A LABORATORY TEST FOR DIAGNOSIS OF SMALLPOX

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Experience of over 100 cases of smallpox studied in the Middle East has convinced us that a diagnosis of variola on the first and second day of the rash is often extremely difficult and that the differentiation of mild smallpox from varicella may be impossible throughout its course. The purpose of this paper is to describe the clinical application of a laboratory test which has helped us greatly in establishing the identity of variola in the first day or two. The test is based on certain points of distinction between the elementary bodies of variola and varicella found in the skin lesions of these diseases.

Historical

The viruses of variola and vaccinia were discovered in 1886-7 by Dr. Buist, of Edinburgh University Bacteriological Laboratory, who practised as a public vaccinator in the city. Unfortunately Buist failed to appreciate the nature of the tiny bodies which he found and called spores of micrococci, and it was not until 19 years later that Paschen (1906), of Hamburg, stressed their aetiological significance and named them "elementary bodies," followed by Eagles and Ledingham (1932) and others. (For historical notes see Gordon, 1937; and Mackie and van Rooyen, 1937.)

While the existence of the minute organisms has been known for many years, it is strange that few bacteriologists have attempted to utilize them for the laboratory diagnosis of smallpox, despite the fact that their biological characteristics have been extensively studied. (For review of literature see Smadel and Hoagland, 1942.)

In the past, three laboratory procedures—namely, the variola-vaccinia flocculation, the complement-fixation, and Paul's rabbit's cornea tests—have been employed for the diagnosis of human variola in the late stages of the disease. Tanaka (1902) demonstrated that if smallpox vesicle fluid was mixed with vaccinal lymph a precipitate developed; and later Gordon (1925) showed that extracts of variola scabs exhibited a specific precipitation effect when added to vaccinal antisera. The flocculation phenomenon was applied in medical practice by Burgess, Craigie, and Tulloch (1929), who used it as a confirmatory test in mild smallpox as well as a laboratory procedure for the identification of clinically atypical cases occurring in Dundee (see also Craigie and Tulloch, 1931; Tulloch, 1934; Craigie, 1932a and 1932b).

Specific complement-fixation reaction for variola has been practised for many years, and in particular by Beintker (1908), Sugai (1909), and Kolmer (1916). In recent times Gordon (1925) proved that the specific deviation of complement by the interaction of extracted variola scabs (or vesicular fluid), as antigen, with antiserum derived from convalescent smallpox patients or vaccinated rabbits could be of diagnostic help. The modern technique of the variola-vaccinia complement-fixation tests is described by Craigie and Wishart, 1934a, 1934b, 1935, 1936a, 1936b, 1936c; and Tulloch, 1940.

Paul (1919) showed that, if a rabbit's eye was scarified with variolous material, after 48 hours the animal developed acute kerato-conjunctivitis, accompanied by the formation of acidophilic intracellular Guarnieri inclusion bodies (Guarnieri, 1892) in the epithelial cells of the cornea. Paul's test has been extensively used by Scott and Simon (1923), who have recommended a technique whereby the test can be completed within three days.

The present report deals with the prompt recognition of variola by identifying the causal agent of the disease in human

skin lesions during the early stages of the malady, when the virus is easily visible.

Method of Examination

Apparatus.—A sharp-pointed scalpel and two highly polished slides cleaned with potassium bichromate and sulphuric acid solution are required for each case. **Microscope.**—The best instrument available should be employed, and should be fitted with a good substage condenser, a 1.4 N.A. apochromatic 1/12-inch oil-immersion objective, and a $\times 10$ or $\times 12$ compensating eyepiece giving a total magnification of 1,000 diameters. The ideal source of light for critical microscopy is the low-voltage high-intensity electric light bulb, used in conjunction with a light-blue ground-glass filter and condensing lens. For technical details of optical equipment suitable for virus research see van Rooyen (1937).

Staining Reagents Required.—**Löffler's flagellar mordant**, which is made up as follows: 20% aqueous solution of tannic acid, 100 c.cm.; saturated solution of ferrous sulphate, 50 c.cm.; and saturated alcoholic solution of basic fuchsin, 20 c.cm. It should be allowed to stand for three days and then be filtered before use. **Carbol Fuchsin Stain.**—Two stock solutions are stored separately; they consist of (a) 0.3 g. of basic fuchsin in 10 c.cm. of ethyl alcohol, and (b) 5 g. of phenol dissolved in 95 c.cm. of distilled water. One part of solution a and nine parts of b are mixed together and filtered before use.

Collection of Specimens.—Six early lesions are selected, preferably fresh vesicles containing clear fluid, and these are opened with forceps. The base of fully formed papules can also be scraped, and when opening these one must try to avoid drawing blood. If the lesion is a vesicle the fluid is first absorbed with cotton-wool. The base of the exposed cavity is then firmly scraped with a sharp knife or the corner of a glass slide, and the material obtained is rubbed with a rotary motion on the surface of a glass slide. Three such smears can be made on each slide. The best results are obtained by scraping the earliest papular and vesicular lesions of smallpox; consequently pustules should be avoided, as these often contain artefacts.

Paschen's Staining Method.—Slides are placed upright in distilled water for from 2 to 15 minutes, according to thickness of the smear, and then allowed to dry in air. They are treated with ether, and then with alcohol for two minutes. The alcohol is poured off and the films allowed to dry. **Filtered mordant** is added, the films are heated very gently, and the mordant is allowed to act for three minutes. They are washed repeatedly and very thoroughly with running water for two minutes. Diluted and **filtered carbol fuchsin** stain is poured on and gentle heat is applied until the steam rises; the films are left for seven to ten minutes, and then washed with water. (If necessary any excessive deposition of stain is removed by a rapid application of a few drops of alcohol and by washing in water.) They are dried, blotted, and mounted in neutral Canada balsam.

Bad results are attributable to failure to wash off all traces of the mordant with water before application of carbol fuchsin, or to drying of the mordant or stain by overheating and precipitation of carbol fuchsin dye so as to leave a granular background. (Such films should be discarded.)

Essential Control Preparations

Before searching a stained slide from a suspected case of smallpox for elementary bodies it is imperative to familiarize oneself with the appearance of control slides—two negative and one positive. The former should consist of: (a) a slide showing a pure culture of *Staph. aureus* or *albus* stained by Paschen's method; (b) a good film showing the elementary bodies of varicella: these are not easy to demonstrate, and it may be necessary to make several scrapings before a characteristic film of varicella elementary bodies can be obtained from a case of chicken-pox. The positive control should be a film which is made from a typical case of variola stained by Paschen's technique and which shows "myriads" of elementary bodies.

Points to be Remembered when Searching Films

In interpreting the results of stained films one should *never* state that elementary bodies are present unless masses of them are found. Moreover, these should be of uniform shape and of regular size and be clearly stained a deep red, identical in appearance with the positive control. Pleomorphic granules, deposited stain, and isolated single or scattered elementary bodies, however suggestive in appearance, should be ignored. To diagnose variola on the presence of scanty elementary bodies in a suspected film will inevitably lead to disastrous conclusions.

In the event of doubt one must discard that particular film and examine the next preparation: hence the reason why at least six smears should be made from every suspected case. In the average case of variola at least three of the slides will show numerous elementary bodies without much searching, and their appearance is so striking as to leave no room for hesitation.

If all the slides are doubtful or completely negative then that can be interpreted as strong presumptive evidence against the diagnosis being one of smallpox. In such circumstances the laboratory examinations should be repeated the next day. An attempt can also be made to isolate the virus by rabbit inoculation, but this procedure is both uncertain and slow.

The Elementary Bodies of Varicella, Variola, and Vaccinia

When viewed under the same optical conditions the elementary bodies of variola and vaccinia are identical in size but are larger than those of varicella. Certain other subtle differences can also be detected, such as avidity for carbol fuchsin dye and relative numbers in infected tissues.

The accurate measurement of stained microscopical objects of the dimensions of elementary bodies is a difficult task. Several technical methods have been evolved for the purpose, but none of them is perfect. One principle, advocated by Coles (1929), is Nelson's (1909) method of micrometric extinction performed with a dry high-power objective fitted with a graduated iris diaphragm calibrated against an apertometer. Using such a device, van Rooyen and Rhodes (1940) estimated vaccinia virus to be 0.25μ in diameter. In the present studies the elementary bodies of variola and varicella have been measured. The former were found to vary from 0.3 to 0.24μ , and thus were of approximately the same size as vaccinia virus. We have also repeatedly noticed that the elementary bodies were not only easier to demonstrate during the papular eruption of smallpox but appeared to be larger than those demonstrated later in the same case, at the stage of pustulation.

Smears showing elementary bodies of varicella were also measured and compared with those of variola, and were found to range from 0.125 to 0.175μ in diameter. This difference can easily be appreciated by the naked eye, and was first noted by Paschen (1933), who referred to the "essentially smaller size of the elementary bodies of varicella," as well as their poor staining affinity, in the following paragraph:

"Vor 15 Jahren habe ich auf der Versammlung der Vorstände der Staatlichen Impfanstalten berichtet, dass ich in Ausstrichen von ganzfrischen Varizellen-Bläschen E.K. gefunden hätte die wesentlich kleiner als die bei Variolavakzine beobachteten waren und sich schlechter färben liessen."

We have confirmed Paschen's findings and have appreciated that the difference in size between these two varieties of elementary bodies is great enough to permit it to be utilized as a laboratory diagnostic principle. Subsidiary points of distinction observed by ourselves are as follows:

In variola they appear in "myriads," whereas the elementary bodies of varicella are relatively scanty, are hard to detect (stained by Paschen's method), and usually leave some doubt in the microscopist's mind whether they are real or artefacts. Moreover, only a small proportion of films made from typical vesicles in a case of varicella show elementary bodies, whereas in variola the reverse is true. In the early smallpox rash elementary bodies occur intracellularly in masses and clumps as well as in the form of clusters of extruded bodies in the proximity of ruptured epithelial cells. Another common appearance is the tendency to form long rows of

bodies stretching from ruptured infected cells across the film. These appearances are rarely observed in varicella.

Varicella cannot be diagnosed by the film method, and one must resist the temptation to try to do so. The elementary bodies of varicella are exceedingly small, are too scanty, stain with difficulty, are often hard to find in films prepared from vesicles, and are readily confused with stain deposit.

The elementary bodies of variola and varicella can also be demonstrated under the dark-field-illumination microscope. Unfortunately artefacts are impossible to identify by this method, and consequently oblique illumination of unstained material should be restricted to the examination of infected tissue cultures and other special purposes. This unreliable method must never be employed in routine smallpox diagnosis, and far less for attempting to differentiate between the elementary bodies of variola and varicella. (See van Rooyen and Rhodes, 1940.)

Vaccinia.—Since the elementary bodies of vaccinia are morphologically indistinguishable from those of variola, the direct film method is not applicable to their differential diagnosis. However, this is not a serious obstacle, because the natural disease is confined to agricultural communities, and large outbreaks of cowpox are (at least in Egypt) uncommon. Even if an occasional case were wrongly labelled as smallpox no damage would result from any preventive measures which might have been instituted at the time. As for generalized vaccinia following vaccination, the history of inoculation, together with the patient's clinical state, should be enough to exclude variola.

Correct Wording of Laboratory Reports

It is imperative to adopt a uniform phraseology in recording results, so that both the bacteriologist and the clinician may serve the best interests of the patients. Two stereotyped examples of laboratory answers are recommended: (1) Positive for variola-vaccinia group. (2) Nothing found. If the case remains in doubt scrapings are repeated next day. Repeated negative results are suggestive of varicella—if the clinical diagnosis justifies it.

The Test in Varicella

Egypt

(a) During March–April, 1943, approximately 2,000 cases of chicken-pox and 3 cases of smallpox occurred in an isolated group of West African troops during sea transit from the Belgian Congo to Egypt. The R.M.O. in charge, Capt. Logie Bain, R.A.M.C., informed us that the whole unit had been well vaccinated within a year and that, except for the three typical instances of smallpox mentioned, the cases were in every way characteristic of varicella. Skin scrapings were duly obtained from 60 typical cases of varicella by Major Hartston, R.A.M.C., D.A.D.H. to the camp, as well as from the 3 cases of variola. The results showed that whereas in the cases of smallpox elementary bodies were easily demonstrable, they were usually absent or difficult to find in the patients suffering from chicken-pox.

(b) A group of 6 cases with a vesicular rash not due to smallpox were investigated in another military hospital. Four of these had chicken-pox, one a vesicular rash of unknown cause unlike smallpox, and one a fine pemphigoid rash very closely resembling smallpox. The test was of great value in these cases. In this area smallpox is ten times as common as chicken-pox, and a diagnosis of chicken-pox had to be made with great caution in view of the prevalence of mild modified smallpox in vaccinated personnel. One of the cases of chicken-pox had a prodromal scarlatiniform rash and three vesicular lesions on the sole of one foot; but these lesions were not hard and shotty as those of smallpox, and the subsequent fully developed distribution of the rash showed that the case was one of chicken-pox. The absence of elementary bodies of smallpox in the scrapings was a help in diagnosis. Another case of chicken-pox had an unusual number of lesions on the forearm, giving rise to doubt about the diagnosis; but the absence of elementary bodies in the scrapings helped the diagnosis, which was confirmed by the distribution of the rash when it was fully developed. The pemphigoid rash was at first thought to be smallpox, but repeated scrapings from

lesions failed to reveal the elementary bodies of smallpox, and the subsequent course of the rash showed that it was septic in nature. The negative laboratory finding was therefore of as much value in these cases as were the positive findings in some of the cases of small pox.

England

Although the cases affecting West African and Belgian Congo soldiers were unquestionable examples of adult varicella, studied before epidemic smallpox had begun in Egypt, to be doubly assured of our facts we decided to obtain a second series of control scrapings from genuine chicken-pox cases from independent reliable sources in Great Britain, where all chance of possible contact with variola could be excluded. This was done in June and July, 1944, when Great Britain was free from smallpox according to published information on infectious diseases and vital statistics. Major (Miss) Janet Niven, R.A.M.C., and Dr. E. M. Young provided us with skin scrapings from a total of 16 different typical cases of adult and juvenile chicken-pox contracted in Wiltshire and the S.E. London area. All these were carefully stained and examined under identical conditions with similar scrapings obtained from cases of variola acquired by British troops in Egypt. The results showed that the morphological picture presented in stained skin scrapings from cases of varicella, whether acquired in Egypt or England, were the same. Of a total of 88 well-prepared smears from 16 patients, typical exceedingly minute varicella elementary bodies were found in 12 smears from 4 cases. The latter were compared with typical Paschen bodies of early smallpox, and the difference was immediately recognized.

The Film Test in 80 Cases of Variola

Results in 50 Test Cases affecting British and Other Soldiers

Scrapings from lesions were taken in 50 out of 100 cases of smallpox seen by one of us (R. S. L.) at a military hospital in the Middle East. The cases chosen for the test were of all degrees of severity. Eight of the 50 were extremely mild throughout, while 10 were of the very severe type, of which seven were haemorrhagic. In 10 of the 50 cases the rash was in the papular stage at the time when scrapings were taken, in 39 the rash was vesicular, and in 1 it was in the early pustular stage. In 47 (94%) the scrapings showed the elementary bodies of smallpox, and in 3 (6%) they did not. Every positive laboratory diagnosis was subsequently confirmed clinically. In 21 of the 50 cases the diagnosis of smallpox was obvious at the time when the scrapings were made. Scrapings were taken from these cases, which were early ones in the series, in order to check the validity of the method. In 23 of the cases the diagnosis was not obvious at the time when the scrapings were taken, but became so later. Thus the laboratory diagnosis was made before the clinical diagnosis was possible. In 6 cases the clinical diagnosis was not clear when the scrapings were taken, and from the appearance of the rash did not become so later, but the laboratory result was confirmed by other means—notably by the history of close exposure to infection at the appropriate interval before the development of the disease. Three of these six cases figured in an interesting local outbreak in which the laboratory test was of real value in diagnosis.

A Jamaican was admitted with a very sparse vesicular rash of about 20 to 30 spots, none of them on the palms or soles, which had been preceded by three days of fever. He had scratched almost all the spots, but an attempt was made to obtain scrapings from the remaining one or two unscratched lesions. The scrapings did not show the elementary bodies of smallpox, and it was not possible to repeat them as there were no remaining lesions suitable for the test. No new lesions developed, and it was never possible to distinguish the rash from chicken-pox, but the three-day prodromal fever made the diagnosis of smallpox likely. He was kept in an observation tent separate from known cases of smallpox, vaccinated, and notified as having smallpox so that unit precautions could be instituted. Twelve days after his admission two more Jamaicans were admitted with a still more sparse rash, which had appeared 1 to 2 days after the onset of fever. Chicken-pox was diagnosed, but scrapings showed the elementary bodies of smallpox. Neither developed further lesions, and it was never possible to distinguish them from chicken-pox by their appearance. But two days later three others were admitted—natives of Jamaica and British Guiana—

all with a vesicular rash, two of them characteristic of mild smallpox, with lesions on palms and soles, and the other indistinguishable from chicken-pox. All had developed the rash on the same day as the fever. Scrapings were taken from one of these cases, and they showed the elementary bodies of smallpox. It was then found that all these six cases had come from the same hut in the same unit. Twelve days later three others were admitted from the same hut, all with obvious mild smallpox, and the scrapings from all three showed elementary bodies of the disease. One of these three had the classical three-day prodromal fever before the rash developed. All the 9 cases had been very recently "vaccinated," and the clinical diagnosis was difficult at first in all and impossible throughout in four. The laboratory test was here of great value in establishing the nature of the outbreak.

The test was of great service in the early diagnosis of many of our cases. In 22 of them the scrapings were taken on the first day of the rash, at a time when a clinical diagnosis was usually difficult or impossible, and all these scrapings showed the elementary bodies of smallpox. In 4 of these 22 cases the laboratory diagnosis was actually made on the first day of the disease, because the rash had developed on the same day as the fever. One of our early cases which developed the rash—not a prodromal one—on the same day as the fever while in this hospital presented great difficulties in diagnosis. This patient developed a very severe form of the disease, which was fatal, and the laboratory report was a great help in the diagnosis, which was subsequently obvious.

In many of the cases the rash was extremely sparse when the scrapings were taken, there being 12 to 24 lesions in all. In a Cingalese boy there were only 12 vesicles when the test was made, the rash having appeared on that day. In another case 12 to 15 papules were found on the back of a man who had had fever for three days. They were non-specific in character, but, by carefully opening the papules without drawing blood, scrapings were obtained which showed the elementary bodies of smallpox. It was not possible to make a clinical diagnosis in this case until 36 hours after the scrapings had been taken, and he was therefore kept in an observation-room until the diagnosis became clinically obvious.

In 3 of the 50 cases elementary bodies of the disease were not found in the scrapings. Only one series of scrapings was taken in each of these cases, and it is likely that if the series had been repeated a positive result would have been obtained. One was the Jamaican patient mentioned above, in whom there was a scarcity of lesions suitable for the test. The other two were typical mild cases of smallpox.

Independent Observations in a Further 10 Selected Cases

At another military hospital the film method of diagnosis was put to a critical trial by Lieut.-Col. H. L. Wallace and Major R. W. Eason in 12 patients who had been admitted to the general wards of the unit, labelled pyrexia of unknown origin, and who subsequently developed a rash in hospital. Skin scrapings were made at the first signs of the appearance of the rash, usually on the second or third day of illness. Ten were reported as positive, and all ten cases subsequently proved to be typical smallpox. One patient had been admitted to hospital at the height of the typhus season, and the rash was strongly suggestive of epidemic typhus, but the diagnosis was changed to variola on the findings of the film test. This man later proved to be a case of haemorrhagic smallpox, and died. Another case was of particular interest. The illness was exceedingly mild, and the patient, who had been under treatment for another complaint, had fully recovered and, while waiting to be discharged as cured, was working as a voluntary helper in the wards distributing bedding and linen to others, when it was noted that he had developed a few red spots on the arms and trunk. A film scraping proved to be positive, and seven days later the diagnosis became apparent. In the two remaining cases the film test was repeatedly negative; both ultimately proved to be septic skin conditions, and no secondary cases developed from them.

Results in 20 Civilian Cases of Late Smallpox

Twenty late fully developed severe cases of pustular semi-confluent smallpox affecting Egyptians were investigated. Films

were prepared from these subjects, duly stained and examined. The results showed that, although elementary bodies were present in considerable numbers, they are difficult to detect after pustulation and secondary infection has occurred. Prolonged immersion in distilled water is required to rid the film of excess serofibrinous pus, and the film tends to over-stain. Nevertheless even in late cases, by scraping the base of the pock, it is possible to obtain excellent stained preparations showing elementary bodies.

Discussion

To perform either the complement-fixation test or the variola-vaccinia flocculation test vesicular fluid or dried scabs are required, or the serum of patients 7 to 10 days after infection. In consequence their use is restricted to cases in the stage of pustulation or desquamation. Moreover, both tests demand a high standard of serological technique with proper controls, and about 24 hours must elapse before results can be read. Paul's test likewise suffers from the disadvantage of the time taken to obtain an answer. Moreover, our experiences with Paul's test in true smallpox have been disappointing, and it is not as easy to do as the literature indicates it to be. The slide method provides a quick and easy test for recognition of smallpox from the first day of the rash, when it is in the papular stage before vesiculation. A smear preparation can be made from a suspected case of smallpox, stained, examined, and a reply handed to the physician in half an hour. To assess the relative merits and percentage of experimental error in different laboratory tests would be unfair without comparative trials over a large series of cases. However, we believe that if all the laboratory diagnostic methods for variola were put to a rigid trial and judged according to their merits the slide technique would surpass the remainder in speed and accuracy.

Summary

We have confirmed Paschen's (1933) observation that the elementary bodies of variola are larger than those of varicella, and have utilized this finding to form the basis of a laboratory test for the identification of smallpox.

In Egypt, skin scrapings from 80 cases of smallpox ranging in severity from mild to fatal haemorrhagic variola were subjected to the film test for elementary bodies.

The laboratory and clinical findings corresponded in 77 cases (i.e., 96%). A negative result was returned in three cases of smallpox.

Variola elementary bodies were demonstrated with ease in the papular and vesicular stages of the illness, but after the onset of pustulation they tended to disappear.

In 32 cases of early smallpox the laboratory answer was positive on the first day of the rash, when it was very sparse and the clinical diagnosis was difficult or impossible to establish.

In no instance did a positive laboratory verdict disagree with the final clinical diagnosis of the case.

Control skin scrapings from 64 clinical cases of varicella occurring in Egypt, together with a further 16 cases of chicken-pox contracted in England, have been studied under identical conditions and compared with similar material from 80 cases of variola in Egypt.

The film test is not applicable to the diagnosis of chicken-pox, because the elementary bodies of varicella are too small, are too scanty, and show feeble affinity for Paschen's stain.

The principal conclusion reached is that the film method, when used with caution and discretion, can be of great value in the early recognition of variola, especially on the first day of the rash, when the clinical picture may so closely simulate chicken-pox.

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AIR EMBOLISM AS A COMPLICATION OF VAGINAL DOUCHING IN PREGNANCY

BY

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The potential dangers of air embolism during surgical procedures are well known, and are being constantly emphasized by all those responsible for surgical teaching. Within recent years the importance of this subject has increased proportionately to the frequency with which intravenous resuscitation therapy is employed. According to Hamilton and Rothstein (1935) the first case was reported by Beauchesne in 1818, and since then many other accounts of fatal accidents due to air embolism have been placed on record.

The Causes of Air Embolism

When any large vessel containing blood under less than atmospheric pressure is opened and cannot collapse owing to rigidity of its walls; partial severance, or external fixation, the entry of air is possible and the mechanism easily understood. This may happen during operations in the region of the great vessels of the neck, as a sequel to their traumatic severance, and through opening the dural sinuses during operations on or within the skull. A somewhat similar situation exists when air enters the uterine venous sinuses during or after delivery. This may happen in normal healthy women during labour when the sinuses are open after detachment of the placenta (Osborn and Dawson, 1938), or up to 11 days post partum where retained membranes, clot, or placental fragments have kept the sinuses open and the entry of air into the uterine cavity has been facilitated by the adoption of the knee-chest position (Gough, 1924; Quigley and Gáspár, 1936; Stroh and Olinger, 1938; Redfield and Bodine, 1939). In these circumstances the air is sucked into the open vessel. Criminal abortions are often attempted by the injection of fluid into the uterus. If the fluid passes the cervix its force detaches the membranes or placenta and air may be driven into the open uterine sinuses, as precautions are seldom taken to ensure that the syringe is completely filled with fluid before use (Gonzales, Vance, and Helyer, 1937; Gough, 1924; Hamilton, 1936).

The fact that even quite small veins may, if damaged, serve as a point of entry for air is perhaps not so widely known.