284. *Amino-acids and Peptides. Part III. The Solubility Criterion of Chemical Purity with Special Reference to Amino-acids. The Use of Differential Vapour-pressure Measurements.*

By I. W. Hughes, E. W. Williams, and G. T. Young.

The solubility of a pure substance is independent of the amount of excess solute present, and this " solubility criterion " of purity is of great value for substances for which more normal criteria are inapplicable. The use of a direct differential procedure for detecting differences in solubility offers many advantages, and in this paper a method is described in which differential vapour-pressure measurements are used to detect small amounts of impurities in materials such as amino-acids. The limitations of the method are discussed.

During work on the isotope dilution method of amino-acid analysis, we have encountered the difficulty of establishing the purity of the sample isolated, on which the accuracy of the determination depends. Observation of the sharpness of melting point is of little value for amino-acids and, since the contaminants may be of closely similar composition or even isomeric, elementary analysis is of limited use. In such cases, a valuable criterion has been derived from the fact that the solubility of a pure substance is independent of the amount of excess solute present; this has been used to examine the homogeneity of proteins (Kunitz and Northrop, *J. Gen. Physiol.*, 1930, **13**, 781; Butler, *ibid.*, 1940, **24**, 189) and of amino-acids (Dunn, Frieden, Stoddard, and Brown, *J. Biol. Chem.*, 1942, **144**, 487). In these cases, conventional methods for solubility determination were applied.

It is not, however, necessary to obtain absolute measurements for this purpose and differential procedures present many advantages. An interesting approach was that by Halford (*J. Amer. Chem. Soc.*, 1931, **53**, 2640) who observed the elevation in boiling point of successive saturated extracts of the substance to be examined. We have developed a method, suitable for use with materials of relatively low molecular weight, by which the difference in solubility is detected by a direct comparison of the vapour pressures of the solutions. Essentially, two saturated solutions are prepared, one containing the minimum excess of solute and the other a large excess; the difference in vapour pressure is then observed by means of a differential tensimeter. A preliminary note (Hughes and Young, *Nature*, 1949, **164**, 503) reported this development and its use in establishing the purity of amino-acids. In this way it is possible to detect contamination by enantiomorphs, and proof of identity, analogous to that by mixed melting point determinations, may also be obtained. We now describe fully the apparatus and procedure, and present the results of a more detailed examination of the sensitivity of the method in certain cases.

Properties other than vapour pressure may, of course, be used as the basis of a differential solubility test. Since the publication of our note, the use of radioactivity measurements for this purpose has been described (Gutmann and Wood, *Science*, 1949, **110**, 662). We have developed an alternative general method, in which the refractive indices of the solutions are compared. This will be described later.

The sensitivity of the vapour-pressure method under typical conditions has been determined for three series of mixtures, consisting of known amounts of (*a*) potassium nitrate in " AnalaR " sodium chloride, (*b*) DL-alanine in " AnalaR " glycine, and (*c*) L-aspartic acid hydrochloride in L-glutamic acid hydrochloride. The results are shown in Figs. 1—3. Over the range of concentration investigated and under the conditions specified in the Experimental section, the difference in vapour pressure is proportional to the amount of impurity present, and in each case *ca.* 0·2% of the contaminant was readily detected.

In the preliminary work (Hughes and Young, *loc. cit.*), we had difficulty in obtaining a satisfactory sample of glycine; during the present work several samples of " AnalaR " glycine were examined and each appeared to be of high purity.

The Significance and Sensitivity of the Differential Solubility Test.—The application of the solubility criterion of purity to proteins has been discussed by Kunitz and Northrop and by Butler (*locc. cit.*). In brief, the observation that the solubility is independent of the amount of excess solid leads to the deduction from the phase rule that the number of components (including the solvent) is equal to the number of phases; any number of insoluble components might

therefore be present. In practice, such a test is normally applied to materials purified by repeated crystallisation, and the contaminants are likely to be of similar solubility. In order to be able to deduce from this test that the sample is pure, it is however essential that the smaller excess of solid should consist of a single phase, and for this reason emphasis is laid on the use of the minimum excess of solid in one vessel. If two substances are present in amounts exactly proportional to their solubilities, they will behave for this purpose as one component; impurity

FIG. 1.

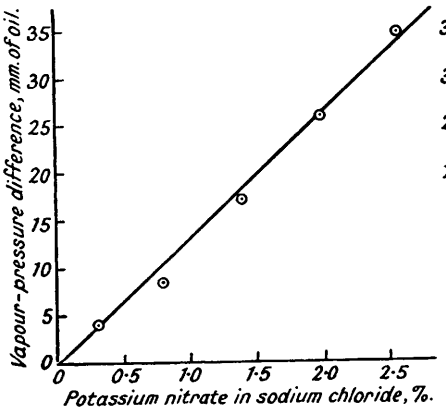


FIG. 2.

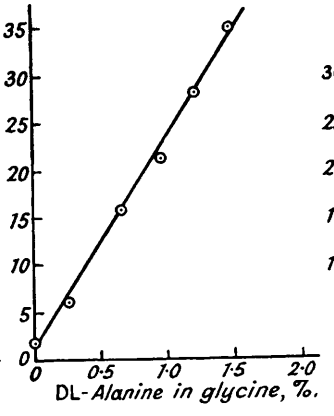


FIG. 3.

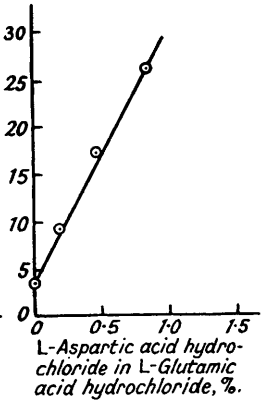
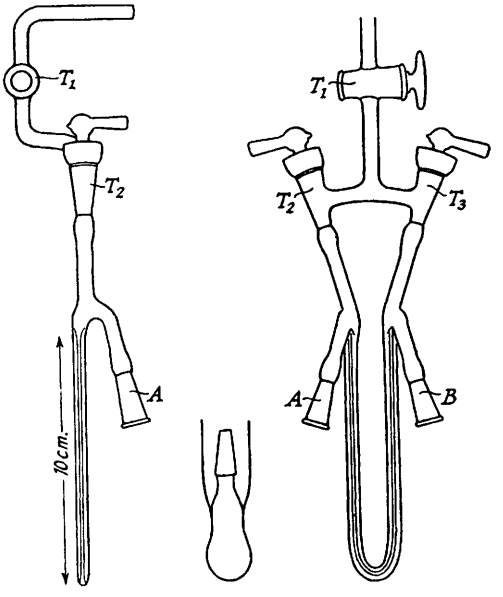


FIG. 4.



should however be detected by changing the temperature or by examination in a second solvent; in either case it is unlikely that the solubilities will change in the same proportion. In the case of solid solutions, equilibrium with the solvent may be difficult to establish, but unless again the substances are present in the same ratio as their solubilities, the criterion should be applicable. It must be noted that contamination by the solvent in which the test is made will not be detected.

The sensitivity of the method depends on the difference in concentration of the impurity in the two solutions. With purified materials, this concentration will be low in one solution, whereas in the other the amount of solid may be increased until the solution is saturated both

with the main component and with the impurity. The greatest sensitivity may therefore be expected when the solubility of the impurity is high. The sensitivity clearly depends also on the molecular weight of the impurity; small amounts of macromolecules would not be detected by the present method. The use of higher temperatures will also increase the sensitivity, but practical difficulties, such as the prevention of leaks, also become greater.

The use of the differential vapour-pressure test is not, of course, restricted to the examination of amino-acids, and it may be found of value for the establishment of the purity or of the identity (Hughes and Young, *loc. cit.*) of many other types of substance to which more normal criteria are inapplicable.

Experimental.

Apparatus.—The apparatus, of Pyrex glass, is drawn to scale in Fig. 4. The U-tube manometer is of uniform capillary tubing, 1·5 mm. in diameter. Taps T_2 and T_3 are right-angled, with mercury seals. Two small vessels (volume *ca.* 2 ml.), which contain the solutions, have B.10 " Quickfit " cones (ground further if necessary) to fit sockets *A* and *B*; these vesels also are provided with mercury seals. It is advisable to keep the volume of the apparatus as small as practicable, to diminish the effect of slight differences in the temperature of the arms. For the same reason, the apparatus is so constructed that the vessels are as close to each other as possible, lying immediately behind the manometer. For the taps and joints, we have found " Silicone " grease satisfactory, provided that it is thoroughly removed after each experiment. The apparatus is supported in a glass-walled thermostat bath maintained at 50° ± 0·1°; the barrels, but not the handles, of taps T_2 and T_3 are immersed. We have found Apiezon Oil C (d^{50}, 0·80) a more satisfactory manometer fluid than the xylene mentioned in our preliminary note. The difference in level of the oil in the two arms was measured sufficiently accurately for the present work by means of a graduated scale mounted behind the manometer.

Method.—The following procedure is typical of that adopted to examine the purity of a substance, when using aqueous solutions and a temperature of 50°.

Into one vessel is weighed just sufficient of the material to saturate 0·1 ml. water and to leave a visible excess, and approx. ten times this weight is placed in the second vessel; water (0·1 ml.) is then added to each. It is helpful to mix the solvent and solute with a thin glass rod. The joints are carefully greased and both vessels attached firmly to the apparatus, all taps being open. Mercury is placed in the seals and the apparatus is transferred to the thermostat. After some minutes (to allow the air to expand) tap T_1 is closed, and then taps T_2 and T_3. After several hours, when considerable dissolution has occurred, the attainment of equilibrium in the two solutions may be hastened by reducing the pressure gradually to *ca.* 10 cm., by connecting T_1 to a water-pump (provided with a manometer and air-leak), with T_2 and T_3 open. The taps are then immediately closed in that order. The manometer liquid may again require levelling at intervals by opening and closing taps T_2 and T_3.

We have usually found that saturation is reached within 24 hours, after which the apparatus is re-evacuated to *ca.* 10 cm. pressure as before. Tap T_1 is closed and the manometer liquid should then be level in both arms. Taps T_2 and T_3 are now closed. The manometer level should become steady in about an hour, and the difference in height is noted. The evacuation and subsequent procedure is repeated after a further hour, until the pressure difference is reproducible to within 1 mm., indicating that equilibrium has been attained.

It is advisable to test new apparatus by carrying through similar operations with both vessels empty, in order to detect leaks. It is, of course, essential that, when the final reading is taken, no pure solvent should be present in any part of the system; this should not occur if the apparatus is adequately immersed and if equilibrium has been reached. It is often desirable to carry out the final recrystallisation with the solvent to be used in the vapour-pressure test; traces of residual solvent will not then affect the observations.

Examination of Synthetic Mixtures.—Three series of mixtures were prepared: (*a*) potassium nitrate in " AnalaR " sodium chloride, (*b*) DL-alanine in " AnalaR " glycine, and (*c*) L-aspartic acid hydrochloride in L-glutamic acid hydrochloride; the percentage compositions and vapour-pressure differences (expressed in mm. of Apiezon Oil C) are shown in Figs. 1—3. In each experiment, one vessel contained 0·1 g. of mixture and the other 1·0 g.; 0·1 g. of water was added to each. The temperature of the thermostat was 50°.

We thank the Department of Scientific and Industrial Research for a grant during the tenure of which this work was initiated, and the Royal Society for a grant for materials.

The Dyson Perrins Laboratory, Oxford University. [*Received, January 19th*, 1951.]