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# Bacterial Flora of the Gastro-intestinal Tract of Man: A Review

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*Received for publication, August 1972*

Within the last few years knowledge concerning anaerobic bacteria of man and other monogastric animals has increased and changed a great deal. With the development of improved culture techniques for the isolation of the more sensitive anaerobic organisms it has become possible to study more effectively these anaerobes. Studies carried out with these methods have shown that *no* area of the gastro-intestinal tract is sterile. Also, the predominant kinds of bacteria in the different areas of the tract are not the same, and that these bacteria are metabolically active. It has been further demonstrated, using these improved techniques, that these same species of anaerobic bacteria are also found rather frequently in infected tissue.

The following is an attempt to present some of the observations that have led to the above statements.

Classically, it has been taught that micro-organisms could not survive in the low pH created by the stomach acids. Any stomach and small intestine contents that were obtained (usually from patients at operation), were deemed to be sterile when no organisms could be cultured from them by the usual laboratory methods.

In the early 1960's some groups of workers (notably Hungate *et al.*[9, 10], during their rumenology research), realised that the culture media used in routine laboratory work may not be detecting all the organisms present. From this realisation came new techniques of anaerobic culturing, and with these techniques came a whole new concept of intestinal flora and their relationship with their host.

Animal models have been used extensively in studying intestinal flora. Schaedler *et al.*[14] showed that in healthy mice lactobacilli and anaerobic streptococci are extremely numerous in the stomach, small intestine, and large intestine. In contrast, the bacteroides group proliferates only in the large intestine. These three bacterial species persist at approximately constant levels in their characteristic localisation throughout the lifespan of healthy animals. The organisms were shown to be closely associated with the walls of digestive organs and are probably concentrated in the mucous layers.

Gnotobiology, the study of germ-free animals, was used to demonstrate the effects of colonisation of the gastro-intestinal tract by these organisms. Germ-free mice were given food contaminated with pure cultures of various bacterial species isolated from ordinary healthy mice. Cultures were given singly, or in association, or consecutively at weekly intervals. Whatever the technique of administration, the lactobacilli and anaerobic streptococci immediately established themselves throughout the gastro-intestinal tract and became closely associated with the walls of the animal's intestine. In contrast, the organisms of the bacteroides group were found in large numbers only in the large intestine.

Within a week of exposure the population of these three bacterial species reached levels similar to those found in ordinary mice. They remained at these characteristic levels throughout the period of observation (several months). Their presence resulted in a progressive decrease in the size of the caecum, which eventually became normal in appearance, having been abnormally large and thin-walled in the germ-free state. This is one of many observations that the autochthanous flora seems to affect the tissues in the organism's environment and has interesting parallels when certain human intestinal diseases are studied, such as blind loop syndrome.

Coliform bacilli multiplied extensively and perished at high rates in all parts of the gastro-intestinal tract in germ-free mice, even after these became colonised with lactobacilli, anaerobic streptococci and bacteroides. However, the coliform count fell precipitously within a few days after the animals were fed the intestinal contents of healthy, pathogen-free mice.

The authors suggest the components of the gastro-intestinal flora have become symbiotic with their host in the course of evolutionary development, and this constitutes a true autochthanous flora. The other components of the indigenous flora are acquired early in life, either through accidental contact or because they are ubiquitous in the environment. The "normal" flora is that which is always present in the environment of the animal colony under consideration.

Williams-Smith[7] reported a study on the pig stomach. The organisms appeared early after birth at a time when the pH was very low. They showed that there were organisms present in the muscularis mucosae, and that these were different from organisms in the stomach content. For example: Peptostreptococcus was present in the cardiac mucosae only, while Veillonella was present in the tissue from all regions of the stomach, but never in the stomach contents. *Candida albicans* was present in oesophageal and cardiac regions, and in the contents too.

Donaldson[2] points out that the techniques used for sampling humans are limited and difficult, and the culture techniques will probably not give the full picture. With the organisms being given fresh food and the waste food being removed, the human gut is essentially a continuous flow system. The influence of diet of the microbial population of the gastro-intestinal tract has long been under discussion. Speck *et al.*[14] showed that changes in the diet do not account for other variabilities seen in the bacterial flora. He reported that a marked stability still seems to be the dominant feature of the normal bacterial flora of the lower intestinal tract.

Gorbach[4], one of the leading figures in the research into gastro-intestinal flora, carried out quantitative and qualitative studies of microflora of the faeces of normal individuals aged 20 to 100 years. They showed that elderly subjects were found to harbour few anaerobic lactobacilli, and larger quantities of fungi and coliforms than young persons. Great variations in the numbers of bacteria were noted in people of the same age. Careful analysis of two small groups of individuals demonstrated that these differences were not due to errors or difficulties in sampling, or to variations in diet. Despite the differences between the faecal microbial flora of different individuals, the numbers of bacteria recoverable from the same person over a seven-week, or seven-month, period were noted to be remarkably stable.

Gorbach[5] then carried out a study comparing the microbial flora of ileostomy effluent, normal ileal contents, and faeces. He showed that there were significant differences in the numbers and relative prevalence of micro-organisms in each material, and concluded that ileostomy excreta appears to harbour a unique microbial ecology that is different from the micro-flora of the large or small intestine.

Many workers have studied and identified the micro-organisms which are present in the gastro-intestinal tract, and the following is a list of the major types present:

Staphylococci
Lactobacilli
Neisseria
Yeasts
Bacteroides
Streptococci
Fusobacteria
Bifidobacteria
Clostridia
Veillonella

Somewhat surprisingly it has been shown that the Enterobacteria contribute less than 0.1 percent of total bacterial population in the faeces. The dominant organisms were bacteroides and bifidobacteria, and these comprised more than 99 percent of the total organism population of the faeces. The enterococci and clostridia were very minor components of the flora (Drasar *et al.*[3]).

It became obvious that the study of experimental animals and human faeces was an inadequate approach for determining the microbial flora of the gastro-intestinal tract. Savage *et al.*[13] reported that gram-positive and gram-negative bacteria can be seen in the mucin on the mucosal epithelium in histological sections of biopsies of the human jejunum. Numerous spiral-shaped organisms can be seen attached to the epithelium and deep in foveae in histological preparations of human stomachs viewed by light or electron microscopy. Comprehensive examination of biopsy specimens from hospitalised persons revealed that micro-organisms are present in the mucins on the epithelium of the ileum and colon. Also Nelson *et al.*[11] reported that bacteria were closely applied to walls of cells lining the jejunum, appendix and colon, and that these bacteria appeared to be set in the mucus on the walls.

The most commonly seen types in descending order of numbers present were gram-positive bacilli, gram-positive cocci, and gram-negative bacilli.

Gorbach[6] concentrated on obtaining direct samples from the stomach and small intestines.

The surgical approach to studying normal flora of the gastro-intestinal tract is open to criticism because the bowel is in an unphysiological state. For example,

(a) The patient has been starved, which leads to diminished numbers of micro-organisms.

(b) Anaesthesia is known to alter intestinal mobility, which may also alter the numbers of micro-organisms.

(c) The method of aspirating intestinal juices from the bowel introduces unknown dilution factors.

(d) Subjects studied at surgery are, by definition, not healthy individuals.

They attempt to overcome some of these criticisms by using tube aspirations. The validity of this method was established in animal experiments and then by duplication sampling studies in man. The flora of the small intestine was found to be distinctive and to consist of small numbers of streptococci, lactobacilli, staphylococci, and fungi. These gram-positive elements often grew in gastric contents and appeared to increase in number in the distal area of the small intestine.

They also demonstrated a marked difference in number and types of micro-flora present on the proximal and distal sides of the ileo-caecal valve. This structure appears to demarcate two distinct microbial ecologies. The upper ileum contains small numbers of gram-positive bacteria, while bacteroides, anaerobic lactobacilli and coliforms predominate in the colon. The distal ileum harbours a variable micro-flora, and they suggest that this appears to be a transitional zone, between these two microbial populations.

Plaut *et al.*[12] also reported experiments where gram-positive cocci and rods were found in the mucosal layer adherent to the washed jejunal mucosa, but no structures resembling micro-organisms were demonstrated in the internal wall itself. These micro-organisms were cultured after vigorous washing of specimens of tissue removed by biopsy. The predominant microbial flora consisted of anaerobic streptococci and lactobacilli.

It has been established by Smith and Holdman[15] that there are 90-95 percent of gastro-intestinal tract bacteria which are obligate anaerobes, and the most commonly found are: Lipidobacteria, Bacteroides, Eubacteria, Proprionobacteria and Peptostreptococci—present in different concentrations in different parts of the tract. These bacteria are metabolically active; lactic acid (2.6mEq/100ml) is present in stomach contents when the stomach is full, produced by acid-resistant flora, and lactic acid is also produced in the small intestine but appears to be rapidly absorbed. In lower areas of the tract other fatty acids are produced by the microbial population; viz., butyric, proprionic, isovaleric, valeric, and caproic acids, also ethyl and other alcohols and amines are produced. Presumably these are absorbed from the tract and metabolised by the host.

**Effect on Host**

Gorbach and Tabagehali[7] studied the microbial populations of the small bowel and bile salt metabolism in 15 patients with lesions of the stomach and small intestine. The types of micro-organisms could be correlated with the site and extent of stasis in the small bowel and the presence of a normally functioning stomach. They could correlate the presence of obligate anaerobes (bacteroides) and free bile acids with areas of stagnation.

When these abnormalities were detected throughout the small bowel steatorrhoea was also noted and they suggest that the steatorrhoea may be related to bacterial decomposition of bile salts within the lumen of the small intestine. If the lesion remained localised, normal fat excretion occurred.

After treating three of these patients with antibiotics they suggest that Lincomycin may be especially effective in eradicating the anaerobic flora and restoring intestinal function to normal.

Smith and Holdman[15] found strict anaerobic bacteria in large numbers in so-called "sterile" abscesses from which bacteria were not established by other methods. In studies of 300 infections of the lung, urogenital tract, brain, blood and other sites, over 85 percent of specimens contained obligate anaerobes. Sometimes, but not always, facultative anaerobes were present.

The important piece of information to be obtained from their work was that these different anaerobic organisms had different antibiotic sensitivity patterns, hence the desirability of isolating all anaerobes present in the wound. For example, they isolated *Eubacterium lentum* from blood cultures and

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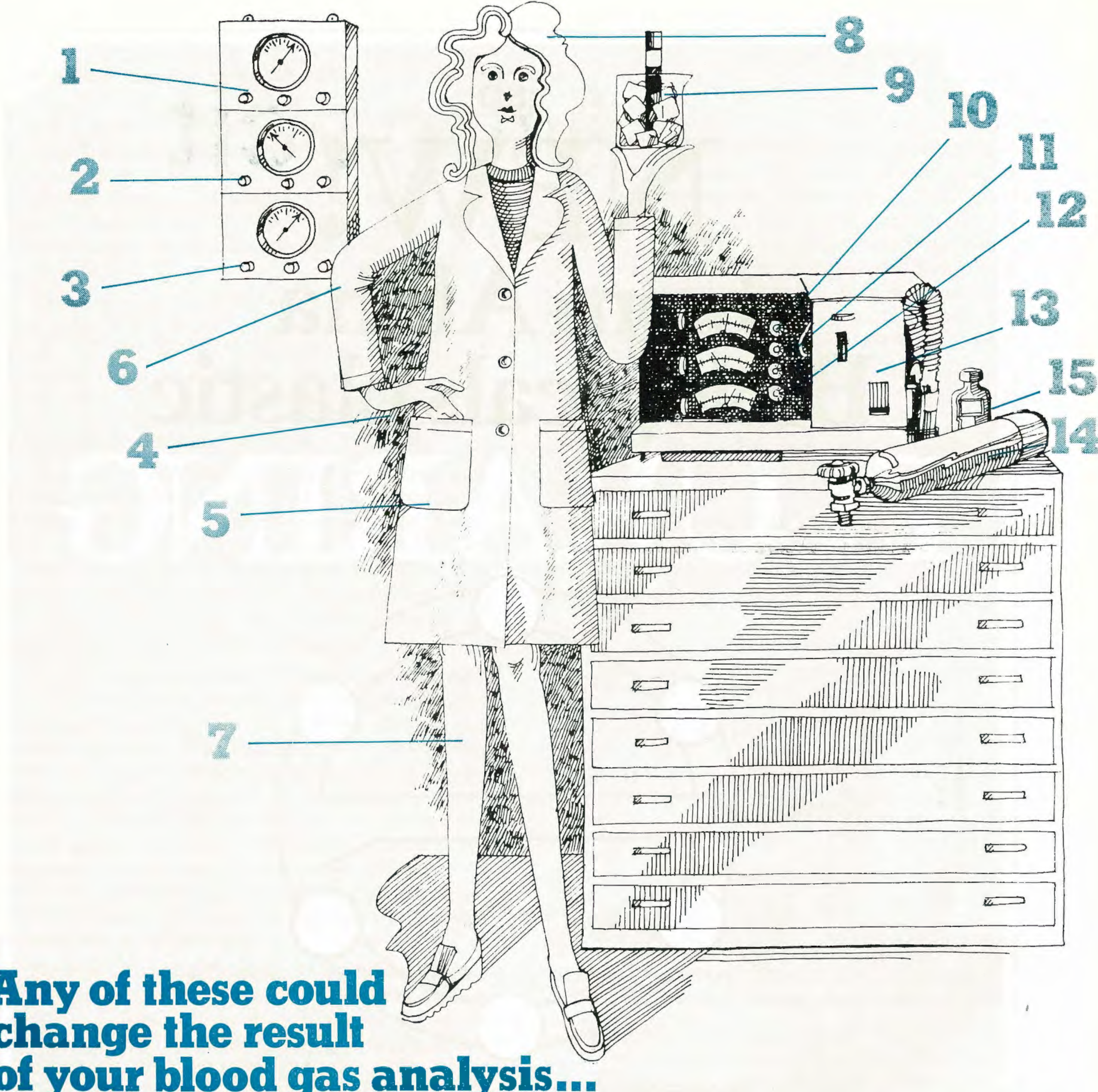


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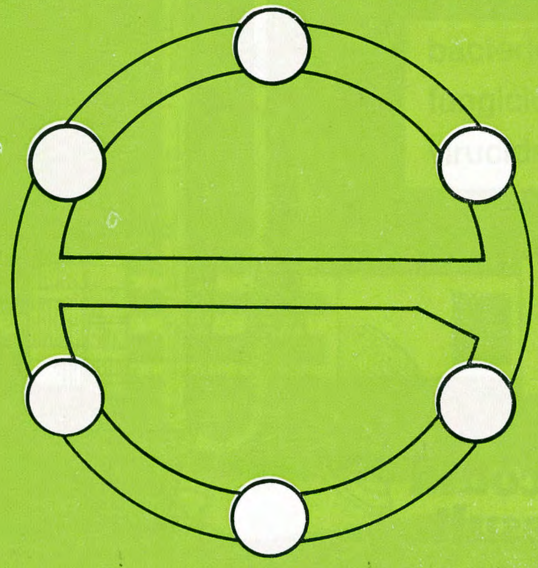

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from abscesses. This organism is resistant to tetracyclines, whereas many anaerobic organisms are sensitive. Also, they suggest the pathogen *Ramibacterium alactolyticum* is often overlooked because it is superficially similar to catalase-negative strains of anaerobic Corynebacterium.

They also showed in experimental animals the selective passage of anaerobic organisms from the gut to the bloodstream in sufficient quantities to cause disease, if the gut was first damaged by virulent *Streptococcus faecalis*.

## Structure Related to Function

Hudson *et al.*[8] showed, using germ-free and normal mice and rats, that poor lymphatic systems developed in germ-free animals. Since the lymphatic system is intimately involved in cellular and humeral responses to infection it is assumed that the absence of demonstrable flora is a prime factor in the maldevelopment of lymphatic tissue in these animals.

In the lamina propria of the human stomach there is a delicate network of collagenous and argyrophil fibrils that is almost devoid of elastic elements. The meshes of the fibres contain numerous small lymphocytes. In the lamina propria, especially in the pyloric region, small spherical accumulations of lymphatic tissue occur normally. The lamina propria of the small intestine contains great numbers of isolated lymphatic nodules scattered all along the intestine, but are more numerous and larger in the distal part.

Groups of many solitary nodules massed together are called Patches of Peyer. They occur as a rule only in the ileum, but occasionally may be found even in the duodenum. They consist of dense lymphatic tissue, with large lymphocytopoietic centres in their interior (Bloom and Fawcett[1]).

Although there is a danger of applying the findings of animal experiments directly to humans, it is not unreasonable to suggest that the presence of lymphatic tissue in the stomach and proximal end of the small intestine is due to the presence of microbial flora in these regions.

## Conclusions

The techniques used at the moment in routine diagnostic laboratories are totally inadequate to detect and identify the potentially pathogenic flora present in the gastro-intestinal tract, and this identification and interpretation must at the moment remain in the few highly specialised research laboratories that exist. However, as these workers collect more and more data on the identification and role of these micro-organisms, it may be possible to show that a true symbiosis between man and his gastro-intestinal flora exists, and that disease is an upset in this balance.

As more evidence accumulates on the role of anaerobic gastro-intestinal flora in the diseases of man it will become impossible for the physicians to ignore the presence of these organisms when considering the treatment of the patient.

Like most changes of " traditional thinking " it will be a slow awareness, but will have far-reaching implications for man and his continuing study of himself.

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# A Review of the Literature on *Vibrio parahaemolyticus*—Its Isolation and Identification

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## Summary

*Vibrio parahaemolyticus* is a Gram negative, halophilic pleomorphic rod which has been isolated from shellfish and sea water. It is probably widely distributed throughout the world especially in warm coastal waters. It has proved to be the causative organism in numerous outbreaks of gastroenteritis particularly in Japan. The organism was first isolated in 1951 by Fujino and his co-workers but was not classified as a Vibrio species until 1963 after a comprehensive study on over 1,700 isolates was carried out by Sakazaki.

*Vibrio parahaemolyticus* may be readily isolated from shellfish and specimens from patients by liquid enrichment followed by plating on to selective agar. It may be distinguished. from members of the Enterobacteriaceae by its positive cytochrome oxidase test, and from species of Pseudomonas and Aeromonas, which it closely resembles, by its fermentative anaerogenic metabolism.

At present, strains of the organism derived from food poisoning patients have been classified into 12, " O " antigen groups and 52, " K " antigen types, and new " O " and " K " antigens will probably be added to the scheme in the future as the number of untypable strains increases.

## Introduction

*Vibrio parahaemolyticus* is a halophilic marine vibrio known to be the major cause of summer food poisoning in Japan, where it causes 60-70 percent of diagnosed outbreaks[18]. It has also been associated with gastroenteritis outbreaks in Australia in 1968-69, Asia in 1970, U.S.A. in 1971, and among airline passengers returning from the Far East to Britain in 1972 [7, 28, 15, 29]. *Vibrio parahaemolyticus* has been so far isolated in Asia, the Far East, North America,[27, 2, 3, 9, 6, 22] parts of Europe[16, 4, 11] and in Australia, and we have recently received confirmation from Dr Barrow in Great Britain that several vibrio cultures isolated from Bay of Islands (New Zealand) oysters in March and April 1972 are typable strains of *Vibrio parahaemolyticus*[5]. The organism has also been isolated from localised tissue infections contracted in coastal areas of the U.S.A.[23], and caused leg gangrene and endotoxin shock in a male who acquired this infection in New England coastal waters[17].

*Vibro parahaemolyticus* was first isolated in 1951 by Fujino *et al.* while investigating an outbreak of food poisoning associated with eating of shirasuboshi (semi-dried young sardines)[10]. It was isolated on conventional media and not then recognised as a halophile or as the prime cause of the food poisoning. Later Takikawa isolated a similar organism in 1958 from patients with acute gastroenteritis[21], and subsequent studies by other Japanese investigators established that the organism was an enteropathogenic facultatively halophilic marine bacterium found in fish and shellfish. Comprehensive studies on the morphological, cultural and biochemical properties of this organism were carried out by Sakazaki *et al.* in 1960, it was classified into the genus *Vibrio,* and the species *parahaemolyticus* was proposed[19].

Most of the research before 1967 on *Vibrio parahaemolyticus* was performed in Japan, and therefore the published papers, some 1,100 of them, are in Japanese journals and largely inaccessible to us. There has been considerable research in Western countries since 1957, particularly in the U.S.A., the first reported Western isolation of the organism being in 1967 from American estuarine sediments by B. A. Ward[27]. This followed a suggestion in 1966 by Dack in *Food Technology* that organisms of significance in other parts of the world should be considered in American outbreaks of food-borne disease of unrecognised causes. In less than 35 percent of gastro-intestinal disorders in the U.S.A. is an aetiologic agent identified.

## Materials and Methods

There are two isolation procedures, Japanese and American. The former employs a liquid enrichment phase in glucose-salt-teepol broth which is plated after overnight incubation at 37°C on to a commercially available selective agar, thiosulphate-citrate-bile-salts-sucrose agar, TCBS, which may also be inoculated directly. The high pH, salt content and inhibitory substances present in the agar prevent most organisms other than the pathogenic vibrios from growing. Colonies of *Vibrio parahaemolyticus* after 18-24 hours' incubation are round, 2-3mm in diameter and have a green or blue centre. A related vibrio, *Vibrio algynolyticus,* commonly found in seafoods, forms yellow colonies due to fermentation of sucrose, as does *Vibrio cholerae.*

The American isolation method of Liston and Baross, described in the *Bacteriological Analytical Manual,* 1969[1], involves direct plating of the specimen on to a semi-selective saltwater-starch-agar, which is incubated anaerobically for up to three days. *Vibrio parahaemolyticus* hydrolyses starch and should appear as white non-spreading colonies with a halo round them, however we found that the organism grew poorly on this medium anaerobically, the halo was difficult to see, and isolated colonies hard to obtain as a large number of other organisms grew on this medium. In 1971 Twedt and Novelli modified this agar by employing partially soluble corn starch instead of soluble potato starch, and incubating aerobically for 18 hours[24]. Shortly after this Vandezant and Nickelson modified Twedt's medium, they increased the concentration of corn starch, used 7 percent sodium chloride instead of 3 percent and incubated at 42°C[25]. We were unable to obtain corn starch so substituted maize or Indian corn starch. The three strains of *Vibrio parahaemolyticus* we received from Dr Barrow in Great Britain would not grow on this medium at 42°C, and only one would grow at 37°C, starch hydrolysis was exhibited but growth was slow. All our oyster isolates resembling *Vibrio parahaemolyticus* have been obtained by the Japanese isolation method.

*Vibrio parahaemolyticus* may be distinguished from members of the Enterobacteriaceae by its positive oxidase reaction, and from species of Pseudomonas by its fermentative metabolism, demonstrated in the Liston-Baross modification of Hugh and Liefson's glucose broth. The organism may be differentiated from anaerogenic Aeromonas strains by its sensitivity to the vibriostatic agent pteridine 0/129 to which the genus Aeromonas is resistant. The *Bacteriological Analytical Manual* gives a broad outline of the biochemical tests for the organism, of particular importance is its halophilism, it grows in broth containing 3 percent and 7 percent sodium chloride but not 0 percent or 10 percent, so 3 percent sodium chloride is employed in the testing media. *Vibrio parahaemolyticus* is motile, produces indole, lysine decarboxylase but not arginine dihydrolase, hydrolyses starch and gelatin, anaerogenically ferments maltose, trehalose, mannitol and often arabinose, but not sucrose, utilises citrate, and does not produce acetylmethylcarbinol.

Since the *Bacteriological Analytical Manual* was published, a report by Twedt *et al.* comparing Japanese strains with related cultures isolated in the U.S. has shown that there are biochemical differences between *Vibrio parahaemolyticus* isolates from gastroenteritis outbreaks, tissue infections, and marine sources and a set of differential reactions for these three groups is given[23].

Miyamoto, Kato *et al.* found that there was a correlation between haemolytic activity in *Vibrio parahaemolyticus* and human pathogenicity[13], however, Twedt *et al.* found that the three groups of cultures in their study could not be differentiated by haemolysis on sheep blood agar. Vanderzant and Nickelson used three similar groups of *Vibrio parahaemolyticus* isolates to those used by Twedt, and in their investigations tested haemolytic ability with sheep, human and rabbit blood, with 0.5 percent, 5 percent and 7 percent soduim chloride, and also in the medium proposed by Wagatsuma for the "Kanagawa phenomenon" the Japanese test for haemolytic activity. The results of their study indicate that haemolysis varies greatly, depending on the type of blood and sodium chloride concentration, and is not a measure of pathogenicity. No difference could be established between human and marine isolates on any of the blood media tested[25].

Serological typing of isolates of *Vibrio parahaemolyticus* has provided epidemiological and ecological data in food poisoning outbreaks, but at present appears to be of little value for identification of the organism, since a large proportion of strains isolated from seafoods do not type. Zen Yoji *et al.* report that about 90 percent of isolates obtained from food poisoning patients in Japan are typable[30], and Twedt *et al.* found that 11 out of 17 isolates from tissue infections agglutinated with K antisera, showing a serological relationship with the enteropathogenic strains of *Vibrio parahaemolyticus*[23]. However, Fishbein *et al.* were able to type only 22 of 56 strains of *Vibrio parahaemolyticus* from crab meat[9]. There are at present 12 "O" groups and 52 "K" types derived from strains from gastroenteritis patients, and typing has been mostly based on the K antigen as it involves only a simple slide test. However, "O" antigen identification has recently been simplified from a tube test to a slide test developed by Japanese microbiologists[14]. New "O" groups and "K" types will probably be added to the scheme in the future as the number of untypable strains increases.

Several researchers in the U.S. have investigated the survival of *Vibrio parahaemolyticus* at various temperatures, pH and salt concentrations in seafoods, as this is of importance in determining its potential as a cause of gastroenteritis associated with their consumption[12, 8, 26]. Generally speaking, the organism would appear to be more sensitive to heating and freezing than other food poisoning organisms, is sensitive to pH values below 6.0 and is unstable in the absence of sodium chloride.

Research has shown that the organism has a very short generation time, about 10 minutes[31], almost twice as fast as *E. coli,* and the infective dose is high, in the region of 1,000,000 organisms[20]. Raw seafood prepared well in advance of consumption has proved to be the commonest source of *Vibrio parahaemolyticus* gastroenteritis in Japan, and outbreaks in other parts of the world would probably be discovered if the organism was suspected and its selective isolation media were used. Suspicion should be aroused if the gastroenteritis follows the consumption of raw *or* cooked seafoods, since the latter may be cross-contaminated[15].

## Acknowledgments

I am indebed to the late Dr D. P. Kennedy, Diretor-General of Health, Department of Health, Wellington, for permission to publish this paper, and to Miss D. M. Norris, Senior Bacteriologist, National Health Institute, Wellington, for her assistance.

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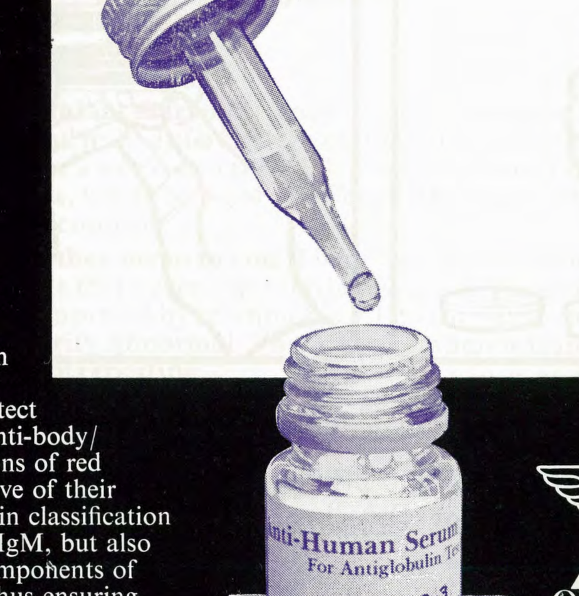


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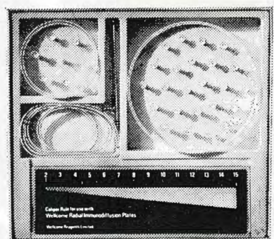

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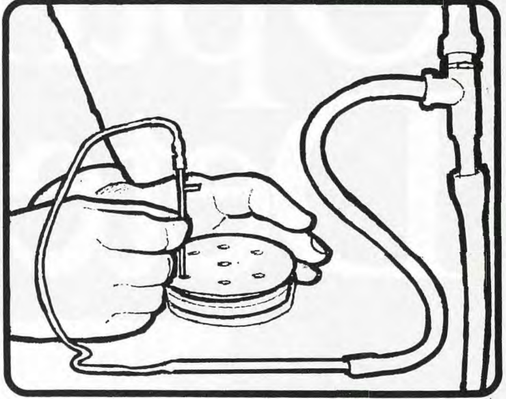

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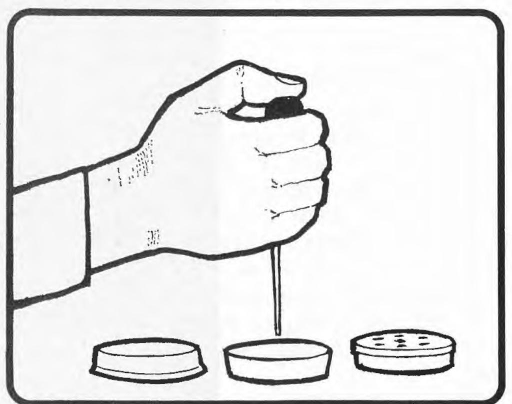
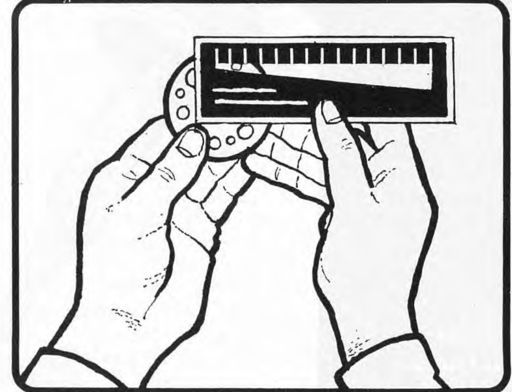


3 Sample dilutions introduced into freshly-prepared wells.



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# Biochemical and Carbohydrate Fermentation Tests Using the Replicating Device

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*Received for Publication, December 1972*

## Introduction

Non-pathogenic or saprophytic organisms are being reported with increased frequency as the causative organisms of serious infection in patients whose defence mechanisms have been impaired by medication, radiation or by the disease itself. Practically every member of the family Enterobacteriaceae has been implicated in clinical infection, Fields *et al.* (1967)[2] of both hospital associated and non-hospital associated origin.

All these organisms have clinical and epidemiological importance and the clinical laboratory should be prepared to identify them rapidly and accurately, never discarding any organisms as a " contaminant ".

Many papers have been written on methods devoted to the isolation of pathogenic enterobacteria and some media described have come to have a fairly wide usage, e.g., Xylose Lysine Deoxycholate agar for the isolation of salmonellae and shigellae.

However, methods for the identification of enterobacteria are diverse and range from one tube screening—Triple Sugar Iron agar: two tube screening—Kohn's medium modified by Gillies and Robertson; the medium recently described by Fuscoe (1972)[3]: three tube screening—Lee *et al.* (1972)[6]: to commercial packets, R-B; Analytab; Enterotube; API; and to commercial impregnated papers—Pathotec. All claim varying degrees of accuracy, McIlroy *et al.* (1972)[7], Smith *et al.* (1972)[9], and naturally, this reflects the personal preference of the user at the time of testing.

This paper is written to describe a simple method of testing isolates for biochemical activity on a wide range of media at minimal cost, ease of handling and can be readily assimilated into current methodology.

## Materials

The use of the replicating device as described by Lederberg and Lederberg (1952)[5] is the basis of the system, using agar plates containing the various biochemical substrates. The replicator will accurately transfer a constant volume of inoculum from wells to defined positions on the test plate, thus eliminating any inaccuracies due to poor techniques in subculture inoculation.

## Media

*Carbohydrates* are incorporated in Cystine Trypticase Agar Base (Ortali)-CTA. This medium was originally designed for plate carbohydrate fermentation testing with corynebacteria but has proved a useful solid medium for this purpose. The carbohydrates used are lactose, sucrose, mannite, raffinose, arabinose, rhamnose, sorbitol, dulcitol, salicin, adonitol, inositol. For ease of handling it is convenient to dispense the CTA in 100ml amounts, sterilise, add 1g of the respective carbohydrate, dissolve and pour five plates. This will then suffice for one week and ensures the use of fresh media. There has been no contamination problems to date.

*Citrate*: The agar of Simmons (BBL, Difco) is prepared according to the manufacturers' instruction.

*Urease*: The agar medium of Christensen 1946 (BBL Difco) is prepared according to the manufacturers' instruction.

*KCN*: KCN broth, Rogers and Taylor (1961)[8], is prepared according to the formulation given and solidified by the addition of 1.25 percent agar. For use 100ml of base is melted and 1.5ml of a freshly prepared 0.5 percent KCN solution in sterile distilled water is added. One ml of 0.1 percent triphenyltetrazolium chloride is added prior to pouring as suggested by Kelly and Fulton (1953)[4].

*Malonate*: Malonate broth Ewing Modification (BBL, Difco) is prepared according to the manufacturers' directions and solidified by the addition of 2.5 percent agar.

*Phenylalanine*: Phenylalanine agar Ewing, *et al.* (1959)[1], is prepared following the original formulation.

All these above media are prepared weekly and each poured into five plates giving a constant volume of 20ml/plate and an even depth of 5mm (± 0.5).

**Methodology**

The plates are inoculated using the replicating device and a head with 2mm pins, the exception being the KCN medium. The broth cultures that have been prepared for sensitivity testing are used, approximately four hours after initial inoculation. With one exception all the inoculated plates are incubated at 37°C overnight and read the following morning.

The production of acid from the carbohydrates is readily observable as yellow colonies against a red background.

Citrate utilisation is indicated by the presence of a blue colony against a green background.

Malonate utilisation is indicated by the presence of a colony surrounded by a deep blue colour as opposed to the greenish colour of the base medium.

Phenylalanine deamination is determined by flooding the incubated plate with a solution of 10 percent $FeCl_2$ and standing for 2-3 minutes. A positive test will reveal a deep green coloration in the agar immediately surrounding the colony.

Urease activity: if the plates are incubated at 37°C the rapid hydrolysis of urea by Proteus species is apparent within 2-3 hours. If left overnight the colour change will spread across the plate and mask any late urea hydrolysis. However, if incubated at 27°C the Proteus species will show as a neatly defined pink area immediate to the colony and the slow urea hydrolyser will show as a pinkish colony.

KCN. Initially, difficulty was experienced in demonstrating consistent results with this medium. However, if control is exercised on the inoculum preparation the medium will work satisfactorily. For use, the colony to be tested is touched with a sterile wire and this minute inoculum is emulsified in 5ml of sterile distilled water. This inoculum is then immediately inoculated into the KCN plate using a replicator head with 1mm pins. After incubation overnight at 37°C any KCN positive organisms will show as red colonies due to the reduction of TTC.

DNA. DNA medium (Difco, BBL) is prepared according to the manufacturers' instruction, inoculated in the routine method, incubated and tested using either 2N HCl acid or 0.1 percent toluidine blue.

*Cost*: The cost per plate of the basal CTA is approximately 1.4 cents, 1 gram of the carbohydrate ranges from 1.5 cents for lactose to 40 cents for rhamnose giving a total cost of between 7 cents and 48 cents to provide the medium to test up to 25 organisms in one operation. The cost per plate of the remaining substrates ranges between 11 cents for malonate to 15 cents for urease medium.

*Controls*: The *E. coli* NCTC 9001 strain, and *Klebsiella aerogenes* NCTC 418 strain have always been included in the antibiotic sensitivity control and these two organisms act as positive and/or negative controls for malonate, citrate, urea (slow), KCN and fermentation plates. *Proteus sp.* are almost inevitably isolated in the daily routine and will control the urea (rapid) and phenylalanine.

**Conclusions:** The use of the replicating device has allowed antibiotic sensitivity testing to become a standardised and precise test procedure. By modification of the base medium for biochemical testing it is also possible to test a number of organisms for biochemical activity on a number of substrates at minimal cost, and using accepted methodology. This allows the recognition of more factors ensuring the use of more precise taxonomical application of tables presented for identification of the Enterobacteriaceae.

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# A Simple Method of Protein Preparative Electrophoresis

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*Received for publication, October 1972*

## Introduction

There are many instances where the preparation of a pure protein or group of proteins is a necessary first step for subsequent work. The choice of a protein for a particular study is biased to a greater or less degree by its ease of purification. The increased interest in the properties, functions and uses of particular proteins has been paralleled by the development of a wide spectrum of preparative methods. Some of these include: salt fractionation, column chromatography, column electrophoresis, ultracentrifugation, and zone electrophoresis.[5]

Generally it is necessary to use a combination of methods to obtain a protein in a pure form. A number of these techniques require special and often expensive equipment as well as considerable time and experience to get them operating satisfactorily. We have found zone electrophoresis on PVC/PVA copolymer blocks to be an exceptionally simple and inexpensive technique that has given excellent results.

## Method

The method is based on that outlined in Smith[6] and Williams and Chase.[7] However, in this instance the powder used is Breon AS 70/40. Other similar materials can also be obtained in New Zealand. (See discussion.)

1. *Preparation of the Powder*

In some instances it may be necessary to wash the powder free of small amounts of metal ions. This can be done on a Buchner funnel or by decantation using HCl. This is followed by washes with distilled water and finally equilibration with electrophoresis buffer. In most cases, however, a simple equilibration with buffer with removal of some of the fines by decantation is sufficient.

2. *Pouring the Block*

The slurry is simply poured into the mould which has pads of blotting paper at the electrode ends to remove the excess buffer and cause the block to solidify. The size of the mould may vary from 5 x 20 cm up to 50 x 60 cm or even larger, although the thickness should not exceed 1.5 cm.

3. *Loading the Sample*

The position of the origin depends on the type of material to be separated and the buffer system used.

A narrow sample well is cut out and the sample is mixed with the powder to make a slurry of similar moisture content to the block. It is then packed into the well and the top of the block carefully smoothed out. Alternatively, particularly with heavy loadings, a narrow slit is cut and the sample loaded directly from a Pasteur pipette. The area either side of the slit is first moistened with several drops of buffer to ensure a narrow band. The slit is closed and the top of the block smoothed over. Prior dialysis of the sample may be necessary in some cases.

4. *Electrophoresis*

The moistened paper pads are removed and the ends connected with the electrode troughs by means of filter paper wicks. The block surface is covered with polythene or thin glass to prevent evaporation. Electrophoresis is usually carried out in a cold room. A voltage of up to 5V/cm is generally used and times of up to 24 hours or more. When serum is used,

an albumin dye, e.g., bromophenol blue, is added to show the movement of this band and the evenness of the advancing front. We have found that the normal starch gel size moulds and tanks are very suitable for trial runs and smaller preparative runs.

5. *Location of Bands and Recovery of Fractions*

The position of the bands after electrophoresis can be found without actually staining any of the block. A piece of filter paper is placed edgewise on to the block and the buffer with dissolved sample soaks up into it. The paper is dried and then stained with an appropriate stain. The required band is then cut out and the compound eluted using a Buchner funnel or similar piece of apparatus. The powder may be completely washed and reused.

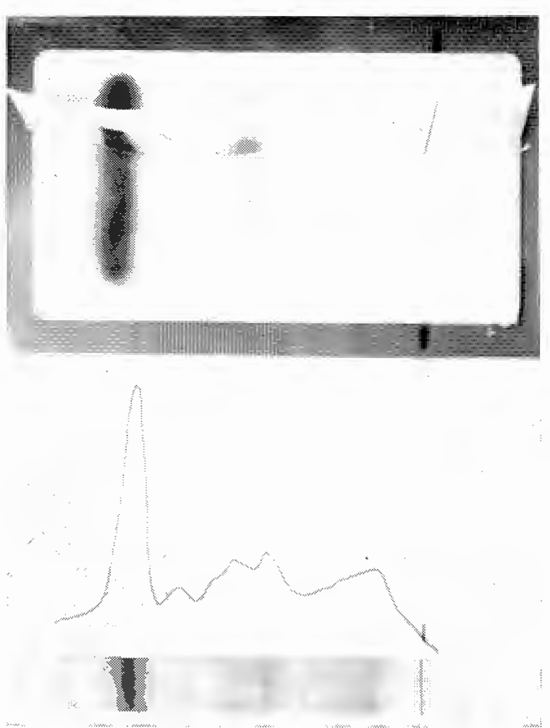

FIG. 1.—Location of Bands Following Electrophoresis. In this instance serum proteins were separated on Breon AS 70/40 using pH 8.6 barbitone buffer with an ionic strength of 0.1. Electrophoresis was carried out for 20 hours at 5V/cm. The paper was stained with amido black and scanned on a densitometer. The block dimensions are 18 x 10 x 0.8 cm.

## Results

We have used the method in this laboratory as a first step in the separation of albumin or $\alpha_1$-antitrypsin from the other serum proteins. (See Fig. 1.) Barbitone buffer at pH 8.6 with an ionic strength of 0.1 gives an excellent separation of the $\alpha_1$ band from albumin.[7] In general it is possible to use the same buffer for PVC/PVA electrophoresis as is used for paper, e.g., those haemoglobins that are separated by paper electrophoresis can be separated on a preparative scale using the same buffer on PVC/PVA.

## Discussion

Zone electrophoresis on powder blocks represents one of the simplest preparative electrophoretic methods. Starch block and powdered cellulose have been the most common supporting media used. They have enjoyed considerable success but have a number of disadvantages. These arise from the fact that neither of them are completely inert or insoluble. Furthermore they both give rise to considerable electro osmotic flow arising from the negative charges on the carbohydrate. These are very significant points when glycoproteins are being separated.[1, 4] Considerable improvement was obtained when polyvinyl chloride was substituted as the supporting media.[2] Being inert there was negligible tailing and yields of over 90 percent were routinely obtained. But it also gave rise to considerable electro osmotic flow. This is generally not desirable although, however, it can be an advantage in cases where the mobility difference between the proteins to be separated is small.[7]

A copolymer of polyvinyl chloride and polyvinyl acetate, however, has minimal electro osmotic cathodal flow yet it gives the same excellent separation as PVC.[3]

This copolymer is used commercially in the production of floor tiles and footwear and can be obtained very cheaply in New Zealand under the trade name Breon AS 70/40 or Breon AS 70/42. However, some industrial users of this resin are replacing it by Nipeon A4J which has a lower acetate content than Breon (Table 1.) The lower percentage results in an electro osmotic flow for Nipeon intermediate between Breon or Pevikon and PVC. Pevikon C-870 (Shandon) is the original copolymer used and marketed for scientific purposes. There are probably other products

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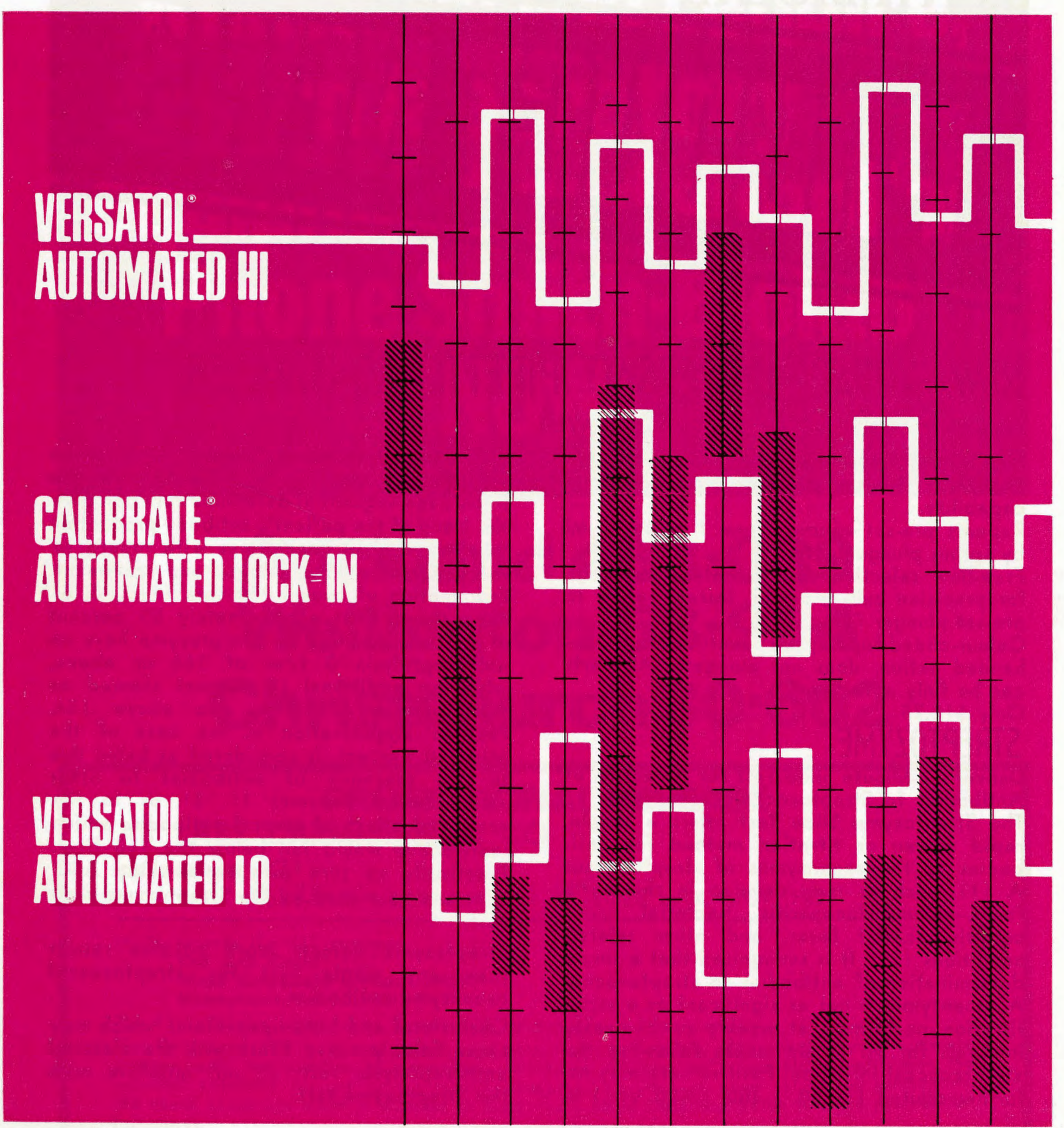


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of similar specification which could undoubtedly be used in their place.

**Summary**

A simple preparative electrophoretic method is described. Buffer conditions similar to those for paper electrophoresis are used and electrophoretic apparatus like that employed for starch gel or certain cellulose acetate runs is ideal for most separations. The polyvinyl chloride/polyvinyl acetate copolymer resin is inert and may be reused after washing.

**Table 1: Acetate Content of Three Resins**

| Resin | % Acetate |
|---|---|
| Breon AS 70/40 | 16 |
| Pevikon C-870 | 13 |
| Nipeon A45 | 5 |

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# The Laboratory Characterisation of Serum Lipoproteins

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## Introduction

Ischaemic heart disease is one of the leading causes of premature death in the " advanced " countries of the world accounting today for about one-third of such deaths under the age of 60. For this reason the causes have come under increased study in recent years, in the hope that prevention may prove to be feasible. Although a positive correlation has been shown between the serum (or plasma) cholesterol and the incidence of ischaemic heart disease, the risk for the individual with a high serum cholesterol remains difficult to predict. This risk depends on many variables, some related to the subject of this article (serum cholesterol, serum lipoprotein pattern, family history or signs of hyperlipoproteinaemia) and some relevant but unrelated (smoking history, blood pressure, glucose tolerance, obesity, etc.), all of which should be taken into account[2]. It is assumed but not proven that the risk of developing ischaemic heart disease or of dying from established ischaemic heart disease is diminished by lowering elevated serum lipids, and this forms a cornerstone of therapy for those at risk. The laboratory worker plays an essential part in the evaluation and treatment. He should be able to measure serum cholesterol and triglycerides and report the appearance of the serum, and also should be able to give the clinician an estimate of the different lipoproteins in the serum sample if required. This latter may be done directly, using electrophoresis, or indirectly, by using precipitation techniques. The physician should have a measure, not only of the blood lipids but also of the lipoprotein pattern if this is abnormal, and he will therefore be better able to initiate rational therapy.

## Nomenclature

Lipoproteins are complex particles made up of protein, triglycerides, phospholipids, and esterified and non-esterified cholesterol; albumin, which carries an extremely important load of non-esterified fatty acid, is by common consent excluded from this classification. As shown in Table 1, several systems of nomenclature are used. One of the most prevalent, based originally on paper electrophoresis, recognises chylomicrons, beta, pre-beta, and alpha lipoproteins. This system is equally applicable to electrophoresis on cellulose acetate. An alternative, and in many ways preferable, system is that based on density, which is in practice largely determined by the percentage of triglyceride making up the lipoprotein particles. Chylomicrons have a density less than 1 g per ml and float in serum which has been stored overnight, very low density lipoproteins

are of density less than 1.006 g per ml and float in serum only when spun at 100,000 $\times$ *g*, low-density lipoproteins have a density between 1.006 g per ml and 1.063 g per ml, and high-density lipoproteins have a density between 1.063 g per ml and 1.21 g per ml. It can be seen from Table 1 that chylomicrons and very low-density lipoproteins carry most of the triglyceride, and hence are present in increased concentration if the serum triglyceride is raised, whereas most of the serum cholesterol is carried on low-density lipoproteins. The latter fraction is that most strongly implicated in the genesis of atherosclerosis.

A specimen of serum from a normal fasting patient should show no chylomicrons and few, if any, very-low-density lipoproteins on electrophoresis. It appears clear because it contains no large light-scattering particles. Some sera may demonstrate a pre-beta band in the presence of a normal triglyceride concentration. This is a low-density lipoprotein variant of no pathological significance.

## Serum Sample

In this paper the words *serum* and *plasma* are in general interchangeable. Lipoproteins and the individual lipids may be estimated equally well in either type of sample. Samples should not be frozen but should be kept at 4°C; samples which have been frozen or kept too long at room temperature or which have undergone bacterial contamination behave abnormally on electrophoresis. In such samples chylomicrons and very-low-density lipoproteins may aggregate or lose some of their components. On the other hand the serum total cholesterol and triglyceride is usually stable for some days at room temperature, and for some months if frozen, although the specimen should be well mixed before sampling. Lipoproteins are denatured after storage for about one week at 4°C without EDTA (the time depending on the class of lipoprotein), but samples can be stabilised at this temperature for many weeks by the addition of EDTA at a concentration of 1 mg per ml.

The conditions under which blood samples are taken for lipoprotein estimations are very important and should be standardised. After a meal chylomicrons will appear in the serum, and the serum triglyceride may be double or treble the fasting level. This should reach a peak by two to four hours and have largely disappeared by six to eight hours. A high level of serum very-low-density lipoproteins may be induced by a high carbohydrate or alcohol intake. The type or quantity of fats in the diet (especially cholesterol and certain saturated fatty acids) may affect the low-density lipoprotein levels. Recent dietary and medical history exerts a strong influence on plasma lipoprotein levels. Following a myocardial infarction low-density lipoprotein levels may be lowered by a quarter or a half of their usual value for two or three months. Pregnancy and the contraceptive pill in women, exercise, emotional stress and smoking will all affect the concentration of lipid in serum. Venostasis may elevate the serum cholesterol by up to 15 percent and a 10 percent increase has been reported when the subject moved from the recumbent to the standing position.

To obtain a truly representative baseline sample, the patient should have been on his normal diet for at least two weeks before the sample is taken. If he has had a myocardial infarct, two or three months should be allowed to elapse after the infarct. He should have been fasting for at least twelve to fourteen hours, should be reasonably calm and perferably reclining. All this is too much to ask of many patients, but the requirements of a reasonably stable diet and twelve to fourteen hours prior fasting are important; as deviations from ideal conditions increase, so will the difficulties in interpretation. If a tourniquet is used it should be released before collection.

## Methods

### *Appearance*

The laboratory should comment on the appearance of the serum. A milky or turbid serum usually indicates a triglyceride concentration over 400 to 500 mg per 100 ml. Any such serum should be left in the refrigerator overnight and a further comment made on its appearance next morning. If chylomicrons are present a layer of " cream " will be found floating on the surface of the specimen, whereas very-low-density lipoproteins will remain dispersed, conferring a uniform turbidity on the specimen. If the low-density lipoproteins are raised the serum remains clear.

### *Cholesterol*

Many people put undue reliance on the accuracy of serum cholesterol measurements, not realising the lack of precision in this test as it is done in most laboratories. The cholesterol

fraction of low-density lipoprotein is probably of greatest importance, but the total serum cholesterol is normally measured. One reason for the variable results obtained by different analytical methods may be the finding that about 10 percent to 20 percent of serum "cholesterol" consists of cholesterol derivatives; another is that only about 25 percent of total serum cholesterol is "free", the remainder being esterified.[15]

The first major problem in the analysis of cholesterol is the provision of a suitable standard. Cholesterol is a sterol with a double bond between carbon atoms 5 and 6, and because of this it slowly oxidises when stored. The usual pure cholesterol off the shelf may be 90 percent to 95 percent pure, the remainder consisting of a complex mixture of sterols. Reasonably pure cholesterol can be formed from the dibromide, which is turn can be derived as crystals from ordinary commercial AR cholesterol, and this simple method of preparation is recommended[11]. The dibromide is resistant to oxidation. Several commercial primary standards are available, and a standard of known purity is available from the U.S. National Bureau of Standards; these should be stored at —20°C in the dark. It is certainly cheaper to store one's standard as the dibromide, and convert to cholesterol as necessary. Precision can be monitored using a frozen serum pool or commercial reference sera (remembering to allow overlap between one control and another when changing), but these are poor substitutes for a primary

Methods for cholesterol estimations vary from very simple to most complex, and these are discussed in the standard texts[16, 9]. Briefly, they may be classified as single step, involving a colour reaction directly on serum, generally rather inaccurate or prone to inaccuracies; two step, involving prior extraction of lipid before the colour reaction, satisfactory for most laboratories; three step with extraction, saponification of esters and a colour reaction, and four step involving digitonin precipitation of the free cholesterol after saponification. The reference procedure usually accepted is a three-step procedure, that of Abell and others[1], but this is somewhat tedious, the colour development may be unstable, and it requires attention to detail by a good trained laboratory worker. Different methods give quite different results, which are not always easily predictable. A look at some commercial reference sera exemplifies this; the data in Table 2 are from five different boxes of control serum chosen at random from our control serum refrigerator. These estimations are said to be done by reference laboratories, and the spread of results obtained would probably be wider if the tests were carried out by ordinary laboratories not specialising in lipid analysis. These limitations are not generally realised by clinicians, who may attach some importance to a drop in serum cholesterol of say, 20 to 30 mg per 100 ml; the laboratory should point out that in cases where there has been a change in the method used such a drop may merely reflect this change, and alternatively the fall is probably within the range of error of the method used.

A reference method such as the Abell method in good hands in a reference laboratory has been said to have confidence limits of ± 6 percent (2 SD), and this seems to be the optimum precision available, though problems of accuracy are still major and bedevil any comparisons. In our laboratory precision on the SMA 12/60 cholesterol channel is not good, being about 15 percent to 20 percent (2 SD), although this fluctuates from day to day. For this reason the SMA 12/60 cholesterol method will be withdrawn from use in our laboratory in the near future. The two-step procedure involving extraction which Technicon have largely automated[4] gives improved figures in our hands with precision varying between 7 percent and 12 percent (2 SD). There seems little doubt that this method is preferable if a laboratory has a sufficient number of specimens to justify the use of an autoanalyser channel, but in most laboratories a manual method will continue to be used for some time. A two-step method, for instance that of Carr and Drekter[8], is recommended as these methods have a minimal number of manipulative steps and are capable of reasonable precision. Any reference laboratory that wishes to upgrade its methods should apply to enter the programme of evaluation initiated by the Centre for Disease Control, Atlanta, Georgia.

*Triglycerides*

Measurements of total lipids and total esterified fatty acids do not usually give the physician much meaningful information, and these methods should probably be discontinued.

Although in theory an estimation of serum cholesterol with quantitation of an electrophoretic strip stained for lipid might be satisfactory, in practice the errors involved are so great that measurement of triglycerides is strongly recommended as a standard screening method.

We use an automated technique for triglyceride estimation[3], using the same extract from serum for simultaneous cholesterol estimation. This is a fluorometric procedure in which the glycerol is oxidised to formaldehyde before the final fluorometric reaction. Phospholipids are removed by preliminary treatment with zeolite, a mixture containing zeolite, Lloyd's reagent and copper lime. Colorometric procedures are also available, and quite satisfactory estimations can be made after phospholipids have been adsorbed[7] or extracted[20]. There are kitsets to measure triglycerides by an enzymatic procedure, but these require an instrument to read absorbance at 340 nm and as in any enzymatic procedure there are many potential pitfalls involving enzyme and coenzyme storage, heavy metal trace contamination, etc.

Again, acceptable primary standards and serum reference materials are a problem. because the molecular weight of triglycerides varies according to the fatty acid moiety. A WHO report[5] recommends that triglycerides be reported on a molar basis rather than on a weight basis, but this is unlikely to find favour generally for many years. A triolein standard is usually used, though tripalmitin would be equally acceptable.

*The Translation of Hyperlipidaemia Into Hyperlipoproteinaemia*

With these two tests, serum cholesterol and triglycerides, together with a report on the appearance of the serum and if necessary its appearance after twelve hours standing at 4°C, an estimate of the presence or otherwise of any abnormality can be made. If either of these results are outside the normal limits for sex and age an estimation of the lipoproteins present should be made. The concept of " normality " insofar as cholesterol and triglycerides are concerned is difficult to define, as the normal range of values (as usually defined in clinical biochemistry) is not the desirable range. It seems likely that the range of serum cholesterol found in the New Zealand population is much the same as that in similar populations in other countries, judging by the data of Evans, Prior and Cook[10], some of which is shown in Table 3. I would suggest that estimates of lipoprotein concentration in serum would be desirable and helpful to the clinician if the serum cholesterol in a young adult was over 250 mg per 100 ml, or over 300 mg per 100 ml in an older man or woman, or if the fasting triglyceride level is over 150 mg per 100 ml in adults of any age group.

*Lipoprotein Concentrations by Electrophoresis or Precipitation*

Both these techniques ideally give the same information, but when a densitometer is not available to quantitate electrophoretic strips a precipitation technique can be a valuable adjunct.

A number of media may be used for zone electrophoresis; the most commonly used are paper, with albumin present in the buffer[17], and cellulose acetate, though agarose and polyacrylamide are also used. Paper electrophoresis is the traditional method, but it is expensive and may be subject to a number of difficulties which are not always easy to correct. Because the sample takes sixteen to twenty-four hours to run, then another twelve to sixteen hours to stain, a series of electrophoretic strips may not be recognised as being defective until the specimen is rather old. While electrophoresis is in progress the apparatus should be in a secluded preferably dark and cool spot.

For these reasons and others concerned with details of technique. I would not recommend paper electrophoresis as a new method for a laboratory, but rather would suggest microzone electrophoresis on Beckman or Gelman apparatus, using Cellogel or Sepraphore III cellulose acetate membranes for example. In our hands these have provided good estimations of all lipoprotein classes, with separation of the pre-beta band usually superior to that with paper electrophoresis. A serum sample with a very high lipid level, in particular one with a high triglyceride level, can easily overload these membranes and precise evaluation in these cases is difficult. Old serum specimens may also give quite abnormal artifactual patterns on cellulose acetate. Nevertheless, because of speed and cheapness I would recommend cellulose acetate for the routine hospital laboratory

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* ASO, AH, ASK, ADNase, ANADase

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rather than paper, unless of course, there is anyone in the laboratory with a particular interest in lipoproteins and a desire to perfect the paper method, as an adjunct to typing patients with hyperlipoproteinaemias, for epidemiological or research purposes.

On paper, cellulose acetate and agarose, the position of the four primary lipoprotein bands is the same; that is, chylomicrons stay at the origin, and beta-lipoproteins, pre-beta lipoproteins (which may trail back towards the origin) and alpha lipoproteins are seen sequentially towards the anode. Polyacrylamide gel is a little different, in that size criteria as well as electric charge decide the distance migrated; the very low density lipoproteins therefore migrate a shorter distance than low density lipoproteins.

Another method of low density lipoprotein quantitation is by the method of Walton and Scott[23] marketed commercially as the Serum Low Density Lipoproteins Kit by BDH, in which low density lipoproteins are precipitated by dextran sulphate in the presence of calcium ion at pH 9.0. Low density lipoproteins measured are equivalent to the beta and pre-beta electrophoretic fractions. An alternative method is that of Burnstein and Samaille[6, 14]. In this case very low density and low density lipoproteins are precipitated from a 3 ml aliquot of plasma by addition of 0.15 ml of 1.0 M manganese chloride and 6 mg of sodium heparin. The cholesterol content of the supernate from this procedure and that of whole serum are measured, and the cholesterol in the alpha lipoprotein is derived from the difference. The cholesterol in the very low density lipoprotein fraction can be taken to be approximately 1/5th the value of the triglyceride level (in mg per 100 ml), and the remaining cholesterol, that in the low density lipoprotein, can be derived by subtraction. Precipitation methods become unreliable in the presence of large quantities of chylomicrons or very low density lipoproteins.

*Other Methods*

Other methods used in lipoprotein analysis are legion. These include nephelometry, immunological estimation of low density lipoprotein concentrations and other precipitation techniques. The preparative ultracentrifuge is an essential research tool and its use is necessary whenever Type III hyperlipoproteinaemia is suspected. An analytical ultracentrifugal study may also be necessary at times. Enzymes studies include post-heparin lipolytic activity (reduced in Type I disease) and occasonally lecithin cholesterol acyl transferase which is deficient in a very rare hereditary syndrome.

## Abnormalities of Serum Lipoproteins

Several syndromes exist in which different serum lipoprotein classes are found in abnormally high concentrations. The group at the National Institute of Health at Bethesda, Maryland, led by Dr Donald Fredrickson, some years ago proposed a system of nomenclature[13] which has now been accepted with one or two minor changes as an official classification by WHO[5]. Classification has important implications for the patient insofar as treatment and prognosis for himself and his family are concerned, but it should be remembered that many factors other than the lipids in a sample of serum should be taken into account in assessing the patient.

A detailed discussion of the hyperlipoproteinaemias is outside the scope of this paper and summaries can be found in many papers[5, 12, 18, 19, 21, 22]. It is not always apparent from these summaries that in fact several types are extremely rare, and for this reason it is not out of place to stress the common types here. In addition, each type may be primary, or secondary to other disorders, and the serum lipoprotein pattern in any individual may change from time to time for a variety of reasons. Although these are described as syndromes, in fact, it should be remembered that the laboratory can only report a pattern, e.g., Type IV pattern.

Type I is a very rare syndrome of which one or two cases are known in New Zealand. In this there is a deficiency in post-heparin lipolytic activity, and there are very high concentrations of chylomicrons in fasting serum.

The Type II syndrome is that in which there is a high serum level of beta-lipoprotein or low density lipoprotein. Data on prevalence are sparse, and depend upon the limits of normality adopted, but 3 or 4 percent of our adult population may come into this category. This has been subdivided into two classes, IIa in which elevation of low density lipoprotein is the only abnormality and IIb in which there is also some very low density lipoprotein with a mild elevation of serum

triglyceride. Type II pattern is associated with a high risk of premature ischaemic heart disease, and therefore in general is treated vigorously with regimens designed to lower the serum cholesterol. The congenital form of this syndrome is uncommon, but any physician with an interest in lipid disorders has a number of such patients. The acquired form is more often seen in middle-aged men and women, and may be due to an abnormal sensitivity to excess dietary cholesterol or other lipids (on the premise that the normal Western diet includes an excessive amount of saturated fatty acids and cholesterol).

Type III hyperlipoproteinaemia is another rare syndrome of which four cases are known in New Zealand. The serum is turbid, and a small creamy layer may separate on standing. On paper electrophoresis a rather broad beta-band is seen, and definitive diagnosis depends on an ultracentrifugal study, which demonstrates an abnormal, very low density lipoprotein with beta-mobility on electrophoresis. This disorder is generally suspected initially on clinical grounds.

Type IV hyperlipoproteinaemia is that in which the very low density lipoproteins are raised. This is found particularly in obese people, who may or may not drink alcohol too heavily and who may or may not have diabetes mellitus. Whereas the serum in Type II patients is generally clear, that in Type IV patients is uniformly turbid on standing. This syndrome, like Type II is not particularly uncommon in this country.

Type V hyperlipoproteinaemia is an uncommon and less well understood syndrome which combines the features of Type I and Type IV. On paper electrophoresis this usually presents with a massive overload of lipid stretching from the origin to the pre-beta position.

Two other rare syndromes deserve mention. One is abetaliproproteinaemia and the other is Tangier disease, in which beta-lipoproteins and alpha-lipoproteins respectively are absent. One family with Tangier disease has been recognised in New Zealand, and there have been at least two cases of abetalipoproteinaemia. These patients are likely to have been provisionally diagnosed before reference to the lipid laboratory.

It should be stressed again that all these patterns merely reflect serum lipoproteins at the moment the blood sample was taken; the laboratory is justified in saying for instance, that the sample displays a "Type II pattern" but diagnosis of Type II hyperlipoproteinaemia with all its implications requires a thorough examination by a physician and full evaluation of all available evidence.

**Table 1**

Lipoproteins in human serum, showing differing systems of nomenclature and the different constituents in each class. All except alpha-lipoproteins are involved in syndromes of lipoprotein excess.

| Class | Chylomicrons | VLDL | LDL | HDL |
|---|---|---|---|---|
| Density | <0.95 | 0.95-1.006 | 1.006-1.063 | 1.063-1.21 |
| Electrophoresis | Origin | pre$\beta$ | $\beta$ | $\alpha$ |
| Diameter (Å) | 2000-10,000 | 200-600 | 150×300 | 50×300 |
| Approximate Mol. Wt. $\times 10^6$ | $10^3$-$10^4$ | 5-100 | 2-3 | 0.25 |
| *Composition* | | | | |
| Protein | 2% | 8 | 22 | 50 |
| Triglyceride | 86% | *54* | 11 | 8 |
| Cholesterol | 7% | 19 | *46* | 19 |
| Phospholipid | 5% | 19 | 21 | *23* |

**Table 2**

Cholesterol estimations from a set of control sera, carried out by reference laboratories from the manufacturers. Data are from the package sheets accompanying samples.
Serum Cholesterol (mg per 100 ml).

| *Method* | Dade A | Dade B | Dade C | Metrix | Hyland |
|---|---|---|---|---|---|
| Q-PAK Ferro-Ham (single step) | 175 | 140 | | | |
| Hycel (single step) | | | 321 | | 210 |
| SMA 12/60 (single step) | 164 | 128 | 315 | 176 | 185 |
| Autoanalyser (N-24a) (two step) | 139 | 101 | 290 | 185 | 185 |
| Abell (three step) | 150 | 101 | 291 | | |
| Zak-Henly (three step) | | | | | 166 |
| Monitor "cholesteright" | | | | 171 | |

**Table 3**

Serum cholesterol levels (mg per 100 ml) in men and women of Carterton, a New Zealand country town. From Evans, J. G., Prior, I. A. M. and Cooke, N. J. (1969). *New Zealand Medical Journal,* 69, 346.

Serum Cholesterol in mg per 100 ml

| Age (years) | MALES No. | Mean | SD± | FEMALES No. | Mean | SD± |
|---|---|---|---|---|---|---|
| 20-29 | 37 | 223.6 | 35.1 | 38 | 228.1 | 44.5 |
| 30-39 | 42 | 245.5 | 49.2 | 47 | 236.3 | 41.9 |
| 40-49 | 42 | 251.8 | 43.0 | 39 | 261.8 | 41.5 |
| 50-59 | 31 | 268.5 | 46.6 | 41 | 273.8 | 50.9 |
| 60-69 | 29 | 254.1 | 49.8 | 31 | 299.5 | 49.6 |
| 70 + | 21 | 230.1 | 52.5 | 26 | 287.8 | 31.8 |

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# The Incidence Of Hepatitis Associated Antigen In The Waikato

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## Introduction

An attempt as made to conduct a survey in the Waikato on the incidence of HAA amongst Maoris and Europeans. For the purpose of the test all those with Maori features were classed as Maori. A local borstal, and local schools and colleges co-operated by allowing their students to donate blood on a voluntary basis.

## Procedure

Reagents:

(a) Veronal Buffer, pH 8.6
3.12gm barbituric acid,
17.1gm sodium barbiturate, per litre.

(b) Oxoid Ionagar No. 2, made up with buffer.

(c) Stain—Amido black, 0.8 percent made up in; Methanol, Water and Acetic Acid in the proportion, 45 : 45 : 10. Also used as slide wash.

(d) Lyphogel polyacrylamide gel (Gelman).

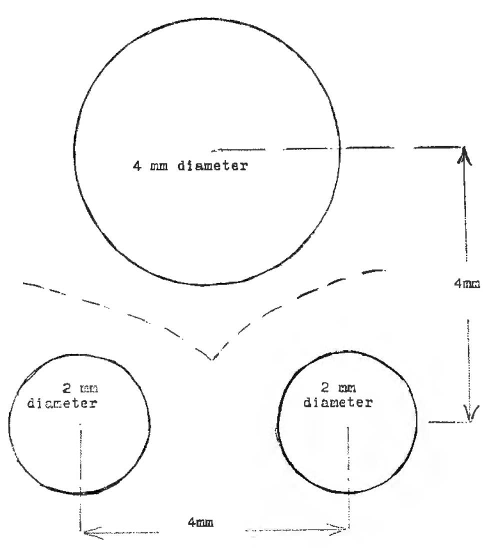
The principle used was counter immunoelectrophoresis, with a Savant electrophoresis tank; 200ml of buffer was placed in each trough, the troughs being alternated after each run. The buffer was stored at 4°C and changed every other day. Lantern slides, $2\frac{1}{2}''$ x $2\frac{1}{2}''$ were painted with a thin layer of 1 percent

molten agar which was allowed to dry[3]. The purpose of this was two-fold; to prevent the gel from leaving the slide whilst diffusing out, and to stop serum diffusing under the agar. Four ml of molten agar was pourned on each slide. The plates could be used one hour after pouring, and kept in a moist chamber for two to three days. Wells were punched in the agar using an ordinary giving set needle ground to external size 2mm. The wells were 4mm apart. Twenty-six tests plus a control fitted on one slide. As a template, a piece of paper was used with the distances marked on it.

The needle was attached to a vacuum pump which evacuated the agar plugs. The serum to be tested for the antigen was placed in the well to the left, and the antisera in the opposing well. When testing for antibody, the process was reversed. Whatman 3mm paper was used as wicks. The plates were run for 45 to 50 minutes at 250 volts. Twenty-five milliamperes were allowed for each plate. If the milliamperes started to rise, the cold power was turned on to 20°C. After 50 minutes the positive precipitin line was clearly visible, and also any strong positives. The plates were washed in saline for a few hours to eliminate any fuzziness caused by plasma proteins. Weak positives often became more apparent. Reading was facilitated by using an angle poise lamp. The slides were then stained for 10 seconds and washed twice in the methanol acetic acid wash. They were dried on filter paper at 37°C for half an hour.

Each test was also run on concentrated serum using polyacrylamide gel[1]. The test serum was concentrated by adding 0.05gm of the gel to five drops of serum. This was allowed to stand at room temperature for two hours before testing, parafilm being placed over the tubes to prevent evaporation. By this method, weak positives became apparent, but prozone phenomena occurred. Positive sera was checked for identity. The test serum was placed in a well adjacent to a known HAA, and run against an antibody. The precipitin lines fused, forming a line of identity[2].

Two commercial antisera were used: Biotest and Behringwerke, weak antigens often appearing stronger with the other antisera.




## Results

In testing 16,000 blood donors, 48 positives were found, giving an overall incidence of 0.3 percent HAA positive. All SGOTS from the positives were within normal range.

When testing Waikeria, a local borstal institution, much higher figures were obtained; 303 of the inmates donated blood samples, the average age being 18.2 years. They were approximately 50 percent European, 50 percent Maori. An incidence of 18.5 percent positivity was found (Table I).

Subsequently, two of the HAA positives developed hepatitis. Both were Maori, neither were tattooed.

A boys' boarding school was tested next, an attempt being made to emulate the close conditions at Waikeria. All the boys were European, and the total number tested was 372, the ages ranging from 13 to 19 years. Only one positive was found, a 16-year-old with a history of nephritis. This gave an incidence of 0.3 percent positivity (Table II).

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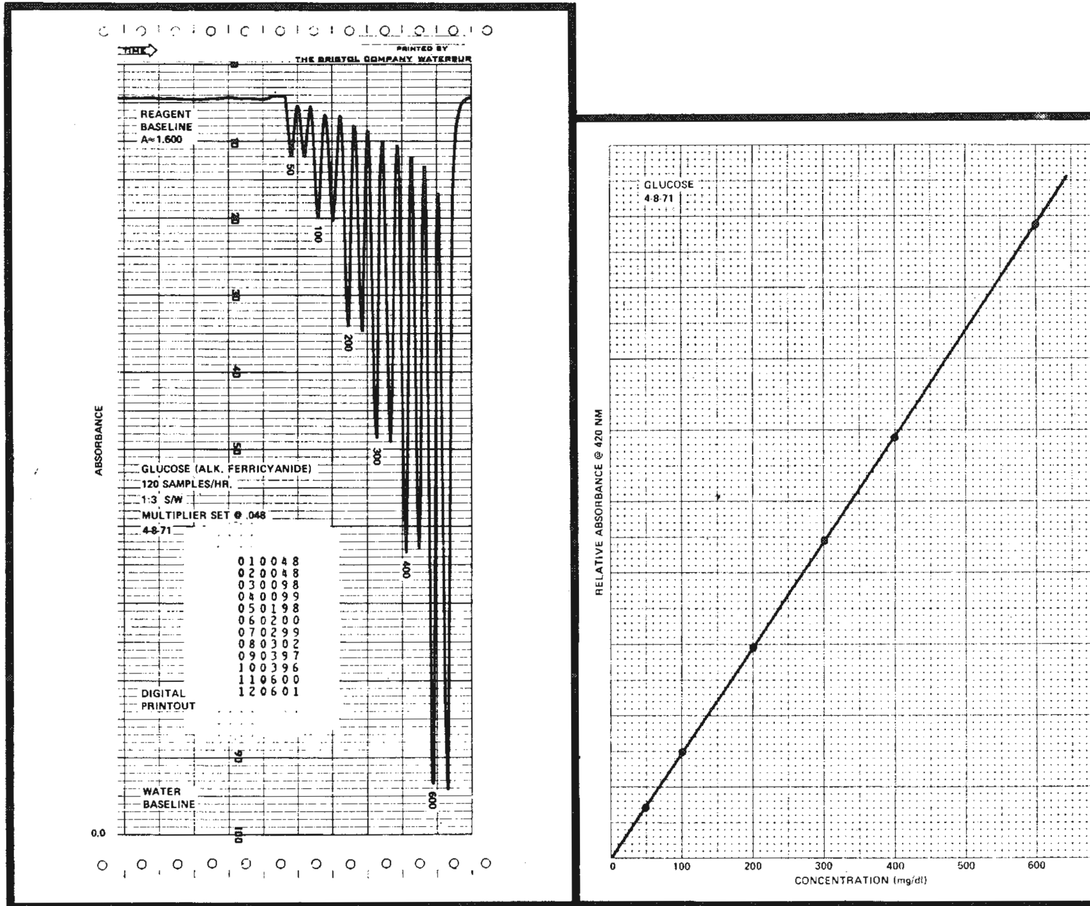


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A mixed college was tested, comprised of non-boarders, and a small proportion of Maori samples were obtained. The ages ranged from 13 to 17 years, the total number tested being 546. There were four positives, three Maori and one European, giving an overall figure of 0.7 percent HAA positive. Five hundred and nine Europeans were tested, but only 37 Maoris came forward. One girl was positive, the other positives being male (Table III).

Another mixed school of non-borders contributed 592 samples, 70 Maori and 525 European. Three HAA positives were found amongst the Maoris. The ages ranged from 13 to 18 years (Table IV).

A church college volunteered 40 Maori students, boys and girls, the ages ranging from 13 to 17 years. All were non-boarders, and four HAA positives were found (Table V).

Of the four positives, three were boys, and two of the positives were brother and sister (Table VI).

## Summary

The main difficulty encountered was in obtaining samples of blood from Maoris living in a natural home environment. Only small numbers were obtained. No correlation could be found between tattooing and HAA. Of the HAA positives, discounting Waikeria, none were tattooed. Tattooing at the borstal was generally done by the inmates themselves, using unsterile needles.

From the data obtained it would seem that the Maori in the Waikato area possesses a higher percentage of HAA than the European, On this basis possibly the Maori has had insufficient opportunity in terms of time, to develop adequate immune response.

| | No. tested | Average Age | Tattooed | HAA | SGOT | % positive |
|---|---|---|---|---|---|---|
| **TABLE I** | | | | | | |
| Maori | 153 | 18.2 | 83 | 55 | Normal | 36 |
| European | 150 | 18.2 | 70 | 1 | Normal | 0.7 |
| **TABLE II** | | | | | | |
| | 372 | 15.5 | Nil | 1 | Normal | 0.3 |
| **TABLE III** | | | | | | |
| Maori | 37 | 15.2 | 4 | 3 | Normal | 8.1 |
| European | 509 | 15.2 | 11 | 1 | Normal | 0.2 |
| **TABLE IV** | | | | | | |
| Maori | 70 | 15.5 | 7 | 3 | Normal | 4.3 |
| European | 525 | 15.5 | 4 | – | — | Nil |
| **TABLE V** | | | | | | |
| Maori | 40 | 15.5 | Nil | 4 | Normal | 10 |

TABLE VI

| | Total number of non-boarders tested | % positive |
|---|---|---|
| Maori | 147 | 6.8 |
| European | 1034 | 0.2 |

## Acknowledgments

I should like to thank the Waikato Blood Transfusion Centre for their invaluable help, and also Dr C. M. David and Dr R. G. Stephens for their advice and assistance.

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# Some Family Studies on an Unusual Rhesus Gene -$r''^{G}r$

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*From a paper presented at the 28th Conference of the NZIMLT, Tauranga, August 1972*

The Rhesus antigen G is normally present whenever C or D is present on a chromosome. It is occasionally present when both C and D are lacking[1, 5] and very rarely a person who is C or D positive may lack G[3, 4].

Anti-G is often produced in conjunction with anti-C or anti-D and most "anti-CD" sera are in fact anti-C+D+G or anti-D+G. Anti-G reacts against all cells which are C or D positive.

The antigen G was discovered in 1958[1] when cells of an apparently Rhesus negative (rr) woman reacted with most "anti-CD" sera. These cells were labelled $r^{G}r$. The presence of G antigen explained how Rhesus negative women sensitised by an $R_2$ fetus could produce an apparent anti-CD; they were in fact producing anti-D+G. It also explained why rr persons produce anti-C more frequently than D positive, C negative persons, as the former lack G.

This paper does not intend to delve into the various theories on the place of the G antigen in the Rhesus system. Interested persons are advised to consult the latest edition of "Race and Sanger". Also, as a result of much study on one of the families mentioned in this paper (the South Otago Weirs), a paper was presented at the International Blood Transfusion Congress in Washington (1972) which deals with a new hypothesis on the place of G antigen[2]. This paper will be published shortly. (Personal communication, Mr J. Case.)

The aim of this paper is to present the manner in which C and D negative, G positive cells may be identified and to give details of three family studies.

## Case Studies

Mrs Looker was first grouped at Laboratory Diagnostic Services, Auckland, using "Ortho" typing sera, and was found to be D negative with a probable genotype of $r''r$ (C and D negative, E positive). There was an unexpected positive result with the anti-CD serum, which was confirmed by repeat tests. This result was puzzling considering that the cells were negative with pure anti-C and anti-D and that tests for $D^u$ were also negative.

The specimen was referred to the Otago Regional Blood Transfusion Service at Dunedin Hospital for further investigation. The cells were routinely tested for ABO and Rhesus group and Rhesus genotype. The results obtained confirmed those found earlier at Laboratory Diagnostic Services and are shown in Figure 1.

The finding of the unusual Rhesus genotype cdEG/cde ($r''^{G}r$) in a South Otago donor some months previously that gave similar results suggested that this gene could be the cause of the anomaly.

The cells were then tested against six anti-CD sera; four pure anti-C sera and two pure anti-G sera. The anti-G sera were obtained by absorption/elution techniques using the previously discovered $r''^{G}r$ cells and an anti-CD

FIG. 1

PRELIMINARY TESTS ON HEATHER LOOKER'S CELLS

| ANTISERA → | Saline D | Alb. D | CD | DE | $D^u$ | AB | C | c | E | e | $C^W$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| LOOKER | - | - | ++ | V | - | - | - | V | V | V | - |
| Orr | - | - | - | - | - | - | - | V | - | V | - |
| $Or''r$ | - | - | - | V | - | - | - | V | V | V | - |
| $OR_2{}^{u}r$ | - | + | + | V | +++ | - | - | V | V | V | · |

FIG. 2

FURTHER TESTS ON HEATHER LOOKER'S CELLS

| ANTISERA → | C | C | C | C | CD | CD | CD | CD | CDG | CDG | G1 Eluate | G2 Eluate | $D^u$ | $D^u$ | $D^u$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LOOKER | - | - | - | - | + | ++ | ++ | (+) | V | V | V | V | - | - | - |
| Or″r | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| $Or''^{G}r$ | - | - | - | - | + | ++ | ++ | + | V | V | V | V | - | - | - |
| $OR_2{}^{u}r$ | - | - | - | - | (+) | + | + | (+) | V | V | ++ | V | +++ | +++ | +++ |

serum containing potent anti-G. The results obtained with Mrs Looker's cells are shown in Figure 2.

The anti-CDG serum in Figure 2 was then used for absorption/elution tests on the patient's cells. In addition a pure anti-D was tested in parallel. The eluate obtained from the anti-CDG absorption reacted against all C or D positive cells and against three examples of known $r''^{G}r$ cells. The absorbed serum showed a marked decrease of anti-C and a lesser decrease of anti-D. The pure anti-D serum eluate showed no activity and the absorbed serum showed no decrease in titre.

The fact that Laboratory Diagnostic Services were using separate anti-CD and anti-DE in preference to anti-CDE to check all Rh(D) negative cells was instrumental in detecting the rare Rhesus genotype, as the latter serum would have been positive due to the presence of the normal E antigen. The recognition of the unexpected positive results with the anti-CD serum and the determination to follow them up and not write them off as "contamination" resulted in confirmation of an unusual Rhesus genotype. Positive results with anti-CD serum with such cells will depend on the amount of anti-G the serum contains, and reactions may be much weaker than with C or D positive cells. For this reason it is advisable to read such tests microscopically. It is now practice in Dunedin to test all new batches of anti-CD against C and D negative, G positive cells to determine the amount of anti-G content.

With the assistance of Mr G. Weston and Mr B. Millar of L.D.S. Auckland, a family study was performed on Mrs Looker's relatives. This showed that the $r''^{G}$ gene was inherited in the normal manner through three generations, and revealed the presence of an even more unusual Rhesus genotype ($r''^{G}r''$) in the paternal grandfather of the propositus (See Figure 3).

It is a pity that, in the second generation, the father of the propositus is the only surviving member, as it would have otherwise been likely to have demonstrated the presence of both the normal r″r and the more unusual $r''^{G}r$ in that generation. In the third generation, Ross and Gary both typed as CcDEe; normally the most probable genotype would be $R_1R_2$ but in this case the presumptive genotype must be $R_1r''^{G}$.

An interesting fact emerged when the family study specimens were received in Dunedin in that the maiden name of the propositus (Mrs Looker) was WEIR. The previous case of $r''^{G}r$ was discovered in a South Otago donor named Mrs Pringle. A family study which had been carried out on this case showed that the unusual gene had been inherited through the paternal side of the family, and that the family name was also WEIR. The family study is shown in Figure 4.

Questioning of the families failed to reveal any definite knowledge of relationship, but Mr Weir Snr. of Auckland said that his grandfather had a brother who had emigrated from Scotland to New Zealand and it was thought that he did marry in the South Island. It would be very coincidental that such a rare gene should be present in two completely independent families of the same name, and the probability of inter-relationship must be strong. Unfortunately any connection was so

FIG. 3

THE AUCKLAND WEIR FAMILY

John WEIR
cdEG/cdE
$(r''^{G}r'')$

Nina Graham WEIR
cde/cde
(rr)

John Scorgi WEIR
cdEG/cde
$(r''^{G}r)$

Joyce Beryl WEIR
CDe/cde
$(R_1r)$

not tested

Heather Joy LOOKER (nee WEIR)
cdEG/cde
$(r''^{G}r)$

Glenys Jean WEIR
cdEG/cde
$(r''^{G}r)$

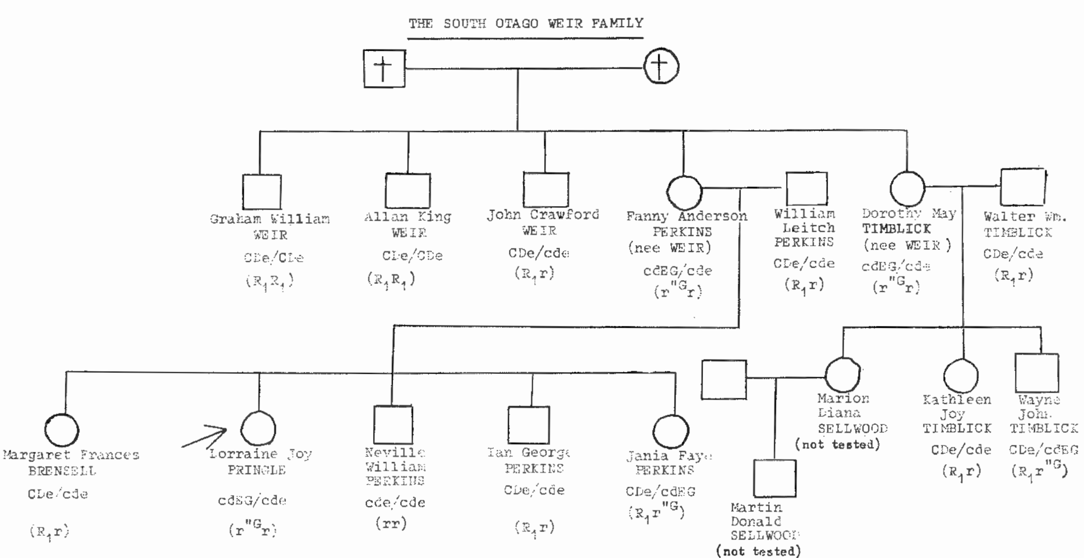
Ross Andrew WEIR
CDe/cdEG
$(R_1r''^{G})$

Gary James WEIR
CDe/cdEG
$(R_1r''^{G})$

Colin John WEIR
cdEG/cde
$(r''^{G}r)$

?

FIG. 4



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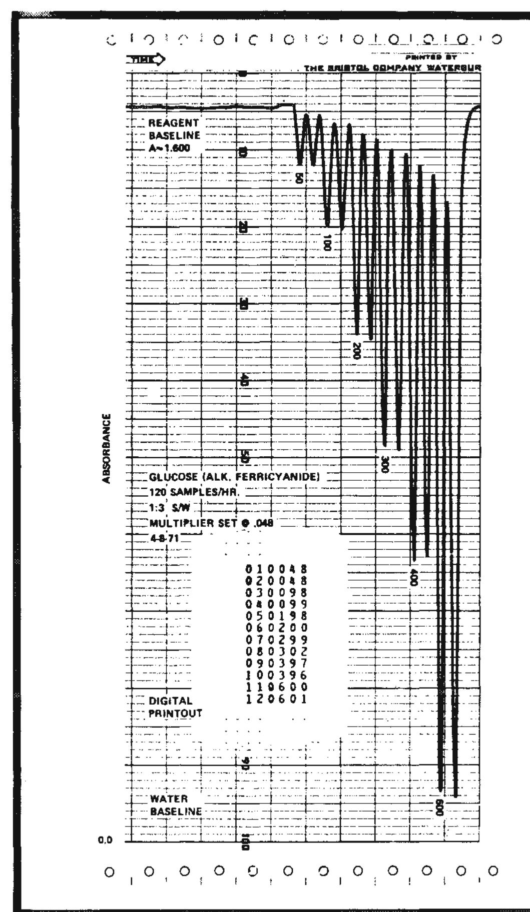


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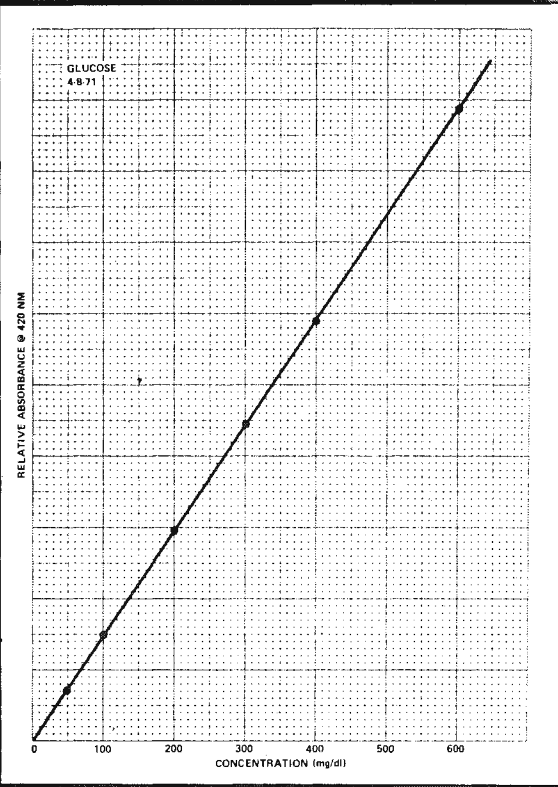


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far back in time that the New Zealand Registrar of Births was unable to be of assistance, and recourse would have to be made to Somerset House in England. Insufficient details of the propositus' great-grandfather's family made this impossible.

The finding of a probable connection between these two families caused a re-investigation of another $r''^{G}r$ which has been found during routine ante-natal testing of a Dunedin patient (Mrs McEwan) in 1971. At that time the patient had given a very brief family tree and there was no obvious connection with the South Otago Weirs. However, on re-questioning the patient's family it was found that the maternal side of the family was connected—her great-grandmother being a Catherine Weir, and one of her great uncles having the family name Weir as a forename. The patient's great-great-great-grandfather emigrated from Scotland and married a Scottish girl in Dunedin. It is known that he had brothers in Scotland, and it seems highly likely that he was the great-uncle referred to by Mr Weir Snr. of Auckland.

Thus it appears that these three $r''^{G}r$ cases are probably related to a Scottish family who emigrated amongst the first white settlers in New Zealand. Amazingly none of the propositi knew of their relationship with the other branches of the family.

The literature shows that $r''^{G}r$ is more common that $r^{G}r$ although the reason for this is open to debate amongst the authorities on Rhesus. The fact that it is more common is sufficient reason that blood bank staff should be aware of it and use techniques capable of detecting its presence. The use of separate anti-CD and anti-DE and a technique capable of detecting weaker degrees of agglutination should be sufficient to show the anomalous anti-CD positive reactions which would hopefully arouse the suspicions of the technologist.

## Summary

This paper deals briefly with the discovery of the G antigen and describes how three cases of $r''^{G}r$ were detected. Subsequent family studies showed a high degree of probability that the three cases were inter-related. The importance of using techniques capable of detecting the G antigen is discussed.

## Acknowledgments

I wish to thank Mr G. Weston and Mr B. Millar for collection of samples from the Auckland family; Mr J. Case for supplying information on the South Otago family, and the families themselves for so readily agreeing to take part in the studies.

## Addendum

Recently Mr B. Millar of L.D.S. Auckland discovered yet another $r''^{G}r$. Family studies have not yet been initiated on this case, but it will be most interesting to see whether a connection can be found with the families dealt with above.

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# Neutrophil Function Tests

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*Based on a paper presented at the 28th Annual Conference of the NZIMLT, August 1972*

## Summary

Neutrophil bactericidal activity is preceded by adhesion to the organism, phagocytosis, the formation of a phagocytic vacuole and the release of lysosomal granules into the vacuole. Tests have been devised to measure these various functions of the neutrophils and of these, a neutrophil adhesion test, the bactericidal activity test, the Nitroblue-Tetrazolium test and the role of complement and myeloperoxidase are described.

## Introduction

Recent advances in immunology have led to a greater understanding of the mechanisms whereby an individual protects himself from the environment. These defence mechanisms include the lymphocyte-plasma cells systems (B lymphocytes generating humoral antibodies and T lymphocytes being concerned with cellular-mediated immunity) and the neutrophil monocyte systems (responsible for bacterial ingestion and killing). Methods of assessment of the lymphocyte-plasma cell systems are now fairly well established and general agreement has been reached on a proposed scheme of investigation (Fudenberg *et al.,* 1971). The classification of the primary immunodeficiency diseases, however, remains confused. The development of neutrophil function tests, in order to assess the phagocytic-cidal properties of leucocytes, is more recent (see review of Nathan and Baehner, 1971) and standard methods are not as yet available. Useful tests, however, attempting to measure various aspects of neutrophil function, have been described and have a place in laboratory medicine.

This paper describes our attempts to set up a series of tests to cover all aspects of neutrophil function. Some of these tests are now being evaluated in the laboratory and others are in the planning stage only. The place of these tests in the overall assessment of neutrophil function can best be illustrated by a short synopsis of the physiology of neutrophil phagocytosis and killing.

Phagocytosis of bacteria requires the presence of complement either on the surface of the ingesting cell or on the organism itself. The complement component most commonly measured and related to phagocytosis is the third component C′3. It is also known that a deficiency of C′5 leads to depressed neutrophil bactericidal activity.

Firstly, the neutrophil moves towards the bacterium, the attraction probably being a chemical one. Pinocytosis then takes place and a phagocytic vacuole is formed within the cell. Now the bactericidal events take place and the two major systems involved are the peroxide generation system and the lysosome system. The neutrophilic (lysosomal) granules, which contain both iodide ions and myeloperoxidase, move towards the phagocytic vacuole and by a process which is not yet understood, empty their contents into this phagocytic vacuole. Hydrogen peroxide, which is produced by the neutrophil, then combines with the iodide ions, mediated by the myeloperoxidase, yielding iodine; it is the iodine which is thought to kill the bacteria.

Thus in order to study neutrophil function, we perform the following tests: neutrophil adhesion to assess their adhesive properties, bactericidal activity tests to measure their killing ability and the Nitroblue-Tetrazolium (NBT) test as another test of total neutrophil function. In addition, complement activity is assessed quantitatively and qualitatively. The myeloperoxidase activity of neutrophils is also measured. Other tests of neutrophil activity not discussed include the Rebuck skin window test (Rebuck and Crowley, 1955), the phagocytic capacity of neutrophils (Miller, 1969) and bactericidal activity using a gram negative organism.

## Methods and Comments

*Neutrophil Adhesion Test* (*Spitler et al., 1971*)

A suspension of total leucocytes equivalent to $5 \times 10^6$/ml is prepared in Hanks solution to which 200 mg percent glucose has been

added (method described below). Two to 3 ml of this leucocyte suspension is passed through 3 ml of glass wool column in a plastic syringe, the glass wool having been washed in distilled $H_2O$ and dried for 24 hours. The leucocyte suspension is allowed to pass through the glass wool under gravity and at room temperature. A white cell count is then carried out on the filtrate and the percentage adherence of neutrophils to the glass wool is calculated.

Normal adherence is approximately 95 percent or more but as we have only carried out this test a limited number of times, we have not yet determined a normal range for this laboratory.

*Bactericidal Activity Test* (*Azimi et al., 1968*)

The principle of the test consists of incubating equal numbers of leucocytes and bacteria, in this instance *Staph. aureus,* for two hours to allow phagocytosis and killing to proceed and to estimate the bactericidal activity by the number of organisms remaining alive after two hours.

Firstly, a suspension of the patient's total leucocytes equivalent to $1 - 2 \times 10^7$ leucocytes per ml is prepared as follows:

20 ml of venous blood is collected from the patient into two sterile siliconised bottles (10 ml into each bottle which contains 4 ml of dextran of molecular weight 70,000 plus 100 units of heparin). The blood is allowed to sediment at room temperature for one hour and the plasma is separated aseptically into two siliconised bottles, using a sterile siliconised pasteur pipette. 0.05 ml of streptokinase containing 2000 I.U./ml (i.e., 100 units of streptokinase) is added to each bottle to prevent clumping of the leucocytes. The leucocyte-rich plasma is then centrifuged at $500 \times g$ for five minutes; the supernatant is poured off, leaving behind a pellet of leucocytes. Some degree of red cell contamination occurs which does not interfere with the test. The leucocytes are washed twice in 10 ml of Hanks solution plus 100 units streptokinase. The number of white cells is counted and, provided the white cell count of the patient is within normal limits, approximately $1 - 2 \times 10^7$ leucocytes/ml are obtained. The volume of Hanks solution added may be varied to obtain the required leucocyte concentration.

Secondly, a bacterial suspension is prepared. The organism used is *Staph. aureus* strain SV5 which is subcultured into Trypticase Soy broth the night before and incubated at 37°C for 18 hours. It is necessary that the bacterial suspension be equivalent to $1 - 2 \times 10^7$ organisms/ml. The number of organisms/ml may be ascertained in two ways: (1) with the use of Brown's turbidity tubes and (2), using observations that SV5 grows to $10^9$ in Trypticase Soy broth in 18 hours at 37°C. Hence, 5 ml of Trypticase Soy broth suspension is transferred into a sterile bottle and centrifuged; the deposit is then washed twice with sterile normal saline and finally resuspended in 5 ml of Hanks solution. A 1 : 10 dilution of this suspension is made in Hanks solution so that we now have a suspension of SV5 equivalent to $1 - 2 \times 10^8$/ml. As was mentioned previously, complement is necessary for phagocytosis and is added in the form of fresh pooled serum.

Each time this test is carried out, a normal control and a "blank" are set up. Firstly, a mixture is prepared in sterile siliconised bottles containing:

0.5 ml bacterial suspension
0.5 ml fresh pooled serum
4.0 ml leucocyte suspension

When setting up the "blank", 4 ml of Hanks solution is substituted for the leucocyte suspension. Immediately this mixture has been prepared, dilutions of the mixture are made in sterile distilled water equivalent to 1 : 10, 1 : 100, 1 : 1000 and 1 : 10,000. For convenience, these dilutions are called "Zero Time" dilutions.

The original mixture is now put on to a reciprocating shaker and placed in the 37° incubator for two hours. After two hours, dilutions in distilled water are again prepared, these being labelled "two-hour" dilutions. The addition of water disrupts the leucocytes and releases the organisms whether dead or alive. Agar pour plates are now prepared from each of the zero time and two-hour dilutions as follows: 1 ml of each dilution is placed in a clearly labelled petri dish to which is added approximately 15 ml of melted Trypticase Soy agar, taking care that the agar is not too hot (to avoid killing *Staph. aureus*). The agar and distilled water dilution are well mixed and the agar allowed to set. The plates are now incubated at 37°C for 48 hours after which time the colonies are counted over a light box

in those plates where the dilutions allow for easy counting. Corrections are made for dilutions and the original number of organisms at zero time and that after two hours can be calculated. These results may be either plotted on a graph or expressed as a percentage killing and are compared with the normal control. The "blank" generally shows a rise in the number of organisms after two hours. Normal bactericidal activity in our hands using this method is more than 94 percent killing.

*The Nitroblue Tetrazolium Test*

The NBT test can be used in two ways: (1) to test for the presence in neutrophil granulocytes of NADH (?oxidase) which is said to be deficient in chronic granulomatous disease. The method described by Gifford and Malawista (1969) is used and (2) to test for the presence of bacterial infection as described by Park *et al.* (1968).

First we will describe the NBT reduction test by stimulated neutrophil granulocytes (Gifford and Malawista, 1969): a large drop of whole blood is obtained by fingerprick; the drop is placed on a coverslip and incubated in a moist chamber for 20 minutes. During this time the neutrophils, eosinophils and monocytes adhere to the glass. The clotted drop of blood is then gently washed away with saline, leaving the neutrophils, monocytes and eosinophils stuck to the coverslip. Excess saline is removed with a filter paper but the preparation is not allowed to dry. The coverslip is now immediately inverted on to some NBT solution (equal amounts of 0.2 percent NBT in physiological saline and 0.15 m phosphate buffered saline pH 7.2) on a clean slide and replaced in the moist chamber at 37°C for 20 minutes. The coverslip is removed, drained and dried. It is fixed in methanol for 60 seconds, washed in running tap water for 10 seconds and again dried. It is counterstained in 1 percent safranin for five minutes and again rinsed in water, dried and finally mounted.

One hundred neutrophils must be counted and the number of NBT positive cells (containing black formazan granules) expressed as a percentage. Normal values vary between 70-90 percent. It is most important that the slides and coverslips used are either acid-washed or soaked in xylol overnight and rinsed in ethanol to permit maximal adhesion of leucocytes. The high percentage of NBT positivity is explained by the glass activation of neutrophil phagocytosis; the membrane of the neutrophil is modified in a way not yet understood and the NBT enters the cell. Phagocytosis also accelerates the hexose monophosphate shunt, resulting in the production of hydrogen peroxide. Once inside the cell, the NBT is reduced to formazan which appears as black granules in the cell cytoplasm. NBT + NADH → NAD + NBTH or formazan. Reduced NBT positivity implies a relative lack of NADH in neutrophils and poor function.

The second NBT test is used to detect the presence of infection (Park *et al.,* 1968). One ml of venous blood is drawn into a plastic syringe and transferred to a plastic tube (or acid washed and siliconised glass tube) to which has been added 75-100 units of heparin. Approximately 0.1 ml of this is added to an equal amount of NBT solution in a plastic (or siliconised) tube and incubated at 37°C for 15 minutes; it is then kept at room temperature for a further 15 minutes. At the end of this period the tube is mixed and very thin films are made. These are then fixed in methanol, stained with a Romanowsky stain and mounted. The slides are examined under oil immersion; 100 neutrophils counted and the percentage of positive cells recorded.

Park reports that in the absence of bacterial infection, the mean percentage of NBT positive cells was 8.5 percent. Thus, patients that had 8.5 percent or less positive NBT cells did not have bacterial infection and those that had more than 8.5 percent were suspected of having an infection. Difficulties have been encountered with this method as increased NBT positivity due to infection has been associated with leucocyte clumping. Separation of NBT positive from negative cells has proved somewhat difficult and an exact percentage of positivity cannot be reported. Nevertheless, marked clumping of leucocytes would indicate NBT positivity and the presence of bacterial infection. As in the previous method, the glassware is either acid-washed or soaked in xylol overnight and rinsed in ethanol. Note also that in this method, great care is taken to put the blood into siliconised tubes in order to prevent any artefactual changes of the cell membrane which would allow the NBT to enter the cell in the absence of phagocytosis and yield a false positive result.

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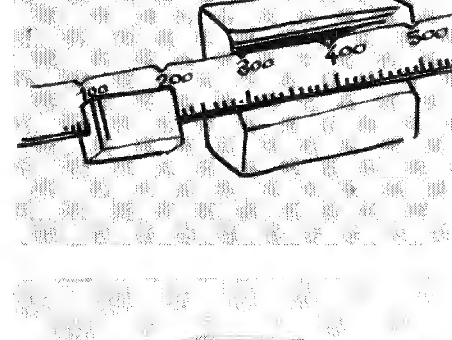


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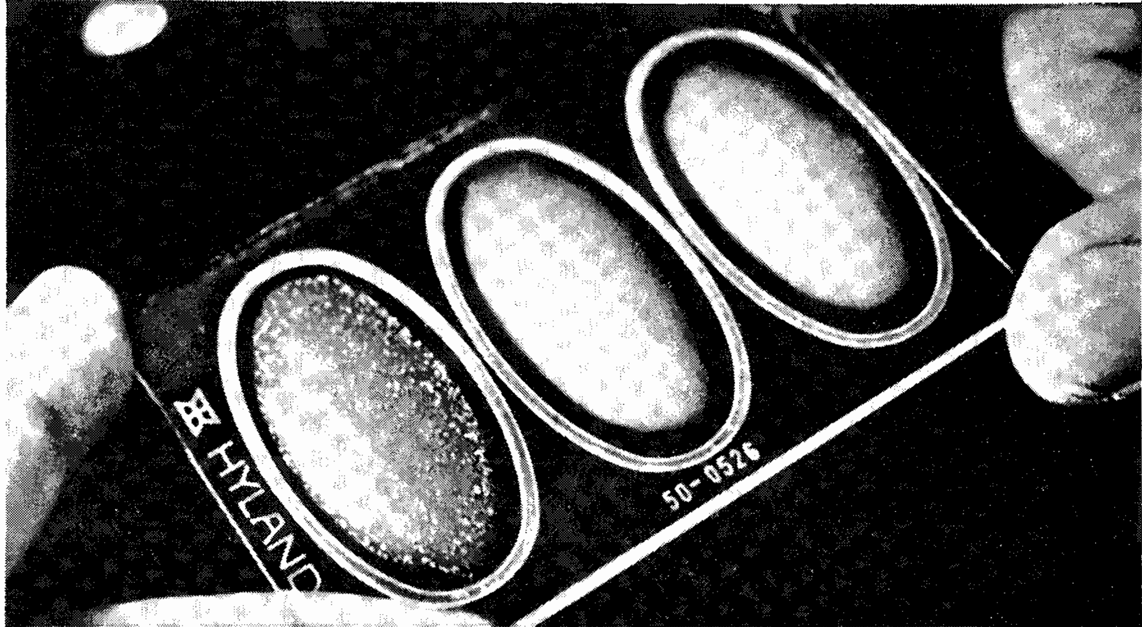


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Serum complement is tested qualitatively by comparing the bactericidal activity of normal neutrophils in the presence of normal serum (complement) and the serum of the patient under investigation. A prior estimation of C′3 in the patient is done using commercial immunodiffusion plates supplied by Hoechst (Austr.). Nathan and Baehner (1971) state that deficiency of either C′3 or C′5 may result in poor bactericidal activity of neutrophils.

Myeloperoxidase activity is assessed on the peripheral blood film. It has been stated by Catovsky *et al.* (1972) that neutrophils lacking myeloperoxidase were found in 12 out of 28 cases of acute myeloid leukaemia and in two out of seven cases of "blast-cell transformation" of chronic granulocytic leukaemia. They suggested that these abnormalities could result from a disturbance in the function of the Golgi complex in cells derived from leukaemic stem cells. This lack of myeloperoxidase impairs the bactericidal capacity of the neutrophils. An apparent inherited deficiency of neutrophil myeloperoxidase has also been reported (Salmon *et al.,* 1970). Our normal range of myeloperoxidase positive cells varies from between 95-100 percent and we have confirmed the reduction in myeloperoxidase activity in some cases of leukaemia.

It is apparent that the methods described to assess neutrophil function are early attempts only to put some order in the field. The tests available are numerous, but they have not been used by enough workers or for long enough to permit them to be judged critically. Problems such as excess clumping of NBT positive neutrophils and what is the right ratio of organisms to leucocytes to clearly differentiate normal from abnormal bactericidal activity remain problems to be resolved. Moreover, the development of these tests has led to the description of new neutrophil dysfunction syndromes. These diseases are as yet poorly understood and cannot be satisfactorily classified. Future work in this field is bound to yield more information of great interest.

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## BAGG Broth and Group D Streptococci

Buffered azide glucose glycerol (BAGG) broth has been found to be a reliable test for the routine presumptive identification of *Streptococcus faecalis* and its variants. In a recently completed study 217 streptococcal isolates from 213 urine samples were cultured in BAGG broth and grouped serologically. Antigen extracts were reacted against Lancefield Groups A, B, C, D, and G antisera and 210 (96.8 percent) were identified accordingly; seven could not be assigned to any of the above groups.

By this means 161 isolates were found to belong to Group D, 28 to Group B, 18 to Group C, two to Group A and one to Group G. On 206 occasions (95 percent) the serological and cultural tests were in agreement, i.e., in 160 instances both were positive and in 46 both were negative. Of the remainder 10 gave cultural results which could be considered falsely positive. In this regard it seems that Group C strains (*Streptococcus equi*) are commonly capable of growth in BAGG broth for of 18 strains that demonstrated Group C—specific C carbohydrate eight (44.4 percent) acidified the culture medium. Two of the isolates found to be serologically ungroupable also gave a positive BAGG result. No disparity in cultural and serological testing was noted with Group A, B and G isolations. On only one occasion did the cultural test fail in the presence of a positive D grouping.

M. J. Gratten,
Pathology Services,
Christchurch Hospital.

December, 1972.

# SELECTED ABSTRACTS

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

## CHEMICAL PATHOLOGY

**Application of Fe (11)-S-Pyridyl Benzodiazepin-2-ones to the Manual or Automated Determination of Serum Uric Acid.** Klein, B. and Lucas, L. B. (1973), *Clin. Chem.*, 19, 67.

A new coupled-ferricyanide redox reaction has been developed for the manual and automated determination of uric acid. The automated procedure has a simple manifold with dialysis. The violet-blue colour formed a $Fe^{2+}$ — 5 pyridyl benzodiazepin-2-one chelate, is proportional to uric acid concentration and is read at 580nm.

The authors demonstrated excellent accuracy, precision, and recovery of added uric acid. They also showed that formaldehyde commonly used to preserve uric acid solutions interfered with recovery in the automated procedure.

Results compared favourably with carbonate phosphotungstate and uricase methods.

—A. G. W.

**Some Divergent Results of Protein Standardisation.** Booij, J. (1972), *Clin. chim. Acta*, 38, 355.

The author has demonstrated the different results obtained by different methods for determining the protein content of human albumin, bovine albumin, or fresh serum as a standard. Only biuret procedures, which are suitable for standardisation with human albumin can be recommended. The difficulty of standardising the dye binding methods for albumin is mentioned. The author suggests the use of fractionation method using fresh sera.

—A. G. W.

**Modifications to SMA 6/60 and SMA 12/60 Dialysers.** Farrance, I., Culross, J., and Dennis, P. M. (1972), *Clin. chim. Acta*, 38, 477.

The newest dialyser of the SMA 6/60 and 12/60 series has a fixed inlet and outlet nipple which tend to split, causing leakage. The authors detail a solution to the problem with the use of standard Technicon fittings.

—A. G. W.

**An Improved Method for the Concentration of Bromosulphthalein in Blood.** Culter, M. G. (1972), *Clin. chim. Acta*, 40, 503.

The existing methods can give rise to errors due to turbidity, haemolysis and other serum pigments. This method is claimed to eliminate such errors. Pigments and proteins are precipitated from serum by a mixture of acetone, saturated magnesium sulphate, and sodium hydroxide. The inclusion of ammonia solution with sodium hydroxide severs the protein binding of BSP in serum and liberates the BSP from the precipitate into a clear solution.

—A. G. W.

**The Effect of Haemolysis on the Determination of Plasma Constituents.** Brydon, W. W. and Roberts, L. B. (1972), *Clin. chim. Acta*, 41, 435.

For a number of constituents commonly affected by haemolysis the authors have calculated the error per gram haemoglobin and compared them with results calculated by the formula derived by Caraway. The results are shown in tables.

The authors conclude that a corrective procedure could find application in some circumstances.

—A. G. W.

**An Improved Rapid Automated Method for the Estimation of Blood Glucose.** Moore, G. R., Barnes, I. C. and Pennock, C. A. (1972), *Clin. chim. Acta*, 41, 439.

The method of Brown using copper neocuproin has been studied and modified. The colour development time has been reduced by using a 10ft coil of 1.6mm internal diameter. They found the three double mixing coils used to mix dialysate and neocuproin unnecessary and substituted a single mixing coil. The flow rate was modified to take more solution through the flow cell thereby improving the wash characteristics.

With the improvements a 10mg/100ml solution of glucose gave 7-10 percent and 300mg/100ml standard gave 90-95 percent full scale recorder deflection. The total "sample retention time" from sampler to recorder is less than three minutes.

From the analysis of the standard profile they calculated a sample rate of 100/hr at a 1/1 sample to wash ratio would give discrete peaks. Carryover is said to be less than 1 percent when a sample is 8.3 percent of the value of the preceding peak. The authors found they could run the test at 120/hr at a 2/1 sample wash ratio with carryover of less [illegible] percent.

—A. G. W.

**On a Standard Temperature for Clinical Enzymology.** King, J. (1972), *Ann. clin. Biochem.*, 9, 197.

The need for a standard temperature for clinical enzyme assays is discussed. The inadequacy of temperature correction factors is demonstrated and from this it is inferred that reaction and standard temperature must be the same. The advantages and disadvantages of various temperatures are examined and it is concluded that 37°C is the most practical of the alternatives.

—A. G. W.

**A Non Mercurimetric Automated Method for Serum Chloride.** Fingerhut, B. (1972), *Clin. chim. Acta*, 41, 247.

A new automated chloride method for AA II systems is described. It is based on the reaction between ferric perchlorate and chloride ion in dilute perchloric acid. The method offers the advantage of greater specificity and eliminates mercury pollution. The method gives a linear reaction in respect to concentration.

—A. G. W.

**Biliverdin Appearing in a Case of Malnutrition.** Prichard, J. S. (1972), *Br. J. clin. Pract.*, **26**, 481.

In the case reported in this article, biliverdinuria and biliverdinaemia occurred in a patient with megaloblastic anaemia. The appearance of billiverdin during haemolysis has been reported only rarely although it has been more commonly recognised in obstructive jaundice.

Both urine and serum showed a green colouration —the serum faintly, the urine strongly. The urine, tested by Fouchet's method, gave a green spot suggestive of biliverdin and a similar result was obtained with serum after deproteinisation and concentration.

—J. H.

**Tests for Glucosuria: An Analysis of Factors That Cause Misleading Results.** Feldman, J. M. and Lebovitz, Francine L. (1973), *Diabetes,* **22**, 115.

In preliminary studies, 25 urine samples free of glucose as tested by Tes-Tape (Lilly), Clinistix (Ames), Diastix (Ames) and Clinitest (Ames) were obtained from normal volunteers. The specimens were then fortified with glucose to a concentration of 0.05 percent and retested. This small amount of glucosuria produced a positive test with Tes-Tape in 100 percent of the specimens, with Diastix in 40 percent and with Clinitest in 20 percent. The glucosuria was not evident with Clinistix.

Study of urine specimens from 513 diabetic and non-diabetic patients indicated that the incidence of potentially misleading urine tests for glucose is high.

Tes-Tape was found to be the most sensitive test, consistently detecting as little as 1/20 percent glucose. It is the least affected by reducing metabolites. It is suggested that Tes-Tape is able to avoid falsely negative tests because it acts as a minichromatography system, separating glucose from reducing metabolites which interfere. It is very satisfactory both for screening for diabetes and for following the adult onset diabetic patient whose disease is fairly well regulated. It is less useful in the case of patients with brittle or juvenile diabetes because the discrimination of shades of green (with glucose concentrations >1/2 percent) is sometimes difficult to make.

—J. H.

**Alpha Foetoprotein in Maternal Serum: A New Marker for Detection of Foetal Distress and Intrauterine Death.** Seppälä, M. and Ruoslahti, E. (1973), *Amer. J. Obstet. Gynec.*, **115**, 48.

The authors used radioimmunoassay to determine maternal serum alpha foetoprotein (AFP) levels in normal and high-risk pregnancies and found that the AFP test is useful in predicting foetal distress and intrauterine death. The AFP levels of 13 women whose foetuses died *in utero* were from 280 to 9000 μg/litre. Nine of these (69 percent) were above 530 μg/litre, which was the upper limit of normal in 65 uncomplicated cases. The increase in maternal AFP took place before the foetal death. Clinically established foetal distress was correctly predicted by the AFP test in two women whose oestriol excretion was normal.

—J. H.

**Clinical Role of Serum Gastrin Measurements in the Zollinger-Ellison Syndrome.** Thompson, J. C., Reeder, D. D. and Bunchman, H. H. (1972), *Am. J. Surg.*, **124**, 250.

Zollinger and Ellison in 1955 described a clinical syndrome which is now recognised to consist of massive gastric hypersecretion, peptic ulceration, and a nonbeta cell islet tumour of the pancreas. Gregory *et al.*, 1960, demonstrated that the active secretagogue of the Zollinger-Ellison (Z-E) tumour was similar to gastrin, and radioimmunoassay measurements of serum gastrin concentrations have shown hypergastrinaemia to be a characteristic of the Z-E syndrome.

The initial complaints can be relatively innocuous and the development of the full-blown picture insidious. The importance of establishing the diagnosis, however, is clear; the mortality in patients treated without surgery is reported to be 78 percent but the mortality after primary total gastrectomy is only 13 percent.

Early bioassay techniques used to measure gastrin concentrations were relatively insensitive and the results were largely qualitative. The development of sensitive radioimmunoassay techniques for gastrin has allowed a definitive diagnosis to be made much earlier, and thus patients may be operated on sooner.

—J. H.

## CYTOLOGY

**Sex Chromatin in Gynaecologic Cancer, Incidence and Limitation of its Clinical Interpretation.** Sinacky, J. (1972), *Acta cytol.*, **16**, 105.

A study was made of the sex chromatin incidence in 651 gynaecologic cancers investigated in Feulgen stained histologic sections. The five year survival rate is, in general, worse in sex chromatin negative cancers (162 cases), than in sex chromatin positive group (48 cases). Nuclear size studies performed in epidermoid cancer of the uterine cervix and endometrial cancer revealed that sex chromatin was found predominantly in the range below 10 microns.

On the basis of karyometric studies some explanation of cytokinetic problems of the sex chromatin negativity has been sought.

—W. S.

**Cytologic Findings in Vaginal and Oral Smears from Pregnant Women.** Hugoson, A., Winberg, E. and Angstrom, T. (1972), *Acta cytol.*, **16**, 111.

Vaginal, gingival and buccal smears for cytohormonal examination were obtained from the 12th to 38th week of pregnancy and two days and eight weeks after delivery from 26 healthy pregnant women.

The vaginal smears showed a normal pregnancy picture. The oral smears obtained during pregnancy showed a constant cell picture without any significant variation. The buccal smears were dominated by intermediate cells, while gingival smears contained anucleated superficial cells.

Eight weeks after parturition the gingival epithelium showed increased maturation. In order to decide whether this represents the normal gingival picture in non-pregnant women, it would be worthwhile to carry out a similar study of normally menstruating women.

—W. S.

**Vaginal Cytology as a Monitor of Foetal Well-being in Early Pregnancy.** Sen, D. K. and Langley, F. A., (1972), *Acta cytol.,* **16**, 116.

A method is presented of grading lateral vaginal wall smears for assessing the outcome of pregnancy. Four cytologic features: eosinophilic and karyopyknotic indices, bacterial flora and cell clumping, are used in combination to give a more accurate assessment than a single feature. Five grades of smear were described. In threatened abortion the incidence of foetal loss bears a direct relation to the grading of the smears. Smears are more reliable as an indication of foetal loss in threatened abortion than in complications occurring in late pregnancy.

—W. S.

## HAEMATOLOGY

**Reliable Routine Estimation of Small Amounts of Foetal Haemoglobin by Alkali Denaturation.** Pembrey, M. E., McWade, P. and Weatherall, D. J. (1972), *J. clin. Path.,* **25**, 738.

This technical method described fully, is a modification of the method of Betke *et al.* (1959). Some of the haemolysate is converted to cyanmethaemoglobin and exposed to the action of 1.2N sodium hydroxide for two minutes before saturated ammonium sulphate is added. The authors claim excellent reproducibility and increased sensitivity and accuracy. The concentration of the haemolysate at 8-10g/100ml is discussed together with anticoagulants and storage effects.

—M. J. G.

**A Simple Method for the Detection of Unstable Haemoglobins.** Carrell, R. W. and Kay, R. (1972), *Brit. J. Haem.,* **23**, 615.

A simple test is described for the detection of unstable haemoglobins by the addition of haemolysate to an isopropanol/buffer solution at 37°C. Over 200 normal haemolysates have been tested giving no precipitation up to 30 minutes incubation whilst six samples containing an unstable haemoglobin gave a precipitate within 5 minutes, which was flocculent by 20 minutes. As well as being a simple and effective screening test, the procedure is suitable for the purification of unstable haemoglobins for further studies but suffers from limitations, probably in common with the heat precipitation method, in the quantitation of the abnormal haemoglobin. Its use as a research tool is illustrated by comparative measurements of the stabilities of various forms of haemoglobin. These show deoxyhaemoglobin to be considerably more stable than oxyhaemoglobin thus supporting suggested interchain bondings in deoxyhaemoglobin.

—M. J. G.

**Megaloblastic Anaemia in Pregnancy and the Puerperium.** Tan, P. M. (1972), *Ann. Acad. Med., Singapore,* **1**, 79.

In recent years, there has been an increased awareness of the importance of megaloblastic anaemia as a cause of maternal ill-health in pregnancy and the puerperium. Its frequent association with pregnancy complications is generally accepted. It has also been found to adversely affect the course of labour. An increase of foetal malformation in cases of megaloblastic anaemia has also been reported. The importance, therefore, of early diagnosis of this condition cannot be overstressed.

The peripheral blood lobe index (percentage of polymorphonuclear leucocytes with five or more lobes out of 200 polymorphonuclear leucocytes examined) was correlated with the marrow picture in 112 cases. A good correlation was found between megaloblastosis and the lobe index when it was 3 percent. This correlation was even more accurate when the lobe index was 5 percent or above in this study of 2,569 antenatal and 1,007 postnatal patients.

—J. H.

**The Nitroblue Tetrazolium Dye Test.** Rubin, B. E. and Tramont, E. C. (1972), *Med. Ann. Distr. Columbia,* **41**, 422.

Reduction of nitroblue tetrazolium (NBT) dye by polymorphonuclear cells was found originally to be a useful test for diagnosing and studying patients with chronic granulomatous disease. Later it was adapted to the study of patients with a variety of febrile illnesses. Preliminary reports showed it to be very promising in distinguishing febrile patients with acute bacterial and fungal infections from those patients with fever from other causes. This article is a report of the authors' experience with the NBT test over a six-month period in the study of 117 patients.

A mixture of heparinised blood and NBT was incubated for 30 minutes at 37°C. A thin film was then stained with Wright's and the number of neutrophils containing reduced black formazan deposits was determined after viewing 100 neutrophils.

The authors' results agree in general with the previous studies reported in the literature. The test appears to be helpful in alerting the physician to the possibility of an acute bacterial infection. In this sense, it appeared to be more helpful than the WBC count and differential. However, it is stressed that the NBT test must be interpreted in each case in the light of the clinical examination and other laboratory findings.

—J. H.

**Pyknocytotic Haemolytic Anaemia of the Newborn.** Petersen, Helena S. (1972), *Acta paediat. scand.,* **61**, 362.

Pyknocytes are RBCs which are usually smaller than normal; they are distorted, contracted and fragmented, with irregular cell borders, often with pointed processes, and they stain densely.

The presence of a small number of pyknocytes seems to be common both in full-term and premature infants during the first three months of life. Other workers have found 0.5-1.9 percent pyknocytes in full-term infants aged 5-8 weeks and 0.3-5.6 percent in prematures aged 1-83 days. The number increased within the first 2-3 months of life and fell rapidly after the age of three months. In adults, pyknocytes invariably constitute $<$0.3 percent. However, in infantile pyknocytosis the number of these cells is considerable, up to 50 percent.

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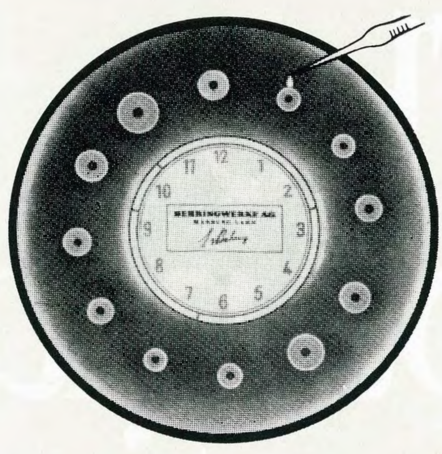


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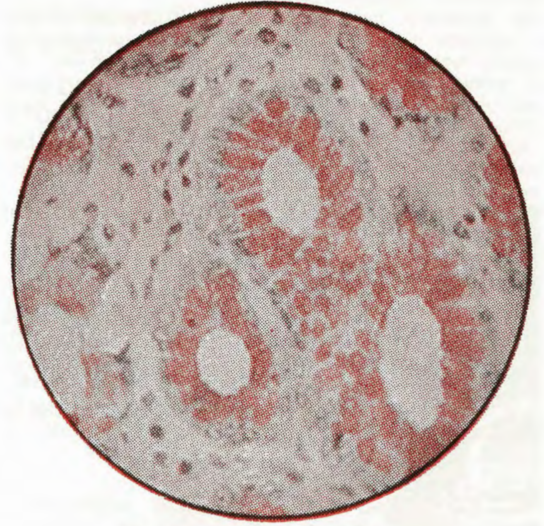

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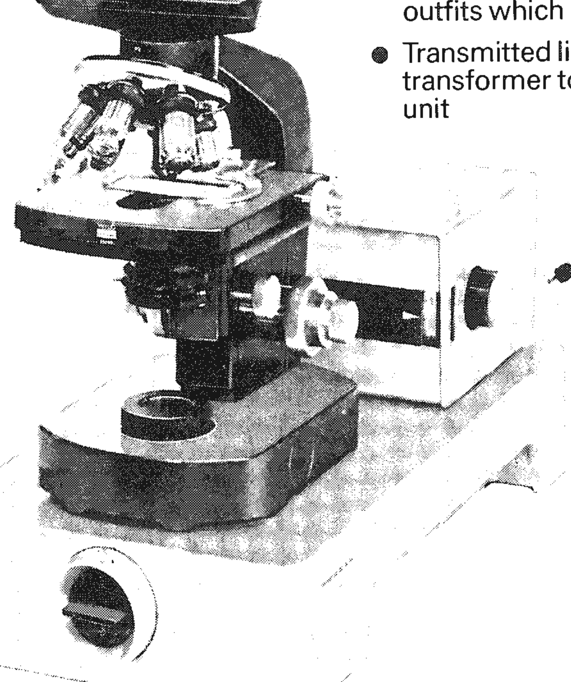

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At present, infantile pykocytosis is conceived as a haemolytic anaemia which is characterised by a morphological abnormality of the RBCs, probably brought about by an extracorpuscular factor. It is thus apparently not a hereditary defect, but a transient abnormality which may be encountered in infants within the first few months of life. As the abnormal cells may occur unaccompanied by clinical symptoms, the condition is presumably an accentuation of an apparently normally occurring developmental phenomenon.

The RBC abnormalities are highly reminiscent of those seen in toxic haemolytic anaemia, hepatic or renal insufficiency and in micro-angiopathic haemolytic anaemia.

Only a few cases of pyknocytotic haemolytic anaemia are on record.

—J. H.

**Utilisation of Leukocytes for the Study of Inborn Errors of Metabolism.** Hsia, D. Y. Y. (1972), *Enzyme,* **13**, 161.

Some 175 "inborn errors of metabolism" have been described. Of these, some 35 can be diagnosed through the use of WBCs. Depending upon the problem to be investigated, one can prepare lysates of total WBCs, lysates of specific fractions, such as lymphocytes or polymorphonuclear leucocytes, or lysates of lymphocytes stimulated by phytohaemagglutinin.

Since it is considerably easier to work with WBCs these techniques offer obvious clinical advantages over the use of fibroblasts cultivated from skin biopsies or from tissues obtained by biopsies of other organs.

—J. H.

**The Lymphocyte in Immunobiology.** Ritzmann, S. E., Daniels, J. C., Sakai, H. and Beathard, G. A. (1973), *Ann. Allergy,* **31**, 109.

The lymphocyte, once considered an end-stage cell devoid of known functions, stands at the forefront of new developments pervading the entire field of biology. Profoundly influencing such areas as infection, oncology, autoimmunity and transplantation, it has emerged as the healthy person's chief guardian against myriads of invading organisms, as well as the enemy that bears from within, the mutant cell with neoplastic potential. For the execution of its multi-faceted task, the lymphocyte possesses unique dual functions, namely, those of recognising the disparate antigenic composition of foreign or nonself material, and of subsequently eliminating it, either directly or indirectly.

During the last decade we have probably gathered more information about the lymphocyte than during the entire preceding time period. The primary interest of the modern investigator lies not in areas of staining or chromatin patterns, the battleground of haematologists in times past, but rather in the functional aspects of the lymphocyte, a cell which is now recognised as an incredibly complex factory turning out dozens of biologically active products.

—J. H.

## HISTOLOGY

**A Soft Epon for the Rotary Microtome.** Püschel, Mónica, Herrera, Anita and Alvarez, J. (1973), *Acta anat.,* **84**, 71.

The histological processing of tissue produces different sorts of artefacts. It is generally accepted that the techniques used for electron microscopy reduce these artefacts to a minimum. The main shortcoming of the straight use of this method for light microscopy is the small area of tissue that can be sectioned with the ultramicrotome.

Several plastics have been developed for the steel knife with greater or less success. The authors have developed a soft Epon for the rotary microtome which has been used in several investigations. The main advantages of this embedding method are: better image and details, easy handling of small specimens, easy orientation of specimens and suitability for "problem" tissues. Not all staining methods devised for wax sections are successful with this soft Epon; complete directions are given. —J. H.

**Microchemical Analysis of Human Tibial Growth Cartilage in Various Forms of Dwarfism.** Stanescu, V., Stanescu, R. and Szirmai, J. A. (1972), *Acta endocr., Copenh.,* **64**, 659.

Morphologic and biochemical analysis of the growing cartilage in various forms of dwarfism is an important prerequisite for the understanding of abnormal human growth. Microchemical determinations of glycosaminoglycans and collagen were performed in isolated histological zones from sections of tibial epiphysial plate biopsies obtained from children with growth disorders (pituitary dwarfism, congenital myxoedema, Turner's syndrome, Noonan's syndrome, mucopolysaccharidosis type VI, vitamin D resistant rickets and achondroplasia). Alternate sections were used for histochemical localisation of glycosaminoglycans and proteins.

In some of the pathological biopsies the concentration of chondroitin sulphate was slightly decreased whereas that of collagen was slightly increased. A marked increase in the collagen concentration was found in achrondroplasia. In several pathological biopsies there were indications of possible deviations from the normal molecular characteristics of chondroitin sulphate. —J. H.

## MICROBIOLOGY

**Significance of Coagulase-negative Staphylococcus in Urine.** Bailey, R. R. (1973), *J. Infect. Dis.,* **127**, 179.

Twenty-six episodes of urinary tract infection (UTI) due to coagulase-negative staphylococci were studied. These represented 16.7 percent of all urinary tract infection seen in a clinic over a 21-month period.

The number of organisms in urine may be considerably less than is usually encountered with Gram-negative UTI; the colony count may be in the range of 5,000 to 50,000/ml. It is not generally appreciated that the carefully evaluated statistical concepts and criteria proposed by Kass for significant bacteriuria were derived from meticulous studies of Gram-negative bacterial infections and are not generally applicable to UTI with the Gram-positive cocci. Coagulase-negative staphylococci grow with difficulty in urine, and unreliably on some popular culture

media (*e.g.,* MacConkey agar) or at room temperature.

It is completely unjustifiable to consider the presence of coagulase-negative staphylococci in urine as always an insignificant contaminant. A careful bacteriologic diagnosis is essential, and it is preferable to obtain urine for culture by suprapubic aspiration.

—J. H.

**Acute Candida Arthritis: Report of a Case and Use of Amphotericin B.** Noyes, F. R., McCabe, J. D. and Fekety, F. R., Jr. (1973), *J. Bone Jt. Surg.,* 55-A, 169.

Candida involvement of bones and joints has been exteremly rare, the first having been reported in 1960. With more liberal use of antimicrobial and antimetabolite agents, the occurrence may rise.

The diagnosis of a deep Candida infection is often difficult. Peripheral blood films may contain Candida blastospores and pseudohyphae either as a sign of candidaemia or, if an intravenous catheter is used, an indication that an infected thrombus exists at the catheter tip, which is the source of the organism. A precipitin test may aid in the diagnosis of candidiasis. The sudden appearance of the organism in the urine may indicate renal involvement which is known to be common with dissemination.

—J. H.

**Bacteroides Liver Abscess.** Futch, C., Zikria, B. A. and Neu, H. C. (1973), *Surgery,* **73**, 59.

During 1970 and 1971, five cases of liver abscess in which the infecting organism was *Bacteroides fragilis* were seen at the authors' medical centre. Improved anaerobic culture procedures have now shown that a variety of anaerobic organisms of non-sporiferous types probably have been undetected in infections of the liver.

—J. H.

**A Comparison of Culture Media for the Isolation of** *Trichomonas vaginalis.* Lowe, G. H. (1972), *Med. Lab. Technol.,* **29**, 389.

A comparison of Lowe's (1965) media and Oxoid Trichomonas medium number 2 is presented. When comparisons of the cases where one medium was positive and the other negative were made by the method of sequential analysis suggested by Crone and Widdowson it was obvious that Lowe's medium was superior to the Oxoid medium. Full details of the preparation of Lowe's medium are given

—D. G. B.

**Streptococcal Grouping. Heat Extraction of C Substance from beta Haemolytic Streptococci.** Hamilton, W. J. (1972), *Med. Lab. Technol.,* **29**, 385.

A discussion of methods for the extraction of C substance from streptococci prior to Lancefield grouping. The author demonstrates the efficiency of heat alone as a releasing agent and suggests two methods of extraction by heat. Autoclaving at 121°C for 15 minutes and heating in an oil bath at 180°C for one minute. The author appears unaware of the widespread use of the autoclave technique outside Great Britain and the publication of Rantz and Randall (1955) which gave details of this method.

—D. G. B.

**Immunoelectroosmophoresis in the Diagnosis of Meningococcal Infections.** Tobin, B. M. and Jones, D. M. (1972), *J. clin. Path.,* **25**, 583.

A discussion of the role of immunoelectroosmophoresis in the diagnosis of meningococcal infections is presented. It is concluded that the method may be useful and the saving in infections due to organism groups A and C but is of less value in organisms of group B due to the difficulty of preparing good type B antisera. The usefulness as a diagnostic aid of immunoelectroosmorphoresis is greatest in cases where antibiotic therapy has been commenced and traditional diagnostic methods may well prove useless.

—D. G. B.

**Preparation of Plaque-free Pyocin Extracts for use in the Typing of** *Pseudomonas aeruginosa.* Rampling, A. and Whitley, J. C. (1972), *J. Med. Microbiol.,* 5, 305.

This interesting paper describes a method of preparing plaque-free pyocins for use in typing *Ps. aeruginosa* by a modification of the method of Osman. All who have experienced the limitations of the Gillies technique of pyocine typing will welcome this new devolopment. Full details are given of the technical methods involved.

—D. G. B.

**A Modification of the Oxidation Fermentation Test for the Classification of Micrococcaceae.** Chalmers, A. (1972), *Med. lab. Technol.,* **29**, 379.

Bromcresol Purple used as an indicator in the OF medium described by the international committee on the Micrococcaceae was found to have an inhibiting effect on certain strains of Micrococcaceae. Marked batch to batch differences were found and the product marketed by Difco was found to be the most satisfactory, when used at half the concentration recommended by the international committee. It is suggested that sealing fermentation tubes with soft paraffin rather than liquid paraffin gives a more effective seal.

—D. G. B.

**Experiences in the use of Commercial Antisera for the Capsular Typing of Klebsiella species.** Caswell, M. W. (1972), *J. clin. Path.,* **25**, 734.

A description of the author's experiences using the recently introduced Klebsiella typing antisera marketed by Difco Laboratories. The paper is interesting but hardly useful to the routine hospital microbiology department.

—D. G. B.

# BOOK REVIEWS

**The Gastoenterology Assistant, A Laboratory Manual.** Melvin Schapiro, MD and Joel Kuritsky, AB (1972), 96 pages illustrated. Charles C. Thomas, Springfield, Illinois. Price $US6.75.

The authors describe this manual as presenting in step-by-step detail the basic diagnostic procedures used in the gastroenterology laboratory. They suggest that it could be used as a convenient reference work in the gastrointestinal laboratory and that it would help to establish standardised procedures. It has been produced primarily as an instructional text for in-service training in the clinical setting and for this purpose it succeeds admirably. Details of commonly used tests are given concisely and in correct sequence and with frequent graphic illustrations. Where a great variety of instruments are available as in endoscopy of the upper and lower alimentary tract the authors are careful to point this out and to indicate that the methods suggested will serve only as a framework with additional instruction being required for assistance with the use of particular instruments. For many of the tests there will clearly be a considerable variation in precise detail adhered to in different clinical laboratories but with this proviso this training manual can be highly recommended as providing a basic training framework for the gastroenterology assistant.

—B. P. M.

**Microbiology for the Small Laboratory.** Dorothy Branson, PhD (1972). Charles C. Thomas, Publisher, Springfield, Illinois. Contains 70 pages, spiral binding. Price $US3.95.

The stated aim of this book is to provide guidance to the worker in a small laboratory where no one person is concerned solely with bacteriology. This is a situation directly relevant to many small New Zealand laboratories. Despite this relevance, I do not feel that this book can be wholeheartedly recommended as the author has fallen into the trap which awaits all of those who attempt to write short, introductory, basic textbooks; namely that of leaving out too much of some aspects of the subject and of writing too much on others. In this book such points as environmental culturing are emphasised to the neglect of the sections on media, inoculation and incubation. On the credit side the book gives a clear warning of the dangers of attempting too much with insufficient time and equipment and gives suggestions for levels of service. (The first two levels suggested are inappropriate in New Zealand conditions.)

The book gives a reasonable list of essential books for the microbiology laboratory and a comprehensive and critical bibliography of the relative merits of the numerous kitset identification tests for bacteria available in the United States of America. The very comprehensive identification keys for gram-negative organisms which are based on initial differentiation using triple sugar iron agar, oxidase and urease tests use a dichotomous key system, this can lead to difficulty in identifying enterobacteriaceae if the great biochemical variability of this group is not kept in mind.

Sensitivity testing methods are not described, reference being made to the literature available dealing with the Kirby-Bauer technique. In the reviewers opinion this is not the best method for the smaller laboratory, the Stokes method being both technically simpler and more easily controlled.

There are several rather questionable techniques in the sections dealing with Bacteriology. The practice of sub-culturing broth cultures on to the plates used for primary cultures if no growth has occurred is a practice to be condemned, admittedly the author does not recommend the technique, but to mention it as acceptable if suboptimal is in a book of this type unacceptable. For urine cultures, non-quantitative aerobic, anaerobic and broth culture are advocated together with aerobic quantitative culture. This is contrary to current practice as a perusal of the author's own book list shows and certainly appears impracticable for the smaller laboratory. The value of routine anaerobic culture of urines is questionable and in fact the only use the author seems to make of this type of culture is to distinguish faecal contamination of urine by the isolation of *Cl. perfringens.*

The sections dealing with parasitology and mycology are extremely brief in conformity with the stated intention of concentrating on

Bacteriology. What is described appears to be sound although the author unaccountably fails to mention the cellophane flag technique for examining dermatophyte cultures but does describe a more complicated and less satisfactory method of preparing cultures for microscopic examination.

A comprehensive section on environmental control is included, seven pages of the 61 pages of text are devoted to this subject compared to the two pages dealing with media. Some of this material is not relevant to New Zealand conditions.

This book cannot be recommened to the smaller laboratory as the information it contains is in itself insufficient to enable adequate microbiology to be carried out and such information as it does contain is better presented in an amplified form in the standard laboratory textbooks which all laboratories carrying out microbiological examinations should already possess.

—D. G. B.

**Basic Hematology.** First Edition. Arthur Simmons, Assistant Professor, Department of Pathology, College of Medicine, University of Iowa, and Associate Haematologist, Clinical Laboratories, University of Iowa Hospitals and Clinics. 278 pages. Charles C. Thomas, Springfield, Illinois, USA. Price $US12.75.

The author of this book states "that it is written for the medical technology and certified laboratory assistant student, recent graduate, and paramedical persons who need a basic understanding of the subject that is sufficiently broad and deep so as to enable them to fully comprehend the laboratory results" It is just that. It is compiled from a series of lectures given over a period of years to these students and is not a technical manual, actual methods are excluded.

As such it is a well written and concise volume dealing with the theoretical aspects and interpretation of haematology tests which NZCS students and laboratory assistants would find very useful.

The chapters cover the full range of haematology: origin and development of cells, staining techniques, haemoglobin metabolism, abnormal haemopoietic cellularity, investigation of haemolytic states, blood coagulation and principles of clotting tests, etc. There is a chapter on technical theory and instrumentation (necessarily brief and dealing largely with the Coulter counter and Technicon equipment) and a short one on quality control.

Spelling is naturally American but terminology is in general universal, though the author uses the term "rubricyte" in preference to erythroblast or normoblast which is unusual. On page 79 "stercobilin" is misspelt.

Overall this is a most useful volume for haematology students and for any technologist involved in teaching to NZCS standard and is well worth a place in the laboratory reference collection.

—B. W. M.

**Pathology.** C. P. Mayers, MRCPath (1972). 155 pages. The English Universities Press Ltd. Price in UK £2.25 Boards Edition, £1.20 Unibook Edition.

This little book forms another worthwhile addition to the paperback range of the "Modern Nursing" series, at present covering 11 different aspects of medicine. As the author states in his preface the book has been written to give nurses and nursing students a short account of the common disease processes, their causes, and effects. The trainee technologist may very well be included in this group as the text provides concise and simple descriptions of many clinical conditions which the laboratory is often asked to investigate or confirm.

The book has 17 sections beginning with a general discussion on infection and immunity, miscellaneous disease processes, etc., followed by separate chapters detailing diseases of specific organs, or organ systems, It is a very easy book to read, perhaps in some instances over-simplified, but due mainly to the author's easy style and somewhat unusual analogies. For example the discussion on hypertension is compared with playing the bagpipes in that the heart pumps blood into the system just as a piper pumps air into the bag, the release of pressure being via the chanter and drones rather than through the arterioles of the vascular system.

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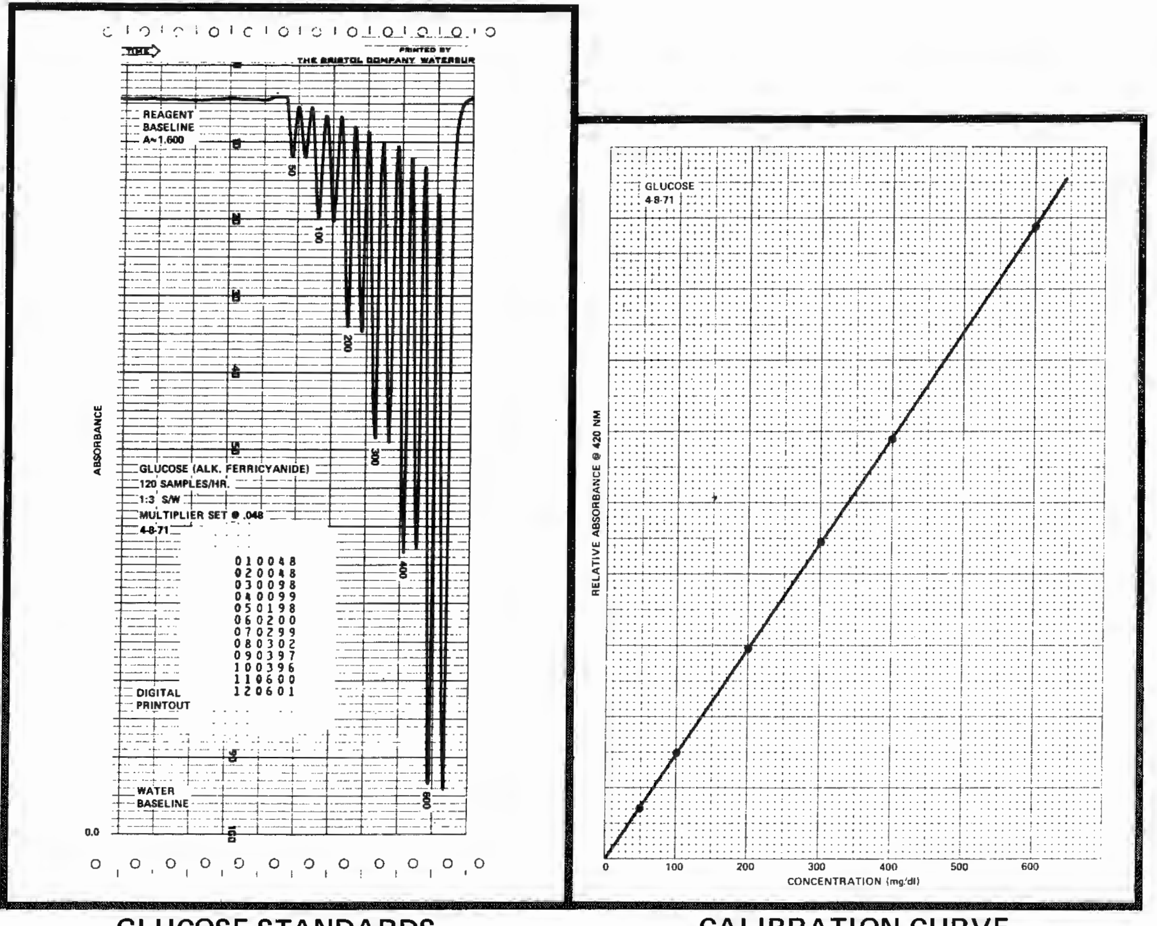


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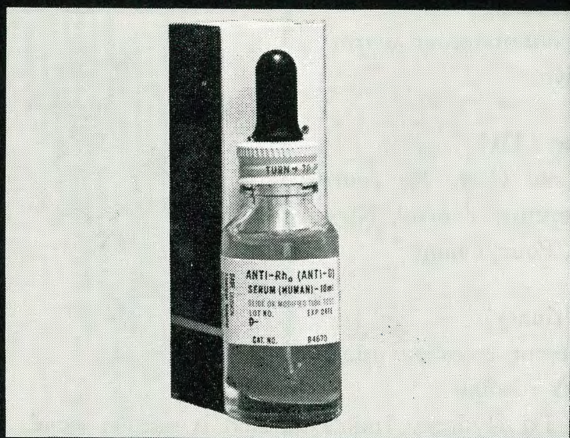

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The book is not a text book or even a reference book, nor does it show any bias towards diagnostic laboratory texts; although the author has been a hospital laboratory director. It is simply an introduction to the science of pathology, briefly explaining what the subject is about, and illustrating the need for continual development and research, by offering several theories or likely causes for unknown disease processes

In all a most interesting little book which could well be used in any introduction to medical laboratory technology.

—D. A. McD.

# CORRESPONDENCE

Sir,—In phenotyping hyperlipoproteinaemias according to Fredrickson, observance of the sample after 12 hours at 4°C is an important factor, especially when there are chylomicrons present.

Even with the best of instructions, we do occasionally receive specimens from patients who have not been entirely fasting for approximately 12 hours. After lipoprotein electrophoresis, using cellogel strips and Oil Red Om staining, a faint chylomicron band appears at the origin of application, but upon inspection of the serum next morning reveals no creamy top layer as is expected. However, by leaving the serum a further 24 hours at 4°C a faint creamy top layer does usually appear in our experience.

In our laboratory we have noticed this five times, no creamy top layer after 12 hours but appearance after 36 hours. I bring this to your attention as it can be misleading or suggestive of poor technique if a faint chylomicron band appears. This is especially so when paper, poor cellulose acetate, prestaining of sample or a hard application of sample is used. The tendency in such cases is to disregard the chylomicron band and to classify the pattern into one of the other types rather than reporting it as a probable non-fasting specimen which is misleading to the physician and can result in the patient being treated for a hyperlipoproteinaemia which may not exist at all. Therefore I suggest that serum specimens be kept at 4°C for 36 hours in such cases and would like to hear from anyone who has experienced the same problem.

R. W. L. Siebers,
Laboratory,
Hawera Hospital.

May, 1973.

Sir,—Michael Gratten's findings of "false positive" hydatid serology in pigeon fanciers, (*N.Z. J. med. Lab. Technol.* 1973, **27**, 13), is of great interest. Doubt was expressed, whether the negative result of the Latex test depends on the nature of this reaction, or in the purity of the antigen used.

Perhaps it may be worthwhile to test all reactive sera with Latex agglutination using antigens different from the commercial reagent used. The original methods described by me in 1960 used whole human or sheep fluid, specially selected. I was unable to get details of the preparation of the commercial antigen (the only commercial one on the market) as the company considers this a secret. However from relevant Italian literature it is obvious that the commercial antigen which coats the Latex, is not whole fluid, but a concentrated product, different from crude fluid.

The two sera originally detected by Gratten, were tested by whole fluid antigen Latex, and also found negative. This does not necessarily hold for all sera. We did find some discrepancies between results with the commercial product and non-commercial Latex tests in routine hydatid testing (unpublished data), when a larger series were compared.

A Latex test has also been used, with scolex extract as a sensitising antigen (Fischman and Allen 1967, *Aust. J. exp. Biol. med. Sci.*, **45**, 221). Here we have a different technique from the scolex complement fixation, which surprisingly also gave "false positives" in pigeon fanciers.

Andor Fischman,
Department of Serology,
Auckland Hospital.

May 12, 1973

# WHAT'S NEW?

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Size of the autosampler is compact and allows two units to be mounted on multi-column gas chromatographs such as Aerograph Models 2100, 2700, and 2800. The same unit may be mounted for either horizontal or vertical injection on either the left- or right-hand side of these chromatographs.

Cost (in the U.S.) of the basic autosampler will be under $4,000, and shipments are expected to begin August, 1973. Information about this unit is available from Varian Instrument Division, 611 Hansen Way, Palo Alto, California, 94303.

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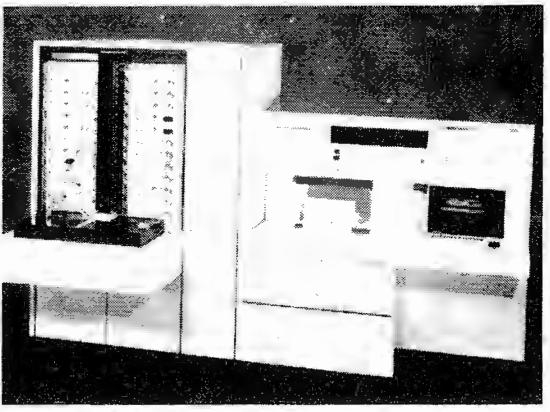

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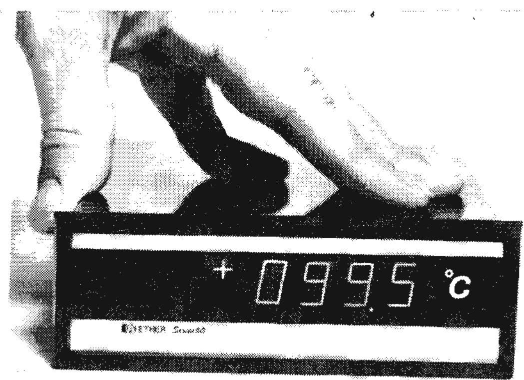


Fig 1

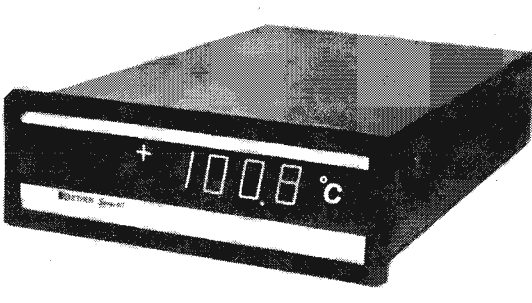


Fig 2 NS 1120

The new Series 60 digital panel meters announced by Pye Ether Limited of Caxton Way, Stevenage, England, can accept linear or non-linear input signals from voltage, current or resistance sources. These inputs are converted into an accurate three and three-quarter-figure digital display presenting in "engineering" units of measurement such as °C or kg/cm².

All the parameters commonly encountered in medical and laboratory instrumentation can be handled—temperatures; gas and liquid pressures; flow rates; pH and density readings; speeds; voltages; currents; resistances.

The instruments may be operated from all normal industrial power supplies, both a.c. and d.c.

The Series 60 instruments are compact, inexpensive and light in weight, suitable either for use singly or for high-density mounting in large panels. Their frontal dimensions of 144 x 48mm conform to DIN 43700; they are 212mm deep and weigh 26oz (740g). Their versatility enables instrumentation designers to standardise on a single type of instrument.

The complete electronic circuitry is mounted on four printed-circuit boards, all readily replaceable for ease of maintenance. The main board, common to all versions of the instrument, carries the circuits for internal power supplies and analogue-to-digital conversion and is a slide fit in the case. The three subsidiary boards plug in to the main board and two of them vary with the particular application. An Input Conditioning board processes the input signal into a form suitable for assessment. It converts current or resistance inputs to voltages, and in the case of thermocouple inputs, uses a temperature-dependent bridge circuit to provide cold-junction compensation. The range of the instrument and the choice of output units are controlled by a Characterising board, which also compensates, during the analogue-to-digital conversion, for any non-linearity between the input and the parameter it represents. Finally, there is a Display board, common to all instruments, which converts the binary-coded output into decimal form for display.

All connections to the instruments are made via a terminal-block at the rear. The power supply is taken to appropriate tappings on an input transformer, from which, via associated rectifier, regulator and inverter circuits, all necessary voltage levels are obtained.

The input signal is first fed to the Input Conditioning printed-circuit board, and is then amplified as necessary (under the control of the Characterising board) to provide 4.0V full-range to the integrator.

Analogue-to-digital conversion is effected by the technique known as dual-slope integration. The measuring cycle is divided into two periods. For 100 milliseconds the amplified input is fed to the integrator, which "ramps up" to a voltage directly proportional to the input. A reference voltage of opposite sign is then applied to the integrator, which "ramps down" again at a rate proportional to this voltage. The time taken to reach zero is recorded by a counter and thence displayed.

This technique gives very accurate measurement and provides the necessary input noise rejection. It also provides a convenient method of linearising non-linear inputs. Whereas for linear inputs the reference voltage remains constant, for non-linear inputs it varies with time under the control of the Characterising circuit-board so as to compensate exactly for the non-linearity.

The instruments can work at ambient temperatures from —5 to +60°C.

# Directions for Contributors

These instructions are provided with the object of ensuring uniformity of presentation. Manuscripts should be typed double spaced, on one side only of good quality paper with one inch margins. Carbon copies are not acceptable. Give the author's name with initials if male, or one christian name if female, and the address of the laboratory where the work was carried out. Use capitals only where indicated and do not underline except where italics are required.

In general, papers other than reviews, should consist of a short summary capable of standing alone as an abstract; an Introduction (outlining the problem and the proposed solution); Material and Methods; Results and Discussion.

### Illustrations

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### Nomenclature and Units

Scientific names of micro-organisms should conform with Bergey's Manual of Determinative Bacteriology. The first time an organism is mentioned the full generic name should be given and underlined to indicate that it is to be printed in italics. Subsequently it may be abbreviated. Trivial or common names are printed in roman, e.g., staphylococci, and should not be underlined.

To conform with the Systemes Internationale D'Unites or SI units it is recommended that the following prefixes and abbreviations be employed.

*Length*: m, cm, mm, μm, nm.
*Area*: $m^2$, $cm^2$, $mm^2$, $\mu m^2$.
*Volume*: litre, ml, μl, nl, pl ('litre' in full avoids confusion with 'l')
*Mass*: kg, g, mg, μg, ng, pg.
*Mass concentrations*: kg/litre, g/litre, mg/litre, μg/litre. For the present concentrations per 100 ml also accepted as are daily outputs in urine and faeces.
*Molar concentrations*: mol/litre, mmol/litre, μmol/litre, nmol/litre. (For the present mequiv/litre may also be used.)
*Temperature*: Express as °C.
*Time*: s, min, h, d, a. The latter two symbols which stand for day and year respectively are best expressed in full to avoid confusion.
*Density*: kg/litre (relative density replaces 'specific gravity')
*Clearance*: litre/s, ml/s (for the present ml/min may also be used).

N.B.:

1. The symbol for a unit is unaltered in the plural and should not be followed by a full stop, e.g., 5 cm not 5 cm. nor 5 cms.

2. No space should be left between the symbol for a prefix and the unit. A space is left between the symbols in derived units,
e.g., ms = millisecond
m s = metre x second

Where ambiguity could arise abbreviations should be written in full.

3. **Numbers.** The decimal is indicated by a full stop. Commas are not used to divide large numbers but a space is left after every third digit.

A zero should precede numbers less than unity. Units which give a number between 0.1 and 1000 should be chosen when possible.

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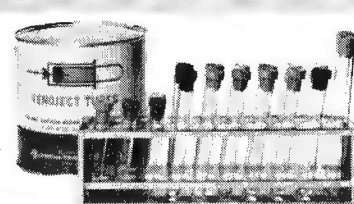

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