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Automation of Steroid Analysis

Direct photometric scanning of thin-media chromatograms can be both accurate and very rapid.

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When insulin was introduced into medical practice in 1926 and it became possible, for the first time in human history, to maintain diabetic patients in good health for long periods, a new era began in the application of chemistry to medicine. Diabetes mellitus was not the first disease of the endocrine organs for which a cure or palliative had been found, but the use of insulin presented a number of features new to the medical profession. Above all, it was a disease for which the principal tools of investigation and control were entirely chemical. The discovery of the optimum regimen of insulin and diet and the final establishment of a diagnosis could, in many cases, be made only through accurate quantitative measurement of glucose concentrations in the blood.

In the early days a physician had rather more difficulty in getting good measurements of blood sugar from the hospital laboratory than he has today in obtaining accurate measurements of the blood concentration of vitamin B_{12}, gamma-globulins, pyruvate, or protein-bound iodine.

Nowadays, the practice of accurate quantitative clinical chemistry is commonplace, and many metabolic and other diseases are continually managed and diagnosed through biochemical methods. Furthermore, we have already reached the stage at which between six and 12 of the commonest types of measurement are now carried out automatically in most large hospitals by instruments specially designed for the purpose. By far the commonest instrument used in this task is the Technicon Autoanalyzer, which may fairly be credited with having caused a major revolution in hospital biochemistry. The substances most commonly measured in this way—either in blood or in urine—include the ions of hydrogen, sodium, potassium, calcium, chloride, bicarbonate, and phosphate, and glucose, urea, creatinine, protein, and cholesterol. It is convenient to class these substances as "simple biochemicals"—that is, biochemically important substances which, because of their relatively large concentrations in the body fluids, or because of the existence of highly specific physical or chemical methods for their measurement, can be measured relatively easily without the use of complicated methods of purification before the final step of actual measurement.

These "simple biochemicals" also include a number of substances which may not be so simple, from the point of view of chemistry, but which are relatively easily measured under some special circumstances in laboratory research. They must be contrasted with "complex biochemicals," whose measurement is difficult, either because of a very low concentration of such substances in the body fluids (or experimental material) or because the substance to be measured—for example, an individual amino acid or steroid metabolite—is one of a large group of similar substances present in the sample to be analyzed. This classification, it should be emphasized, is only an operational one; it is a rough classification of methods rather than of the substances themselves. Thus, for instance, the measurement of small quantities of an individual amino acid in a protein hydrolyzate is a complex procedure, whereas its measurement at moderate concentrations in a purified enzyme system giving rise to no other amino acids is relatively simple. By and large, it is convenient to talk of amino acids, steroids, specific proteins, vitamins, nucleic acids, and nucleotides as "complex biochemicals" because, in the great majority of experimental or clinical situations in which we wish to measure them, complex methods are required.

In this article I describe some of the work that has been done on the problem of automating the measurement of steroid hormones and their metabolites. I regard this problem as one which is not simply of interest to steroid biochemists and endocrinologists but typical of the problems of measuring complex biochemicals on a large scale.

The Problem of Large Numbers

Complex biochemicals, by definition, require a combination of considerable skill, time, and expensive apparatus for their precise measurement by ordinary manual methods. Thus, in nearly all fields in which such substances are of crucial interest, far more man-hours and expense have been spent in devising, improving, using, and maintaining the necessary analytical methods than in performing the experiments in which they are used. There are very few reliable estimates of the concentrations of most steroid hormones in the blood plasma of men and women, although many excellent workers have been trying for 10 years or more to devise adequate methods. Methods developed in recent years (see, for example, *1* and

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2) are now adequate to the task, but not more than ten to 20 measurements can be made per week by a team consisting of two above-average technicians or assistants led by a first-class research worker, who may have devoted most of his effort over 3 years or more to the devising of his method. If we consider the measurements that are at present most desirable in the steroid field—measurements of testosterone, androstenedione, progesterone, estrogens, and aldosterone, all in peripheral blood or plasma—it can be fairly said that, for each substance, there exist no more than five to ten laboratories in the world where reliable measurements can be made on a routine basis, and that the teams in these laboratories cannot carry out more than five to 20 measurements per week, usually of only one substance or of small, closely related groups of substances.

Such a situation recalls the era before the industrial revolution. We nearly all prefer a chair by Chippendale to most modern chairs, but Chippendale's production methods, however admirable in other ways, would not meet the present-day need for chairs. There are good grounds for believing that the complex methods, usually only recently devised, for measuring steroid hormones and their metabolites are inadequate to meet the need for such measurements. I believe this also holds for many other types of complex biochemicals.

First, let us examine a medical problem. There is reason to believe that abnormalities in the secretion or metabolism, or both, of steroid hormones are an important causal factor in the production of cancer of the breast, and are also related to the chances of success in the treatment of such cancers (see, for example, *3*). Few, if any, workers, however, believe that a gross, clear-cut difference in any one hormone is responsible for this disease, and most would guess that cancer of the breast is a disease of multifactorial origin. It is now recognized that retrospective studies of such diseases are unlikely to lead to the discovery of significant causal factors, because it is all but impossible to make an adequately matched selection of control subjects. The desirable approach is to take a presently healthy population large enough so that one can expect to find a significant number of individuals succumbing to the disease over a reasonable period of study.

It is estimated that to study cancer of the breast in this way requires a population of between 5000 and 8000 women (*4*). As a bare minimum we might propose to investigate the hormonal physiology of these women by drawing blood or taking urine samples once a week during two menstrual periods per year—that is, taking eight samples per patient per year. We would certainly wish to measure at least six, and possibly 12, steroids in each blood sample and from 15 to 30 steroid hormones and metabolites in the urine samples.

However many substances we set out to measure, we would face the prospect of handling between 40,000 and 64,000 blood or urine samples a year, or between 160 and 256 samples per working *day*, on the basis of a 5-day week and 11 holidays per year. Present methods, with teams and laboratories of feasible size (that is, capable of making measurements of approximately ten different steroids on one blood sample), are too slow for the job by a factor of about 250.

But, quite apart from the large-scale investigation of diseases of multifactorial origin, it is clear that the methods of measuring complex biochemicals are also the rate-limiting factors in many laboratory investigations. Thus, protein chemists may use three or four automatic amino acid analyzers, of the type first perfected by Spackman, Stein, and Moore (*5*), but still find that their amino acid measurements are not rapid enough to satisfy them. For studying the metabolism and mechanism of action of a steroid anesthetic (*6*), one experiment on a cat yields a minimum of 50 extracts of blood plasma, to be analyzed quantitatively for up to six closely related steroids. Many other examples could be given.

It is uncommon to find more than passing attention being paid to the productivity of analytical methods in the field of complex biochemicals. This is unfortunate, because a consideration of the "numbers problem" leads one inescapably to the conclusion that nothing less than a major breakthrough is needed. Our productivity is inadequate, not by a moderate factor, but by approximately two orders of magnitude. The following discussion is based on the premise that something of the attitude of the first Henry Ford is needed if such a breakthrough is to be made.

Are there any general principles for the development of analytical methods of the power hinted at above? It seems likely that the measurement of complex biochemicals will, in general, continue to depend heavily upon purification—that is, separation—procedures for specificity. Only relatively rarely will it be possible and economical to obtain a major degree of specificity by means of physical, chemical, enzymatic, or immunochemical methods. Nevertheless, the final method of obtaining a measurable "signal" from a substance should be as specific as possible, since we are usually faced with the problem of separating traces of a substance from relatively overwhelming quantities of other substances, and distribution coefficients are not infinite. A more detailed discussion of this problem is given elsewhere (*7, 8*).

Promising and ingenious attempts have been made to use digital computers in conjunction with infrared or mass spectrometry for measurements on relatively impure extracts of urinary steroids (see, for example, *9*). Such methods have been only partially successful, and it seems likely that, for the foreseeable future, complex methods will usually depend on some type of chromatographic technique for a major part of their specificity.

Two broad classes of methods exist for obtaining the requisite sensitivity. One is the method of "labeling" the substance to be measured by a reaction which introduces an easily detectable group. This group can contain either a radioactive element of high specific activity, an element capable of activation in an atomic pile to a radioactive isotope, or an element or group easily detectable by other means (for example, halogens, which give exceptional sensitivity in electron-capture detectors in gas-liquid chromatography). The other class depends upon the generation of a "signal" from the substance itself (or from a derivative) without labeling.

Labeling methods are the only ones at present possessing the sensitivity required for measuring most steroid hormones in blood. They have been found, in practice, to present numerous difficulties, the most serious being the production of high "backgrounds" from the labeling of other substances, or from decomposition or side-reaction products of the labeling reagent. Very extensive chromatographic fractionation—for example, up to five or six thin-layer or paper chromatograms—is needed before the final chromatogram is run.

The quantitative transfer of materials

from one chromatographic medium to another is time-consuming and awkward, rather than difficult, to automate. Even though the time lost in obtaining multiple preliminary chromatograms can be cut down through a little time-and-motion study, through use of labeled material as internal controls on recovery, and through construction of simple apparatus, it constitutes a serious difficulty in attempts to increase the productivity of present analytical methods in which labeling is used. On the other hand, multiple chromatography in some form is almost certainly necessary for achieving the required specificity of *any* method when very small quantities (1 to 100 nanograms) are involved (see, for example, *2*).

It seems likely, therefore, that, for some time to come, analytical methods for measuring steroids and complex biochemicals in general are going to depend upon (i) use of chromatographic methods for the major part of their specificity; (ii) high specificity in the method of obtaining a measurable "signal" at the final stage; and (iii) use of highly sensitive methods of obtaining the final "signal."

In general this has meant, and it seems still to mean, that such methods must remain the province of a relatively few highly skilled research workers, working slowly and patiently "in the back room." My own work has been based on the thought that this need not, and indeed must not, be so. Both experimental research and the future biochemical investigation and control of human disease demand analytical methods for measuring steroids and other complex biochemicals which will be as productive and simple to carry out as present methods for measuring simple biochemicals are. In 5 years or 10, "estradiol tolerance curves" may well be as common and as important a part of clinical diagnosis as glucose tolerance curves are today.

An operational analysis of the several general procedures available suggests strongly that chromatographic methods for quantitative work should, if possible, be based upon the measurement of substances without elution from the chromatographic system. This argument, first advanced in 1963 (*10*), remains valid today despite advances in automatic multiple-ion-exchange columns and in capillary or other forms of gas-liquid chromatography. With most analytical methods in the steroid field the aim is to separate from one to eight substances for measurement on the final chromatogram. In the amino acid field, 20 to 30 components must be separated per final chromatogram. There are theoretical and practical limitations to the speed at which chromatograms of any type can be run. With most gas-liquid chromatography methods for steroids, runs of 0.6 hour to 2.0 hours are usual. The fastest available elution method is the Golay capillary technique of gas-liquid chromatography (*11*), but there is no established technique for measuring steroids in plasma or urine with Golay methods at present. Even if there were, one apparatus would be capable of handling only five to six samples per hour. For a comparable (in fact larger) investment in equipment, systems in which some form of semiautomatic liquid elution is used normally produce less than six chromatograms per day. In contrast, paper, or other thin-media, chromatograms can be scanned at rates of 1 centimeter per second or 80 full-length 45-centimeter strips every hour (*10*).

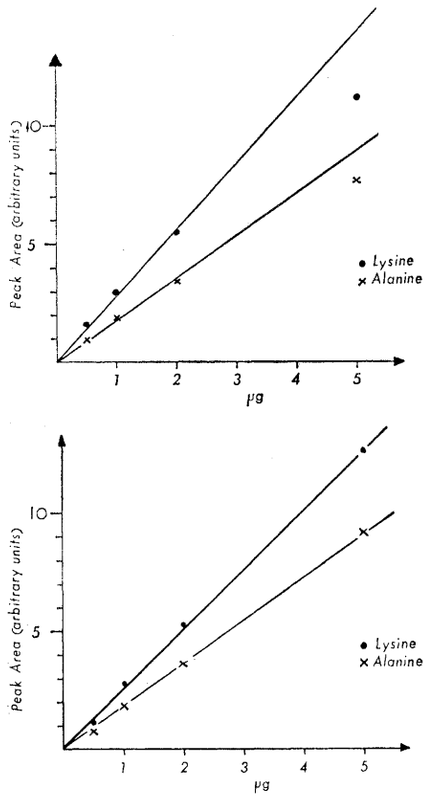

Fig. 1. One of the two most frequent sources of error in direct colorimetry of paper chromatograms is an inadequate concentration of color reagent in orthodox spray reagents. (Top) Calibration curve of peak areas obtained with lysine and alanine, with the Barollier-ninhydrin reagent at twice the originally recommended concentration of ninhydrin and cadmium acetate. The curve is nonlinear for amounts above approximately 2 micrograms. (Bottom) Calibration curve obtained in the same way, but with four times the recommended concentration of ninhydrin and cadmium acetate. The curve is linear for amounts up to at least 5 micrograms. Linearity up to amounts of 20 micrograms per amino acid is achieved only with six times the recommended concentration of ninhydrin and cadmium acetate (see *10*).

In summary, it seems at present that the direct photometric scanning of paper (or thin-media) chromatograms is a far more productive method of measuring complex biochemicals than any other chromatographic technique. This conclusion is based on a comparison of the instrument described below, which has been in routine operation since early 1958, with presently nonexistent but potentially feasible forms of its nearest competitor.

A great advantage of the scanning method of quantitative chromatography over other forms is the fact that, with a sufficiently rapid scanner, an increase in the productivity of a small or medium-sized analytical laboratory is achieved very cheaply. Most chromatographic separations can be run on standard paper strips (about 45 centimeters long) in 3 to 4 hours or, conveniently, overnight (15 to 16 hours). Thus, a moderate-sized laboratory with a scanner capable of handling 500 to 600 strips a day but actually handling only 50 strips a day can double its productivity merely through the purchase of two or three more chromatography jars. A disadvantage of the scanning method, however, is the delay in obtaining results. A fast scanner leads to high productivity because paper or other thin-media chromatograms can be run economically in parallel in large numbers. The gas-liquid chromatography method, while too expensive to be used for multiple parallel operations on a large scale, has potentially the great advantage of a small delay time (the time between receipt of the sample and obtaining of the result—1 hour to 2 hours, compared with 5 to 20 hours). These advantages, however, have not been apparent in the steroid-measurement field because of the large number of preliminary chromatograms on thin media that have been needed, in practice, for gas-liquid chromatography methods. In fact (*12*), thin-media techniques are now practically as fast as existing gas-liquid chromatography methods for steroid measurements (delay time, 0.5 hour to 2 hours).

If this is the best logistic solution to

our problem, is it also acceptable from the standpoint of analytical accuracy? In view of the failure of many workers, in the period before 1954, to obtain reliable results by the direct scanning of paper chromatograms, it was at first thought unlikely that the method could be brought to a reasonable degree of precision and still not require special skills or excessively expensive apparatus. This impression has been reinforced, perhaps, by the failure of most commercially available scanners to improve upon the optics of their forerunners.

Most quantitative chromatographic elution methods, when pure standards are used, have standard errors, at the mean of the working range, of about ± 2 to 3 percent of the estimate. The signal from the system is recorded on a potentiometric chart recorder and integrated over time, peak areas being calculated through subtraction of the value for the background signal integrated over the duration of the peak (*5, 7*). Around 1954 it was generally believed (*10*) that direct densitometric or fluorometric measurement of substances on paper chromatograms could not be improved further, and that a standard error of ± 10 to 15 percent was unavoidable (see, however, *13*).

It was subsequently found that almost all earlier attempts (compare *13* and *14*) to achieve quantitative scanning of paper chromatograms (or electrophoretograms) had been subject to two simple and controllable sources of error. (i) Little or no attempt had been made to achieve the optical pathway appropriate for quantitative absorptiometry or fluorimetry. (ii) The reagents used were too dilute for achieving a stoichiometric reaction over the desired working range of these methods. A detailed account of this problem, with two types of color reactions for steroids, has been given elsewhere (*7, 30*). Figure 1 shows that the same phenomenon can be seen in studies with the well-known Barollier modification of the ninhydrin reagent for amino acids (*15*).

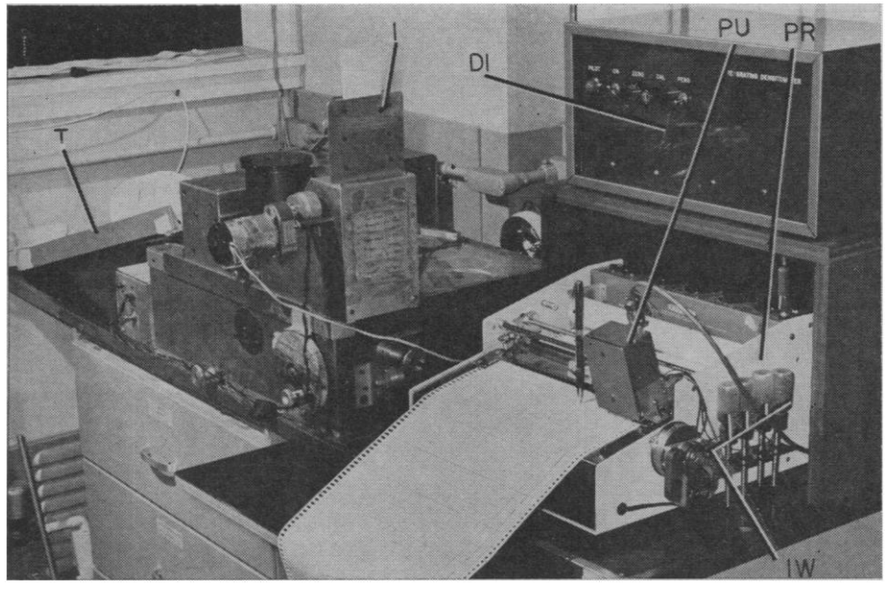

Fig. 2. General view of the scanner for measurement of paper strip chromatograms. A small train of short strips is shown emerging into the collecting tray (*T*); the last strip is just disappearing into the inlet (*I*). Electronic gear is contained in the Heathkit EUW-20A potentiometric recorder (*PR*) and the cabinet above (*DI*). The four-pen digital (decimal) pen unit (*PU*) is attached to the rail on which the standard pen carriage slides, and is fed by ink reservoirs with adjustable levels (*IW*).

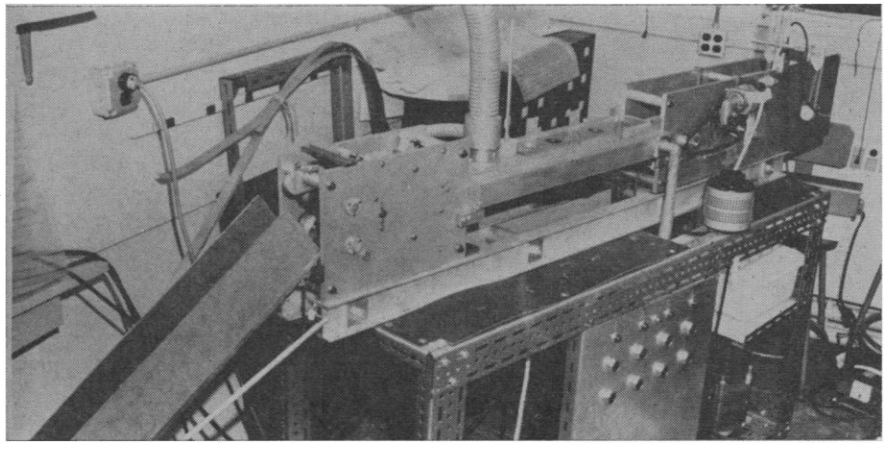

Fig. 3. General view of the apparatus for treating chromatograms with liquid reagents. A train of strips enters the section at right, in which the reagent is applied, and then passes through the reaction chamber, the middle section of the machine. Hot air enters by conduits on each side of the reaction chamber, passing into it from ports whose apertures are controlled by the slide valves which project upward at intervals along the reaction chamber. The exhaust is carried away by the flexible hose at left-hand end of the reaction chamber. The strips emerge from the left-hand section of the apparatus and are collected at the inclined tray at extreme left. The transporting belts are washed in the left-hand section and dried on their return passage through the reaction chamber.

Direct Photometry of Thin-Media Chromatograms

Considerations of this sort based on manual methods [and the promising results of Brown and Marsh (*13*)] led me in 1955 to start developing a machine for the precise direct photometric measurement of paper chromatograms. The aim was to develop an apparatus which would lend itself eventually to a considerable degree of automation, and which would in addition mechanize the carrying out of chemical reactions on paper chromatograms. In accordance with the fashion of the times the machine was given a name—CASSANDRA, for Chromatogram Automatic Soaking Scanning And Digital Recording Apparatus. (Despite her pessimism, Cassandra was the most accurate of the ancient prophets, and a feminine name is appropriate for complicated engineering devices.)

Figures 2 and 3 show the present form of the apparatus. In Fig. 2 the scanner—8 years old, and a little battered from use after having scanned

over 23,000 chromatograms—is at the left, coupled to a solid-state version of Sweet's logarithmic densitometer circuit (*16*), which drives the Heathkit EUW-20A potentiometric recorder (at bottom right). A retransmitting slidewire has been fitted, from which a voltage between 0 and −10 volts is obtained, according to the position of the recorder pen. This secondary output is fed either to a voltage-frequency converter which, via a chain of decades and Schmidt triggers, drives four pens (units, tens, hundreds, and thousands) in the integrator unit attached to the recorder, or is fed directly to an analog-to-digital converter (Digital Equipment Corporation, type 138E) for direct processing by a D.E.C. PDP-8 computer.

In Fig. 3 the treatment part of the apparatus is shown. This is used at present only for reactions in which careful control of heating and drying is necessary (for example, the Zimmermann reaction for 17-keto steroids; the molybdophosphoric acid reaction for hydroxy steroids; the ordinary ninhydrin reagent reaction for amino acids). While the development of this part of the machine was difficult, it is easily understood in principle. It is fully described elsewhere (*17*).

For many methods in which slow reactions (10 to 50 minutes) suffice, the scanner alone is needed. (Examples are the alkaline tetrazolium reaction for reducing steroids; isonicotinic acid hydrazide or sodium hydroxide fluorescence reactions for Δ^4-3-keto steroids; the Barollier-ninhydrin method for amino acids.)

Although not yet commercially available, the scanner could be reproduced in a good workshop for about $1500, and the associated electronics, for $320. The Heathkit recorder is not ideal (the full-scale deflection time is 1.5 seconds, which is a little too long), but it is relatively inexpensive, costing $200. Superb performance is obtained with an Esterline Angus series S-609 (full-scale deflection time, 0.2 second) with a retransmitting slidewire, costing $1400. The whole setup for scanning thus costs rather less than one good analytical gas-liquid chromatography apparatus (for example, the F & M 400) and very much less than an automatic ion-exchange chromatograph of the Moore and Stein type. The chemical-treatment section would cost about $5000 to duplicate in a laboratory workshop. Both sections of the apparatus are capable of processing 500 to 600 45- by 5-centimeter chromatograms per working day (*10*).

If careful attention is paid to certain details of technique, the overall method is not especially difficult to master (*18*). It has recently been adopted by a newly established Central Analytical Laboratory at the Worcester Foundation for Experimental Biology and has been used for just under a year as a routine procedure by a team consisting of one postdoctoral chemist and approximately four technicians. Only two members of the team, both of them technicians, had had any previous experience in extraction and chromatographic methods for measuring steroids. This team now makes 30 or more analyses per week, in which ten individual urinary steroids are measured on three separate chromatograms, together with certain group determinations (for example, total estrogen, total 17-keto steroids). In the first 10 months of routine operation they made over 1000 analyses of this complexity.

The various manual and auxiliary techniques that will be required until a completely automated system has been developed must be made as rapid and as efficient as possible. It pays to examine one's methods very carefully stage by stage. Opinions on what is a

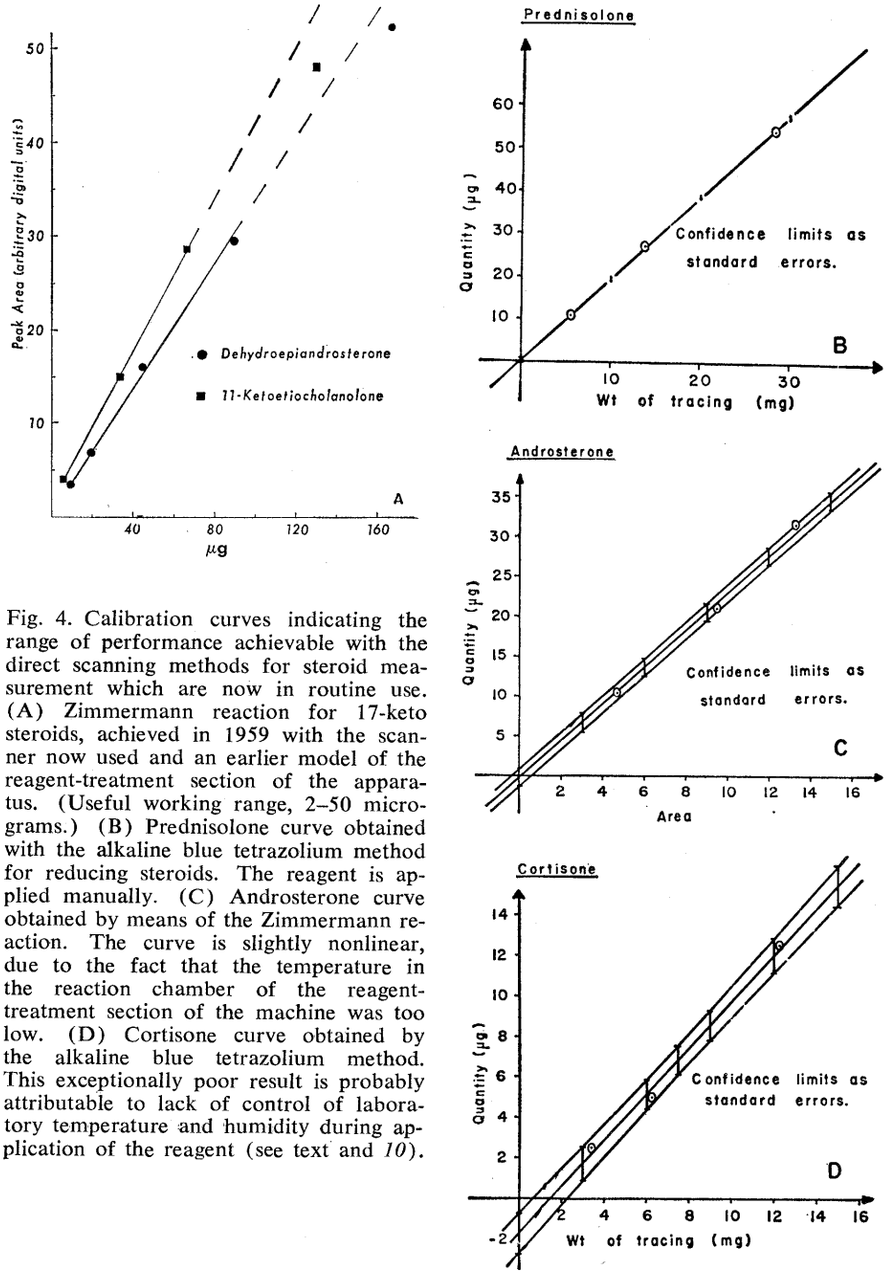

Fig. 4. Calibration curves indicating the range of performance achievable with the direct scanning methods for steroid measurement which are now in routine use. (A) Zimmermann reaction for 17-keto steroids, achieved in 1959 with the scanner now used and an earlier model of the reagent-treatment section of the apparatus. (Useful working range, 2–50 micrograms.) (B) Prednisolone curve obtained with the alkaline blue tetrazolium method for reducing steroids. The reagent is applied manually. (C) Androsterone curve obtained by means of the Zimmermann reaction. The curve is slightly nonlinear, due to the fact that the temperature in the reaction chamber of the reagent-treatment section of the machine was too low. (D) Cortisone curve obtained by the alkaline blue tetrazolium method. This exceptionally poor result is probably attributable to lack of control of laboratory temperature and humidity during application of the reagent (see text and *10*).

"fast" or a "slow" method of carrying out a step in the process should not be accepted until a careful analysis has been made of the temporal organization of the step and of its place in the method as a whole. Parallel and serial operations should be carefully analyzed, for the sake of improving their organization and detecting possible deleterious effects on the quality of results.

In short, some knowledge of the methods of operational research is needed, and a procedure must often be explored in considerable detail if serious flaws are not to be overlooked. Simple time-and-motion studies can often lead to a doubling or trebling of productivity through the adoption of simple changes in procedure or in the layout of laboratory equipment.

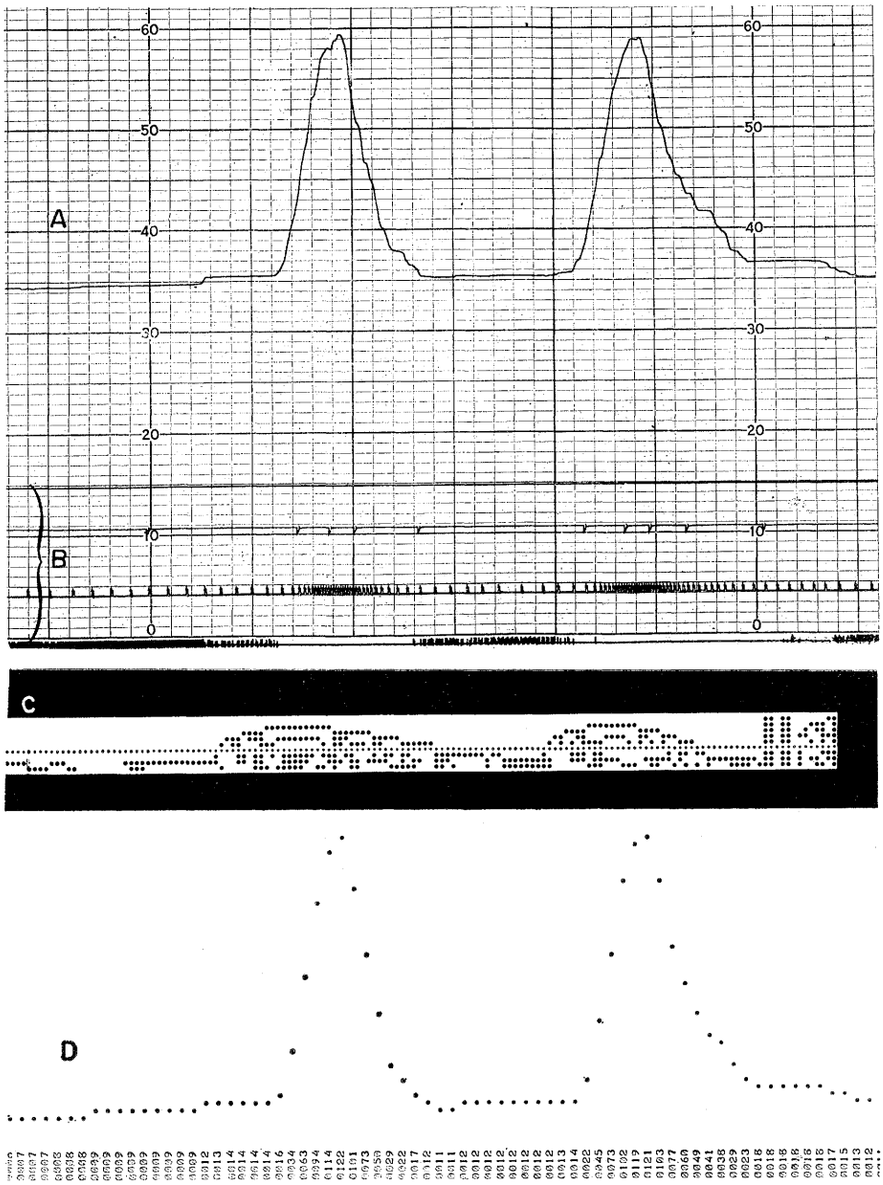

Fig. 5. Four stages of data processing in quantitative chromatography. (A) Direct output of a paper strip scanner on the potentiometric chart recorder. The same type of record is obtained with other types of quantitative chromatography, such as gas-liquid chromatography or liquid-ion exchange chromatography. (B) A four-digit decimal integral record; areas of peaks can be counted directly. (C) A binary-coded punched paper tape produced by an analog-to-digital converter connected to a retransmitting slide-wire fitted to the recorder that produced records A and B. (Two peaks are "seen" but appear distorted because the most significant bit is at the top edge of the tape.) (D) Teletypewriter output of a program which processes the binary tape shown in C. The program smoothes the scanning record, punches a four-decimal figure for the optical density reading, and produces a crude dot profile of the original scanning record. Tape C is produced simultaneously with the scanning of the strip (45 seconds). Production of D takes approximately 3 minutes.

Characteristics and Limitations of the Method

In its present incompletely automated form, this method has a number of limitations but seems to be as precise as any existing method of comparable complexity, while being far more productive.

Figure 4 gives an idea of its analytical accuracy. Calibration curves representing the upper 10 percent of quality usually achieved in 1959 (Fig. 4A) and in 1966 (Fig. 4B) are compared in Figs. 4C and 4D, respectively, with curves representing moderate quality (approximately the middle 80 percent) and poor quality (the lowest 10 percent) as achieved in ordinary routine use. The linear regression was calculated by means of a FORTRAN program on the PDP-8 computer, and the standard errors were corrected for deviation of the independent variable from the sample mean (*19*). The envelopes for standard error could not be shown on the better curves and are indicated by the vertical bars. Replication experiments have shown that the greater part of the error resulted from the manual methods used at the time for obtaining peak areas.

Anyone working with chromatogram records of this sort knows that this last step is a tedious and rate-limiting feature of *all* existing methods of quantitative chromatography. The 4-decade integral record provided by the apparatus shown in Fig. 2 speeds up the work considerably but not sufficiently to meet the "numbers problem." Various integrators are commercially available [for example, the Texas Instrument Company's recorders; the Beckman Model 125 System; the Perkin-Elmer Model 194 GLC integrator; the Infotronics Corporation (Houston) integrator] and have been used with gas-liquid chromatography and liquid-ion-exchange apparatus. There are also a number of systems available for providing a digital output from such instruments via an A-to-D converter. This, as paper or magnetic tape, can then be processed by a computer.

The problems involved in such data-processing methods have been well discussed in outline by Jones and Spence (*20*). With slowly processed chromatograms (0.5 hour to 12 hours per chromatogram, as in gas-liquid or liquid elution chromatography), an on-line specialized computer (cost, around $6500) is capable of smoothing noise from the record and correcting for base-line drift

and "plateaus." Such systems are not sufficiently sophisticated for scanners working at the speeds we use (one 40-centimeter strip in 40 seconds), and an off-line system in which paper or magnetic tape is used seems likely to give the best results. Partial or complete systems of this type have been described (*21*), but more experience is needed for their evaluation. Few serious difficulties of principle exist in devising data-processing methods capable of processing the scanning records.

Four levels of sophistication are shown in Fig. 5. Record A is part of a paper chromatogram record; accurate calculation of the peak areas by geometrical and graphical methods requires approximately 1.5 minutes per peak, with an average number of overlapping peaks to be resolved (*7*) per batch of chromatograms. For record B, the 4-digit decimal integral record, the time is reduced to approximately 0.8 minute per peak. Record C is a binary-coded digital record of a similar chromatogram, punched out by means of a D.E.C. 138E A-to-D converter using a very simple program (55 12-bit instructions). Such a tape is produced simultaneously with the chart record, and a large batch can be processed off-line at a rate of 1 to 15 seconds per peak according to the sophistication of the program and the computer used. Such programs are designed to take in raw data from chromatograms as binary coded paper or magnetic tape, and to print out the results in the desired format. According to the scale of such an operation, a teletypewriter, card-printer-punch, or line-printer can be used for recording the output (see, for example, Fig. 5D).

While the bottleneck that occurs after the scanning operation is being eliminated, the bottleneck that precedes it is as bad as ever. Thus, all methods for measuring steroids require more or less laborious procedures whereby the steroids are extracted from blood or urine and the extracts are then partially purified and concentrated to small volume by evaporation under reduced pressure. This is a bottleneck common to *all* methods, whatever form of chromatography or detection is used. We are embarking on the automation of such procedures. It is a complex task, but no strikingly new theoretical problems stand in the way of its accomplishment.

A serious limitation of the existing scanner is its inadequate sensitivity for measuring steroids at normal concentrations in peripheral blood plasma. For many problems in clinical endocrinology, it is necessary to have methods giving reasonable precision with extremely small quantities of steroid (10^{-7} to 10^{-5} micromole) per blood sample. The working range of the present scanner, with color reactions having effective molar extinction coefficients of the order of 2×10^4, is from 5×10^{-3} micromole upward. With some classes of steroid favorable for fluorescence reactions the working range is from 5×10^{-5} micromole upward. The limitation here, however, is not in photomultiplier sensitivity but in signal-to-noise ratio. A new model of the strip scanner, incorporating cross-scanning and correction for background (*10*), is now nearly completed; this, it is hoped, will be sufficiently sensitive to bring the excessively low concentrations of many steroids in plasma into the working range of the instrument.

For many purposes, even the present instrument has more than adequate sensitivity. By and large, the working range for an absorptiometric method is comparable to that achieved only with 0.5-milliliter, 1-centimeter-path cuvettes, with standard spectrophotometers (*10*). Thus, our routine methods for measuring the major urinary steroids require only 1/100 of a 24-hour sample for the main metabolites of cortisol and the 11-deoxy-17-keto steroids and approximately 1/25 of a 24-hour sample for 11-oxy-17-keto steroids and pregnanetriol. The measurement of amino acids with the Barollier-ninhydrin reagent is reliable over the range 3×10^{-3} to 1×10^{-1} micromole, with different sensitivity settings, and measurement of the fluorescent DANSYL derivatives of amino acids (*22*), reliable to 0.5×10^{-3} micromole or less.

Color and fluorescence reactions have the important advantage that a considerable fraction of the specificity of the overall method resides in the final step of measurement itself. Labeling methods have a good deal less specificity, since the label is usually introduced by a group-specific reaction —for example, esterification, or condensation with carbonyl groups. Gas-liquid chromatography methods in which labeling is not used are the least specific of all in their method of generating a "signal." There seems to be at least as good a chance of improveing the instrumental sensitivity of thin-media scanners up to the standard required for measuring steroids in blood as there is of improving the specificity of labeling methods, or of gas-liquid chromatography detectors. On the other hand, the methods now being worked out for the automation of steroid extraction from blood and urine could equally well be used to speed up very greatly the numerous steps required in the more successful labeling methods. Time will tell which of these approaches is the more successful for the measurement of steroids in blood.

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23. The number of scanners is enormous, and full reference to this literature was not possible in the space available. Strictly speaking, most of the work described here is concerned with the "mechanization" of procedures as an essential preliminary to automation, rather than with automation itself. I am extremely grateful to Mr. Clarence Barber, Miss Rowena Owen, and Mr. John Grist for help in preparing the figures.