The Extra-floral Nectaries of Hevea brasiliensis, Müll.-Arg. (the Para Rubber Tree), an Example of Bud-Scales[1] serving as Nectaries.

BY

JOHN PARKIN, M.A.,

Trinity College, Cambridge.

With Plate XVI.

WHILE engaged in economic work on india-rubber in Ceylon during 1898–9, the Para Rubber Tree (*Hevea brasiliensis*)[2] was constantly under my observation, and peculiar nectaries occupying the position of bud-scales on its young shoots attracted my attention. Less conspicuous nectaries also occur on the foliage leaves proper. Though these latter are incidentally mentioned by systematists, the bud-scales generally, as well as their nectariferous nature, appear to have escaped their notice. This is not surprising, for the adult tree only puts forth fresh foliage annually, and the bud-scales being caducous, are merely evident while the shoots are in the immature condition; thus, unless the tree be examined during the short period of leaf-renewal, no bud-scales would be seen.

In the account of the genus *Hevea* in Martius' Flora of Brazil[3], the nectaries of the foliage leaves are mentioned, but no reference is made to the bud-scales. Delpino[4] in his elaborate work on extra-floral nectaries dismisses this genus in a few words, referring apparently only to the nectaries of the foliage leaves.

In a recent paper by Huber[5] on the periodicity in growth of *Hevea*

[1] The term 'bud-scale' is here used in the sense of a reduced leaf-structure situated on the shoot below the true foliage leaves. The author does not necessarily wish to imply that such structures in *Hevea* serve or have ever served as protective coverings to the bud.

[2] Introduced into Ceylon in 1876.

[3] Martius, Flora brasiliensis, vol. xi, pars. II, 1873. On p. 298 the genus (nine species including *H. brasiliensis*) is described as having 'petioli communes apice supra glanduligeri.'

[4] Delpino, Mem. Accad. Bologna, viii, 1887, p. 635. In giving examples of extra-floral nectaries in the Euphorbiaceae he refers to *Hevea* as follows: '8 specie di Hevea (petioli ima basi patellari-glanduligeri).'

[5] Huber, Bot. Centralb. lxxvi, 1898, pp. 259–64.

(Annals of Botany, Vol. XVIII. No. LXX. April, 1904.)

brasiliensis the bud-scales are just mentioned, but their nectariferous nature is not pointed out.

The description to follow is the result partly of observations made while resident at the Royal Botanic Gardens, Peradeniya, Ceylon, and partly of the examination of some spirit-preserved young shoots from adult trees brought back to England. The following account does not claim to be at all exhaustive. The object of this paper is chiefly to bring to notice a somewhat peculiar type of extra-floral nectary.

Morphology of the shoot. The adult trees at Peradeniya shed their leaves early in the year and remain bare for some days before the new foliage appears. On February 16, 1899, the new shoots had almost gained their full length, but their foliage leaves were still very immature. At this stage of growth the bud-scales are fully developed and their nectaries active. On the leaves attaining maturity these structures shrivel and drop off. According to Huber[1] the adult trees in their natural habitat, the Amazon valley, produce likewise only one crop of leaves in the year, but the time they are bare is about June; hence the trees in Ceylon appear to have changed the time of the annual renewal of their foliage. This may be due to climate. The early months of the year constitute a moderately marked dry season in that part of Ceylon where Peradeniya is situated, and dryness is considered to have a direct bearing on leaf-fall. Yet on this idea the *Hevea* trees at Peradeniya ought not to burst into fresh leaf till about April, when the rains of the little monsoon commence: as it is they renew their foliage about the driest time of the year, while they cast off the old in January, a wetter and cooler month than either February or March.

Though mature trees produce only one set of leaves during the year, young trees—saplings—put forth several, showing a periodicity, which has been described by Huber[2]. Such saplings may produce fresh shoots about every month.

My attention was first called to the nectariferous bud-scales by noticing one day insects busy on the young shoots of some saplings growing in a plot. At a short distance away they looked as if they were devouring the immature foliage, leaving behind the stumps of the petioles, but on closer inspection I saw that they were a hairy kind of ant (?) imbibing the honey secreted by special foliar organs situated on the lower part of the shoot. Owing to the internodes between these structures having lengthened considerably, the general impression conveyed a little distance away was that of short petioles with the foliaceous part nibbled off. The true foliage leaves, however, were quite intact on the upper part of the shoot with their laminas as yet feebly developed. My first observations were made on these saplings, as I had to wait till the

[1] Huber, loc. cit.

[2] Huber, loc. cit.

proper time of the year to see if the young shoots of mature trees likewise possessed these nectariferous scales. Such was found to be the case.

The foliage leaves of *Hevea brasiliensis* are not evenly distributed along the whole length of the shoot, but are crowded together on the upper portion. The stretch of stem, which in the mature shoot appears to be a long internode below the foliage leaves, is really composed of several, the nodes of which were occupied in the young state by the nectariferous scales. That is to say, the internodes between the upper nectariferous scales have increased considerably in length—this is especially well seen in saplings. The leaf is trifoliate with a long petiole. The leaflets are large and lanceolate in shape, and are joined to the apex of the petiole by very short stalks (Plate XVI. Fig. 3). As a rule the length of the petiole and the size of the leaflets of a shoot decrease from the base upwards. For example, the petiole and leaflet of the lowest leaf may have a length of 30 cm. and 23 cm. respectively; whereas these measurements for the uppermost leaf may be only 3·5 cm. and 4·5 cm. respectively. The direction of the petioles is such that their laminas tend to be in one plane, and thus do not overshadow one another. The number of foliage leaves to a shoot varies, but is commonly twelve. They as well as the scales have a three-eighth arrangement on the stem-axis.

The nectary of the foliage leaf is situated on the upper surface just at the point of union of the leaflets with the petiole (Fig. 3 *n*). It may consist of either three contiguous saucer-shaped glands, one corresponding to each leaflet, or of only two of these, as in the figure, or it may assume the form of an irregular depression due to their fusion. In any case, they are not prominent structures, and do not differ as a rule in colour from the surrounding surface.

The bud-scales permit of division into two categories, viz. (1) the basal scales which are very small, non-nectariferous, and usually few in number; (2) the upper scales which are conspicuous, nectariferous, and numerous. Both kinds of scales as well as the foliage leaves possess each a pair of insignificant stipules (Figs. 1 and 2 *st*).

The basal non-nectariferous bud-scales. In the spirit material brought home for examination two types of young shoots could be distinguished, viz. those which had very few—one to three—non-nectariferous scales, and those possessing a great number, twenty or so. A drawing of each kind of shoot is shown in Figs. 1 and 2 respectively. A few shoots were intermediate in this respect, having several non-nectariferous scales, but not such an imbrication of them as represented in Fig. 2 *s*. No mention is made in my Ceylon notes of any large number of these basal scales having been noticed, but the shoots are referred to as possessing not

more than two or three each. Whether or not the possession of a large number of these scales be a common feature of young *Hevea* shoots cannot well be decided from the few examined, but the supposition is that such a shoot as the one shown in Fig. 2 is exceptional, and that as a rule only two or three non-nectariferous scales occur.

The nectariferous bud-scales. These vary considerably in number. The average for twenty-two shoots of adult trees examined was seven, ranging from five to twelve. The shoot from which Fig. 1 was drawn possessed eight, while that of Fig. 2 was exceptional in having twelve. Naturally, only part can be represented in the drawings. The lower nectariferous scales are small and short. The middle ones are usually the largest and possess the best developed nectaries, while the upper ones, though quite as long, are not so thick, and have the honey-secreting part reduced in extent; in fact in the uppermost one of all this part may be restricted to the apex, or perhaps even absent. The internodes increase in length as a rule from the base upwards; thus the lower nectariferous scales are near together, while the upper ones are some distance apart.

The inflorescences are borne in the axils of the nectariferous scales as well as in those of the lower foliage leaves (Figs. 1 and 2 *fl*).

Sapling. The young shoots of saplings resemble in most respects those of the adult trees, but being longer the internodes between the middle and upper nectariferous scales are more marked. From an examination of thirty-eight young sapling shoots the following numbers were obtained:—

non-nectariferous basal scales ranged in number from 0–3, aver. 1.
nectariferous scales „ „ 4–7, „ 5.
foliage leaves—average 10.

Nine of these thirty-eight young shoots possessed each an arrested leaf between the nectariferous scales and the foliage leaves proper. This bore three leaflets well defined but quite small, while the nectariferous scales have mere points to indicate the remains of the leaflets. The nectary appeared to be absent[1]. This vestigial leaf did not persist, but withered and fell off with the scales.

Seedling. In germination the two cotyledons remain in the testa in the soil, so that what looks like a hypocotyl is really the epicotyl; it is quite long, 25 cm. or so in length. The first two foliage leaves formed quit the stem about the same level, and are similar in shape to those of older plants. Then comes an internode of about 3 cm., followed by two more foliage leaves situated at nearly the same level on the stem and alternating with the first pair; sometimes there may be only one

[1] Not microscopically examined—might possibly possess a trace of glandular tissue invisible to the naked eye.

leaf, or even three at this point. Occasionally the first pair of leaves may be vestigial, or only one of them fully developed.

If the plumule be fatally injured then the bud in the axil of one of the cotyledons develops into a shoot, bearing first three to four reduced leaves apparently without nectaries, before the true foliage leaves appear; sometimes the buds in both axils so sprout. The shoot arising from the axillary bud of the cotyledon simulates that derived from the plumule, but in the one case the length of stem produced before the foliage leaves are emitted is really composed of several internodes, the nodes being occupied by inconspicuous scale-leaves; while in the other it consists of one internode only, the epicotyl.

Unfortunately my notes do not connect the seedling with the sapling-stage, so as to see when the nectariferous scales first arise. This is probably at the second period of foliation. They apparently do not appear in the seedling, but rather later in the development of the plant.

Structure of the individual bud-scales. The structure of the non-nectariferous scales requires little description. A glance at Fig. 4 *s*, shows their size and shape. They are each accompanied by a pair of lateral bodies—stipules. In the mature or sprouting bud they are brown dead objects.

The nectariferous scales are fairly long, often bent structures and somewhat circular in transverse section; they project from the stem at right angles or with a downward inclination. Each bears at its apex three minute points, the sole remains of the leaflets. Their upper convex surface is covered with yellow honey-secreting tissue, and has often a median longitudinal groove. In the lower and middle scales the whole length of the upper surface is glandular. In the upper scales the glandular portion tends to recede from the proximal part, and in the uppermost one it is confined to the apex (Fig. 6 *ne*).

From a structural point of view the nectar-secreting tissue of plants can be divided into two classes[1], viz. (1) that consisting of small epidermal cells of the usual shape with thin hardly cuticularized outer walls, overlying a mass of closely packed cells full of contents, and secretory in function, and (2) that in which the epidermis itself assumes the form of a secretory epithelium with greatly thickened cuticle. In the first class the nectar reaches the surface by passing through the thin walls, while in the second class it escapes by bursting the cuticle.

The extra-floral nectaries of *Hevea brasiliensis* present a modification of the second type of structure, in that many of the original epithelial cells become divided in the mature nectary by tangential walls into two or three daughter-cells. That is, in the immature state the epidermis

[1] Bonnier, Les nectaires, Étude critique, anatomique et physiologique, Ann. d. Sci. Nat., 6e sér., T. viii, 1879, p. 96.

is a simple epithelium, but on approaching maturity it becomes in places two or three layered (Fig. 7). Conspicuous nuclei and much cytoplasm without prominent vacuoles are present in the epithelial cells, as well as in the small cortical cells below. The cuticularized part of the outer wall is quite thick, as is shown in the drawing (Fig. 7 *ct*).

Examples of extra-floral nectaries with an epithelium divided in places are to be met with in *Homalanthus populifera* and *Clerodendron Bungei*[1]; also a regularly two-layered epithelium exists in *Prunus avium*[2].

The diagram (Fig. 5) shows the position of the nectar-secreting epithelium in a transverse section of a typical median bud-scale; while that of Fig. 6 represents the epithelium as restricted to the apex in the uppermost scale.

The minute structure of the nectaries of the foliage leaves is similar to that of the scale ones.

General Remarks. This case of *Hevea brasiliensis* is about the first example cited of bud-scales—cataphyllary leaves—serving as nectaries. The only other instance I have found at all comparable is that mentioned by Reinke[3]. He points out that the bud-scales, as well as the foliage leaves of *Prunus avium*, have glandular teeth which are honey-secreting. But here the transformation is very partial. The scales are not so modified as to be merely nectaries. Their primary function is still that of bud-protection.

The Euphorbiaceae are rich in examples of plants with extra-floral nectaries. Baillon[4], in his work on this natural order, enumerates the various types, showing that their situation may be various, such as on the stem, petiole or lamina; and that different organs may be wholly transformed into them, such as stipules and leaflets. *Hevea brasiliensis* affords a still further type, viz. that of bud-scales serving as nectaries.

Two or three questions suggest themselves as to the origin of these cataphyllary nectaries of *Hevea.* Are they connected by descent with the petiolar glands, or are they a fresh production of glandular tissue in the evolution of the plant? What is the relationship between the non-nectariferous and nectariferous scales? Have they been derived independently at different periods from foliage leaves, or have the former arisen by further retrogression from the latter? From an identity in structure between the petiolar and scale nectaries and from the situation of the glandular tissue in the uppermost scale it looks as if the two classes of nectaries were directly connected. The petiolar glands have perhaps become much more developed in the scales, so that the function of these latter is now wholly that of secreting honey.

[1] Morini, Contributo all' anatomia ed alla fisiologia dei Nettarii Estranuziali, Mem. Accad. Bologna, 1886, vii.

[2] Reinke, Secretionsorgane, Pringsh. Jahrb., 1876, p. 125.

[3] Reinke, loc. cit. [4] Baillon, Étude générale des Euphorbiées, p. 250.

The view of the evolution of the shoot of *Hevea* that suggests itself to the author is as follows. Originally the base of the shoot had one, two, or three non-nectariferous bud-scales such as occur now; the rest of the foliar organs were true foliage leaves arranged equidistantly along the axis. Assuming that their laminas gradually increased in size towards the middle of the shoots and then decreased, the lowest and highest leaves would in consequence be the smallest and the middle ones the largest—a condition often occurring in shoots. That of the Beech (*Fagus sylvatica*) is a case in point. Providing that the *Hevea* shoot had an upward tendency, as it has at the present day, the large median leaves would tend to overshadow the lower smaller ones, and thus render these latter to a great degree functionless as assimilating organs, and through disuse a gradual reduction in their laminas might follow. The nectaries on the petiolar apices still remaining would be the first to secrete. Viewing their service as one of attracting ants to keep off leaf-destroying insects, it would be an advantage to the plant to retain the nectaries on these retrograde leaf-structures, and further to increase their size and consequently their secretion, in order to protect the expanding foliage leaves, till their nectaries became functional. Thus gradually a condition which now occurs would be brought about.

The Beech shoot has a few scales at its base without any lamina, which may be comparable, though not homologous as they are stipules, to the non-nectariferous scales of *Hevea*; then come the foliage leaves increasing in size as far as the middle of the axis, and then diminishing towards the apex. There is a tendency in some of its shoots for the small lower leaves to wither and fall early. This may be partly due to their being shaded by the higher leaves, though this overshadowing is largely guarded against by the shoot as a rule having a horizontal direction, and as a consequence the leaf-blades are in one and the same plane. If the shoot, on the other hand, were inclined considerably to the vertical as in *Hevea*, then the middle leaves would shade the lower ones much more effectually. Such a shoot as that of the *Rhododendron* demonstrates this. It is obliquely erect and has the foliage leaves crowded together on its upper part, thus resembling the shoot of *Hevea*. The length of stem below the rosette of foliage leaves is not a single internode, but composed of several, the nodes of which in the young state were occupied by small leaves which have shrivelled and disappeared. These, being perhaps originally smaller than the middle leaves and thus subject to shade, now no longer persist as functional foliage leaves; they have most likely decreased still further in size, and now apparently serve as protective scales to the bud. Consequently as a rule in horizontal shoots the lowest foliage leaves are the smallest, or at any rate smaller than the middle ones; while in shoots inclined to the vertical the lowest leaves are the largest, because

they represent probably the middle leaves of the primitive shoot, the lowest having ceased to act as foliage leaves.

The reason why in an ordinary shoot such as that of the Beech the middle leaves should generally be the largest is perhaps owing to the intensity of growth during development, first rising gradually to a maximum, then falling again till growth ceases. This would result in the first and last formed leaf-blades being the smallest.

The only other species of *Hevea* I have been able to examine is *H. spruceana*, Müll.-Arg., a very closely allied one. It possesses similar nectariferous scales.

Summary.

1. *Hevea brasiliensis* possesses two kinds of extra-floral nectaries:—

(*a*) Small inconspicuous glands situated on the upper surface of the foliage leaves, where the three leaflets join the petiole (Fig. 3 *n*).

(*b*) Large conspicuous glands borne on vestigial foliar structures—'bud-scales'—which are situated on the shoot below the foliage leaves proper (Figs. 1 and 2 *ns*).

2. The 'bud-scale' nectaries are a prominent feature of the young expanding shoot, and are functional till the foliage leaves are mature, when they wither and drop off. They are present in saplings, as well as in adult trees, but were not observed in seedlings.

3. Besides these nectariferous structures, one or more insignificant bud-scales without nectaries may be present at the base of the shoot (Figs. 1 and 2 *s*).

4. The minute structure of the foliar and 'bud-scale' nectaries is the same. Each consists of a well-defined secretory epithelium with a thick cuticle. The original cells of this epithelium may be divided here and there by one or two tangential walls to form in places a two- or three-layered epidermis (Fig. 7). The nectar escapes by the bursting of the cuticle.

5. The two kinds of extra-floral nectaries are considered as homologous; that is to say, the 'bud-scale' one may be regarded as a further development of what was at one time a petiolar nectary.

6. These nectariferous structures, occupying relatively the same position on the shoot as ordinary bud-scales, probably never had a protective function, but have been derived directly from what were once foliage leaves by the disappearance of the lamina and an increase in size of the nectary.

7. According to the usual view taken of the function of extra-floral nectaries, the 'bud-scale' glands may be looked upon as attracting ants to keep off insects injurious to the developing foliage. As soon as the foliage

leaves mature, their own nectaries become functional, and the scale ones being no longer required wither and drop off.

8. This case of *Hevea brasiliensis* is the first striking instance recorded, as far as the author is aware, of bud-scales—cataphyllary leaves—serving solely as nectaries.

POSTSCRIPT.

Just on the completion of this paper a communication on the extra-floral nectaries of *Hevea*, read before the Academy of Sciences, Paris, on November 9, 1903, came to my notice. The article[1] resulting from it in the corresponding number of the 'Comptes Rendus' deals wholly with the structure of the petiolar nectaries, and makes no reference whatsoever to the nectariferous bud-scales; hence the chief subject-matter of my paper is not in the least affected. The authors state that the number of individual glands composing the petiolar nectary of *Hevea brasiliensis* may vary between two and five, but is usually three. They point out that the secretory epidermis of the nectary is two-layered in places, and lay stress on the two following structural features: (1) the presence of a ring of lignified parenchyma in the interior of the raised border which surrounds the secretory surface of each gland; (2) the laticiferous tubes, occurring in fair abundance in the specialized parenchyma of the gland, either end just below the secretory epidermis, or even pass between the epidermal cells to the exterior. J. P.

CAMBRIDGE, *December*, 1903.

[1] Daguillon et Coupin, Sur les nectaires extra-floraux des *Hevea*, Comp. Rend. cxxxvii, No. 19, 1903, pp. 767-9.

EXPLANATION OF FIGURES IN PLATE XVI.

Illustrating Mr. Parkin's paper on the Extra-floral Nectaries of *Hevea brasiliensis*.

Fig. 1. Young shoot from adult tree bearing the immature foliage leaves on its upper part. Natural size. *s*, single non-nectariferous basal bud-scale; ns_3, ns_5, ns_6, ns_8, represent respectively the 3rd, 5th, 6th, and 8th nectariferous bud-scales; this shoot possessed eight of these structures; the other four are not depicted in the drawing; the shaded areas show the position of the nectar-secreting parts of the scales; in ns_8, the uppermost scale, this part is restricted to the apex; *l*, lowest foliage leaf; *pn*, position of the petiolar nectary; *st*, stipules; *fl*, young inflorescences in the axils of the upper scales and lower foliage leaves; *ls*, leaf-scars of previous year's shoot.

Fig. 2. Young shoot from adult tree with many basal bud-scales. Natural size. *s*, imbrication of non-nectariferous basal scales; *ns*, length of stem bearing nine nectariferous bud-scales (the shoot possessed twelve altogether); *fl*, inflorescences in the axils of the nectariferous scales; *l*, lowest foliage leaf; *st*, stipules; *ls*, leaf-scars of previous year's shoot.

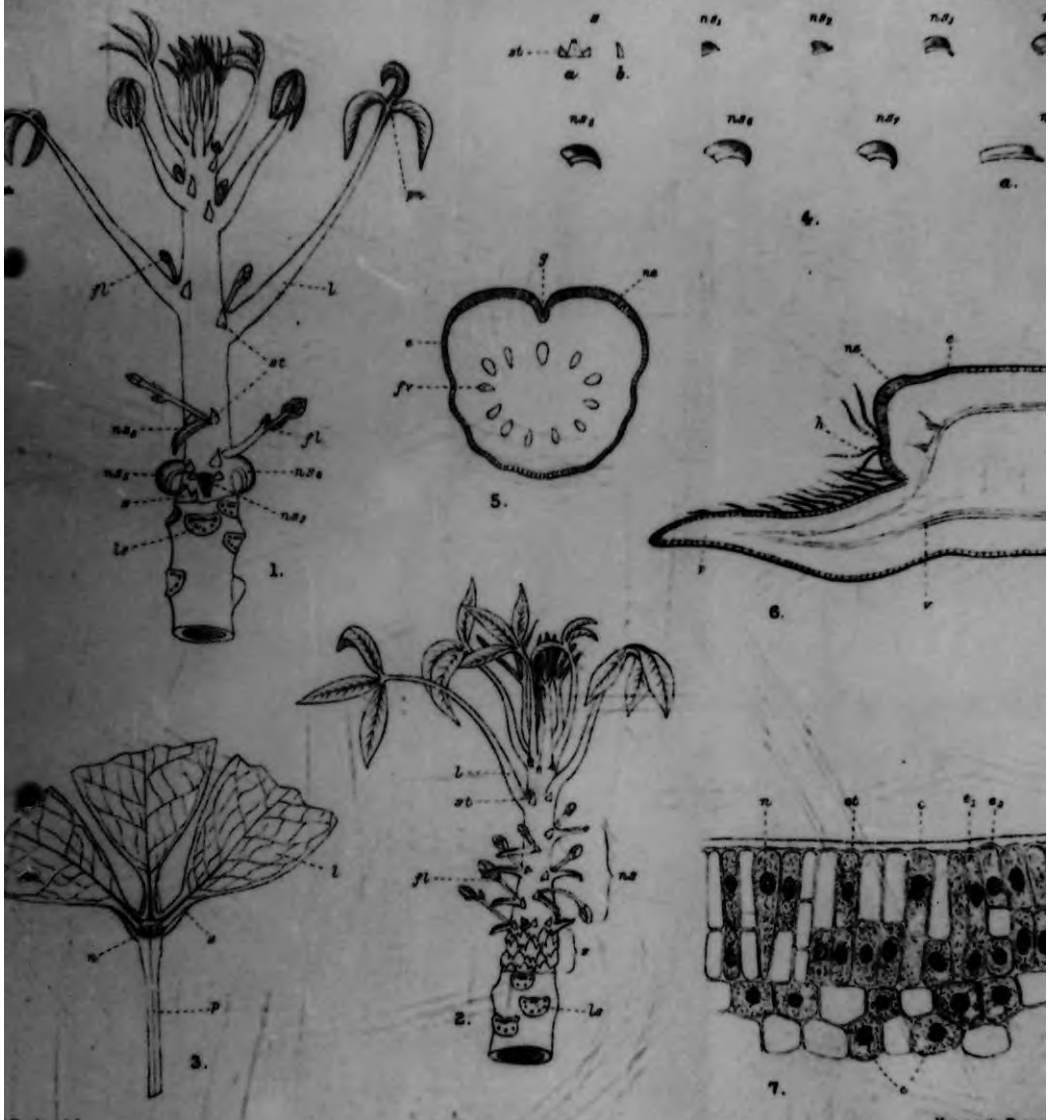
Fig. 3. Part of the upper surface of a foliage leaf. Natural size. *n*, twin-nectary; *p*, upper part of petiole, *l*, lower part of one of the three leaflets; *s*, short stalk of leaflet.

Fig. 4. Individual bud-scales from shoot represented in Fig. 1. Natural size. *s*, single non-nectariferous basal scale, *a*, dorsal view showing pair of stipules, *b*, side view; ns_1—ns_7 (inclusive), side views of seven nectariferous bud-scales in order of succession from base upwards, the position of the honey-secreting tissue is indicated by shading; ns_8, uppermost nectariferous bud-scale, *a*, side view, *b*, ventral view showing three points, the vestiges of the three leaflets—the nectar-secreting part is restricted to the apex and is shown by the small shaded area in this region.

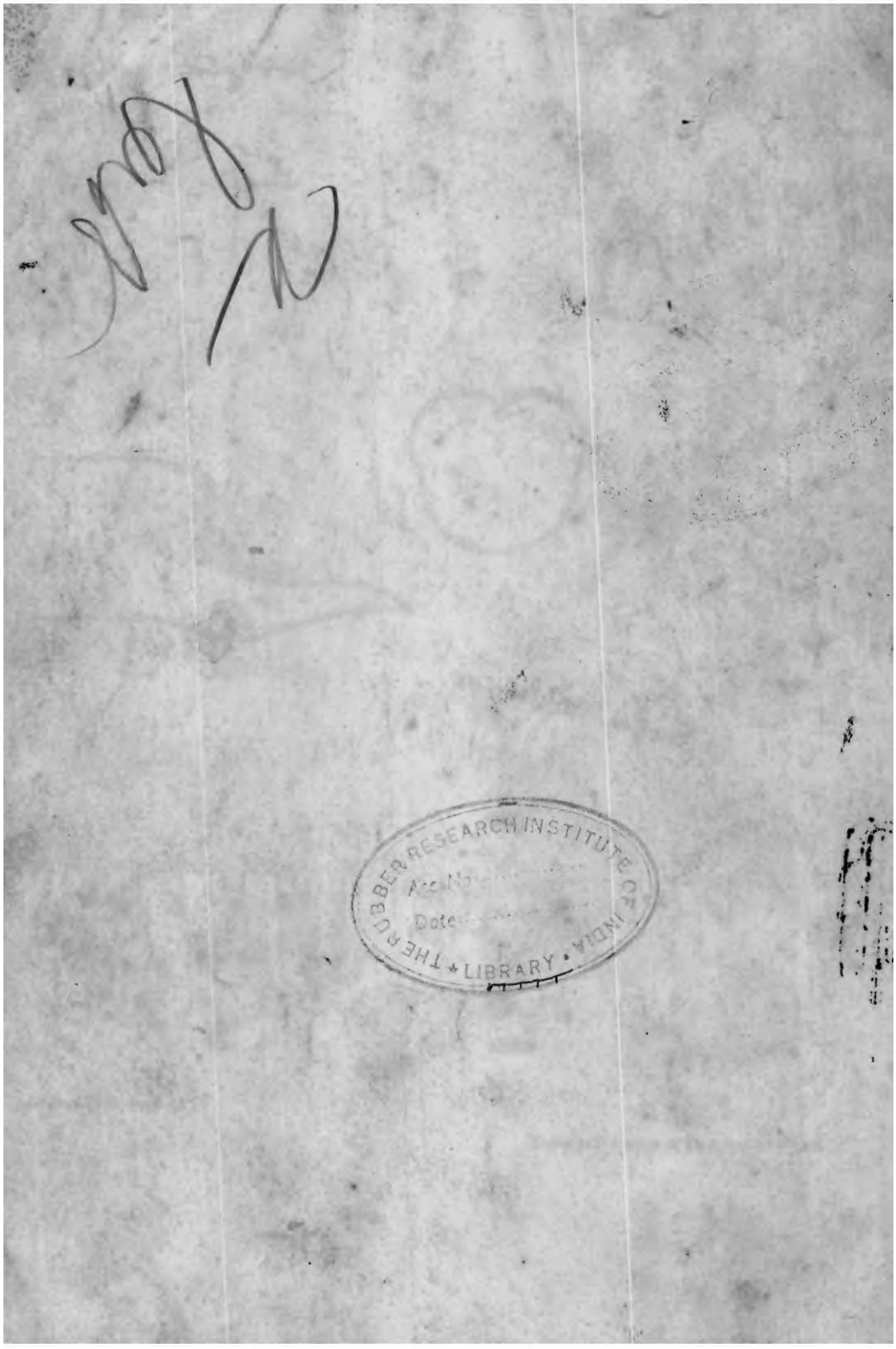
Fig. 5. Diagram of a median transverse section of a nectariferous bud-scale. × 30. *e*, ordinary epidermis; *ne*, honey-secreting epithelium on the upper surface with groove, *g*; *fv*, ring of fibro-vascular bundles.

Fig. 6. Diagram of a longitudinal section of the apical part of the uppermost nectariferous bud-scale (ns_8 in Figs. 1 and 4) showing the restricted distribution of the glandular tissue. × 30. *e*, ordinary epidermis; *ne*, honey-secreting epithelium in a position corresponding to that occupied by the nectary of the foliage leaf; *p*, one of the three apical points—vestige of a leaflet; *h*, hairs; *v*, vascular strands.

Fig. 7. Section of the honey-secreting epithelium of a bud-scale. × 400. *e*, undivided epithelial cell; e_1, epithelial cell divided into two daughter-cells; e_2, epithelial cell divided into three daughter-cells; *ct*, the thick cuticle; *n*, nuclei; *c*, small cortical cells full of contents and without intercellular spaces. The empty areas in the figure represent cells in the section from which the contents had accidentally disappeared during preparation.



PARKIN.—HEVEA BRASILIENSIS.



DIVISION S-2—SOIL CHEMISTRY

Extraction and Colorimetric Determination of Urea in Soils[1]

L. A. DOUGLAS AND J. M. BREMNER[2]

ABSTRACT

A simple colorimetric method of determining urea in soils is described. The soil sample is extracted with 2*M* KCl containing a urease inhibitor (phenylmercuric acetate), and the extract is analyzed for urea by measurement of the red color formed when an aliquot is heated with diacetyl monoxime and thiosemicarbazide under acidic conditions (phosphoric acid-sulfuric acid medium). The method is sensitive and precise, and it gives quantitative recovery of urea added to soils. The extraction performed is satisfactory for determination of exchangeable ammonium, nitrite, and nitrate by steam distillation methods.

Additional Key Words for Indexing: diacetyl monoxime, thiosemicarbazide, phenylmercuric acetate.

ALTHOUGH numerous studies of urea transformations in soils have been reported, very little attention has been given to the problems in satisfactory performance of the urea, ammonium, nitrite, and nitrate analyses usually required in these studies. One problem, which has been overlooked in most investigations, is that significant hydrolysis of urea by soil urease can occur during extraction of soils for determination of urea (5). A second is that the colorimetric methods available for determination of urea have low sensitivity and do not permit analysis of soil extracts containing less than about 50 ppm of urea. A third is that no soil extraction procedure has been developed that is satisfactory for determination of exchangeable ammonium, nitrite, and nitrate as well as of urea, which means that two or more extractions must be performed to carry out these analyses.

Previous work in our laboratory showed that urea could not be recovered quantitatively by extraction of soils with 2*M* KCl for 60 min as in the procedure developed for determination of exchangeable ammonium, nitrite, and nitrate by steam distillation methods and that this was due to hydrolysis of urea by soil urease during the extraction with 2*M* KCl (5). Tests showed that this hydrolysis problem could be overcome by use of 2*M* KCl containing compounds that inhibit urease activity, but use of such inhibitors vitiates urea analysis of the extract by urease techniques (5).

The colorimetric method of Watt and Chrisp (8) has been used to determine urea in soil extracts, but this method does not have the sensitivity needed for studies of urea transformations in soils (it was designed for analysis of solutions containing 50–240 ppm of urea), and we found that some soil extracts contain substances that interfere with this method. We attempted, therefore, to modify the 2*M* KCl extraction procedure so that it does not lead to hydrolysis of urea, and to develop a sensitive colorimetric method of determining urea that can be applied satisfactorily to extracts obtained by this modified procedure. The requirements of the analytical procedure sought were: (i) The compound used to inhibit urease activity during extraction of soil with 2*M* KCl should not interfere with extraction of exchangeable ammonium, nitrate, nitrite, or urea, or with analysis of the extract for these forms of nitrogen; (ii) The colorimetric method used for determination of urea should be accurate, sensitive, and precise, and it should not be subject to interference by substances normally present in 2*M* KCl extracts of soils.

The procedure described here meets these requirements. In this procedure, the soil sample is extracted with 2*M* KCl containing a urease inhibitor (phenylmercuric acetate), and the extract is analyzed for urea by measurement of the red color formed when an aliquot is heated with diacetyl monoxime and thiosemicarbazide under acidic conditions. The colorimetric method used for determination of urea has been described (3). It is about 50 times as sensitive as the method of Watt and Chrisp, and it permits accurate and precise urea analysis of soil extracts containing less than 20 ppm of urea.

MATERIALS

The soils used (Table 1) were surface (0- to 15-cm) samples selected to obtain a wide range in pH (5.5–8.0), organic-matter content (0.30–8.92% organic C), and texture (3–78% clay, 1–94% sand). Before use, each sample was air-dried and crushed to pass a 2-mm screen. In the analyses reported in Table 1, organic carbon was determined by the method of Mebius (7), total nitrogen by a semimicro Kjeldahl procedure (1), pH by a glass electrode (soil/water ratio, 1:2.5), and particle-size distribution by pipette analysis (6) after dispersion by sonic vibration (4). When analyzed by the method described in the next section, none of the samples selected contained a measurable amount of urea.

Table 1—Analyses of soils

Soil type*	pH	Organic carbon	Total nitrogen	Clay	Sand
		%			
Clyde sil	5.5	4.30	0.402	25	16
Buckner sa	6.1	0.30	0.032	3	94
Cresco l	6.2	2.32	0.205	22	32
Grundy sicl	6.7	2.21	0.201	27	5
Glencoe sic	*6.8*	*8.92*	*0.860*	*41*	*8*
Clarion sal	6.9	2.19	0.203	18	51
Nicollet l	7.0	2.27	0.204	20	44
Primghar sicl	7.0	3.41	0.313	38	6
Hayden sal	7.7	2.46	0.164	10	53
Regina c	*8.0*	*2.30*	*0.241*	*78*	*1*

* sil, silty loam; sa, sand; l, loam; sicl, silty clay loam; sic, silty clay; sal, sandy loam; c, clay.

[1] Journal Paper no. J-6484 of the Iowa Agr. & Home Econ. Exp. Sta., Ames. Project no. 1070. This work was supported in part by the Tennessee Valley Authority. Received Feb. 24, 1970. Approved Sept. 1, 1970.

[2] Research Associate and Professor, respectively, Department of Agronomy, Iowa State University, Ames.

The urease used was obtained from Sigma Chemical Co., St. Louis, Mo.

METHOD FOR DETERMINATION OF UREA

Reagents

Phenylmercuric Acetate (PMA) Solution—Dissolve 50 mg of phenylmercuric acetate (Eastman Organic Chemicals, Rochester, N.Y.) in 1 liter of water.

Potassium Chloride-Phenylmercuric Acetate (2M KCl-PMA) Solution—Dissolve 1,500 g of reagent-grade KCl in 9 liters of water, and add 1 liter of PMA solution.

Diacetyl Monoxime (DAM) Solution—Dissolve 2.5 g of diacetyl monoxime (Fisher Scientific Co., Chicago, Ill.) in 100 ml of water.

Thiosemicarbazide (TSC) Solution—Dissolve 0.25 g of thiosemicarbazide (Eastman Organic Chemicals, Rochester, N.Y.) in 100 ml of water.

Acid Reagent—Mix 300 ml of 85% phosphoric acid and 10 ml of concentrated sulfuric acid, and dilute the mixture to 500 ml.

Color Reagent—Prepare this reagent immediately before use by adding 25 ml of DAM solution and 10 ml of TSC solution to 500 ml of acid reagent.

Standard Urea N Solution—Dissolve 0.4288 g of urea in 2*M* KCl-PMA solution, and dilute to 2,000 ml with 2*M* KCl-PMA solution. If pure, dry urea is used, this solution will contain 100 μg of urea N per ml. Store in a refrigerator.

Procedure

Place 10 g of soil in a 250-ml, wide-mouth bottle and add 100 ml of 2*M* KCl-PMA solution. Stopper the bottle, shake it on a mechanical shaker for 1 hour, and filter the resulting suspension (Whatman no. 42 filter paper). If the extract cannot be analyzed soon after its preparation, store it in a refrigerator.

To determine urea N, pipette an aliquot (1–10 ml) of the extract containing up to 70 μg of urea N into a 50-ml volumetric flask, make the volume to 10 ml with 2*M* KCl-PMA solution, and add 30 ml of color reagent. Swirl the flask for a few seconds to mix the contents, and place it in an oven at 120C. After 30 min, remove the flask from the oven, cool it immediately in running water (13–20C) for 15 min, make the contents to 50 ml by adding water, and mix thoroughly. Then transfer about 10 ml of this solution to a Klett-Summerson colorimeter tube (1.3-cm light path), and measure its red color intensity with a Klett-Summerson photoelectric colorimeter fitted with a green (no. 54) filter. Calculate the urea N content of the extract by reference to a calibration graph plotted from the results obtained with standards containing 0, 10, 40, and 70 μg of urea N. To prepare this graph, dilute 10 ml of the standard urea N solution to 100 ml with 2*M* KCl-PMA solution in a volumetric flask, and mix thoroughly. Then pipette 0, 1, 4, and 7-ml aliquots of this diluted standard solution into 50-ml volumetric flasks, adjust the volumes to 10 ml by adding 2*M* KCl-PMA solution, and proceed as described for urea N analysis of the soil extract.

Comments

Any colorimeter or spectrophotometer that permits color intensity measurements at 500–550 mμ can be used for the procedure described. The maximum absorption of the color is at 527 mμ (3).

A bath of boiling water can be used instead of an oven for the heating stage of the method described. If a water bath is used, the time required for maximal color development is 27 min (3). It is important that the flask used for color development be cooled immediately after it is removed from the oven because some loss of color occurs if the flask is not cooled rapidly as specified (3).

Calibration graphs prepared from urea standards as described show a linear relationship between urea N concentration and color intensity, but they differ slightly from day to day. It is recommended, therefore, that urea standards be included in each series of analyses.

The color developed in the method described is photosensitive, but this is not a problem if color intensity measurements are performed shortly after color development, because color fading is negligible if the time between color development and color intensity measurement does not exceed 1 hour. Color intensity measurements can be postponed for several hours if the colored solutions are stored in the dark (3).

Nitrite interferes with the colorimetric method used for determination of urea if the concentration of nitrite N in the extract analyzed is more than five times the concentration of urea N. Nitrite interference can be eliminated very easily by treating the aliquot of extract taken for urea analysis with 1 ml of 2% (w/v) sulfamic acid solution and by allowing the treated aliquot to stand for 5 min before addition of the color reagent.

The color reagent is unstable and should be prepared immediately before use. The other reagents used are stable for several weeks if stored in a refrigerator.

RESULTS AND DISCUSSION

Preliminary work showed that hydrolysis of urea by soil urease during extraction of soils with 2*M* KCl (10 ml/g of soil) could be inhibited completely by use of 2*M* KCl containing small amounts (less than 100 ppm) of silver sulfate, mercuric chloride, or phenylmercuric acetate. We found, however, that both silver sulfate and mercuric chloride interfered with determination of urea by the diacetyl monoxime method even when the 2*M* KCl used for extraction contained the minimal amount of silver sulfate or mercuric chloride needed for complete inhibition of urease activity (ca. 5 ppm). Phenylmercuric acetate (PMA) also interfered with analysis of soil extracts by the diacetyl monoxime method when added at the rate of 10 or 20 ppm to the 2*M* KCl used for extraction. But tests showed that complete inhibition of soil urease activity was achieved with 2*M* KCl containing 5 ppm of PMA (equivalent to 50 μg of PMA/g of soil) and that the diacetyl monoxime method could be applied satisfactorily to extracts obtained with this 2*M* KCl-PMA solution.

The effect of phenylmercuric acetate on hydrolysis of urea by soil urease during extraction of soils with 2*M* KCl is illustrated by Tables 2 and 3, which show the recoveries of urea obtained by extraction of soils and urease-treated soils with 2*M* KCl and with 2*M* KCl containing 5 ppm of phenylmercuric acetate (the extractant recommended). The data show that phenylmercuric acetate completely inhibits urease activity during extraction of soils with 2*M* KCl and that the 2*M* KCl-PMA extraction procedure adopted gives quantitative recovery of urea even with soils treated with urease. The tests with urease-treated soils were included to check that the 2*M* KCl-PMA extraction procedure would be satisfactory for soils having abnormally high urease activities.

The accuracy and precision of the method described are illustrated by Table 4, which gives the results of replicate analyses of soils treated with different amounts of urea.

Table 5 gives the results of recovery tests in which soils treated with urea, ammonium, nitrite, and nitrate were

Table 2—Recovery by method described of urea N added to soils

Soil	Recovery of urea N, %*
Clyde	100.2 (95.8)
Buckner	100.4 (96.6)
Cresco	99.8 (89.0)
Grundy	99.3 (93.2)
Glencoe	99.8 (86.2)
Clarion	99.3 (98.0)
Nicollet	100.4 (95.0)
Primghar	100.4 (91.2)
Hayden	99.3 (94.6)
Regina	100.2 (96.0)
Average	99.9 (93.6)

* Recoveries were determined by analyzing 10-g samples of soils before and after treatment with 5 ml of urea solution containing 500 μg of urea N. Urea solution was added to soil immediately after addition of 2*M* KCl-PMA solution. Figures in parentheses are recoveries obtained when 2*M* KCl was used instead of 2*M* KCl-PMA solution.

extracted with 2*M* KCl-PMA solution as in the method described, and the extracts were analyzed for urea by the diacetyl monoxime procedure and for ammonium, nitrite, and nitrate by the steam distillation methods described by Bremner and Keeney (2). The data reported show that the phenylmercuric acetate in the 2*M* KCl-PMA solution adopted for extraction of urea from soils does not interfere with extraction of exchangeable ammonium, nitrite, or nitrate and does not affect the steam distillation methods developed for ammonium, nitrite, and nitrate analysis of 2*M* KCl extracts of soils.

Attention should be drawn to the fact that, in the recovery tests reported in Tables 2–5, the soil samples were treated with 2*M* KCl-PMA solution immediately before addition of urea or ammonium. Tests in which urea and ammonium were added to soils before addition of 2*M* KCl-PMA solution showed that some hydrolysis of urea or fixation of ammonium occurred with several soils even when the time between addition of urea or ammonium and addition of 2*M* KCl-PMA solution was only a few minutes. With most soils, however, the recovery of urea was quantitative (> 99%) if the time between addition of urea and addition of 2*M* KCl-PMA solution did not exceed 5 min.

Tests showed that the following substances did not form colored products under the conditions of the method described (tests were performed with 10-ml aliquots of aqueous and 2*M* KCl-PMA solutions containing 50 μg of N as substance listed) and that they did not interfere with urea N analysis by this method when added to urea solutions in amounts such that the concentration of N added as substance was equivalent to the concentration of urea N (50 μg of N/10 ml of solution): ammonium sulfate, sodium nitrite, potassium nitrate, biuret, thiourea, glutamine, asparagine, creatine, creatinine, alanine, glycine, aspartic acid, glutamic acid, lysine, arginine, glucosamine, and galactosamine. Nitrite, biuret, and thiourea interfered when the amount of N added as these substances was increased to 500 μg/10 ml of solution (10 times the amount present as urea), but the other substances listed did not interfere at this level of addition. If the extract under analysis contains significant amounts of nitrite, interference by this substance can be eliminated very easily by the sulfamic acid technique described.

Tests with a wide variety of soils have shown that the colorimetric method described gives no trace of red color with 2*M* KCl-PMA extracts of soils not previously treated with urea and that it gives quantitative recovery of urea added to these extracts.

A study of the effect of storing extracts obtained by shaking urea-treated soils with 2*M* KCl-PMA solution showed that, if stored in a refrigerator (5C), these extracts can be stored safely for at least 3 weeks before analysis for urea N (Table 6).

Table 3—Recovery by method described of urea N added to urease-treated soils

Soil	Recovery of urea N, %*
Clyde	99.9 (53.8)
Buckner	100.4 (28.6)
Cresco	99.3 (2.5)
Grundy	99.9 (0)
Glencoe	99.3 (0)
Nicollet	100.0 (5.0)
Hayden	99.3 (15.3)
Average	99.7 (15.0)

* Recoveries were determined by analyzing 10-g samples of urease-treated soils (1 ml of 0.3% urease solution/10 g of soil) before and after treatment with 5 ml of urea solution containing 4,000 μg of urea N. Urea solution was added to soil immediately after addition of 2*M* KCl-PMA solution. Figures in parentheses are recoveries obtained when 2*M* KCl was used instead of 2*M* KCl-PMA solution.

Table 4—Accuracy and precision of method

Soil	Urea N added	Urea N recovered*		
		Range†	Average	SD
	ppm			
Grundy	10	9-11	10	0.7
	25	24-26	25	0.8
	100	98-101	100	1.2
	250	248-252	251	1.7
	500	498-504	501	2.2
Nicollet	10	9-10	10	0.7
	25	24-25	25	0.7
	100	98-101	100	1.4
	250	248-251	249	1.6
	500	496-502	499	2.2

* Recoveries were determined by analyzing 10-g samples of soils before and after treatment with 5 ml of urea solution containing 20 to 1,000 μg of urea N per ml. Urea solution was added to soil immediately after addition of 2*M* KCl-PMA solution. SD, standard deviation.
† Five analyses.

Table 5—Recovery of urea N, ammonium N, nitrite N, and nitrate N added to soils

Soil	Recovery of N added to soil, %*			
	Urea	Ammonium	Nitrite	Nitrate
Grundy	99.3	99.8	99.7	99.4
Glencoe	99.4	100.1	99.9	99.3
Clarion	99.5	99.8	99.8	99.6
Nicollet	99.3	99.7	99.5	99.7
Average	99.4	99.9	99.7	99.5

* Recoveries were determined by analyzing 10-g samples of soils before and after treatment with 5 ml of standard solution containing 2,000 μg of urea N, 2,000 μg of ammonium N (as ammonium sulfate), 2,000 μg of nitrite N (as $NaNO_2$), and 2,000 μg of nitrate N (as KNO_3). Standard solution was added to soil immediately after addition of 2*M* KCl-PMA solution.

Table 6—Urea N analyses of stored extracts of urea-treated soils*

Soil	Time of storage of extract at 5C, days		
	0	10	21
	μg of urea N/10 ml of extract		
Clyde	49.9	49.6	49.9
Buckner	50.0	49.9	49.7
Grundy	49.9	49.6	49.8
Glencoe	49.7	49.5	49.6
Nicollet	50.0	49.7	49.8
Primghar	49.8	50.0	49.7
Hayden	49.9	49.7	49.6

* Extracts were prepared by shaking 10-g samples of soils with 100 ml of 2*M* KCl-PMA solution containing 500 μg of urea N for 1 hr. Filtered extracts were stored in a refrigerator at 5C.

LITERATURE CITED

1. Bremner, J. M. 1960. Determination of nitrogen in soil by the Kjeldahl method. J. Agr. Sci. 55:11–33.
2. _____, and D. R. Keeney. 1966. Determination and isotope-ratio analysis of different forms of nitrogen in

soils. 3. Exchangeable ammonium, nitrate, and nitrite by extraction-distillation methods. Soil Sci. Soc. Amer. Proc. 30:577–582.
3. Douglas, L. A., and J. M. Bremner. 1970. Colorimetric determination of microgram quantities of urea. Anal. Letters. 3:79–87.
4. Edwards, A. P., and J. M. Bremner. 1967. Dispersion of soil particles by sonic vibration. J. Soil Sci. 18:47–63.
5. Keeney, D. R., and J. M. Bremner. 1967. Determination and isotope-ratio analysis of different forms of nitrogen in soils: 7. Urea. Soil Sci. Soc. Amer. Proc. 31:317–321.
6. Kilmer, V. J., and L. T. Alexander. 1949. Methods of making mechanical analyses of soils. Soil Sci. 68:15–24.
7. Mebius, L. J. 1960. A rapid method for the determination of organic carbon in soil. Anal. Chim. Acta 22:120–124.
8. Watt, G. W., and J. D. Chrisp. 1954. Spectrophotometric method for determination of urea. Anal. Chem. 26:452–453.

Adsorption of Polysaccharides by Montmorillonite[1]

R. L. PARFITT AND D. J. GREENLAND[2]

ABSTRACT

Amylose and a dextran of MW 2 million were adsorbed by montmorillonite. However, two dextrans of lower MW did not adsorb on montmorillonite. It is suggested that entropy changes arising from changes in configuration of the macromolecules and the association of water molecules with clay and polymer control the adsorption behavior of uncharged polysaccharides. Poly(galacturonic acid) was adsorbed by aluminum montmorillonite but showed negative adsorption on sodium montmorillonite. The uronic acid components of a soil polysaccharide were adsorbed by montmorillonite if aluminum ions or hydroxy aluminum species were present on the clay; neutral sugar components were adsorbed irrespective of the exchangeable cations present or the pH.

Additional Key Words for Indexing: **adsorption isotherms, soil organic matter, polymers, clay surfaces.**

THE ADSORPTION of soil polysaccharides on montmorillonite has been studied by several authors in attempts to demonstrate that polysaccharides can interact with soil particles and thereby influence soil physical properties (Dubach et al., 1955; Greenland, 1956; Lynch et al., 1956; Finch et al., 1966; Swincer et al., 1969).

Hydrogen bonding has often been invoked as the mechanism of interaction between uncharged polysaccharide molecules and montmorillonite (Emerson, 1963; Emerson and Raupach, 1964; Lynch et al., 1956), but recent studies have shown that hydrogen bonding between organic molecules and the montmorillonite surface is not necessary for adsorption to occur (Dowdy and Mortland, 1967, 1968; Parfitt and Greenland, 1970).

The aims of the present investigation were to examine the adsorption of some specimen and microbial synthesized polysaccharides to obtain further information regarding the mechanism of adsorption, and to study the adsorption of fractions of a soil polysaccharide in an attempt to obtain further information regarding the interactions which occur between such materials and clays in soils.

MATERIAL AND METHODS

Clay Minerals—The montmorillonite was obtained from Wards Natural Science Establishment, Inc. It originated from the John C. Lane tract, Upton, Wyoming, and was a sample of the standard clay mineral number 25 b of the American Petroleum Institute, project 49. The sample was hand ground with a pestle and mortar and a 2% suspension was made by shaking with distilled water. The less than 2μ esd fraction was separated by repeated sedimentation and decantation. The suspension was washed three times with sodium chloride solution (1*N*, pH 3), twice with sodium chloride solution (1*N*, pH 5.7), and then repeatedly with distilled water until the clay dispersed. The sodium montmorillonite was washed further until the conductivity of the suspension was of the same order as that of distilled water (10^{-5} mhos cm^{-1}). During the washing procedure the clay was separated by centrifugation at 38,000*g* in a Lourdes centrifuge. Calcium montmorillonite and aluminum montmorillonite were prepared by washing the dispersed sodium montmorillonite three times with equal volumes of calcium chloride and aluminum chloride solutions (1*N*), respectively. These suspensions were further washed with distilled water by centrifugation and decantation until the resistance of the suspension was of the same order as distilled water. The suspensions contained approximately 1 g of clay in 100 ml of water.

Polysaccharides—Dextran 2 was a sample provided by Dr. C. E. Clapp with a weight average molecular weight of 2 million. It was produced by the B512 F strain of *Leuconostoc mesenteroides*. Dextrans 10 and 110 were commercial dextrans obtained from Pharmacia, Sweden, with weight average molecular weights of 11,200 and 100,000, respectively. They also originated from *Leuconostoc mesenteroides* B512 but were separated after partial hydrolysis and have narrower molecular weight distributions. Dextran 2 contains 95% $\alpha(1 \rightarrow 6)$ glucose units and 5% $\alpha(1 \rightarrow 3)$; whereas the two fractions of lower molecular weight are stated to have 5 to 10% of $\alpha(1 \rightarrow 3)$ and $\alpha(1 \rightarrow 4)$ linkages.

Amylose was supplied by Calbiochem, Los Angeles. A saturated solution was prepared by boiling in distilled water for 15 min. The solution was cooled for 5 hours in ice and was centrifuged at 38,000*g* for 20 min at 2C. The supernatant was separated and used in the adsorption experiments. A solution of poly(galacturonic acid) obtained from NBC, Ohio, was prepared by a similar method as for amylose.

The approximate MW of amylose was determined by gel filtration with Biogel P100 (Calbiochem, Los Angeles). After gel swelling and removal of fine particles, the column was prepared and equilibrated with sodium chloride solution (0.5*N*) and calibrated using Blue Dextran (Pharmacia, Sweden). One-half milliliter samples of amylose were loaded in NaCl solution

[1] Contribution from the Dept. of Agri. Biochem. and Soil Sci. Waite Agri. Res. Inst. Univ. of Adelaide, S. Australia. Received