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ASSAY OF HISTAMINE ON THE ISOLATED GUINEA-PIG INTESTINE BY THE METHOD OF SUPERFUSION

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Histamine assays are commonly performed on an isolated strip of guinea-pig intestine suspended in 2 to 5 ml. Tyrode solution. This method is satisfactory if the concentration of histamine in the test solution is about 100 ng./ml. The assay may then be conducted with doses usually not exceeding one tenth of the bath volume. In this way, effects of small differences in composition between the test and Tyrode solution are likely to disappear on dilution. However, when the concentration to be estimated is 1 to 5 ng./ml., the test solution itself must be allowed to act directly on the intestine to obtain a measurable response. This can be done by surrounding the strip of ileum with the test solution in a small bath (Mongar and Schild, 1950) or by superfusion (Gaddum, 1953a). The solution containing the dose should then be similar in composition to that bathing the ileum in the interval between the doses.

Our interest in superfusion arose from the need to estimate minute quantities of free histamine in the plasma. The purification of the histamine will be described elsewhere. The main object of the present work was to devise a method of performing histamine assays on the superfused ileum with the aid of a semi-automatic apparatus and to determine the precision of this method. We have also studied methods by which incomplete solutions, that is, solutions lacking some or all of the Tyrode salts, may be converted into solutions suitable for assay.

METHODS

The apparatus (Fig. 1) is semi-automatic in the sense that the dose has to be placed by hand into a tube from which it is later automatically released. Its various parts are fixed to a strip of " Dexion " which also holds the lever, a trembler and a light signal for the dose. The relays (Schild, 1946; Gaddum and Lembeck, 1949) are connected to points on a uniselector (Schild, 1946) and are energized at times determined by the discharge of current from a condenser (Boura, Mongar, and Schild, 1954). In the resting position R1 stops the flow from bottle B but not from bottle A; on being energized it does the reverse. R3 controls the flow from bottles B and C in a similar way, but can act only when current is diverted from R1 by a two-way switch. R2 releases the dose from tube F and can be made to act simultaneously with R1 or R3, or be excluded from the circuit. The electrical part of the apparatus is the subject of an addendum.

Temperature.—This is maintained in the manner described by Boura *et al.* (1954). Tube G is 17 cm. long and 1.2 cm. wide (O.D.): the inner tube is 3 mm. wide (I.D.) and is flared at each end. The lower end carries a solid glass arm (2 mm. diam.) which curves forward and ends in a hook. This design avoids retention of fluid by capillarity, and the hook, which encloses the thread, ensures a drop of constant size. The temperature of the Tyrode solution was measured at the lower end of the gut, where the drops re-formed. The air temperature at this point was always 1° C. higher. The assays were performed at temperatures in the range 33° to 36° C.

Application of the Dose.—The dose is placed in tube F, which consists of a 1 ml. all-glass tuberculin syringe barrel (" Accoson "). The nozzle has been reduced to 6 mm. and the inner surface ground to provide an efficient seating for the valve (J). This is made of polythene tubing (2 mm. O.D.) loaded with a thin steel wire to give it rigidity. The tip of the polythene is heated gently, drawn out and cut to a length of about 5 mm. The tip passes into the narrow stem and so keeps the valve centred. The upper end of the polythene rod passes through a metal guide clipped to the tube. At the top is a flat metal disc of sufficient weight to ensure closure of the valve.

The tube is calibrated in the range 0.5 to 0.8 ml.: the volume of the dose is usually 0.6 ml., but within these limits it is not critical. The time taken for the tube to empty depends on the distance between R2 and the weighted top of the valve. This distance is set at 3 to

4 mm. by turning screw E. The tube empties by gravity. It is important that none of the dose remains in the tube. Drainage is complete if the nozzle touches the inside of the cup of tube G.

Rate of Flow of Solutions.—The dose (0.6 ml.) flows out of tube F at an average rate of 4.5 ml./min. Since the initial flow is faster than the flow of Tyrode solution, the temperature falls by about 0.5° C. Tyrode solution (bottle A) flows at a constant rate of 4.5 ml./min. This gives a drop rate of 58/min. with the thread *in situ*; the average drop size is therefore 0.078 ml. Tyrode solution containing mepyramine maleate (bottle C; 4 to 8 ng./ml.), and histamine (bottle B; 3 ng./ml.), flows at the same rate.

Dose Cycle.—A suitable period is 90 sec. during which the dose acts for 8 sec.

Lever.—Magnification, 9.2; tension, about 500 mg. (uncorrected for the weight of the gut; this is usually 150 mg. ±10 mg.).

Solutions.—The composition of the Tyrode solution is, in g./l., NaCl 8.0, NaH_2PO_4 0.05, $NaHCO_3$ 0.36, KCl 0.2, $MgCl_2$ 0.1, $CaCl_2$ 0.14, glucose 1.0; atropine sulphate is added to give a concentration of 0.1 μg./ml. The *p*H of this solution is 7.7.

The Tyrode stock solutions are also used in very small volumes to adjust the composition of incomplete test solutions to that of Tyrode solution. They have the following concentrations, in g./l. of the anhydrous salts: NaCl 80.0, NaH_2PO_4 2.0, $NaHCO_3$ 36.0, KCl 8.0, $MgCl_2$ 4.0, $CaCl_2$ 5.5.

Standard histamine solutions are prepared from a stock solution of 10 ng./ml. in Tyrode. All the histamine values are expressed in terms of the base on the assumption that this represents 36.16% of the acid phosphate (British Drug Houses, Ltd.). The concentration of mepyramine maleate (May and Baker, Ltd.) refers to the salt.

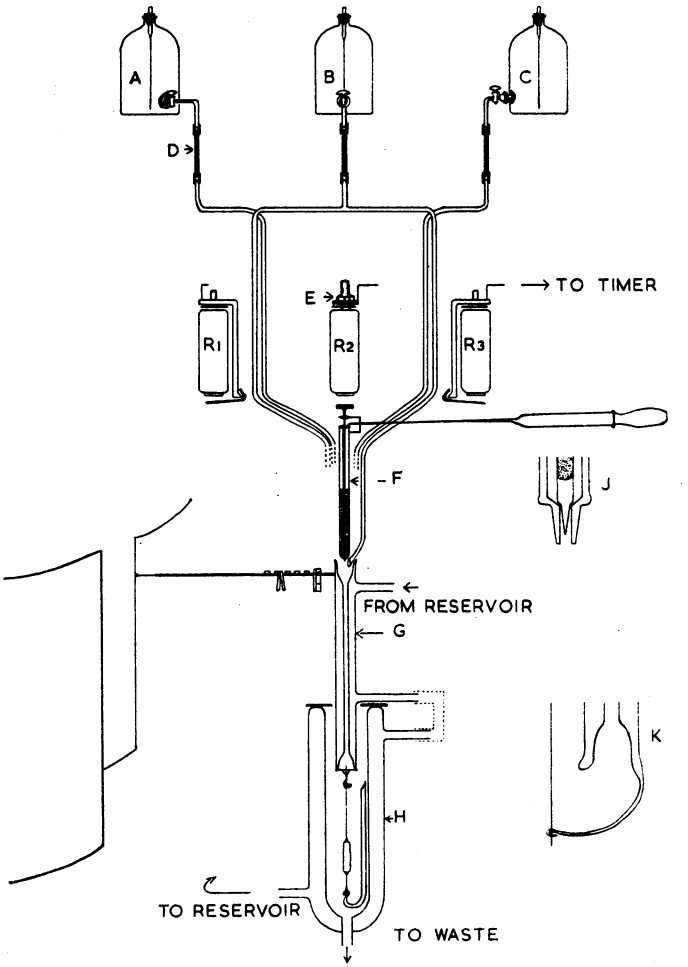

FIG. 1.—Apparatus for superfusion. (A) Tyrode solution, (B) Tyrode+histamine, (C) Tyrode+mepyramine, (D) capillary resistances, (R 1, 2, 3) Post Office relays, (F) tube for dose with valve (J), (E) screw for adjusting aperture of valve, (G) tube for warming solutions, (K) lower end of same tube with glass arm and hook, (H) warm chamber for gut. A centrifugal pump circulates warm water from a reservoir the temperature of which is thermostatically controlled.

Intestine.—A strip 3 to 4 cm. long is taken from the terminal ileum of guinea-pigs weighing between 150 and 200 g. It is tied with the ends left open and suspended in the flow of Tyrode, at a distance of at least 3 cm. below the hook.

Procedure.—Tension is applied to the gut, which is then left for 30 min. At the end of this time automatic dosage is begun from bottle B and allowed to continue until the gut becomes sensitive and gives regular responses. The tap of bottle B is then closed and the assay begun. After the assay has been completed, the gut may be used for qualitative tests by replacing the flow of Tyrode by that of Tyrode containing mepyramine.

Assays.—A number of 2 and 2 assays were performed with histamine solutions of known concentration. The design of the assay and the calculations from the data followed those of Schild (1942). In nearly all the experiments the low and high doses of the "standard" were 1 and 2 ng./ml. and of the "unknown" 1.25 and 2.5 ng./ml. These doses correspond to S1 and S2 and U1 and U2. Four groups of four doses were used in each assay.

The test of significance for departure from linearity of regression was applied in the following way. A special index of curvature H (Gaddum, 1953b), applicable to the spacing of doses actually used, was calculated for each assay from the formula $H=a-b-c+d$, where a is the mean of the effects of the four doses of S1, b of

U1, c of S2 and d of U2. Thirteen estimates of H were used to obtain the mean, $\bar{H}$. The standard deviation of $\bar{H}$ was calculated from the formula

$$s_{\bar{H}} = \sqrt{\frac{4V}{N}}$$

where V is the variance of a single estimate (a, b, c, or d) and N the number of estimates of H. The ratio $\bar{H}/s_{\bar{H}}$ gave t. The value of P was obtained from df at infinity.

Reconstitution of Incomplete Test Solutions for Assay.—The following solutions are prepared from the Tyrode stock solutions (abbreviated to SS).

Solution A. NaH_2PO_4 5 ml.+$MgCl_2$ 5 ml.+KCl 5 ml.+$CaCl_2$ 5 ml.
Total volume 20 ml.

Solution B. Solution A 10 ml.+$NaHCO_3$(SS) 1 ml.+water 89 ml.
Total volume, 100 ml.

Solution C. Solution A 10 ml.+NaCl(SS) 20 ml.+$NaHCO_3$(SS) 1 ml.+water 69 ml.
Total volume, 100 ml.

Procedures.—Two cases are considered.

(i) *A Salt-free Solution.* If the volume of this solution is 8 ml. or less, add: solution A 1 ml.+NaCl(SS) 1 ml.+$NaHCO_3$(SS) 0.1 ml. The final volume of the test solution is 10 ml.

It is convenient to use an "Agla" micrometer syringe for adding volumes less than 0.5 ml.

(ii) *A Solution Containing an Unknown Quantity of NaCl.* It is assumed that no other salt is present except that of the active substance. If the volume of the solution is 8 ml. or less, reconstitute it partially by adding solution A 1 ml., $NaHCO_3$(SS) 0.1 ml., and water to 10 ml. Take two 1 ml. lots of this solution and titrate the chloride against 0.1 N $AgNO_3$; 8 ml. of the solution are left. In the same way, titrate two 1 ml. portions of the Tyrode used for superfusion. The difference between the average values of these titrations is taken to represent the excess or deficiency of sodium chloride in the reconstituted solution. Subtract the average value for Tyrode solution from the average value for the reconstituted solution. Let this difference be $\pm$X ml. and the remaining volume of the solution be V ml. Then the volume of solution B or C to be added is given by the expression

$$\text{Vol}_{B \text{ or } C} = \frac{5.8\ X \cdot V \text{ ml.}}{8}$$

The value 5.8 is the factor for the titration and the denominator is the concentration of NaCl in Tyrode solution expressed in mg./ml. If X is positive, add solution B; if negative, solution C. A correction should be applied for dilution of the active substance by the solution added.

Effect on the Ileum of Varying the Tonicity, Composition and pH of Test Solutions.—10 ml. volumes of Tyrode solution were prepared as follows: Solution A 1 ml.+NaCl(SS) 1 ml.+$NaHCO_3$(SS) 0.1 ml.+water 7.9 ml. Glucose and atropine were not usually added; their omission made no difference to the response. The *p*H and chloride concentrations of these solutions did not differ significantly from those of Tyrode solution prepared for superfusion. The concentrations of KCl and $CaCl_2$ were varied by changing their concentration in solution A. The *p*H was altered by adding more or less bicarbonate. Histamine solution was added to give a concentration of 2 ng./ml.

RESULTS

Behaviour of the Ileum

(*a*) *Sensitivity to Histamine.*—The number of doses applied to the gut before this became stable and sensitive varied in 58 experiments from 15 to 75, with a mean of 32. The base line was then usually steady and the response rose steeply with the dose in the range 1 to 2.5 ng./ml.

(*b*) *The Response to Various Solutions.*

Tyrode.—A dose of 0.6 ml. seldom produced an effect. This shows that the intestine tolerates the slight fall in temperature that accompanies the dose. Between the end of the dose and start of the flow of Tyrode there is an interval of 1 to 2 sec. when no fluid passes over the gut. During this interval the gut may begin to contract, but the effect is cut short by the return of the flow. This contraction tends to increase with the interval, which should therefore be kept as short as possible.

Histamine.—The contraction starts 3 sec. after the dose begins to flow over the gut and reaches its maximum in 10 to 11 sec.; it therefore continues for 2 to 3 sec. after the flow of Tyrode has returned. The muscle relaxes completely in the ensuing 25 to 30 sec.

Modified Test Solutions Containing Histamine (2 ng./ml.).—Reconstituted Tyrode solutions in which the concentration of NaCl or KCl or $CaCl_2$ was varied by $\pm$8% were assayed in comparison with standard histamine solutions and found to be equivalent to 2 ng./ml. Nevertheless, hypotonic Tyrode and solutions containing an excess of KCl or $CaCl_2$ (8%) were by themselves slightly active in comparison with standard Tyrode solution. When the *p*H of the Tyrode test solution was lowered by 0.4 unit (i.e. from 7.7 to 7.3) the response was identical with that of the standard solution containing 2 ng./ml. When it was raised by 0.3 to 0.5 unit the value for the assay rose to 2.5 and 2.7 ng./ml.

2 and 2 Assays.—The gut usually remains in good condition for 4 or 5 hours. A 2 and 2 assay may be completed in 24 min. and it is therefore possible to carry out several assays on one preparation. The results of assays with known concentrations of histamine in Tyrode solution are presented in Table I. They are arranged in

TABLE I
ASSAY OF HISTAMINE ADDED TO TYRODE SOLUTION

Expt. No.	Temp. °C.	Added ng./ml.	Found ng./ml.	Limits of Error (P=0·99)	λ
1	36	(a) 2·50	2·60	2·10–3·20	0·052*
		(b) 2·50	2·64	2·28–3·06	0·035
		(c) 2·50	2·48	2·26–2·70	0·023†
2	36	(a) 2·50	2·61	2·24–3·06	0·038†
		(b) 2·50	2·64	2·38–2·96	0·027
		(c) 4·00	4·08	3·66–4·54	0·028
3	36	2·50	2·72	2·42–3·08	0·029
4	36	2·50	2·48	2·32–2·64	0·017
5	36	2·50	2·58	2·18–3·06	0·043
6	36	2·50	2·44	2·22–2·70	0·025
7	36	2·50	2·48	2·26–2·74	0·027
8	32	2·50	2·50	2·36–2·60	0·012
9	33	3·00	2·84	2·36–3·42	0·045
10	34	2·50	2·60	2·12–3·22	0·052‡
11	34	2·50	2·46	2·28–2·64	0·018
12	34	2·50	2·52	2·36–2·70	0·020
				Mean	0·030
				Range	(0·012–0·052)

* Assay performed too early: base-line still unsteady.
† Tubes F and G washed with Tyrode solution between doses.
‡ Assay performed too late: gut fatiguing.

chronological order. Each number refers to an assay on intestine from a different guinea-pig. Most of these assays were performed between or, more often, after the assay of samples by the simpler method of direct comparison. The tracing obtained in Expt. 8 is shown in Fig. 2. Expts. 1 and 2 were performed with Tyrode solution at *p*H 8 ; in the remainder the *p*H was 7.7. Reduction of the *p*H by this amount diminished spontaneous activity without impairing the sensitivity of the ileum. Assay 1 (a) was performed soon after the gut had become sensitive, but before the base-line was regular. In Expt. 10, the assay was performed after the gut had been working for 4½ hr. In both these experiments the value for λ (s/b) was high. In Expts. 1 (c) and 2 (a) the tube containing the dose was washed between the doses with Tyrode solution, to remove traces of the previous dose. This procedure did not increase the accuracy of the result. In Expt. 8 the temperature fell accidentally to 32° C.

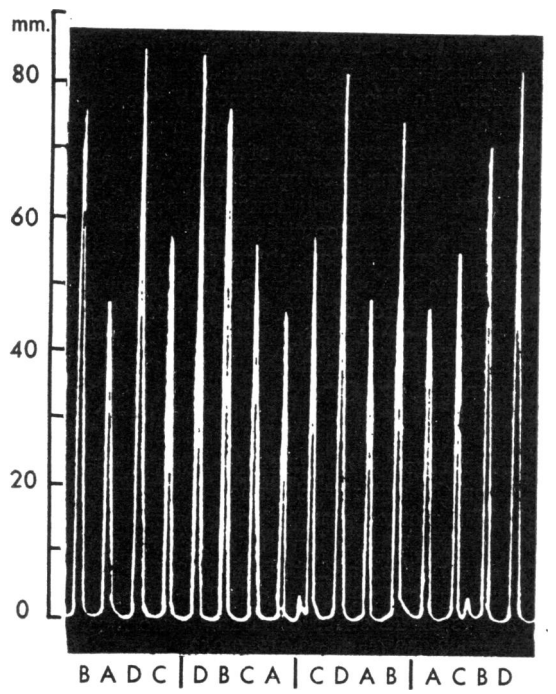

FIG. 2.—Tracing of a 2 × 2 assay with histamine solutions of known composition (Expt. 8, Table I). A (S1) 1.0 ng./ml., B (S2) 2.0 ng./ml., C (U1) 1.25 ng./ml., D (U2) 2.5 ng./ml.

The results of assays in which histamine was added to reconstituted solutions are shown in Table II. In some of these experiments part of the sodium chloride was formed by neutralizing 0.25 N-HCl with N-NaOH. The blank solutions were slightly active, but in 8 out of 9 experiments the activity was equivalent to <0.5 ng./ml. Nevertheless, the recoveries were nearly all slightly higher than the theoretical concentration (2 ng./ml.). This difference could not be accounted for by an effect of *p*H. In Expt. (a) 4, the amount of histamine added was 2.2 ng./ml.

TABLE II
ASSAY OF HISTAMINE ADDED TO RECONSTITUTED TYRODE SOLUTIONS. VOLUME OF SOLUTIONS, 10 ML.

Expt. No.	Blank Value ng./ml.	Found ng./ml.	Limits of Error (P=0·99)	λ
(a) *Reconstituted from Salts of Stock Solutions Only. Histamine added to give Concentration of 2 ng./ml.*				
1	<0·5	2·38	1·95–2·92	0·052
2	<0·5	2·20	1·95–2·48	0·032
3	0·5	1·81	1·62–2·00	0·028
4	<0·5	2·37*	2·21–2·54	0·018
(b) *Sodium Chloride formed by Neutralizing 5 ml. 0·25N-HCl with N-NaOH. Histamine added to give Concentration of 2 ng./ml.*				
1	<0·5	2·30	2·14–2·48	0·019
2	<0·5	2·20	1·86–2·64	0·046
3	0·5	2·50	2·20–2·90	0·037
4	<0·5	2·20	1·90–2·50	0·032
5	<0·5	1·87	1·60–2·10	0·033

* Concentration of added histamine 2·2 ng./ml.

DISCUSSION

The technique of superfusion and its applications have already been discussed by Gaddum (1953a). The present apparatus, which employs timing devices similar to those described by Schild (1946), makes it possible to standardize more rigorously the conditions of the assay. It has the practical advantage that a constant dose can be given until the gut becomes fully sensitive and stable. This could also be done by hand, but would be tedious. Since the solutions pass through a heated tube before they reach the ileum, the standard and the test solution can be kept at room temperature. They are therefore less likely to lose activity.

In our experience, the success of the assay also depends on the weight of the guinea-pig and the care taken in the preparation of solutions. It is important to use gut from small guinea-pigs, since that from pigs weighing much over 200 g. tends to be thicker and to show more spontaneous activity; it does not become sensitive so quickly and the base-line is less stable. It may be that the thicker gut takes longer to become permeable to histamine and that active substances formed or liberated in its walls are removed at a slower rate as compared with thinner gut. We have not investigated the effect of varying the rate of superfusion on the time needed for the preparation to become sensitive and stable. Temperature may also influence the time taken for the gut to become sensitive. In recent experiments, we have observed that fewer doses of histamine (at 3 ng./ml.) were needed after the temperature was reduced from 36° to 34° C. The base-line may be unsteady in the first hour, but the temptation to straighten it by increasing the load should be resisted, since this would decrease the sensitivity of the preparation. If enough time is allowed, and regular dosing is maintained, the base-line eventually becomes steady and the dose-response curve steep. The preparation may then be used until it shows signs of fatigue, when the responses become smaller, the dose-response curve becomes less steep, and the base-line tends to rise. These signs generally appear after the muscle has performed 200 to 250 contractions.

The method of applying different doses from the same tube is open to the criticism that a trace of the previous dose may alter the concentration of the dose actually placed in the tube. For example, a high dose may increase the effect of the low dose that follows it. This interference of one dose with another does not influence the result of the assay provided the differences between the concentrations are less than fivefold. In the present experiments most of the assays were performed with concentrations of histamine within the range 1 to 2.5 ng./ml.; moreover, washing the tube with 0.6 ml. Tyrode solution between the doses did not improve the accuracy of the assay.

The results of assays performed with histamine solutions of known concentration provide statistical evidence that the method is reliable for concentrations as low as 1 to 2.5 ng./ml. In this range the dose-response curves were steep and the values of λ agree well with those recorded by Schild (1942), and more recently by Boura *et al.* (1954). In none of the assays, either with histamine added to Tyrode or reconstituted Tyrode solution, was there significant ($P=0.95$) deviation from parallelism of the regression lines. Further, it was possible to conclude from the test applied to a special index of curvature, that deviation from linearity of regression was insignificant ($P=0.95$). This is surprising, since the doses were small, and it might therefore be expected that the assays were carried out on a low, and possibly curved, portion of the dose-response curve. However, the actual portion of the curve used may have been so small as to make it difficult to detect curvature.

The effect of reconstituted Tyrode solutions on the recovery of added histamine is to make the values somewhat higher than the theoretical values. This is because the blank solutions are slightly more active than Tyrode solution alone and the effect may be additive. In this respect, the results obtained in 2 and 2 assays do not wholly accord with those of experiments in which the concentration of neutral salts was deliberately varied and shown, within the limits tested, not to influence the response to histamine. The reason for the slight activity remains obscure, but the results confirm the importance of estimating the effect of a blank solution by adding the drug to it.

Chromatographic methods of purification often make it possible to separate and concentrate an active substance in aqueous solution. If this solution is free from salts it can be prepared for assay on the superfused ileum by adding Tyrode salts to it. Similarly, if the solution contains only NaCl its composition can be adjusted to that of Tyrode solution. In this way, it is possible to avoid loss of the active substance which might otherwise occur during the removal of water by evaporation. In reconstituting incomplete solutions for assay, the most important adjustment appears to be that of *p*H. Under the conditions of the assay, the intestine becomes perceptibly more sensitive to histamine in the test solution when the *p*H of this solution is raised from 7.7 to 8.1, but not less so when the *p*H of the solution is reduced to 7.3. Hence, the *p*H of the test solution should not exceed that of the standard solution.

Summary

1. A method is described for the assay of histamine on the superfused guinea-pig ileum.

2. The method was tested by performing 2×2 assays in which the lowest dose of histamine was usually 1 ng./ml. and the highest 2.5 ng./ml. The index of precision, λ, was calculated from the data of assays on 12 strips of ileum. The mean value of λ was 0.030 (range 0.012–0.052).

3. Details are given of methods by which incomplete solutions containing an active substance may be prepared for assay by this method.

We are grateful to Professor J. H. Gaddum, F.R.S., for introducing us to the technique of superfusion, and for advice on the statistical treatment of some of our data.

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ADDENDUM

SEQUENTIAL TIMING UNIT

BY

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The purpose of the instrument is to activate relays periodically during a cycle of operations. In the present instance a sequence of five operations was chosen. A diagram of the unit is shown in Fig. 3.

The unit comprises a timing circuit, a multiple switch (uniselector) and a means of resetting the timing device after each cycle. The timing circuit consists of an *R.C.* network which excites a Schmitt Trigger when the voltage reaches a critical value.

A stock four-bank uniselector is modified by converting the interrupter switch (S_2) from normally closed to normally open, so that it "makes" when the uniselector armature is energized. One of the four banks of 25 contacts is used to time the period of each operation. For continuous running all 25 contacts must be connected to the timing circuit. The remaining banks are available for external switching.

The P.O. relay in the anode circuit of V_3, which switches the armature current of the uniselector, must have heavy-duty contacts capable of carrying 5 A and the relay coil should be of high impedance (10,000–20,000 Ω).

Power Supplies.—About 300 V H.T. at 60 mA from a conventional power supply, 6.3 V for valve heaters and a 24 V source of 5 A which may be from accumulators or a d.c. rectifier such as a battery charger. Low impedance working relays requiring 24 V are the most useful. If the relays are of high impedance a further source of high voltage is needed.

Operation

When switch S_2 is closed, C_1 is discharged, the control grid of V_2 is at earth potential, and the

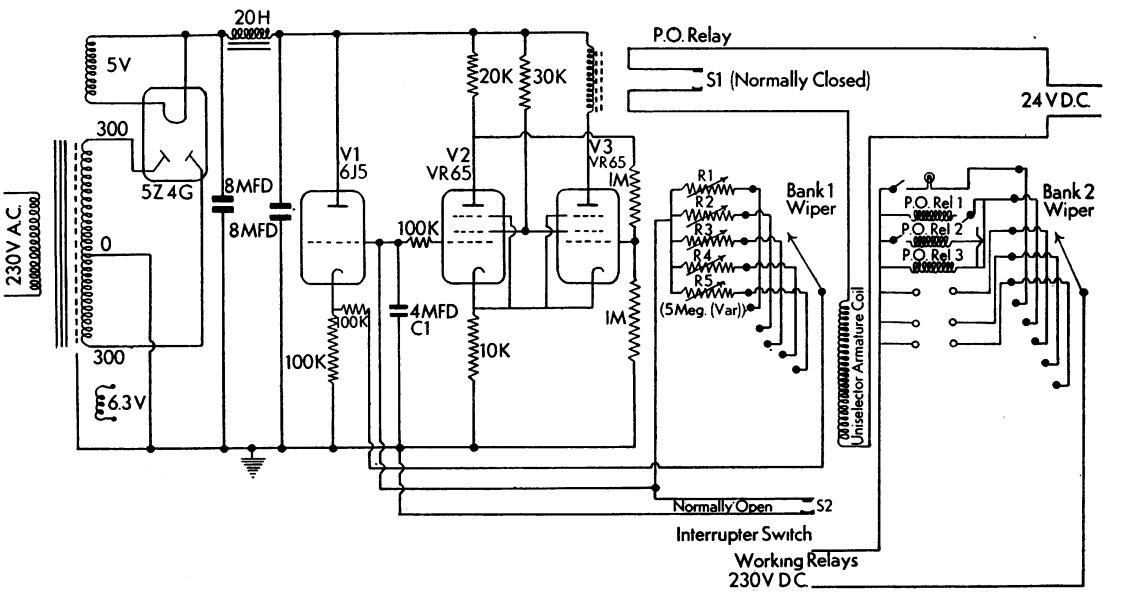

FIG. 3.—Sequential timing unit. Only two of the four banks of the uniselector are shown. The first is used to time the period of each operation; the second, to energize the working relays (P.O. Rel. 1, 2, 3).

anode of V_2 at high tension; hence the control grid of V_3 is at high potential and V_3 conducts; this causes the P.O. relay to be energized and S_1 to be broken. When S_2 breaks, C_1 charges via R_1 from the grid-cathode potential of V_1. When the voltage on the control grid of V_2 approaches cathode potential, V_2 conducts and the cumulative Schmitt action results in V_3 being cut off and S_1 be closed. The uniselector armature is energized to move the wipers one step further, R_2 being now the charging resistance. S_2 is now closed, C_1 is discharged, and the timing action repeated with a duration dependent on R_2. The values of C and R in the diagram may be altered as required. Condenser C_1 must be of good quality since leakage paths affect the times obtainable. To ensure complete discharge of C_1, it may be necessary to increase the "make" time of S_2. This can be done by connecting a condenser of, say, 0.25 mF between the cathode of V_1 and the control grid of V_3.

If a particular pattern of five operations is to be repeated continuously, the five variable resistances, R_1–R_5, are connected consecutively and in the same order to all 25 contacts of the timing bank.

External Switching.—The second bank of the uniselector feeds the signal lamp and the working relays previously described (relays 1, 2, 3). The two-way switch between relays 1 and 3 makes it possible to replace the flow of Tyrode solution by that of Tyrode solution containing an antagonist. The third bank operates five pilot lamps which indicate the stages of the cycle; the fourth bank is unused. Only the first and the second banks are shown in the diagram.

The variable resistances were set as follows:

. R_1 at 15 sec. (to maintain the light signal before the release of the dose).

R_2 at 8 sec. (to stop the flow of Tyrode and simultaneously release the dose).

R_3 at 65 sec. (interval).

R_4 and R_5 were set at zero and accounted for 2 sec. The dose cycle was therefore 90 sec. The longest cycle attainable with the present unit is 10 min.