The importance of the H-ion concentration in the colour-preservation of museum specimens.*)

By

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„Our cells are washed with the water of the eternal ocean". Abderhalden employs this poetical simile in one of his books, when referring to identity of the salt-concentration of the tissue-fluids of the body and of sea-water. The phenomenon thus described was perhaps first of all noticed by those who attempted to preserve the colour of specimens containing blood, and perceived that in this respect no success could be attained by means of the simple hypotonical formaline-solution. They paid practically no attention to the further physico-chemical properties of the fixatives and preservative fluids. Accordingly we find no reason to gainsay the opinion of some biochemists who say that the methods employed in colour-preservation have from the chemists point of view scarcely reached more than that attained by cooking. — Indeed, if we think of potassium nitrate, long ago used as a meat-preservative and one of the ingredients of the mixture of salts used in the fixatives, or if we consider the Talajew procedure of embedding the specimens in gelatine — a process, which has been adapted in various places during the last 10 years — or the practice of preservation in a 50% sugar-solution which has recently again come into fashion — we must allow that there is more or less truth in the above mentioned dictum.

It seems, that the preservation of organs in approximately natural colours under conditions approaching the natural or-

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ganic ones, is a problem only to be solved by means of some fluid medium for hitherto we have not been able to preserve the form and the customary degree of transparency of the organs by any other means, such for example, as the uncommon and therefore favoured Talalajew method for embedding the organs in gelatine or in agar. Convincing proof of this is supplied by the dull-brown specimens presented by Talalajew himself to Aschoff's collection in the Freiburg museum.

The solutions, however, have likewise their own „internal life", characterised by physico-chemical phenomena; by changes in their structure and appearance which proceed with the regularity of natural law. Can it be doubted that these labil systems exercise an effect on dead organs placed in them for a period longer than the duration of a man's life? I have visited 45 different Pathological Institutes in Europa and America, and have looked through a large number of their specimens, collected since the so-called colour-preservation procedure first came into general use — let us say over a period of 30 years — and I have come to the conclusion, that standardisation has evidently not been achieved. Faded, discoloured specimens lead us to suppose that the preservative solutions have also their own „internal life". And perhaps also we may suppose that in these dead organs we have to reckon with a continuation of biological processes.

What can be the change, the „internal life" of these preservative fluids, and of the organs they surround, which destroys the colour of specimens which have been prepared with so much care, trouble and expense? Is it not possible to determine any conditions of equilibrium in the fluids and the organs they contain, that will give us a favourable standard for the preservation of colour? In what follows, my aim will be to provide an answer to these questions.

The Kaiserling- procedure most generally employed consists, as is well-known, of three phases. If, with the lapse of time, specimens subjected to this process have lost their colour, this change may be ascribed to all three of these phases, or only to one or two of them. The first possibility seems to be excluded, because it is well-known that, with the Kaiserling process, specimens have been prepared, even if in very few cases,

which have successfully retained their colour. I myself have found that, if after phase II. of the alcohol treatment I enclosed a number of specimens, [all without exception of the same bright colour and all treated at the time in exactly same manner] in separate glass jars, only same of the specimens retained their colour, while the majority became faded or discoloured, after a period of less than ten years: although all were placed in hermetically closed vessels containing Kaiserling III. preservation fluid of precisely the same composition. As the specimens which retained their colour and those which lost it had been treated in exactly the same way in the first two phases of the Kaiserling method, it seemed safe to conclude that the loss of colour was not due to the first two phases, but only to phase III. that is, to changes in the preservative fluid.

Would it be possible to find differences in the fluid preserving these specimens, some of which retained while others lost their colour, although all were treated at the same time and in exactly same manner? And if so, what might be the cause of these differences? The desire to find an answer to these questions urged me to perform the work, which I now describe.

I started with the investigations of the unpleasant, acid-smelling preservative fluid of the old Kaiserling specimens, which had become completely faded and discoloured. The character of the odour itself leads one to suppose a marked acid reaction, developed in the old solution, since there is never any such odour to observe in the fresh one. The determination of the H-ion concentration presented a suitable method of observing the changes due to any acid reaction.

This I performed colorimetrically. — Here, as also in the cases when I examined the coloured specimens, I obtained, on an average, very satisfactory results with three indicators: methyl-red (pH 4·4—6), bromcresol-purple (pH 5·4—6·4), and bromthymol-blue (pH 6—7·2). Among these the methylred and the bromthymol-blue especially proved to be most useful for the sharp determination of the transitions which were of most frequent occurence (pH 5·2—5·6—5·8 respectively pH 6—6·2—6·4—6·6—6·8). As standard buffer-solutions of comparism I employed a freshly prepared scale of Sörensen's primary and secondary phosphate-mixtures. By means of a pipette on every

occasion thoroughly washed with distilled water and provided with a small rubber bulb- I transferred 10 cm^3. of the preservative fluids to test-tubes of equal width. To the 10 cm^3. of fluid I added the indicators in the following quantities: methyl-red (0·02% solution) 0·3cm^3., bromchresol-purple (0·04% solution) 0·5 cm^3., and bromthymol-blue (0·04% solution) 0·5 cm^3. For the investigations I employed only preservative fluids which were perfectly clear and transparent. I took care to choose only those specimens of which the preservative fluid had not been changed and which therefore had always remained in the original solution. The tests were always made and the records noted by daylight.

With the bromthymol-blue, I found the H -ion concentration for the Kaiserling preservative-fluid (water-glycerin-potassium acetic-mixture, briefly „Ka III.", made freshly with distilled water in the prescribed manner) to be on an average about pH 6·8. However I had the opportuntiy, in the Pathological Institute of the Boston City Hospital, to examine Ka III.-fluid, which had stood unused for a longer period, more than one year — in a half-closed bottle, and this showed pH 6·2—6·4. But here we have to take into account the effect of the air and its CO_2 contents — an effect which is practically excluded in the case of specimens in hermetically-closed bottles and glass-jars.

Examining the preservative fluid of some discoloured specimens in the Pathological Institute of Professor Mallory in Boston, I found that the fluid of the completely faded specimens nearly always showed an H -ion concentration of pH 5·0—5·4. I made similar observations in the case of old discoloured Kaiserling specimens in the Pathological Institute No. I. of the Budapest University. It appears from these results that in comparison with the hydrogen-ion concentration (about pH 6·8) of freshly prepared Ka. III. fluid, the Ka. III. fluids in which faded specimens have stood, disclose an H -ion concentration of pH 5·0—5·4. —

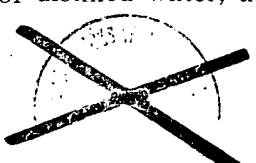
In the case of faded specimens, especially on the freshly made cut-surface of larger parenchymateous organs, I frequently observed that the structure had quite disappeard, and that there was, moreover, a softening in the centre accom-

panied with complete absence of colour. When viewed microscopically, the characteristic histomorphological changes of autolysis were present.

Desiring to follow the changes in the H-ion concentration in the course of the Kaiserling fixation process, I used as specimen for this purpose a freshly amputated femur sarcoma in the Pathological Institute Professor Turnbull of the London Hospital. — I placed this specimen, after cutting through its length, in the original Kaiserling I. salt-formalin solution (at 12^{30} p. m. on June 9, 1925). This fixative had a fairly constant pH 6·0—6·2. My observations are summarised in the following table:

Day of examination	VI. 19. 1925.	VI. 10. 1925.	VI. 11. 1925.	VI. 12. 1925.
pH of the Fluid	4. p. m. pH=5.8–6.0	10. a. m. pH 6.0—6.2	9.45. a. m. pH = 6.2—6.4.	10. a. m. pH = 6.8.
Correction of the pH approximately to the neutral point, and other remarks:	4.30 p. m. with circa 400 ccm. n/10 Na OH. pH=cca 7.0.	10.30 a. m. with cca 200 ccm. n/10 Na OH. pH = 7.0. 5 p. m. pH = 6.2. 5.30 p. m. with 300 ccm. Na OH.: pH=7.0.	1. p. m. I cut the muscles in several sections and rinsed with water 4. p. m. with 160 ccm. n/10. Na OH: pH = 7.0.	3.30 — 4. p. m. abs. alcohol 4. p. m. Ka. III. which showed ph 6.8.

It appears from this Table that especially in the case of larger organs, the Kaiserling I.-fixative proved to be constant by preliminary tests, can undergo a change in the sense that its H-ion concentration continually increases, even if, from day to day, or twice in one day, we corrected the pH-value approximately to the neutral point, for instance by the use of $\frac{n}{1}$ NaOH. Although during the investigation, after the manipulations exhibited in the Table given above a number of deeper incisions were made in the specimen, we were able to make the following noteworthy observations. From different layers of the specimen we cut pieces of about the size of a small finger-joint. These portions of muscle we placed separately in test-tubes, each containing 10 ccm. of distilled water, and after 15 minutes we



examined the above distilled-water for its H-ion concentration. The examination showed that the H-ion concentration of the water surrounding those pieces of muscle which appeared to be best fixed, was about pH 6·6, while the H-ion concentration of the water above the apparently quite raw, unfixed portions of muscle, showed pH 5·6. — The former excised pieces of muscle were very well coloured, but the latter were faded and grayish.

The remarkable results obtained by the observations described above, may be summarised as follows.

The H-ion concentration of the preservative fluid of faded Kaiserling specimens increases considerably. This begins in the first phase of the Kaiserling-process (during the fixation) and it is then of a progressive character. In spite of this occurence we may obtain a bright red colorisation for a longer or shorter time on the superficial layers of many specimens treated with alcohol (during the so called II. stage of the Kaiserling-treatment and later). In the „III. stage" however, when the specimen is placed in a relatively small quantity of preservative fluid, it may in the course of time increase very considerably. In the case of such small quantities of preservative fluid their H-ion concentration may under unfavorable conditions approach pH5.0, chiefly as a result of autolytic changes.

Let us now turn to the examination of the Ka. III. fluid of specimens with their colour preserved by the Kaiserling process. The colour of the organs, if we consider only the colour of the organ itself and the colour due to its blood-contents, may be said to be of a highly complicated character. Kaiserling seems to find a statement for Takayama's spectroscopical observations in the formaline + alcohol treatment of animal-blood. Kaiserling described namely that using human-blood (obtained at autopsy) in phases I.—II. of his fixing process, „kathemoglobin" (λ 567—556 and λ 546—516) is developed, under the effect of the succeeding treatment with the alcohol, from the formaline-methemolglobin.

According to Hoppe-Seyler, in the organs of dead bodies, or at least on the surface of them, hemochromogen is also developed, under the influence of the alcohol. Pick does not in reality come into conflict with Hoppe-Seyler when he, Pick,

assumes that under the influence of the alcohol after the salt-formalin fixation, there is a formation of alkaline-hematin. According to Kaiserling there is also a formation of oxyhemoglobin in the case of the Jores-method of fixing with salt-formalin-chloral-hydrat, which I could also perceive — at least in my spectroscopical examinations of sheep's blood. There can, however, be no doubt that spectroscopical examinations carried out with blood only, in vitro, cannot be accepted, without further consideration, as also applicable to the colour of the organs. It might perhaps be best to attempt the spectrum-analysis of light reflected from the cut-surface ot the organs. But this gives such a complicated and confused spectrum that even Dr. Bovie (Boston) has considered its exact analysis to be quite hopeless. One may recall in this connection Hyrtl's cynical remark: „Colour still remains imperfect light".

My investigations carried out with the colourmetric method of determining the H-ion concentration, extended to more than 100 different museum-specimens, hermetically inclosed in separate glass-jars. From the point of view of colour-preservation I can summarise the results hitherto obtained with specimens of different origin, by stating that I did not find, among the specimens which had to any extent retained their colour a single one whose preservative fluid showed the high degree of H-ion concentration (that is, the pH 5·0, found in the case of discoloured specimens). The value of the H-ion concentration for the Ka. III. solutions of more or less coloured specimens, was pH 6·2—6·4, and in the case of specimens which showed the colour of the blood, through darker, in bright shades, this value was pH 6·4—6·6. On the other hand, the preservative solution of the best-preserved specimens of bright colour proved consistently to be of about

pH 6·8!

For reasons mentioned above I could not complete the spectroscopical analysis of all these specimens and therefore I cannot yet say to what extent the „Kathaemoglobin", the hemochromogen, or the oxyhemoglobin played a role in the composition of their colours. But apart from this, I should now like only to emphasise the fact that, according to my obesrvations, the colour given to an organ by the blood it contains can

only be preserved if the H-ion concentration of the preservative fluid does not increase to much more than pH 6·0. Further I should like to point out particularly that, according to my observations, the Kaiserling III. fluid in which the specimens most approximately retained their natural pretty colour, was characterised by a H-ion concentration of pH = 6·8, or, in other words, such a *H-ion concentration which Michaelis determined to be the isoelectrical point not only of the reduced hemoglobin but also of the oxyhemoglobin, the conditions are such that both are at the same time least dissociable and least soluble.*

According to my observations bearing on coloured specimens I can corroborate the conclusions indirectly given above, namely that a discolouration of Kaiserling specimens takes place in the course of time there is a marked increase in the H-ion concentration of the Ka. III. fluid. In the development of this acid reaction the autolysis (as indicated histomorphologically, especially in the centre of the organs) may, ceteris paribus, play a very considerable and, as it seems, important role. In consequence of the autolysis — as also proved by H. G. Wells (Chicago) — organic acids may develop; and autolysis of tissues proceeds furthest in a pH range between 5·0 and 6·0.

How could this autolysis be prevented? First of all, by getting the organs for preservation in the freshest possible condition, before the autolytic process has begun in them. But this in itself is not sufficient, as is shown by the experiment I made with the perfectly fresh femur-sarcoma. It is also necessary that the organ shall be saturated in every part by the fixative, a postulate best attained by injecting the fluid through the blood-vessels. Otherwise, we cannot count on perfect fixation with the Ka. I. salt-formalin solution, especially in the case of the more bulky specimens; neither therefore on the prevention of all sorts of fermenting processes in the interior parts of the organs. Talalajew, for instance, having embedded in formol-gelatine a specimen of „peribronchitis-tuberculosa", which had previously stood for 25 years in formalin solution, perceived, six month after its embedding, that the gelatine had become peptonised. He says: „In the specimens which contained elements of inflammatory products of different origin, there finally sets a liquefaction of the gelatine, in spite of its formalinisation".

But we also want to emphasise the experiences of other investigators in connection with the autolysis. Among these I would now only mention an observation made by Bradley who pointed out that even a slightly alkaline reaction (pH 7·4—7·8), approximately equal to that of blood, almost completely prevents autolysis. Thus we can understand the advantage of the Jores (pH 7·4—7·6) or of the Pick (pH 8·2—8·4) salt-formalin-mixtures, using them instead of the Kaiserling I. fixative (ca pH 6·2—6·4). Perhaps this would also partly account for the circumstance that there exist, as I perceived, so many well-preserved coloured specimens in Professor Pick's Pathological Institute (at Berlin-Friedrichshain-Hospital) although some of them are of a deeper red colour than the organs have been in nature. — Pick's fixative (1000 ccm. distilled water 50 ccm. conc. formalin + 50 gr. artificial Carlsbad salt) is strongly alkaline. As Pick says: „The high contents of the artificial Carlsbad salt in sodium-bicarbonate appears especially favourable to the formation of the stabile alkaline hematin"; to which I would add that this is undoubtedly the reason why it can act so effectively against the autolysis. After the organs have been placed either in the Jores or in the Pick fixatives, the fluid may undergo a change, but nevertheless the H-ion concentration of the fluid does not increase to the same extent as that of the Kaiserling I. mixture. Although I myself observed that in Pick's Museum at Friedrichshain there are very many beautifully coloured specimens, yet, as others before me, I could see that in spite of the treatment either with the Jores or with the Pick fixing agents the specimens had in many cases become discoloured. Therefore I believe it would be advisable to resort also in this case to the help of Nature, „the greatest of all instructors". Nature preserves the bright red colour of the oxyhemoglobin during the life. Nature controls and keeps the acidbase-equilibrium of the blood with marvellous regularity. Could we not attempt to apply the same for our preservation-process?

The attempts I made in this direction — which would furnish an answer to the second question proposed in the introduction to this paper viz. the question of the possibility of finding such equilibrating conditions for the preservative fluids, were

closely connected with the investigations mentioned above and therefore spontaneously suggested making use of the „Buffer-solutions". During my work I read that E. L. Judah had already in 1922. recommended taking care of the neutralisation of the Kaiserling — fixative by means of litmus. But this procedure which, was at an earlier date also already employed by Dr. Julius Schuster, (formerly Assistant of the Psychiatric Clinic of the University of Budapest) in his histological work, could not be regarded as a method for the determination of hydrogen-ion concentration. On the other hand it has been suggested, by the authors above cited for the „neutralisation" of the formaline-fixatives and not for the standardisation of preservative fluids, which was our purpose to do.

For standardising the H-ion concentration of the Ka. III. fluid at pH 6·8 which my own experiences had shown to be the best I took as most the suitable the Sörensen primary and secondary phosphate-solutions. If from these we take the Na_2HPO_4 in its anhydrous form, we must take 50 ccm. of each of the normal solutions in order to obtain a Buffer-system, corresponding to pH 6·8. As I found that the m/15 solutions were just as effective as the m/1 and m/2 solutions, I dispensed with the latter.

I carried out my preliminary experiments with small portions of organs in test-tubes. In the Kaiserling three-stages-process, instead of Ka. I, I used the Jores mixture. After my experience this is composed somewhat like a Buffer system. Perhaps in this way is also favourable in contrast to the formaline which tends continually to increased H-ion concentration. But the chloral-hydrat as a compoud in the Jores mixture seems to be of good effect in other ways, and especially in preventing the hemolysis. However, I consider that it is much more important — a point not mentioned by any other investigators — to add to the final Ka. III. preservative fluid, after correcting its H-ion concentration to pH 6·8, a mixture of equal parts of n/15 Sörensen phosphate-solutions in the proportion of 100 : 25. The colours of specimens thus treated, proved to have hitherto kept satisfactorily after the lapse of two years. — The Sörensen standard solutions are sufficient to account for the lasting condition of equi-

librium in the KaIII. fluid. In these experiments I obtained the desired state of equilibrium for the specimens by taking the material which I desired to preserve in small portions $^1/_2$ cm. in thickness and only a few. cc. in volume, and these small portions were thoroughly penetrated not only with the fixative but also with the buffered preservative fluid. The stabilisation of bigger specimens and of entire organs can, I believe, be best secured by treating the organs in the above manner, but either the organs must be cut in thin sections [cca. $^1/_2$ ccm. thick], or, if treated as a whole, the fixing mixture and the preservative fluid, saturated with the Sörensen standard-solutions after phase II., must be injected into the bloodvessels etc. and the specimens kept protected from sunlight in glass jars hermetically sealed. The specimens which I prepared by adopting this method have hitherto remained well-preserved.

I am of opinion that if we carefully adhere to the perscriptions of Kaiserling and Pick in every technical details and supplement them with the details I have mentioned above, we shall not have to be content with the putrid, discoloured specimens in our museums which have for generations demanded so much work, perseverance, material sacrifice in their preparation, we shall obtain well-preserved coloured specimens which will be of real value in medical education. If the modest work which I have performed on my own initiative should to any small extent contribute to the hoped for successful solution of the problem, this would indeed be only an indication of the small value which is at present attached to its wider theoretical interest.

I wish to express my best thanks to Prof. F. B. Mallory, of Boston, Mass., and to Prof. H. Turnbull, of London, for their extreme kindness in enabling me by placing at my disposal the necessary chemicals to carry out a great of this work.

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