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Preserving Supravital Staining with Neutral Red in Paraffin Sections of the Lung.

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The method to be described is a modification of that used by McJunkin[1] which proved to be inapplicable for use on lung tissue. After our technique had been perfected Cash[2] reported a similar method, but ours, we believe, offers certain advantages. Two pathways are employed for injection of the vital stain, either the intratracheal, the intravenous or a combination of the two. In using the former the animal is first killed by air embolism. For the latter method the dye is injected into a vein 10 minutes before its death. In rabbits intravenous injections are made into the ear vein; in guinea pigs, the jugular veins are exposed under local anesthesia with 3 per cent cocaine. Ether cannot be used, as it destroys the capacity of the pulmonary cells to react specifically to the dye.

Supravital Staining—(a) Intratracheal injection: 30 to 40 cc. of a warm 1:1500 solution of neutral red made up in physiological salt solution is injected into the guinea pig's trachea, which is then tied off. The whole animal is incubated at 37° C. for 5 to 30 minutes. After this interval the dye ceases to react specifically and stains the nuclei. (b) Intravenous injection: 60 to 80 cc. of the same solution are slowly injected into the jugular vein or carotid artery of a guinea pig. If the animal survives it is allowed to live 10 minutes before being killed by air embolism. Occasionally it dies and the whole body is incubated as just described.

Fixation—This is accomplished by dropping the lungs and heart into alkaline Zenker-formol solution. After intravenous staining the lungs are distended with air. To prepare this fixative add sufficient solid NaOH to U. S. P. formaldehyde solution to give a pH value of 7.6. Dissolve by gentle heating. Keep in the incubator at 37° C. Of this alkaline formaldehyde add 15 per cent to the usual Zenker's fluid (without acetic acid). The sodium not only neutralizes the acidity, but it has a specific mordanting effect upon staining. Merk's Reagent neutral formaldehyde is not as effective as that prepared in this manner. Allow fixation to continue for 12 to 18 hours. With a razor blade cut blocks not over 2 mm. in thickness. These are washed under a vacuum in fresh Zenker's solution without formaldehyde or acetic acid. This ensures rapid removal of the

formaldehyde and exhausts the air from the lung. Tissues may be used immediately or within two days.

Dehydration and Embedding—Selected blocks are washed for 15 minutes in running water and blotted on paper. They are then immersed for 10 minutes in each of the following solutions: 80 per cent alcohol, followed by graded mixtures of 95 per cent alcohol and benzene (Mark's Reagent) in the following proportions: 9:1, 8:2, 7:3, 5:5, 3:7, 2:8, and 1:9. It is often necessary, particularly with dense pathological tissues, to introduce double changes of alcohol-benzene in the dilutions of 5:5 and thereafter. The tissues are cleared for an hour or two in one or more changes of pure benzene and placed in benzene saturated with paraffin at 37° C. for an hour. Finally they are embedded in pure paraffin at 56° C. (4 changes in 30 minutes). Sections are cut, fixed to the slide and dried in the usual manner.

Counter Staining and Clearing—The paraffin may be removed from 10 or more slides at a time in a dish of xylol, but thereafter they must be treated individually, preferably by the drop bottle method. Water is more injurious than alcohol to neutral red stains. Slides are treated successively with absolute and 95 per cent alcohol and then dropped into a jar of 1 per cent iodine in 95 per cent alcohol for 15 seconds. The iodine is removed by several changes of 95 per cent alcohol.

Without washing in water they are counterstained in Harris' haematoxylin (without acetic acid) for a sufficient time to overcome the surface tension of the alcohol and to allow the dye to spread evenly on the slide. They are drained and quickly washed in 95 per cent alcohol, followed by water in a beaker, only long enough to thoroughly wet their surface. Finally the sections are dehydrated in 95 per cent and absolute alcohol, cleared in xylol and mounted in neutral balsam. Sections so prepared are clear and show deep nuclear and specific staining which is permanent and resists the action of light.

The successful application of the method depends upon the presence of an excess of dye in contact with the tissues, the slow fixation and mordanting action of the alkaline Zenker-formaldehyde solution, the rapid dehydration and clearing in the alcohol-benzene mixtures, and the use of water in counterstaining only after the excess of haematoxylin has been removed by alcohol.

The intratracheal injection of neutral red is itself an irritating process, and as such it causes the migration of many cells into the air spaces even after the death of the animal. Before employing the

method to study pathological processes one should therefore familiarize himself with the reaction excited in the normal animal.

We have found the Cash method of injecting a 1 per cent solution of the dye into the veins of the living animal (10 cc. for a rabbit and 2 to 4 cc. for a guinea pig) 10 minutes before death particularly satisfactory when combined with the special methods of fixation and dehydration above described. The fixed cells in the alveolar septa are deeply stained and any stimulating effect by the dye resulting in migration of cells is avoided. However, most of the phagocytes free in the alveoli do not stain. As these are the cells of most interest in pathological processes, it would therefore seem preferable to combine intravenous with intratracheal staining, particularly where massive lesions are present.

Extrapulmonary Tissues—When neutral red is injected into the ear or jugular veins it is largely absorbed by the pulmonary tissues and the cells of other organs remain unstained. Therefore the local circulation of the spleen, liver or lymph nodes must be injected directly (20 to 30 cc. of dilute solution of dye).

Furthermore, fixation must not progress too rapidly or the dye will be dissolved. This may be overcome by placing the injected tissues in a parchment sack of neutral red solution and suspending the sack in a jar of fixative. After 30 to 40 minutes the Zenker-formol dialyses through the parchment and precipitates the dye inside the sack. The tissues are then removed and placed directly in the fixative.

In sections from organs prepared in this manner supravital staining is well preserved but the other properties of the cells naturally suffer.*

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[1] McJunkin, F. A., *Am. J. Path.*, 1925, i, 305.

[2] Cash, J. R., PROC. SOC. EXP. BIOL. AND MED., 1926, xxiv, 193.

* This work was begun with the assistance of Dr. Benjamin Sacks, who was unable to bring it to completion.