5. Of the methods tried, the calcium hypochlorite method seems best suited for the assay of methenamine and certain mixtures containing methenamine. The method is simple, comparatively rapid and accurate.

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# Electrophoretic Separation of the Blood Pressure Principles of Hog Kidney Extracts*

*By Raymond Jonnard and Marvin R. Thompson*

It has been shown by various authors that the ischemic kidney of various animals yields, under suitable conditions, a vaso-pressor substance called "renin" (1, 2, 3, 4, 5). This substance is also present in the normal organ (4, 6) and is somewhat specific (8, 10). It is neutralized by a vaso-depressor substance extractable under certain conditions (7, 9). The work done to date indicates that the chemical properties of these substances have some similarity, so that their complete separation is difficult. Thus they are usually obtained together in solution during the preliminary stages of the extraction of the kidneys.

Much stress has been laid upon the biological properties of these two antagonistic principles, but the literature contains only very limited information on the physical and chemical properties of the substances involved. Most of the alleged properties of these substances have been inferred from solubility data and from the conditions of their extraction. Although it can be safely assumed, to date, that "renin" is a protein-like substance, little can be said as yet of the nature of the depressor principle.

In view of the findings by du Vigneaud and co-workers (11) that the vaso-depressor factor extracted from the hypophysis gland could be isolated in the catholyte compartment of an electrophoretic apparatus, and that this substance seems to have an iso-electric point far in the alkaline range, it was thought desirable to submit certain kidney extracts to a similar electrophoretic analysis.

In view of the uncertainty in the identification of the various kidney depressor principles claimed by various authors (6, 12, 13, 14), we have used several different types of kidney extracts.

## EXPERIMENTAL

### METHOD

*I. Electrophoresis.*—Due to the minute amount of active material contained in the hog kidney, this active material is usually obtained in the form of dilute solutions as will be discussed later. Therefore, a rather large electrophoretic cell had to be designed in order to separate the components of a volume of solution large enough to yield material for repeated bioassays. The apparatus was made of large flanged Pyrex glass fittings (15); it was somewhat similar to that of Russell and Stauffer (16). The clamps holding the various parts were also used to squeeze removable diaphragms of either paper or cellophane. Both anodic and cathodic compartments had a capacity of 90 cc. The medium cell held 200 cc. The electrode compartments held 300 cc. The anodic and cathodic compartments were separated from the central cell by paper diaphragms and from the electrode compartments by cellophane diaphragms preventing the escape of the practically non-dialyzable vaso-motor principles in the present series of experiments. The whole apparatus was held on a "flexaframe" and could be immersed in a bath of running water at any given temperature. The assembly is shown in Fig. 1.

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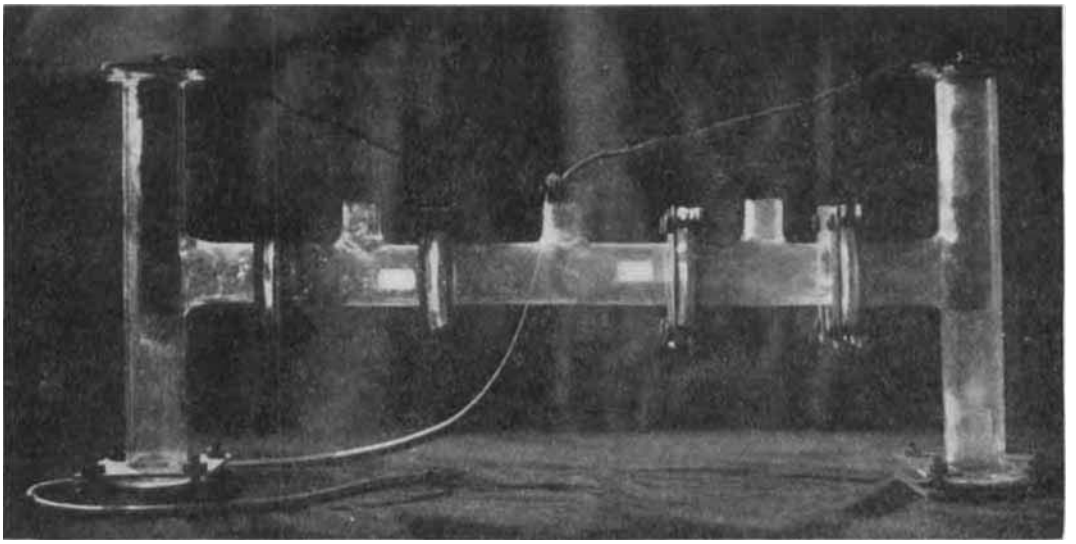

Fig. 1.—The Electrophoretic Apparatus.

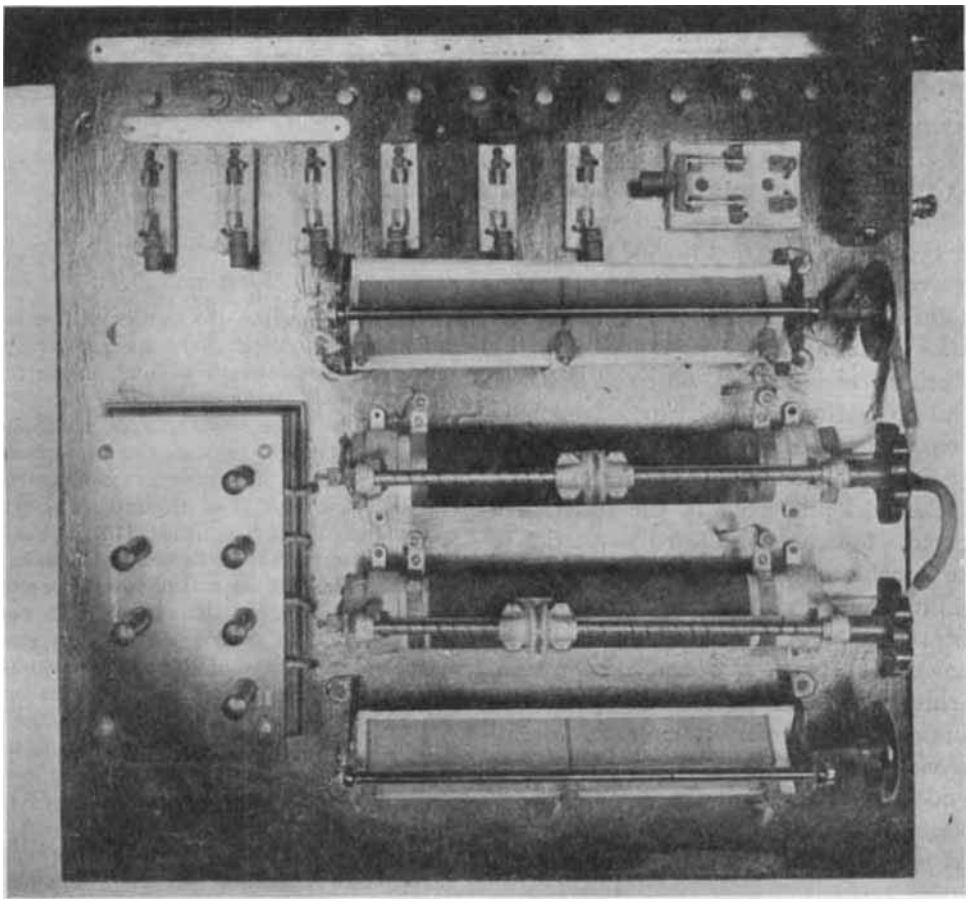

Fig. 2.—Electrical Control Panel for the Electrophoretic Cell.

The electrical circuit included a voltmeter and an ammeter used for preliminary adjustment, and a control board comprising a twin sliding nichrome wire potentiometer in parallel with a twin carbon resistor-potentiometer, thus providing a very great sensitivity in the adjustment of both potential and intensity together with an almost complete independence of the resistor values from the effect of external thermic and hygrometric factors. The current source was a constant speed d.-c. generator. The control panel is shown in Fig. 2. Accurate measurements of both potential and intensity were also made with a Leeds and Northrup type K potentiometer set-up.

The potential gradient $p$ in our apparatus is given by the formula used by Tiselius (17): $p = (i/q.k)$ (volt/cm.), where $i$ = intensity in amp.; $q$ = cross-section area of the electrophoretic tube in cm.$^2$ (here = 7.5 cm.$^2$); $k$ = conductivity of the solutions in ohms $-1$. Notwithstanding the inherent sources of inaccuracy in our set-up particularly designed more for separating large volumes of material than for determining boundary positions or velocities of migration, no attempt has been made at determining the $k$'s separately. Rather the $k$ values have been approximately obtained from a direct measure of both the potential $v$ and the intensity $i$ by the relation $r = v/i$ and $k = (l/r \times C^{te}$, where $C^{te} = r/0.1118$ if $N$ KCl is used for the calibration of the apparatus. The values of $p$ thus found are given in the accompanying table. This method is somewhat similar to that of Briggs (18).

With such a form of electrophoretic apparatus, it might be possible that a poor heat transfer would bring about disturbances due to the thermal gradient inside the tubes. It is known that in such a type of apparatus the thermal gradient, $dt/dr = wr/2k$, considerably increases with the radius $r$ since $t_0 - t = w.r^2/4k$ ($r$ = radius of the tube, here 12 mm.; $k$ = thermal conductivity of the liquids; $w$ = load, in watt/cc.). Here again the effect of the thermal gradient upon the velocity of the migrating molecules and the completeness of the separation could be neglected, but, nevertheless, this gradient was kept low by using a very low load, $w$ (see table), to avoid a thermal alteration of the active principles, which have been shown to be thermolabile (Page (6), Braun-Menendez (10)). Such precaution was important in this experiment when the joule effect $w^{watt} = ri^2t$ could have been important due to the large value of both $r$ and $t$ (see table for values of $t$).

Finally it should be pointed out that in our instrument, the relationship indicated by Tiselius between the volume of the electrode compartment and the volume of solution to be separated (17), was not satisfied with the result that the ionic composition of the liquids in the various compartments of the apparatus underwent slight progressive change during the experiments. It has been verified, however, that the pH remained constant within 0.1 unit, while the load = $w^{watt}$ could be continuously adjusted. Since in most cases the active principles were further isolated, sometimes in the dry form, as will be discussed later, this source of error was neglected during this preliminary investigation.

*II. Preparation of the Solutions.*—(*a*) The saline extracts were prepared at 0° C. using 1 liter of saline per Kg. of fresh kidney. One cc. represented 1 Gm. of fresh kidney.

(*b*) The defatted kidney extracts were prepared according to the method of Page and others: extraction of the minced kidneys with four parts of acetone per weight, and with one part of ether. The prepared kidney powder thus obtained was finally extracted with one part of saline solution at 0° C. which was made up to 1 mol. in sodium phosphate and filtered: 1 cc. represented 0.5 Gm. of fresh kidney.

The "renin D" was prepared according to Page's method (8). Doses representing 5 Gm. of fresh kidney were uniformly used for the bioassay.

The "renin E" was Page's "renin D" further purified by dialysis at pH = 4, neutralization, precipitation by $^1/_2$ saturation with ammonium sulfate at pH = 3.5, re-precipitation by saturation with sodium chloride at pH = 2.0, and drying. Doses of 0.1 mg. per Kg. of body weight were uniformly used for the bioassay.

All the solutions were finally made up to 1 mol. in sodium phosphate and adjusted at various pH values recorded in the table. Finally they were dialyzed 16 hours in cellophane bags against the same buffer solution at the chosen pH value just before the electrophoresis.

*III. Isolation of the Separated Material.*—In some cases the anolyte and catholyte were directly tested. When the isolation of the active material was attempted, the liquid from both compartments was brought to pH = 2.0 with lactic acid and saturated with ammonium sulfate at 0° C. It is known that under such conditions the "renin" precipitates, while vaso-dilator substances of the type isolated by Grollman (19), for instance, are also rendered insoluble.

*IV. Identification of the Isolated Products.*—The most practical criterion so far available for the identification of the substances involved in this study is the effect upon the blood pressure of anesthesized animals. The tests were conducted upon either normal cats or normal dogs under pentobarbital anesthesia. The blood pressure was recorded from the carotid artery in the conventional manner, the solutions under test being injected intravenously. In many instances rapid control experiments have been made by inserting a needle into the femoral artery of the animal, the needle being connected directly to a direct reading Bourdon or Thycos manometer, the test solutions being injected into the jugular vein. Although the latter method does not leave any record of the experiment, it has been found rapid, convenient and reliable, and gives results identical to those obtained with the former method.

Figure 3 shows a few kymographic records which have been considered typical of the substances under investigation: (*a*) a typical increase of blood pres-

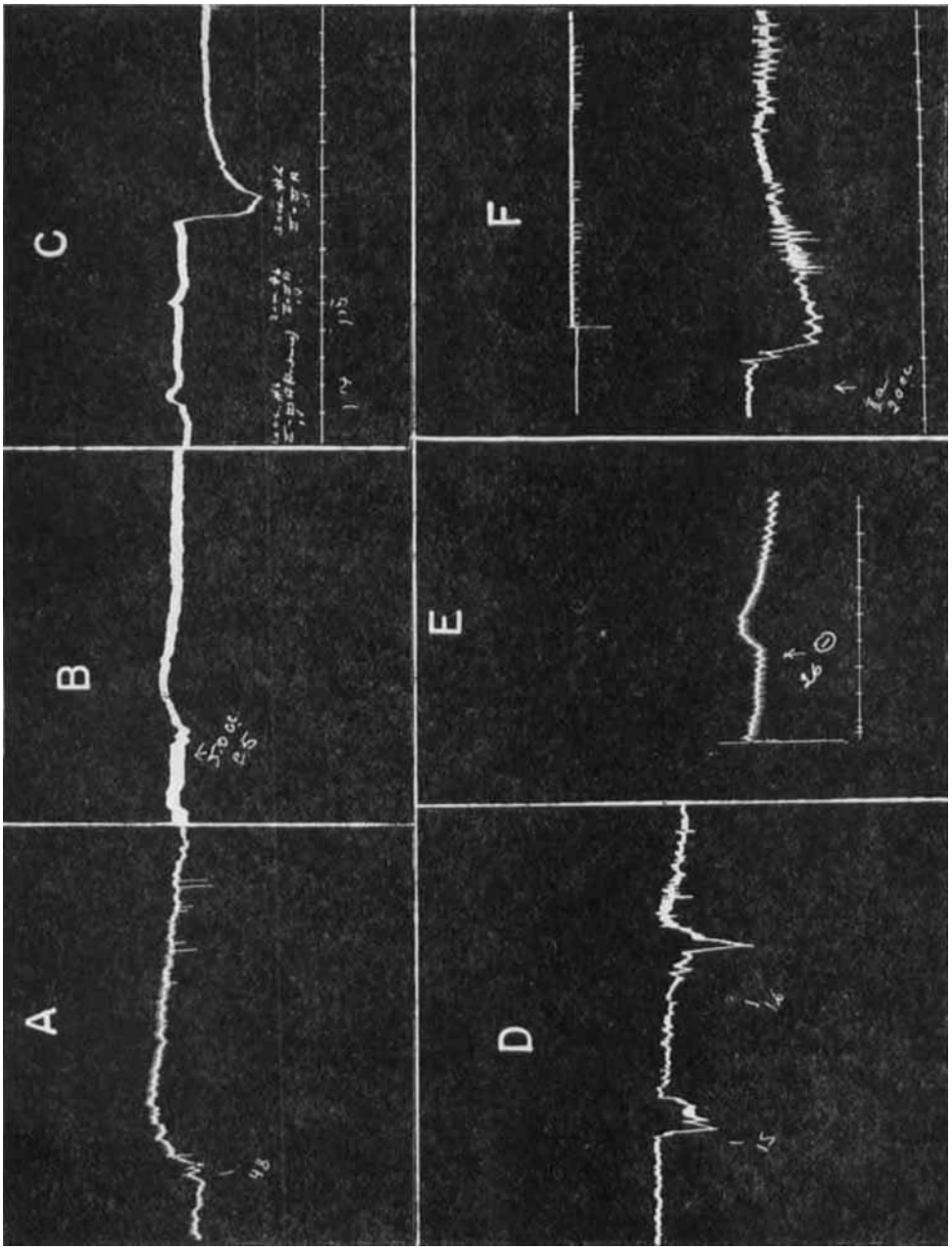


Fig. 3.—Blood Pressure Tracings (see Text).

sure obtained with "renin E" (0.2 mg./Kg.), (*b*) a similar curve obtained with "renin D," (*c*) a typical decrease of blood pressure obtained with a kidney depressor fraction obtained by Grollmann's method, (*d*) a non-typical decrease of blood pressure frequently obtained with depressor or toxic substances of various origin (such results must not be confused with the typical response to the injection of "renin" or of the specific kidney hypotension factor), (*e*) an increase of blood pressure obtained with a cathodic electrophoretic fraction at pH = 6.5, (*f*) a decrease of blood pressure obtained with an anodic electrophoretic fraction at pH = 5.0. The records were similar for cats and dogs.

## RESULTS

The series of measurements was necessarily limited because of (*a*) the length of time required for preparing potent kidney extracts in any reasonable quantity, and the fact that such extracts are rather unstable, (*b*) the time required for the many biological tests which must of necessity be done without delay after electrophoretic separation or isolation of the separated active materials.

Table I.—Effect of the Electrophoretic Fractions upon the Blood Pressure of Anesthesized Dogs (Expts. I to V) and Cats (Expts. VI to IX)

| Preparations Tested | Experiment No. | Temperature, °C. | *t*, Hours | *v*, Volts | *i*, Amp. | *k* | *p* | pH | Blood Pressure Anolyte | Blood Pressure Catholyte |
|---|---|---|---|---|---|---|---|---|---|---|
| One mol. sodium phosphate solution of kidney extract; final precipitation with 30% sodium chloride at a pH of 2.0 and redispersion in saline | I | 20 | 24 | 10 | 0.1 | 0.062 | 0.21 | 6.5 | 0 | +45 |
| | II | 20 | 48 | 10 | 0.1 | 0.062 | 0.21 | 6.5 | +30 | −35 |
| | III | 20 | 36 | 10 | 0.1 | 0.062 | 0.21 | 6.5 | −30 | +15 |
| | IV | 15 | 72 | 10 | 0.1 | 0.062 | 0.21 | 6.5 | −30 | +10 |
| Same, with addition of chloroform | V | 15 | 72 | 10 | 0.1 | 0.062 | 0.21 | 6.5 | −20 | +25 |
| Saline extract of non-defatted kidneys | VI | 15 | 48 | 5 | 0.05 | 0.062 | 0.11 | 7.5 | 0 | −15 |
| | | | | | | 0.062 | 0.11 | 6.5 | −40 | +45 |
| | | | | | | 0.062 | 0.11 | 5.0 | −20 | +45 |
| | | | | | | 0.062 | 0.11 | 3.0 | 0 | 0 |
| Saline extract of defatted kidneys | VII | 15 | 48 | 5 | 0.05 | 0.062 | 0.11 | 7.5 | −20 | 0 |
| | | | | | | 0.062 | 0.11 | 6.5 | −50 | +50 |
| | | | | | | 0.062 | 0.11 | 5.0 | −20 | +60 |
| | | | | | | 0.062 | 0.11 | 3.0 | 0 | 0 |
| Renin D | VIII | 15 | 36 | 7.5 | 0.1 | 0.083 | 0.15 | 6.5 | −25 | +30 |
| Renin E | IX | 15 | 36 | 7.5 | 0.1 | 0.083 | 0.15 | 6.5 | 0 | +35 |
| | | | | | | 0.083 | 0.15 | 5.0 | −10 | +55 |
| | | | | | | 0.083 | 0.15 | 3.0 | 0 | 0 |

The results are shown in the accompanying table. The sign (−) indicates a decrease of blood pressure, and conversely; the amplitude of the blood pressure variation observed is expressed in terms of mm. of Hg.

## DISCUSSION

The conditions of the experiments would eventually permit the evaluation of the mobilities $u+$ and $u-$ of the active substances investigated. Indeed, the velocity of migration in cm./sec. is given by: $i.u/q.k = pu$, where $p$ is the potential gradient defined above. The total distance traveled by each of the charged particles after a time $t$ sec. is given by: $d$(cm.) $= p.u.t$, so that after the time: $t$ sec., each particle has swept through a volume $V = p.u.t.q = u.i.t./k$. Unfortunately, in the absence of a basis for a quantitative evaluation of the active components from either chemical data or the bio tests, such calculation cannot be exactly related to the findings reported in the accompanying table. Nevertheless, the above-reported results lead to some interesting conclusions.

The essential results of this investigation show the possibility of a sharp separation of the antagonistic vaso-motor principles contained in the kidney extracts, by means of an electrophoretic field. Quite consistently the pressor factor has been found in the catholyte compartment (from which it could be further isolated in the dry form) when the migration took place at pH values ranging between 3.0 and 7.0. It has also been shown that "renin D" and "renin E" behave similarly. At a pH below 3.0 the pressor factor can no longer be detected under the conditions of the experiment.

On the other hand, the anolyte has likewise been found quite consistently to contain a substance producing a decrease of blood pressure, when the electrophoresis was conducted at pH values ranging between 5.0 and 7.5. From a comparison of Experiments I, V, VI and VII it seems that this depressor substance has an isoelectric point not far from neutrality. A comparison of Experiments I, III and VIII makes it appear that the pressor principle, and probably the "renin," migrates much more rapidly than the depressor substance at pH values ranging between 5.0 and 6.5.

The similarity of the blood pressure curves obtained with the "renin D" and "renin E" and the catholyte on one hand, and with the Grollman depressor substance and the anolyte on the other hand, is very striking and suggests a likely chemical relationship.

If the above results are confirmed, it would appear that the vaso-motor substances extractable from the kidney are fundamentally different from those of the hypophysis (11) or the brain (20).

The work is being pursued with the object of determining the exact isoelectric points of the substances considered and the conditions of their quantitative isolation.

## SUMMARY AND CONCLUSIONS

1. The electrophoretic field has been used for the separation of the kidney vaso-pressor from the vaso-depressor principles.

2. The vaso-pressor substance migrates to the cathode at pH values ranging between 3.0 and 6.5 under the conditions of the experiment. It has been found that "renin D" and "renin E" have a similar behavior under the same conditions.

3. The vaso-depressor factor consistently migrates to the anode at pH values

ranging between 5.0 and 7.5, and its migration velocity is much slower than that of the pressor factors.

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# The Determination of Theobromine in Tablet Mixtures*

*By A. G. Richardson and Y. C. Campbell*

In an effort to find a suitable rapid method for the determination of the alkaloid theobromine in tablet mixtures containing other nitrogenous organic compounds it became plainly evident that the ordinary shake-out methods using two immiscible solvents were unsatisfactory. This was confirmed by E. C. Deal (1) who recommended the Emery-Spencer (2) method wherein the theobromine in a tablet mixture is precipitated with iodine; phenobarbital or other barbiturates being uneffected. He pointed out, however, that starch, which is present in most tablet mixtures, would interfere and produce inaccurate results.

The A. O. A. C. volumetric method (3), in which the silver salt of theobromine is precipitated and the nitric acid formed from a known amount of standard silver nitrate

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