AUTOMATION OF URINALYSIS

by

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TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>ix</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>REVIEW OF LITERATURE</td>
<td>4</td>
</tr>
<tr>
<td>I. BILIRUBIN</td>
<td>7</td>
</tr>
<tr>
<td>II. CREATININE</td>
<td>9</td>
</tr>
<tr>
<td>III. GLUCOSE</td>
<td>11</td>
</tr>
<tr>
<td>IV. HEMOGLOBIN</td>
<td>13</td>
</tr>
<tr>
<td>V. KETONE BODIES</td>
<td>14</td>
</tr>
<tr>
<td>VI. TOTAL PROTEIN</td>
<td>16</td>
</tr>
<tr>
<td>VII. COLOR AND APPEARANCE</td>
<td>17</td>
</tr>
<tr>
<td>VIII. pH</td>
<td>18</td>
</tr>
<tr>
<td>MATERIALS AND METHODS</td>
<td>21</td>
</tr>
<tr>
<td>I. APPARATUS</td>
<td>21</td>
</tr>
<tr>
<td>II. FEASIBILITY TESTING</td>
<td>22</td>
</tr>
<tr>
<td>III. ALTERNATIVE EQUIPMENT</td>
<td>27</td>
</tr>
<tr>
<td>IV. TEST PROTOCOL</td>
<td>27</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------</td>
<td>------</td>
</tr>
<tr>
<td>V. TEST SPECIMENS</td>
<td>28</td>
</tr>
<tr>
<td>VI. CREATININE</td>
<td>29</td>
</tr>
<tr>
<td>A. Reagents</td>
<td>29</td>
</tr>
<tr>
<td>B. Procedure</td>
<td>30</td>
</tr>
<tr>
<td>C. Comments</td>
<td>30</td>
</tr>
<tr>
<td>VII. GLUCOSE</td>
<td>32</td>
</tr>
<tr>
<td>A. Reagents</td>
<td>32</td>
</tr>
<tr>
<td>B. Procedure</td>
<td>33</td>
</tr>
<tr>
<td>C. Comments</td>
<td>35</td>
</tr>
<tr>
<td>VIII. HEMOGLOBIN</td>
<td>35</td>
</tr>
<tr>
<td>A. Reagents</td>
<td>35</td>
</tr>
<tr>
<td>B. Procedure</td>
<td>36</td>
</tr>
<tr>
<td>C. Comments</td>
<td>38</td>
</tr>
<tr>
<td>IX. KETONE BODIES</td>
<td>38</td>
</tr>
<tr>
<td>A. Reagents</td>
<td>38</td>
</tr>
<tr>
<td>B. Procedure</td>
<td>39</td>
</tr>
<tr>
<td>C. Comments</td>
<td>39</td>
</tr>
<tr>
<td>X. TOTAL PROTEINS</td>
<td>41</td>
</tr>
<tr>
<td>A. Reagents</td>
<td>41</td>
</tr>
<tr>
<td>B. Procedure</td>
<td>41</td>
</tr>
<tr>
<td>C. Comments</td>
<td>43</td>
</tr>
<tr>
<td>XI. BILIRUBIN</td>
<td>43</td>
</tr>
<tr>
<td>A. Reagents</td>
<td>43</td>
</tr>
<tr>
<td>B. Procedure</td>
<td>44</td>
</tr>
<tr>
<td>C. Comments</td>
<td>44</td>
</tr>
<tr>
<td>EXPERIMENTAL RESULTS</td>
<td>46</td>
</tr>
<tr>
<td>I. URINE ANALYZER</td>
<td>46</td>
</tr>
<tr>
<td>II. CREATININE</td>
<td>47</td>
</tr>
<tr>
<td>III. GLUCOSE</td>
<td>48</td>
</tr>
<tr>
<td>IV. HEMOGLOBIN</td>
<td>54</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.</td>
<td>COMPARISON OF CREATININE VALUES</td>
<td>49</td>
</tr>
<tr>
<td>II.</td>
<td>PATIENT ANALYSIS</td>
<td>52</td>
</tr>
<tr>
<td>III.</td>
<td>COMPARISON OF NORMAL VALUES</td>
<td>53</td>
</tr>
<tr>
<td>IV.</td>
<td>COMPARATIVE THRESHOLD DETECTION</td>
<td>67</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Urine analyzer</td>
<td>23-24</td>
</tr>
<tr>
<td>2</td>
<td>Electronic module and printer</td>
<td>25-26</td>
</tr>
<tr>
<td>3</td>
<td>Automated determination of urine creatinine</td>
<td>31</td>
</tr>
<tr>
<td>4</td>
<td>Automated determination of urine glucose</td>
<td>34</td>
</tr>
<tr>
<td>5</td>
<td>Automated determination of hemoglobin in urine</td>
<td>37</td>
</tr>
<tr>
<td>6</td>
<td>Automated determination of ketones in urine</td>
<td>40</td>
</tr>
<tr>
<td>7</td>
<td>Automated determination of urine protein</td>
<td>42</td>
</tr>
<tr>
<td>8</td>
<td>Automated determination of urine bilirubin</td>
<td>45</td>
</tr>
<tr>
<td>9</td>
<td>Glucose, normal value distribution</td>
<td>50</td>
</tr>
<tr>
<td>10</td>
<td>Hemoglobin, normal value distribution</td>
<td>54</td>
</tr>
<tr>
<td>11</td>
<td>Ketones, normal value distribution</td>
<td>56</td>
</tr>
<tr>
<td>12</td>
<td>Protein, normal value distribution</td>
<td>57</td>
</tr>
<tr>
<td>13</td>
<td>Bilirubin, normal value distribution</td>
<td>59</td>
</tr>
</tbody>
</table>
ABSTRACT

In recent years there have been logarithmic upsurges of test demands placed on medical laboratories. Currently, there is a pre-existent and progressive shortage of medical technologists. The widening gap has in part been closed by automation.

Automation is being heavily utilized to an increasing degree in most areas of the laboratory with enhanced accuracy and precision, decreased professional effort and significantly decreased cost to the patient as coincidental returns. Through automation mass screening is being performed and maximum quality control can be exercised. The urinalysis may be the last major hiatus still remaining.

Inasmuch as the clinical laboratory scientist is dedicated to providing maximum excellence in patient care and because present methodologies (spot testing) in the area of urinalysis provide suboptimal clinical information, a study was undertaken to devise methodologies and essential equipment to improve laboratory diagnosis in the area.

The routine urinalysis consisting of tests for pH, specific gravity, protein, sugar, hemoglobin and ketones was studied. Two additional tests, creatinine and bilirubin, were
also incorporated. Color determination was investigated colorimetrically and pH was studied with an electrometer.

Methods utilizing microquantities of specimen were devised and adapted to two systems. One system was a urine analyzer designed and built especially for the study. The second system was fabricated from existing laboratory apparatus.

The feasibility of automating urinalysis was established for both systems. Using healthy volunteers, the normal ranges and other pertinent data were established for each method. Pathologic specimens were included in the study. Comparison with existing spot tests proved the value of the automated quantitative approach.

Several innovations resulted from this study. The use of a double beam colorimeter eliminated possible background errors introduced by reagents or intrinsic pigments in the specimens. A new test for glucose was designed using a previously unreported coenzyme analog which permitted linear colorimetric readings up to 1000 mg/100 ml without pretreatment of the specimen. Creatinine was determined for the first time on undiluted urine with a linear range up to 375 mg/100 ml. A novel approach for analyzing bilirubin was developed. A relatively "noise free" turbidimetric protein test system was adapted to continuous flow automation. Hemoglobin was estimated using O-dianisidine, with a degree of sensitivity five times greater than previously attainable. An electronic system was proposed and fabricated which enabled three orders
of signal magnitude to be compressed into one scale sweep.

In summary, the automation of the urinalysis was achieved. The quantitative automated approach appears technically and clinically superior to the qualitative manual method.
INTRODUCTION

There are two basic approaches to clinical laboratory testing. These are quantitative analyses and qualitative estimations. In general, the cost and effort input of quantitative approaches are high. Qualitative, or "spot testing," has the advantage of low cost, but sacrifices accuracy (Feigle, 1966). In recent years, the trend has been away from qualitative approaches. Spot testing has been used mainly in mass screening efforts where the emphasis has been on early detection of disease processes, rather than in establishing definitive diagnosis and monitoring progress of disease states. Urinalysis, the assay of urine constituents, is the last major area in which qualitative testing is still widely performed (Kark et al., 1963).

Automation is being increasingly utilized in virtually all areas of the laboratory, excepting that section concerned with urine studies (U. S. Government Printing Office, 1966). Direct benefits resulting from automation include enhanced accuracy and precision, decreased professional effort, and significant decrease in cost to the patient (Lab World, 1968). Automation also provides rapid mass screening and maximum quality control capability. In addition, with the computer data acquisition available today, equipment can be monitored
and involved calculations may be derived. Data may be rapidly collated, retrieved and made available for vital differential diagnostic information (Pribor, Kirkham and Hoyt, 1968).

Progress in the laboratory sciences has been characterized in recent years by logarithmic upsurges of test volume. Growth and expansion efforts have been compounded by existent shortages of medical technologists to handle the increased work demands and research development has lagged (U. S. Dept. Health, Ed. and Welfare, 1969; U. S. Dept. of Labor, 1967). Automation has helped alleviate this situation in all sections of the laboratory field; however, minimal progress has occurred in the field of urinalysis.

This static state of urinalysis invited studies on the feasibility of enhancing the accuracy, precision and clinical value of conventional test systems. It was soon recognized that these aims could best be achieved through automation and extended multiphasic analysis of urine metabolites. Since existing automated apparatus are primarily designed to analyze serum metabolites, experiments were initiated into the design and fabrication of new equipment and/or the total renovation of existing apparatus to handle the test systems. This was necessary because the chemical constituents and physical characteristics of urine differ greatly from serum. Challenging problems were repeatedly encountered, defined and for the most part solved. Promising new methodology and equipment was
devised. Although extended confirmatory experimentation re-
mains, initial findings indicate that quantitation of the
urinalysis by an automated approach is feasible and has clini-
cal value.
REVIEW OF LITERATURE

Automation was first introduced into the clinical laboratory in 1957 (Skeggs, 1957). This prototype system performed a glucose analysis on samples of blood using a continuous flow principle. Subsequent improvements led to the utilization of this scheme for multiple analysis, i.e., the performance of several tests simultaneously on a given specimen (Skeggs and Hochstrasser, 1964). Several automated chemical systems have been developed more recently. Each one entails a distinct method of handling the sample in a wet form. The Technicon Auto-Analyzer (Technicon Instruments Inc., Tarrytown, N. Y.) uses a flow-through method that allows sequential multiple analysis, whereas the Robot Chemist (Newman et al., 1968), Beckman DSA-560 (Rohte and Meyer, 1968), Hycel Mark X, du Pont ACA (Nadeau, 1968) and others have discrete systems in specific packets for the performance of tests in a wet phase. Instruments have also been developed for partial semiautomation performance of a given test, e.g., chloride is determined on an automatic titrator (Cotlove, Trantham and Bowman, 1958); a semiautomatic lithium, sodium and potassium system has been developed by Instrumentation Laboratory Inc., Boston, Mass.; the PBI and T₄ Autoanalyzer for thyroid function studies has been marketed and total protein may be read refractometrically (Sunderman, 1944).
The automation of hematology progressed rather slowly. However, automation is evidenced in this area by slide strain-ers, mechanized complete blood counts and indices (Gochman, Negersmith, and Weschler, 1966; Barnard et al., 1969), semi-automated coagulation studies (Miale, 1965) and the automatic recording of osmotic cell fragility (Allen and Shields, 1966).

Mass-screening for veneral diseases provided the impetus for automation in the serology sector (McGrew et al., 1968). The need for accurate and precise evaluation of drug therapy and antibiotic sensitivity resulted in further mechanized pro-cedures (Pagarno, Haney and Gerke, 1962). Automation has also been extended to such time consuming tasks as changing air mixtures for anaerobic culture conditions (Cohen, 1967) and in typing specific organisms (Hochberg et al., 1966). Bacteria are now being studied by analysis of metabolites using gas chromatography (Mitruka and Alexander, 1968).

Automatic blood typing instruments and antibody titer measurements are available to detect and quantitate abnormal antibodies and identify red cell antigens (Morton and Pickles, 1966; Sturgeon et al., 1966). These instruments may be used maximally in institutions where the blood banking volume is high. Time is conserved and the chance of gross error occurring in typing and crossmatching is lessened.

Multiple channel systems made possible multiphasic test-ing or mass screening (Reece, 1967). The term "multiphasic
testing" implies the performance of chemical determinations, a complete blood count and a urine analysis on every patient evaluated on an out-patient basis. Reports of such studies have indicated favorable response by the physicians (Young and Duabe, 1966; Whitehead, 1966).

Urinalysis, one of the most common test batteries, is performed on every hospital admission, in every doctor's office and in all out-patient clinics. It is an integral component of every health examination performed. The routine urinalysis consists of a pH determination, a specific gravity measurement, an occult blood or hemoglobin screen, a carbohydrate test, a test for albumin and a microscopic examination of the sediment (Hepler, 1963). Baird in 1958 developed a test for the detection of phenylketonuria, which has been included in pediatric urinalysis routine.

Many clinicians feel that the urinalysis is the single most important examination performed on a given patient. Insurance companies require that such an analysis accompany all physical examinations. Yet, with the exception of qualitative or crude semiquantitative tests, few advances have been made within the last decade. The lack of sensitivity in current testing procedures may have resulted in failure to detect incipient and subclinical disease processes.

Urine has over 300 constituents (Scott, Attrill and Anderson, 1967). These constituents result from normal body
metabolism or metabolic alterations. Fluctuations of these constituents can be related to a specific organ dysfunction, e.g., bile is associated with liver dysfunction, protein may indicate the beginning of existence of renal malfunction and ketone bodies are associated with metabolic acid-base imbalance. Constituents and properties are cited below. Various methodologies are reviewed and evaluated.

I. BILIRUBIN

Ordinarily bilirubin is not present in the urine but in certain diseases the conjugated component may be excreted. Accordingly, tests for urine bilirubin have been shown to be useful in the diagnosis and prognosis of biliary obstruction or diseases causing regurgitation of conjugated bile into the circulatory system.

There are many tests for the detection of bile in the urine. These may be classified into four groupings:

1. Observation of urine for the typical color of bilirubin, i.e., yellow, brown or green.

2. Oxidation of bilirubin to give characteristically colored derivatives, e.g., bilicyanin blue and biliverdin green. Specific colors are produced with iodine or nitric acid (Godfried, 1934). Various adaptations of the Fouchet test, e.g., Harrison spot test and Watson strip test, wherein barium chloride is used to concentrate the bilirubin and Fouchet's reagent (ferric chloride-trichloroacetic acid) to
oxidize the material, have been proposed (Giordano and Winstead, 1953; Musser, 1962).

3. Diazotization, which is the coupling of bilirubin to a diazo compound. Barium chloride is again used to concentrate the bilirubin and diazo reagents are then added to form azobilirubin, which may turn blue, pink, or purple depending on the pH (Godfried, 1934). A tablet form of diazo reagent has been described (Free and Free, 1953) and in 1969 a dip stick method was introduced (The Bili Labstix, Ames Co., Inc., Elkhart, Indiana) which incorporates a bilirubin test, utilizing the diazotization reaction.

4. Dye dilution procedures which involve the blending of the yellow-brown color of bilirubin with some dye, such as methylene blue or methyl violet (Gellis and Stokes, 1945).

Until recently the aforementioned qualitative tests were run only on request and not as part of the routine urinalysis. Furthermore, their specificity and sensitivity vary. For example, ferric chloride, which is a component of Fouchet reagent, also reacts with salicylates, and pigments other than bilirubin interferes with many tests (Thoma and Kitzberger, 1948). Dye dilution tests are sensitive only in the 2 mg/100 ml range, as opposed to the 0.05-0.1 mg/100 ml range of the other tests. Diazo reactions appear to be the most specific and sensitive methods available for qualitative and semi-quantitative estimations but do not provide reliable results
in urine. All qualitative tests are generally formulated to
detect abnormal presence of bilirubin (Henry, 1964b).

Quantitative estimations of urine bilirubin have been
made utilizing the principles of oxidation and diazotization.
A combination oxidation and diazotization method was intro­
duced which attempted to increase the specificity of the diazo
reaction. Sodium hypochlorite was used as an oxidizer and
Erhlich's reagent for the diazo reaction (Brereton and Lucia,
1948). The procedure involves precipitation techniques and,
therefore, is not readily automated. Direct diazotization of
the urine yields brown colors and not true azobilirubin color.
A recent modification of the diazo reaction using a copper­
azobilirubin complex shows promise (Michaelson, 1961).

Automation of presently available techniques involves
considerable preliminary handling of the specimen by way of
precipitation, centrifugation or filtration. Investigations
using a hypochlorite technique have shown possibilities. A
modified direct hypochlorite technique was developed and
adapted to automation.

II. CREATININE

Creatinine is a nonprotein nitrogenous hydrolytic product
of creatine catabolism. Determination of creatinine is of
diagnostic value in a host of disease states (Searcy, 1969b).
Increased amounts are found in the urine following muscular
injury and decreased amounts are associated with glomerular
damage. It is present in the urine in amounts of 15-30 mg/kg body weight per day. Since the amount of creatinine formed under normal conditions is reasonably constant, determination of creatinine in 24 hour urine specimens serves as a rough estimate of the completeness of urine collection. The concentration of certain urine constituents are frequently equated to and reported in terms of units per milligram of urine creatinine. The very important creatinine clearance test requires both urine and serum creatinine values (Peterson, 1967).

There are four methods in use for the estimation of creatinine:

1. Modification of the Jaffe reaction, wherein creatinine treated with an alkaline picrate solution yields a bright orange to red color (Slot, 1965; Owen et al., 1954).

2. The reaction of creatinine with 3,5 dinitrobenzoic acid in alkaline solution to give a purple rose color (Langley and Evans, 1936).

3. The conversion of creatinine to methylguanidinone by O-nitrobenzaldehyde (van Pilsum et al., 1956).

4. The estimate based upon the reaction of creatinine with 1,4-naphthoquinone-2-potassium sulfonate (Cooper and Briggs, 1961).

The Jaffe reaction appears to be most suitable for adaptation to an automated urine system. 3,5 dinitrobenzoic acid is
purported to be slightly more specific for creatinine; however, the purification of reagents and the length of the procedure make it impractical. The conversion of creatinine to methyl-guanidine and the naphtoquinone reactions are too lengthy and difficult for routine laboratory uses or for automated procedures.

### III. GLUCOSE

Determination of glucose in both blood and urine has long been a challenge to clinical chemists. As a result, considerable effort has been exerted to develop better quantitative and qualitative tests. The clinical value of urine glucose studies needs little amplification.

Many of the test methods are based on the ability of glucose to reduce copper and ferricyanide. Techniques employing the ferricyanide reaction are gasometric (Hawkins, 1929), photometric (Hoffman, 1937) and titrimetric (Rappaport and Eichhorn, 1950). Copper reduction methods have also used titration and photometry (Gray and Millar, 1953; Benedict, 1909). Unfortunately, these methods are not highly specific for glucose and the presence of other reducing substances may interfere (Searcy, 1969a). Another entry in the field of sugar chemistry is the condensation of aldosaccharides with primary aromatic amines, namely orthotoluidine. A colored compound is formed and its transmittance follows Beer's law and is relatively specific for glucose. Protein free filtrates
are not necessary unless high quantities of protein are present (Dubowski, 1962). An incubation period of ten or more minutes at high temperatures is required in most reducing tests.

Enzymatic systems employing glucose oxidase have proven to be more specific but removal of interfering substances, such as enzyme inhibitors, e.g., urates and free drugs, is necessary in urine. This system has also been automated (Logan and Haight, 1965) and employs O-dianisidine or O-tolidine as the chromogenic oxygen acceptor. Both the copper reduction technique and the glucose oxidation technique have been adapted to commercial tablet and paper forms for qualitative studies (Kasper and Jeffrey, 1944; Free et al., 1957).

A newer system uses adenosine triphosphate (ATP) and hexokinase (HK) to convert glucose to glucose-6-phosphate, which then reacts with nicotinamide adenine dinucleotide phosphate (NADP) in the presence of glucose-6-phosphate dehydrogenase (G-6-PDH). 6-phosphogluconate and reduced nicotinamide adenine dinucleotide phosphate (NADPH) are formed. The reduction of NADP to NADPH is measurable and relates on a molar basis to the amount of glucose present. The system is reportedly interference free and highly specific (Peterson, 1968) and requires no pretreatment of the specimen. This makes it a logical choice for utilization in an automated urine system. Sensitive enzyme procedures such as this have
indicated that about 90 percent of healthy people excrete glucose in amounts of 1-15 mg/100 ml (Fine, 1965).

Investigation using Thio-NADP is place of, or in conjunction with, natural NADP has shown great promise in providing sensitivity over a wide range. Furthermore, the test can be read in the visible range, making it possible to perform with conventional equipment. Experimental data was gathered and evaluated for the automation of the procedure.

IV. HEMOGLOBIN

Hemoglobinuria is defined as the presence of hemoglobin, or hemoglobin pigment derivatives, in the urine. Although hemoglobin molecules readily filter through the glomeruli, free molecules are rapidly complexed by haptoglobin and only limited amounts will be present in urine, except when plasma concentrations exceed 100 mg/100 ml (Laurell and Nyman, 1957). When red cells have been shed into the urine, hematuria results and hemoglobin tests will be positive. Hemolytic episodes (Lynch et al., 1969), genito-urinary hemorrhage and glomerular disease are the most common causes of hemoglobinuria.

A number of chemical tests have been devised to demonstrate the presence of submicroscopic quantities of blood in biologic fluids. Several methods are based on the color produced by oxidation reactions of iron-containing derivatives of hemoglobin in the presence of hydrogen peroxide. These are the benzidine hydrochloride test (Cook, Free and Free, 1956),
benzidine base test (George, Thornton and Illingworth, 1955), O-tolidine test (Stone and Burke, 1934), Zwarenstein's test (Zwarenstein, 1949) and guaiac test (Reich, 1956). These techniques have been incorporated into a tablet (Watson-Williams, 1955) and paper stick (Leonards, 1962). Although the reactions are similar, they differ markedly in sensitivity (Cook, Free and Free, 1956).

The use of a compound related to O-tolidine has been shown to be effective in staining electrophoretic patterns for haptoglobin and hemoglobin-haptoglobin complex quantitation. Color stability and sensitivity are both enhanced by use of the highly reactive O-dianisidine, whereas colors conferred by benzidine and O-tolidine fade within minutes and guaiacol is unstable (Neale, Aber and Northam, 1958; Owen, Silberman and Got, 1958; Jermyn and Thomas, 1954). O-dianisidine was selected for use in adapting a hemoglobin method to automation.

V. KETONE BODIES

The three metabolic end products produced by lipolysis are usually referred to as ketone bodies. B-hydroxybutyric acid, acetoacetic acid and acetone are normally produced in small amounts and pathologically in large amounts. Approximately 25-35 percent of these ketones are acetoacetic acid and acetone.

Excretion of ketones in excess of 50 mg/day is considered pathologic (Van Slyke, 1917). Increased levels of ketone
bodies are encountered in conditions in which the metabolism of glucose is impaired or in which increased fat metabolism occurs. Increased concentrations of these are associated with acidotic states and from this standpoint the early detection of ketonuria is of value is therapeutic management.

Quantitative methods developed to date are too complex to be of immediate value and may not be specific enough for the ketones concerned, nor adaptable to automation. The following reactions have been employed:

1. Bisulfite binding, which is not specific for the ketone bodies (Klein, 1940).

2. Iodometric titration, which again is not specific and requires lengthy distillation (Rappaport and Baner, 1943).

3. Mercuric sulfate precipitation is supposedly acetone specific but the length of time for performance is prohibitive (Van Slyke, 1917).

4. The salicylaldehyde test is used to determine total ketone bodies by converting B-hydroxybutyric acid to acetone with sulfuric acid but the procedure is too lengthy (Behre, 1940).

5. An enzymatic test using B-hydroxybutyric dehydrogenase is the only true specific test for B-hydroxybutyric acid (Williamson, Mellanby and Krebs, 1962).

The quantitative tests mentioned are involved and non-specific. Qualitative tests have been developed for quick
detection and semiquantitative estimations of ketone bodies (Riekers and Miale, 1958). Ferric chloride, mercuric dyanide (Wallhauser, 1928), salycilaldehyde (Behre, 1940) and the widely accepted nitroprusside test (Rothera, 1908) have been used.

Nitroprusside reactions have been incorporated into a tablet and paper strip form (Riekers and Miale, 1958). Both forms have gained wide acceptance in the laboratory as part of the urinalysis test and for serum estimations (Nash, Lister and Vobes, 1954). A colorimetric method for estimating acetoacetic acid using the nitroprusside test has also been introduced (Schilke and Johnson, 1965) and automated (Klein and Oklander, 1966). Since acetoacetic acid is fairly stable in urine at room temperature, the nitroprusside reaction was adapted to the proposed automated urinalysis system providing a quantitative, yet expeditious procedure.

VI. TOTAL PROTEIN

Proteinuria is the presence of protein in the urine. Normally, urine contains trace amounts of proteinaceous material but the amount is usually less than 250 mg for 24 hours, or 40 mg/100 ml. This amount is generally undetectable by any of the simple spot tests now in use (Oser, 1965). Routine determination of the protein concentration in the normal range would be useful in that minor changes may occur which could be clues to the beginning of a pathologic condition.
Routine determinations for protein are qualitative or semiquantitative and serve only to differentiate the normal concentrations from an abnormal elevation. Sensitivity varies between methods. Qualitative tests used include the acid ring test, heat and acetic acid test, sulfosalicylic and trichloroacetic acid precipitation tests. Commercial tests are available which incorporate the principles of precipitation and the colorimetric effect of protein error of indicators (Free, Rupe and Metzler, 1957; Exton, 1923; Watson, 1964). Quantitative estimations are performed by the turbidimetric reading of protein, which has been precipitated with such agents as trichloroacetic acid, sulfosalicylic acid and polythioniate. Methods incorporating the Biuret reaction are used but the sensitivity is not sufficient at low protein concentrations (Henry, 1964a). This prohibits use of the reaction in an automated urinalysis system. In this study, the sulfosalicylic acid reaction was adopted as a routine test system.

VII. COLOR AND APPEARANCE

Color determinations are a very important aspect of the routine urinalysis. Normal urine color varies from very yellow to dark amber. Urochrome is the chief pigment present and accounts for the yellow to reddish-yellow spectrum. The intensity of color provides a quick means of determining the concentrating capability of the kidney and the patient's state of hydration. Dilute urines are pale and
concentrated ones are darker. Description of the color for a given specimen will vary with individual interpretation at least within the normal color range and, to some extent, in pathologic ranges (Frankel, 1963). A probable cause is difference in learning experiences in conjunction with varied vision capability.

In physiologic conditions, urine may be of unusual color. Certain vegetative foodstuffs, such as rhubarb, senna and beets, confer pigment ranging from brown-yellow to red-brown. Medications are also responsible for certain abnormal colors.

Pathologic conditions present abnormal colors ranging from dark yellow to brown-red in acute febrile diseases, to milky color seen in chyluria or purulent diseases of the urinary tract. Hemoglobin confers a red-brown color if present. Bile yields an orange color (Bauer, Toro and Ackermann, 1962).

Elimination of error in color reporting requires the use of standards. Since color reporting is traditional and of some clinical significance, an automated urinalysis system should include it. Therefore, a colorimetric scan at three wavelengths was tested to ascertain the value of automated estimation.

**VIII. pH**

The determination of pH in urine has long been considered a valuable clinical parameter. Both mono and dibasic
phosphates are mainly responsible for the reaction. Monobasic phosphates contribute acid reaction, while dibasic forms allow for a more alkaline reaction. Organic acids contribute but a minor part in the overall pH. Twenty-four hour specimens have a pH range of 4.8 to 8.0 with a mean around 6. Women tend to have a higher pH in the morning specimen than men (Waters, Sussman and Asscher, 1967).

Diet plays an important role in the reaction of the urine. After ingestion of meals the urine may be neutral or alkaline. This is due to the increased utilization of acid radicals in the digestive process and is often referred to as the alkaline tide. Furthermore, ingestion of carbonates and citrus fruits also cause alkaline reaction in urine. Acid reactions are caused by cereals, cranberries, meats and plums. Therefore, the time of day and type of diet contribute to the reaction of urine and should be considered in the overall clinical investigation (Oser, 1965).

Measurement of the urine reaction is helpful in determining some metabolic disorders and in the regulation of the reaction for therapeutic purposes (Bauer, Toro and Ackermann, 1962). Respiratory and metabolic acidosis decrease urine pH and in cases where alkalosis is present, the urine is alkaline.

Treatment of genito-urinary infections with sulfalthiazole, sulfadiazone, sulfamerazine or certain antibiotics require that an alkaline medium be maintained for more effective
action. Also, patients prone to renal calculi must have an acid or an alkaline urine maintained depending on the type of calculi being formed. Phosphates and calcium are precipitated in alkaline urine and uric acid, calcium oxalate and cystine precipitate in acid urine.

At present, nitrazine or similar pH indicator paper is used for pH estimation. This is generally sufficient for purposes of screening and rough estimation. Fresh urine should be used to avoid shifts in pH due to bacterial action. A pH meter with single probe was used with the proposed automated system (Bradley and Benson, 1969).
MATERIALS AND METHODS

I. APPARATUS

Since most commercially available automated equipment has been designed to analyze serum metabolites and constituents, these apparatus were considered suboptimal for the analysis of urine constituents. For example, urine normally contains only trace amounts of certain materials such as glucose, protein, bile and hemoglobin, which are present in substantial quantities in blood and serum. Yet, in pathologic states urine levels of many substances will exceed blood levels. Color, turbidity, inhibitors and physical characteristics of urine are so variable as to be unsuited to conventional analysis without considerable modification of technique.

Therefore, a major effort was initiated to design and construct a unique, automated device particularly suited to analyze normal and abnormal urine components and properties. New and existing test methodologies were perfected to interface with the proposed apparatus.

The proposed apparatus was designed and built in collaboration with Bio-Logics, Inc. of Salt Lake City, Utah. It was comprised of the following modules: 1) a disc sample tray that rotated the specimens into position for sampling and testing; 2) a sampling system, consisting of a pneumatically
driven arm that drew a specimen of urine and deposited droplets (beads) of equal quantities in parallel rows on a moving belt; 3) a nonwettable surface belt that served the purpose of conveying blank and test beads through the reagent bank to the readout; 4) a reagent bank, consisting of a series of containers fitted with plastic tubing that carried the reagents to small syringes. A pneumatic pump operated each syringe, allowing preadjusted quantities of liquid to be dispensed at timed intervals; 5) a micro-cuvette double beam spectrophotometer was designed and fabricated. The test beads were simultaneously picked up by capillary action and the optical density was recorded on a strip recorder. Filters of appropriate wavelengths were indexed into position for each test; and 6) a dual purpose pH electrode connected to an electrometer measured the pH of the specimen (see Figures 1 and 2).

A control cam programmed each functional component. After the beads had been deposited on the belt, the reagents were added at intervals designed to allow effective reaction to occur as needed for each test. The apparatus had capability to process 60 specimens per hour.

II. FEASIBILITY TESTING

The various modules were tested individually and, following assembly, as an integral unit. The final testing consisted of serial analysis of creatinine, protein and
Figure 1
hemoglobin standards. Fabrication delay and sensitivity problems precluded definitive, in depth study, of the total system.

III. ALTERNATIVE EQUIPMENT

Since certain of the new concepts incorporated into the equipment design were untested in clinical situations, a secondary system was designed using available laboratory modular components. A continuous flow system was constructed from basic Technicon (Technicon Instruments Inc., Tarrytown, N.Y.) and Beckman (Beckman Instruments Inc., Fullerton, Calif.) elements. Special manifolds were constructed for use with a single channel autoanalyzer. Manifold pumps were used to deliver reagents and specimens. An autoanalyzer sampler was used to sequence and time specimen sampling. Dialysis was performed where needed using either a long or short SMA-12-60 transit dialyzer.

Optical density readings were obtained using a Beckman DB-G spectrophotometer with a ten inch linear-log recorder. The recorder was set for log mode at one inch per minute. Fifteen mm quartz flow-through cells were used with the apparatus.

IV. TEST PROTOCOL

Following initial feasibility studies using the urine analyzer, it was elected to use the modified flow system
approach to generate experimental data and to evaluate the merits of the automated urinalysis and its associated refinements. Each test system was assembled and requisite reagents and standards were prepared in accordance with procedure detailed in the respective technical section. The apparatus was activated and a zero base line was established. Standards were processed and calibration curves were constructed. Normal and pathologic centrifuged specimens were tested and reproducibility and recovery studies were performed. Baseline drift, intrinsic noise, specimen "carry over," possible chemical interference and optimal incubation and related sensitivity problems were studied for each procedure.

All specimens were analyzed by one or more reference procedures. Glucose, ketones, protein, bile and hemoglobin were all presumptively tested using the Ames Bili-Lab-Stix. Creatinine was assayed by an automated method (Hunter, Degn and McGuire, 1968) and glucose was, in addition, estimated by a colorimetric procedure (Dubowski, 1962). All specimens in which a discrepancy was encountered were assayed by an additional reference procedure. These confirmative procedures included the Acetest for ketones, the sulfosalicylic acid turbidimetric test for protein, the Ictotest for bile and the Hematest for hemoglobin.

V. TEST SPECIMENS

Primary aqueous standards were prepared from reagent
grade chemicals. These were made up in serial concentrations and processed as specimens. Readings were then plotted against concentration and a calibration curve was drawn for each method.

Standards were interspersed at various times to validate the stability of the methodology. Preparation of standards for each metabolite is described in the respective methodology section.

Twenty-four hour urine collections from 17 normal subjects were used to establish normal values. Sixteen patients were studied to determine the capability of the system to detect pathologic conditions. These patient specimens were assayed in the routine manner. Trained, experienced personnel independently performed the prescribed qualitative tests. These tests included a dip stick test for protein, hemoglobin, ketone, glucose and bilirubin.

VI. CREATININE

Creatinine yields a measurable red color when reacted with picric acid in the presence of a strong alkali.

Creatinine + Alkaline Picrate → Red Tautomer of Creatinine

A. Reagents

1. Sodium hydroxide, N/2. Dissolve 200 grams NaOH pellets in H₂O. Dilute to 1000 ml.

Cool and dilute to one liter and use the clear solution. Store in a brown bottle away from light.

3. Stock creatinine solution. Transfer five grams of pure creatinine to a 100 ml volumetric flask and dilute to 100 ml with N/10 HCl. Add two drops toluene. One ml contains 50 mg creatinine. Dilute 10 ml of stock to 100 ml with distilled water. Use this as a working stock standard.

Diluted standards were prepared as follows:

<table>
<thead>
<tr>
<th>Working Stock ml</th>
<th>Distilled H₂O ml</th>
<th>Concn. mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>500</td>
</tr>
<tr>
<td>75</td>
<td>25</td>
<td>375</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>200</td>
</tr>
<tr>
<td>25</td>
<td>70</td>
<td>125</td>
</tr>
<tr>
<td>12.5</td>
<td>87.5</td>
<td>62.5</td>
</tr>
</tbody>
</table>

B. Procedure

1. Assemble flow system in accordance with Figure 3 (see Figure 3).

2. Establish a baseline with reagents.

3. Perform single cycle analysis on standards and test specimens.

4. Plot a calibration curve of optical density vs. concentration for each standard. Extrapolate unknowns from this graph.

C. Comments

The undiluted specimens were mixed with NaOH as the diluent stream. Dialysis occurred into a recipient stream comprised of
one part picric acid and one part NaOH. The dialysis eliminated the need for a blanking cycle. The transit time should be approximately six minutes.

**VII. GLUCOSE**

Hexokinase and ATP phosphorylate glucose to glucose-6-phosphate. Glucose-6-phosphate dehydrogenase then oxidizes glucose-6-phosphate to gluconolactone-6-phosphate. The coenzymes NADP and TNADP (Thio-NADP) act as hydrogen acceptors and are reduced to NADPH-TNADPH. This reduction bears a direct molar ratio to glucose conversion. Absorbance of NADPH is read at 340 nm and TNADPH is read at 400 nm. The basic system using NADP only was described in detail by Peterson (1968). TNADP was used to facilitate visual reading and regulation of sensitivity.

\[
\text{Glucose + ATP} \xrightarrow{\text{HK}} \text{G-6P + ADP} \\
\text{G-6-PDH} \xrightarrow{\text{G-6P+NADP-TNADP}} \text{Gluconolactone-6-phosphate+NADPH-TNADPH (Colorless)} \xrightarrow{} \text{(Yellow)}
\]

**A. Reagents**

1. Nicotinamide adenine dinucleotide (NADP) 1 mg.
2. Thionicotinamide adenine dinucleotide phosphate (TNADP) 12 mg.
3. Adenosine triphosphate (ATP) 70 mg.
4. Hydrochloric acid .1N.
5. Buffer: Tris buffer pH 8.0 2.43 Tris to 100 ml with
distilled water. Twenty-five ml of this solution mixed with 29 ml .1N HCl will give a solution of pH 8.

6. Hexokinase (HK). Ten international units.

7. Glucose-6-phosphate dehydrogenase (G-6-PDH). Ten international units.

The above may be purchased in prepackaged units from Calbiochem Co., Los Angeles, California (Catalog No. 869204). The TNADP solution must be made separately. The lyophilized material is available from Boehringer-Mannheim Co., San Francisco, California. The other reagents are also available commercially (Sigma Chemical Co., St. Louis, Mo.) and may be prepared individually.

The reagent mixture contained the following: NADP, ATP, HK and G-6-PDH. Additional NADP was added with TNADP. 15.5 ml of buffer was added to the vial containing this material. The substrate should be prepared daily.

8. Standards: Stock solution contained 5 gm glucose per 100 ml. Dilutions were as follows:

<table>
<thead>
<tr>
<th>Working Stock ml</th>
<th>Distilled H₂O ml</th>
<th>Concn. mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>95</td>
<td>250</td>
</tr>
<tr>
<td>2.5</td>
<td>97.5</td>
<td>125</td>
</tr>
<tr>
<td>1.25</td>
<td>98.75</td>
<td>62.5</td>
</tr>
<tr>
<td>.625</td>
<td>99.375</td>
<td>31.25</td>
</tr>
</tbody>
</table>

B. Procedure

1. Assemble flow system in accordance with Figure 4 (see Figure 4).
AUTOMATED ENZYMATIC DETERMINATION OF URINE GLUCOSE

WASTE

SINGLE SHORT DIALYSIS

MIXING COIL

cc/min.
0.10
SAMPLER
30/hr

0.32
H2O

0.16
AIR

0.42
SUBSTRATE

0.16
AIR

WASTE

0.32

DELAY COIL

BECKMAN
DB-G
400 nm

4 Min.

Fig. 4
2. Establish a baseline with reagents.

3. With the use of dialysis a blank cycle was unnecessary. Standards were cycled first, followed by specimens.

4. Plot graph of optical density standards vs. concentrations. Extrapolate unknowns.

C. Comments

Linearity up to 250 mg/100 ml was achieved. This may be extended to 1000 mg/100 ml, by changing the NADP-TNADP ratio. A ratio of 15 parts NADP to one part TNADP will provide this range. The substrate acted as the recipient stream. The reaction proceeded rapidly and was read in four minutes.

VIII. HEMOGLOBIN

Hemoglobin acts as a peroxidase to catalyze the oxidation of colorless oxygen acceptors to chromogenic states in the presence of hydrogen peroxide.

\[
\text{O-Dianisidine} \xrightarrow{\text{Hgb (peroxidase)}} \text{Oxidized O-Dianisidine} \\
\text{(Colorless)} \xrightarrow{} \text{(Red-Yellow)}
\]

A. Reagents

1. Absolute ethyl alcohol.

2. 0.5% hydrogen peroxide made from stock 3% solution. Should be prepared daily.
3. Buffer pH 4.0 (Coleman certified buffer tablets).

4. Working reagent: a) .1 gm ortho-dianisidine in 70 ml ethanol; b) 10 ml buffer solution; c) 18 ml distilled water.

5. Standards: Fresh blood cells were washed several times with normal saline. The cells were then lysed by repeated freeze and thaw. A hemoglobin content was then determined by a standard cyanometh-hemoglobin method and proper dilution made with 0.1 M saline to establish 10 gm/100 ml. One ml of this stock solution was then diluted to 100 ml to make the working standard solution. Diluted standards were prepared as follows:

<table>
<thead>
<tr>
<th>Working Stock ml</th>
<th>Distilled $H_2O$ ml</th>
<th>Conc. mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Standards and reagents were refrigerated when not in use.

B. Procedure

1. Assemble flow system in accordance with Figure 5 (see Figure 5).

2. Establish a baseline with reagents.

3. Recycling for a blank was not necessary due to the extreme specimen dilution involved. Sampling was performed at 50 specimens/hour.

4. Plot a calibration graph to extrapolate unknown values.
AUTOMATED DETERMINATION OF HEMOGLOBIN IN URINE

cc/min.
0.60 H2O
0.05 SAMPLER
0.80 50/hr
AIR
2.02 REAGENT
(SOLVAFLEX)
2.50 H2O2
0.5%

WASTE
2.50 (SOLVAFLEX)

BECKMAN
DBG
450 nm
2.5 Min.

Figure 5
C. Comments

Baseline drift may occur due to reagent darkening. Allowance should be made for this by interspersing water washes between series of tests. If mixing problems occur, the alcohol content of the O-dianisidine reagent may be decreased.

IX. KETONE BODIES

Sodium nitroferricyanide reacts with acetoacetic acid in the presence of an alkali to form a lavender to deep purple color (Rothera, 1908).

\[
\text{Na} \cdot \text{Nitroprusside} + \text{Acetoacetic} + \text{Oxidized acetoacetic acid (permanganate colored)}
\]

Aminoacetic acid (glycine) is used to stabilize the color and to provide anions for the reaction to reach completion (Madonia, 1963; Schilke and Johnson, 1965). Ammonium hydroxide potentiates the reaction and prevents interference by creatinine (Rothera, 1908).

A. Reagents

1. Ammonium hydroxide - concentrated.

2. Dissolve 10 grams nitroferricyanide in distilled water and dilute to 100 ml.

3. Buffer-glycine-alkaline complex. Dissolve 4.26 gm of sodium phosphate dibasic anhydrous and 7.5 gm of glycine in distilled water and dilute to 300 ml. Add 7.5 ml of concentrated ammonium hydroxide to the solution.

4. Standards: Three ml of ethyl acetoacetate were
diluted to 100 ml with 0.2 N sodium hydroxide and refrigerated 48 hours at 4°C. The hydrolysate contains 236.3 mM/liter or 30.8 grams per liter. A working stock solution of 0.308 gm/100 ml was prepared by diluting one ml of the hydrolysate to 100 ml. The following standard solutions were prepared from this stock solution.

<table>
<thead>
<tr>
<th>Working Stock ml</th>
<th>Distilled H₂O ml</th>
<th>Concn. mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>.1</td>
<td>99.9</td>
<td>3.081</td>
</tr>
<tr>
<td>.2</td>
<td>99.8</td>
<td>6.16</td>
</tr>
<tr>
<td>1.0</td>
<td>99.0</td>
<td>30.18</td>
</tr>
</tbody>
</table>

The standard solutions were stable for one week under refrigeration.

B. Procedure

1. Assemble flow system in accordance with Figure 6 (see Figure 6).

2. Establish the baseline with reagents.

3. Begin sampling at 40 specimens per minute.

4. Plot a calibration curve of optical density vs. concentration for each standard. Extrapolate unknowns from this graph.

C. Comments

Using a dialyzer eliminated the necessity of a blank cycle. The nitroferricyanide, however, began to darken rapidly after two to three hours of use. Since this is the main color producing reagent, baseline drift may become an increasing
problem unless corrected for by use of controls and water washes.

X. TOTAL PROTEINS

Proteins are precipitated by sulfosalicylic acid. The degree of turbidity is proportional to the quantity of proteins present (Henry, 1964a).

A. Reagents

1. Saline. 0.85%.

2. Sulfosalicylic acid 3%. Three grams sulfosalicylic acid in 100 ml of water.

3. H₂O, pH 1 with hydrochloric acid.

4. Standard: A working standard of 100 mg/100 ml was prepared from a stock human albumin standard of 25 gm/100 ml. The following dilutions were then prepared:

<table>
<thead>
<tr>
<th>Working Stock ml</th>
<th>Distilled H₂O ml</th>
<th>Concn. mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>1.0</td>
<td>75</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>50</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>25</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
<td>12.5</td>
</tr>
</tbody>
</table>

B. Procedure

1. Assemble flow system in accordance with Figure 7 (see Figure 7).

2. Establish baseline with water and reagents.

3. Begin sampling 50 specimens per hour.

4. Recycling of the specimens was necessary to obtain a
AUTOMATED DETERMINATION OF URINE PROTEIN

cc/min.

0.6 AIR

0.23 SAMPLER 50/hr

2.50 REAGENT or H_2O pH 1

.80 TRAP RETURN

BECKMAN DB-6 660-nm

4 Min. WASTE 1.60

Fig. 7
blank. In the first cycle water of pH 1 was pumped through the reagent line. The optical density inherent in each urine is thus established. 3% (w/v) sulfosalicylic acid is used in the second cycle.

5. A graph was constructed with the standards. After subtraction of the blank optical density, the unknowns were extrapolated from the curve.

C. Comments

A trap (inverted debubbler) was positioned prior to the flow cell to promote convection and eliminate large particles. Noise may be a problem if the trap is not positioned properly in the system. Linearity was established through 100 mg/100 ml using a continuous flow system. Excessive noise is indicative of high protein content and the sample should be diluted and repeated.

XI. BILIRUBIN

Bilirubin absorbs maximally in the 440 nM range. The conjugated form is very labile and is readily oxidized with an ablation of red-yellow maxima. Dilute hypochlorite was used to effect this oxidation. The difference in absorption at 440 was equated to bilirubin concentration.

\[
\begin{align*}
\text{Bilirubin} & \quad \text{Oxidizing Agent} & \quad \text{Urobilinogen} \\
440 \text{ nM} & \quad \rightarrow & \quad \text{(Colorless)}
\end{align*}
\]

A. Reagents
1. Sodium hypochlorite 1% w/v solution.

2. Standards: An ox bile solution containing .08 mg/100 ml of bilirubin was used as a primary standard. Standards were stored in dark bottles and away from light. The following dilutions were then prepared:

<table>
<thead>
<tr>
<th>Working Stock ml</th>
<th>Distilled H$_2$O ml</th>
<th>Conc. mg/100 ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>.08</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>.032</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>.016</td>
</tr>
</tbody>
</table>

B. Procedure

1. Assemble flow system in accordance with Figure 8 (see Figure 8).

2. Baseline was established with reagents.

3. A sampling rate of 30 specimens per hour was used. Both specimen and reagent streams were divided. One portion contained hypochlorite as clorox, the other, water. The blank and test specimens were run through a Beckman D-BG spectrophotometer. The usual reference side had the reaction and the sample side acted as the blank.

4. Curves were drawn and unknown values were extrapolated.

C. Comments

Phasing of the two flow through cells presented little difficulty. Sufficient time must be allowed for the oxidation to occur. This should not be less than three minutes.
AUTOMATED DOUBLE BEAM DETERMINATION OF URINE BILIRUBIN

BECKMAN DB-G 440 nm

MIXING COIL

WASTE

AIR 0.42

SAMPLER 30/hr 1.0

CLOROX 1:30 0.60

WASTE 1.2

H₂O 0.60

Fig. 8
EXPERIMENTAL RESULTS

I. URINE ANALYZER

Due to unpredictable developmental and fabrication delays, the urine analyzer was delivered in an untested, unfinished mode. Minor functional difficulties and limitations were encountered in several components including: 1) modular interface was approximate; 2) the cuvet system filled imprecisely; 3) build-up required frequent cuvet clearing; 4) light source, path and window were suboptimal; 5) reagent delivery system lacked precision; 6) clean-out mechanism activated prematurely and removed unread specimens from the conveyor belt; 7) the sensing mechanism was below electronic noise levels, making it difficult to achieve test sensitivity at low concentrations; and 8) color, pH and conductivity channels were not incorporated at the time of testing.

However, the evaluation study yielded invaluable information including: 1) the merits of the double beam system were confirmed; 2) specimen and reagent utilization was minimal; 3) a novel electronic system was developed with capability to measure optical density over three orders of magnitude; and 4) feasibility of automating color and pH in a single modular operation was demonstrated.

Color was read manually in the DB-G spectrophotometer at
660 nm, 520 nm and 450 nm. Preliminary results indicate that yellow color yielded elevated absorbance in the 520 nm range, reddish colors in the 450 nm range and blue pigments and turbidity at 660 nm in accordance with the concept of reciprocal wavelength absorption (Henry, 1964c). Further evaluation is needed to establish the clinical value and feasibility of reporting color in terms of wavelength absorptivity.

Comparative studies of pH were done using standard pH paper and pH meters. Accuracy and sensitivity were enhanced by use of the electrometer.

The urine analyzer is in the process of minor redesign to eliminate certain of the problems cited above. Initial testing has shown the feasibility of this approach. The ensuing results were acquired using the alternative system.

**II. CREATinine**

Undiluted specimens were dialyzed in a short transit dialyzer. The dialysate was reacted for six minutes with alkaline picrate. Three deviations from the conventional method are noted: 1) the use of undiluted urine; 2) a short incubation period; and 3) a fore shortened dialysis.

Sampling at a rate of 40 specimens/hour linearity was established through 375 mg/100 ml. The normal distribution was calculated at 20-340 mg/100. Values expressed for a 24 hour period showed a range of 300-600 mg/100 ml. A correction was made for body surface area in order to reduce
randomization of the values which were then expressed in grams per 24 hour period. Results indicated a somewhat lower value for women and children (see Table I).

Replicate specimens were tested and yielded a precision of 3.61%. Additive studies indicated 100% recovery. Sensitivity of the procedure is 10 mg/100 ml based on the calculated precision.

Comparative tests were performed with an SMA 12/30 Auto-Analyzer (Technicon Instruments Inc., Tarrytown, N.Y.) (Hunter, Degen and McGuire, 1968). A 1:20 specimen dilution is required. Results were lower than those obtained with undilutes specimens. Dilution error and the increased alkalinity of the final reaction mixture may account for the elevated values in the new procedure.

Using the standard regression coefficient formula, \( Y = a + b \tilde{x} \), \( a \) was calculated at -3.74 mg/100 ml and \( b \) was calculated at 0.82. \( a \), the \( Y \) intercept, indicates excellent correlation between the test and reference techniques; a slope of 0.82 suggests a somewhat greater recovery and correlation between standard and specimen reactivity in the test procedure over the reference procedure.

III. GLUCOSE

A modification of the ATP-Hexokinase method with Thio-NADPH allowed for establishment of a system which may be observed in the visible spectrum. Dialysis enhanced the
TABLE I
COMPARISON OF CREATININE VALUES

<table>
<thead>
<tr>
<th>SPECIMEN NUMBER</th>
<th>24 HOUR VOLUME</th>
<th>mg/100 ml</th>
<th>mg/TOTAL</th>
<th>gm/VOL</th>
<th>gm/VOL SURFACE AREA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M</td>
<td>1925</td>
<td>187</td>
<td>3620</td>
<td>3.62</td>
<td>1.61</td>
</tr>
<tr>
<td>2 M</td>
<td>2050</td>
<td>98</td>
<td>2010</td>
<td>2.01</td>
<td>1.15</td>
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<tr>
<td>3 M</td>
<td>1650</td>
<td>102</td>
<td>1680</td>
<td>1.68</td>
<td>1.03</td>
</tr>
<tr>
<td>4 M</td>
<td>775</td>
<td>325</td>
<td>2520</td>
<td>2.52</td>
<td>1.40</td>
</tr>
<tr>
<td>5 M</td>
<td>1810</td>
<td>98</td>
<td>1773</td>
<td>1.773</td>
<td>1.04</td>
</tr>
<tr>
<td>6 M</td>
<td>1110</td>
<td>167</td>
<td>1854</td>
<td>1.854</td>
<td>1.04</td>
</tr>
<tr>
<td>7 F</td>
<td>1175</td>
<td>63</td>
<td>.740</td>
<td>.740</td>
<td>.456</td>
</tr>
<tr>
<td>8 F</td>
<td>860</td>
<td>153</td>
<td>1.316</td>
<td>1.316</td>
<td>.77</td>
</tr>
<tr>
<td>9 M</td>
<td>1000</td>
<td>252</td>
<td>2.520</td>
<td>2.520</td>
<td>1.23</td>
</tr>
<tr>
<td>10 M</td>
<td>910</td>
<td>260</td>
<td>2.370</td>
<td>2.370</td>
<td>1.18</td>
</tr>
<tr>
<td>11 C</td>
<td>480</td>
<td>77</td>
<td>.370</td>
<td>.370</td>
<td>.513</td>
</tr>
<tr>
<td>12 F</td>
<td>1010</td>
<td>120</td>
<td>1.212</td>
<td>1.212</td>
<td>.637</td>
</tr>
<tr>
<td>13 M</td>
<td>1120</td>
<td>225</td>
<td>2.520</td>
<td>2.520</td>
<td>1.43</td>
</tr>
<tr>
<td>14 M</td>
<td>1275</td>
<td>217</td>
<td>2.766</td>
<td>2.766</td>
<td>1.32</td>
</tr>
<tr>
<td>15 F</td>
<td>580</td>
<td>195</td>
<td>1.131</td>
<td>1.131</td>
<td>.765</td>
</tr>
<tr>
<td>16 M</td>
<td>825</td>
<td>315</td>
<td>2.598</td>
<td>2.598</td>
<td>1.27</td>
</tr>
<tr>
<td>17 F</td>
<td>1150</td>
<td>147</td>
<td>1.680</td>
<td>1.680</td>
<td>1.12</td>
</tr>
</tbody>
</table>

M = Male, F = Female, C = Child. Note that randomization seen in values for mg/100 ml is almost eliminated when corrected for surface area. Twenty-four hour values are similar to those reported in the literature.
already sensitive and specific test and made blanking unnecessary.

A normal range of 0-13.5 mg/100 ml (Figure 9) and 0-153 mg/24 hr. was calculated for 16 patients. Beer's law is followed up to 1000 mg/100 ml. A precision value of 7.6% was established with replicate specimens. Additive studies indicated a 90% recovery. Sensitivity of the procedure is

**GLUCOSE, NORMAL VALUE DISTRIBUTION**

![Graph showing glucose distribution](image)

Fig. 9
± 5 mg/100 ml and may be increased or decreased by baseline shifts or by changing the ratio of NADP to TNADP in the reaction mixture.

Comparison studies were performed using the O-toluidine test as a reference test (Dubowski, 1962). Lower results were obtained with the enzymatic system by approximately one-half. It is probable that there are other substances present which react with ortho-toluidine and yield slightly increased values.

Using a standard form for the coefficient of regression, \( Y = a + b \bar{x} \), a \( b \) value of 1.54 and an \( a \) value of 1.72 were obtained. These indicated a close correlation between the two tests and any deviation from unity may be explained on the basis of experimental error and differences in specificity between manual and automated procedures.

Sixteen randomized patient specimens were evaluated with the new procedure. These were run in series with the normal specimens. The enzymatic procedure detected two elevations, one at 25 mg/100 ml and the other 250 mg/100 ml. Studies performed with the routine procedures indicated one elevated glucose (see Table II).

Experiments indicate that this test may be used for measuring quantities of glucose up to 1000 mg/100 ml provided proper ratios of coenzymes are used. The values or normal range of 0-13.5 mg/100 ml are comparable to the values reported by Fine, 1965 (see Table III).
Table II
Patient Analysis

<table>
<thead>
<tr>
<th>Patient Number</th>
<th>Observed Abnormality</th>
<th>Reference Test</th>
<th>Confirmatory Test</th>
<th>Diagnosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>Glucose 25 mg/100</td>
<td>Routine Neg.</td>
<td>Tablet Neg.</td>
<td>Cholystectomy, UTI Sepsis</td>
</tr>
<tr>
<td></td>
<td>Protein 9.43 mg/100</td>
<td>Routine Neg.</td>
<td>Salicylate Neg.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hemoglobin 5.0 mg/100</td>
<td>Routine Neg.</td>
<td>Positive</td>
<td>Fractured Tibia</td>
</tr>
<tr>
<td></td>
<td>Bilirubin 1.014 mg/100</td>
<td>Routine Neg.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Ketones 16 mg/100</td>
<td>Strip Test Neg.</td>
<td>Tablet Neg.</td>
<td>Fractured Tibia</td>
</tr>
<tr>
<td>16</td>
<td>Glucose &gt;250 mg/100</td>
<td>Dip-Stick Pos.</td>
<td>Neg.</td>
<td>Stroke and Urinary Tract</td>
</tr>
<tr>
<td></td>
<td>Protein 2.71 mg/100</td>
<td>Dip-Stick Neg.</td>
<td>Salicylate 4+</td>
<td>Infection</td>
</tr>
</tbody>
</table>

Abnormal results from 16 random specimens are reported. Observed reference and confirmatory tests are shown.
<table>
<thead>
<tr>
<th>METABOLITE</th>
<th>NORMAL RANGE OBSERVED</th>
<th>NORMAL RANGE REPORTED</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/100 ml</td>
<td>mg/24 HR</td>
</tr>
<tr>
<td>Creatinine</td>
<td>20-340</td>
<td>1680-3600 (males)</td>
</tr>
<tr>
<td>Glucose</td>
<td>0-13.5</td>
<td>0-153</td>
</tr>
<tr>
<td>Ketones</td>
<td>4-24</td>
<td>30-285</td>
</tr>
<tr>
<td>Protein</td>
<td>0-0.6</td>
<td>0-9</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>-.08-.03</td>
<td>-</td>
</tr>
</tbody>
</table>

TABLE III
COMPARISON OF NORMAL VALUES OBSERVED WITH VALUES REPORTED IN THE LITERATURE
IV. HEMOGLOBIN

A range of 0-2.4 mg/100 ml (see Figure 10) and 0-35 mg/24 hours with the 17 normal subjects was observed. While most of the activity is probably due to hemoglobin, some may be attributable to nonspecific peroxidase activity. Linearity of the calibration curve was established through 100 mg/100 ml. Precision of the method is 7.1%. Additive
studies yielded a value of 100%, indicating that there are few substances present in urine that would interfere with the procedure. Sensitivity based on the calculated values is approximately ±3 mg/100 ml.

Thirteen patients were tested, one of which had an elevated hemoglobin. Routine analysis by dipstix procedure failed to detect this elevation of 5 mg/100 ml (see Table II, page 52). Again, the low range specificity of this method is validated and the estimated sensitivity is also substantiated.

Baseline shifts were encountered but compensated for by interspersing a water sample between each specimen. The reagent complex manifested a slight increase in absorptance.

V. KETONES

Linearity was followed through 125 mg/100 ml. Normal ranges of 4-24 mg/100 ml (see Figure 11) and 30-285 mg/24 hours were established. Precision was calculated at 4.3% and 100% recovery was obtained. These normal values are above those reported in the literature.

Twelve patient specimens were run in series with the normals. Routine dip stick and a reference test indicated one elevation, which was quantitated by the present test system as 37 mg/100 ml (see Table II, page 52). Deterioration of the nitroprusside reagent resulted in a baseline shift. Breakdown occurs rather rapidly after two to three hours. This deterioration may cause a decrease in sensitivity, which is
estimated to be approximately ±8 mg/100 ml. Correction for baseline shift was successfully achieved by interspersing water samples between a given series of specimens.

VI. TOTAL PROTEIN

The sulfosalicylic acid turbidimetric technique appropriately blanked by a dilute acid cycle gave a normal range of

**KETONES, NORMAL VALUE DISTRIBUTION**

![Graph showing ketone levels](image)
0-0.6 mg/100 ml of protein (see Figure 12). Based on 24 hour volumes, this was calculated to be 0-9 mg/24 hours (see Table III, page 53). The reproducibility study on replicate specimens yielded an 8.6% coefficient of variation. Recovery observed was 96.5%. Based on the noise level and calculated precision, sensitivity to ±5 mg/100 ml could be reliably estimated. The reaction was linear through 100 mg/100 ml.

Sixteen randomly selected patient specimens were also
tested. Fourteen of these showed normal results based on the established range. Two of the specimens had 9.43 mg/100 ml and 2.71 mg/100 ml respectively. These specimens were reported "negative" by the reference procedure (see Table II, page 52).

Large particles cause prohibitive "noise" in continuous flow systems. The trap effectively reduced the background fluctuation to acceptable levels.

VII. BILIRUBIN

A hypochlorite reagent was used to oxidize the bile pigments present. Optical density readings were obtained using a dual flow system with a double beam differential read-out. This was done to avoid recycling the specimens and to enhance the sensitivity and reproducibility of the procedure. Since the blank channel gave a stable color and the reaction channel was associated with a decrease in absorption, the blank stream was diverted through the sample cuvet while the reaction stream was passed through the reference side. This allowed for a positive scale deflection in the presence of bilirubin.

The normal range established was -.08 to +.03 mg/100 ml (see Table III, page 53, and Figure 13). Negative readings indicated the development of turbidity resulting from precipitation of materials insoluble at high pH. Using an ox bile standard of .08 mg/100 ml, a linear curve was established. Precision of 8% was estimated. Recovery tests were not performed, due to impurity and lability of standards.
One patient specimen containing an estimated 1.01 mg/100 ml of bilirubin gave an off scale reading. Comparative studies were performed using the Bili Lab-Stix and Ictotest. The Ictotest detected the presence of bile, but the dip stick was negative (see Table II, page 52). Another patient specimen which had been stored in the freezer was tested and caused full scale deflection on the chart recorder. This specimen was known to contain bile. Such a specimen would require several dilutions.
This study is not yet complete and the data presented is a preliminary investigation. Further investigation is being pursued to define the parameters of the system and to refine the procedure.
DISCUSSION

Routine urinalysis, as presently performed in clinical laboratories throughout the country, has afforded a means for relatively rapid and economic mass screening of patients. Significant changes in metabolism secondary to diseases such as diabetes, kidney infection, hemolytic anemias, liver disease and a variety of pathologic processes that alter the "normal" composition of urine are usually detected. The semi-quantitative "spot tests" are simple to perform and require a minimum of experience or skill interpreting color changes indicative of pathologic processes. Through the conventional approach one technologist can process approximately 20 specimens per hour (Ashton and Lashof, 1964). This includes additional confirmatory tests that may be necessary to further validate a result, e.g., the use of sulfosalicylic acid as a definitive test for albumin when detected on reagent strips.

Reputed sensitivity of spot testing has been disclaimed (Kark et al., 1963; Oser, 1965); similarly, specificity and quantitative accuracy have been generally questioned (Kark et al., 1963). However, it will be presumed that the spot tests are capable of detecting materials when they are present in an abnormal amount. The human element may introduce errors by way of subjectivity or misinterpretation. Furthermore,
present methods necessitate the usually unpleasant and hazard­
our chore of handling the urine. As virtually all other areas
of laboratory science advance, clinical microscopy remains
stationary and progressively its relative importance decreases.

Two systems were evaluated for the purpose of achieving automation. One system, the urine analyzer, was evolved from
cncept to feasible prototype. The apparatus incorporates
several advantages; however, further evaluation and modification are needed before it is fully operational. Improvements
could be made in the reagent dispensing system by increasing
the volumes used or by converting to a hydraulic pneumatic
method of dispensing. Conveyor belts could be changed into a
belt with cups carrying the reaction mixture. Flow-through
cells could be used for the double beam system, enabling a
better wash cycle, constant fill and longer light path. All
modules should be interfaced before any conclusions may be
reached concerning the final operation. With these few modi-
fications, the sensitivity of the chemical systems will be
enhanced and sequential analysis may be accomplished.

The second system evaluated was assembled from existing
apparatus. Component parts were readily interfaced and
adapted to the previously developed chemical test systems.
One immediate disadvantage resulting from this approach was
that sequential analysis could not be performed and, indeed,
recycling to eliminate blank contribution was essential in
one test system. Individually, each test system proved valuable to productive quantitative data. Investigation may be pursued in the area of sequencing the individual system into a single operational mechanism. Both systems generated a considerable volume of vital technical information, which in aggregate will contribute greatly to the final apparatus.

Through automation it was possible to establish normal values for the metabolites tested. These were based on specimens obtained from normal subjects. Furthermore, reporting of results in terms of milligrams of creatinine might eliminate possible dilution variables and preclude the necessity of accumulating a 24 hour specimen. This does not imply that the need for this type of study has been eliminated. Incorporating this approach, the methodology used in the automated process takes on greater import for two reasons: one, this will be a means of determining whether a fairly good 24 hour specimen was obtained; two, once the amount of creatinine per milliliter is determined, observed metabolite levels in random specimens can be corrected for dilution and concentration effect.

The value of precise quantitation may be easily illustrated. Studies may be performed on patients to establish trends of pathologic processes as they may occur. As an example, let us assume that a patient is suspect of having a paroxysmal nocturnal hemoglobinuria. A morning specimen is
obtained and subsequent specimens are taken at given intervals. Serial values will show a fluctuating trend which characterizes the disease. This affords a research tool as well as a diagnostic aid. In cases where proteinuria may be important, serial studies will indicate changes which will enable better diagnosis and management of the disease. Situations where metabolic acidosis may be a significant factor, or even a contributor to some pathologic process, the detection of serial ketone fluctuations will help in the overall diagnosis and therapy.

Physicians will benefit by having absolute values to evaluate. At present, subjective values seen as two or four plus are reported. The availability of metabolite concentration data will permit specific, precise therapy and will enable critical analysis of therapeutic response. It is even conceivable that accurate analytic capability of urine metabolites will preclude some serum determinations.

One of the most important aspects of any automated system over manual procedures is the decrease in overall cost. With the automation of urinalysis a decrease in the total cost for a test should be realized, despite the incorporation of additional important tests. As with chemical screening, the unit cost per test decreases with the number of tests performed.

The laboratory scientist will accrue benefits also. The handling and manipulation of urine will be minimized. Work patterns and specimen flow can be made more efficient.
Statistical data can be gathered more efficiently and evaluated. Accordingly, there will also be a decrease in false positive and false negative results due to human error and length of time before specimen is run. Conservation of personnel effort will enable extension into new laboratory efforts. Automation usually results in economy of space and expendibles.

As an automated device generates results independent of technical observation, more time and study can be devoted to the all important microscopic examination of the urinary sediment. A decrease in the time lapse from collection to microscopic study will help preserve formed elements such as casts and red cells. Furthermore, bacteria, if present in limited amounts, will not be allowed time to multiply and greatly alter small quantities of measurable metabolites such as protein and glucose.

As stated earlier, the introduction of "mass production" into the clinical laboratory has introduced the concept of multiphasic screening. An integral part of this screening procedure is the urinalysis test panel. With the advent of automation in the area of urine study, a more accurate and precise screening can be accomplished. Values from studies of serum, blood and urinalysis together can now present a more meaningful diagnostic profile for the clinician.

Several major innovations have been incorporated in the
feasibility study. Threshold detection has been increased through the photometric readout (see Table IV). Test systems have been modified or developed to meet certain mechanical restrictions or clinical requirements. One completely new modification involves the glucose test system.

The measurement or true glucose by any method has always met with formidable difficulties. Quantitation by the ATP-Hexokinase method of Peterson (1968) has been widely accepted; however, it has not been adapted to urine analysis. The great sensitivity of the test limited the effective range. In addition, readings were made in the ultraviolet range at 340 nm. By adding Thio-NADP to the reaction mixture, the test can be read in the visible 400 nm range. The NADP-TNADP ratio can be adjusted to permit a range excursion to over 100 mg/100 ml without specimen dilution. It is virtually interference free. The capability to detect minute elevations of urine glucose may facilitate the detection of early diabetes.

Another new modification was the quantitation of bilirubin by direct oxidation with sodium hypochlorite. Normal values established for this method are comparable to the sensitivity required of spot tests (Henry, 1964b). This test provides a rapid means for quantitating and detecting incipient cases of hepatitis or other hepatic syndromes (Couch, 1970).

The presence of hemoglobin in urine has great significance
### TABLE IV

**COMPARATIVE THRESHOLD DETECTION**

<table>
<thead>
<tr>
<th>TEST SYSTEM</th>
<th>QUANTITATIVE (mg/100 ml)</th>
<th>QUALITATIVE (mg/100 ml)</th>
<th>OBSERVED UPPER LIMIT OF NORMAL (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine</td>
<td>350*</td>
<td>20*</td>
<td>340*</td>
</tr>
<tr>
<td>Glucose</td>
<td>5</td>
<td>10 (Kark)</td>
<td>26</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>2</td>
<td>2 (Kark)</td>
<td>2.057</td>
</tr>
<tr>
<td>Ketones</td>
<td>5</td>
<td>10 (Kark)</td>
<td>24</td>
</tr>
<tr>
<td>Total Protein</td>
<td>0.6</td>
<td>20 (Kark)</td>
<td>0.6</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>0.02</td>
<td>0.05 (Kark)</td>
<td>0.03</td>
</tr>
</tbody>
</table>

*Creatinine is normally present in high concentration. Conventional methods use great dilution to enable scale reading. Values in the table are presented in terms of upper rather than lower threshold levels.*
in the detection of hemolytic disease, bleeding diathesis and numerous renal and lower urinary tract diseases. O-dianisidine was employed to provide greater sensitivity than benzidine, the commonly used chromogen. Dianisidine is approximately five times more sensitive to peroxidase activity than other benzidine derivatives (Owen et al., 1958). Quantities down to 3 mg/100 ml are detected. In this low range hemoglobin is usually present as the result of shed red cells. Again, this affords an opportunity to quantitatively follow a patient either at the beginning of a process or during therapy.

Ketone bodies are measured by means of a modified nitroprusside test. Ammonia hydroxide is added to prevent interference by creatinine. The test is sufficiently sensitive to detect low grade metabolic acidosis due to starvation, malnutrition or poor glucose metabolism. Debilitated patients with complicating conditions cannot be adequately evaluated using qualitative tests. Being able to follow a patient over a period of time with an inexpensive, but quantitative test, will enable better diagnostic and therapeutic capability.

Total protein is usually measured on random morning specimens. For maximum information a 24 hour specimen is usually analyzed to establish increasing or decreasing pathologic trends. The use of sulfosalicylic acid as the precipitating agent will allow for the detection of proteoses which are excreted during active progressive renal disease.
Furthermore, abnormal proteins, such as Bence Jones protein fragments, are important in the diagnosis of blood dysciasias. Serial urine examinations by quantitative protein tests in the third trimester of pregnancy may pick up early rises and enable the early diagnosis of eclampsia. Numerous other examples could be given.

Creatinine excretion has long been acclaimed as one of the best indicators of renal function. On controlled diet, the amount excreted is constant for a given individual. Its determination has been used to check for the completeness of 24 hour specimens (Oser, 1965). Assuming a normal diet, the average man excretes one to 1.8 gm per day. If this is divided into random specimens and is checked accordingly, a good indication of renal function can be obtained. In addition, the other measurements can be reported out in terms of creatinine concentration. For maximum consistency, the 24 hour creatinine excretion should be corrected for body surface area (see Table I, page 49). The degree of interference, as indicated by recovery studies, was not great.

Quality control may be maintained in the system by running known standards at regular intervals. This allows for correction of baseline drift and monitors the linearity and activity of the reagents. Primary or weighed in aqueous standards were used. They were prepared in stock concentrations from which working aliquots were diluted. Use of lyophilized
standards, with most if not all materials included in one vial is feasible, thus eliminating the need for technical personnel to undertake the preparation of separate standards.

Recovery studies indicate that the tests are reasonably specific and sensitive. Comparative studies between the spot tests and the automated methods also confirmed that automated procedures incorporated increased sensitivity over the manual methods (see Table IV, page 67).

Introduction of automation into urinalysis with digital reporting will no doubt require a certain amount of re-education. As quantitative data is accumulated for normal and pathologic states, the relationship between the various results must be related to the clinical picture. At a future time, the analysis and evaluation of urine metabolites will be of the same degree as that accorded serum findings.

Potential computer interfacing opens up the vast area of collating all information on a given disease process. A patient can then be evaluated in terms of serum chemistry, urine chemistry, spinal fluid chemistry, blood parameters and perhaps even tissue analysis. The multiphasic screening will now be extended by more accurate, quantitative test procedures. With the potential offered by computers, great masses of data may be gathered and intelligently evaluated. This will lead to better utilization of information and enhanced health care delivery. Improved definition or patterns
will result in the identification of new syndromes and disease entities. Furthermore, mass screening programs will allow greater individualization of patient care, i.e., the storage of data on a particular patient from year to year will enable individualized normals to be developed, affording the physician more exact information on each individual patient.

Additional tests which may be adapted to an automated urine analysis system include methods for phenylketonuria and other inborn errors of metabolism; a general ferric chloride test to detect false drug inhibitors; tests for chloride and other ions, urobilinogen, amino acids, bacteria, various enzymes, toxilogical derivatives and medications. This then opens up an area of new methodology to be evaluated on a long term basis.
SUMMARY

A study of the routine urinalysis was undertaken to determine the feasibility of automating the technique to achieve a degree of quantitation, accuracy, precision, sensitivity, specificity, rapidity, volume output and capability comparable to that attained in other laboratory sciences. Furthermore, in keeping with laboratory volume trends and medical scientific advance, it was not only highly desirable but tantamount that these objectives be attained. Although there is considerable justification for extending the test battery, this feasibility study was designed to include only those tests currently included in the routine urinalysis: pH, protein, ketone bodies, color, glucose, hemoglobin, bilirubin and specific conductivity. Creatinine was also incorporated as a potential means to allow more meaningful quantitation.

Significantly modified chemical methods were adapted both to a specially designed automated device and to conventional automated systems. The former apparatus deposited microquantities of specimen and reagent in the form of a droplet (bead) on a nonwettable continuous belt. At various predetermined positions reagents were added. At the conclusion of a given interval the reaction mixture was carried into a double beam microcuvette photometer where the bead
entered the cuvette by capillary attraction and a graphical read-out of the voltage differential was recorded. One turbidimetric test, eight colorimetric reactions and one physical parameter were evaluated. Similarly, all methods used in the urine analyzer were adapted to a Technicon system comprised of existing apparatus, proving the versatility of the methodology and providing preliminary clinical data for evaluation.

Minor imperfections and time restrictions precluded successful testing of the specially designed urine analyzer; however, primary standards, normal and pathologic specimens were successfully tested on the conventional equipment. Normal ranges, precision, recovery and comparative data were compiled and analyzed.

The feasibility of automating urinalysis was proven. Quantitation of urine metabolites may be readily achieved. It appears reasonable that at some future time automated urine analysis will be routinely performed and that considerable patient benefit will accrue.


<table>
<thead>
<tr>
<th><strong>Name</strong></th>
<th>Philip Anthony Fidel</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Birthplace</strong></td>
<td>Santa Fe, New Mexico</td>
</tr>
<tr>
<td><strong>Birthdate</strong></td>
<td>13 April 1938</td>
</tr>
<tr>
<td><strong>Elementary and High School</strong></td>
<td>Saint Michael's High School Santa Fe, New Mexico</td>
</tr>
<tr>
<td><strong>Degree</strong></td>
<td>B.S., College of Santa Fe, 1960</td>
</tr>
<tr>
<td><strong>Certificate</strong></td>
<td>Certificate in Absorption, Spectroscopy Arizona State University, 1966</td>
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<tr>
<td><strong>Licensure and Registration</strong></td>
<td>American Society of Clinical Pathologists California Department of Public Health</td>
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