ON THE ACTION OF IONS ON THE UTERINE MECHANICS

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1. *Introduction*

The still persisting controversy about the influence exerted by inorganic ions upon the uterine mechanics justifies a new experimental approach to the problem. Many of the results up to now obtained, which have been interpreted as probably due to the actions of Na^+, K^+, Ca^{++} and Mg^{++} ions, can be better understood only when we take into consideration other factors which can also act upon the uterus. Among those factors it must be first considered the variation of the ovarian hormones during the sexual activity that induces metabolic changes and can be responsible for the different grades of activity in the various stages of the oestral cycle and pregnancy. Secondly, it is obvious that any study of the activity of the uterine muscle depends also on the consideration of the concentration of the constituents of the bathing or perfusing fluids used in isolated experiments. Thirdly, it must be emphasized that these two just mentioned factors may have mutual influence, that is the variation of the ionic composition of the uterine muscle being correlated with the variation of the hormones (ROSSENBECK, apud REYNOLDS 1949, p. 439). Finally, in the case of action of ions, it is necessary, when a certain ion is subtracted, to consider the variation of the osmotic pressure (*o. p.*). The *o. p.* must be appropriately compensated, in order to obtain results that may really represent the effect of the removal of the ion.

The submaximal isotonic responses of the isolated uterine muscles of guinea pigs to the pituitrin extract was studied by VAN DYKE and HASTINGS (1928). They altered the ionic medium and compensated the difference of the *o. p.* by varying the NaCl content of the perfusing solution. They assumed that the small changes of NaCl would not affect the response of the guinea pig uterus. They did not mention however the phase of the cycle of the uterus used, only saying that the work was performed with inactive muscles. This inactivity may be attributed, as we shall see later, either to the phase of complete restness during anestruous or diestruous observed in the guinea pig, or to the influence of the Mg^{++} ion, which concentration in the solution prepared by those authors is relatively high in comparison with other proposed physiological fluids.

GOMES DA COSTA (1948) discusses the question of *o.p.*, criticizing SIMONART's ideas (1926) who is inclined to assume that all the contractions (initial, after bathing and final) observed in the muscle *in vitro* are due to this factor. He sustains that these contractions should not be due exclusively to *o.p.* Other factors, such as pH, temperature, oxigenation and action of $Ca^{++} + K^{+}$ ions, should also be considered. The same has been quoted by NOVIS (1953, p. 16) who used Jalon's fluid for studying the mechanics of rat's uterus.

In GOMES DA COSTA experiments, the *o.p.* is kept constant by varying the NaCl content. When K^{+} or Na^{+} ions were removed, the Na^{+} was increased to counterbalance the absence of those ions. Inversely when the amount of Na^{+} or K^{+} was doubled, the content of the solution in Na^{+} was decreased.

It is also mentioned that DALE (1913, ap. VAN DYKE and HASTINGS, l. c.) demonstrated that small variations in *o.p.* in the bathing solution alters the responses of the uterus.

As we can see there are still doubts on the mechanism of the uterine contractions. By a method that we think better appropriated for studying the present problem we have attempted to contribute to a bettesr understanding of the mechanism referred to.

2. *Material and Methods*

Virgin female rats (Wistar) were used, weighing 180 g (± 20 g) supplied by the Instituto Butantan, Torres Laboratory and Faculty of Medicine of the University of São Paulo. A Palmer apparatus for perfusion of isolated organs was employed for graphical records at 37° C (± 1° C); the pH of all solutions was adjusted to 8 with Sodium Bicarbonate. Before each experiment, the oestral cycle phase was checked by simple vaginal smear, stained by an aqueous 0,5% methylene blue solution. The animals were killed by a blow in the occipital region followed by isolation of the uterine horns which were immerged in 40 ml of a phsiological solution.

The procedure was the following: several experiments were performed in order to detect the influence of such ions on the uterine fibers. Care was taken to maintain the *o.p.* at the right level, because preliminary experiments have demonstrated that this factor is an essential one in the uterine contraction. After several attempts, experiments have been done as follows: a) a modified Tyrode solution (NaCl-9,00 g/1; KCl-0,42 g/1; $CaCl_2$ 0.06 g/1; $NaHCO_3$ — 0.5 g/1) in which the uterus contracts well, was taken as a standard solution. Whenever it was desired to decrease in the solution the rate of a certain ion, the variation in *o.p.* was compensated by adding glucose.

For each ion 4 solutions were prepared, each with the ion under study in different concentrations, but all of them, with the original *o.p.* and pH. In solution 1 the ion was absent; in solutions, 2, 3 and 4 its concentration was respectively 1/4; 1/2 and 3/4 of the original concentration. With this procedure it was possible to obtain records and duplicates of different stages in the different solutions used and to study the action of a certain ion on the rat's uterine muscle.

3. *Results*

We first tried to recompose Locke's and Tyrode's fluids. Only in one experiment, with Locke, we got a nearly complete return to normality when all components of the fluid were successively added (Gr. 1). Neverthless in experiments with Tyrode, or even with Locke, the muscle did not return to the original state after being bathed in a solution with only one or two of the ions.

A) *Influence of Na^+*

In every stage of the oestral cycle the complete absence of Na^+ provokes on the muscle a tendency to tetany followed by a rising of the basic line and a resting in contracture (Gr. 2). This resting is reversible, that is, by changing from the solution without Na^+ to another with 1/4 of the normal concentration of that ion, the organs recover their normal contractions. It was further observed that as the ratio Na/K approaches the normal, the amplitude of the contractions increases and even becomes more regularly than at the beginning. This relative regularity of the contractions can be atributed to the glucose used to obtain the compensation. The effects due to increasing the Na^+ content above the normal concentration, was not considered because in those cases it is hard to separate the osmotic effects from those produced by the ion.

B) *Influence of* K^+

In all experiments on complete absence of K^+ the uterine contractions are similar to those observed in the case of Na^+, with a tendence to rest in contracture. The frequency is greater than the one observed in the case of Na^+, and the amplitude becomes lower and lower, until no contractions are finally observed (Gr. 3). This blockage of the uterine activity is also reversible, for a return of the contractions is observed when the solution without K^+ is replaced by one with only 1/4 K^+, and so on. The amplitude of the contractions increases as the rate of K^+ in the solution becomes higher and higher until the initial concentration is reached.

C) *Influence of* Ca^{++}

In every case the absence of Ca^{++} caused relaxation of the uterine muscle with a previous stage, in which the frequency increases and the amplitude decreases until complete stopping. This rest is also reversible when the solution without Ca^{++} is immediately substituted by another with Ca^{++} (Gr. 4). Prolonged immersion in a solution without Ca^{++} however, alters the muscle so much that it no longer returns to the normality when it is replaced in solutions tending to the original concentration of the ion. It is still possible to verify, by comparing the record nr. 4 with that related to the experiments with the ion K^+ (Gr. 3), that there is, in fact, an antagonism between those two ions. Actually, the absence of K^+ and the presence of Ca^{++} induced a blockage contracture; inversely the absence of Ca^{++} and the presence of K^+ has caused a relaxation.

The influence of Ca^{++} in different concentrations in the several normal physiological fluids, may be evideneed when we replace a certain solution by another. The organ presents greater concentrations in the Locke's fluid in which Ca^{++} is 4 times more concentrated (0.24 g/1) than in the modified Tyrode (0.06 g/1).

As to the greater amplitude of the contractions in Locke's fluid than in Tyrode's this might be due not only to the different concentrations of Ca^{++} in both solutions as well to the absence of the ion Mg^{++} in Locke's fluid. The presence of Mg^{++} decreases not only the amplitude, but also the frequency of the contractions.

D) *Influence of Mg^{++}*

The absence of Mg^{++} does not alter appreciably the mechanics of the isolated uterus of the rat, when other ions are present in their convenient ratios. The experiments were repeated adding Mg^{++} to the standard fluid. The results are recorded in graph nr. 5 in which the preceeding phenomena can be well observed. It must be only emphasized the important fact that the presence of Mg^{++} only delays the blockage, in contracture, in the absence of Na^{+} and K^{+}, and in relaxation in the absence of Ca^{++}. These conclusions are reinforced by the fact that when the uterine muscle is bathed in a solution without Mg^{++} and Na^{+}, the mentioned stopping in contracture is verified after 65 minutes; and when the Mg^{++} is present in the solution this resting takes place after 122 minutes without modification of the main phenomena.

In the case of K^{+} we have:

Solutions without Mg^{++} — contracture after 54 minutes
Solution with Mg^{++} — ” ” 77 ”

In the case of Ca^{++}

Solutions without Mg^{++} — contracture after 54 minutes
Solution with Mg^{++} — ” ” 124 ”

4. *Discussion*

Excepting the works of VAN DYKE and HASTINGS and GOMES DA COSTA, previous papers on the action of ions on uterus have not taken into consideration the influence of the factor *o.p.*

Accordingly, we shall compare our results, obtained with rat, with those obtained by the authors above mentioned.

In the GOMES DA COSTA's experiments the *o.p.* is maintained constant, as already mentioned, by the variation of the $NaCl$ content the physiological fluid. From the description of the results and the records obtained by this author, the ion Na^+, in the compensation, should have the same influence as the Mg^{++} ion in ours, i. e, it only delays the blockage provoked by the absence of K^+ and the relaxation if Ca^{++} is absent from the fluid. We think that this type of compensation is an incorrect one because when we increase the rate of one of the ions and decrease the rate of another, the ionic equilibrium is broken, and also its respective ratios, as may be seen in the case of the Na^+/K^+ ratio.

The correction of the *o.p.* by glucose has given better results, having in addition the advantage of regulating the rithm and the amplitude of the contractions.

On the other hand, van DYKE and HASTINGS' work differs primarily from ours in the fact that they used guinea pig's uterus in an inactive stage, that is, when it does not contract spontaneously. Using the VAN DYKE and HASTING's fluid, (as they proposed) the rat uterus, active in Locke or Tyrode's, showed very litlte activity. It would be interesting to know the behaviour of the guinea pig uterus in Tyrode, but this was not done by those authors. The inactivity of the rat's uterus in the Van Dyke's solution could be explained by the antagonism between Ca^{++} and Mg^{++}. In fact, analysing the ratios between Na^+, K^+, Ca^{++} and Mg^{++} in the modified Tyrode used by us and the Van Dyke's fluid we have

	Tyrode	*van Dyke-Hastings*
$NaCl$	9.00 gr/1	6.595 gr/1
KCl	0.42 gr/1	0.462 gr/1
$CaCl_2$	0.06 gr/1	0.058 gr/1
$MgCl_2$	0.05 gr/1	0.095 gr/1

Na/K ratio = 21,4   Na/K = 14,2
Mg/Ca " = 0,083   Mg/Ca = 1,637

One can see a marked difference in the ionic relation between the two solutions in relation to the quantities of Na^+ and Mg^{++}. The quantity of Ca^{++} in both solutions is almost the same, but that of Mg^{++} is larger in the Van Dyke-Hasting's solution. During our experiments we observed that Mg^{++} in fact is a modifier of the action of Ca^{++} upon the uterine muscle (Gr. 6), lowering its action. Now, the same quantity of Ca^{++} existing in both solution opposing its action to different quantities of Mg^{++} is likely to provoke different responses on the muscle. Inversely, a greater quantity of Mg^{++} acting on a same quantity of Ca^{++} can bring up the muscle to an inactivity, what, in fact, was verified. As Locke's fluid does not contain Mg^{++}, it is out of consideration here. The greater amplitude of the contraction of the uterus in Locke's solution may be attributed to the absence of the Mg^{++} ion. In this case the great quantity of Ca^{++} does not find any opposition to its action.

In relation to the action of the Na^+ ion on the uterine mechanics, only **VAN DYKE** and **HASTINGS** paper says something about: the increase of the Na^+ ions induces a greater excitability on the guinea pig's uterus to the pituitary extracts, and when the concentration of all ions is reduced to a half, with the exception of Na^+, the response is small but greater than when Na^+ is also reduced to a half. The results obtained in our experiments have been already analysed during the description of the action of this ion.

When the *o.p.* is not counterbalanced, the complete absence of Na^+ or its presence in a concentration equal to the half of the original quantity, provokes a relaxation of the uterine muscle, and this is just the contrary of what is observed when the *o.p.* of the solution is mantained at the right level (Gr. 7). The ratio Na^+/K^+ is equal 13.66 in the experiments in which the concentration of Na^+ is half of the original, and without compensation, and in the ones in which there was compensation. Therefore the results differ when the uterine muscle of the virgin rat is placed in different ionic and osmotic conditions.

When the ratio Na^+/K^+ is varied and *o.p.* is properly compensated, the results are different from the ones obtained in the experiments in which the referred ratio is the same, while *o p.* is not counterbalanced. Our results are similar to those obtained by GOMES DA COSTA in the case of the complete absence of K^+ in Tyrode's solution, although the composition of Tyrode solution then used is not indicated.

It must be emphasized that in those experiments, GOMES DA COSTA did not really compensate the ion withdrawal, because when 0.012% of NaCl was added the expected rest came later. When the rate of K^+ was twofold without compensation was employed the uterus rested after 4.5 hours, and with compensation only after 7.5 hours.

When a twofold, or fourfold of ion concentration is used as it was done by GOMES DA COSTA, the balanced ratio of the ions is strongly altered, mainly the ratios Na^+/K^+ and K^+/Ca^{++}; on the other hand, it seems not reasonable that the compensation should be always made in the same way,, that is, the withdrawal of one ion is compensated by adding 0.012% of NaCl while his twofolding ou fourfolding is compensated with a decreasing of 0.012% NaCl.

In the fourfolding of the ion K^+, a rest is observed after 2 to 2.5 hours. In the experiments without compensation with overpassing normal concentration of the ion, the results were the following:

a) concentrations up to 1.4g/1 (nearly 3.5 times the normal) stimulate the uterine contractions; b) 1.5g/1 induces a blockage and c) 1.7g/1 provokes a sudden and complete blockage (1.7 = fourfold). The time for the complete blockage was never superior to 30 minutes, as can be seen in record nr. 8.

Apud CLARK, KNAUS and PARKES (1926), twofolding ou threefolding the amount of KCl of Locke's fluid (which is the same as in the Tyrode's) has an effect almost of estimulation of the rat's uterus identical with that produced by the pituitary extracts. The excess of KCl causes an increasing in the frequency of the contractions, while its withdrawn provokes also as an early effect, that is, an increasing of the frequency of the contractions.

In the first case there is a facilitation in the conduction, resulting in contractions of higher amplitude; in the second, there occurs a decrease in the rate of conduction, the contractions being of lower amplitude.

BLAIR-BELL and co-workers verified that the increasing or decreasing of theK^+ contents within reasonable limits, do not produce remarkable alterations of the uterine movements. *In vivo* injections 2 ml of a solution 5% KCl have produced discordant results even in the same animal, for they observed either stimulation or inibition of the contraction.

VAN DYKE and **HASTINGS** using the pituitary extracts as stimulator, verified that the reduction or the complete withdrawn of the K^+ ions causes a decreased response to the pituitrine (inactive uterus). They observed that generally an increasing in the K^+ concentration is sufficient to alter the response of the uterus to pituitrin, starting a sequency of rithmic contractions.

Ca^{++} ions are considered specific for the uterus, as already shown. The isolated uterine muscle is more susceptible to Ca^{++} than to the other ions. The addition of Ca^{++} to the bath induces activity in an inactive uterus. Referring to the absence of Ca^{++}, all authors agree that it brings the uterine muscle into relaxation. Above the normal rate Ca^{++} provokes a resting in contracture. **GOMES DA COSTA** doubling the rate of Ca^{++} (without compensation) got a stop in contracture, which was longer when he used compensation. The same consideration about this ion can be made in the case of the ion K^+. **VAN DYKE** and **HASTINGS** also did not detect any response to pituitrin, when Ca^{++} is missing in the solution. The responses decrease when the Ca^{++} content decreases in the fluid. Inversely, increasing the concentration of the ion an increasing of the contractions is also observed.

BLAIR-BELL and co-workers suggested that the quantity of Ca^{++} in the blood below the normal is probably a factor that causes a simple primary inertia.

In vitro it was demonstrated that varying the rate of Ca^{++} to 25%, 33%, 50%, 66% an 90% less than in the normal Locke's fluid, the contractions becomes lower and lower.

The results here obtained with the Mg^{++} ion suggests that that ion is a modifier of the Ca^{++} ion action. It also antagonizes, partially, the Na^+ and the K^+ action, because it is capable of bringing about a relaxation of a muscle in contracture or, at least, to delay the blockage.

REYNOLDS in living unanesthetized rabbits, verified that the Mg^{++} ion inhibits the sustained contraction induced by Ca^{++} and prevents any further answers.

4. *Summary*

1) The action of Na^{+}, K^{+}, Ca^{++} and Mg^{++} on the mechanics of the albino rat (Wistar) uterus was studied. Whenever a certain ion concentration was decreased, the osmotic effect was compensated with glucose so that throughout the experiments a same osmotic pressure of the perfusing fluid was maintained.

2) Of each of the ions studied, four solutions were prepared: normal, one quartel, half and three quarters of the original concentration. Care was also taken to maintain the same pH in all cases. The following results were obtained, when a modified Tyrode (Mg free) was taken as a starting point, and during the various phases of the oestrous cycle.

a) Absence of Na^{+} induces a blockage in contracture, reversible as the concentration of the ion gradually increases again. With 1/4 of its original concentration spontaneous contractions reappear. (Gr. 2).

b) Absence of K^{+} causes a sustained contraction similar to that obtained in the absence of Na^{+}, but whereas in the case of Na^{+} the height of the contractions prior to contracture is not altered, in the case of K^{+} a tetanic type of contracture occurs. As K^{+} increases again contractions reappear. With 1/4 they return to the basal line, but curiously with half and with three quarters there occured a decrease in amplitude and frequency. The complete Tyrode finally restored the normal rhythm.

c) Absence of Ca^{++} induces a complete relaxation of the uterus and prolonged immersion in calcium free Tyrode makes impossible the return to normality. This one can be obtained if immediately after exposing the uterus to a soluction free form Ca^{++} increasing concentrations of the ion are used.

d) Ausência de Mg^{++} não induz mudanças perceptíveis no ritmo do útero pois não foram observadas diferenças quando se usou Tyrode completo. Entretanto, em presença de Mg^{++} as contraturas obtidas com ausência de Na^{+} ou K^{+} e o relaxamento induzido pela ausência de Ca^{++}, se tornam grandemente retardadas.

5. *Resumo*

1) Foram estudadas as ações dos ions Na^{+}, K^{+}, Ca^{++} e Mg^{++} sôbre a mecânica uterina de ratos albinos (Wistar). Sempre que a concentração de um certo ion foi diminuída, o efeito osmótico foi compensado com glicose, a fim de que fôsse mantida a mesma pressão osmótica (o. p.) em tôdas as experiências.

2) De cada um dos ions estudados foram preparadas 4 soluções: normal, 1/4, 1/2 e 1/3 da concentração original. Tomouse cuidado para que o mesmo pH fôsse mantido em todos os casos. Usou-se como solução fisiológica um Tyrode modificado (sem Mg). Os seguintes resultados foram obtidos durante as várias fases do ciclo estral.

a) Ausência de Na^{+} provoca um bloqueio em contratura que é reversível à medida que a concentração do ion aumenta gradualmente. Com 1/4 da sua concentração original as contrações espontâneas reaparecem.

b) Ausência de K^{+} causa uma contratura sustentada semelhante àquela obtida na ausência de Na^{+}, mas enquanto que no caso do Na^{+} a altura das contrações anteriores à contratura não é alterada, no caso de K^{+} ocorre um tipo de contratura tetânica. À medida que a concentração de K^{+} aumente novamente, as contrações reaparecem. Com 1/4 da concentração normal elas voltam à linha de base. Curiosamente, com 1/2 e 3/4 ocorreu uma diminuição da amplitude e freqüência. O Tyrode completo restabeleceu o ritmo normal.

c) Ausência de Ca^{++} induz ao completo relaxamento do útero e a permanência prolongada num Tyrode sem Ca torna impossível a volta à normalidade, a qual pode ser obtida se imediatamente após a exposição do útero à ausência de Ca, se usam concentrações progressivamente maiores do ion.

d) Absence of Mg^{++} induces no noticeable changes in the rhythm of the uterus, for no differences were observed when complete Tyrode was used. However, in the presence of Mg^{++}, the contracture obtained in the absence of Na^{+} or K^{+} and the relaxation induced by the absence of Ca^{++} are greatly delayed.

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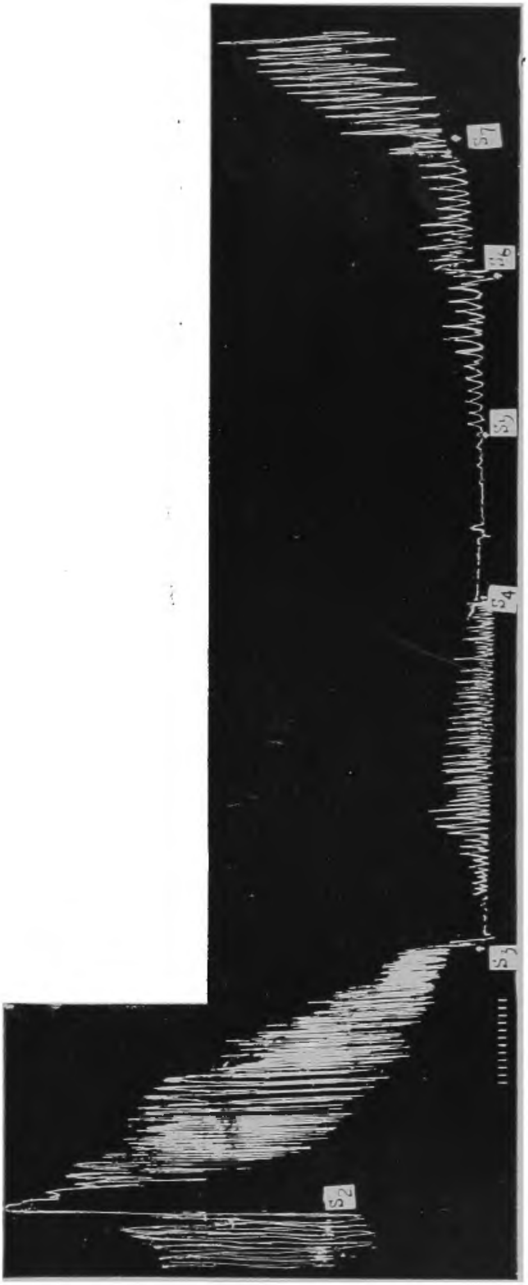
GRÁFICOS

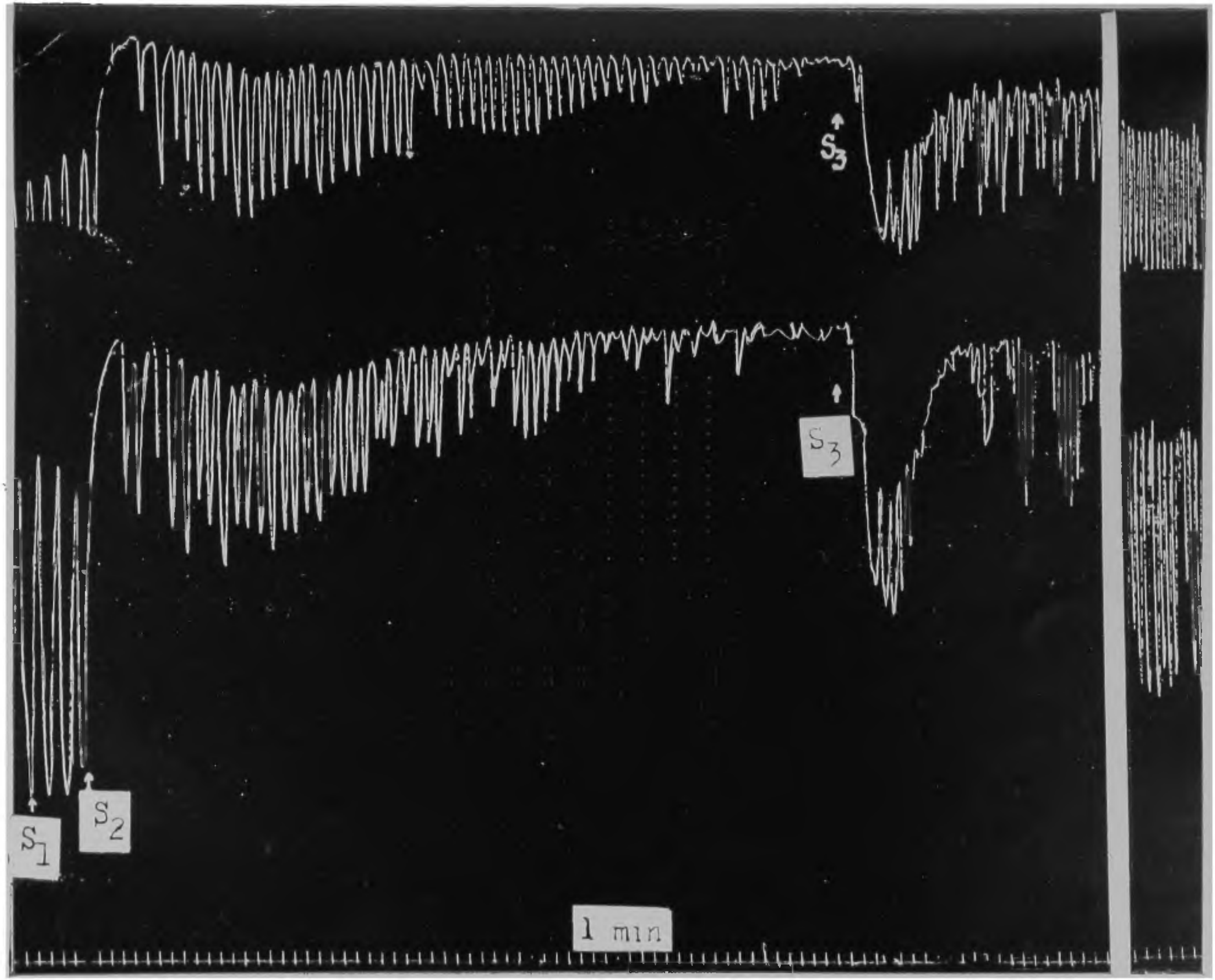
GRAPH 1

S_1 = Locke	
NaCl	9.15 g/1
KCl	0.42 g/1
$CaCl_2$	0.24 g/1
$NaHCO_3$	0.15 g/1
Glicose	1.00 g/1
S_2 = 25 ml NaCl	91.5%.
S_3 = S_2 + 2.5 ml KCl	4.2%.
S_4 = S_3 + 50 ml $CaCl_2$	0.24%.
S_5 = S_4 + 0,25 g glucose	
S_6 = S_1	
S_7 = S_1	


S7
S6
S5
S4
S3
S2

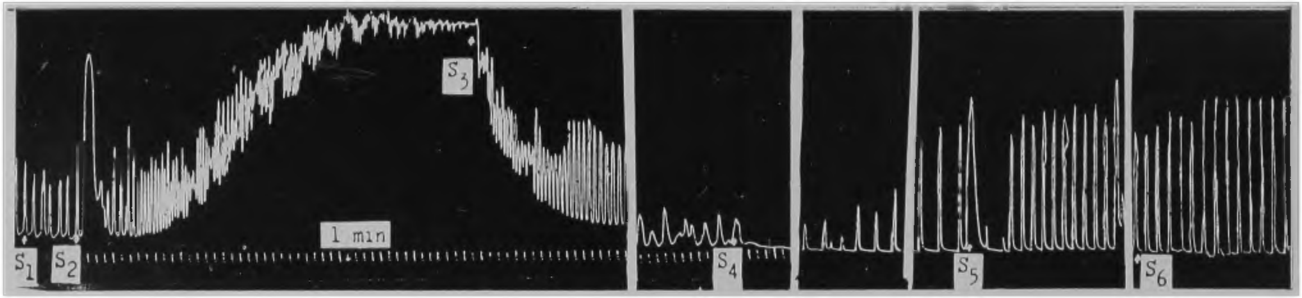
GRAPH 2

S_1 = NaCl 9.00g/1
KCl 0.42g/1
$CaCl_2$ 0.06g/1
$NaHCO_3$ 0.5g/1
S_2 = S_1 − Na + 51.3g/1 glucose
S_3 = S_1 + 1/4 Na + 38.4g/1 glucose


S3
S3
S1
S2
1 min

GRAPH 3

S_1 = NaCl 9.00g/1
KCl 0.42g/1
$CaCl_2$ 0.06g/1
$NaHCO_3$ 0.5 g/1
$S_2 = S_1 - K + 0.18$g/1 glucose
$S_3 = S_1 + 1/4\ K + 0.135$g/1 glucose
$S_4 = S_1 + 1/2\ K + 0.09$ g/1 glucose
$S_5 = S_1 + 3/4\ K + 0.045$g/1 glucose
$S_6 = S_1$


S1
S2
1 min
S3
S4
S5
S6

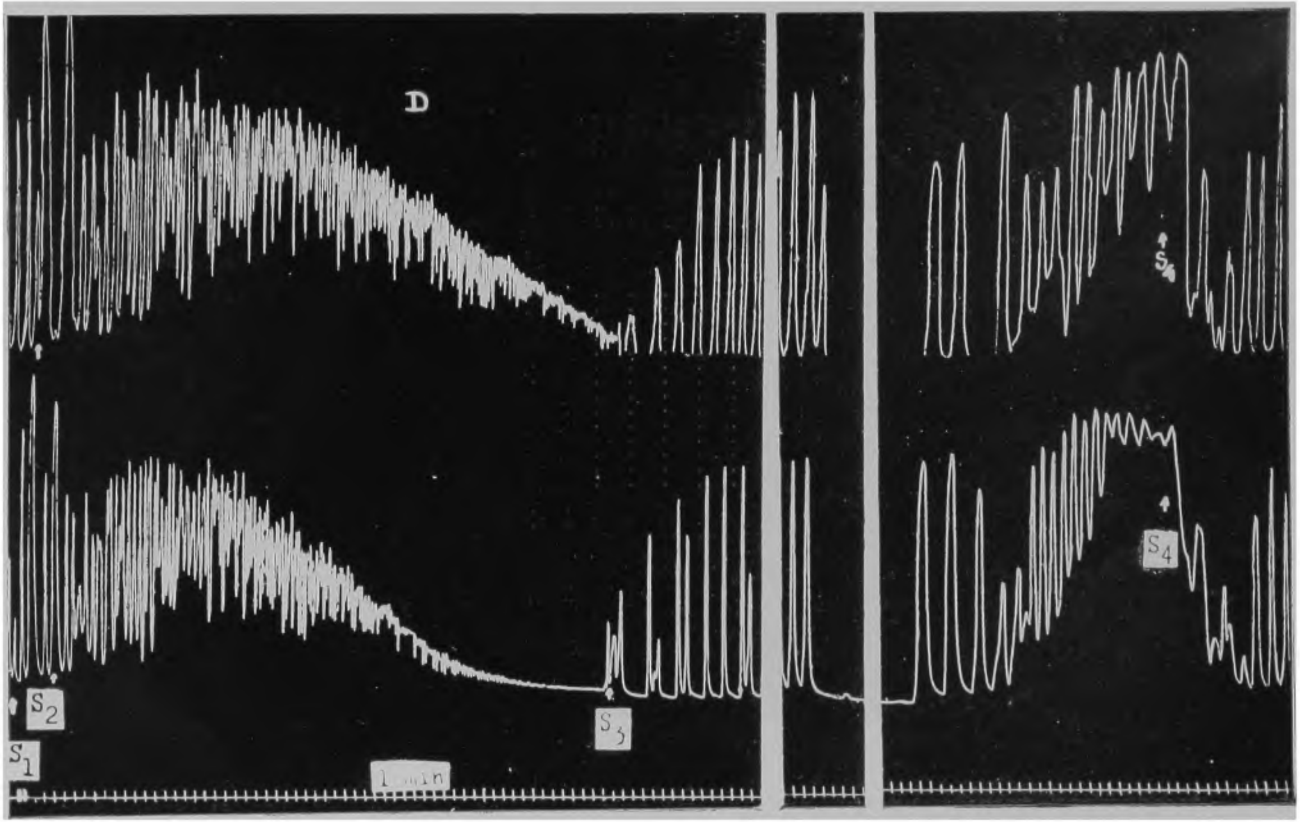
GRAPH 4

$S_1 =$	$NaCl$	9.00g/1
	KCl	0.42g/1
	$CaCl_2$	0.06g/1
	$NaHCO_3$	0.5 g/1

$S_2 = S_1 - Ca + 0.198$g/1 glucose

$S_3 = S_1 + 3/4\ Ca + 0.049$g/1 glucose

$S_4 = S_1$

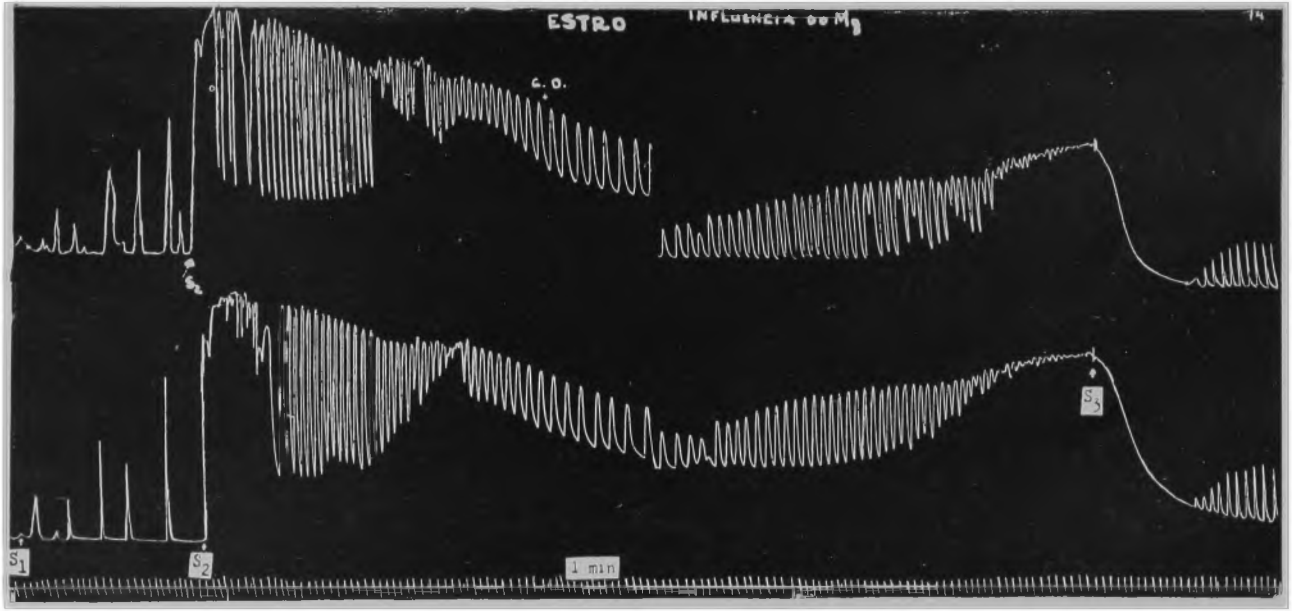

D
S4
S1
S2
S3
S4
1 min

GRAPH 5

S_1 =	$NaCl$	9.00 g/1
	KCl	0.42 g/1
	$CaCl_2$	0.06 g/1
	$MgCl_2$	0.005g/1
	$NaHCO_3$	0.5 g/1

$S_2 = S_1 - Na + 51.3g/1$ glucose

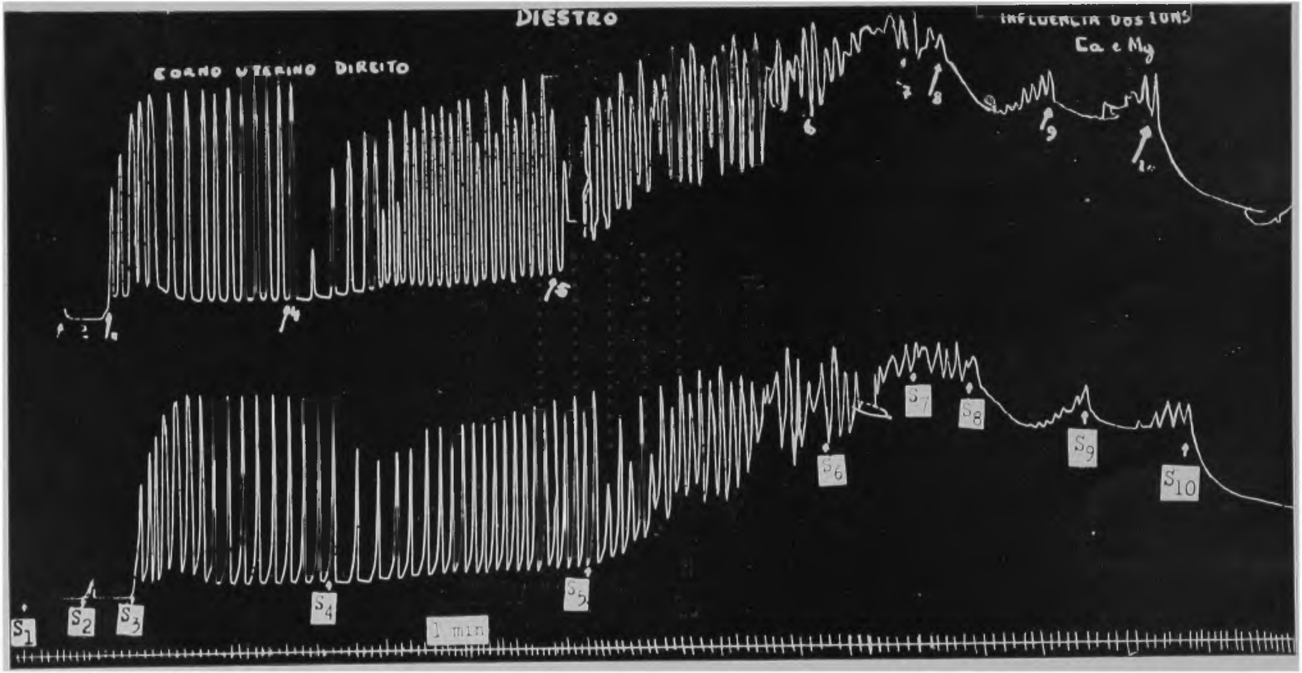
$S_3 = S_1 + 1/4\ Na + 38.4g/1$ glucose


ESTRO
INFLUENCIA DO Mg
14
C. O.
S2
S1
S2
1 min
S3

GRAPH 6

$S_1 = S_2 =$ NaCl 9.00g/1
KCl 0.42g/1
$CaCl_2$ 0.06g/1
$NaHCO_3$ 0.5 g/1

$S_3 = S_2 +$ 1 ml $CaCl_2$ 4%
$S_4 = S_3 +$ 0.5 ml $CaCl_2$ 4%
$S_5 = S_4 +$ 0.5 ml $CaCl_2$ 4%
$S_6 = S_5 +$ 0.5 ml $CaCl_2$ 4%
$S_7 = S_6 +$ 2 ml $MgCl_2$ 2%
$S_8 = S_7 +$ 5 ml $MgCl_2$ 2%
$S_9 = S_8 +$ 5 ml $MgCl_2$ 2%
$S_{10} = S_9 +$ 10 ml $MgCl_2$ 2%

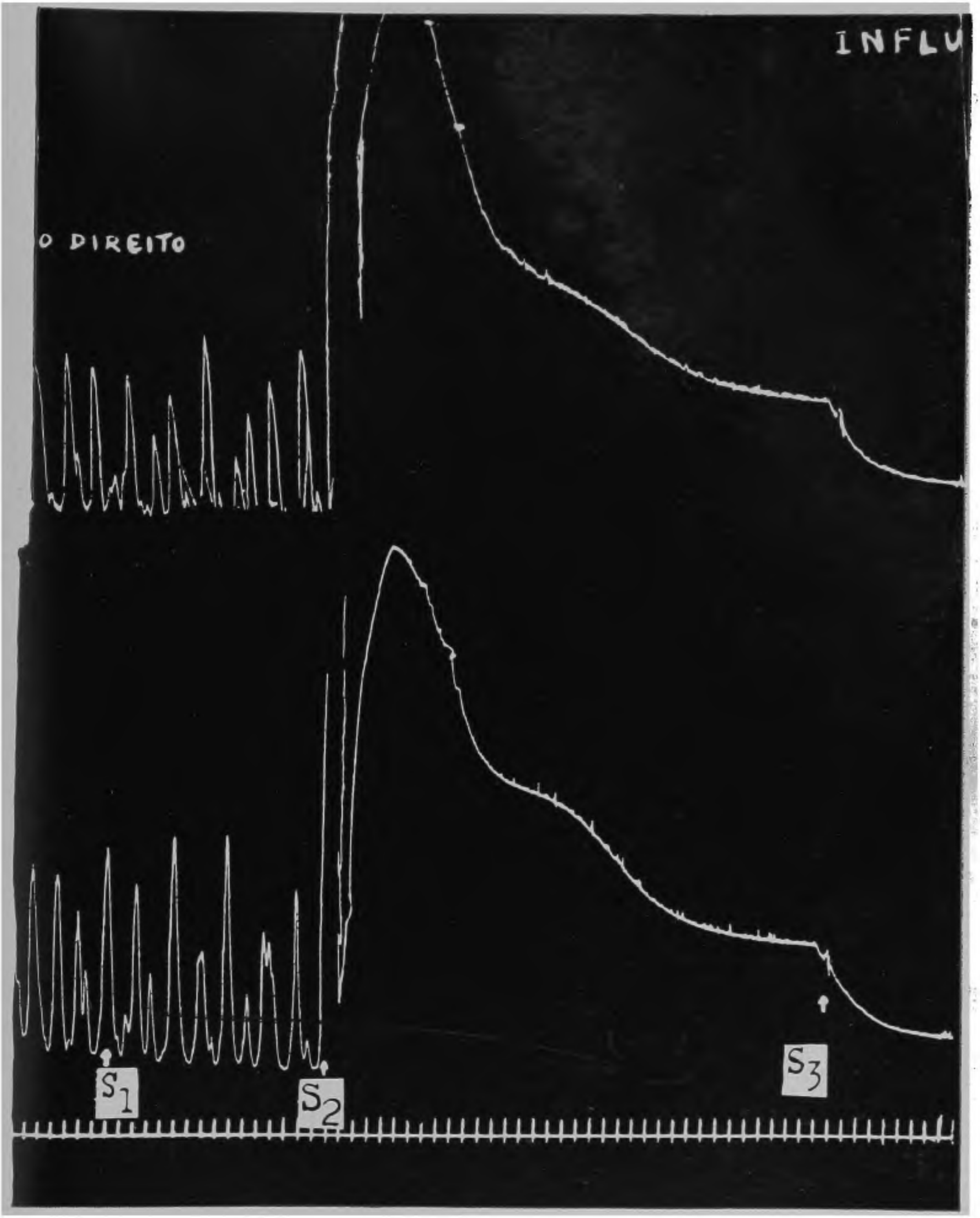

DIESTRO
INFLUENCIA DOS IONS
Ca e Mg
CORNO UTERINO DIREITO
4
5
6
7
8
9
10
S1
S2
S3
S4
S5
S6
S7
S8
S9
S10
1 min

GRAPH 7

S_1 =	NaCl	9.00 g/1
	KCl	0.42 g/1
	$CaCl_2$	0.06 g/1
	$MgCl_2$	0.005g/1
	$NaHCO_3$	0.5 g/1

$S_2 = S_1 - NaCl$

$S_3 = S_2 +$ 1 ml NaCl 9.g/1


INFLU
O DIREITO
S1
S2
S3

GRAPH 8

S_1 =	NaCl	9.00 g/l
	KCl	0.42 g/l
	$CaCl_2$	0.06 g/l
	$MgCl_2$	0.005g/l
	$NaHCO_3$	0.5 g/l

S_2 = S_1 + 1 ml $CaCl_2$ 0.24%

S_3 = S_1 0.5 ml KCl 4.2%

S_4 = S_1 + 0.5 ml KCl 4.2%

L = washing with S_1

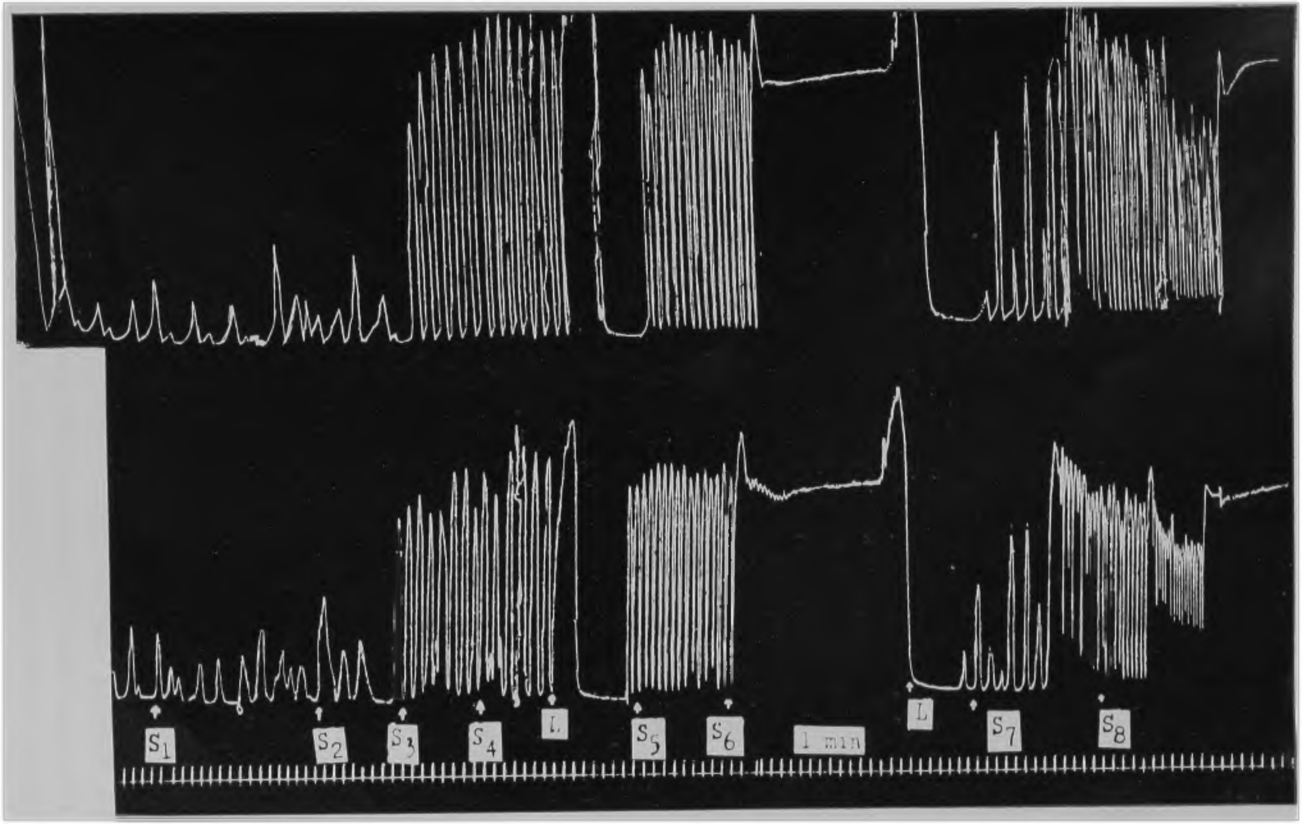
S_5 = S_1 + 1 ml KCl 4.2%

S_6 = S_5 + 0.5 ml KCl 4.2%

L = washing with S_1

S_7 = S_1 + 1 ml KCl 4.2%

S_8 = S_1 + 0.5 ml KCl 4.2%


S_1
S_2
S_3
S_4
L
S_5
S_6
1 min
L
S_7
S_8