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STUDIES ON DEMINERALIZATION OF BONE*

II. The effect of electrolytic techniques in demineralization.

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In 1947, Richman, Gelfand, and Hill[3] introduced the electrolytic method for demineralization of bone. This technique has received widespread acceptance because it is reputed to demineralize bone more rapidly than any other method.

Recently, Lillie, et al.[1], and Rosen and Friedland[4] have presented evidence that the decreased time of demineralization by the electrolytic method was due to an increase in solution temperature.

The present experiments were designed to determine whether electrolysis does shorten the time of demineralization under defined conditions with all known variables, except the electric current, constant.

MATERIALS AND METHODS

1. For the experiments, an electrolytic machine was constructed which controlled the electric current, the temperature of the demineralizing solution, and the rate of agitation. The apparatus was constructed in three parts: (a) a rotating electrode carrier and a solution container, (b) a source of direct current, and (c) a constant temperature bath (cf. Figure 1).

a. Electrode carrier: The electrode carrier was constructed of two lucite discs which were connected by an aluminum shaft and bearing. The lower disc rotated in the bearing mounted in the upper disc; electrical contact was maintained during rotation by a commutator of copper rings and silver brushes. Mounted on the lower disc of the carrier was a thermometer, a mercury thermostat, and two platinum electrodes. The electrodes were made of 16 gauge platinum wire wound to a 1 centimeter diameter; the electrodes were placed 1 centimeter apart. The amount of platinum wire submerged in the solution was the same for both electrodes.

b. Electric current: The source of direct current was from a Heath powerpack which gave controlled wattage from 0 to 12 volts at 0 to 8 amperes.

c. Solution temperature control: The temperature of the electrolytic solution was controlled by circulating water through a coil of 5 mm polyethylene tubing in the container. The polyethylene coil was connected to a constant temperature bath through a pump which was controlled by the thermostat mounted in the electrode carrier. The demineralizing solution was maintained by this method at $\pm$ 1°C. of the desired temperature.

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2. Bone samples for the experiments were dense cortical bone from the shaft of an air-dried, adult, human femur. The chemical analysis of this standard bone has been previously reported (cf. Materials and Methods, Morris and Benton[2]).

3. One hundred milliliters of an acid solution of known molarity were used for demineralization.

4. Determinations of acid concentration, calcium, phosphorus, and nitrogen were made on all solutions before and after demineralization. The technical methods used were described in the section on Materials and Methods of a previous paper (Morris and Benton[2]).

5. Other conditions were:

   a. Bone samples were placed in the coil of the anodal electrode and moved through the solution at 25 revolutions per minute.

   b. X-ray pictures were taken of the bone samples during the process of demineralization.

   c. When the bone samples were removed to be weighed or X-rayed, they were blotted on bibulous paper by the same investigator each time; the weighings were made by the other investigator to keep errors constant. Repeated dryings and weighings have shown this error to be not more than ± 1 mg.

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EXPERIMENTS

Experiment 1: To determine if demineralizing conditions in the electrolysis machine are comparable to those previously used.

Two pieces of the standard bone were shaped to approximately the same weight and surface area.

Data:	Sample 1	Sample 2
Weight	232 mg	230 mg
Surface Area	1.65 sq. cm.	1.63 sq. cm.

The samples were demineralized separately in the electrolytic machine without electrolysis in 1 molar hydrochloric acid for 2 hours at a solution temperature of 25°C. The bone samples were weighed at the end of 2 hours of demineralization and X-ray pictures taken. The depth to which mineral had been removed was measured from the X-ray negatives.

At the end of the experiment, the bone samples were digested in 25 ml of concentrated hydrochloric acid and the calcium, phosphorus, and nitrogen concentrations determined.

RESULTS:

The data are summarized in Table I. At the end of 2 hours of demineralization, Sample 1 had a weight loss of 53 mg and Sample 2 had a weight loss of 49 mg. The weight of mineral removed from the two samples differed by 1 mg. There was no difference in the amount of nitrogen removed or in the depth to which mineral had been removed in the two samples.

The weight of mineral determined after digestion of the two samples differed by 1 mg. The total amount of mineral in each bone sample was the same.

The reduction in the molarity of the acid during demineralization of these bone samples was so low that it was considered to be nil.

Experiment 2: The effect of electrolysis upon the rate of demineralization with hydrochloric acid.

Two pairs of bone samples were used: one pair of bone samples was demineralized at 25°C. with and without electrolysis (Experiment 2A); the second pair of bone samples was demineralized at a temperature of 45°C. with and without electrolysis (Experiment 2B).

Experiment 2A (25°C): Two pieces of standard bone were shaped to approximately the same weight and surface area.

Data:	Electrolytic Sample	Control Sample
Weight	185 mg	174 mg
Surface Area	1.56 sq. cm.	1.56 sq. cm.

Experiment 2B (45°C): Two pieces of standard bone were shaped to approximately the same weight and surface area.

Data:	Electrolytic Sample	Control Sample
Weight	193 mg	191 mg
Surface Area	1.67 sq. cm.	1.66 sq. cm.

The samples were demineralized for 2 hours in 1 molar hydrochloric acid at the experimental temperatures. The electrolysis samples were demineralized with 5 volts at 3 amperes to give a 15 watt electrolysis. Acid changes, weighings, and X-ray pictures were made every half-hour. The depth to which mineral had been removed was measured on the X-ray negatives.

RESULTS:

The data are summarized in Table II. In Experiment 2A (25°C.) after 2 hours of demineralization, the electrolysis sample had a weight loss of 47 mg, and the control sample had a weight loss of 48 mg. The amount of mineral removed from the electrolytic sample was 43 mg, and from the control sample 42 mg. In both weight loss and mineral removed, the electrolytic and control samples differed by 1 mg.

In Experiment 2B (45°C.) after 2 hours of demineralization, the electrolysis sample had a weight loss of 69 mg and the control sample had a weight loss of 66 mg. The amount of mineral removed from the electrolysis sample was 56 mg, and from the control sample 53 mg. In both weight loss and mineral removed, the electrolytic and control samples differed by 3 mg.

The molarity of the acid used for demineralization was determined at the end of each half-hour. The reduction of the acid concentration during demineralization in the electrolysis solutions was from 1.0 to 0.7 molar in Experiment 2A (25°C.) and from 1.0 to 0.6 molar in Experiment 2B (45°C.); the reduction in molarity in the control solutions was from 1.0 to 0.99 molar. There was no difference in the amount of nitrogen removed in the electrolysis and control samples.

The depth to which mineral was removed was the same for both electrolysis and control samples in Experiment 2A and 2B.

Experiment 3: The effect of electrolysis upon the rate of demineralization with phosphoric acid.

Two pairs of bone samples were used in this experiment. One pair of bone samples was demineralized at 25°C. (Experiment 3A), the second pair of bone samples was demineralized at 45°C. (Experiment 3B). One sample from each pair was demineralized without electrolysis.

Experiment 3A (25°C.): Two pieces of standard bone were shaped to approximately the same weight and surface area.

Data:	Electrolysis Sample	Control Sample
Weight	134 mg	130 mg
Surface Area	1.26 sq. cm.	1.28 sq. cm.

Experiment 3B (45°C.): Two pieces of standard bone were shaped to approximately the same weight and surface area.

Data:	Electrolysis Sample	Control Sample
Weight	141 mg	142 mg
Surface Area	1.32 sq. cm.	1.34 sq. cm.

The bone samples were demineralized for 3 hours in 1 molar phosphoric acid at the experimental temperatures. The electrolysis samples were demineralized with 10 volts at 1.5 amperes to give a 15 watt electrolysis. Acid changes, weighings and X-rays were made at half-hour intervals. The depth to which mineral had been removed was measured from the X-ray negatives.

RESULTS:

The data are tabulated in Table III. In Experiment 3A (25°C.) the difference in weight loss between the electrolysis and control samples at the end of 3 hours of demineralization was 2 mg. In Experiment 3B (45°C.), the difference in weight loss between the electrolysis and control samples at the end of 3 hours of demineralization was 3 mg. The mineral determinations were not reported in this experiment because the large amount of phosphorus in the solutions made the results of doubtful value.

The reduction in molarity in the solutions during demineralization was so small as to be within the error of measurement. There was no difference in the amount of nitrogen removed in the electrolysis and control samples. The depth to which mineral was removed was the same for both the electrolytic and control samples.

Experiment 4: The effect of electrolysis upon the rate of demineralization with formic acid.

Two pieces of standard bone were shaped to approximately the same weight and surface area.

Data:	Electrolysis Sample	Control Sample
Weight	404 mg	401 mg
Surface Area	2.33 sq. cm.	2.37 sq. cm.

The bone samples were demineralized in 1 molar formic acid at 25°C. for 3 hours. The electrolysis sample was demineralized with 12 volts at 0.5 ampere to give a 6 watt electrolysis. Weighings and X-ray pictures were made at the end of 3 hours. The depth to which mineral had been removed was measured from the X-ray negatives.

RESULTS:

The data are tabulated in Table IV. The bone samples lost the same amount of weight, 7 mg, and the same amount of mineral was removed, 21 mg.

The weight of mineral removed in this experiment was much greater than the weight loss of the bone samples. This results when mineral acids of low molarity (below 0.5 molar) or weakly ionizing acids are used for demineralization and is due to the hydration of the bone matrix. (For further discussion of this phenomenon see Morris and Benton[2].)

The depth of mineral removal was the same for both samples.

Reduction in molarity of the acid solutions during demineralization was within the error of the titration for both the electrolysis and control samples. There was no difference in the amount of nitrogen removed from the electrolysis and the control samples.

DISCUSSION

The theoretical basis of the electrolytic method of demineralization is that a polarizing current will cause an increased rate of migration of the mineral ions from the bone sample to the electrical pole of opposite charge. During demineralization, the primary mineral element to be removed from bone is the divalent, positively charged calcium ion. The bone minerals cannot be removed any faster than they are released from their combination within the bone. The removal of bone minerals, under any circumstances, cannot be more rapid than the breakdown of the mineral complex by the demineralizing acid. In order to break down the mineral complex in bone, the acid must diffuse through the demineralized bone matrix and come in contact with the mineral complex.

Hydrogen ions are positively charged; calcium ions are positively charged. It is difficult to understand how a polarizing current can cause two positively charged ions to migrate in opposite directions; the hydrogen ion into the bone, and the calcium ion out of the bone.

On theoretical grounds, it must be concluded that the electrolytic method of demineralization cannot accomplish its purpose because:

1. minerals cannot be removed any faster than the minerals are released from their complex within the bone by the acid and,

2. the electrolysis, rather than increasing the diffusion of the acid into the bone, probably tends to the opposite result.

Thus, if the electrolytic method of demineralization does decrease the demineralization time, the result is due to some cause other than the applied polarizing current.

For the present experiments, the range of difference in weight loss and weight of mineral removed from paired samples of standard bone under the conditions used in the electrolytic machine had to be determined. In previous experiments under standard conditions (cf. Morris and Benton[2]), the variation in weight loss per unit time was from 0 to 5 mg for approximately matched bone samples. This difference, 5 mg, is due to the difficulty in shaping bones to identical weight and surface area; the slight differences in surface area, which often are beyond the range of accurate measurement, can cause the small differences found in the experimental results.

Experiment 1 was made to determine whether the difference in weight loss and mineral removed from matched samples of standard bones was the same in the electrolytic machine as that

found under the standard conditions previously reported (cf. Morris and Benton[2]). The data from this experiment showed the difference in weight loss to be 4 mg and the difference in mineral removed from matched samples was 1 mg. The difference in surface area of the two samples was 0.02 square centimeter; the bone with the greatest weight loss had the greatest surface area. These results were comparable to the differences found under the previously established standard conditions.

In Experiment 2, the weight loss and the weight of mineral removed from matched samples of bone, with and without electrolysis, in 1 molar hydrochloric acid was determined. At 25°C. the samples differed in weight loss by 1 mg when the surface areas were the same; at 45°C. the samples differed by 3 mg where the surface areas differed by 0.01 square centimeter. The 3 mg difference at 45°C. was in favor of the bone with the greater surface area. Thus, the bone samples demineralized with equal rapidity with and without electrolysis if all other conditions were made constant.

During electrolysis with hydrochloric acid, hydrogen chloride gas was evolved, and this, as shown by titrations of the remaining acid, reduced the acid concentration. Because this loss of acid might be a possible cause for the lack of effect of the electrolytic method with hydrochloric acid, two non-gas-forming acids were used, phosphoric and formic acids. In both experiments (cf. Experiments 3 and 4) the differences between the electrolytic and control samples were within the expected range of variation. The difference in weight loss with formic acid was 0. A 3 mg difference in weight loss was found with phosphoric acid; the greater weight loss was in favor of the bone with the greater surface area. Thus, the loss of hydrochloric acid during electrolysis did not affect the time necessary for demineralization. Previous experiments (cf. Morris and Benton[2]) showed that the reduction of concentration of hydrochloric acid from 1.0 to 0.6 molar does not appreciably affect the rapidity of demineralization.

Electrolysis produces heat in the acid solution because of the solution resistance to the flow of current. Experiments with the electrolytic machine used in these studies showed that a 0.1°C. rise in temperature was produced per minute per watt of electrolysis in 100 ml of 1 molar hydrochloric acid solution. The amount of heat produced will vary with the resistance of the solution, but 1 molar concentrations of several acids (phosphoric, formic, and trichloroacetic) showed about the same temperature rise of 0.1°C./minute/watt/100 ml of solution during electrolysis. In a previous report (cf. Morris and Benton[2]), the influence of solution temperature on demineralization was determined, and it was found that an increase of 20°C. (an increase from 25° to 45°C.) decreased the time necessary for demineralization by 15 to 20%.

In the present experiments, an increase in temperature also decreased the time necessary for demineralization. Because the

bone samples used for the various experiments were of different weights and surface areas, the data have been expressed as milligrams of weight loss per square centimeter of surface area for comparison (cf. Table V.). These data show that after two hours of demineralization in 1 molar hydrochloric acid, a difference in weight loss of 0.1 mg/cm^2 of surface area was found between the 25° and 45° C. samples. A similar difference of 0.05 mg/cm^2 of surface area was found between the 25° and 45°C. samples demineralized for 3 hours in 1 molar phosphoric acid. Thus, the increased weight loss with the increased temperature was in approximately the same range as previously determined and decreased the demineralization time about 20%.

These experiments have shown that the electrolytic method of demineralization will increase the solution temperature. At 15 watts electrolysis, the temperature of 100 ml of 1 molar hydrochloric acid solution will increast 15° C. in 10 minutes. The demineralization will then proceed at 40° C. or higher. If the time necessary for demineralization at 40° C. is compared with the time necessary for demineralization at 25° C., the electrolytic method will appear to be more rapid simply due to the temperature increase. When the temperature was controlled, the electrolytic and control samples demineralized at the same rate. Electrolysis produces heat but, per se, is ineffective as a demineralizer.

SUMMARY

1. The theoretical concept of electrolysis is unsound because it demands that two positively charged ions migrate in opposite directions in response to the same polarizing current: the H^+ into the bone, and the Ca^{++} out of the bone.

2. Electrolysis per se has no effect upon decreasing the time of demineralization.

3. In electrolytic baths, heat will be produced; increase in solution temperature will decrease the time necessary for demineralization of bone. These controlled experiments demonstrate that the reported decreases in the time necessary for demineralization when using the electrolytic method are not due to electrolysis but are due to the increase in temperature of the demineralizing solution caused by current going through it.

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TABLE I

Data showing bone weight, weight loss, percent weight loss, and weight of mineral removed from two pieces of bone of similar weight and surface area under control conditions as described in Experiment 1.

Time (hours)	Weight Loss (mg)		Percent Wt. Loss		Mineral Removed (mg/100 mg)			
					Ca		P	
	#1	#2	#1	#2	#1	#2	#1	#2
0	(232)	(230)						
2	53	49	23%	22%	34	33	12	12
Complete Digestion					29	30	11	11

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TABLE II

Data showing total weight loss, percent weight loss, and total milligrams of calcium and phosphorus removed from bones of similar weight and surface area after 2 hours demineralization in 1 molar hydrochloric acid at 25° C. and 45°C. with and without electrolysis as described in Experiment 2, parts A and B.

Electrolysis Sample (15 watts)						Control Sample				
Time (hours)	Bone Wt. Loss (mg)	Percent Wt. Loss	Mineral Removed (mg)			Bone Wt. Loss (mg)	Percent Wt. Loss	Mineral Removed (mg)		
			Ca	P	Ca+P			Ca	P	Ca+P
Experiment 2A (25°C.)										
0 c	(185)					(174)				
2	47	25%	30	13	43	48	27%	31	11	42
Experiment 2B (45°C.)										
0	(193)					(191)				
2	69	36%	42	14	56	66	35%	39	14	53

TABLE III

Data showing the weight loss, and percent weight loss of bones of similar weight and surface areas demineralized in 1 molar phosphoric acid at 25° and 45°C. with and without electrolysis as described in Experiment 3A and 3B.

	Electrolysis Samples (15 Watts)		Control Samples	
Time (hours)	Wt. Loss (mg)	Percent Wt. Loss	Wt. Loss (mg)	Percent Wt. Loss
	---------- Experiment 3A (25°C.) ----------			
0	(134)		(130)	
3	24	18%	22	17%
	---------- Experiment 3B (45°C.) ----------			
0	(141)		(142)	
3	31	22%	28	20%

TABLE IV

Data showing bone weight, weight loss, percent weight loss, and weight of mineral removed from two pieces of bone of similar weight and surface area as described in Experiment 4.

	Electrolysis Sample (6 Watts)					Control Sample				
Time (hours)	Wt. Loss (mg)	Percent Wt. Loss	Total Mineral Removed (mg)			Wt. Loss (mg)	Percent Wt.Loss	Total Mineral Removed (mg)		
			Ca	P	Ca+P			Ca	P	Ca+P
0	(404)					(401)				
3	7	1%	14	7	21	7	1%	14	7	21

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TABLE V

Data from Experiments 2 and 3 showing the difference in amount of demineralization of two matched bone samples at 25°C. and 45°C. after two hours of demineralization in 1 molar hydrochloric acid (Experiment 2) or after three hours of demineralization in 1 molar phosphoric acid (Experiment 3). Because the samples varied in weight and surface area, the data are expressed as milligrams of weight loss/square centimeter of surface area for comparison.

Temperature (°C.)	Wt. Loss (mg)/cm^2 of Surface Area	
	Exp. 2	Exp. 3
25°C.	0.30	0.18
45°C.	0.40	0.23

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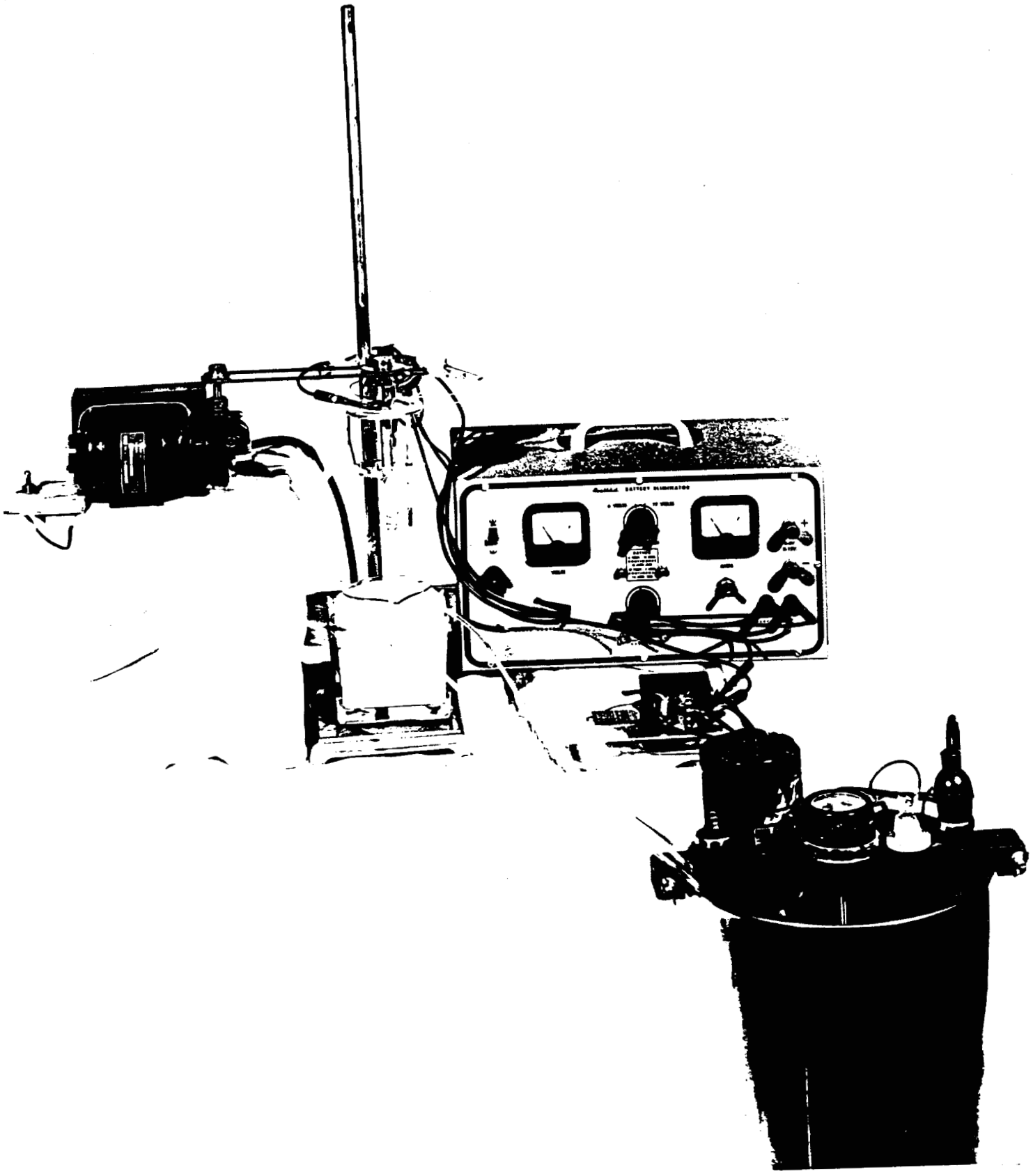

Figure 1.

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TABLE II

Average Nitrogen in milligrams per hundred milliliters determined for various acid solutions in Experiment 2.

Hydrochloric	-	8.5
Formic	-	9.5
Phosphoric	-	12.3
Trichloroacetic	-	28.2

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