

THE STABILITY OF EMULSIONS IN THE CONSTRICTED TUBE AND MARBLE DEVICE FOR ANAEROBIOSIS¹

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In comparing the marble seal in the constricted tube² with a seal of hydrocarbon oil in the cultivation of obligative anaerobic bacteria it was repeatedly observed that growth in the tube sealed by the latter method and especially when inoculated with minute quantities of culture, was considerably less rapid and gas formation in glucose broth less abundant. That inhibition is not due to antiseptic properties of the oil was easily shown by as early growth as usual in media containing an excess of oil but sealed by means of the marble in the broth.

In connection with this fact, and more particularly some experiments as yet unpublished on methylene blue as a criterion of anaerobiosis, I was led to the observation that if the marble is placed in the oil, and the tube heated in a bath of boiling water, on cooling, the oil below the marble becomes markedly opaque, due, as shown by further study, to emulsification of water in the oil. The oil open to the air, however, remains quite clear, or, if an emulsion is formed at all, as it can be by rapid cooling, it persists for a short time only. The emulsion below the marble, on the contrary, is moderately stable, and consists of microscopic drops of water in oil. The excess of water in the base of the tube remains throughout perfectly clear. The development or stability of such an emulsion does not depend upon the presence of any of the usual constituents of culture media, being obtained with distilled water and hydrocarbon oil. Two immiscible liquids are, of course, necessary; oil heated alone in a

¹ From the Hearst Laboratory of Pathology and Bacteriology, University of California.

² Hall: "A New Aerobe—Anaerobic Culture Tube," Univ. of Calif. Pub. in Pathology, 2, 147 (1915).

clean *dry* tube remains clear on cooling. In experiments to test the effect of hydrochloric acid, sodium hydroxide, and sodium chloride solution, instead of distilled water, no difference in the formation of emulsions could be detected. Solutions ranging from normality to hundredth normality showed no difference in the *stability* of the formed emulsions at ice chest temperature (about 6° C) during the first 48 hours. After 96 hours the emulsions formed over N/1 and N/5 NaCl were clearing, at 120 hours also that over N/1 NaOH; all the rest were subsiding slightly. The differences were not marked and seemed to justify the conclusion that the effect of these substances, i. e., acids, bases and salts, is rather a minor one. Since hydrocarbon oil is a non-saponifiable compound even vigorous shaking with strong alkali fails to produce a stable emulsion.

A simple explanation of the *formation* of the emulsion suffices. The solubility of water in oil, though slight at low temperature, is increased as the temperature is raised. When the temperature is reduced, the water precipitates in the oil; on raising the temperature again the emulsion disappears by redissolving. As to why emulsions form so much more readily and persist so much longer below the marble seal constitutes the point of interest in this paper.

Emulsions may be prepared by this method not only with water and hydrocarbon oil but also with water and any one of the following, to wit: gasolene, heptane, chloroform, xylol, ether, benzol, kerosene, carbon bisulphide, commercial lubricating oil, olive oil, and aniline. In every case water forms the internal phase; this is true even with immiscible liquids heavier than water, e. g., chloroform and carbon bisulphide, and also with liquids more volatile than water such as heptane, chloroform, ether, benzol and carbon bisulphide. Some special cases must be considered. For example, olive oil and "zerolene" (a commercial automobile cylinder oil) emulsify very slowly though finally very heavily; this may be attributed to their high viscosity which tends to prevent the coalescence of the precipitated water. A tube of 15 cc of zerolene with 5 cc of distilled water in the base and sealed

with a marble in the oil was still heavily emulsified below the marble after ten months at room temperature. Even without the marble seal there is a tendency to permanent emulsification in the oil near the oil-water surface in this instance. The emulsification of water in ether is very slight, which may be explained partly as due to the impossibility of raising the temperature above the boiling point of ether in the open air. It is to be noted in several cases, however, e. g., carbon bisulphide, benzol and chloroform, that the boiling point of water is not nearly reached, yet the solubility of water in these compounds is sufficiently increased at the lower temperature to produce a marked turbidity on cooling. In all but benzol, xylol, and aniline, water forms the internal phase only; with benzol and xylol water forms also the external phase of an emulsion, and with aniline the emulsion of the oil in the water is more prominent than that of water in the oil though both occur—the latter principally in the depths. Aniline constitutes a very special case; it is lighter than water above 70° and heavier than water below 65° C. Heated in a bath of boiling water, and cooled, the mixed liquids form two thick emulsions, first, of oil in water, then of water in oil. And the position of large oil drops in the water with respect to the air-liquid surface determines whether an emulsion of water shall or shall not form in them, those in the depth emulsifying first and most distinctly.

In all cases tried, emulsions of water in water-immiscible liquids form more quickly and with less cooling under the marble seal in the constricted tube. And when emulsions are formed without the seal, as they can be in most cases by especially rapid cooling, as, for example, in ice water, the stability has been found to be slight as compared with similar emulsions protected by the marble seal.

No doubt many valuable points might be gleaned as to the necessary temperature of the initial heating, the degree and rapidity of cooling, et cetera, but it is necessary to refrain from discussing these interesting quantitative questions for the present. We may pause to mention, however, that the

stability of emulsions of water in zerolene and olive oil under the marble seal is a matter of months and of water in carbon bisulphide, hydrocarbon oil, kerosene, and xylol of days as Table I shows:

TABLE I
Stability of emulsions of water in various immiscible liquids

Substances	Maximum temperature of heating	Stability at ice chest temperature	
		Without marble	With marble seal
Zerolene	98.5° C	Less than 3 days	More than 240 days
Olive oil	98.5° C	Less than 1 day	More than 61 days
Carbon bisulphide	52° C	Less than 1 day	More than 21 days
Hydrocarbon oil	98.5° C	Less than 2 days	More than 7 days
Kerosene	98.5° C	Less than 1 day	More than 2 days
Xylol	98.5° C	Less than 1 day	More than 1 day

The stability of emulsions of water in benzol, ether, chloroform, heptane, gasoline, and aniline, however, is a matter of hours in the constricted tube with marble seal as compared to similar tubes without such seal, but always greater in the former. The possibility of producing emulsions in other immiscible combinations than those involving water is suggested. I have experimented mostly with emulsions of water in hydrocarbon oil (Langley and Michaels, San Francisco).

For the successful preparation of emulsions of water in oil various mechanical devices are suitable. The biconvex disc, a circular cover slip in a constricted tube, or a cover slip in a plain test tube, as previously described for cultivation of anaerobic bacteria, all give results comparable to those obtained by using the marble. A five cent piece or a copper disc (cleaned by dropping while red hot into methyl alcohol) serve equally as well as the glass or porcelain seal. It is only necessary that there be a few drops of water in the base of the tube with the oil overlying and some of the oil below the seal. The system is heated a few moments in a bath of

boiling water, the liquids above and below the seal then being clear. On cooling, the emulsion forms in the oil below the seal.

The Smith fermentation tube, which, barring its objectionable high initial cost, difficult cleaning, handling and heavy breakage, most nearly approaches the constricted tube in availability for anaerobic cultures, serves admirably for demonstrating the persistence of water-oil emulsions. If the water reaches above the bend in the tube so as to separate the oil in the closed arm completely from that in the open arm it is found that the former remains turbid for an indefinite period of months while the latter fails to emulsify except by rapid cooling and such emulsions are very instable. If the water fails to reach the bend in the tube, the emulsion formed in the oil of the closed arm disappears slowly by diffusion into the open arm.

It seemed at first that the formation and especially the persistence of these emulsions was definitely dependent upon the comparative absence of air from the oil and the term "anaerobic emulsions" was tentatively used; my interest as a bacteriologist seemed therefore to be justified, from the standpoint of cultivation of anaerobes, in this physical problem, especially as I had abundant evidence of the inferiority of oil as a means of securing vigorous anaerobic growth. It seemed, indeed, as if the observation that oil layered upon culture broth for anaerobes fails to remain emulsified might be used to explain this inferiority providing it could be shown that the persistence of the emulsion in the constricted tube was dependent upon a superior exclusion of air due to the marble.

But further study shows that temperature and evaporation play more important rôles in determining the instability of the emulsion. We know that as the temperature is raised the solubility of the water in the oil increases. The concentration of water in the deep layers of oil is greater than at the air surface because of constant evaporation of water into the air from the surface of the oil. Therefore when cooling

starts, emulsification begins to be visible near the oil-water surface. Similarly on heating a water-oil emulsion formed by rapid cooling in a tube without the marble seal the turbidity disappears first at the air-oil surface. The particles of water may be seen to diffuse rapidly upward; when they reach the air-oil surface they undoubtedly evaporate and their places are filled by others, resulting shortly in a disappearance of the emulsion. But if a barrier to diffusion, such as the just-described mechanical seal, be interposed, evaporation is prevented and the emulsion is relatively stable.

If evaporation is the main factor in determining the instability of the emulsion, it should be operative even in the presence of air. This is, indeed, the case; emulsions of water in hydrocarbon oil formed in a long tube (1 cm \times 60 cm) open to the air without a seal are moderately stable, whereas in a similar tube only 10 to 12 cm long special precautions for rapid cooling have to be followed to secure an emulsion at all and the stability is short lived. In the Smith fermentation tube also, if a bubble of air be admitted to the closed arm of a tube containing oil with water in the base reaching above the turn in the tube the emulsion formed by heating and cooling is as stable as without the air bubble, while the oil in the open arm freely exposed to evaporation forms only a transient emulsion with the water in the base.

These facts indicate that the presence or absence of an air surface above the emulsion is immaterial and the real factor in the lack of stability in these water-oil emulsions is evaporation.

Further evidence of the importance of evaporation rather than the presence of air was found in some pairs of tubes containing water-oil emulsions in which one tube was sealed while boiling in a water bath and under a vacuum of over 71 cm mercury, the other sealed cold in the open air. Both tubes having finally been boiled and cooled with resultant formation of emulsions, it was regularly observed that the emulsion in the tube having a lowered air pressure was less stable. This is exactly contrary to what would be expected

if the stability depended upon the absence of air and the tendency of emulsions to disappear in such tubes sealed under partial vacuum can be explained best as due to evaporation of the water droplets from the oil surface, as shown by the condensed droplets visible along the sides of the tubes.

Discussion

Interest in these observations lies especially in their bearing upon the theory of emulsification. There is nothing new in the formation of emulsions by cooling two relatively immiscible liquids whose mutual solubility has been increased by heating, Ostwald¹ having noted this method as available in determining the relation between solubility and temperature. But no record of the comparative permanence of emulsions formed in this manner by two immiscible liquids, and especially without the known presence of a so-called emulsifying agent, has come to my attention. The closest suggestion is that in which Pickering² said, "although one of these semi-solid emulsions (of kerosene and soap solution) when kept in a closed vessel will only gradually de-emulsify in the course of months, yet, when exposed to dry air, it will do so in a short time; the edges of the lump assume the appearance of a transparent, slightly blue jelly, and the whole liquefies in the course of some hours. No such change occurs in moist air." Bancroft³ commenting, confidently ascribed the instability of Pickering's emulsions in dry air to evaporation of the water with resultant coagulation of film and release of the kerosene drops.

In my experiments evaporation is one of the fundamental causes of de-emulsification, but the situation is different because in Pickering's petroleum jelly it is the external or dispersing phase which evaporates while in my experiments it is the diffusion and evaporation of the internal or dispersed phase which destroys the emulsion.

¹ Ostwald: "Manual of Physico-Chemical Measurements," p. 175 (1894).

² Pickering: Jour. Chem. Soc., **91**, 2001 (1907).

³ Bancroft: Jour. Phys. Chem., **16**, 475 (1912).

Determination of Phases

It is significant that in these emulsions water forms the internal or dispersed phase with oil as the external or dispersing phase. Bancroft¹ cited Ostwald's² use of the principle that wetted surfaces tend to destroy the dispersity of that phase and conserve the dispersity of the opposite phase, in determining the distribution of two immiscible liquids in an emulsion as follows: "If one dips a piece of filter paper into an oil-water emulsion in which the oil is present as drops, the filter paper becomes wetted with water and the oil drops off. If the filter paper is dipped into an emulsion in which water is present as drops we get an oiled paper from which the water runs off." Newman³ has mentioned three methods of determining the phases of an emulsion, namely (a) inspection, (b) the indicator method of Robertson⁴ and (c) the drop method of Briggs.

In my experiments inspection sufficed in every case to show the distribution of the phases because of an unemulsified excess of each component and the result of inspection was confirmed by each of the other methods in the case of water-hydrocarbon oil emulsions. Of Ostwald's and Briggs' methods the latter gives the more clear-cut result. Robertson's method was easily applied, using not only Sudan III but also such oil-soluble dyes as Scharlach Roth (Grübler) and Dimethyl-amido-azo benzene (Merck). Newman has also mentioned the applicability of iodine and methyl orange in Robertson's method, the first being oil-soluble, water-insoluble, and the last, vice versa.

I made the reverse test also, using the water-soluble, oil-insoluble dye, Nilblau (Grübler); the dye fails to diffuse through the emulsion, but the test is not completely satisfactory, owing to the fact that the water adheres to the glass all along the inside of the tube even when the tube has been

¹ Bancroft: Jour. Phys. Chem., 16, 177 (1912).

² Ostwald: Zeit. Colloidchemie, 6, 103 (1910).

³ Newman: Jour. Phys. Chem., 18, 34 (1914).

⁴ Robertson: "The Proteins," Univ. of Calif. Pub. in Physiology, 3, 115 (1909).

first rinsed in oil, and this film of water becomes stained. Incidentally, this explains a failure to find any difference in stability of emulsions in an oil-wet tube and in a water-wet tube for the oil-wet tube becomes water-wet very soon on heating.

A simple method of determining the phases of not too opaque emulsions was found as follows: A drop of one of the liquids is placed in the emulsion near but not touching the glass. If it is dispersoid it is clearly delimited and moves up or down through the emulsion; if it be the dispersing medium it diffuses quickly into its surroundings making a slightly less opaque area. In the case of water-hydrocarbon oil emulsions, water sinks as a clear drop; oil rises and diffuses. If a water drop is touched to the glass it quickly breaks and runs down the water film into the excess of water below; it was this, in fact, which first drew my attention to the water film on the glass. In this case the wetted surface of the glass apparently does not destroy the dispersity of that phase (water) and conserve the dispersity of the opposite phase (oil). In the constricted tube and marble device this film serves to isolate completely the emulsified oil below the seal from the unemulsified oil above; it therefore insures the retention by the oil of the maximum number of precipitated water globules at any given temperature. The complete isolation of the emulsified oil from the unemulsified oil is to be observed best during the period of cooling, when large agglomerated droplets of precipitated water may frequently be seen to find their way from above to below the marble, there forming an easily visible layer of water over the oil, continuous with the film on the glass and the excess of water in the base. This observation leads one to infer that not all of the water which dissolves in the oil at an increased temperature necessarily becomes emulsified on precipitation.

In the Smith fermentation tube also the mass of emulsified oil is kept saturated with water by the act of its complete boundary of water or water saturated atmosphere which effectually prevents evaporation of the water particles.

Emulsifying Agent

It should be noted particularly that fairly stable emulsions may now be prepared with certain liquids which probably do not react mutually to form an emulsifying agent as in the case of weak alkali and olive oil. Lewis¹ claimed to have prepared stable emulsions of as much as 2 percent mineral oil in water by 48 hours' vigorous shaking but Bancroft² criticized Lewis in his assumption that a two-component emulsion is a thermodynamically stable system, pointed out that "there is nothing in the discussion to show why there should be a 2 percent limit," reached the conclusion that "all the evidence that we have is to the effect that no two-component colloidal suspension is stable," and inferred that the same probably applies to emulsions.

Ellis³ also studied two-component emulsions containing one part of oil in 10,000 of water, which were not very stable. Moreover, in Ellis' work the possibility of oleic acid as an emulsifying agent was not wholly ruled out though it was determined as less than 0.03 percent. Summarizing the work of the various investigators, Bancroft in 1912 concluded that "to obtain a more stable and concentrated emulsion, a third substance must be added as an emulsifying agent. When water is the dispersing phase the emulsifying agent should lower the surface tension of the water (Quincke, Donnan) and should be viscous (Quincke, Hillyer)."

In the case of emulsions formed by heating and cooling it is obvious that the quantity of water which will become emulsified in the oil is limited primarily by the solubility of the water in the oil at the maximum temperature to which the system is heated before cooling. If the explanation given of the stability of these emulsions through the limitation of evaporation is correct, it is difficult to see that an emulsifying agent is necessary. Viscosity, however, appears to be an important factor in determining the stability of such emulsions.

¹ Lewis: *Zeit. Kolloidchemie*, 4, 211 (1909).

² Bancroft: *Jour. Phys. Chem.*, 16, 177 (1912).

³ Ellis: *Zeit. phys. Chem.*, 78, 321 (1911).

Pickering¹ has suggested that surface tension and viscosity are less important factors in some cases than is generally supposed, in fact, certain emulsions can be stabilized by using precipitates or suspensions as emulsifying agents which have but slight effect upon the surface tension of water. He mentions that "dilute soap solutions are not very viscous and yet their power of forming an emulsion may even be destroyed by increasing the concentration and thereby the viscosity above a certain point." While these factors may often facilitate emulsification "the only or rather the chief cause of emulsification seems to be the presence in the liquid medium of small insoluble particles which form a film round the drops of oil and keep these from coalescing. In order that such particles should have the power of forming an emulsion, they must have only a very slight tendency to agglomerate, they must be more readily wetted by the water than by the oil, and they must not be crystalline." This view, essentially similar, as Pickering says, to that formed independently by Ramsden,² is not identical but relates closely to Höber's (quoted by Bancroft) that the emulsifying agent should be a hydrophilic colloid in an emulsion where water is the dispersing medium. Fischer and Hooker³ have recently stated their conviction that the production of stable emulsions is "*always* associated with the discovery of a method whereby the water (or other medium) which is to act as the dispersing agent is *all used* in the formation of a colloid hydration (solvation) compound," and have applied this idea in the explanation of certain pathological and physiological facts. Peculiarly neither Pickering nor Höber recognized the corollary of their explanation, later emphasized by Bancroft, that if water is to be emulsified in oil the emulsifying agent should be a hydrophobic colloid.

Robertson⁴ prepared emulsions of water in oil by the

¹ Pickering: Jour. Chem. Soc., 91, 2001 (1907).

² Ramsden: Proc. Royal Soc., 72, 156 (1903).

³ Fischer and Hooker: Science, 43, 488 (1916).

⁴ Robertson: "The Proteins," Univ. of Calif. Pub. in Physiology, 3, 115 (1909).

addition of NaOH to olive oil so that a critical point near which the transition from oil-water or water-oil took place could be determined for each concentration of alkali. Newman¹ criticized Robertson's work to the effect that "what Robertson thought was an emulsion of water in oil is merely a cracked emulsion in which the appearance of the oil is changed by the presence of solid sodium oleate." Newman concluded, in fact, that no water in oil emulsions are formed with hard soap, sodium oleate or caustic soda. In the belief that hydrophilic colloids tend to make water the external phase, while hydrophobic colloids tend to make water the internal phase, reagents belonging to the latter group were tried with benzene and water. While certain of these gave unstable emulsions success was not achieved by Newman until the trial of, first, magnesium oleate, and more particularly mixtures of magnesium and sodium oleate, produced emulsions of water in benzene stable in some cases for eight weeks. Similar emulsions were prepared of water in carbon bisulfide.

These considerations suggest that if there is an emulsifying agent present in our constricted tube experiments it is soluble in oil but insoluble in water. The glassware used was specially cleaned in strong boiling lye solution, rinsed in tap water and finally soaked in chromic acid cleaning fluid and again thoroughly rinsed and dried. In certain experiments the added precaution was taken of treating the glass with hot oil and cleaning with ether. These procedures would seem to eliminate the possibility of chemical impurities from the glass.

The certain exclusion of impurities from the oil is more difficult. It seems, indeed, as if the most stable emulsions are formed in oils which probably contain impurities, e. g., commercial lubricating oil, olive oil and kerosene. Hydrocarbon oil itself is not a chemically pure substance since it has a variable boiling point. On the other hand, the carbon bisulfide used, which gives moderately stable emulsions, was

¹ Newman: Jour. Phys. Chem., 18, 34 (1914).

Baker's analyzed, sp. gr. 1.27, b. p. 46°-49° with 0.001 per cent non-volatile matter only, and no SO₂, SO₃ or H₂S. And the benzol, though giving emulsions of less stability, was Kahlbaum's cryst., free of thiophene; thus while it is impossible to deny absolutely the presence of a chemical emulsifying agent in my experiments the assumption of such an agent seems unnecessary and the writer is very doubtful of its existence under the particular conditions described. The question then arises as to what prevents the coalescence of the water globules with one another and with the water film covering the glass. The answer is, first, viscosity, which tends to limit movement of the particles, and, second, possibly the possession of electrical charges of like sign which by their repellant action prevent contact between water particles and films. As to the latter we can only mention that Lewis¹ appreciated the importance of the interfacial potential in the stability of certain emulsions. In my few experiments having this latter possibility in mind only imperfect evidence was secured of such charges, that is to say, the effect upon the stability of emulsions having acids, bases, or salts in the aqueous portion of the system is not marked.

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Summary

This paper reaffirms the possibility of forming emulsions of two immiscible liquids by first increasing their mutual solubility through heating and then precipitating the one in the other by cooling. More particularly the stability of such emulsions in certain devices used for cultivation of obligative anaerobic bacteria is emphasized and explained as due to the protection from evaporation which these devices afford rather than to the absence of air. Incidentally this explanation fails to indicate the reason for the demonstrated inferiority of hydrocarbon oil as a means of anaerobiosis in

¹ Lewis: Zeit. Kolloidchemie, 4, 211 (1909).

comparison with certain mechanical seals. The phases of emulsions have been studied by various known means and a new method of determination of phases is described. Viscosity is mentioned as an important element in determining the stability of emulsions formed in the above manner and the likelihood of electrical charges upon the dispersed particles as the only probable emulsifying agent in these experiments is suggested.