A CONTRIBUTION TO THE CHEMISTRY AND PHYSIOLOGICAL ACTION OF THE HUMIC ACIDS

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The physiological action of the humic acids on the living plant organism offers a field of work in which there is room for much investigation.

The present paper, which is preliminary to a larger contribution on the physiological effects of these acids on plant life generally, deals in the first place with the chemical composition of the substances hitherto known collectively under the general name 'Humic Acid.' In addition, in Part II, it is shown that it is possible to use the living organism as an auxiliary, which confirms and emphasizes the points of structural differences in these compounds which are indicated by chemical analysis.

This line of evidence, although seldom utilised by chemists, is of great value in determining minute differences of chemical composition in complex molecules of similar constitution, and is of special service in the case of the humic acids, to which the ordinary methods of organic chemistry cannot well be applied.

PART I. CHEMICAL

The decomposition of complex carbohydrates and albumen by means of mineral acids to give a series of brown amorphous compounds, closely related to the natural humic acid, has engaged the attention of biological chemists for many years, and, until recently, it has been generally accepted that the acid obtained in this way

from cane sugar is identical in chemical structure with the natural acid, which may be extracted from peat or other decaying cellulosic tissues. This claim for identity rested largely on the fact that the general chemical properties of the acids derived from both natural and artificial sources are very similar, and, moreover, in certain cases, the composition of each as determined by elementary analyses, agrees within the limits of accuracy admissible in such work. Both acids are amorphous, sparingly soluble, brown solids, which dissolve easily in caustic alkalis to give highly coloured solutions from which the free acid may be precipitated by means of hydrogen chloride.

Experiments which are at present in progress here on the physiological effect of humic acid on plant cultures suggested that the supposed identity of the acids in question should be subjected to further proof, and accordingly specimens of natural and artificial humic acid were prepared by different methods, and critically compared.

It has already been noted¹ that, when prepared by the decomposition of ammonium humate, the acid contains combined nitrogen, and we therefore prepared the natural acid from heather peat by two methods; in one series of preparations the ammonium salt was precipitated with hydrochloric acid, and in parallel experiments the potassium salt was similarly treated.

The artificial acid was prepared from pure cane sugar by the action of hydrochloric acid, and in this case also the compound was obtained through the medium of both the potassium and the ammonium salt.

It is well known² that the nature of the 'humic acid' obtained from cane sugar varies greatly with the method of preparation, the solubility of the product showing great variation according to the concentration and nature of the acid used. We accordingly adopted conditions which gave a product displaying in appearance, solubility, and general properties the closest resemblance to the natural acid. We thus obtained four distinct yet apparently identical specimens

1. Eggertz, *Centralblatt*, 89, 343; André, *Comptes Rendus*, 127, 414; Festini, *Centralblatt*, 1902, 182.
2. Conrad and Guthzeit, *Berichte*, 19, 2849.

of humic acid (two from natural and two from artificial sources), and the composition of each as obtained in several different preparations was compared.

In addition to estimations of carbon, hydrogen and nitrogen, the methoxyl content (CH_3O) was also determined by Zeisel's process in order to test the suggestion that humic acid contains alkyloxy groups. The results show that considerable uniformity exists in the composition of the different specimens of the acid derived from cane sugar, and that although the acid obtained through the ammonium salt contains a small amount of combined nitrogen, the analytical composition is identical with that obtained from the potassium salt. The natural acids, however, invariably contained considerably less carbon, showing that little reliance is to be placed on the superficial identity of the compounds.

The average compositions of the different specimens deduced from a series of concordant combustions are compared in the following table :—

			C	H	O	N
Artificial	{ Through potassium salt	...	64.70	4.55	30.71	Nil
	{ Through ammonium salt	...	64.74	4.69	29.81	0.76
Natural	{ Through potassium salt	...	56.67	5.16	35.68	2.49
	{ Through ammonium salt	...	54.29	4.94	38.16	2.61

It will be seen that considerable difference exists in the composition of the two specimens of the natural acid, and this is partly accounted for by the somewhat variable nitrogen content of each. A factor of greater importance, however, is the different amount of inorganic impurities invariably present in the two cases. After combustion of the natural acids a quantity of mineral ash containing aluminium and ferric oxides was left behind in the boat, and, although this was allowed for in calculating the results, yet in the absence of any information concerning the form in which this extraneous matter existed in the specimens, it is possible that the analyses might be vitiated to a slight extent by its presence.

A more striking illustration of the different chemical structure of the natural and artificial acids is furnished by the results of the Zeisel determinations. It was found that the former compounds never contained more than from 1·71 to 2·47 per cent. of alkyloxy groups, calculating the result as CH_3O , whilst the artificial acid gave a much higher result, namely, 6·47 per cent. As the compounds resinified with hydriodic acid our results must only be regarded as approximately accurate, but if, as is generally supposed, the molecule of humic acid contains at least twenty-four carbon and twelve oxygen atoms, then in the natural acid the percentage of methoxyl found does not correspond to even one molecular proportion, whilst in the artificial product we must assume the existence of at least one such group. [$\text{C}_{28}\text{H}_{21}\text{O}_{11}(\text{CH}_3\text{O})$, requires $\text{CH}_3\text{O} = 6·15$ per cent.]

Further evidence of a similar nature was furnished by the analyses of the barium salts obtained from the different acids. This method, in fact, affords a much better index of identity or difference than analyses of the acids themselves. Thus the two hypothetical acids, $\text{C}_{20}\text{H}_{16}\text{O}_{10}$ and $\text{C}_{30}\text{H}_{28}\text{O}_{15}$, would give, on combustion, figures which are almost identical, and certainly within the limits of experimental error. Assuming each to be a dibasic acid, their barium salts would show a difference of about 7 per cent. in the barium content, and the values for carbon and hydrogen would also be affected. It will be seen from the table of analyses of the various barium humates described in the experimental part that the identity of the salts of the artificial acids is confirmed, and that the natural barium humates differ in marked fashion from them and from each other.

Our results also throw some light on the problem of the empirical formula of humic acid. In spite of the fact that no crystalline derivatives of the acid are known, investigators have not hesitated to speculate as to the probable complexity of the molecule, and in Lippmann's *Chemie der Zuckerarten* (Vol. II, p. 1245) an excellent summary is given of various possible formulae which have been ascribed to the compound by Mulder, Stein, Berthelot, André and others.

We have been able to test these formulae, which naturally show great variation by application of the analytical results we have obtained.

The best agreement so far as the analyses of the artificial free acids is concerned is shown with Mulder's formula, $C_{40}H_{80}O_{15}$,¹ and also with that ascribed to the compound by Berthelot and André, $C_{18}H_{16}O_7$.² The latter formula is, however, inadmissible when the analyses of the barium humate are taken into consideration, as, assuming the acid to be dibasic, the barium content of the salt would be as high as 28.6 per cent. If, however, the formula be somewhat modified and altered so as to express a molecule of double complexity, then the analyses of both the acid and its salts agree with the calculated figures. Thus the formula $C_{39}H_{32}O_{14}$ requires $C = 64.64$, $H = 4.42$, and assuming the barium salt to have the composition $C_{39}H_{30}O_{14}Ba$ the calculated percentage of barium is 15.98. The mean of several combustions of the artificial acid gave $C = 64.70$, $H = 4.55$, while the barium content of the salts varied between 15.31 and 15.65 per cent., a close agreement considering the nature of the compounds.

It is, of course, more difficult to speculate as to the composition of the natural acid, as the problem is here complicated by the presence of combined nitrogen and traces of inorganic impurity. Our analyses agree, however, approximately with the formula of the acid obtained from Dopplerite,³ $C_{24}H_{28}O_{14}$, although the carbon content is somewhat higher. A barium salt of the composition $C_{24}H_{22}BaO_{12}$ has already been described by Demel,⁴ and it has been suggested that the corresponding acid $C_{24}H_{24}O_{12}$ is a double anhydride derived from the acid in Dopplerite. It should be pointed out here, however, that estimations of barium in natural humates should be accepted with caution, as the inorganic impurities contaminate the ignited residue, and hence increase the apparent barium content. In our experiments the ignited residue was extracted with hydrochloric acid, and the barium estimated by precipitation with sulphuric acid. In this way a result was obtained which was invariably 3 or 4 per

1. *Journal für praktische Chemie*, I, 21, 203; 32, 331.

2. *Comptes Rendus*, 112, 916, 1237.

3. Mayer, *Die landwirtschaftliche Versuchstationen*, 29, 313.

4. *Monatshefte für Chemie*, 3, 763.

cent. lower than that found by merely igniting the residue with sulphuric acid. Disregarding the combined nitrogen, the analyses of the natural acid obtained through the potassium salt stands between the values calculated for the Dopplerite acid, and its anhydride.

$C_{24}H_{28}O_{14}$ requires:—

$$C = 53.33, H = 5.18$$

$C_{24}H_{24}O_{12}$ requires:—

$$C = 57.14, H = 4.76$$

$$\text{Found ... } C = 56.67, H = 5.16$$

The barium salt agrees most closely with the second formula—

$C_{24}H_{22}O_{12}Ba$ requires:—

$$C = 45.07, H = 3.44, Ba = 21.44$$

$$\text{Found ... } C = 46.87, H = 3.27, Ba = 21.60$$

If we take into account the combined nitrogen present, then the analytical figures agree with the anhydro acid in which one oxygen atom is replaced by nitrogen. Thus $C_{24}H_{24}O_{11}N$ contains 2.79 per cent. N, the value actually found being 2.49 per cent.

With regard to the natural acid obtained through the ammonium humate the low barium content of the salt indicates a greater molecular complexity. The hypothetical acid $C_{30}H_{30}O_{17}$ and the corresponding salt $C_{30}H_{28}O_{17}Ba$ require respectively:—

$$\text{Acid ... } C = 54.38 \quad H = 4.53$$

$$\text{Found ... } C = 54.29 \quad H = 4.94$$

$$\text{Salt ... } C = 45.17 \quad H = 3.51 \quad Ba = 17.20$$

$$\text{Found ... } C = 45.31 \quad H = 3.76 \quad Ba = 17.53$$

The amount of combined nitrogen present in the acid is accounted for by the modified formula $C_{30}H_{30}O_{16}N$, for which the theoretical values for carbon and hydrogen are nearly the same; the calculated nitrogen content in this case is 2.12 per cent., the value found being 2.61 per cent.

EXPERIMENTAL

Preparation of Natural Humic Acid from Peat

The usual method of preparation was followed, the well-dried peat being finely divided and thoroughly washed to remove soluble salts. The filtered product was then digested in quantities of 100 grammes with 1500 c.c. water containing 66 grammes hydrogen chloride.¹ The residue was then digested with excess of 5 per cent. ammonium hydrate, and the humic acid precipitated by the addition of a slight excess of strong hydrochloric acid to the filtrate.

The amorphous brown precipitate was filtered on a Büchner funnel, washed with water, and dried first on porous plates and afterwards in a vacuum over sulphuric acid. The average yield is about 4 grammes dry acid from 100 grammes peat.

In a parallel series of preparations a solution of 1 per cent. potassium carbonate was used in place of ammonium hydrate in the above process, the average yields being thereby increased to 5 grammes.

The extensive dilution of the various solutions used in these preparations is rendered necessary by the difficulties experienced in the various filtrations. The effect of alkalis on the cellulosic tissues of the peat is to produce gelatinous syrupy liquids, which filter extremely slowly, even when a vacuum of 3 mm. is used. In very dilute solutions, however, the filtration proceeded more readily, but was nevertheless slow.

Preparation of Artificial Humic Acid from Cane Sugar

We obtained artificial humic acid displaying the closest physical resemblance to the natural compound by boiling a solution containing 200 grammes cane sugar dissolved in 367 c.c. water and 133 c.c. strong hydrochloric acid. The reaction was continued under an inverted condenser for thirty hours, the precipitated solid being filtered off every ten hours. The dark mass thus obtained was finely powdered,

1. *Comptes Rendus*, 1898, 127, 414.

digested repeatedly with hot water, and then extracted with 5 per cent. potassium carbonate (or ammonium hydrate), the humic acid being precipitated and purified exactly as in the previous case. Yield 12 grammes.

All attempts to obtain the acids in a crystalline or colourless state failed. Very little colouring matter was extracted when specimens were boiled with alcohol, acetone, acetic acid or ethyl acetate; and when the alkaline salts were boiled for several days with animal charcoal no decolorisation was effected.

Comparison of the Natural and Artificial Acids

In appearance, solubility, and behaviour towards alkalis the acids appear identical. The solubility in water is very slight, and in the case of the natural acid only amounts to 0.0076 at 20°. Both acids give sparingly soluble calcium, barium or magnesium salts.

The natural acid even when purified by re-precipitation gives a small quantity of mineral ash on ignition, but no residue was obtained from the artificial form. The results of the analyses which are summarised in the theoretical part show that the artificial acids obtained through the medium of the potassium and ammonium salts are practically identical in composition, save that the latter contains a small percentage of combined nitrogen. The natural acids are distinguished by their lower carbon content, higher percentage of hydrogen, and also by the presence of an estimable amount of methoxyl and combined nitrogen. Here again the acid obtained through the ammonium salt contained most nitrogen.

We endeavoured to supplement the above evidence, distinguishing between the natural and artificial acids, by determinations of the specific rotations of each. On account of the extreme dilution of the aqueous solutions the observed readings were very small, and our results are, therefore, only to be accepted qualitatively. Both acids are strongly dextro-rotatory, and so also are their potassium salts. In the latter case also very dilute solutions were used on account of the intense brown colour of the compounds. The artificial acids appeared to be distinctly more active than the natural forms, but

it is possible that the activity of the former may have been partly due to the presence of traces of glucose derived from the decomposition of the cane sugar.

It has already been shewn by Conrad and Guthzeit¹ that fructose is completely decomposed by boiling with 5 per cent. hydrochloric acid, but that quantities of unaltered glucose remained after prolonged boiling with even 20 per cent. acid.

As already explained in the introduction the comparison of the various preparations of the acids was extended by examination of the barium salts derived from each. These were prepared in each case by the addition of excess of barium chloride to solutions of the alkaline salts of the acids. A weighed quantity of acid derived from the ammonium salt was neutralised by the addition of very dilute ammonium hydrate, and precipitated by means of barium chloride. In the case of acids prepared through the potassium salt the method of preparation was similar, save that caustic potash replaced ammonium hydrate. The filtered salt was well washed with water, and dried in a vacuum until constant in weight. Several duplicate preparations were made and analysed. The results tabulated below, which contain the average figures obtained in several concordant analyses, confirm our opinion that the artificial acids are identical, but differ in marked fashion from the natural compounds.

ANALYSES OF BARIUM HUMATES

	Method of preparation :—		Artificial		Natural	
			From ammonium humate	From potassium humate	From ammonium humate	From potassium humate
C	...	...	52.66 %	52.43 %	45.31 %	46.87 %
H	...	...	3.56	3.35	3.76	3.27
Ba	...	...	15.31	15.34	17.53	21.60

The difference in composition shewn in the salts of the natural acids, together with the fact that the acids themselves possess different carbon contents, seems to indicate that the composition of the natural

1. *Berichte* 19, 2509, 2575.

acid varies greatly with the method of preparation, according to whether the potassium or ammonium salt method is used. This result emphasises in marked fashion the structural difference which must exist between the artificial and natural acids, as in the former case the barium salts obtained by both methods are identical in analytical composition.

PART II. PHYSIOLOGICAL

To determine the action of the acids and their salts on the living organism various cultures are in progress both with autotrophic and heterotrophic flowering and flowerless plants. Of these experiments only certain series will be here referred to, in which the effect of acids and salts has been determined on fungi, because these give the nutritive values of the humic compounds for such organisms, and also because they to a certain extent corroborate the points of difference referred to in the analytical part of this paper. We endeavoured to determine the nutritive value of the acids and their salts as carbon and nitrogen compounds respectively, and from the results to draw conclusions as to the relative carbon and nitrogen content of the four series of acids and salts. Artificial cultures were prepared with the usual precautions to secure purity. The acids in these were in concentrated solutions. The salts specially selected were the potassium salts, as their ready solubility allowed of a considerably greater range of concentration than in other cases. In addition we are of the opinion, although we have not yet verified the point, that the potassium salt is the form in which these compounds are presented to plants under natural conditions. In the cultures the potassium salts were used in concentrations varying from 0.0125 to 0.6 per cent. Some of the cultures were in liquid media, to others a sufficient amount of gelatine was added to produce a solid medium for convenience in handling.

In the series to determine the carbon values, the carbon was supplied in the form of the concentrated solutions of the acids or their potassium salts at different concentrations, the nitrogen in the mineral form of potassium nitrate, or organic nitrogen as peptone.

The corresponding controls were potassium nitrate + glucose and peptone + glucose.

In determining the nitrogen values the nitrogen was supplied as 0.15 per cent. of the potassium salts, the carbon as glucose, as against controls of peptone + glucose and potassium nitrate + glucose. In addition to these substances the usual ash constituents were added to the culture media. As a concentrated aqueous solution of the free acid contained only 0.07 gramme per litre of the compound, and we used relatively weak solutions of the potassium salts, it was necessary to equalise the conditions by using proportional amounts of the corresponding nitrogen or carbon compounds in the controls. In these the amount of peptone was 0.7 and of glucose 0.01 per cent.

Pending the completion of investigations into the cardinal points of temperature in relation to nutrition with the acids and their salts, the series of cultures here referred to were carried out at ordinary room temperature, and no very special allowance was made for differences of the osmotic pressure of the media. As a matter of fact, we had evidence which appeared to indicate that the optimum nutritive temperature is higher for the artificial than for the natural acids, and this point was not altogether ignored in judging of results. Further, certain differences in the growth habit of our plants were put down to differences in the osmotic pressure of the media, on the analogy of Beauverie's researches¹ with mineral solutions on moulds. The analogy, however, was not forced, in view of Laurent's observations² that an increase in the concentration of the medium if of organic materials is more readily endured by the plant than if the medium be of mineral matter only.

That we did not more fully allow for the effect of the temperature and pressure factors may be held as militating to a certain extent against the validity of our results. As against this may be placed the fact that we used as our experimental plant *Penicillium glaucum*, which is well known not only to have an extensive range of nutritive

1. *Comptes Rendus*, Vol. CXXXII, p. 226.

2. *Comptes Rendus*, 1902, Vol. CXXXV, p. 871.

materials but also to have marked accommodatory powers to differences of temperature and of osmotic pressure. Further, we aimed here at obtaining not absolute quantitative but relative qualitative results as to whether the acids and their salts could or could not act as carbon and nitrogen food substances respectively. Again, ordinary room temperature is a nearer approximation to the conditions under which the humic substances, the natural compounds especially, act on plants in nature than, say, an artificial temperature of 31°C ., the maximum nutritive temperature for *Penicillium* on sugar.

In determining the nutritive values of our acids and salts as carbon compounds we were precluded in the absence of accurate temperature data, as above mentioned, from utilising as criteria the 'economic coefficients.' After all, this proved to be no disadvantage. We started with the assumption, afterwards shown to be correct, that glucose + peptone had the highest nutritive value, and that the acid + peptone must have a lower coefficient, which, of course, would vary with the position of the optimum nutritive temperature in the scale. We found evidence to show that not only did the optimum for glucose differ from that of the acids, but that the acids themselves had different nutritive optimum temperatures; the relative order of superiority being glucose, artificial acids, natural acids. As there exists this difference in the optima, and as the coefficients are affected by change of temperature, by variations in the respiratory activity consequent on change of temperature, and further, as the pressure of the culture medium also changes with temperature and is not without effect on growth, it is obvious that whether the comparative nutritive values are based on the coefficient at the different optima or on other data from cultures grown at a uniform temperature the results are in neither case absolute owing to the experimental error due to the disturbing factors introduced by variations in the respiratory activity or of the osmotic pressure respectively.

We generally found that the initial growth was most rapid in the natural compounds, next in the artificial, while the glucose controls were usually the last to start. Had we estimated the carbon

values in the usual way by the relative rapidity of growth in the earlier stages, we should have put the natural compounds first, and the others in the above order of nutritive values. What actually occurred was that after the initial retardation the cultures in the artificial compounds and glucose controls made up on the others subsequently, and not unusually surpassed them, producing in a given time colonies of larger size. We compromised matters by noting the average rate of growth, and the ultimate size attained by the colonies after the lapse of a given time.

For convenience in reference the following symbols are used :—

Natural Humic Acid (from peat)—

Prepared through the ammonium salt N_a

„ „ potassium salt N_k

Artificial Humic Acid (from sugar)—

Prepared through the ammonium salt A_a

„ „ potassium salt A_k

The letter K prefixed to either of the above symbols indicates the potassium salt of the acid in question thus :—

The Potassium Salt of the Natural Humic Acid—

Prepared through the ammonium salt KN_a

„ „ potassium salt KN_k

The Potassium Salt of the Artificial Humic Acid—

Prepared through the ammonium salt KA_a

„ „ potassium salt KA_k

In order to emphasise the distinction between the natural and artificial acids, brought out not only by chemical analysis but also by the different physiological action of the acids on the living organism, we suggest that the name humic acid be applied only to the acid as prepared from peat, while the artificial acid prepared from sugar should be known by the new term of *saccharo-humic acid*.

Nutritive Values of the Acids and Salts as Carbon Compounds

These values were based on the results of the following series of cultures :—

Series I.—The nitrogen was supplied in the mineral form of potassium nitrate, the

carbon in the organic form of saturated solution of N_a , N_k , and A_a . (In the latter case, as already noted in the analytical part, traces of glucose are possibly present in addition.)

Series II.—The acids were replaced by KN_a , KN_k and KA_a of concentrations 0.0125, 0.025 and 0.05 per cent. The controls were potassium nitrate + glucose.

Series III.—The nitrogen was supplied in the organic form as peptone, carbon as saturated solutions of N_a , N_k and A_k .

Series IV.—For the acids were substituted KN_a , KN_k , KA_a of concentrations 0.025, 0.05, 0.075 per cent.

Series V contained peptone and KN_a , KN_k , KA_a and KA_k of concentrations 0.1, 0.15, 0.3 and 0.35 per cent. Peptone + glucose formed the controls to III, IV and V.

The general conclusions drawn from the results of these cultures are that, contrary to the experience of Nikitinsky,¹ not only the artificial but also the natural acids as well as their potassium salts serve as sources of organic carbon for *Penicillium*. As evidenced by differences in its growth forms and histological characters, the mould can discriminate between the artificial and the natural acids; the nutritive value varies with the character of the nitrogenous food supply; there is a differential value dependent on the concentration and the chemical nature of the acids and salts, and possibly also on the osmotic pressure of the medium.

When the results of the various cultures are reviewed seriatim the following conclusions are arrived at:—

Series I.—The nutritive combination potassium nitrate + acid is much inferior to nitrate + glucose: not only in its effects on vegetative growth, but also in conidial production; the conidia in the former never develop their characteristic colours. As carbon sources, the natural acids combined with mineral nitrogen are better than the artificial acids. The order of value is N_a , N_k , A_a .

This is exactly the opposite of what might be expected from the carbon contents of the acids, the artificial compound having the highest carbon content. On the other hand, the natural acids are richest in nitrogen, and this additional nitrogen may have had its effect on growth. Besides, the factor of osmotic pressure may enter here also, that of the artificial compounds being presumably

1. *Jabrb. wiss. Bot.*, 1902, Bd. XXXVII, p. 385.

lower than that of the natural acids. This difference in pressure may be inferred from the habit of growth in the various cultures, the mycelia grown in the natural acid showing most submerged growth, while that in the artificial compound shows least. Beauverie,¹ in experiments with mineral cultures on moulds, showed that increased concentration was accompanied by a greater amount of submerged growth of the mycelium.

Series II gave, on the whole, similar results to Series I. While in both series the growth was very scanty even in cultures several weeks old, that in the nitrate + salts was, if anything, even slightly poorer than in nitrate + acid, and *a fortiori* much less than the control.

The variation in growth with varying concentration of the salts is expressed in the following curves :—

At low concentration the artificial acid gave best results; at higher concentration, however, the natural acid yielded the maximum growth.

1. *Comptes Rendus*, Vol. CXXXII, p. 226.

In Series III, IV, and V the nitrogen was supplied as peptone, the carbon as the acid in concentrated solution or as the potassium salts of different degrees of concentration. This formed a very much better nutritive medium than either I or II; the plants grew almost as well as in the control, peptone + glucose, there being good vegetative growth, conidial formation being normal and the spores developing their characteristic colour. Presumably the carbon available in the peptone was drawn upon, but that the acids and salts exerted their influence was obvious from a comparison of the resulting growths. In Series III, peptone + acids, the artificial acids gave best growth, the order being A_k , N_a , N_k . The cultures in the glucose controls and in the artificial acids were late in commencing growth, but ultimately surpassed those in the natural acids, the resulting colonies being much larger in size (Plate I, figs. 1, 2, 3, and 4). Comparison of the subjoined curves illustrating the growth in Series IV (peptone + salts) with those of Series II (nitrate + salts) shows that for the same degree of concentration 0.025 and 0.05 per cent. the relative gradation of growth is similar in both series; at higher concentration, 0.075 per cent., the salts of the acid prepared through the potassium salts— KN_k and KA_k —lead.

Of the three salts in the above, KN_k and KA_k contain the most carbon, and the evidence of this is seen in the rise of their respective curves at concentration above .05 per cent., the KN_a , which has least, falling to third place.

In Series V the salts of the four acids were used at still higher concentration combined with peptone, and these series gave the most satisfactory comparative results of all. The growth was good, although somewhat inferior to that of peptone + acid and *a fortiori* to that of the control.

The following points are brought out in the above curves; above 0.3 per cent. the potassium saccharo-humates have highest value as carbon food compounds, the KA_a leading throughout from 0.1 to 0.35 per cent. Of the two potassium humates the KN_k leads

all through. This corresponds exactly to the relative carbon contents of the four compounds as expressed on p. 466, the order being A_a , A_k , N_k , N_a . Summarising the results as to carbon values of the acids and their salts, we arrive at the following conclusions. The acids and their salts in combination with organic nitrogen serve as good nutrient carbon material, but less so than sugar + peptone; that the acids and salts do act as carbon supply, and that the carbon is not derived entirely from the peptone, is proved by the fact that the combination nitrate + acid or salt also acts as a food material, although of a very much poorer character than peptone + acid or salt.

The nutritive values vary with concentration, chemical character of the acids, and perhaps also with temperature. At and above 0.3 per cent. the saccharo-humates have highest carbon value, at lower concentration there is some variability.

The Nutritive Value of the Humates and Saccharo-Humates as Nitrogen Compounds

This was ascertained by series of cultures (Series VI) in which the carbon was supplied in the organic form as glucose, the nitrogen as 0.15 per cent. humates and saccharo-humates, KN_a , KN_k , KA_k , KA_a —the mineral constituents were added, with the exception, of course, of nitrate. The controls were peptone + glucose. The order of growth here was KN_a , KN_k , KA_k , KA_a . Plate II shows the appearance of ten days' old cultures. In the humates (Figs 1 and 2) there was a luxuriant growth; in the saccharo-humates (Figs. 3 and 4) poor; some growth was, of course, to be expected in the KA_a , but an almost equal development of colonies occurred in the KA_k . When, however, the very great difficulty of keeping cultures quite nitrogen-free, and, further, the suspicion that *Penicillium* may have a slight power of assimilating free nitrogen is remembered, this growth is not inexplicable. The contrast however in the two sets of cultures is a very marked one, and can only be interpreted as due to the very much higher nitrogen content of the humate as contrasted with the saccharo-humate.

Contrasting the results of Series VI with the controls and the Series I-V, we draw the following conclusions. The combination humate + glucose has almost as high a nutritive value as peptone + glucose. Its nutritive value is greater than peptone combined either with the acids or with the humates or saccharo-humates, and *a fortiori* is better than nitrate + acids or salts, or even than nitrate + glucose.

The nutritive value of the acids and their salts as carbon and nitrogen compounds, respectively, for *Penicillium* may be tabulated as follows :—

Source of Nitrogen		Source of Carbon		Value
Potassium nitrate	...	Conc. N or A	...	Very slight
"	"	KN or KA ('0125-'05)	...	" "
"	"	Glucose	...	Fair
Peptone	...	Conc. N or A	...	High
"	...	KN or KA ('025-'35)	...	"
"	...	Glucose	...	Very high
KN	...	Glucose	...	" "

Comparative Histology of the Mycelia in the different Cultures

Microscopic examination of the mycelia grown in media containing humic and saccharo-humic compounds, respectively, confirms the view that these substances are not identical.

To refer here only to the characters of the vegetative mycelium we find that the hyphae grown in humic solutions exhibit the following points of contrast to those cultivated in saccharo-humic media. The former are more robust, septation is more frequent, and consequently the hyphal cells are shorter. At the same time the diameter of the cells is greater, while the cell walls are markedly thicker and generally of a brown colour, especially in the N_k cultures.

The branching is different in the two types. In the saccharo-humic cultures the branches were given off at an acute angle, while those in the humic cultures formed a right angle with the main hyphae.

In the manner of branching, in the delicate transparent character

of its hyphae, the mycelium of the saccharo-humic cultures closely resembled that from the glucose cultures, while the mycelium of the humic cultures by its thicker brown-walled hyphae of shorter cells was distinctly marked off from either of the others.

While holding that these differences indicate and are to be associated with differences in the chemical character of the humic and saccharo-humic compounds acting as food material to the mycelium, we, of course, admit the possibility of difference in osmotic concentration affecting the results to a certain extent. This has been already noted in reference to the relative amounts of submerged and floating mycelium in the fluid cultures containing the same percentages of the various compounds.

Laurent¹ has described for higher plants histological changes, such as increase in cell-wall thickness, shortening and widening of the cells and so forth, caused by differences in concentration of nutrient solutions containing organic substances. These differences are analogous to what we have found in the mycelia from our cultures containing equivalent percentages of the different compounds.

Correlation of Physiological and Chemical Results

The chemical investigations have shown that the artificial acids are richest in carbon, the relative carbon content is expressed in the descending series A_a , A_k , N_k , N_a .

Comparing this series with the growth series as given in Cultures V we find that at 0.3 and 0.35 per cent. the relative nutrient value of the potassium humates and saccharo-humates is according to the order— KA_a , KA_k , KN_k , KN_a . That is to say there is here complete concordance between the chemical and physiological series, and the latter therefore supply auxiliary evidence of the accuracy of the chemical analysis.

This is further borne out by the results of Series III represented in Plate I. Here the growth in the artificial compound stands first. The photographs 2, 3 and 4 may be taken as a series of auxanograms

1. *Comptes Rendus*, 1902, 135, 871.

of the relative carbon content of the acids. The resemblance between figs. 1 and 2, the glucose and artificial acid cultures, respectively, is striking, and seems to support the suggestion already put forward that traces of glucose occur in the specimens of the artificial acids. We endeavoured to test this hypothesis further by yeast cultures in nutrient solutions where the concentrated solutions of the acids were substituted for glucose. From all the cultures traces of CO_2 were given off, appreciably greater in the artificial forms, especially in A_k .

The iodoform test gave evidence of slight traces of alcohol in all the cultures. We, therefore, cannot regard this experiment as decisive of the question, for Nikitinsky (*loc. cit.*) has shown that certain bacteria can decompose humic acid with formation of CO_2 , and it is possible that yeast has the same faculty, and that the acids themselves were undergoing decomposition; on the other hand, there is the possibility that the cultures although carefully prepared were not above suspicion as to purity. The point is being further investigated.

Somewhat better, though not quite conclusive, are our experiments to determine bio-chemically the presence of iron in the natural acids. In nutrient solutions in which the humates and saccharo-humates were substituted for iron salts, various aquatic plants were placed. Up to the present these appear to be going in the right direction, particularly some experiments with *Lemna*. As *Lemna* is an iron-storer we adopt the precaution of removing each old leaf as soon as a new one appears, and thus exhaust the original store of iron in the plant, which then has to depend on the nutrient solution for its supply. So far the experiments lend support to the chemical statement that only the natural acids contain iron. A subsidiary feature not without significance is that micro-algae develop freely in the cultures with the natural humic salts, but not in those containing the artificial salts.

The relative nitrogen content of the acids, as chemically determined, is expressed by the series (highest first) N_a , N_k , A_a (A_k none).

The sixth series of cultures gives a growth series agreeing exactly

with this. In Plate II, which is a series of photographs of the relative growth, we have auxanograms of the nitrogen content. Here the influence of the relatively large percentage of nitrogen in the natural acids (figs. 1 and 2) is evidenced by the very much greater growth, the small development in the artificial forms being correlated with the smaller nitrogen content of the compounds.

In conclusion, we have to express to Professor T. Purdie our great indebtedness, both for his courtesy in placing the ample resources of his Research Laboratory at our disposal for carrying through the chemical part of this research, and also for his advice so kindly given during the progress of the work.

SYNOPSIS OF RESULTS

1. Natural humic acid obtained from peat varies greatly in composition, according to the method of preparation. When prepared through the ammonium salt the percentage of carbon is lower, and that of nitrogen is higher, than when prepared from the potassium salt.
2. Natural humic acid contains more combined nitrogen and less methoxyl than the artificial forms.
3. The form of humic acid obtained from cane sugar, which resembles most closely the natural acid, differs greatly in chemical structure from the latter.
4. The artificial acid is unaltered in composition by conversion into the ammonium salt and reprecipitation.
5. Humic and saccharo-humic acid and their potassium salts are utilisable by *Penicillium* as sources of organic carbon.
6. Potassium humate and, to a less extent, potassium saccharo-humate serve as organic nitrogen food compounds for *Penicillium*.
7. The conclusions drawn from the physiological results of experimental cultures of *Penicillium* in the acids and their potassium salts are in concordance with those arrived at as the result of chemical analysis of the compounds.

EXPLANATION OF PLATES

Plate I. A series of photographs of Petri Dish Cultures of *Penicillium* in

Peptone + glucose (fig. 1)

Peptone + A_a (fig. 2)

Peptone + N_a (fig. 3)

Peptone + N_k (fig. 4)

These represent auxanograms of the relative nutritive carbon values of glucose, the saccharo-humic and humic acids respectively.

Plate II. A series of photographs of Petri Dish Cultures of *Penicillium* in

KN_a + glucose (fig. 1)

KN_k + glucose (fig. 2)

KA_a + glucose (fig. 3)

KA_k + glucose (fig. 4)

These are auxanograms of the relative nutritive nitrogen values of the potassium humates and saccharo-humates respectively.

1

2

3

4

R. A. R., photo.

PLATE I. AUXANOGRAMS OF CARBON NUTRITIVE VALUES OF HUMIC AND SACCHAROHUMIC ACIDS AND *CONTROL GLUCOSE*

1

2

3

4

R. A. R., photo.

PLATE II. AUXANOGRAMS OF NITROGEN NUTRITIVE VALUES OF
0.15% POTASSIUM HUMATES AND SACCHAROHUMATES