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Diffusion Assay by an Automated Procedure

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Equipment is described that allows diffusion assays to be performed automatically in Petri dishes using the punch-hole technique. With a block of six dishes limits of error of approximately ±2% can be obtained consistently. Various sources of systematic errors and their elimination are discussed.

Keywords: *Automation*; *antibiotic assay*; *diffusion assay*; *systematic errors*

A systematic programme of automation of antibiotic diffusion assay was started some years ago by the Antibiotics Division, National Institute for Biological Standards and Control (NIBSC) (then Division of Biological Standards, National Institute for Medical Research). The assay consists basically of three stages: pouring of plates; application of assay solutions; and measurement of zones of inhibition.

It was considered that the third stage had the greatest content of subjective involvement of the operator and was the stage that could most easily allow the introduction of an operator bias in the measurement of potency. Most laboratories at that time measured the diameter of zones of inhibition in millimetres, using some form of magnification, either by projection of an enlarged image on to a screen or indirect magnification of the zone and scale by a lens system.

The use of a television camera in conjunction with a graticule was developed by Tatum and Lightbown[1] and has been used successfully for more than 10 years to quantitate the areas of zones of inhibition on Petri dishes carrying six zones. The six areas were measured consecutively and recorded in arbitrary units. This method removed the need for the operator to make a subjective decision regarding the position of the edge of each zone and allowed zone area measurements to be made that were independent of the operator. It was found, however, that the performance of the image analyser could deteriorate under certain conditions in such a way that a bias was introduced by the machine, which could, for example, result in the first of the six zones measured on a dish being found to be too large or small. The fault was eliminated electronically, but as it could apparently develop again, progressively, any possible effects of this bias were eliminated by reading the replicate dishes in groups of six, as a Latin square, so that any effects of the bias were systematically and evenly distributed to all responses in the assay.

Early in 1969 the Beecham laboratory started measuring inhibition zones by means of a commercial image analyser with a graticule adapted for Petri dishes. The automation of the second stage of the assay, *i.e.*, the application of the solutions to the dishes, was developed subsequently and jointly by the two laboratories and in the course of the work Quantimet Petriscopes, Models 60 and 720, were used for zone measurements (the Optomax, recently available from Micromeasurements Ltd., has also been used).

A decision to base the assay on the use of plastic Petri dishes rather than large square plates, which were currently used by NIBSC, had been taken during the development of the image analyser device for measuring the zones. Various advantages accrued from the use of such dishes. (*i*) They are disposable, thus avoiding problems arising from contamination of assay plates by antibiotics, such as occurs with neomycin and glass surfaces. Experience has shown, in one laboratory, that sufficient neomycin can be adsorbed on to the glass surface of an assay plate so that, after washing three times and dry heat sterilisation, zones of inhibition were still produced by the residual antibiotic on the glass. Steaming in

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dilute acid was necessary to clean the plates. Penicillinase was also adsorbed on to glass plates and resisted washing and dry heat sterilisation, thus interfering with subsequent assays of penicillin. (*ii*) The dishes are readily obtainable with a uniform plane surface. (*iii*) Bias from edge or corner effects is minimised as each zone is equidistant from the edge. (*iv*) Automation is more readily applied to a circular array than to a Latin square. (*v*) Automation of pouring is more easily arranged for Petri dishes.

Consideration was given to the application of the assay solution. A number of possible procedures were tried, *viz.*, application of fish-spine beads and subsequent filling with solutions, delivery of precise volumes of assay solutions to the surface of the agar (fixed volume and several concentrations, or fixed concentration and several volumes), injection of assay solutions into the agar layer, assay solutions solidified with agar in tubing, then extruded and cut into plugs, and punching holes in the agar layer with subsequent delivery of assay solutions into the holes. As a result of these preliminary experiments, and in consideration of the wide variation in the nature of possible assay solutions, particularly variations caused by differences in surface tension, it was decided to concentrate on the development of a system based on holes punched in the agar. The size of the zone of inhibition that develops from the hole containing antibiotic solution is affected by a number of factors, which may vary from hole to hole: size of hole, diameter and surface area of agar to solution interface; seal of agar to surface of dish; volume of assay solution added; and period of diffusion before the zone of inhibition is defined (critical time).

Various procedures for punching six holes per dish were examined: using a single punch and rotating the dish in six steps; punching six holes simultaneously; drilling the holes with a twist drill; and removing the agar plug whole by suction, by blowing and by fragmentation and suction. Probably the most difficult problem was the removal of the plug without disturbing the seal between the agar and the dish. If this seal was disturbed the assay solution spread in the interface between agar and dish, producing distorted or enlarged zones of inhibition.

Various means of delivering the assay solutions into the holes were considered and examined using single or multiple devices; a minimum precision of $\pm 0.2\%$ was considered desirable for repeated deliveries of the chosen volume. Delivering the six volumes simultaneously had the advantage that variation in diffusion time between the assay solutions was eliminated and this design was therefore adopted.

Using Petri dishes with a nominal diameter of 90 mm, an apparatus was constructed that would cut six holes (6 mm in diameter) in the contained agar, remove the plugs and introduce equal volumes of six assay solutions, simultaneously, into the six holes.

Description and Use of Apparatus

The apparatus[2] shown in Fig. 1 consists of two units, the punch head (right) and the dispensing head (left). Both units move vertically, enabling the punches and pipette tips, both of which are plastic and disposable, to perform the various operations. The assay dish together with the reservoirs for the test and standard solutions are transported horizontally underneath the heads on a carriage. A container for the spent agar plugs is placed under the cutting head.

The apparatus normally performs automatically the sequence of operations, but can be controlled manually if required. Operation is electro-pneumatic, and vertical movements of the punch and dispenser heads are independently adjustable. The dispensed volume can readily be adjusted by means of a calibrated micrometer. Likewise, the action of the punches is adjusted to suit a wide range of gel types and thicknesses.

The sequence of operation begins with the operator placing a dish prepared for assay on to the right-hand position of the carriage. The carriage moves to the right until the dish is under the punch head and the reservoirs of test and standard are under the dispensing head. The heads move vertically down, the dispenser pipette tips are immersed in the solutions in the reservoirs and the punches penetrate the agar to the required depth. The dispenser pistons then draw a pre-determined amount of solution into each of the pipette tips, while the punching assembly rotates slightly to free the agar plugs.

Both heads are retracted, the punches carrying with them the plugs removed from the agar. The carriage returns to the left until the holes in the assay agar are under the

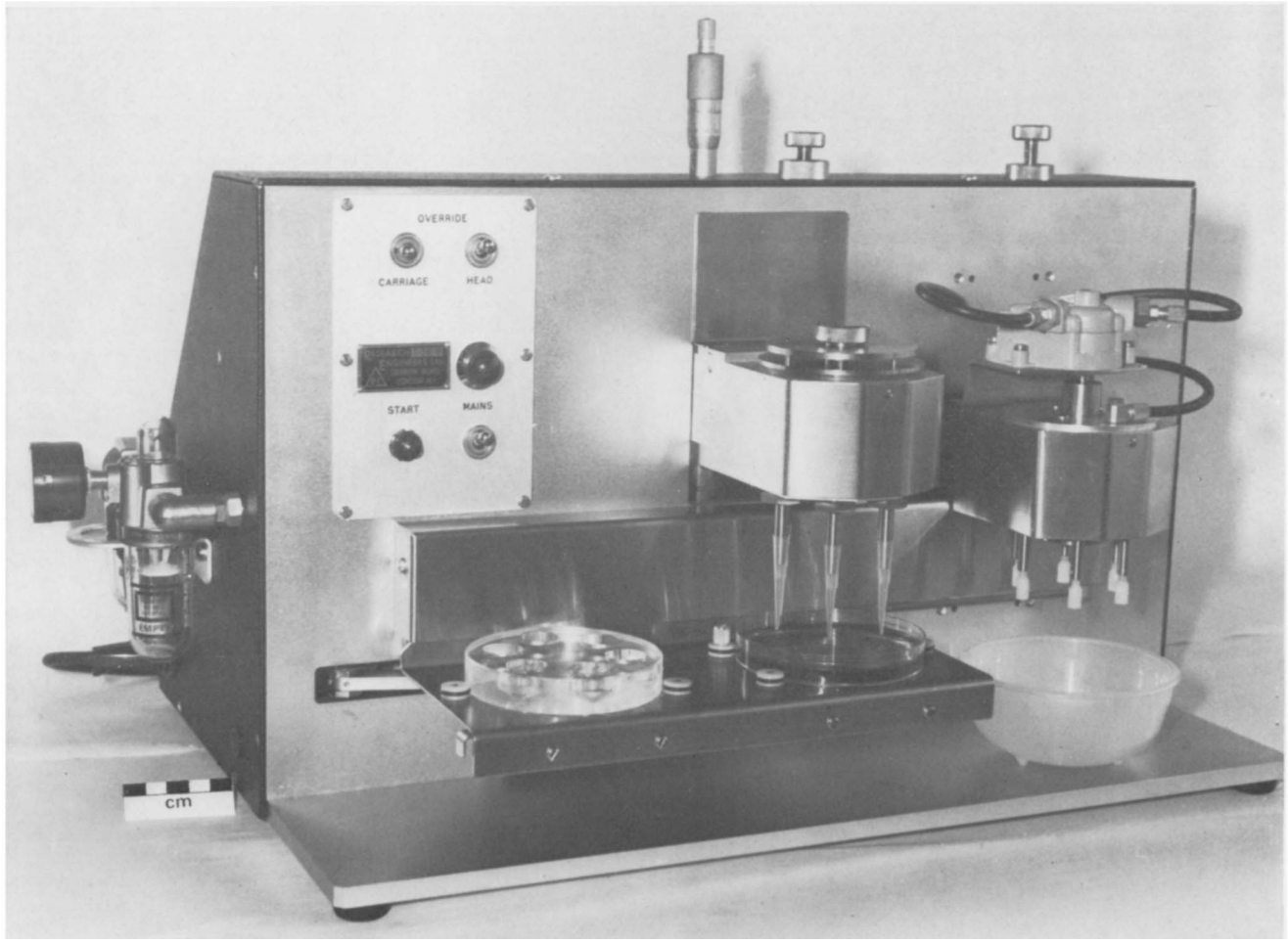

Fig. 1. Automatic bioassay machine.

[to face page 203

dispenser pipettes. The dispenser head descends, the pipettes discharging the solution into the holes in the agar, while the plugs remaining in the punches are ejected into the container below the punch head. The dispenser head retracts and the punched and filled assay dish is removed by the operator. The complete cycle takes approximately 15 s.

Dispensed volumes of 50 and of 70 μl are currently being used. The apparatus can be adjusted to deliver a maximum of approximately 100 μl. The dispensing unit is constructed in such a way that the tips are not completely emptied at each operation. Experience has shown that it may not be necessary to replace pipette tips or to sterilise the punches between assays of different antibiotics. In certain conditions, however, it has been found necessary to treat the punch tips with alcohol, *e.g.*, when changing from *Pseudomonas aeruginosa* to *Bacillus subtilis* as assay organism. The plastic pipette and punch tips are readily replaceable, if this is necessary.

Evaluation of the Apparatus

Variations in volumes delivered into the six holes were measured initially by collecting repeated simultaneous deliveries of an aqueous solution of albumen labelled with iodine-131 and measuring the activity in each sample. With the prototype machine (three micrometers) and using pipette tips of various sources of manufacture, coefficients of variation for a single delivery point ranged from 1.0 to 4.0%. Measurement of the movement of the six pistons showed that these were all well within the 0.1–0.2% target. Measurement of the changes in pressure within a plastic tip during the cycle of filling and emptying showed that the changes were complex and that surface tension was a major factor affecting the volume delivered. Variations were noted in the size of orifice of the pipette tip and in the effectiveness of the seal at the cone joint of the tip. It was important to adjust the movement of the pipette head so that the tips at filling and delivery were as close as possible to the base of the dish. When the base of the dish was not plane and parallel to the six tips, then variable interference with the flow of liquids occurred either at the filling or emptying stages. The adjustments had to ensure that the tips were immersed as soon as possible during delivery and, in addition, the operator had to be careful to observe that liquid remaining on the outside of the pipette tips between emptying and re-filling did not vary greatly. By careful choice of tips, control of cleanliness and care in fitting, it was readily possible to obtain coefficients of variation of approximately 1% for the volumes delivered from the six tips, as is shown in Table I. This variation was greater than hoped, but could not be improved and it was considered that by means of replication, effects of the error could be reduced.

Variation between the mean volumes delivered by the six pipettes was found to be less than 0.5% by mass. The prototype machine was designed with three micrometer adjustments in order to allow any measured bias affecting the six zones (in a circular pattern), arising from any source, to be removed by the application of an opposite bias to the volumes delivered at the six points. Experience proved that this was not practicable because bias arising independently of the machine procedures was not necessarily constant from day to day. For this reason the final engineering design ensured a uniform displacement of the six pistons and a single micrometer head allowed all six to be varied equally and simultaneously. The results given in Table I were obtained with this design of equipment and it is seen that the variation between the mean volumes delivered by each pipette was approximately 0.2%.

Variations in the size of punched holes were examined by using dishes containing nutrient

TABLE I

VARIATIONS IN DELIVERY OF PIPETTES USING WATER

	Positions					
	1	2	3	4	5	6
Mean mass of 20 volumes delivered from pipette/mg	51.59	51.55	51.71	51.52	51.60	51.61
Coefficient of variation	1.26	0.029	1.32	1.02	1.37	1.46

Grand mean mass/mg 51.60
Range of means 1–6 .. .. .. 99.90–100.2% of grand mean.
Range of individual deliveries for all positions .. 50.1–53.2 mg.

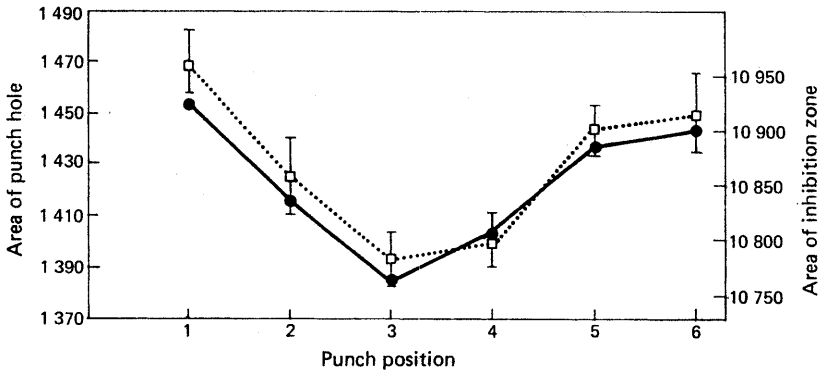

Fig. 2. Systematic bias originating from punches. □ . . . □, Area of punch holes in arbitrary units; mean of 12 plates ± 2 standard deviations of the mean for areas of punch holes. ●——●, Area of inhibition zones in arbitrary units; mean of 18 plates.

agar, flooding the plates with water and measuring the cross-sectional areas of the six holes by means of the plate reading device. The results shown in Fig. 2 were obtained using the prototype assay machine. On the same day, and with the same conditions, 18 plates were prepared and filled with solutions for a tetracycline assay (3 + 3 design). The solutions were applied to three blocks, each of six dishes, in a Latin square within each block, so that within a block each solution was delivered once into a hole produced by each cutter, as shown in Table II.

TABLE II

TEST FORMAT

	Identification of punch hole*					
Petri dish	1	2	3	4	5	6
1	SH	TM	SL	TH	SM	TL
2	TM	SL	TH	SM	TL	SH
3	SL	TH	SM	TL	SH	TM
4	TH	SM	TL	SH	TM	SL
5	SM	TL	SH	TM	SL	TH
6	TL	SH	TM	SL	TH	SM

* Solution identity: SH = standard high; SM = standard medium; SL = standard low; TH = test high; TM = test medium; TL = test low.

After normal incubation the zones were measured. The difference in response between columns represents the effects of differences in the six punch positions. Fig. 2 shows that the variation in size of the zones produced around holes from different punches follows closely the variation in the size of those holes. If holes from one punch are always used for the same assay solution, then with the pattern of distribution of assay solutions used in the two laboratories (1 SH, 2 TM, 3 SL, 4 TH, 5 SM, 6 TL) the two dose response lines for standard and test would be expected to be biased as shown in Fig. 3, with the possible introduction of non-parallelism and curvature.

Assays carried out on a proposed standard preparation, using the machine, were each composed of 36 dishes. The assay design was 3 + 3 in six blocks of six dishes per assay. The dishes within a block had a fixed relationship between the six pipette and punch stations and the six assay solutions, one block of six dishes for each of the six relationships shown in Table II for the six Petri dishes. Analysis of separate blocks of six dishes showed invalidities of curvature and non-parallelism within a number of blocks, but when the 36 dishes were taken together, as was intended, the assays were usually statistically valid. Results from six such assays are reported in Table III. Valid assays of a high degree of precision could be obtained with this assay system, but the procedure was very cumbersome in performance and statistical analysis.

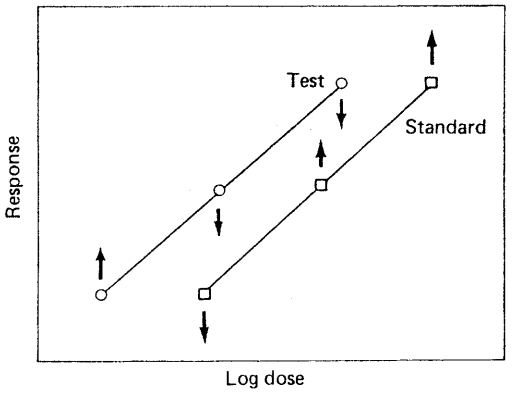

Fig. 3. Effect of systematic bias on assay under conditions shown in Fig. 2. Arrows indicate direction of bias.

One of the two laboratories developed the use of the automated apparatus in the form of two independent units, one to punch holes and the second to dispense the assay solutions. This procedure had the advantage that it allowed dishes to be punched at a more rapid rate than could be accommodated by the dispensing unit. In this way one punch unit could supply dishes for several dispensing units. After punching the holes, the dishes were mixed so that, at subsequent dispensing, a particular punch position was not tied to a particular assay solution. The randomisation thus introduced at this stage had the advantage of reducing or removing the bias which resulted from the punches. Assays performed in this way were usually free from invalidities of curvature or non-parallelism, but there was an expected reduction in precision (see Table IV). The precision obtained with 10 dishes per assay was, however, adequate for the purpose required. A higher precision could be obtained by increasing the number of dishes, but the use of the linked units, together with the systematic rotation of the solutions, is more economical if the higher precision is required.

Later assays with linked units have used a total of six Petri dishes per assay instead of 36, with one dish for each of the six positions (punch hole relative to solutions). With this arrangement valid assays with confidence limits of ± 2 to 3% are regularly obtained (Table V) with a number of different antibiotics. In this way, the precision obtained with a unit of six Petri dishes (36 zones) is better than was previously obtained using a large plate with a 6×6 or 8×8 Latin square arrangement and applying the solutions by means of fish-spine beads.

The greater part of the effort in the assay goes into the preparation of the solutions and

TABLE III

ASSAY OF CHLORTETRACYCLINE PROPOSED STANDARD USING PUNCH AND PIPETTE UNITS LINKED

$3 + 3$ design. Six blocks of six dishes per assay. Each solution was delivered in turn by each station to six dishes within one assay.

Day	Assay	Potency ratio	Fiducial limits ($P = 0.95$)
1	1	1.024	1.012 to 1.035
	2	1.023	1.010 to 1.036
2	3	1.023	1.006 to 1.041
	4	1.022	1.005 to 1.040
3	5	1.039*	1.025 to 1.053
	6	1.016*	1.000 to 1.033

* Significant non-parallelism.

TABLE IV

AUTOMATED BIOASSAY UNIT IN USE FOR ROUTINE ASSAY USING PUNCH AND PIPETTE UNITS SEPARATE

Ten dishes employed per assay.

Antibiotic	Assay	Penicillin content/ μg mg^{-1}	Departure from parallelism, F	Quadratic curvature, F	Difference of curvature, F	Limits ($P = 0.95$), %
Ampicillin trihydrate ..	A (1)	843	0.02	1.08	1.76	1
	A (2)	837	0.94	0.26	1.86	5
	A (3)	838	0.63	0.81	0.38	5–6
	B	858	0.09	1.83	0.03	3–4
Flucloxacillin— syrup preparations ..	A	36.3	0.02	0.41	1.29	3–4
	B	35.1	0.02	0.88	0.03	5
	C	35.9	0.05	1.82	0.02	4–5

dishes and there is a temptation to use a large number of replicate dishes in the assay by means of automation, in order to obtain an even higher degree of precision. This temptation should be resisted; if greater precision is necessary it should be obtained by repetition of completely independent assays. Under our own conditions there is little to be gained by using more than six dishes in a single assay with the linked apparatus.

Statistical analysis commonly used to determine confidence limits from the internal evidence of the assay is in any event of doubtful significance in such automated procedures, where the random error has been reduced to a very low level by a systematic distribution of known errors to all doses. The residual error can be reduced to an extent such that the usual analysis of variance may invite false conclusions.

TABLE V

ASSAYS WITH PUNCH AND PIPETTE UNITS LINKED

Antibiotic	Potency/ U mg^{-1}	Departure from linearity	Departure from parallelism	Fiducial limits ($P = 0.95$)	Limits, %
Neoymcin ..	95.8*	$P > 0.05$	$P > 0.05$	93.5 to 98.2	2.5
Gentamicin ..	649	$0.05 > P > 0.01$	$P > 0.05$	626 to 672	3.6
Tetracycline ..	970	$P > 0.05$	$P > 0.05$	942 to 999	2.9
Oxytetracycline ..	102.6*	$P > 0.05$	$P > 0.05$	100.5 to 104.9	2.2
Tobramycin ..	104.9*	$P > 0.05$	$P > 0.05$	102.7 to 107.2	2.2
Streptomycin ..	771	$P > 0.05$	$P > 0.05$	746 to 797	3.3
Erythromycin ..	933	$P > 0.05$	$P > 0.05$	914 to 952	2.1
Amikacin ..	926	$P > 0.05$	$P > 0.05$	906 to 947	2.3
Rolitetracycline ..	780	$0.05 > P > 0.01$	$P > 0.05$	762 to 798	2.3
Spectinomycin ..	98.2*	$P > 0.05$	$0.05 > P > 0.01$	97.4 to 98.9	0.7
Doxycycline ..	874	$P > 0.05$	$P > 0.05$	855 to 893	2.2
Nystatin	5075	$0.05 > P > 0.01$	$P > 0.05$	5045 to 5263	2.1

* Potency expressed as percentage of claim on label.

Discussion

The apparatus described has been in constant use for 6 years in the two laboratories, one concerned mainly with assays where a high precision was desirable, *e.g.*, in calibration of new standards, and the other which had the additional need for a high throughput of less precise assays. The performance of the machines has gradually improved over this period as general operating experience has been gained, both with the prototype and commercial machines. They have been operating over the past 3 or 4 years constantly and routinely with no significant operational failures, producing results in line with those described in the tables. This has involved the use of a wide range of different assay media and solvents as can be seen from Table V.

Perhaps the most important lesson to be learnt was the practical impossibility of reducing

the systematic errors to an insignificant level. It is perhaps surprising that a systematic variation in the size of the punch holes within a single dish of only approximately 0.05 mm can produce a zone error of approximately 2%; when converted into potency error this would become approximately 3%. The systematic variation in size of holes shown in Fig. 2 is difficult to explain. It is of interest that a similar circular variation was found when the holes were cut automatically and successively with a single punch. It is possible that a rapid change in the gel properties commences following the first rupture of the agar surface and is progressive during the time period necessary to complete the punching of the six holes.

Although the volumetric displacement of the six dispenser pistons was probably within 0.1% of target (represented by an error of the piston movement of 0.01 mm or an error in diameter of the pistons of 0.001 mm) the six volumes delivered varied by up to 4%. However, the dispensed volume from a single pipette was consistent to within approximately 1%.

Hence, the combined errors arising from the punching and the dispensing can be considered in two parts; systematic errors related to each station are 2 and 1%, respectively, and the random errors for the punches and pipettes approximately 1% in each instance. If no attempt is made to eliminate the deterministic errors, then an inaccuracy of up to 4% may be introduced and not revealed by statistical analysis, which may, however, demonstrate apparent invalidities arising from these deterministic errors.

If the deterministic errors are systematically eliminated as described, only the random errors remain and a precision of approximately 2% may be achieved.

Alternatively, when pre-punched plates positioned randomly under the dispenser are used, larger random errors are generated although the dispenser deterministic errors are also retained; in this instance traditional statistical analysis tends to produce valid estimates. There is a narrow dividing line between these two situations arising from the use of the machine in the two modes (punch and pipette linked or separate) and any particular assay may fall into either category.

Measurement of true machine errors is extremely difficult and therefore seldom recognised, let alone quantitatively assessed. It is, however, likely that there are many similar situations in automated biological assays where repeated multiple volume measurements and repetitive quantitative physical observations are made. These effects can only be observed in experiments specifically designed to show them. Once the deterministic and random errors within a particular assay have been quantified, it is possible by careful design of the assay systematically to remove the former. However, in weighing, solution preparation, etc., this will not usually be possible. Little is gained by using more than the minimum number of replicates; for example, in the assay described where there are six positions it is possible to remove all deterministic errors by allowing each punch position to be filled in turn from a different dispenser or with a different solution. Thus, only six dishes are required to eliminate all deterministic machine errors and assays that previously required four large 12 × 12 in plates can be replaced with six Petri dishes.

The pipette tips on the apparatus are those commonly used manually in clinical and analytical laboratories for repetitive delivery of small volumes of reagents. It is likely that the variability obtained with manual operation will be much greater than that shown in Table I.

Thanks are due to the staff of the Worthing Research Division laboratories during the long development and proving period of the equipment. Research Engineers Ltd., Orsman Road, London, N1 5KD, have been associated with the further development of the prototype machine to the commercial stage.

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