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PARTITION COEFFICIENTS OF 27 GIBBERELLINS

R. C. DURLEY and R P. PHARIS
Biology Department, The University of Calgary, Calgary 44, Alberta, Canada

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Abstract—The effect of pH and buffer molarity on the partition coefficients between phosphate buffer solution and ethyl acetate have been examined for 27 of the characterized gibberellins and *ent*-kaur-16-en-19-oic acid Partition coefficients were used to estimate the pK_a of gibberellins A_3 and A_8 The use of other solvents commonly employed in the purification of gibberellins in plant extracts is discussed Application of partition coefficients to the countercurrent distribution of gibberellins is described

INTRODUCTION

THE INITIAL stages of the purification of gibberellins in plant extracts usually involve partitioning between aqueous and organic solvents, and a search of the literature revealed that there has been considerable variation in the methods employed. Ethyl acetate has been extensively used[1] to remove inhibitors and other extraneous material from aqueous gibberellin solutions at pH 7 (and at higher pH values) Chloroform,[1] petroleum ether[1,2] and ether[3] have been used for a similar purpose Gibberellins have been separated[4] from 'weaker acids' by back extraction from ethyl acetate into phosphate buffer at pH 6·2 Ethyl acetate,[4] chloroform[5] and methylene chloride[6] have been used to extract gibberellins from aqueous solution at pH 2 0–4 0.

Very little information has so far been presented[5,7] concerning the partition of the gibberellins between aqueous and organic solvents. It will become apparent from the data presented in this paper that most of the procedures utilizing partition as a method of purification may have resulted in at least partial loss of some of the gibberellins present. The normal detection methods for gibberellins, bioassay,[8] thin layer chromatography (TLC)-fluorescence,[9] gas liquid chromatography (GLC)[9] and combined gas chromatography–mass spectrometry (GC–MS)[10] are often inaccurate or even inapplicable to crude extracts from plants (particularly from vegetative tissue) due to large amounts of impurities, and the use of these methods to follow extraction techniques is of limited value. A knowledge of the partition of gibberellins between aqueous and organic solvents will enable procedures to be used that involve minimal loss of any gibberellins present.

Two other aspects of gibberellin distribution between aqueous and organic media are of

[1] D KÖHLER and A. LANG, *Plant Physiol* **38**, 555 (1963)
[2] G W M BARENDSE, H KENDE and A LANG, *Plant Physiol* **43**, 815 (1968)
[3] R C. DURLEY and R. P PHARIS, unpublished work
[4] J MACMILLAN, J C SEATON and P J SUTER, *Tetrahedron* **11**, 60 (1960)
[5] F. HAYASHI, S. BLUMENTHAL-GOLDSCHMIDT and L RAPPAPORT, *Plant Physiol* **37**, 774 (1962)
[6] L C LUCKWILL, P. WEAVER and J MACMILLAN, *J Hort Sci* **44**, 413 (1969)
[7] G W. ELSON and A R PETERSON, Brit Patent 1174924 (*Chem Abs* 72, 55702X), F J WOLF, *Separation Methods in Organic Chemistry and Biochemistry*, pp 31 Academic Press, New York (1969)
[8] A CROZIER, C C KUO, R. C DURLEY and R P PHARIS, *Can J Bot* **48**, 867 (1970)
[9] B D CAVELL, J MACMILLAN, R J PRYCE and A C SHEPPARD, *Phytochem* **6**, 867 (1967)
[10] R BINKS, J MACMILLAN and R J PRYCE, *Phytochem* **8**, 271 (1969)

importance First, countercurrent distribution (CCD) using phosphate buffer as the stationary phase and ethyl acetate as the moving phase has been used[11,12] to purify gibberellins in plant extracts and if this method is to be generally applied, a knowledge of the distribution of the gibberellins would be desirable Secondly, in a study of 'neutral' gibberellins,[13] confusion could arise between truly 'neutral' gibberellins and acidic gibberellins which are weakly polar but may well behave in a manner similar to neutral compounds

In view of the widespread use of partition procedures, particularly during the initial extractions stages, we report herein the partition coefficients of the gibberellins between phosphate buffer and some commonly used organic solvents The applicability of CCD to the purification of gibberellin extracts is discussed

RESULTS AND DISCUSSION

The partition coefficients of 27 of the 35 characterized gibberellins and *ent*-kaur-16-en-19-oic acid* between ethyl acetate and 1 5 M phosphate buffer solution at five pH's are given

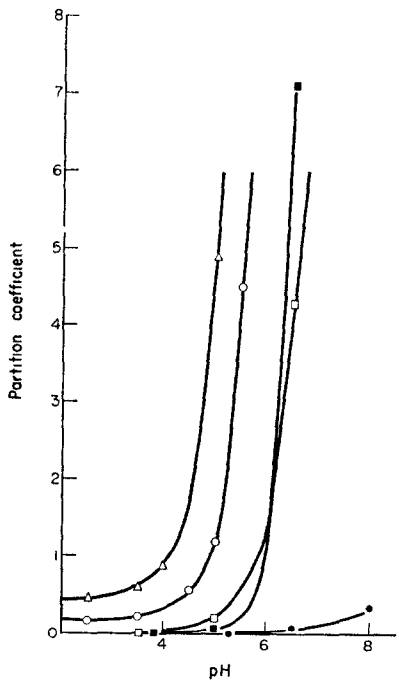

FIG 1 EFFECT OF pH ON PARTITION COEFFICIENTS OF GIBBERELLINS A_3, A_5, A_8, A_9 AND A_{13} BETWEEN ETHYL ACETATE AND 1 5 M PHOSPHATE BUFFER SOLUTION
○ A_3, □ A_5, △ A_8, ● A_9, ■ A_{13}

* The nomenclature used is that proposed in *The Common and Systematic Nomenclature of the Cyclic Diterpenes* (edited by J W ROWE), Forest Products Laboratory, Forest Service, U S Department of Agriculture, Madison, Wisconsin 53705, U S A

[11] (a) N MUROFUSHI, S TRIUCHIJIMA, N TAKAHASHI, S TAMURA, J KATO, Y WADA, E WATANABE and T AOYAMA, *Agric Biol Chem* **30,** 917 (1966), (b) N MUROFUSHI, N TAKAHASHI, T YOKOTA and S TAMURA, *Agric Biol Chem* **32,** 1239 (1968), (c) N MUROFUSHI, N TAKAHASHI, T YOKOTA, J KATO, Y SHIOTANI and S TAMURA, *Agric Biol Chem* **33,** 592 (1969), (d) T YOKOTA, N TAKAHASHI, N MUROFUSHI and S TAMURA, *Planta* **87,** 180 (1969)

[12] A CROZIER, H AOKI and R P PHARIS, *J Exptl Bot* **20,** 786 (1969)

[13] (a) F HAYASHI and L RAPPAPORT, *Nature, Lond* **195,** 617 (1962), (b) F HAYASHI and L RAPPAPORT, *Plant Physiol* **41,** 53 (1966), (c) T HASHIMOTO and L RAPPAPORT, *Plant Physiol* **41,** 623 (1966)

TABLE 1. PARTITION COEFFICIENTS ($K_d = c_{aq}/c_{org}$)* OF THE GIBBERELLINS AND *ent*-KAURENOIC ACID BETWEEN ETHYL ACETATE AND 1 5 M PHOSPHATE BUFFER SOLUTION AT FIVE pH VALUES

Gibberellin	pH K_d				
	8 0	6 5	5 0	3·5	2 5
A_1	∞	∞	1 2	0 17	0 11
A_2	∞	7 9	0 97	0 19	0 15
A_3	∞	∞	1 2	0 21	0 17
A_4	2 2	0 29	0 05	0	0
A_5	∞	4 8	0 19	0	0
A_6	∞	5 4	0 49	0 05	0
A_7	3 2	0 56	0 10	0	0
A_8	∞	∞	4 9	0 64	0 45
A_9	0 34	0 06	0	0	0
A_{10}	11 3	1 6	0 33	0	0
A_{12}	0 56	0 04	0	0	0
A_{13}	∞	7 1	0 06	0	0
A_{14}	∞	0 41	0	0	0
A_{16}	∞	3 2	0 16	0	0
A_{17}	∞	∞	0 50	0 04	0
A_{18}	∞	∞	0 42	0	0
A_{19}	∞	4 6	0 81	0 10	0
A_{20}	∞	2 1	0 09	0	0
A_{21}	∞	∞	9 1	0 89	0 08
A_{22}	∞	15 1	1 4	0 51	0 19
A_{23}	∞	∞	19 4	1 0	0 17
A_{24}	∞	0 83	0	0	0
A_{25}	13 1	0 66	0	0	0
A_{26}	∞	∞	3 2	0 44	0 21
A_{27}	∞	1 6	0 18	0 05	0
A_{28}	∞	∞	12 7	0 81	0 07
A_{29}	∞	∞	1 9	0·21	0 15
ent-kaurenoic acid	0 24	0 04	0	0	0

* K_d's < 0 02 are taken as 0, values > 20 are taken as ∞

in Table 1 and shown in graphical form for purposes of discussion for certain of them in Fig 1. The partition coefficients are expressed as

$$K_d = \frac{\text{concentration in aqueous phase}}{\text{concentration in organic phase}}.$$

To prevent emulsions forming during extraction and countercurrent distribution we have found the use of high molarity buffer solutions (1 5 M and 0·5 M) to be advantageous. Therefore a 1·5 M phosphate buffer was employed to obtain the coefficients given in Table 1. Concentration of the gibberellins in the organic phase was calculated by measuring the GLC peak areas of the methyl ester or methyl ester trimethylsilyl ether derivatives Comparison was always made with injections of standard amounts of the gibberellins in question It can be seen that the partition coefficients vary widely and we have classified the gibberellins into four main groups:

(a) Gibberellins A_4, A_7, A_9 and A_{10} at buffer pH 8 were significantly partitioned into the ethyl acetate phase whilst at pH 3·5 or less were totally partitioned into the ethyl acetate phase

(b) The di- and tricarboxylic gibberellins A_{12}, A_{13}, A_{14}, A_{17}, A_{18}, A_{21}, A_{23}, A_{24}, A_{25} and A_{28} (exception A_{19}) exhibited a rapid increase of K_d with increasing pH, more so than the monocarboxylic acids and may exhibit a more complex curve than that indicated.

(c) Gibberellins A_5, A_6, A_{16}, A_{19}, A_{20} and A_{27} were not partitioned into the ethyl acetate phase at pH 8, but were completely partitioned into the ethyl acetate phase at pH 2 5

(d) Gibberellins A_1, A_2, A_3, A_8, A_{22}, A_{26} and A_{29} were not appreciably partitioned into the ethyl acetate phase at pH 8 or 6 5, and were only partially removed into the ethyl acetate phase at pH 2 5 Gibberellins A_{21}, A_{23} and A_{28} may also be placed in this group.

Another general observation is that monohydroxylated gibberellins having a C-13 hydroxyl group are more soluble in aqueous solution than those which are hydroxylated in ring A. For example, compare the K_d's of the following pairs of isomers. A_5 with A_7; A_{17} with A_{13}; and A_{20} with A_4

To determine whether the molarity of the buffer solution significantly altered gibberellin distribution, gibberellins A_3, A_5, A_9 and A_{13} (one from each of the above general classes) were partitioned between ethyl acetate and phosphate buffer solutions of 0 5, 0·1 and 0 05 M. The results are given in Table 2. It is evident that the higher the molarity of the buffer the

TABLE 2 PARTITION COEFFICIENTS ($K_d = c_{aq}/c_{org}$)* OF GIBBERELLINS A_3, A_5, A_9 AND A_{13} BETWEEN ETHYL ACETATE AND FOUR MOLARITIES OF PHOSPHATE BUFFER SOLUTION AT FIVE pH'S

		pH K_d				
	Molarity	8 0	6 5	5 0	3 5	2 5
Gibberellin A_3						
	1 5	∞	∞	1 2	0 21	0 17
	0 5	∞	∞	2 8	0 63	0 41
	0 1	∞	∞	7 2	1 4	0 86
	0 05	∞	∞	19 2	2 7	1 31
Gibberellin A_5						
	1 5	∞	4 8	0 19	0	0
	0 5	∞	14 2	0 41	0 02	0
	0 1	∞	∞	0 80	0 05	0
	0 05	∞	∞	1 2	0 09	0
Gibberellin A_9						
	1 5	0 34	0 06	0	0	0
	0 5	0 98	0 13	0	0	0
	0 1	2 9	0 29	0 02	0	0
	0 05	3 8	0 37	0 03	0	0
Gibberellin A_{13}						
	1 5	∞	7 1	0 06	0	0
	0 5	∞	20 0	0 13	0 02	0
	0 1	∞	∞	0 20	0 04	0
	0 05	∞	∞	0 28	0 08	0

* K_d's < 0 02 are taken as 0, values > 20 are taken as ∞.

greater the tendency of the gibberellins to move into the ethyl acetate phase, due to the 'salt effect'[14] of the buffer increasing the activity coefficient of the gibberellins. By calculating the ratio of the K_d's of any one of the four gibberellins above at a given pH using 1·5 M buffer, to the K_d's of any other gibberellin falling within the same 'group' at the same pH using 1 5 M buffer (i e the K_d ratio of A_3 to A_1, A_2, A_8, A_{21}, A_{22}, A_{23}, A_{26}, A_{28} and A_{29}; A_5 to

[14] F. A LONG and W F MCDEVIT, *Chem Rev* **51**, 119 (1952).

A_6, A_{16}, A_{19}, A_{20} and A_{27}; A_9 to A_4, A_7 and A_{10}; A_{13} to A_{12}, A_{14}, A_{17}, A_{18}, A_{24}, A_{25}) and comparing these ratios to the K_d's obtained for the test gibberellins at that pH using either 0·5, 0·1 or 0·05 M buffers, the K_d for the other gibberellins can be estimated for 0·5, 0·1 and 0 05 M buffer. This was found to be a reasonable approximation in test cases. For example gibberellins A_3 and A_8 using 1·5 M buffer at pH 2 5 have K_d's of 0 17 and 0·45 respectively. Since A_3 has a K_d of 0 41 using 0·5 M buffer at pH 2 5, A_8 would be expected to have a K_d around $0\,45/0{\cdot}17 \times 0\,41 = 1\,1$ using the same buffer at the same pH. The experimental value was found to be 1 0.

The variation of K_d with pH depends on the pK_a of the acid. For an acid partitioning between an aqueous and organic system, which may be depicted as

$$\text{HA (organic)} \overset{K_r}{\rightleftharpoons} \text{HA (aqueous)} \overset{K_a}{\rightleftharpoons} H^+ \times A^-,$$

the following equation has been derived:[15]

$$K_d = K_r \left(1 + \frac{K_a}{[H^+]}\right) \tag{1}$$

where K_d is the observed partition ratio, K_r the true partition ratio of the un-ionized forms, K_a the dissociation constant for the acid and $[H^+]$ the hydrogen ion concentration in the aqueous phase. Therefore, knowing the variation of K_d with $[H^+]$, the pK_a of a gibberellin can be calculated. This was attempted for two gibberellins, A_3 and A_8.

The equation depends on the following assumptions:

(1) The acid contains only one ionizable group. Hence only monocarboxylic gibberellins can be considered since the variation of K_d with pH for di- and tricarboxylic gibberellins may well be complex.

(2) The ionized molecules cannot be extracted into the organic phase.

(3) The acid does not form association complexes in either phase. It was found by osmometry that gibberellin A_3 did not dimerize in ethyl acetate solution at concentrations of 0·01 M and 0·008 M. Furthermore, if dimerization occurs to any significant extent in either phase, it can be calculated that the partition coefficient is concentration dependent.

TABLE 3. EFFECT OF CONCENTRATION ON THE PARTITION COEFFICIENT OF GIBBERELLIN A_3 BETWEEN ETHYL ACETATE AND 1·5 M PHOSPHATE BUFFER SOLUTION AT pH 5 0

Concentration (μg ml^{-1})*	K_d
5	1 4
10	1 3
20	1 1
30	1 2
40	1 1
50	0 94
60	0 81
80	0 53

* Concentration of the total gibberellin dissolved initially in the ethyl acetate phase

[15] (a) L C CRAIG, *J Biol Chem* **150**, 33 (1943), (b) J CYMERMAN-CRAIG and A A. DIAMANTIS, *J. Chem. Soc* 1619 (1953)

Therefore the partition coefficient of gibberellin A_3 between ethyl acetate and 1 5 M phosphate buffer at pH 5 0 was determined at seven concentrations between 5 and 80 μg ml^{-1} (initially dissolved in the ethyl acetate phase) The results are shown in Table 3 It can be observed that K_d does not vary appreciably with concentration between 5 and 40 μg ml^{-1}, indicating that dimerization is not occurring and hence equation (1) can be applied to this range However, K_d decreases with increasing concentration above 40 μg ml^{-1} This may be due to a lack of solubility of the gibberellins at these concentrations in 1 5 M phosphate buffer at pH 5 0, hence forcing them into the ethyl acetate phase and thereby decreasing K_d

(4) If a buffer is used, the effect of the buffer on partition does not vary with pH No account of the 'salt effect' has been taken into consideration It is known[14,16] that the addition of a salt to an aqueous solution of a nonelectrolyte will increase or decrease the activity coefficient of the nonelectrolyte and this will alter an equilibrium constant such as K_d If the salt effect is taken into account equation (1) becomes complex However in the range pH 3 5–6 0 (the range over which the equation was tested) the ionic strength of the solution varies by only a small amount (1 0–1 2) and hence the salt effect may remain approximately constant For this reason the above simple equation was used to calculate a value of K_a in phosphate buffer Two gibberellins were tested, A_3 and A_8

Equation (1) may be rewritten in the form

$$\log (K_d - K_r) = \mathrm{pH} + K_r - \mathrm{p}K_a \qquad (2)$$

Hence by plotting a graph of log $(K_d - K_r)$ against the pH, a straight line of gradient unity should be obtained A value of K_r can theoretically be obtained since from equation (1) $K_d \simeq K_r$ when $[H^+]$ is large It can be seen from Fig 1 that for gibberellins A_3 and A_8, K_d is almost a constant in the range pH 2 5 and less, indicating that at pH 2 5 ionization of the gibberellins is almost repressed and hence $K_d \simeq K_r$ The reported pK_a's of gibberellins A_3 and A_8 in water are 3 97 and 4 04 respectively [17] If these can be applied to aqueous buffered solutions, this would also indicate that these gibberellins would exist almost completely in the un-ionized form at pH 2 5 Since the results given in Table 1 were insufficient to test the applicability of equation (2) to gibberellins, further K_d's were obtained for gibberellins A_3 and A_8 between ethyl acetate and 1 5 M phosphate buffer at various pH's, and are given in Table 4 Taking $K_d = K_r$ at pH 2 5, a plot of log $(K_d - K_r)$ against pH for each gibberellin is

TABLE 4 FURTHER PARTITION COEFFICIENTS ($K_d = c_{aq}/c_{org}$) OF GIBBERELLINS A_3 and A_8 BETWEEN ETHYL ACETATE AND 1 5 M PHOSPHATE BUFFER

pH	K_d	
	Gibberellin A_3	Gibberellin A_8
6 0	12 5	
5 5	4 5	10 8
5 0	1 2	4 9
4 5	0 53	1 8
4 0	0 33	0 90
3 5	0 21	0 64
2 5	0 17	0 45

[16] P DEPYE and J MCAULAY, *J Physik Z* 26, 22 (1925)
[17] B K TIDD, *J Chem Soc* 1521 (1964)

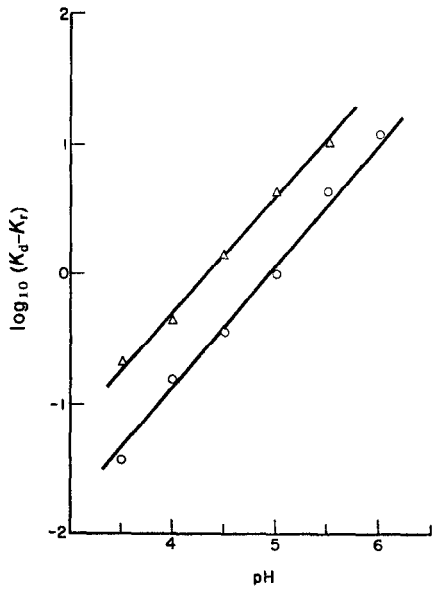

FIG 2 GRAPH OF LOG ($K_d - K_r$) AGAINST pH FOR GIBBERELLINS A_3 AND A_8

○ A_3; △ A_8.

shown in Fig 2. This plot gave straight lines of gradient 0 93 for gibberellin A_3 and 0·89 for gibberellin A_8 The discrepancy between these values and the theoretical gradient of 1 00 may be due to the 'salt effect' or a slight inaccuracy in the estimated value of K_d, but it was thought that the values were close enough to the theoretical value to indicate that equation (2) may be applied to these gibberellins

Estimates of the pK_a values of these two gibberellins can be made It is evident from equations (1) and (2) that when

$$pK_a = pH$$
$$K_d = 2K_r.$$

For gibberellin A_3, when

$$K_d = 2K_r = 0{\cdot}34,$$

from Fig 1,

$$pK_a = pH = 4\,1.$$

For gibberellin A_8, when

$$K_d = 2K_r = 0\,90$$

from Fig. 1,

$$pK_a = pH = 4{\cdot}0.$$

It is also evident from equation (2) that when

$$\log (K_d - K_r) = 0,$$
$$pK_a = pH + \log K_r$$

From Fig 2, pK_a values for gibberellins A_3 and A_8 of 4 1 and 4·0 respectively were calculated Both of these pK_a's of gibberellin A_3 and A_8 in 1·5 M phosphate buffer solution are in approximate agreement with the previously reported[17] values in water of 3 97 and 4 04 respectively, although in the present work, gibberellin A_3 has a slightly higher pK_a than gibberellin A_8

Equation (2) was not tested with gibberellins other than A_3 and A_8 However preliminary results indicate that the equation does not apply to a number of gibberellins and hence would have to be modified in the general case

TABLE 5 PARTITION COEFFICIENTS ($K_d = c_{aq}/c_{org}$)* OF GIBBERELLINS A_3, A_4, A_5, A_9, A_{13} AND *ent*-KAURENOIC ACID BETWEEN DIETHYL ETHER AND 0 5 M PHOSPHATE BUFFER AT 6 pHs

Gibberellin	pH K_d					
	9 0	8 0	6 5	5 0	3 5	2 5
A_3	∞	∞	∞	18 5	7 0	3 1
A_4	∞	∞	4 1	0 72	0 12	0
A_5	∞	∞	∞	3 1	0 32	0 12
A_9	∞	6 2	0 28	0 07	0	0
A_{13}	∞	∞	∞	1 1	0 33	0 15
ent-kaurenoic acid	1 0	0 39	0 12	0	0	0

* K_d's < 0 02 are taken as 0, values > 20 0 are taken as ∞

Since diethyl ether[3] and light petroleum[1,2] have been employed to wash aqueous gibberellin extracts in order to remove chlorophyll, inhibitors etc., and the former also employed to isolate auxins[18] and abscisic acid[19] from aqueous plant extracts, it was of interest to determine the extent of distribution of the gibberellins between phosphate buffer and these solvents. Gibberellins A_3, A_4, A_5, A_9, A_{13}, *ent*-kaur-16-en-19-oic acid and a 0·5 M buffer solution were used. The results are given in Tables 5 and 6. In the case of the ether-buffer partitions, no gibberellins were partitioned into the ether phase at pH 9, whereas at pH 2 5–3·5 considerable amounts of all compounds were partitioned into the ether phase. Therefore auxins and abscisic acid could not be separated from most of the gibberellins merely by partition. In the case of petroleum-buffer partitions gibberellin A_9 was the only gibberellin to be partitioned into the organic phase even under acidic conditions. The results

TABLE 6 PARTITION COEFFICIENTS ($K_d = c_{aq}/c_{org}$)* OF GIBBERELLIN A_9 AND *ent*-KAURENOIC ACID BETWEEN PETROLEUM ETHER b p 65–77° AND 0 5 M PHOSPHATE BUFFER SOLUTION AT 6 pH VALUES

	pH K_d					
	9 0	8 0	6 5	5 0	3 5	2 5
Gibberellin A_9	∞	∞	∞	5 6	1 4	0 39
ent-kaurenoic acid	∞	1 2	0 49	0 06	0	0

* K_d's < 0 02 are taken as 0, values > 20 are taken as ∞.

[18] D. BURNETT, L J AUDUS and H D ZINSMEISTER, *Phytochem* **4**, 891 (1965)
[19] B V MILBORROW, *Planta* **76**, 93 (1967)

for *ent*-kaurenoic acid must be treated with caution since it is almost insoluble in aqueous solution at pH 2·5–7·0. To avoid a concentration effect, concentrations of less than 1 μg ml^{-1} of this acid (dissolved initially in the buffer phase) were employed. Chloroform was not examined as a partition solvent since we have found it to readily form emulsions when shaken with concentrated aqueous plant extracts

Crozier *et al*[12] have discussed the use of CCD of gibberellins, using ethyl acetate as the upper (moving) phase and phosphate buffer as the lower (stationary) phase, as a means of purifying gibberellins in plant extracts. The CCD formula[20] relates the distribution of a compound throughout the CCD tubes with its partition coefficient

$$T_r = \frac{n!\ K^r}{r!\ (n-r)!\ (K+1)^n} \tag{3}$$

where T_r is the fraction of the total in tube number r (0, 1, 2 . . . . . n), n is the total number of *transfers* and K is the distribution coefficient (equal to $1/K_d$ above). Hence the centre of distribution and the extent of distribution of any compound may be calculated from its partition coefficient As pointed out by Crozier *et al*,[12] the most efficient purification of a gibberellin extract by CCD is accomplished by localizing the gibberellins (if possible) in the centre tubes, since a high proportion of the dry weight of a crude plant extract will most likely be located in the lower phase of the first tubes and the upper phase of the last tubes. It will be readily observed that if a compound has a partition coefficient of approximately 1·0 it will be distributed in the centre tubes. Hence by using Tables 1 and 2, and graphs drawn therefrom, it should be possible to determine the pH and the molarity of the buffer solution which would localize the gibberellin(s) in question in the centre tubes.

The partition coefficients of gibberellins A_3, A_4, A_5, A_7, A_8 and A_9 were checked by CCD using ethyl acetate and phosphate buffer at two molarities (0·5 and 1·5 M) and at three acidities (pH 8·5, 5·0 and 2 5). The distribution of each gibberellin agreed well with its calculated distribution from equation (3) using the partition coefficients in Tables 1 and 2. By using a combination of CCD's at various acidities as described previously,[12] it should be possible to considerably purify any plant extract containing a combination of the gibberellins given in Table 1 with minimal loss of those gibberellins, provided the distribution of each gibberellin at any given buffer pH and molarity is calculated from its partition coefficient at that buffer pH and molarity. This would avoid bioassaying each tube, which would almost certainly be inaccurate (or impossible) due to the low activity of some of the gibberellins and the presence of inhibitors However, extract dry weight loaded onto CCD should be kept to a minimum since the added molarity effect may alter gibberellin distribution to some extent.

EXPERIMENTAL

GLC

A dual column F & M 402 gas chromatograph was used with heated injectors and flame ionization detectors Silanized 6 ft × 1/8 in i d glass columns were packed with 2% QF-1 or 2% SE-30 on Gaschrom Q (Applied Science Laboratories Inc). Columns were pretreated by baking for 24 hr at 250° with carrier gas N_2 flowing at 30 ml min^{-1} Chromatography was carried out at oven temperature of 200°, injector and detector temperatures of 240° and 250° respectively, and nitrogen flow rates of 60 ml min^{-1} and 70 ml min^{-1} on the 2% QF-1 and 2% SE-30 columns respectively Efficiency of the 2% QF-1 and 2% SE-30 columns were 1950 and 5090 theoretical plates respectively as calculated by 5α-cholestane The methyl esters and the methyl ester trimethylsilyl ethers were prepared for chromatography by the method of Cavell *et al*[9]

[20] B Williamson and L. C Craig, *J Biol Chem* **168**, 687 (1947)

Partition Experiments

All experiments were performed at 22°

With ethyl acetate and 1 5 M *buffer solution* The gibberellins (50–1000 μg, according to availability) were dissolved in EtOAc and 10 aliquots (each 10 ml) of these solutions were shaken for 1 hr with 10 aliquots (each 10 ml) of 1 5 M Na_2HPO_4–KH_2PO_4 buffer at five acidities of pH 8 0, 6 5, 5 0, 3 5 and 2 5 Buffer solutions at pH 3 5 and 2 5 were prepared by acidifying pH 5 0 buffer Additional acidities (see text) were used for gibberellins A_3 and A_8 Each experiment was duplicated The EtOAc phases were separated and recovered, and the residues esterified In some cases the remaining buffer solutions were further shaken with EtOAc (10 ml each) and the residues from the EtOAc esterified to obtain an additional check on the results A control for each set of experiments was prepared by taking a further aliquot (10 ml) of the original EtOAc solution and preparing the gibberellin esters in the same way In the case of gibberellins A_8, A_{26} and A_{27} the methyl ester trimethylsilyl ethers were used for chromatography A known quantity of each methyl ester or methyl ester trimenthylsilyl ether mixture was chromatographed on GLC and the area under each peak was measured The distribution coefficient was calculated by

$$K_d = \frac{a - b}{b}$$

where a is the peak area of the control gibberellin derivative and b is the peak area of the gibberellin derivative obtained from the partition experiments Dilution and reinjection of the controls was necessary since it was found that GLC peak areas did not exactly correspond to gibberellin derivative concentrations The error in this method was estimated to be approximately 6% for gibberellins A_{1-10}, A_{12-14}, A_{16-18} and *ent*-kaur-16-en-19-oic acid, and 8–9% for gibberellins A_{19-29}

With ethyl acetate and 0 5, 0 1 *and* 0 05 M *buffer solution* The above partition experiments were repeated with gibberellins A_3, A_5, A_9 and A_{13} using 0 5 M, 0 1 M and 0 05 M phosphate buffers

Concentration of each gibberellin in each phase was calculated as above

With diethyl ether and 0 5 M *buffer solution, and petroleum ether and* 0 5 M *buffer solution* Gibberellins A_3, A_4, A_5, A_9, A_{13} and *ent*-kaur-16-en-19-oic acid were dissolved in 0 5 M phosphate buffer at pH 8 and aliquots (10 ml each) were acidified to the required pHs by adding known amounts of conc HCl The aq gibberellin solutions were shaken with an equal amount of Et_2O or petrol b p 65–77° The control was extracted 5 times at pH 2 5 with EtOAc The concentration of each gibberellin in each phase was calculated as above Initial concentration of *ent*-kaur-16-en-19-oic acid in the buffer phase was 1 μg ml^{-1}

Countercurrent Distribution

This was carried out using a twenty tube Pope 200 ml manual extractor * The lower (stationary) phase was 100 ml of phosphate buffer saturated with EtOAc and the upper (moving) phase was 100 ml of EtOAc saturated with phosphate buffer Five countercurrent distributions were carried out with a mixture of gibberellins A_1, A_4, A_5, A_8 and A_9 (100 μg of each) using 0 5 M buffer at pH 8 5, 5 2 and 2 5, and 1 5 M buffer at pH 5 2 and 2 5 After the completion of nineteen transfers the upper and lower phases were separated The lower phase from each tube was adjusted to pH 3 0 and extracted with EtOAc (4 × 100 ml), which was combined with the upper phase, dried and recovered A TLC of aliquots of the residues on silica gel (Kieselgel H) developed with isopropyl ether–HOAc (19 1) followed by spraying with 50% H_2SO_4 and examination of fluorescence under UV light enabled an estimate to be made of the concentration of each gibberellin in each tube

Osmometry

The instrument used was a Mechrolab Osmometer model 301 Gibberellin A_3 was dissolved in EtOAc at molarities of 0 01 M and 0 008 M Mol wt found was 355 (theory 344)

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