBL) on one of the flat slides, and 30ml of brain heart infusion broth (BBL). Medium II consists of a universal container filled with Brewer modified thioglycollate (BBL). Up to 3ml of blood is added to two bottles of each medium, a total of 12ml of blood being sampled.

Identification procedures are carried out mainly in accordance with the tables and methods laid down by Cowan and Steele². Some modifications have been introduced to conform to local preferences. Antimicrobial susceptibility testing is done on pure cultures using the method described by Stokes¹.

Two sets of cultures from one patient, taken on successive days grew what appeared to be a gram negative rod, approximately 1 x 4 microns, with rounded ends. It grew in both media in each set. A preliminary report, was issued, stating the presence of gram negative rods, with identity and sensitivities to follow. Routine identification procedures were set up, the results of which appear in Table I. Disc susceptibility tests showed the organism to be sensitive to tetracycline, gentamicin, ampicillin, cephalosporin, sulpha/trimethoprim, carbeni-

cillin and resistant to kanamycin and chloramphenicol.

On the basis of the results of the initial identification tests, the organism was provisionally identified as *Alcaligenes* sp, and a report was issued to this effect. The lack of growth on MacConkey agar was noted by a senior staff member, and the test was repeated to check viability of the first inoculum. In addition the gram stain was repeated, and the negative reaction confirmed. Next day, the organism had again failed to grow on MacConkey agar.

This anomalous reaction posed some questions as to the validity of the initial identification, and further tests were done. The results of these tests appear in Table II. In addition, the organism was tested for sensitivity to benzyl penicillin. It proved to be sensitive.

The results of the tests ruled out the possibility of a *Pasteurella*, and strongly suggested a gram positive organism. Gram stains were repeated on all cultures held and again these proved negative. However, the stains of the original plate—now four days old—showed pleomorphic forms with distinct gaps in the cell cytoplasm. Spore stains were done using the method of Schaeffer and Fulton³. These demonstrated abundant spores.

A search of Bergey's Manual¹ showed that the organism fitted the identification *Bacillus sphaericus*.

The reporting of *Alcaligenes* sp, from two sets of blood culture bottles could have meant the administration of potentially toxic, and certainly expensive drugs had the clinical situation been appropriate. The correct

identification of this organism as a member of the genus *Bacillus*, however, would have presented a situation that required much more critical evaluation by the clinical staff before such therapy was instituted. The recorded incidence of septicaemia or bacteraemia due to *Bacillus* is extremely low, and the most likely explanation for the presence of this organism in the blood cultures is that it was colonising the patient's skin at the time of collection. The possibility that this was a transient bacteraemia can not be ruled out, however. Subsequent blood cultures failed to recover this or any other organism from this patient.

It is evident that but for the single aberrant result—no growth on MacConkey agar—this organism would have been wrongly labelled.

The value of sets of biochemical tests chosen to give a simple guide to identification is self-evident. We all use them, and probably will continue to do so into the foreseeable future. Without intelligent and careful application however, such schemes can be the source of misinformation.

TABLE I

ONPG: Negative
Indole: Negative
O/F Glucose (open): Alkaline (closed): Negative
Oxidase: Positive
Catalase: Positive
Growth on MacConkey: Negative
Motility: Positive

Partial Thromboplastin Kaolin Time (PTTK), in Children

Sir,—Prompted by a letter by Dr C. S. Norman in the *Lancet* of March 18, 1972, referring to PTTK results in children we have recently completed a survey of 267 children between the ages of one and 14 years. They were referred to our laboratory either for screening or minor surgery, or with clinical particulars that suggested coagulation tests be performed.

The PTTK results of the 267 children showed a mean of 35.2 seconds with an SD of 4.6 seconds, a range of 25.6 to 35.2 seconds, a mean \pm 2 SD of 26 to 44 seconds. (Table I.)

TABLE II

TSI: Alkaline/Alkaline
H ₂ S: Negative
Citrate (Simmonds): Negative
Phenylalanine Determination: Negative
Ornithine Decarboxylation: Negative
Urease: Positive
MR/VP: Negative
Nitrate Reduction: Negative
Gelatinase: Negative
Dn'ase: Negative
Gluconate: Negative
Growth at 40°C: Positive
Growth at 4°C: Negative
Growth on Cetrimide: Negative

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G. L. CAMERON AND
HELEN BRENTON-RULE,
Department of Microbiology,
Wallace Laboratory,
Auckland Hospital.

October, 1972.

A comparable group of 267 controls, consisting of pooled plasma taken each day from three different healthy adults between the ages of 20 and 40 years, were also processed.

They showed a mean of 33 seconds, with an SD of 2.4 seconds and a mean \pm 2 SD of 28-38 seconds. (Table III.)

Sixteen children (6%) of the group studied showed at least one factor assay of less than 40 percent. (Factors VIII, IX, X, XI and XII were assayed.)

Thirteen children (nine male, four female aged 1 to 12 years), showed mild Factor V deficiencies.

One male showed a Factor IX assay of nine percent and a PTTK of 50.6 seconds.

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Table I

PTTK Results of 227 Children up to the age of 14			
Seconds			
Less than	025.0	000.00	0000
	026.0	000.36	0001
	027.0	001.44	0003
	028.0	004.69	0009
	029.0	005.42	0002
	030.0	007.58	0006
	031.0	014.44	0019
	032.0	023.47	0025
	033.0	035.02	0032
	034.0	045.13	0028
	035.0	054.51	0026
	036.0	067.51	0036
	037.0	077.26	0027
	038.0	084.48	0020
	039.0	089.89	0008
	040.0	091.70	0007
	041.0	093.14	0005
	042.0	094.22	0004
	043.0	095.31	0003
	044.0	096.03	0003
	045.0	096.75	0002
	046.0	097.11	0002
	047.0	097.47	0001
	048.0	098.19	0001
	049.0	098.19	0002
	050.0	098.19	0000
More than	050.0	100.00	0005
Mean		035.17	
SD		004.89	
Mean \pm 2 SD		025.39-044.94	
5-95 Percentile		028.45-044.05	
2.5-97.5 Percentile		027.33-048.53	
Range		025.60-058.3	

Table II

PTTK Results of 251 Children up to the age of 14 (16 children with Abnormal Factor Assays)			
Seconds			
Less than	025.0	000.00	0000
	026.0	000.40	0001
	027.0	001.59	0003
	028.0	005.18	0009
	029.0	005.98	0002
	030.0	008.37	0006
	031.0	015.54	0018
	032.0	024.30	0022
	033.0	036.25	0030
	034.0	046.61	0026
	035.0	056.97	0026
	036.0	070.92	0035
	037.0	080.88	0025
	038.0	088.05	0018
	039.0	091.24	0008
	040.0	093.63	0006
	041.0	095.62	0005
	042.0	096.41	0002
	043.0	097.61	0003
	044.0	098.41	0002
	045.0	098.80	0001
	046.0	099.20	0001
	047.0	099.60	0001
	048.0	099.60	0000
	049.0	100.00	0001
	050.0	100.00	0000
More than	050.0	100.00	0000
Mean		034.51	
SD		003.61	
Mean \pm 2 SD		027.29-041.74	
5-95 Percentile		027.96-040.88	
2.5-97.5 Percentile		027.26-043.35	
Range		025.60-048.60	

Table III

PTTK Results of 267 Control Samples			
Seconds			
Less than	025.0	000.00	0000
	026.0	000.37	0001
	027.0	000.37	0000
	028.0	001.12	0002
	029.0	004.12	0008
	030.0	010.49	0017
	031.0	022.10	0031
	032.0	037.08	0040
	033.0	055.81	0050
	034.0	068.91	0035
	035.0	083.52	0039
	036.0	089.51	0016
	037.0	095.88	0017
	038.0	097.38	0004
	039.0	099.25	0005
	040.0	099.63	0001
	041.0	100.00	001
	042.0	100.00	0000
	043.0	100.00	0000
	044.0	100.00	0000
	045.0	100.00	0000
More than	045.0	100.00	0000
Mean		033.00	
SD		002.38	
Mean \pm 2 SD		028.24-037.76	
5-95 Percentile		029.14-036.92	
2.5-97.5 Percentile		028.46-038.26	
Range		025.20-040.80	

One male and one female showed Factor XII assays of 31 percent and 35 percent with PTTKs of 45 and 43.5 seconds respectively.

Excluding the results of the 16 children who had assays less than 40 percent, the mean PTTK then became 34.5 seconds, with an SD of 3.6 seconds and a mean \pm 2 SD of 27 to 42 seconds. (Table II.)

It is interesting to note that of the 13 children studied who had Factor VIII assays less than 40 percent, four had PTTK times within this normal range, i.e., less than 42 seconds. These showed assays of 23 percent, 38 percent, 39 percent and 35 percent with PTTK times of 37.5, 39.3, 36.5 and 41.5 seconds respectively, which would support the view of Dr Norman in that there is no linear relationship between PTTK values and individual assay results.

It is also interesting that during the survey nine children (or 3.3%) showed PTTK times outside the normal range of 27-42 seconds who had completely normal factor assays of VIII, IX and XI. These times ranged from 42.2 to 48.6 seconds. No explanation could be

found for their prolonged times and all other routine coagulation tests were normal.

To summarise, 21 or 7.9 percent of the survey had PTTK times outside of our normal range for children of 27.0 to 42.0 seconds, and of these 12, or 4.5 percent were subsequently found to have factor assays of less than 40 percent.

We would be interested in comments and findings from other laboratories on this subject.

C. S. SHEPHERD,
E. OUDYN,
Medical Laboratory,
Hamilton.

November, 1972.

Selected Abstracts

Contributors: D. G. Bolitho, Alison Buchanan, M. Jeannette Grey, J. Hannan, A. G. Wilson.

CHEMICAL PATHOLOGY

Drug Interferences with Clinical Laboratory Tests. Young, D. S., Thomas, D. W., Freidman, R. B., Pestoner, L. C. (1972), *Clin. Chem.* 18, 1041.

This issue of *Clinical Chemistry* is entirely devoted to this comprehensive bibliography of over 300 pages of close typed entries. As well as an introduction the contents are listed as follows: Generic/proprietary drug name directory, abbreviation and short form directory, alphabetic listing by drug name, alphabetic listing by laboratory test, and a comprehensive list of referees. This issue would be a useful reference book in the laboratory.

—A. G. W.

Observations on the Heat Stability of Alkaline Phosphatase Isoenzymes in Serum. Moss, D. W., Shakespeare, M. J., Thomas, Dilys M. (1972), *Clin. Chim. Acta*, 40, 35.

Serum specimens which apparently contain the same isoenzyme of alkaline phosphatase nevertheless show wide variations in the stabilities of the phosphatase activities on heating at 56°C. The authors have studied the factors which may influence the stability of bone and liver phosphatases and have assessed their contributions to the observed variability of serum phosphatase heat stabilities. The authors found that differences in protein or urea content between specimens had little effect, but found that pH changes caused some variability in heat stability. They conclude that when differences in sample content have been taken into account the range of variation which remains suggests that different individuals produce a particular isoenzyme which does not have constant stability.

—A. G. W.

An Early Warning Clot Detector for the SMA 12/60. Melley, D., Little, A., Rothfield, R. D. (1972), *Clin Chim. Acta*, 40, 273.

This photodiode device detects the small bubble which enters the sample line each time the probe passes between the sample and the wash well. This bubble causes the light reaching the detector to change and as a result the signal from the photodiode also. This signal is amplified and a normal sampling pattern is heard as a constant series of short "beeps". Blockage disrupts this audible signal.

This device would be suitable for conventional autoanalyser channels. The total cost of components was approximately \$A20.

—A. G. W.

Detection of Interrupted Bubble Flow in the Technicon Automated Immunoprecipitin System. Hansen, R. J., Tschida, A. R., Markowitz, H. (1972), *Clin Chem.* 18, 677.

A simple unit is described for detecting interrupted flow in an automated immunoprecipitin system. The principle involved is conductivity change resulting from interruption of bubble flow. A more complex device than the above.

—A. G. W.

Factors Affecting the Results of Serum Cholesterol Determinations. An Interlaboratory Evaluation. Fasce, C. F., Vanderlinde, R. E. (1972), *Clin. Chem.* 18, 901.

The results of an interlaboratory survey are presented in which methodology, standardisation techniques, type of standard, reagent source, and control sera used are considered. Each of these factors is evaluated on both clear and icteric sera, with respect to accuracy, precision, and random error. Most laboratories used direct techniques and did not use correction procedures when needed. It was found that not all extractants effectively prevent bilirubin interference. The authors found that direct procedures have best precision but not in the presence of bilirubin. Participants uniformly exhibited better precision than interlab agreement regardless of the variable being studied. They suggest the interaction of variables should be studied when a method is being evaluated.

—A. G. W.

Estimation of Normal Ranges from a Control Sample Survey. 1. Sex and Age Related Influence on the SMA 12/60 Screening Group of Tests. Reed, A. H., Cannon, D. C., Winkelman, J. W., Bhasin, Y. P., Henry, R. J., Pileggi, V. J. (1972), *Clin. Chem.* 18, 1, 57.

A panel of 13 clinical chemical test was performed on sera from 1,419 clinically normal adults, utilising the SMA 12/60 Autoanalyser to obtain practical normal range estimates by nonparametric statistical methods. Serum samples were obtained over a period of 14 months from eight geographic areas of the United States. Normal range estimates are derived for the entire group surveyed, for subgroups of males and females and for various age categories within each of the two subgroups. Derived normal ranges that are practical for clinical laboratory usage are proposed. Finally, a measure of validity of our estimates of normal range is applied.

—A. B.

Effect of Some Drugs on Clinical Laboratory Values as Determined by the Technicon SMA 12/60. Singh, H. P., Herbert, M. A., Gault, M. H. (1972), *Clin. Chem.* 18, 2, 137.

The quantitative *in vitro* effect of 45 drugs on 12 automated biochemical laboratory tests done on the Technicon SMA 12/60 were examined. Of these, 25 drugs in 1m mol/litre concentration interfered in one or more of the assays.

Only eight drugs (N-acetyl-p-aminophenol, p-aminosalicylate, isoniazid, L-dopa, a-methyldopa, 6-mercaptopurine, sulphathiazole, ascorbic acid) reacted positively with the test reagents at concentrations likely to occur in plasma after administration of a maximum single therapeutic dose.

A slight false elevation of plasma uric acid and a major false increase in urine values occurred after ingestion of N-acetyl-p-aminophenol and phenacetin, corresponding to the greater concentrations of these drugs and their metabolites in urine.

The relative importance of various factors affecting the influence of drugs on biochemical test values is discussed. —A. B.

Alkaline Phosphatase: Lability in Fresh and Frozen Human Serum and in Lyophilised Control Material. Massion, C. G. and Frankenfeld, Jean K. (1972), *Clin. Chem.* 18, 4, 366.

On storage the activity of alkaline phosphatase (AP) in human serum increases.

Activity of 10 fresh sera increased by an average of 0.9, 2.7 and 6.1 percent in 6, 24 and 96 hours: that of pooled serum frozen and thawed, increased by about 1 percent per hour. For 22 lots of reconstituted lyophilised material the increase ranged from 3 to 22 percent in six hours. Refrigeration greatly decreased, but room temperature restored the rates of change. Freezing decreased the activity of some lots, and most had a smaller rate of increase. —A. B.

Serum Gamma-glutamyl Transpeptidase Activity as an Indicator of Disease of Liver, Pancreas or Bone. Lun, K. and Gambino, S. R. (1972), *Clin. Chem.* 18, 4, 358.

Serum Gamma-glutamyl transpeptidase (GGT) activity was above normal in all forms of liver disease studied (viral hepatitis, cirrhosis, cholecystitis, metastatic carcinoma to liver, pancreatic carcinoma, liver granuloma and acute pancreatitis) GGT more sensitively indicated hepatic disease than did alkaline phosphatase. GGT was disproportionately more active in relation to the transaminases in cases of intra- or extra-hepatic biliary obstruction: the reverse was true in cases of viral hepatitis. GGT activity was normal in children, adolescents and pregnant women, and in cases of bone disease and renal failure. —A. B.

Detection of Serum Hepatitis Antigen by Charcoal Particle Agglutination-inhibition. Stevens, R. W., Ziegler, F. D., Kelly, J. H. (1972), *J. Immunol.* 108, 1123.

A method was developed for the detection and quantitation of the antigen related to serum hepatitis (SH). The test reagent is SH antigen adsorbed to particulate charcoal. These particle-antigen com-

plexes are macroscopically aggregated by homologous antisera and inhibition of this agglutination by a serum specimen forms the test. This communication describes the method and reports the findings of a serologic evaluation.

The results indicate that the particle technique is sensitive, reproducible, simple and rapid. The test reagents may be standardised and preserved for convenient use.

Alternative procedures, such as complement fixation, haemagglutination inhibition, and radioimmunoassay have the advantage of greater sensitivity, but technical complexities and reagent instabilities limit their application.

The described technique may serve as a model for the study of other virus antigen-antibody reactions, and for the development of automated particle test systems. —J. H.

Amniotic Fat Embolism: New Laboratory Diagnosis and a Case Presentation. Huaman, A., Nice, W., Merkel, R. L., Meidinger, R., Gillesse, T., (1972), *J. Kans. med. Soc.*, 73, 441.

The symptoms of pulmonary microembolisation require immediate diagnosis and treatment if a fatal outcome is to be averted. The fat component of amniotic fluid has been neglected both as an embolising particle and as a clue for diagnosis. In this publication the authors present their methods, based on oil red staining, for the qualitative investigation of fat in urine and in sputum, as well as a fat-particle counting technique for venous blood. —J. H.

The VMA Test Strip: A New Tool for Mass Screening, Diagnosis, and Management of Catecholamine-secreting Tumors. Leonard, A. S., Roback, S. A. Nesbit, M. E. Jr., Freier, Esther (1972), *J. Pediat. Surg.*, 7, 528.

Neuroblastoma is among the most common and highly malignant varieties of cancer in the paediatric age group. During the five-year period 1960-1964, 1,535 children in the United States are known to have died of this neoplasm. Progress towards increasing long-term survival in childhood neuroblastoma has been limited by the fact that between 50 percent and 75 percent of the patients have disseminated disease at the time of their original diagnosis. Yet if patients are operated upon early, before spread has occurred, long-term survival in up to 80 percent of the patients is possible.

The authors have developed a 3-methoxy-4-hydroxy-mandelic acid (VMA) test strip for urine, based on the LaBrosse spot test. If such a "dip stick" can be put into wide useage by physicians seeing large numbers of infants and children, neuroblastoma can be diagnosed at increasingly early periods in its evolution. —J. H.

Reagent Strip and Sulphosalicylic Acid Tests as Screening Methods for Proteinuria. Joramo, T. A. (1972), *Amer. J. Med. Technol.*, 38:3, 298-301.

The paper compares the Labstix and Sulphosalicylic acid tests for detecting proteinuria. In view of 12.4 percent discrepancy between results with the two methods the author suggests that more than one test should be used for the detection of proteinuria. —D. G. B.

HAEMATOTOLOGY

Active Extrusion of Heinz Bodies in Drug-induced Haemolytic Anaemia. Amare, M., Lawson, B., Larsen, W. E. (1972), *Brit. J. Haem.*, 23, 215.

This paper reports a case of a sulphonamide-induced haemolytic anaemia with formation of Heinz bodies (70-80% of red cells) which had the exceptional feature of being actively extruded from the red cells in a manner resembling the normal physiological denudation of a maturing erythro- or normo-blast. Photomicrographs and scanning electron microscope pictures are included to show this phenomenon. Red cell biochemical studies included normal glutathione and glutathione stability tests. Heat stability tests for unstable haemoglobin and haemoglobin electrophoresis showed no abnormalities.

—M. J. G.

Use of the Cellscan/GLOPR System in the Automatic Identification of White Blood Cells. Preston, K. (1972), *Bio-Med. Engineer*, 7, 226.

A completely automatic system of differentiating blood leucocytes is described. Initially the Perkin-Elmer Corporation of the United States developed a blood smear scanner which automatically detected the presence of binucleated lymphocytes as indicators of radiation damage. Eight years later Perkin-Elmer produced a system consisting of a completely automated television microscope associated with three digital computers used in various stages of blood cell recognition.

The stained slide is placed under an oil-immersion objective and focused manually. Then the Leucocyte Finding Computer operates with automatic focus. As soon as a leucocyte is sensed, a signal goes to the Main Control Computer and the TV scanner relays both red and green images so a photometric histogram is constructed and a third computer (GLOPR) analyses cell topography, including total cell area, nuclear area, nuclear fine structure, and fine and coarse irregularities (concavities), colour of cytoplasm and nuclear elongation. The results showed excellent correlation with visual differential counts, and the system can also be extended to the cytology of cervical smears.

—M. J. G.

Re-assessment of the Reliability of the Haematocrit. England, J. M., Walford, D. M., Waters, D. A. W. (1972), *Brit. J. Haem.*, 23, 247.

Trapped plasma accounts for approximately 3 percent of the normal red cell column in a micro-haematocrit but this paper emphasises that abnormal red cells, particularly hypochromic cells and sickle cells, trap considerably greater amounts of plasma

in the haematocrit than do normal cells and this is responsible for the marked fall in MCHC in hypochromic anaemia. Over 20 percent of trapped plasma may occur in sickled blood.

These authors suggest that automatic blood counters of the Coulter S type be set to record a true haematocrit without trapped plasma.

—M. J. G.

Study of In Vitro Lysis of Paroxysmal Nocturnal Haemoglobinuria, (PNH) and PNH-like Red Cells. Ferrone, S., Marubini, E., Mercuriali, F., Sirchia, G. (1972), *Brit. J. Haem.*, 23, 5.

In paroxysmal nocturnal haemoglobinuria (PNH) the intensity of haemolysis obtained in the acidified serum and sucrose-haemolysis tests—the two procedures most commonly used for the laboratory diagnosis of the disease—varies according to the red cells, serum and test used. The influence of these three variables has been investigated on both PNH and PNH-like lysis, that is lysis of PNH cells and of normal red cells treated *in vitro* with the sulphhydryl compound 2-amino-ethylisothiuronium bromide (AET). The conclusions, almost identical for PNH and AET lysis experiments, suggest that for the laboratory diagnosis of PNH: (1) it is advisable to use both tests in parallel; (2) sera to be used in the two tests should be selected; (3) for this selection AET cells can be employed when PNH cells are not available.

—M. J. G.

The Zeta Sedimentation Rate. Bull, B. S., Brailsford, J. D. (1972), *Blood*, 40, 550.

The zeta sedimentation ratio (ZSR) is a measurement similar to the determination of the erythrocyte sedimentation rate (ESR) but possesses several advantages. It is unaffected by anaemia and responds in a linear manner to increase in fibrinogen and/or gamma globulin. The normal range is identical for males and females. A blood sample, contained within a vertically oriented capillary tube, is subjected to four cycles of dispersion and compaction. At the conclusion of this process, the haematocrit of the blood in the red cell-containing portion of the capillary tube is measured. This haematocrit, a measure of the closeness with which red cells will approach each other under a standardised stress, is the ZSR. The test requires a specific piece of new equipment, the Zetafuge and special capillary tubes. A photograph of this equipment is featured in the paper. The ZSR is obtained within five minutes which is a considerable advantage compared with the conventional ESR.

—M. J. G.

MICROBIOLOGY

A Review of Some Common Sources of Error in the Preparation of Agar Media. Barry, A. L., Fay, G. D. (1972), *Amer. J. Med. Technol.*, 38, 241.

The authors present an interesting series of observations of the normally uncontrolled variables

in culture medium preparation. These are introduced by inadequate control of the varying stages of rehydration and sterilisation of dehydrated commercially prepared media. The importance of the correct storage of opened jars of dehydrated media

is discussed and experimental evidence is produced indicating that six months is the maximum period which should elapse between opening a container of dehydrated media and reconstituting it. The water loss by evaporation from plates poured at 50°C and in excess of 90°C was measured and the authors conclude that an undesirable water loss takes place if plates are poured at high temperatures. This interesting paper is required reading for all interested in quality control in microbiology.

—D. G. B.

A Simple Concentration Method for the Detection of Parasitic Ova and Cysts in Faeces. Thomson, R. G. (1972), *J. Clin. Path.*, 25, 546-547.

This technical method describes a technique using formalin, alcian blue and nigrosin which is claimed to be simple and economical in time and to enhance the chances of detecting ova and cysts of common intestinal parasites.

—D. G. B.

Selective Medium for the Isolation of Bordetella Pertussis and Parapertussis. Sutcliffe, E. M., Abbott, J. D. (1972), *J. Clin. Path.*, 25, 732.

Cephalexin has a wider spectrum of activity than the benzylpenicillin usually used as a selective agent in media for the isolation of *Bordetella* species. *Bordetella pertussis* is not inhibited by 80mg/ml and *Bordetella parapertussis* by 40mg/ml. The suggested medium comprises Oxoid charcoal agar (CM 119) with 10 percent horse blood, 1 percent Difco Proteose peptone No. 3 and 40mg/ml cephalixin. Comparative data with the same medium being benzylpenicillin as an inhibitor is presented.

—D. G. B.

Rapid Differential Testing of the Enterobacteriaceae Using Modified Standard Media. Brooks, W. F., Goodman, N. L. (1972), *Amer. J. Med. Tech.*, 38, 429.

Another method for the rapid identification of members of the Enterobacteriaceae is presented. The basis of the scheme is the use of a very heavy inoculum of the organism to be tested and standard media modified by incorporating twice the concentration of substrate. The modified media used were lysine and ornithine decarboxylase, urease, tryptophan broth, glucose and lactose broth—a Kligler iron agar slope is also used. The modified media were found as reliable as conventional tests with the exception of the lactose broth, and an identification scheme is presented together with evidence of its efficiency. The great advantage of the method is that results can be read after four hours' incubation.

—D. G. B.

Quantitative Sputum Culture as a Means of Excluding False Positive Reports in the Routine Microbiology Laboratory. Wilson, M. J. B., Martin, D. E. (1972), *J. Clin. Path.*, 25, 697.

A technique for the homogenisation and dilution of sputum specimens is described. The improvement in the accuracy of bacteriological diagnosis when pathogens present at least 10^7 per ml are regarded

as of no significance is stressed. Although the homogenisation and dilution technique is described as simple, this reviewer feels that it is not simple enough for routine use in smaller laboratories requiring as it does two weighing operations.

—D. G. B.

Resistance of Staphylococcus aureus to Sulphamethoxazole and Trimethoprim. Makhla, L. S. (1972), *J. Clin. Path.*, 25, 708.

An investigation into the incidence of resistance to sulphamethoxazole trimethoprim by *Staphylococcus aureus* is described. The organisms were clinical isolates from hospital patients in the London area. Of 675 strains tested 18.5 percent were resistant to sulphamethoxazole and 1.6 percent to trimethoprim. All trimethoprim-resistant organisms were resistant to sulphamethoxazole, these resistant organisms were also resistant to several other antibacterial agents. The most usual phase pattern was 84/85, 84 or 85.

—D. G. B.

Normal Electrolyte Broth—A New Blood Culture Medium. Burton, R., Freake, R. (1972), *Amer. J. Med. Technol.*, 38, 437.

A new blood culture medium having many parameters adjusted to approximate to human blood is described. It contains glucose, urea and inorganic salts besides a nutrient broth. The results achieved in comparison with three commonly used blood culture broths in a large series of patients are impressive. The authors also mention as an incidental finding the important fact that washed reused blood culture bottles appear to be inhibitory, some possible reasons for this are discussed.

—D.G.B.

Anaerobic Bacteriology in the Clinical Laboratory. Marymont, J. H., Holdeman, L. V., Moore, W. E. C. (1972), *Amer. J. Med. Tech.*, 38, 441.

A brief résumé of the methods used to isolate and speciate anaerobic organisms in the authors' laboratory is given. The main points are: the use of pre-reduced media prepared under O_2 free conditions. Plating of specimens within 30 minutes of collection and the use of gas chromatography in the speciation of isolates. The paper is thought provoking and should be read by all engaged in clinical microbiology.

—D. G. B.

Evaluation of a Technique for the Estimation of Serum Bactericidal Activity against Gram-negative Organisms. Taylor, P. W., Roberts, A. P., Gowen, P. E. (1972), *Med. Lab. Tech.*, 29, 272.

The authors describe a technique for estimating the *in vitro* antibacterial activity of human serum against gram-negative bacteria. The method is fully described and the effects of varying components of the system described. The complex pattern of bacterial response to the antibacterial effect of serum is also discussed.

—D. G. B.

Observations on the use of Anaerobic Jars and the Methods of their Control. Jarvis, J. D., Bruten, D. M. (1972), *Med. Lab. Tech.*, 29, 325.

An interesting paper discussing the control of anaerobiosis using McIntosh and Fildes jars. The authors discuss the methods used in their laboratory to reduce the failure rate using these jars from 12 percent to 2 percent. The methods are mainly common sense, such as the use of freshly prepared anaerobic indicators, the use of cultures of *Cl. Tetani*, etc., but they can bear repeating. A most useful paper for all concerned with the control of quality in bacteriology.

—D. G. B.

The Deoxyribonucleases and Hyaluronidases of *Clostridium septicum* and *Clostridium chauvoei*. Princewell, T. J. T., Oakley, C. L. (1972), *Med. Lab. Tech.*, 29, 243.

1. An agar plate method for testing for deoxyribonuclease, 255-260.
2. An agar plate method for testing for hyaluronidase.

These two papers describe plate methods for the qualitative and quantitative estimation of deoxyribonuclease and hyaluronidase in *Clostridium* species. The methods appear more applicable to research than diagnostic work.

—D. G. B.

A Dual Composite Medium for the Differentiation of the Pathogenic Enterobacteriaceae and Coliform Organisms. Fuscoe, F. J. (1972), *Med. Lab. Tech.*, 29, 261.

An interesting system for the identification of Enterobacteriaceae is described. Two of three component media are used depending on the organism suspected. The media are capable of detecting the following characteristics: Motility, fermentation of glucose, mannitol indole production, H₂S production, B-galactosidase and phenylalanine deaminase. Urea may also be incorporated into one of the media. The results presented are impressive and as a screening technique the media may be of some use. The media are difficult to prepare.

requiring the aseptic addition of several constituents and are of fairly limited shelf life and expensive.

—D. G. B.

Effect of Antibiotic Resistance on the Survival of *Staphylococcus aureus*. Lacey, R. W. (1972), *J. Clin. Path.*, 25, 713.

The survival of staphylococci on glass slides at room temperature and at 37°C showed no significant difference regardless of the antibiotic resistance pattern of the organisms.

—D. G. B.

Evaluation of Various Media for Growth of Selected Pathogenic Fungi and *Nocardia asteroides*. Dolan, C. T. (1972), *Amer. J. Clin. Path.*, 58, 339.

As a number of known infections by *Nocardia asteroides*, *Histoplasma capsulatum* and *Blastomyces dermatitidis* appear to be increasing the author thought it worth while to investigate common laboratory media for their ability to grow these organisms. He concludes that brain heart infusion or heart infusion supplemented with 5 percent human blood are satisfactory but that trypticase soy agar similarly supplemented is clearly inferior.

—D. G. B.

A Simple Quantitative Urine Culture Method using an Internally Coated Plastic Pipette. Kerim, C. M., Bergerson, J. A. (1972), *Amer. J. Clin. Path.*, 58, 371.

Yet another semi-quantitative method for the enumeration of bacteria in urine is described. This particular technique relies on coating the inside of sterile polystyrene pipettes with a small quantity of media. The urine is sucked into the pipette and discharged at a controlled rate. After incubation colonies are counted. Although ingenious this method suffers from several disadvantages not the least being the difficulty of subculturing colonies. I feel that it offers no advantage over the Dip slide, media coated container or blotting paper techniques already in use. Full technical details are given in the paper.

—D. G. B.

Book Reviews

Problem Solving, Systems Analysis and Medicine. Ralph Raymond Grams, MD (1972). 225 pages. Charles C. Thomas, Springfield, Illinois. Price \$US19.75. Also a Systems-analysis Workbook co-ordinated with the textbook. Price \$US4.75.

I experienced a quickening of interest upon the arrival of this text. It promised enlightenment of a subject which is starting to impinge upon our daily work and as it tends to question the conduct of our affairs it would seem timely and appropriate to become better acquainted with the science.

The author is medically qualified and his sphere of interest includes laboratory medicine and medical problem solving. The intention is that the text should act as a stimulus for those interested in the design and management of medical systems and in the application of systems analysis to this end. Under the circumstances it seemed desirable that the book should be evaluated by a lay person rather than a practitioner already converted to the technique. Of its intrinsic value there can be little doubt and a prime example of the use of "SA" was that of transporting men to the moon. The bias is towards a textbook presentation. The first five chapters explaining the structured format, flowcharting, the production of a systems book and the practical applications. The second half is devoted to working through procedures resulting in a systems book containing propositions for a biomedical library. There is also a specimen composite flowchart for general use and a plastic template of the conventional shapes employed.

The accompanying workbook, of some 60 pages, is divided into two phases. The first is objective and consists of a series of questions on factual matters which can be derived from the text and are in effect an examination of the knowledge derived from it. The second phase is subjective and calls for judgment and evaluation of different situations. The problems are thoroughly discussed and commented on and answers are provided in the last part of the book.

The introduction starts by quoting a poem of Rudyard Kipling which effectively outlines

the spirit of enquiry required and indicates the six basic areas.

"I keep six honest serving men

(They taught me all I knew);

Their names are What and Why and When

And How and Where and Who."

It is obvious that the crucial step is always to define the problem and the method provides for constant re-evaluation of the problem. This may be modified in the course of the investigation perhaps out of all recognition! Initially free rein may be given to the possible solutions by avoiding "tunnel vision". Peripheral issues must be discarded and ambiguity avoided. Stress is laid on the importance of using "the man on the job", whose intimate knowledge and experience is indispensable in problem solving.

A chapter is devoted to the various figures and conventions used and a number of these relate to computers, although "SA" is not necessarily involved in this way. The technique can become quite complex and where time dependent and if there is a rate limiting step the concept of zero order, first order and second order reaction may be brought in. The situation may also be event-dependent or interdependent but best considered separately.

The text was not really easy to follow. This was due to unfamiliar terminology and insufficient information. The various flowcharts are accompanied by an explanatory table or "operations statements" some of the expressions such as "level 2", were strange and the meaning of the symbols was not always clear. The symbol for offpage connection contained unexplained information and the tables which were numbered consecutively did not correspond with the flowchart number. The information was provided later on in the chapter but logically should have preceded the flowcharts, or some form of cross-reference given.

The tenth and eleventh lines from the bottom of page 93 are not as I first imagined an example of "SA" English but a simple transposition! In general the book has been carefully produced. There is no specific

example of "SA" application to the problems of the clinical laboratory nor I suppose could one expect this in the context of a textbook approach. After reading the text and applying oneself to the workbook it should be possible to make a start at using the technique in work situations.

—R. D. A.

Chemical Instrumentation: A laboratory Manual based on Clinical Chemistry. Gary T. Bender, PhD, 291 pages, illustrated. W. B. Saunders Company, Philadelphia, London, Toronto. Obtainable from N. M. Peryer, Christchurch. Price in N.Z. \$9.50.

This book written by an associate professor in chemistry has attempted to use the unusual approach of teaching instrumentation by using medical laboratory methods in experiments. Each chapter has a theoretical introduction to the instrument followed by two or three experiments. For example in the first chapter on visible absorption spectroscopy, methods on total protein, cholesterol, and uric acid are detailed using a simple spectrophotometer. The theoretical knowledge on each analytical system is fairly thorough and well illustrated. References for further reading, and of the methods used are given.

The book has five sections: Section I deals with absorption spectroscopy, with chapters on visible, ultraviolet, infra red, and fluorescent spectroscopy. Section II, Introduction to Flame Methods, describes flame and atomic absorption spectroscopy. Section III, Multi-stage Separation, has chapters on column chromatography, paper chromatography, thin layer chromatography, ion exchange methods, gel permeation chromatography, gas chromatography, and electrophoresis. The fourth section, Electroanalytical Methods, has chapters on potentiometric methods, coulometry, and voltometry.

The final section is entitled Novel Methods in Clinical Chemistry, and has chapters on kinetics, and the use of enzymes in clinical chemistry.

One regrettable feature is the author's adherence to obsolete expressions such as mg% and ppm. No attempt to use International Units has been made.

The safety appendix is compiled with reference to the chemicals used in the book so it has a limited use.

I conclude that from the medical laboratory point of view this book might only be of use to a tutor technologist. In the hands of a trainee it could be confusing. Using the index to find how a particular test is performed directs the reader to only one section on instruments. Many methods are not mentioned at all. I would also regard this book as outdated. Many of the instruments described are early models. For example the section on flame photometry describes the use of the "Coleman 21" flame photometer and no mention is made of instruments using an internal lithium standard.

However, the author's intent is to provide a guide to a practical course in laboratory instrumentation and it may well fulfil this purpose.

—A. G. W.

Ultramicrotomy. R. L. Griffin. Baillière Tindall, London, 93 pages, illustrated. Price in U.K. £1.80.

This book is one of a series of laboratory monographs and deals with the practical aspects of preparing tissues for viewing in an electron microscope. One does not have to read very far before realising that the book has been written by an expert with a great understanding of his subject.

Tissue fixation and the preparation of the two most widely used EM fixatives are described in detail, brief mention being made of other fixatives. Epikote (Epon) and Araldite are the embedding resins in general use and embedding schedules and formulae for the above resins are given in detail. Knifemaking by hand and using the LKB knifemaker is described. Siting of ultramicrotomes and microtomy to produce thick sections for light microscopy and thin sections for electron microscopy is described in detail. A short section on troubleshooting when sectioning contains a wealth of information. Staining sections with heavy metals is covered along with a short chapter on problems encountered with difficult tissues such as skin, blood cells, etc. A final chapter on the techniques to

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Of necessity a book of this nature must be concise and contain the "nitty gritty" of the subject being dealt with; the author succeeds in both respects. One slight disappointment for me was the omission of details about phosphate buffers and Porter-Blum ultramicrotomes but this is probably a personal fad. It is usual to designate an area of laboratory or library space for new books and I would recommend that this one be moored to the laboratory ultramicrotome.

—D. T.

Practical Automation for the Clinical Laboratory. Wilma L. White BA, Marilyn M. Erickson BS, and Sue C. Stevens BA MA PhD FRIC. 591 pages, profusely illustrated. C. V. Moseby Company, Saint Louis (1972). Price \$US22.50.

Four years ago I had the opportunity to review the first edition of this book and in that time sufficient additional material has been added to increase the size and price by a half.

The book is divided into sections. That dealing with general aspects assumes no previous knowledge and gives a well-illustrated exposition of mechanical and electrical aspects of automation very suitable for practical instruction.

The second section, Chemistry, comprises more than two-thirds of the book and the bulk of this is devoted to Autoanalyser systems. The other systems dealt with are the Beckman DSA 560, GEMSAEC, Hycel Mark X, Instrumentations Laboratory Clinicard System and the Sherwood Digecon System. Atomic absorption and flame emission are also included.

Without attempting to assess the relative merits, it is interesting to note that only the Autoanalyser continues to use continuous flow. Some of the older discrete systems have been dropped from this edition and those mentioned are gaining increasing acceptance. One trend is the incorporation of direct computer control or interfacing facilities but there is considerable diversity in the approach. The DSA 560

is a dual channel modular pneumatic system using discrete sampling, paper filtration and up to 160 tests per hour. The GEMSAEC (automatic fast analyser) is a "revolutionary" system using centrifugal mixing and transfer with multiple readings as the cuvettes revolve before the spectro-photometer. The Mark X could be described as an automated duplication of manual procedures. The Clinicard analyser is programmed by punch card and the reactions carried out in disposable plastic cuvettes prefilled with reagent. The Digecon is essentially constructed to measure rate reactions and is capable of measuring the initial maximum rate. All the Autoanalyser systems up to the expected *début* of the SMAC are described. Further chapters discuss linear conversion and continuous *in vivo* monitoring. There are a number of smaller sections covering automatic blood bank procedures, serology tests for syphilis, an antibiotic susceptibility system, the Hema-Tek slide stainer, the Coulter Counter Model S and the Cyto- and Histo-Tek slide stainers.

There is a final chapter on computer application in the clinical laboratory using the Clinical Lab 12 system for illustration. A substantial glossary is appended.

In my experience this has proved a useful book. It is true that a great deal of the material has been gleaned from working instructions of the various systems but it is convenient to have all this information under the same cover. I found the first edition useful to give to people seeking advice on what to read, to learn something about automation. The wider spectrum of apparatus covered in this edition will increase its usefulness in this respect.

Although I did not often refer to the previous edition for help in practical maintenance I found the expositions clear and simple to follow (the chapter in the present edition on data processing is particularly good), and very suitable as a text for teaching purposes.

I experienced a burning sensation on inspecting the extracurricular-Christmas-party-drink-producing manifold in the epilogue. I think this was because the line for diluting the gimlet with lemon and paeroa was missing!

—R. D. A.

What's New?

AUTOMATIC SAMPLING EQUIPMENT CUTS ANALYSIS COSTS

A sampling unit designed to provide continuous unattended operation for long periods, of the Pye Series 104 chromatographic analysis equipment, has been introduced by Pye Unicam Limited of Cambridge England. It enables the chromatograph to be left working not only overnight but for a complete weekend if necessary.

Known as the Pye Autojector S4, the new sampler is supplied with standard programmes that, once started, will automatically sample and inject into the chromatograph up to 100 samples for analysis, and at the end of the run will switch itself off. These features enable users to increase utilisation of their chromatographic equipment and at the same time reduce costs by cutting down on operator time.

The Autojector S4 consists of a removable sample carrier of the turntable type; a pneumatically operated syringe, mounted vertically, to perform the functions of flushing, filling and injection; and two timing units which control the sequence of operation.

Standard programme tapes for various syringe sequences are supplied with the sampling equipment, but alternatives can be cut by operators if required. Correct operation can be checked manually. The unit can handle samples of any liquid that can be dealt with in manual microlitre syringes.

FILTRATION USING GLASS-FIBRE PAPER

by
John C. Meakin,

Technical Service Manager, W. & R. Balston Ltd.,
Maidstone, England
(manufacturers of "Whatman" filter media)

Borosilicate glass is converted into fibrous form by subjecting it simultaneously to heat and a high-velocity gas blast.

The resulting glass fibres are unique among the many fibrous materials used commercially for filtration. They differ from naturally occurring cellulose fibres in that they are circular in section, straight and of uniform diameter; they are also of considerably greater density and can be made far finer. The fibres range in diameter from 0.05 micron upwards, and by careful choice of conditions the actual diameter can be closely controlled. Furthermore, glass fibres do not suffer a change of form when prepared for processing, retaining their original diameter and cylindrical shape without the swelling of fibrillation (longitudinal splitting) exhibited by natural cellulose and asbestos fibres.

Binderless Paper

Glass micro-fibres can be formed into paper using conventional papermaking equipment. To achieve satisfactory strength without adding binders, great care in handling is required and the fibres must be dispersed under acid conditions. Some manufacturers do add organic binders, but since these materials lack the chemical and thermal stability of the glass, they severely limit the application of such papers.

The all-important factor controlling the filtration characteristics of glass-fibre paper is the average diameter of the fibres used. To produce a more retentive filter, a finer grade of fibres is chosen; the resulting paper has a much larger surface-area of glass on which particles can be deposited, and the gaps between fibres are smaller (see Fig. 1). However, the number of gaps is much greater, and hence through-flow of liquid or gas remains rapid.

In contrast, to produce a more retentive filter from cellulose, the fibres are beaten or swollen or the sheet is pressed—which reduces the size of the existing pores without increasing their number. This explanation is over-simplified, but it indicates one basic reason for the higher permeability of glass-fibre papers at a given particle-retention efficiency.

Types of Filter

Apart from glass-fibre and cellulose, other forms of fibrous filter available include asbestos/cellulose sheets, and asbestos pads formed *in situ* as in the Gooch filter. Next, there are sintered materials—including metals and plastics as well as glass, which is the most common form in the laboratory. And finally, membrane filters, commonly made from cellulose esters, are available in many pore-sizes. The membrane filter is, essentially, a very thin film with fine, non-interconnecting pores running from one side to the other.

Fibrous and sintered materials both filter through-out their depth and are known as depth filters. The concept of pore-size is here very theoretical. The particle-retention efficiency of a depth filter is dependent upon flow rate, fluid viscosity, type and concentration of the contaminant, and the complexity of the spatial arrangement within the filter matrix. In practice, efficiency can be realistically determined only by direct measurement; several standard methods are used.



Three general effects are found with all depth filters. Increase in filter thickness at constant density results, firstly, in increased resistance to flow (i.e., lower flow rate for the same pressure differential), but, secondly, in increased total load capacity before the filter becomes choked. And thirdly, a depth filter always removes particles more efficiently from gases than from liquids—an effect largely due to viscosity, the particles in a gas being less firmly “bound” to the streamlines of flow, and hence more easily captured. This last effect occurs to only a slight degree with membranes.

Surface Retention

Membrane filters are surface retention filters. Their particle-retention efficiency is easily defined in terms of pore-size. This is usually determined by measuring a related physical property, e.g., the pressure required to force an air-bubble through the sheet when immersed in a standard liquid. Membrane filters are capable of extremely high standards of filtration, but they suffer the disadvantages of a lower total load capacity than depth filters and of a resistance to fluid flow considerably greater than for glass-fibre papers of equivalent particle-retention efficiency.

Furthermore, because the pores do not interconnect, very little lateral diffusion can occur within a membrane. Suction applied at a few widely spaced points is therefore not satisfactory, and special filter supports which leave a large free area beneath the membrane have to be used—such as sintered glass or stainless-steel mesh. An edge-clamp is also required since membranes, unlike cellulose or glass-fibre papers, do not seal readily to flat surfaces. This special apparatus can add considerably to the already high cost of membrane filters.

Other important comparisons concern thermal and chemical stability. Whereas cellulose paper, membrane filters and sintered plastics have severely limited temperature resistance, binderless glass-fibre has a working temperature range up to 500°C. Asbestos and sintered metals have even higher limits. Chemically glass-fibre paper has excellent resistance to organic solvents, its wet-strength increasing in non-polar solvents. It is biochemically and biologically inert and exhibits low protein-binding—an important factor in laboratory radio-immunoassays. It is also non-hygroscopic and thus stable in weight. In comparison, ordinary cellulose-ester membranes have poor solvent resistance, but membrane manufacturers use other polymeric materials to overcome this.

Applications—Liquid Filtration

One of the most common practical filtration problems is the removal of solid particles from aqueous systems, as in gravimetric analysis or the determination of suspended solids in waters or effluents. If the particles are smaller than 5 microns, the choice of filter media is limited.

Table 1 compares initial flow rates through different filter media suitable for this purpose,

giving the time required to pass 100ml of clean water. Filter areas were equal and the pressure difference across the filter was 30mm Hg in each case.

TABLE I

Filter Type	Thickness (mm)	Time (secs)
Retentive cellulose paper	0.20	760
Asbestos/cellulose sheet (“indicated porosity 3”)	3.50	410
Sintered glass (“porosity 4”)	2.00	250
Gooch filter (asbestos pad)	—	148.5
Membrane (3µm)	0.15	69.3
Glass-fibre paper—		
Whatman GF/B	0.73	72.0
Whatman GF/A	0.25	33.6

The new generation of minicomputer makers

A new generation of minicomputer manufacturers is gaining on the United States suppliers who have dominated the business until now. Manufacturers based outside the United States are growing in technical strength, while prices continue to fall and the choice for the customer is becoming more varied than ever. These three trends are vitally important to both manufacturers and users of minicomputers round the world. They emerge from the new “ERA Guide to Minicomputers”, a report designed to ease the problem of selecting a minicomputer.

“The ERA Guide to Minicomputers” published for ERA by Ovum Ltd., 22, Gray’s Inn Road, London WC1, at \$US69, includes details of these and 40 other machines available outside the United States. For the first time it makes available to a wider audience some of the information accumulated by the major research project, “Minicomputers and Europe” which was begun by a team in ERA’s Computers and Automation Division in 1970. Since then the team has gained a unique knowledge of the minicomputer scene, based on numerous personal visits to both suppliers and users.

In that time they have seen prices falling by a steady 10 percent to 20 percent a year on average—although, of course, cuts in the cost of particular models may be much sharper. Recent examples include the introduction of the Texas Instruments 960A, at about \$5,000, the cheapest machine covered in the guide, and the Digico 16-V at about \$6,300, little more than half the price of the essentially similar 16-P which is superseded.

On the technical side, probably the most important development from the user’s point of view is the continuing trend towards more varied and flexible programming facilities. One outstanding example is the Dietz Mincal 621, where a 256-byte semiconductor random access memory is used to take over the functions conventionally performed by registers. This allows a big gain in register capacity and programming facilities at some cost in speed.

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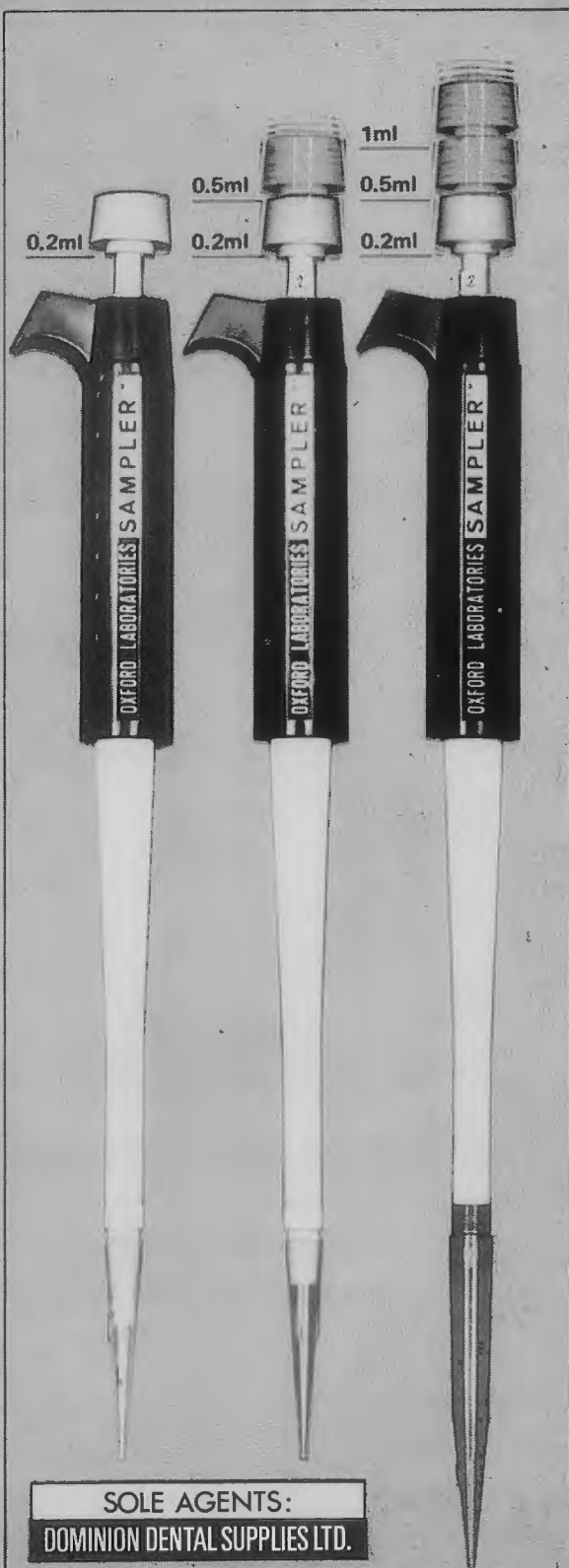
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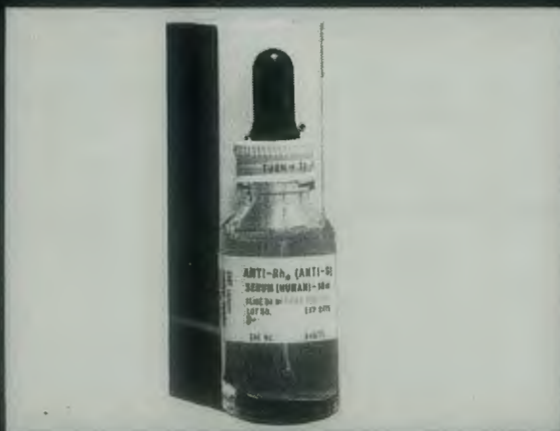
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