EXPERIMENTS ON THE SOLUBILITY OF HEMOSIDERIN IN ACIDS AND OTHER REAGENTS DURING AND AFTER VARIOUS FIXATIONS *

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Virchow [1] first in 1847 reported on the solubility of blood pigment in acids, finding that the granular intracellular form was soluble in warm concentrated sulphuric acid, but resisted cold sulphuric acid, alkalies and other reagents. The pigment was reprecipitable from its sulphuric acid solution with an excess of ammonia as brown floccules, which after ashing and solution in hydrochloric acid gave the Prussian blue reaction. Previous treatment of the pigment with alkali made it soluble in nitric acid and more quickly soluble in sulphuric.

Following the introduction of the ferrocyanide reaction by Perls [2] in 1867, and of the ammonium sulphide reaction by Quincke [3] in 1880, little attention was paid to the acid solubility of hemosiderin until the publication of Hueck's monograph in 1912. Neumann [4] in 1888 had noted the pigment showed a quite notable resistance to mineral acids. Hueck [5] stated that hemosiderin may require a very thorough treatment with aqueous solutions of acids, less with alcoholic solutions, to dissolve it, and published a table in which hemosiderin was designated as "soluble in acids" without qualification. This table was reprinted and the statements repeated by Hueck in Krehl and Marchand's Handbuch der allgemeinen Pathologie in 1921,[6] and since then the bald statement that hemosiderin is soluble in acids has appeared in various texts on general pathology and on pathological technique (Oberndorfer,[7] Mallory and Wright,[8] Mallory,[9] Schmorl,[10] and von Gierke [11]).

Acid extraction has been used by various workers to remove hemosiderin when studying other pigments. Brown,[12] Seyfarth,[13] and Mayer [14] each used oxalic acid to remove hemosiderin while studying the possible iron content of malaria pigment. Brown and Seyfarth used 12 hours extraction with 2 per cent oxalic acid and

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found iron-reacting pigment afterward. Mayer prolonged this to 18 hours and found the reaction positive in some cases and not in others, but the iron-containing pigment was found only in phagocytes, not in red corpuscles. Glasunow [15] extended the extraction to 24 hours and found no remaining iron. He showed that concentrated oxalic acid did not dissolve malaria pigment in 48 hours and speculated that 12 hours treatment with 2 per cent oxalic acid might be insufficient to remove hemosiderin, but did not try extraction of known hemosiderin. He further noted the

TABLE I

Hemosiderin Remaining in Formalin-Fixed Paraffin Sections after Varying Exposures to Varying Concentrations of Various Acids

Stock acid	Dilution	Time	Temperature (room)	Material	Amount of hemosiderin remaining
	%	*hrs.*	*C.*		
None	—	—	30°	S–6896	++++
Conc. HCl	1	48	30°	S–6896	+++
Conc. HCl	10	48	30°	S–6896	+++
Conc. HCl	20	48	30°	S–6896	o – trace
Conc. H_2SO_4	10	48	30°	S–6896	o
Conc. H_2SO_4	10	24	30°	S–6896	o
Conc. H_2SO_4	10	16	30°	S–6896	Trace
Conc. H_2SO_4	10	8	30°	S–6896	+
Conc. HNO_3	5	48	30°	S–6896	+ – +++
Conc. HNO_3	10	48	30°	S–6896	+ – ++
Glacial acetic	20	48	30°	S–6896	++++
90% Formic	20	48	30°	S–6896	+ – ±

insolubility of hemosiderin in aniline, pyridine and 4 per cent quinine in chloroform and the slow solubility of malaria and formalin pigments in these reagents (3–14 days).

In a study of melanosis of the appendix,[16] I used extraction for 2 hours with 20 per cent sulphuric acid to exclude hemosiderin, and we have generally relied on similar extractions with 20 per cent sulphuric acid or a hydrocholoric acid alcohol containing about 2.5 per cent hydrogen chloride to remove hemosiderin.

In the summer of 1937 a section of bone marrow which had been decalcified 2 or 3 days in a 20 per cent formic acid-sodium citrate fluid was included among several sections being stained for iron by the ferrocyanide method, and a considerable amount of hemosiderin was demonstrated.

This finding raised a question as to the degree and rapidity of solution of hemosiderin in acids. Accordingly, a series of experiments was initiated to explore the problem, primarily in connection with establishing reliable technical procedures for our own use.

In the first experiment a series of sections of a heavily hemo-

TABLE II

Hemosiderin Remaining in Paraffin Sections of Formalin-Fixed Material from a Skin Tumor and a Liver from a Case of Hemochromatosis after Varying Exposures to Various Acids

Stock acid	Dilution	Time	Temperature	Material	Amount of hemosiderin remaining
	%	*hrs.*	*C.*		
Conc. H_2SO_4	10	1	37°	S–6896	+
				A–1228	++
Conc. H_2SO_4	10	2	37°	S–6896	±
				A–1228	+
Conc. H_2SO_4	10	4	37°	S–6896	Trace
				A–1228	Trace
90% Formic	20	8	37°	S–6896	+++
				A–1228	++++
90% Formic	20	16	37°	S–6896	± most brown
				A–1228	+ most brown and in portal area
90% Formic	20	24	37°	A–1228	+ most brown and in portal area
Glacial acetic	20	48	37°	S–6896	++
				A–1228	++ – +++
Conc. HCl	10	8	37°	S–6896	+ part brown
				A–1228	++ part brown
Conc. HCl	..	16	37°	S–6896	± part brown
				A–1228	+ most brown and in portal area
Conc. HCl	..	24	37°	S–6896	± part brown
				A–1228	± most portal

siderotic cutaneous sarcoma, fixed routinely in 10 per cent formalin, was utilized. The paraffin sections were brought to water as usual and then soaked in acid solutions for varying periods. Sections were then washed in water and stained by Dr. Maude Abbott's modification of Perls' reaction (Mallory and Wright,[8] 1924, p. 207), using dilute fuchsin as a counterstain. The results are presented in Table I.

Table I shows that 20 per cent hydrochloric acid for 48 hours at about 30°C., or 10 per cent sulphuric acid for 16 hours, barely removed all the demonstrable iron, and that 10 per cent nitric

TABLE III

Persistence of Hemosiderin in Formalin-Fixed Tissues from Various Cases on Soaking in Sulphuric and Other Acid Solutions

	A–1747 Brown induration of lung (a)					10% Sulphuric acid							
											A–1695		
Time in acid at 25°C.	5% oxalic	20% acetic	HCl alcohol	20% H_2SO_4	10% H_2SO_4	A–1743 Lung CPC(a)	A–1735 Lung CPC(a)	A–1716 Spleen	A–1593 Liver cirrhosis	A–1367 Spleen (b)	Liver Kc(b)	Spleen (c) pulp (b)	Lymph node (c)
Control	+++	+++	+++	+++	+++	+++	++	+++	++	+++	+++	+++	+++
½ hr.	+++	..	+++	+++	+++	+++	++	+++	++	+++	++ (c)	++	+++
1 hr.	++	..	+++	+++	+++	+++	+.++	+++	+	+++	++ (c)	++	+++
2 hrs.	+	..	+++	++ (c)	+++	+ (*)	+	+++	+	+++ (c)	+ (c)	++	+++
4 hrs.	– (c)	..	+++	+ (c)	++	+++	+	++	+	++ (c)	+ (c)	+	+++
8 hrs.	– (c)	..	+++	– (c)	+	+++	–	+.++	±.+	± (c)	± (c)	±.+	++
16 hrs.	– (c)	..	+++	– (c)	±	++	– (c)	+	– (c)	– (c)	± (c)	±	±
1 day	– (c)	+++	+++	–	–	–	–	±.+	–	– (c)	– (c)	–	–
2 days	– (c)	+++	+++										
3 days	– (c)	+++	+++										
4 days	–	+++	++										
5 days		++.+++	++										
7 days		++	+										

Kc = Kupffer cells.
CPC = Chronic passive congestion.
(a) = Some carbon present in addition to hemosiderin.
(b) = + formalin pigment in hemolyzed areas.
(c) = Some brown granular iron-free pigment.
(*) = Unexplained irregularity.

acid and 20 per cent formic acid did not quite remove it all in 48 hours, and that 20 per cent acetic for 48 hours was almost without appreciable effect.

The next experiment was made at 37°C. and showed more rapid removal of demonstrable iron. However, often part of the brown pigment remained, though it failed to give the ferrocyanide reaction. The results are shown in Table II.

Table II shows that 4 hours at 37°C. in 10 per cent sulphuric acid removed practically all of the pigment, that 10 per cent hydrochloric acid or 20 per cent formic acid removed most of the iron from the pigment in 16 to 24 hours at 37°C. but left much brown pigment, and that acetic acid was a little more active in pigment removal or iron removal than at 30°C.

Further human formalin-fixed material from 7 cases was selected for testing to see whether there was any great variation in acid solubility of hemosiderin in various cases. One of these was used also to see whether longer exposure to 20 per cent acetic acid would be effective and to test the solvent action of oxalic acid on hemosiderin.

Table III shows that the limits of persistence of hemosiderin in 10 per cent sulphuric acid at room temperature (25°C.) were 16, 16, 4, 24, 8, 8 and 16 hours for the 7 cases, 4 hours in 20 per cent sulphuric, and 2 hours in 5 per cent oxalic acid in the 1st case. Hydrochloric acid alcohol (2.5 cc. HCl in 100 cc. 70 per cent alcohol) and 20 per cent acetic acid were very slow, still showing some hemosiderin after 15 and 30 days respectively.

Apparently iron-containing pigment in formalin-fixed material is relatively resistant to acids.

The next experiment was to test the solubility of the pigment in various acid-containing fixatives applied to fresh unfixed tissue from the spleens of guinea pigs previously given several intraperitoneal inoculations with sheep erythrocytes. The results are presented in Tables IV, V and VI.

From these tables it seems evident that formalin protects hemosiderin against the solvent action of formic and acetic acids, but not sulphuric, while Zenker's potassium bichromate and corrosive sublimate mixture, which by itself preserves hemosiderin, fails to prevent solution of the iron from the pigment by formic, acetic and nitric acids.

TABLE IV

Amounts of Hemosiderin Shown in One Spleen Fixed by Various Fixing Fluids

Fixing solution	Time	Amount of hemosiderin remaining	Red cells
	hrs.		
10% aqueous formalin, neutral	48	+++	Preserved
10% formalin, 5% acetic in water	48	+++	Laked
10% formalin, 5% formic in water	48	+++	Laked
10 pts. formalin, 90 pts. 95% alcohol	24	+++	Partly laked
10% formalin, 5% acetic in alcohol	24	+++	Laked
10% formalin, 5% formic in alcohol	24	+++	Laked
Helly-Maximow ($HgCl_2$ 6, $K_2Cr_2O_7$ 2.5, H_2O 100, formalin 10)	22	+++	Preserved
Zenker ($HgCl_2$ 6, $K_2Cr_2O_7$ 2.5, H_2O 100, glacial acetic 5)	22	+ brown + green ± brown	Laked
Zenker-formic ($HgCl_2$ 6, $K_2Cr_2O_7$ 2.5, H_2O 100, formic 5)	22	+ green + blue	Mostly laked
Zenker-nitric ($HgCl_2$ 6, $K_2Cr_2O_7$ 2.5, H_2O 100, HNO_3 5)	22	+++ brown	Partly laked

TABLE V

Further Tests of Fixing Solutions on Hemosiderin in One Spleen

Fixing solution	Time	Amount of hemosiderin remaining	Red cells
	hrs.		
Sublimate ($HgCl_2$ sat. aq. 85, H_2O 15)	24	−	−
Sublimate-nitric (sat. aq. $HgCl_2$ 85, H_2O 10, HNO_3 5)	24	−	Laked
Sublimate-acetic (sat. aq. $HgCl_2$ 85, H_2O 10, acetic 5)	24	−	Laked
Sublimate-formol-acetic (sat. aq. $HgCl_2$ 85, formalin 10, acetic 5)	24	++	Preserved
Sublimate-formol (sat. aq. $HgCl_2$ 85, formalin 10, H_2O 5)	24	++	Preserved
Sublimate-sulphuric (sat. aq. $HgCl_2$ 85, H_2O 10, H_2SO_4 5)	24	−	Laked
Sublimate-formol-sulphuric (sat. aq. $HgCl_2$ 85, formalin 10, H_2SO_4 5)	24	−	Slight hemolysis
Zenker-without-acetic ($HgCl_2$ 6, $K_2Cr_2O_7$ 2.5, H_2O 100 + 15)	18	++	−
Zenker-formol-hydrochloric ($HgCl_2$ 6, $K_2Cr_2O_7$ 2.5, H_2O 100, formalin 10, HCl 5)	18	+++	Subtotal hemolysis
Zenker-formol-acetic ($HgCl_2$ 6, $K_2Cr_2O_7$ 2.5, H_2O 100, formalin 10, acetic 5)	18	++	Partial hemolysis

Following these fixation experiments paraffin sections from blocks of tissue fixed in fixatixes not containing formaldehyde and showing preservation of hemosiderin were treated with acids for varying periods as in the first experiments and the results are presented in Table VII.

From this table it is evident that hemosiderin fixed with potassium bichromate and corrosive sublimate (Zenker-without-

TABLE VI

Tests of Fixing Solutions on Preservation of Hemosiderin in One Spleen

Fixing solution	Time	Amount of hemosiderin remaining	Red cells
	hrs.		
		± blue	
Picro-sublimate (5% $HgCl_2$ in sat. aq. picric acid)	24	+ green + brown	Preserved
Picro-sublimate-sulphuric (5% $HgCl_2$ in sat. aq. picric + 5% H_2SO_4)	24	+	Partial hemolysis
Picro-sublimate-formol (5% $HgCl_2$ in sat. aq. picric + 10% formalin)	24	+++	Preserved
Picro-sublimate-formol-sulphuric (5% $HgCl_2$ in sat. aq. picric, 10% formalin, 5% H_2SO_4)	24	+	Partial hemolysis
Bouin (sat. aq. picric 75, formalin 20, acetic 5)	24	+ brown	Partial hemolysis
Carnoy (abs. alcohol 60, $CHCl_3$ 30, glacial acetic 10)	24	++	Laked
Formol-Carnoy (abs. alcohol 60, $CHCl_3$ 30, formalin 10)	2	+ green + brown	Partial hemolysis
Carnoy-formol (abs. alcohol 60, $CHCl_3$ 25, formalin 10, glacial acetic 5)	2	−	Partial hemolysis
Absolute alcohol	18	+ green	Laked
10% formalin	48	+++	Preserved
Formol-sulphuric (10% formalin, 5% H_2SO_4)	48	++ brown	Laked

acetic) is materially less resistant to acid extraction than is formalin-fixed pigment, and that Carnoy-fixed pigment is intermediate in its resistance. The same differences in extracting power among sulphuric, formic and acetic acids as noted previously were seen in this series. Mercuric chloride, as previously indicated in the fixation experiments, apparently shows feeble extracting power, again more in the Zenker-without-acetic material than in the Carnoy-fixed specimens.

To confirm and extend these results a further series of rats

was injected subcutaneously with phenylhydrazine hydrochloride in single doses of 80 mg. per kg. and killed 4, 8, 16 and 24 hours, and 2, 3, 4 and 6 days later, and blocks of the spleens, kidneys and livers were fixed each by 7 fixation methods as follows: 85 per cent alcohol, 24 hrs.; Carnoy's fluid (absolute alcohol 6, chloroform 3, glacial acetic acid 1) 2 hrs.; corrosive sublimate (saturated $HgCl_2$ 85, H_2O 15) 24 hrs.; Zenker-without-acetic, 24 hrs.; 10

TABLE VII

Test of the Solubility of Hemosiderin in Paraffin Sections in Various Reagents at 25°C. after Fixation with Fluids not Containing Formalin

Fixation	Extracting agent	Time	Amount of pigment remaining	Color with acid ferrocyanide	Erythrocytes color with ferrocyanide
		hrs.			
Zenker-without-acetic	10% H_2SO_4	4	–	–	Greenish
		8	–	–	–
		16	–	–	Faintly greenish
		24	–	–	–
	20% formic	24	–	–	–
		48	–	–	Pale blue
	20% acetic	24	++	Blue	–
		48	++	Blue	–
	Saturated $HgCl_2$	24	+	Blue	–
		48	+	Faint blue	–
Carnoy's	10% H_2SO_4	4	±	Blue	Laked
		8	–	–	Laked
		16	–	–	Laked
		24	–	–	Laked
	20% formic	24	+	Blue-green	Laked
		48	+	Blue, green,brown	Laked
	20% acetic	24	++	Blue	Laked
		48	++	Blue	Laked
	Saturated $HgCl_2$	24	++	Blue-green	Laked
		48	++	Blue-green	Laked

per cent formalin, 48 hrs.; 10 per cent formalin and 5 per cent formic acid, 48 hrs.; and the last at 56°C. for 48 hours.

Hemosiderin was best preserved by potassium bichromate and corrosive sublimate (Zenker-without-acetic), 10 per cent formalin, and corrosive sublimate, fairly well with Carnoy's fixative, somewhat less with 85 per cent alcohol, particularly in the central portions of the blocks, and about one-third with the formic acid-formalin at room temperature. The last reagent acting at 56°C. for 48 hours not only removed all of the hemosiderin, but seriously impaired nuclear staining as well.

In passing it may be noted that appreciable quantities of iron-

reacting pigment appeared in the spleen in 16 hours and in the liver in 48. Only 1 animal, killed after 4 days, showed blue granules with ferrocyanide in the epithelium of the renal convoluted tubules.

Sections fixed in several of the foregoing solutions were further tested for the solubility of the hemosiderin in 10 per cent sulphuric acid at 25°C. and at 37°C. (Tables VIII and IX).

At 25°C. small amounts of pigment coloring blue to green with ferrocyanide were evident after 1 hour in the Carnoy and

TABLE VIII

Extraction of Pigment by 10% Sulphuric Acid at 25°C. after Various Fixatives

Fixative	No. of rat	Organ	10% H_2SO_4 at 25°C.			
			1 hr.	2 hrs.	4 hrs.	Control
Carnoy's	38 2d	Liver	–	–	–	±
		Spleen	± blue s.c.	–	–	+
	43 3d	Liver	–	–	–	++
		Spleen	± blue-green	–	–	+
$HgCl_2$	38 2d	Liver	–	–	–	±
		Spleen	–	–	–	++
	43 3d	Liver	–	–	–	++
		Spleen	–	–	–	++
Zenker-without-acetic	38 2d	Liver	± brown	± brown	–	+
		Spleen	± brown-green	–	–	++
	43 3d	Liver	± brown	–	–	++
		Spleen	± gray-brown	–	–	++
10% formalin	38 2d	Liver	–	–	–	±
		Spleen	–	–	–	++
	43 3d	Liver	–	–	–	++
		Spleen	–	–	–	++

Zenker-without-acetic material, but not later, while at 37°C. some brown iron-free pigment remained for 1 and 2 hours but no free iron remained.

Comparing Table VIII with Table II, it is seen that the pigment in the last experiment, fixed within a few days of its appearance, was more readily removed by sulphuric acid than was the probably older pigment in the human material.

Following this indication that recently formed hemosiderin might be more readily soluble in acids than older pigment, further experiments with phenylhydrazine * were carried out.

* Mice averaging 21 gm. were used. A dose of 5 mg. (240 mg. per kg.) of phenylhydrazine hydrochloride killed 92 per cent of 25 mice in less than 24 hours, most of them in 30 minutes. Doses of 2 and 3 mg. (95 and 145 mg. per kg.) were

With phenylhydrazine, traces of iron-containing pigment appear in the spleen in 24 hours. Very considerable amounts are evident in the splenic follicles in 48 hours, less in the pulp. The intrafollicular pigment persists only for 5–6 days, and reacts with ferrocyanide throughout that period. Considerable amounts of brown pigment which fail to react with ferrocyanide persist in the pulp and less in the follicles for as long as 25 days. This pigment also fails to react with potassium ferricyanide.

On testing sections of those spleens showing considerable amounts of iron-reacting pigment for solubility of the pigment

TABLE IX

Extraction of Pigment by 10% Sulphuric Acid at 37°C. after Various Fixatives

Fixative	No. of rat	Organ	10% H_2SO_4 at 37°C.				
			1 hr.	2 hrs.	3 hrs.	5 hrs.	Control
Carnoy's	272 4d	Liver	−		−	−	++
		Spleen		−	−	−	+
	271 6d	Liver	−	−	−		++
		Spleen	−	−	−	−	++
$HgCl_2$	272 4d	Liver	−	−			++
		Spleen		−	−	−	++
	271 6d	Liver			−	−	±
		Spleen	−		−	−	++
Zenker-without-acetic	272 4d	Liver	± brown	± brown			±±
		Spleen		−	−	−	++
	271 6d	Liver		± brown	−	−	++
		Spleen	++ brown		−	−	++
10% formalin	272 4d	Liver	++ brown	++ brown		−	++
		Spleen		−	−	−	++
	271 6d	Liver		−	−	−	++
		Spleen	++ brown		+ brown	−	++

in 10 per cent sulphuric acid at room temperature, it was found that in all animals the intrafollicular iron-containing pigment was removed in one-half to 1 hour, or perhaps persisted for some hours as an iron-free brown pigment. In any case, no iron reaction was positive after more than 1 hour in acid.

As iron-containing pigment disappeared in this series in a short period no indication was given as to alteration of acid solubility of hemosiderin with age.

better tolerated, 92 and 78 per cent respectively surviving for 24 hours, and 84 and 62 per cent for 48.

A series of 7 formalin-fixed spleens containing considerable quantities of hemosiderin in the pulp, from animals intoxicated with organic selenium compounds for periods varying from 25 to 380 days were studied in respect to the acid solubility of the pigment. The exposures to organic selenium were 25, 32, 156, 342, 372, 373 and 380 days, the time necessary for solution of the iron-containing pigment in 10 per cent sulphuric acid at room temperature was 2, 2, 2, 8, 2, 8 and 4 hours.

A series of 24 formalin-fixed spleens from rats intoxicated with sodium vanadate for periods varying from 2 to 53 days were tested as to the solubility of the pigment in 10 per cent sulphuric acid at room temperature. Regardless of the length of exposure to vanadium, the pigment disappeared in approximately 4 hours in all animals.

Apparently the age of the pigment is not a determining factor in its resistance to solution in 10 per cent sulphuric acid.

From the foregoing, it was noted that the iron of hemosiderin is quickly soluble in oxalic acid and very slowly if at all soluble in acetic acid. Standard references on solubilities of inorganic salts note that ferric oxalate is highly soluble in water and basic ferric acetate insoluble, while ferrous oxalate is insoluble in water and ferrous acetate quite soluble. This would appear to indicate that the iron content of hemosiderin is largely ferric.

In studies on the toxicology of sodium formaldehyde sulphoxylate done in 1933, I found that none of the copious iron-containing pigment reacted directly with potassium ferricyanide. Gömöri [17] also noted that he has never obtained a direct reaction with ferricyanide and concludes that hemosiderin does not contain ferrous compounds thus demonstrable.

Having noted that ferrous sulphate in solution is almost immediately converted to the ferric form by hydrogen peroxide, I decided to try various oxidizing and reducing agents on heavily hemosiderotic lung, liver and spleen, considering that the first would theoretically contain the least ferrous iron, and the two latter more, if any such were to be found. The results are presented in Table X.

From this table it is noted that there is no evident increase in demonstrable ferric iron on oxidation with H_2O_2, that no ferrous iron is demonstrable with ferricyanide, even after reduction with

TABLE X

The Effect of Oxidizing and Reducing Agents on the Reactions of Hemosiderin after Formalin Fixation with Potassium Ferrocyanide and with Potassium Ferricyanide

Preliminary treatment	Results with ferrocyanide			Results with ferricyanide		
	Amount	Form	Color	Amount	Form	Color
None	+++	Granular	Dark blue	+++	Granular	Brown
Hydrogen peroxide USP 1 hr. 25°C.	+++	Granular	Dark blue	+++	Granular	Brown
4% elon * 1 hr. 25°C.	+++	Granular	Dark blue	+++	Granular	Brown
4% hydroquinone 1 hr. 25°C.	+++	Granular	Dark blue	+++	Granular	Brown
10% pyrogallic in half saturated sodium carbonate 1 hr. 25°C.	+++	Granular	Dark blue	+++	Granular	Brown
10% sodium sulphite 1 hr. 25°C.	+++	Granular	Dark blue	+++	Granular	Brown
Yellow ammonium sulphide sol. in 3 parts alcohol 17 hrs. 25°C.	++	Granular	+ blue halos	++	Granular	+ blue halos

* Elon = trade name for monomethylparamidophenol sulphate, a photographic developer.

elon, hydroquinone, alkaline pyrogallol or sodium sulphite, and that after treatment with ammonium sulphide part of the iron still reacts with ferrocyanide, and part with ferricyanide. The quantity in both cases is evidently less than with the direct ferrocyanide procedure or with the Quincke iron sulphide reaction, and marked diffusion of the blue stain out from the granules is noted.

This agrees completely with Gömöri's [17] findings and further indicates that, as part of the iron still reacts as ferric after the ammonium sulphide treatment, the Tirmann-Schmelzer sulphide Turnbull blue reaction affords an incomplete and partial demonstration of the iron content of the pigment.

Discussion

Hemosiderin after fixation with formalin is rather slowly soluble in dilute acids, best in oxalic, then sulphuric, then nitric, formic and aqueous hydrochloric acids. It is very slowly soluble in hydrochloric acid alcohol and almost insoluble in 20 per cent acetic acid.

Higher concentrations of acid increase the speed of solution, and elevation of temperature facilitates solution of at least the iron component of hemosiderin.

Fixatives that contain acids but no formalin often completely remove hemosiderin or alter it so that brown pigment containing no demonstrable iron is produced. The presence of formalin in the fixative often protects hemosiderin against acetic, formic and hydrochloric acids, but not sulphuric. Alcohol is inferior to formalin as a fixative for hemosiderin.

Formalin in the fixing fluid tends to make hemosiderin more resistant to subsequent extraction with acids.

The alteration of the solubility of the iron in hemosiderin by certain fixatives as compared with others and with unfixed material indicates that iron exists in a protein combination. This concurs with the views of Hueck,[5] Schmidt,[18] Neumann,[19] and Arnold.[20]

That brown granular pigment containing no demonstrable iron may persist in areas of old hemorrhage after the disappearance of hemosiderin has often been noted. The present findings show that a similar pigment may be produced from hemosiderin by

the presence of acids in the fixing fluids, or by the action of warm acids on previously fixed material. This indicates that hemofuscin may be derived from hemosiderin by the loss of its iron content.

It seems clearly indicated that the iron of hemosiderin is in the ferric state. Even powerful reducing agents fail to reduce it to the ferrous form, and even after conversion into iron sulphide, part of the iron appears to remain in, or very promptly to revert to, the ferric state.

Considerable variation in the speed of solution of hemosiderin in acids is noted from case to case, and in experimental hemosiderosis with the mode of its production. The age of the pigment appears to have little influence on its speed of solution. Granule size has a material influence on speed of solution, coarser granules resisting longer.

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