Botany. — *The value of plasmolytic methods for the demonstration of the active asparagine intake by Vallisneria leaves.* By W. H. ARISZ and P. J. S. VAN DIJK. (Communicated by Prof. J. C. SCHOUTE.)

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§ 1. *Introduction.*

In an investigation into salt intake (1927) STILES pointed out that there is a certain discrepancy in the results with regard to the intake of salt into the cell obtained by plasmolytic methods and by those employed by himself, with which the amount of salt taken in by a piece of tissue is determined quantitatively, either directly by analysis of the substance absorbed or indirectly by determining the loss of substance of the external solution. His experiments indicated a fairly large intake of salt, whereas FITTING and others had found with the plasmolytic method that the permeability for salt was slight.

STILES points out that it is not known how the salts taken up by pieces of tissue in his experiments are distributed over protoplasm and vacuole, and to what extent, owing to their being bound by adsorption to colloidal substances in the cell sap, they are osmotically inactive.

One difference between his method and the plasmolysis method is that with the latter much stronger concentrations of the salt solutions are used. HOMÈS has ascribed the divergent results to this fact. The problem has not yet been solved. What has become known regarding the intake of salt indicates that this is quite a different process from the passive permeation of organic substances.

Whereas this latter process is a diffusion process, depending on the difference in concentration of the substance in the external solution and in the vacuole, it has been found that with the intake of salt a state of equilibrium between the concentration of the external solution and of the cell-sap is not attained (STEWARD). It is an accumulation process, dependent on the aerobic respiration, and in STEWARD's opinion occurs only in the case of growing cells. COLLANDER and HOLMSTRÖM have pointed out that the intake of sulphonic acid dyes is also an active accumulation process. The probability, moreover, that practically the whole of the substances forced out of the tissue under pressure were previously present in the vacuole has been demonstrated by STEWARD and confirmed by ROSENFELS and others.

A second process of salt permeation is the exchange of ions (BRIGGS, PETRIE), which brings about a replacement of ions already present in the

vacuole. In this case an increase in the osmotic concentration needs not be expected, so that this process is not to be investigated by plasmolytic methods and is not further considered here. BROOKS has called attention to still another complication, viz., that a substance is first taken in by the protoplasm and only afterwards penetrates into the vacuole. He found this with the intake of rubidium by Nitella cells.

ARISZ and OUDMAN found that Vallisneria leaves were able to take in asparagine, and showed that this process is in many respects similar to the active intake of salts by growing cells. (HOAGLAND and BROYER, STEWARD). The leaf-cells of Vallisneria grow considerably during intake, and the intake of asparagine is likewise dependent on the aerobic respiration of the leafcells.

As it is possible in the case of Vallisneria to determine the intake of a substance by the vacuole by means of plasmolytic methods, the permeation can be investigated with one and the same object both by plasmolytic methods and by quantitative analytic methods, and the results of the different methods can be compared. The problem dealt with in this publication is therefore whether, in the case of Vallisneria, asparagine penetrates into the vacuole and whether this is demonstrable by plasmolytic methods.

The problem may be divided into two parts:

1. is it possible to show that the osmotic value of the cell-sap of Vallisneria cells is increased after the intake of asparagine, and can it be shown to be probable that this increase is the result of the osmotic action of the asparagine taken in?

2. is it possible with the aid of plasmolytic methods, e.g. the deplasmolysis method of COLLANDER and BÄRLUND or the plasmometric method of HÖFLER, to demonstrate the intake of asparagine into the vacuole?

Both these questions will be discussed below.

The result of our experiments is that the first question can be answered in the affirmative, that asparagine does actually penetrate into the vacuole, and may there be present in greater concentration than in the external solution (accumulation).

The answer to the second question, however, is in the negative. Neither with COLLANDER and BÄRLUND's nor with HÖFLER's method is it possible to demonstrate the intake of asparagine in the vacuole.

In accordance with this with the quantitative analysis method also only a very slight uptake of asparagine was found to occur if so much saccharose had been added to an asparagine solution that plasmolysis occurred.

Valuable assistance was lent in these experiments by Miss J. W. E. VAN WEERDEN, analyst.

§ 2. *Increase in the osmotic value of the cell-sap by the intake of asparagine.*

The leaves of Vallisneria spiralis are very suitable for the carrying out

of osmotic determinations, both the epidermal cells and the adjacent mesophyll cells being of sufficiently regular shape to admit of plasmometric determinations being made with them. HUBER and HÖFLER found that the epidermal cells were more permeable to water than the mesophyll cells. HURCH found a greater permeability of the epidermis cells for ureum and glycerine. We will revert to an investigation by SCHMIDT into the influence of plasmolysis on permeability (§ 6).

The experiments carried out with regard to the active intake of asparagine (ARISZ and OUDMAN) have demonstrated the conditions in which the intake of asparagine must take place. In the present experiments, as in these previous ones, we divided a number of Vallisneria leaves into pieces of 8 mm, from which we made series of 12 pieces of leaves, eliminating the variability resulting from the use of different leaves and of different parts of the same leaf. Quantitative analysis by the micro-Kjeldahl method shows such series to have an equal amount of nitrogen. Some of these series were analysed directly for the nitrogen present. With another series the osmotic value was determined at incipient plasmolysis, by putting the leaf-pieces into saccharose solutions of different concentrations.

Since with Vallisneria leaves saccharose does not penetrate into the vacuole in the dark, it is allowed to use saccharose as a plasmolytic. The percentage of plasmolysed epidermis cells was determined by estimating, and from the values obtained was calculated in what concentration 50 % of the number of cells would show incipient plasmolysis.

The remaining series were put into aerated asparagine solutions for 24 hours or longer. The intake took place in a dark room at a temperature of 25° C. It is necessary to make the experiments in the dark, because Vallisneria, like Elodea (BÄCHER 1920), increases its osmotic value when in the light, whilst in the dark only a very slight reduction takes place (A. VAN SCHREVEN, unpublished results).

After the intake of asparagine had taken place, the amount of nitrogen was determined with the micro-Kjeldahl method in the case of a number of series. From the difference in the amount of nitrogen of the series analysed before and after intake, the amount of nitrogen absorbed by one series was determined. If it is assumed that all the nitrogen taken up is present in the form of asparagine, the amount of asparagine absorbed can be calculated from this. In order to make an estimate of the increase in the osmotic value which this asparagine would give if it were present in solution in the vacuole, it is necessary to know how much water was present in the tissue. This was determined as the difference between fresh and dry weight of a series of leaf pieces. The fresh weight is not a constant value, since the leaf pieces grow, i.e. increase in weight, during the experiment. Nor is the water present in the leaf pieces to be considered as equal to the cell sap present, since part of the water will be bound to the wall and protoplasm. We did not try to determine how large this

amount was, as it was only intended to calculate an approximate value for the asparagine increase which can lay no claim to accuracy, as it is unknown whether the epidermis and mesophyll cells relatively absorb equal amounts of asparagine. The value obtained by calculation is therefore no more than an approximation, and on the strength of the points here dealt with it is to be expected that the actual increase in osmotic value of the epidermis cells will necessarily be considerably higher. Since with plasmolysis the length of the cells shows comparatively little decrease, and since it is a question of differences in the osmotic value, a determination of the osmotic value at incipient plasmolysis will suffice.

In order to determine the osmotic value of the cell sap after absorption of asparagine, one would be inclined again to place the leaf pieces into saccharose solutions of different concentrations. This, however, is not allowed, as in that case exosmosis of the asparagine just taken in would occur. Experiments on exosmosis described elsewhere (ARISZ and OUDMAN, unpublished results) have shown that with leaf pieces which have absorbed asparagine and which are then put either into water or into sugar solution, exosmosis of the asparagine first taken in occurs. The exosmosis can, however, be prevented by adding asparagine to the sugar solution in the same concentration as that in which the leaf pieces were during intake.

For the purpose of determining the osmotic value the leaf pieces were therefore put into sugar solutions of various concentrations, to which so much asparagine was added that the concentration, so far as asparagine was concerned, was equal to that of the solution from which they had absorbed asparagine. As these plasmolysis tests only lasted for a short time, the intake of asparagine in that time, in so far as that is possible (§ 6), may be neglected.

We give below the description of an experiment with absorption of $^1/_{80}$ m asparagine solution during 22½ hours in a dark room with constant temperature at 25° C. The observation of the plasmolysis took place by green light (Lifa filter 200 b Fitting)

	Fresh weight in mg	Dry weight in mg	Nitrogen content in γ	Nitrogen increase in γ
Control	104	5	161	
Intake in aerated solution . .	117		209	48
Intake in sol. without oxygen	102		170	9

From these data it was calculated that the asparagine might be present in the tissue in a concentration of 0.017 m.

During the plasmolysis determinations the osmotic value of the epidermis cells was found to be 0.285 m before the intake. When oxygen was present

during the intake a value of 0.315 m was found after absorption, that is, an increase of 0.030 m. When no oxygen was present during the intake no increase in the osmotic value was found. The increase in osmotic value found by plasmolysis tests is considerably larger than that calculated from the quantitatively determined nitrogen absorption. This, as we have already stated above, is entirely in accordance with the expectations.

In another experiment a $^1/_{80}$ m asparagine solution was absorbed during 48 hours. Here the increase in the osmotic value was 0.047 m. After absorption the leaf pieces were put into water for $4^1/_2$ hours to allow exosmosis to take place. The osmotic value of the cell sap was then found to have fallen by 0.031 m. From the quantitative nitrogen analysis an increase of 0.026 m and an exosmosis of 0.014 m were calculated.

From these determinations it is seen that the actively absorbed asparagine very largely finds its way into the vacuole and can there be found in a higher concentration than in the external solution. With the first experiment the concentration had risen 0.03 m and with the second one 0.047 m, whilst in both experiments the concentration of the external solution was only $^1/_{80}$ m. There has therefore been an accumulation in the vacuole. The first experiment further shows that this accumulation does not occur in an environment free from oxygen, the second one, that when the leaf pieces are put into water after absorption the increase again recedes to a great extent as a result of exosmosis. This latter fact indicates that the increase in the osmotic value is not brought about by anatonosis.

§ 3. *Demonstration of the asparagine taken in by the cell sap.*

The question now is whether it can be shown that the increase in the osmotic value is due to the presence of asparagine in the cell sap. It is not a priori certain that the asparagine remains unchanged after the absorption. The numerous investigations into the metabolic processes in leaves point to there being a great probability of the asparagine being concerned in the metabolism and either being used in the protein synthesis or broken down into simpler substances such as ammonia. Although experiments which we cannot discuss here in detail indicate that the asparagine can be used in more protracted experiments as respiratory material, we consider that the experiments discussed below justify our assuming that, at any rate in the first 24 hours, only a very small part of the asparagine is involved in the metabolism; we are thinking here only of experiments carried out in the presence of oxygen.

In the first place we tried to determine whether the amount of protein had increased after the absorption of asparagine. The protein was precipitated with trichloro-acetic acid. In the case of a number of series of leaf pieces the total nitrogen, protein nitrogen and soluble nitrogen expressed in γ was determined before and after the intake. The intake took place during 24 hours at 25° C out of a $^1/_{20}$ m solution of asparagine.

The same determinations took place after 4 hours' exosmosis in water.

	Total nitrogen	Protein nitrogen	Soluble nitrogen
Before intake	175	158	36
After 24 hours' intake.	260	152	128
After 4 hours' exosmosis	192	150	66

The amount of protein therefore remains practically unchanged with intake and exosmosis, and at all events does not increase.

The changes in the soluble nitrogen correspond with the changes in total nitrogen.

In another experiment 74 γ nitrogen was absorbed; the protein-nitrogen was unchanged after the intake of asparagine; in the filtrate a determination of the amid nitrogen according to SACHSSE was carried out. The amount found was 41 γ amid nitrogen, that is therefore 82 γ asparagine nitrogen, a value which agrees very well within the limits of error of the method with the amount of asparagine absorbed, calculated from the increase in the total nitrogen. This indicates that chiefly asparagine is present after the intake.

The ammonia was either determined titrimetrically after destillation at 40° C after the addition of CaO or colorimetrically with a Cencophotelometer after addition of NESSLER's reagent. In untreated leaves a small quantity of ammonia, 3—5 γ ammonium nitrogen, was found per series of 12 leaf-pieces. After absorption of asparagine about the same amount was found.

If, after taking in asparagine, the leaf-pieces are put into water, exosmosis takes place. The water in which the exosmosis of a number of series had taken place was tested for ammonia. With these tests various sources of error had to be inquired into. It was found that the stripes of tulle in which the leaf-pieces were sewn up gave off ammonia when put into water. An exosmosis of 3—5 γ nitrogen per series was found; an amount little larger than the limit of error of the determination, and at any rate much smaller than the amount of nitrogen excreted during exosmosis.

SACHSSE's method was then employed to determine how much amid nitrogen is present in the water in which the exosmosis has taken place. From this can be calculated how much asparagine has been given off by exosmosis and this amount can be compared with the loss of total nitrogen from the pieces of tissue by exosmosis.

An intake of 86 γ nitrogen was found per series. Owing to exosmosis 74 γ of this has been given off. The water, in which there had been 4 series of leaf pieces during the exosmosis, was found to contain 146 γ amid nitrogen. This agrees well with what is to be expected, if nothing but asparagine is given off during exosmosis. For 146 γ amid nitrogen corres-

ponds to 292 γ asparagine-nitrogen. According to the Kjeldahl tests 74 γ per series of the total nitrogen had been given off, that is, 296 γ nitrogen for 4 series. An equally good result was obtained with the other experiments. The slight exosmosis of ammonia and the agreement of the amid nitrogen found in the water with the loss of total nitrogen of the leaf-pieces shows convincingly that with the exosmosis practically only asparagine comes into the external solution.

After the exosmosis, however, a greater or lesser part of the nitrogen absorbed remains in the leaves. It is conceivable that this nitrogen is not excreted, because part of the asparagine taken in is bound by adsorption or converted into a non-diffusing substance. No certain pronouncement can as yet be made on this point. The results of the protein, amid and ammonia nitrogen determinations, however, indicate that chemical conversion of the asparagine taken in has not taken place within 24 hours. It is advisable, however, to be cautious here, since it is of course possible that at other times of the year (these experiments were carried out in the winter months) a different result may be obtained.

The fact, however, that not all the asparagine taken in was excreted may be accounted for in a different way. The tissue has recovered (ARISZ and OUDMAN, unpublished results) after a few hours from the shock caused by transferring from one solution to another, and the passive exosmosis has changed into an active process, in which the asparagine given off can again be absorbed.

Although, therefore, it would be premature to say that the asparagine taken in in the first 24 hours always remains unchanged, the conclusion may be drawn from the above data that most of the asparagine in the above experiments is not involved into the metabolism.

§ 4. *Determination of the intake of asparagine with the aid of deplasmolysis methods.*

Most investigations into permeability to salts and to organic substances have been carried out by means of deplasmolysis methods. Both with the method of DE VRIES, improved by FITTING, as carried out by COLLANDER and BÄRLUND, and with HÖFLER's plasmometric method, many reliable results have been obtained.

It was therefore obviously desirable to ascertain whether the permeation of asparagine could be investigated with these methods.

It is unnecessary to discuss these experiments in detail, as there was not a single case in which deplasmolysis could be observed. As asparagine is not soluble in water in a stronger concentration than $^1/_{10}$ m., the plasmolysing solutions were prepared with the addition of saccharose or glucose. A difficulty with these experiments is the sensitiveness of Vallisneria to impurities which are present in chemically pure preparations of saccharose and glucose. For this reason the sugars are, if necessary, purified by re-crystallisation. Asparagine also proved to be not unharmful

to plasmolysed cells. For that reason asparagine solutions of low concentration were used. The large mesophyll cells of the leaf of Vallisneria lend themselves particularly well to plasmometric determinations, and a slight permeation of asparagine would certainly have been easily observable.

As it was known that the intake of asparagine takes place only in an environment containing sufficient oxygen, an adequate supply of oxygen during the plasmolysis was arranged for, and this was checked by determination of the oxygen present in the liquid.

The leaf-pieces were investigated in open dishes with a water immersion objective.

The method of COLLANDER and BÄRLUND was also employed to ascertain whether asparagine permeates. The tests were made in a room for constant temperature at 20° C. Use was made of $^1/_{50}$ m asparagine with addition of saccharose in various quantities. The experiments were not continued for longer than 18 hours, as some decay of a few epidermis cells began to occur in the strongest concentrations. Otherwise the leaf-pieces gave the impression of being still vigorous and showed a normal streaming of the protoplasm. It was, however, impossible to demonstrate any penetration of asparagine.

§ 5. *Influence of oxygen on the permeability to glycerol.*

The indispensability of oxygen for the intake of asparagine suggested the question as to whether the influence of oxygen is specific for the intake of a particular group of substances, such as salts and asparagine, or whether this influence also makes itself felt with the permeation of other substances, even if only to a slighter extent, since these substances permeate so much more rapidly. To get some idea of this, glycerol was chosen, and tests were made with the object of determining whether the permeation of glycerol was affected by withdrawal of oxygen. With this object leaf-pieces were investigated in glycerol solutions with and without oxygen in a dark room for constant temperature by the COLLANDER and BÄRLUND methods. The leaf-pieces were in glass chambers through which the glycerol solutions flowed. These chambers were covered with a glass plate, so that the leaf-pieces could be inspected whilst they remained in the solutions.

The glycerol solutions were led to the chambers from supply bottles by means of siphons. Six aerated bottles were placed side by side and 6 bottles through which nitrogen was passed. The amount of oxygen present was determined by means of the micro-WINKLER method according to VAN DAM. In the chambers with a deficiency of oxygen 0.06—0.09 cc O_2 per L was found. In fig. 1 two curves are reproduced of the course in the time of the osmotic concentration giving incipient plasmolysis both for leaves in water containing oxygen and in water deficient in oxygen. Except in the case of the first two points, after 15 and 30 minutes, the values for 60, 120, 180 and 240 minutes coincide.

This experiment, which was confirmed by a number of other observations, showed that the supply of oxygen has no appreciable effect on the permeation of glycerol. This indicates that under the influence of the

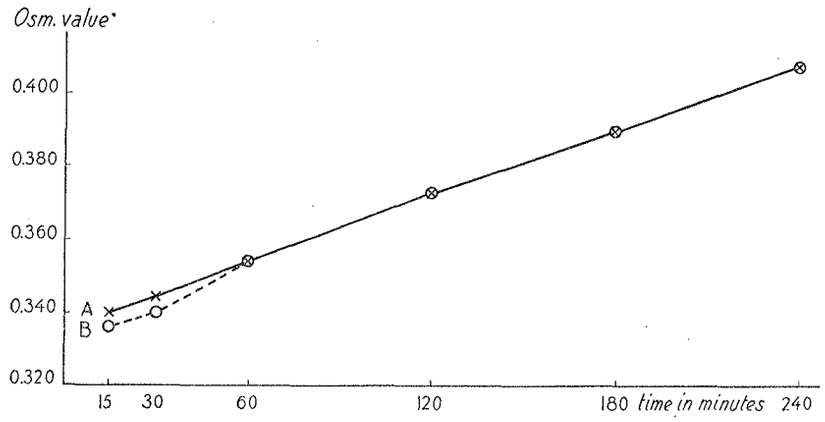

Fig. 1. Permeation of glycerol A under aerobic and B under anaerobic conditions in the epidermal cells of leaves of Vallisneria.
Osmotic value at incipient plasmolysis at the beginning was 0.289 m.
on the abscissa time in minutes
on the ordinate conc. of glycerol giving incipient plasmolysis.

aerobic respiration no alteration in the distance between the pores in the membrane occurs, by which the ordinary permeability for non-electrolytes would necessarily be affected.

§ 6. *Influence of plasmolysis on the permeation of asparagine.*

Various investigators have pointed out the possibility of plasmolysis having an influence on permeability. The most detailed investigation of this problem was carried out by SCHMIDT in 1936. She found that with Vallisneria a faint inhibition of the penetration of ureum and glycerine was caused by plasmolysis. With other objects, such as Majanthemum and Allium cepa the inhibition was greater. Her investigation therefore shows that the permeability of the protoplasm in the case of Vallisneria is but little affected by plasmolysis. Yet we have seen that the penetration of asparagine into the vacuole cannot be demonstrated. The question now arises whether it is possible to ascertain with the quantitative analysis method that the addition of sugar to the asparagine has any influence on the process of active intake. Preliminary experiments had shown that with strong asparagine concentration no clear results were to be obtained. This can readily be understood, since with plasmolysis the asparagine penetrates into the walls and the space between wall and protoplast, whilst it is impossible to wash out this asparagine. The already mentioned toxicity of higher concentrations of the asparagine solutions where sugar is present renders such experiments impossible. Accordingly the only remaining possibility was to make experiments with a diluted solution of asparagine.

The choice fell on a $^1/_{80}$ m asparagine solution to which saccharose in various quantities was added. The influence of $^1/_{80}$ m asparagine was investigated with 0.1, 0.2, 0.3, and 0.4 m saccharose. From the data in table 1 is seen that the addition of 0.4 mol saccharose produces a great

TABLE I.
Influence of a gift of saccharose on the absorption of asparagine from a 1/80 m asparagine solution. 24 hours at 25° C in the dark.

	Increase of nitrogen in γ				
	no sugar	0.1 m sugar	0.2 m sugar	0.3 m sugar	0.4 m sugar
experiment 109	82	73	50	62	24
„ 111	78	69	68	78	28
„ 113	57	58	51	60	23
average	72	67	56	67	25

decrease in the intake. With the larger concentrations a specific influence of the addition of sugar reveals itself; this will not be discussed more fully in this connection. From these experiments one therefore also gets the impression that a higher osmotic concentration, which causes plasmolysis, strongly inhibits the active asparagine intake.

§ 7. *Summary of the results.*

The active intake of asparagine demonstrated by ARISZ and OUDMAN with the aid of quantitative analysis methods, is seen to lead to an increase of the osmotic concentration of the cell sap. It was possible to demonstrate, by analysis of the substances absorbed, that the largest part of the asparagine taken in is present unchanged in the cell sap, and is not bound by adsorption to colloidal substances, as a result of which it would no longer be able to act osmotically.

By deplasmolysis methods the intake of asparagine in the case of Vallisneria cannot be demonstrated. Although according to SCHMIDT plasmolysis in the case of Vallisneria has only a slight influence on the permeability to ureum and glycerol, the addition of sugar to an asparagine solution to such a degree of concentration that marked plasmolysis occurs, has the effect of strongly inhibiting the intake.

As discussed in § 5, the penetration of glycerol with Vallisneria is actually found to occur with osmotic concentrations which cause plasmolysis, and it is independent of the presence of oxygen. Here we have two points of difference between what may be called "active permeation" of asparagine and "passive permeation" of glycerol; in the first place, oxygen is required for "active permeation"-passive permeation is not affected by oxygen; and in the second place, high osmotic concentrations

have an inhibitive effect on active permeation- they have but little effect, in the case of Vallisneria, on passive permeation.

Now that these particulars of the asparagine absorption of Vallisneria are known, the question arises whether they also apply to other plants, and whether they are applicable to other substances, such as salts, in addition to asparagine. With regard to the first point it will be necessary to await the results of further investigations. With plasmolytic investigations it is not usual to pay any attention to the supply of oxygen to the tissue; to make them more transparent the preparations are even in many cases freed from air by means of an air-pump. It is plain that under these circumstances it will never be possible to observe active intake. As to the question whether a high osmotic concentration inhibits active intake in the case of other plants also, only further research can throw light. It will be necessary to distinguish between two effects; in the first place, that strong concentrations of a particular substance may behave differently with regard to permeation than weak ones, and secondly, that strong concentrations produce plasmolysis and thus bring about important changes in the protoplasm.

The mechanism of the asparagine intake will be discussed more fully elsewhere. Here it may suffice to point to the fact that according to BJERRUM a "Zwitterion" is formed by the dissociation of asparagine, and that it is possible that the active intake of asparagine is connected with the polar structure of this ion.

With regard to the active intake of salts there is as yet no agreement amongst investigators as to whether the salt is absorbed in a dissociated state or in an undissociated one. It seems likely that salts, also, are only taken in when they have an electric charge. This, however, does not exclude the possibility that asparagine and salts can penetrate into a cell in an undissociated state also. (ARISZ and OUDMAN, Exosmosis, unpublished results). It seems, however, improbable that this intake is then dependent on oxygen. It is then an ordinary passive permeation.

The conception given here of the intake of asparagine agrees in some respects with that given by STILES in 1927, who, without knowing the difference between active and passive permeation, was able to reconcile the contradictory results of his method of investigation, chiefly investigating active permeation and those obtained by plasmolysis methods, with which passive permeation was demonstrated.

Groningen, September 1939. *Laboratory for Plant Physiology.*

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Fig. 1

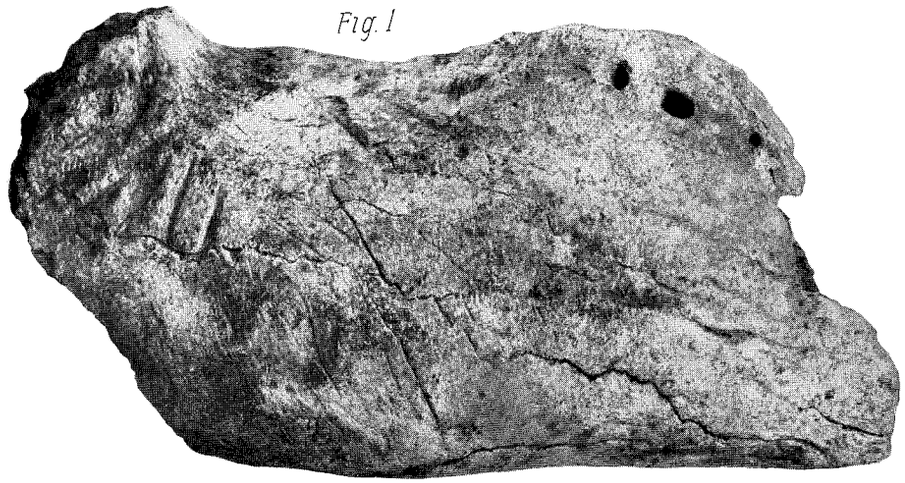

Fig. 2