methods. The presence of particles in the inorganic portion of bone and teeth which range in size below 100 Å has been demonstrated by low angle X-ray scattering techniques[1]. However this technique does not tell us exactly what crystalline phase is in that size range. Thus

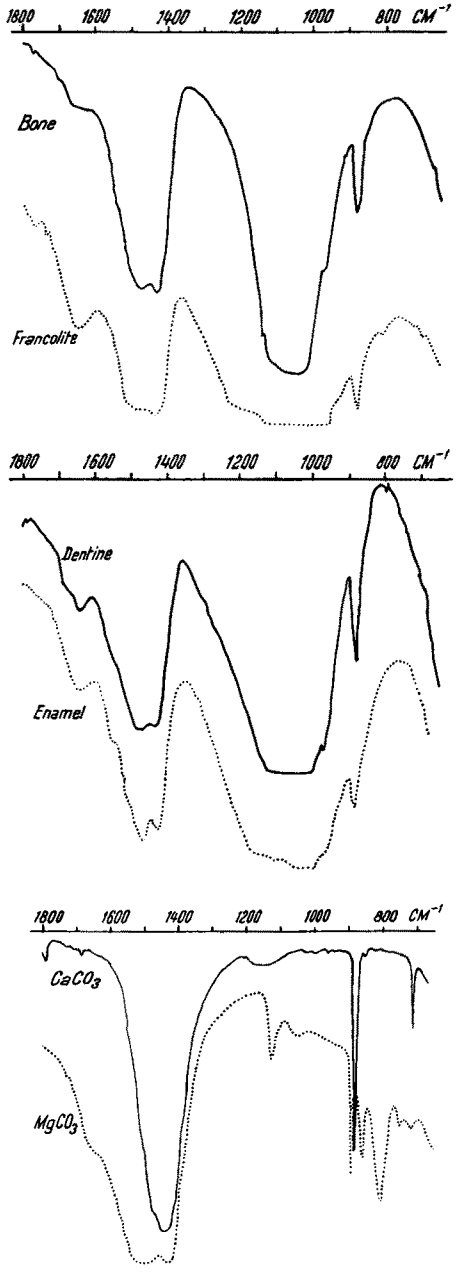

Fig. 2.–A comparison of the infra-red spectrum of dentine, enamel, bone and francolite with the standards calcite and magnesite. The large band appearing at about 1050 cm^{-1} is the orthophosphate band of apatite.

X-ray diffraction techniques alone have been unsuccessful in positively elucidating the character of the carbonate containing apatites. This is illustrated in Figure 1 which compares the X-ray patterns of the mineral francolite before and after about one third of the CO_2 has been leached out by lactic acid treatment. The two patterns are identical, showing the absence of the carbonate in the apatite phase, in a sort of negative manner.

[1] A. S. Posner and S. R. Stephenson, J. Amer. Dent. Assoc. *46*, 257 (1953).

Infra-red studies

It has been shown in the case of calcite that, as its particle size decreases, the intensity and resolution of the characteristic infra-red absorption spectrum increases[1]. This makes the infra-red absorption method ideal for characterizing a possible mixture of amorphous calcite and well crystallized apatite. Due to excessive broadening of the maxima, X-ray diffraction is unable to show crystals which are below about 100 Å in diameter. On the other hand, infra-red spectroscopy can demonstrate the presence of fine crystals.

The samples were prepared for examination by making a dry mixture of chemically pure KBr containing 2·5% of the apatite. After mixing in a hand mortar, the mixture was pressed into a pastille of wafer shape (about 12 mm diameter and 1 mm thick) in a press which was constantly evacuated to avoid air and moisture entrapment and kept at a pressure of 10 tons for 5 min. The pastille was balanced against a standard pastille of pure KBr in a double beam Perkin-Elmer infra-red spectrometer and the spectrum taken between 1800 cm^{-1} and 650 cm^{-1}. The results are shown in Figure 2.

Conclusions

It seems apparent that there exists in francolite, enamel, bone and dentine a chemical bond between calcium and carbonate and between magnesium and carbonate which is identical to these bonds in calcite and magnesite. This would tend to support the negative X-ray diffraction evidence which shows no change in the apatite phase when the CO_2 is removed.

A. S. Posner and G. Duyckaerts

Institute of experimental Therapeutics and Laboratory of analytical Chemistry, University of Liège, March 23, 1954.

Résumé

La diffraction des rayons X et la spectrographie infrarouge ont été employées pour rechercher la présence de calcite et de magnésite dans la fraction minérale de l'os et des dents ainsi que dans le minéral qui lui est le plus proche au point de vue chimique, la francolite. Le résultat positif obtenu dans l'infra-rouge indique la présence de liaisons $CaCO_3$ et $MgCO_3$ associées à la matrice fondamentale du type apatite de ces substances.

[1] G. Duyckaerts and R. Lejeune, Spectrochimica Acta (in publication).

Multiple Spots on Paper Chromatograms

It is normally assumed that pure organic compounds give single spots on paper chromatograms and there is no doubt that, in the majority of cases, the assumption is justified. There are, however, various recorded instances of pure compounds giving multiple spots. In some cases the development of several spots is due to the formation of a new molecular species under the conditions of chromatography. Glucuronic acid gives spots corresponding to the original compound and the

cognate lactone, glucurone[1]. Sugars may give multiple spots[2] and there is evidence in one case that this is caused by the formation of new compounds under the influence of alkaline impurities in the paper[3]. The multiple spots obtained with lysine[4], using phenol-water, are attributed to the formation of association complexes with the solvent. Vitamin B_{12} may give spots corresponding to both Vitamin B_{12} and Vitamin B_{12b}[5]. In the case of phosphate esters it appears that ghost spots, which remain at the origin after the bulk of the ester is eluted in the normal way, are due to the presence of alkaline impurities in the paper[6]. A similar explanation applies in the case of salicylic and sulphosalicylic acids[7]. The ghost spots do not appear when paper washed with dilute hydrochloric acid is used. Certain antibiotics may give two zones in the presence of salts. The existence of tautomers was offered as a possible explanation in the case of streptomycin[8].

Fig. 1.

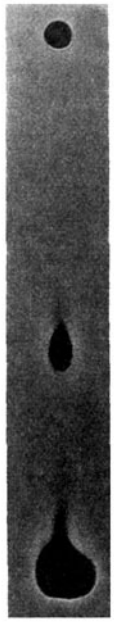

Fig. 2.

Fig. 1.–Normal chromatogram of monamycin (chloroform-water) showing ghost spot (R_F 0·00) and mobile spot (R_F 0·92).

Fig. 2.–Interrupted chromatogram showing third spot.

There are other cases, however, where it is not possible to account for the results by postulating the formation of new compounds. SMITH has drawn attention to the possibility of false separations in paper chromatography similar to those obtained in partition chromatography on columns and indicates that this may occur in the paper chromatography of Vitamin B_{12}[9]. Double-fronting, which, under particular conditions may lead to the development of double spots has been observed with proteins[1]. One of the earlier publications on paper chromatography has indicated that ghost spots may be formed when mixtures of amino acids containing inorganic salts are chromatographed. The effect was attributed to water-logging of the paper at the origin[2].

We have observed another case which cannot be attributed in any simple way to the formation of new compounds. In the course of a study on the paper chromatography of seven cardiac glycosides[3] it was shown that when chloroform-water was used as solvent a ghost spot always remained at the origin. We have obtained a similar result with the antibiotic monamycin. In this case the very high degree of sensitivity of the biological procedure for locating the antibiotic[4] made possible a closer study of the phenomenon. Two spots (R_F values: 0·00, 0·54, and 0·00, 0·92) were obtained in experiments using benzene-water and chloroform-water respectively at 30°C. Toluene, xylene and n-butanol saturated with water also gave two spots. The ghost spots obtained in all these cases were eluted readily with methanol. The development of the ghost spot was not influenced by the moisture content of the paper or of the eluting solvents. After prolonged periods of equilibration before the solvent was allowed to run, the ghost spot remained but covered a larger area. The effect persisted after the paper had been thoroughly washed, chromatographically, with dilute hydrochloric acid, with an aqueous solution of the disodium salt of ethylene diamine tetra-acetic acid (Versene), and with an ethanolic solution of 8-hydroxyquinoline, to remove metallic ions. Two simple experiments using monamycin and the solvent chloroform-water excluded the possibility of the effect being due to trace impurities. When the solution obtained by running the mobile spot off the end of the paper was concentrated and applied again to a paper strip, the ghost spot appeared on the second chromatogram. In the second experiment a chromatogram was interrupted at an intermediate stage so that the mobile spot passed approximately half-way down the paper. When the solvent had evaporated from the paper the chromatography was resumed in the usual way. In this case, in addition to the normal spots (R_F 0·00, 0·92) (Fig. 1), there was a new distinct spot at a point corresponding to that occupied by the mobile spot at the time of interruption (Fig. 2)[5]. Similar results were obtained using the cardiac glycoside digitoxin with chloroform-water as solvent except that when prolonged equilibration was involved the RAYMOND reaction[6] was not sufficiently sensitive to detect the relatively diffuse ghost spot.

We have confirmed that two spots (R_F 0·00, 0·95) are produced when azobenzene is chromatographed with hexane. It appears likely that the process involved in the formation of ghost spots with azobenzene and with certain alkaloids is similar to that which applies in the case of monamycin and cardiac glycosides.

The results we have obtained do not permit any decision concerning the mechanism of this effect. They may be most simply attributed to partial irreversible

[1] S. M. PARTRIDGE, Biochem. J. *42*, 238 (1948).
[2] R. J. BAYLY, E. J. BOURNE and M. STACEY, Nature *168*, 510 1951).
[3] R. B. DUFF, Chem. Ind. *1953*, 898.
[4] S. ARONOFF, Science *110*, 590 (1949).
[5] G. COOLEY, B. ELLIS, V. PETROW, G. H. BEAVEN, E. R. HOLIDAY, and E. A. JOHNSON, J. Pharm. Pharmacol. *111*, 271 (1951).
[6] C. S. HANES and F. A. ISHERWOOD, Nature *164*, 1107 (1949).
[7] J. B. SCHUTE, Nature *171*, 839 (1953).
[8] D. H. PETERSON and L. M. REINEKE, J. Am. Chem. Soc. *72*, 3598 (1950).
[9] E. L. SMITH, Nature *169*, 60 (1952).

[1] H. G. BOMAN, Nature *170*, 703 (1952).
[2] R. CONSDEN, A. H. GORDON, and A. J. P. MARTIN, Biochem. J. *38*, 224 (1944).
[3] C. H. HASSALL and S. L. MARTIN, J. Chem. Soc. *2766* (1951).
[4] D. H. PETERSON and L. M. REINEKE, J. Am. Chem. Soc. *72*, 3598 (1950).
[5] We are grateful to Dr. G. BRAS for the photographs, Figures 1, 2.
[6] W. D. RAYMOND, Analyst *64*, 113 (1939).
[7] J. B. SCHUTE, *Mededelingen van de Vlaamse Chemische Vereniging 1*, 15 (1953).

adsorption of the solute on a cellulose phase[1] when the mobile solvent phase is absent.

We are indebted to the Colonial Products Research Council for a grant in support of this work.

C. H. HASSALL and K. E. MAGNUS

Chemistry Department, University College of the West Indies, Jamaica, December 12, 1953.

Zusammenfassung

Bei der papierchromatographischen Prüfung bestimmter reiner Stoffe wurde die Bildung mehrerer Flecken beobachtet. Derartige «ghost»-Flecken entstehen mit gewissen herzaktiven Glykosiden und auch mit dem Antibiotikum Monamycin.

[1] A. J. P. MARTIN, Ann. Rev. Biochem. *19*, 517 (1950).

Paper Electrophoretic Separation of Tuberculin Constituents

In the course of our investigations into the immune response of the host to tuberculous infection and anti-tuberculosis vaccination, the problem arose as to how to determine the purity of the isolated antigenic fractions by a quick method and on a microscale. The recent successful application of zone electrophoresis on paper for the separation of serum proteins, enzymes, etc.[1] induced us to investigate this method for our purposes. Some of the results are presented in this preliminary note.

Two types of apparatus were used – one similar to that described by KUNKEL and TISELIUS[2] in which the paper is placed horizontally between two glass plates, and the other similar to the one used by GRASSMANN and HANNIG[3] but with room for 6–8 horizontally placed paper strips (35 × 4 cm).

Of the different buffers used, phosphate buffer pH 8·3, ionic strength $\mu = 0{\cdot}1$ and veronal/veronal sodium buffer pH 8·6, ionic strength $\mu = 0{\cdot}11$, gave the best results, the latter yielding better separations in the GRASSMANN and HANNIG apparatus. Citrate buffers of pH 5·5 and 6·3 afforded no good separations, and in addition some of the proteins tended to precipitate out from their solutions in these buffers, whereas 2–10 % solutions of most fractions could easily be dissolved in the alkaline buffers. Buffers of much higher pH values have not been tried since denaturation of tuberculo-proteins occurs under such conditions. A thick filter paper (Munktell 20/150) was used.

The experiments were run for 19 h at 200 V and 14–24 mA at + 4°C, applying 10–20 μl of 2–10 % solutions of the antigens. The proteins were identified by the bromphenol blue method according to DURRUM[4], and for quantitative measurement eluted with 0·01 *n* NaOH and measured in the BECKMAN spectrophotometer at 595 mμ. Crystalline bovine albumin was chosen as standard substance. In a parallel run the polysaccharides were directly identified on paper after periodate oxidation with fuchsin sulphite[5], or eluted with water and determined with anthrone according to MORRIS[6].

[1] For a review, see A. TISELIUS and P. FLODIN, Adv. Protein Chem. *8*, 461 (1953).

[2] H. G. KUNKEL and A. TISELIUS, J. Gen. Physiol. *35*, 89 (1951).

[3] W. GRASSMANN and K. HANNIG, Hoppe-Seyler's Z. Physiol. Chem. *290*, 1 (1952).

[4] E. L. DURRUM, J. Amer. Chem. Soc. *72*, 2943 (1950).

[5] E. KÖIW and A. GRÖNWALL, Scand. J. Clin. Lab. Invest. *4*, 244 (1952).

[6] D. L. MORRIS, Science *107*, 254 (1948).

The photographic reproduction in Figure 1 illustrates the separation of two fractions and a mixture of these into their protein components. The result suggests that there is one component which is common to both fractions.

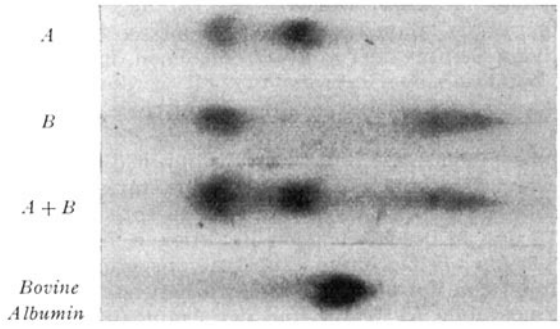

Fig. 1

Figure 2 indicates the usefulness of the method, in showing the heterogeneity of one particular fraction, thus confirming serological results which will be published shortly[1]. It is obvious that the polysaccharide part of the fraction moves rather slowly towards the anode, thereby affording a separation from proteins with higher mobilities. The curve for the proteins suggests the presence of three different components.

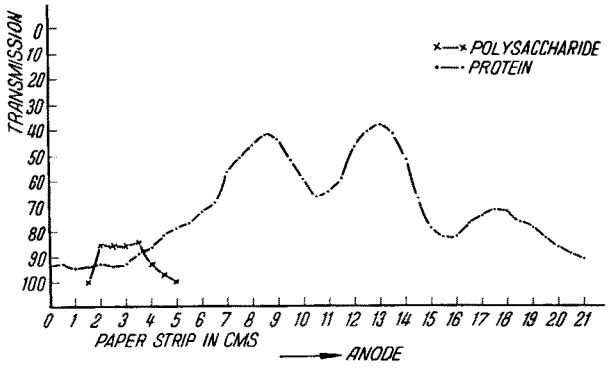

Fig. 2

There are, however, some antigens or antigenic mixtures which cannot yet be satisfactorily separated under these conditions. Heated fractions, e.g. PPD, are not separated into single spots, although they are known to contain at least two proteins with distinct serological specificities. Some unheated proteins, such as a highly purified protein corresponding to SEIBERT's C protein, although serologically nearly pure, show considerable tailing, probably due to strong absorption into the paper. They do not separate into single spots with the electrophoresis method as described above.

J. M. RHODES and E. SORKIN

Tuberculosis Immunization Research Centre, c/o Statens Seruminstitut, Copenhagen, July 9, 1954.

Zusammenfassung

Methoden zur papierelektrophoretischen Trennung von antigenen Komponenten von Tuberkulin werden beschrieben.

[1] S. V. BOYDEN and E. SORKIN, To be published.