THE RELATIVE REACTION WITHIN LIVING MAMMALIAN TISSUES.

V. (a) Influence of Lymph-Soluble Tissue Materials on the Significance of the Coloration with Some Phthalein Indicators.

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Living tissues of many sorts can readily be stained with indicators of the phthalein series;[1,2] and one can follow local alterations in tissue reaction by means of the color changes. Not infrequently the alterations become pronounced, as in the condition to which we have given the name "outlying acidosis."[3] Even in the healthy animal there would appear to be marked differences in the reaction of some of the organs, judging from the hues they manifest when stained.[2] But do the observed hues mean what they would if encountered under controlled conditions? And are the color alterations which occur in the tissues under abnormal circumstances to be taken at face value, or is this value wholly relative? The present paper and a succeeding one are concerned with these questions.

The phthaleins are less subject to error than are most indicator substances; yet there can be no doubt that in the case of some of them errors of considerable magnitude occur on occasion. True, Clark and Lubs[4] report that salt errors are slight, that phenol red and brom cresol purple in Dunham's solution, beef infusion, and peptone broths behave nearly as they do in buffer solutions, and that brom cresol purple can safely be utilized in the titration of media containing milk; but Clark has been careful to emphasize the fact that indicator may be removed from the field of action by proteins in solution,[5] with result in erroneous colorimetric

[1] Rous, P., *Science*, 1924, lx, 363; *J. Exp. Med.*, 1925, xli, 451.

[2] Rous, P., *J. Exp. Med.*, 1925, xli, 739.

[3] Rous, P., and Drury, D. R., *J. Am. Med. Assn.*, 1925, lxxxv, 33.

[4] Clark, W. M., and Lubs, H. A., *J. Bact.*, 1917, ii, 1.

[5] Clark, W. M., The determination of hydrogen ions, Baltimore, 1920.

readings. Kolthoff[6] states that the phthaleins give dependable readings in the presence of the decomposition products of proteins but cannot be used when a solution contains proteins as such. The data responsible for this dictum are not presented. Cullen[7] has successfully employed phenol red for the determination of the pH of blood; but he found dilution necessary to rule out the influence of the serum proteins upon the virage, and his method is not to be relied upon in all pathological conditions.[8] Jaumain[9] has pointed out that brom thymol blue is subject to gross error as an indicator when in contact with serum, even if this latter is in high dilution; though he further noted that with fluids containing some other proteins (gelatin, white of egg) the colorimetric error is relatively inconsiderable.

The literature on factors affecting the dependability of the phthaleins seems astonishingly scant, when one considers the popularity of these indicators. But the observations cited suffice to give grounds for caution in the interpretation of the hues that they assume when distributed through the living body. Some theoretical objections to the employment of indicators in the determination of the reaction within living organs will be considered in a succeeding paper.

Our studies have been restricted to three phthaleins possessing ranges that experiment has shown to be well adapted for a color differentiation of tissues on the basis, presumably, of differences in reaction. These three are phenol red, brom cresol purple, and chlor phenol red. Brom thymol blue possesses the proper range for *in vivo* work but is so highly toxic that it cannot be used;[2] and consequently, its errors, as demonstrated by Jaumain, may be dismissed from account in the present connection.

Influence of Phosphate Buffers on the Tissue Coloration.

We made, to begin with, a few observations on the hues of the indicators in solutions of egg albumen or gelatin as compared with potentiometric readings on the same material. But it was evident that one could not generalize from data thus obtained. Only by utilizing ingredients of the tissues themselves for the work could one hope to control in some part the findings in the living creature. Consequently tissues and tissue derivatives were employed in the later experiments.

[6] Kolthoff, I. M., Der Gebrauch von Farbenindicatoren, Berlin, 1923.

[7] Cullen, G. E., *J. Biol. Chem.*, 1922, lii, 501.

[8] Bennett, M. A., *Proc. Soc. Exp. Biol. and Med.*, 1924–25, xxiii, 114, 115.

[9] Jaumain, D., *Compt. rend. Soc. biol.*, 1925, xciii, 860.

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Mice were injected with phenol red or brom cresol purple; they were exsanguinated under ether when staining had become pronounced; and slices of several of the organs were placed in phosphate buffer solutions (Sörensen) of known pH. The color assumed by the slices after they had been immersed for some hours in the buffer fluid was compared with that of the fluid itself into which the stain had diffused,—far more slowly and imperfectly, by the way, than into water or salt solution. Needless to say the tissue slices had to be thin, else interior conditions, not the surrounding medium, largely determined their color; and the buffer mixtures had to be sufficiently concentrated for their reaction to prevail everywhere. In the experiments as at length elaborated N/5 solutions of the phosphates were used and in them were placed 25μ to 100μ sections of liver, as prepared with the freezing microtome, small pieces of shredded tendon, thin sheets of connective tissue, as such, and of cartilage from the ear. In some cases the liver had been washed out with salt solution. The other tissues were always practically bloodless. To render the staining more pronounced a small amount of the phthalein material with which the animals had been injected was often added directly to the buffer solutions.

In brief it was found that cartilage, connective tissue, and tendon, stained with phenol red during life and placed in strong buffer solutions, manifested the same hues to all intents and purposes as did the latter when colored with the dye. They behaved in this respect like so much absorbent paper stained with indicator. Such trifling deviations from the tint of the medium as came to attention were obviously due to the color proper to the tissue itself. No tests were made with chlor phenol red or brom cresol purple, for the reason that when stained with these dyes under the conditions of life, the tissues just mentioned assume hues indicative of a reaction beyond the available phthalein range, on the alkaline side.

The color of sections of liver stained with brom cresol purple or chlor phenol red corresponded less well with those of the surrounding buffer solutions. Preparations submitted to buffers of reactions that ensured the presence of a considerable proportion of the indicator in acid form showed deeply stained sections, the hue being that associated with a frank acidity, whereas in more alkaline solutions, with the indicator all, or nearly all, in the alkaline form, it dissolved out with result that there was either no staining or an anomalous one which might conceivably have been the combined result of a slight accumulation upon the liver slices of such portion of the dye as was present in the acid form and a suffusion of them with buffer contain-

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ing the dissolved, alkaline form of the dye. The results suggested that the intense staining of some of the tissues during life might possibly be referable to the accumulation upon them of the undissociated portion of an injected phthalein out of body fluids in which the dissociated and undissociated forms of the indicator were circulating together. Kendall[10] had already noted that slight acidosis increases the absorption of phenol red by the tissues, as also that they hold it more firmly. To widen the observations some further tests were performed.

Mice were totally exsanguinated under ether by the introduction of warmed Ringer's solution into the beating heart after the inferior vena cava had been severed. The kidneys and portions of the liver and skeletal muscle were weighed and ground separately in a mortar with washed sand. The material deriving from each organ was separated into two portions which were mixed, the one with a large excess of N/5 phosphate buffer of pH 5.29, the other with a similar buffer solution at pH 7.17. To these buffers a phthalein solution had been added in identical proportion. The tissue was distributed in the fluid by shaking and the mixture poured out into a Petri dish so large that the tissue and sand formed but a thin sheet over the bottom. After 5 hours at room temperature the material was again collected into a tube, and centrifuged at high speed until the sediment was firmly packed and nothing more would come down. The supernatant fluid was pipetted off as completely as possible; 1 cc. of it was diluted with many times its bulk of water alkalinized by the addition of 1 cc. of 40 per cent NaOH for every 99 cc.; and the entire sediment was similarly treated, on the basis that all of the ground tissue originally employed was present therein and that 1 gm. of this tissue had a bulk of 1 cc. After 24 hours or more in the ice box, to allow for complete extraction of the phthalein, readings of the amount of it in sediment and supernatant fluid respectively were made with the Duboscq colorimeter against a standard containing the appropriate phthalein in known amount. The alkaline solution was too weak to destroy the phthalein; and none had been adsorbed upon the sand. It was regularly noted that the fragments of ground tissue submitted to the buffer at pH 7.17 became somewhat swollen, gelatinous, and sticky, whereas those in the acid buffer remained discrete.

Besides the organs above mentioned, portions of adipose tissue were employed in the work but they lent themselves poorly to the purpose in hand, most of the fat rising to the surface in the centrifuge instead of packing, while furthermore the supernatant fluid was so turbid as to give but a poor match in the colorimeter. It will suffice to say of the results that they accorded with those yielded by the other tests in showing that much more phthalein became fixed on the tissue in the

[10] Kendall, E. C., *J. Am. Med. Assn.*, 1917, lxviii, 343.

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acid menstruum. Even under these favorable circumstances, though, relatively little indicator accumulated upon the fat itself.

In the tests made with brom cresol purple so much phthalein had been provided as compared with tissue that the amount taken out of solution by this latter was inconsiderable as compared with that left

TABLE I.

The Distribution of Phthaleins in Mixtures of Tissue with N/5 Phosphate Buffer Solutions.

Tissue.	Buffer reaction.	Amount of indicator in the undissolved tissue materials.							Remarks.
		Brom cresol purple.			Cresol red.		Thymol blue.		
		Cc. of buffer solution per gm. of tissue.	Concentration.	Proportion of total for mixture.	Concentration.	Proportion of total for mixture.	Concentration.	Proportion of total for mixture.	
	pH			*per cent*		*per cent*		*per cent*	
Liver.	7.17	13.6	1	—	1	30.6	1	33	1 cc. of 1 per cent cresol red and thymol blue at approximately pH 7.4 was added to 44 cc. and 50 cc. respectively of buffer solution. 1 cc. of 2 per cent brom cresol purple was added to 30 cc. of the buffer solution that was to be mixed with liver tissue, and 1 cc. to 50 cc. of that employed with the other tissues. 20 cc. of the solution colored with cresol red or thymol blue was used for every gm. of tissue.
	5.29	13.6	4.04	—	2.6	66	2.35	76	
Kidney.	7.17	37.3	1	—	1	46	1	41	
	5.29	37.3	5.16	—	2.24	81	2.24	72	
Muscle.	7.17	18.75	1	—	1	29	1	49	
	5.29	18.75	2.95	—	2.09	65	1.76	72	

behind; but in the later experiments with cresol red and thymol blue the quantity added to the buffer solutions was better judged, and it was possible to check the influence of weak acidity and alkalinity respectively, not alone by the proportion of dye actually found in the sediment, but by the amount of it remaining in the fluid. The discrepancies between the findings with sediment and fluid respectively

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(Table I) can be sufficiently explained by difficulties in the colorimetric readings, though it is conceivable that there was some destruction of phthalein by the tissues similar to that Kendall has noted in the case of phenol red.[10] Experiments to be described further on speak against the importance of any such occurrence in the present instance, as do further the conditions of the tests themselves. The destruction, of phenol red at least, is largely conditioned upon an oxygen lack, and our preparations were well oxygenated.

On consulting the table (Table I) it will be seen that, in consonance with our previous observations upon sections of liver, far more brom cresol purple accumulated upon the tissues out of the acid buffer solution than out of the alkaline one, several times as much indeed. Clark's chart[5] shows that a watery solution of brom cresol purple at pH 5.29 contains more than 75 per cent of the indicator in the undissociated, or acid, form; whereas at pH 7.17 less than 20 per cent is in this condition. To determine whether these proportions had been responsible for the outcome of our tests cresol red was utilized, a phthalein which is all in the acid form at pH 5.29 and nearly all (about 90 per cent) at pH 7.17. The proportion of cresol red taken out of the buffer solution at pH 5.29 by the tissues averaged more than twice that at pH 7.17, as shown by the titration of both fluid and sediment. This finding suggested that the results with brom cresol purple were referable, not to the proportion of phthalein present in acid form in the buffer-tissue mixtures but to some effect of the phosphate solutions upon the tissues themselves, a possibility further supported by the differing state of the tissues in the two buffers, as above described. That such was the actual case was shown by tests with thymol blue, an indicator which is practically all in the alkaline form both at pH 5.29 and pH 7.17. The results with it were essentially the same as of those with cresol red, and, due allowance being made for the differing quantities of indicator provided, as of those with brom cresol purple. Approximately twice as much indicator accumulated on the tissues at pH 5.29 as at pH 7.17.

Influence of Lymph-Soluble Tissue Materials on the Phthalein Colors.

This demonstration of the extraneous character of the findings in stained tissues exposed to artificial buffer solutions led us to abandon

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their use and to employ fluids that might yield a closer approach to conditions as they exist within the body. Two sets of experiments were devised, one to control the influence upon the phthalein colors of substances brought into solution out of the tissues, the other,—dealt with in a succeeding paper,—to demonstrate the effect of the remaining, undissolved, tissue materials upon the color of indicators adsorbed or in other ways accumulated upon them.

In tests of the sort first mentioned a concentrated extract of fresh tissue was made, phthalein added, and readings carried out with the colorimeter and the potentiometer upon the same specimens. In some tentative observations water or salt solution served for the extraction; but lymph of the animal furnishing the tissue was ultimately adopted as a more natural menstruum. In order to bring the reaction of the extracts within the ranges of brom cresol purple and chlor phenol red it was necessary to acidify them slightly. This was done by adding dilute lactic acid. A summary of the colorimetric and potentiometric comparisons obtained by the method is given in Table II.

The protocols which follow, and which find further record in the table, are those of all of our experiments in which sound working conditions were attained. These involved among other things a practically complete absence of hemoglobin from the extracts, the development in the latter of acidity without precipitation when lactic acid was added, a strong and constant light source for colorimetric purposes, and electrodes which did not show a deterioration on contact with the extracts, such as Cullen has noted in his studies with serum.[7]

To prepare the extracts the kidneys and one or more lobes of the liver were perfused with water, or 0.9 per cent salt solution, or lymph,—according to whichever was to be used for the extraction,—followed in some instances, by an injection of air to empty the vessels. The perfusions were made into the renal arteries and branches of the portal vein of the organs as removed from the etherized animal. The most satisfactory washings were those carried out with lymph. When it was used all the blood was readily flushed out and the tissue appeared unaltered otherwise, whereas when water or salt solution had been employed there was frequently some hemoglobin left behind and the tissue itself sometimes appeared swollen. In an instance in which salt solution had been employed for the washing but lymph for the extraction, a prompt precipitation occurred in extracts to which lactic acid was added. This did not take place when lymph had been used at every step in the preparation.

In order to get sufficient lymph for our purposes it was necessary to use large dogs. To ensure that it should be clear the animals were not fed for 24 hours before the operation; but they were given 500 to 750 cc. of water by gavage a few

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TABLE II.

Comparative Colorimetric and Electrometric Determinations of the Reaction of Tissue Extracts.

	Tube.	Colored buffers.		Extract.			Colorimetric error.	Color readings.	Total nitrogen.	Protein (calculated).	Remarks.
		Electrometric.		Colorimetric.	Electrometric.						
		mv.	*pH*	*pH*	*mv.*	*pH*	*pH*		*per cent*	*per cent*	
Liver + Water + Phenol Red.											
Experiment V. Guinea pig livers. Tubes *a* and *b* and their controls were derived from different animals.	*a*	633 645	6.37 6.56	Between 6.37 and 6.56.	642	6.52	Approximately −0.06.	Fairly easy.			Saturated calomel electrode; N/10 HCl = 307 millivolts.
	b	645	6.56	6.56	634	6.39	+0.17				
Experiment VI. Dog liver.	*a*	633	6.37	6.37	632	6.36	+0.01	Easy.			Saturated calomel electrode; N/10 HCl = 307 millivolts.
	b	633	6.37	Just below 6.37.	631	6.34	None?	"			
	c	633	6.37	Just above 6.37.	631	6.34	Slightly more than +0.03.	"			
Liver + Salt + Phenol Red.											
Experiment VI. Dog liver.		645	6.56	6.56	629	6.31	+0.25	Easy.			Saturated calomel electrode; N/10 HCl = 307 millivolts.

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Liver + Lymph + Phenol Red.											
Experiment IX. Dog liver.	a	657 666	6.70 6.84	Between 6.70 and 6 84.	658	6.71	Approximately +0.6.	Fairly easy.	1.62	10.1	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
Double strength extract.	b	666	6.84	6.84	659	6.73	+0.11	Fairly easy.	1.70	10.6	
		646	6.52	6.52	653	6.63	−0.11	Difficult.			
Experiment X. Dog liver. Drifting necessitated frequent changing of electrodes.	a	634.5 646	6.33 6.53	Between 6.33 and 6.53.	652.5	6.62	Approximately −0.19.	Very difficult.	1.605	10.05	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	622	6.13	6.13* or less!	652.5	6.62	−0.49 or more.	Very difficult.			
Double strength extract. Twice as much P.R. in extracts and buffers as usual.	a	647	6.54	6.54	640	6.42	+0.12	Very difficult.	1.68	10.5	
	b	647 657	6.54 6.70	Between 6.54 and 6.70.	638	6.39	Approximately +0.22.	Very difficult.			
Experiment XI. Dog liver. Double strength extract.	a	655 664	6.66 6.81	Between 6.66 and 6.81.	645	6.50	Approximately +0.24.	Fairly easy.	1.29	8.05	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	646 655	6.51 6.66	Between 6.51 and 6.66.	644.5	6.49	Approximately +0.09.				

The "half strength" extracts were made with twice as many cc. of lymph as there were gm. of tissue, the "double strength" with half as many cc. as gm. Ordinarily a cc. to gm. ratio was employed.

* The specimen contained so much phenol red that it was possible to make colorimetric readings which involved this degree of acidity.

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TABLE II—*Continued.*

	Tube.	Colored buffers.		Extract.			Colorimetric error.	Color readings.	Total nitrogen.	Protein (calculated).	Remarks.
		Electrometric.		Colorimetric.	Electrometric.						
Liver + Lymph + Brom Cresol Purple.											
		mv.	*pH*	*pH*	*mv.*	*pH*	*pH*		*per cent*	*per cent*	
Experiment XII. Dog liver. 5 *gtt.* lactic acid.	*a*	583 602.5	5.48 5.81	Between **5.48** and **5.81.**	589	**5.59**	Approximately +0.06.	Easy.	1.46	9.15	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	583	5.48	Slightly less than **5.48.**	577	**5.39**	Less than +0.09.	Easy.			
Experiment XV. Dog liver. 5 *gtt.* lactic acid. Drifting necessitated frequent changing of electrodes.	*a*	595	5.69	Just above **5.69.**	611.5	**5.96**	Less than −0.27.	Easy.	1.11	6.96	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	603.5	5.82	**5.82**	605	**5.86**	−0.04	Easy.			
Double strength extract; 5 *gtt.* lactic acid.	*a*	623	6.14	**6.14**	—	—	—		1.35	8.45	
	b	623	6.14	**6.14**	619.5	**6.09**	+0.05	Easy.			
	a	623	6.14	Just above **6.14.**	620	**6.10**	Slightly more than +0.04.	Easy.			
Liver + Lymph + Chlor Phenol Red.											
Experiment XII. Dog liver. Double strength extract; 2 *gtt.* lactic acid.	*a*	619	6.08	**6.08**	621	**6.11**	−0.03	Rather difficult.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.

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Experiment XII—*Continued.* The same; but 3 *gtt.* lactic acid.	*a*	582.5 602	5.49 5.81	Between **5.49** and **5.81**	613	**5.98**	Approximately −0.33.	Rather difficult.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	582.5 602	5.49 5.81	Between **5.49** and **5.81.**	609	**5.91**	Approximately −0.26.	Rather difficult.			
Experiment XV. Dog liver. 5 *gtt.* lactic acid.		622	6.12	Just below **6.12.**	604	**5.84**	Less than +0.28.	Rather difficult.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.
Kidney + Water + Phenol Red.											
Experiment V. Guinea pig kidney. Half strength extract.		633	6.37	Just above **6.37.**	634	**6.39**	−0.02	Fairly easy.			Saturated calomel electrode; N/10 HCl = 307 millivolts.
Experiment VI. Dog kidney. Half strength extract.		645 655	6.56 6.73	Between **6.56** and **6.73.**	650	**6.61**	Approximately +0.04.	Easy.			Saturated calomel electrode; N/10 HCl = 307 millivolts.
Kidney + Salt + Phenol Red.											
Experiment VI. Dog kidney. Half strength extract.		645	6.56	**6.56**	634	**6.39**	+0.17	Easy.			Saturated calomel electrode; N/10 HCl = 307 millivolts.
Kidney + Lymph + Phenol Red.											
Experiment IX. Dog kidney.		677	7.02	Just below **7.02.**	673	**6.95**	None?	Fairly easy.	1.055	6.60	3.5 N calomel electrode; N/10 HCl = 311 millivolts.

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TABLE II—*Concluded.*

	Tube.	Colored buffers.		Extract.			Colorimetric error.	Color readings.	Total nitrogen.	Protein (calculated).	Remarks.
		Electrometric.		Colorimetric.	Electrometric.						
Kidney + Lymph + Phenol Red—*Continued.*											
		mv.	*pH*	*pH*	*mv.*	*pH*	*pH*		*per cent*	*per cent*	
Experiment XI. Dog kidney. Extra strength extract. Two-thirds as many cc. of lymph as there were gm. of tissue.	*a*	655 664	6.66 6.81	Between 6.66 and 6.81.	653	6.61	Approximately +0.13.	Fairly easy.	1.28	8.00	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	655 664	6.66 6.81	Between 6.66 and 6.81.	653	6.61	Approximately +0.13.	Fairly easy.			
Kidney + Lymph + Brom Cresol Purple.											
Experiment XII. Dog kidney. Double strength extract; 5 *gtt.* lactic acid.	*a*	583 602.5	5.49 5.81	Between 5.49 and 5.81.	584	5.51	Approximately +0.14.	Easy.	1.12	7.0	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	583 602.5	5.49 5.81	Between 5.49 and 5.81.	585	5.52	Approximately +0.13.	Easy.			

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Experiment XV. Dog kidney. 4 *gtt.* lactic acid.	*a*	595 603.5	5.69 5.82	Between 5.69 and 5.82.	611	**5.95**	Approximately −0.20.	Easy.	0.74	4.6	3.5 N calomel electrode; N/10 HCl = 311 millivolts.
	b	587	5.56	Slightly above 5.56.	607	**5.89**	Less than −0.33.	Easy.			
Double strength extract; 5 *gtt.* lactic acid.	*a*	595 603.5	5.69 5.82	Between 5.69 and 5.82.	—	—					
	b	587 595	5.56 5.69	Between 5.56 and 5.69.	597	**5.72**	Approximately −0.10.	Easy.	0.90	5.6	
	a	603.5	5.82	5.82	593.5	**5.66**	+0.16	Easy.			
Kidney + Lymph + Chlor Phenol Red.											
Experiment XV. Dog kidney. 4 *gtt.* lactic acid.		622	6.12	Just below 6.12.	604	**5.84**	Less than +0.28.	Rather difficult.			3.5 N calomel electrode; N/10 HCl = 311 millivolts.

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minutes prior to it. Under ether the thoracic duct was cannulated and lymph collected during several hours until, in some cases, 200 cc. or more had been got. This was rid of the thin clot by stirrings with a rod, and centrifuged when it contained red cells. The renal arteries were now tied, then the renal veins, and both kidneys were rapidly removed and perfused through the arteries under slight pressure. A 20 cc. syringe was used, both to inject fluid and to force air in after it. Leakage backward from the needle was prevented by clamping it and the enveloping vessel wall between the rubber-covered jaws of a hemostat forceps. During the washing the kidneys were gently kneaded. Next the animal was bled to death by cutting the inferior vena cava above the diaphragm; and the lateral mass of the liver, comprising the right lateral and caudate lobes, was perfused with lymph through the branch from the portal vein.

To prepare the extracts the pallid tissues were cut into blocks which were squeezed between folds of washed and dried gauze to remove free fluid and then forced through a barrel-and-plunger sieve provided with round openings less than 1 mm. in diameter. The cortex only of the kidney could be used, the medulla proving too tough for easy pulping. The pulp was mixed with lymph, or salt solution, or water, as the case happened to be, in the proportion, ordinarily, of as many cc., or half as many, as there were gm. of tissue. The mixtures were shaken violently in a machine for 20 minutes and centrifuged at high speed for from $\frac{3}{4}$ of an hour to an hour, that is to say until everything had come out of suspension that would. The supernatant fluids,—the extracts,—were pipetted off and distributed in portions of $2\frac{1}{2}$ or 3 cc. in colorimeter tubes. To some of the portions 0.2 cc. or more of a dilute solution of phthalein indicator was added, while others were kept for control purposes. Ordinarily the phthalein had been made up in 1 per cent or 2 per cent solution isotonic with 0.9 per cent NaCl and at pH 7.4; and it was further diluted with water to 0.1 per cent or 0.2 per cent. A number of duplicate mixtures of tissue extract and phthalein were usually made. It was deemed best in most cases to add the indicator, as also any lactic acid required, to the small portions of extract as such rather than to the whole quantity prior to distribution, since in this way opportunity was given for some slight individual variation in the pH of the specimens. N/15 phosphate buffers (Sörensen) were employed in the colorimetric readings, and phthalein was added to them in the same proportion as to the extracts. The readings were made in the usual way by contrasting the color of a tube of the tinted extract plus one of water, in an ordinary colorimeter block, with that of a tube of untinted extract plus one of tinted buffer. Tubes of 12 mm. diameter, carefully cleansed and dried beforehand, were employed. The readings were at 38°C., like those in the potentiometer. Always the control tubes of buffer and of extract were warmed at the same time as the tinted extract; and when lactic acid had been added to the latter the same amount was added to the control specimen as well. The acid caused some gradual change toward opacity in the already turbid extract and for this reason those tinted duplicate specimens containing it which had been separately warmed for reading, were usually not read against the same control specimen, warmed for both, but against different ones treated like themselves.

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The phthalein hues could be much more readily distinguished in the kidney extracts, which were a creamy buff, than in the brownish buff liver extracts. All were far too opaque for color comparisons by daylight. Instead an arc light was used having a lens of 9.5 cm. aperture that rendered the rays parallel.

In the earlier work a single observer carried out both sets of readings, the colorimetric series first, and then, as rapidly as might be, the electrometric ones, on the same series of specimens. Since the potentiometer readings were strictly quantitative in character it was felt that the personal equation as involved by a knowledge of the colorimetric pH could have no part in them. Only a short time elapsed between the two series of readings as compared with the period during which the extracts had been in preparation; and such extracts were found to alter little in reaction on standing for the necessary period at room temperature. Nevertheless, as a check on the influence of this factor, the order in which the extracts were tested electrometrically was varied from that of the colorimeter observations, some specimens being tested at once after the color reading and other duplicate ones not until a considerable time had elapsed. The readings on the liver specimens of Experiment VI, Table II, furnish an instance in point. During the later work (Experiments IX to XV inclusive), a potentiometric reading on each specimen was made immediately after the colorimetric and by a different observer unapprised of the color findings. Since the duplicate specimens were regularly tested in succession by both methods and they were found to differ but little in pH, it can safely be assumed that the time element as involved in a rapid transfer from the colorimeter to the potentiometer had no hand in the results. As a further check on the influence of the time factor, however, two colorimetric readings on the same specimen were sometimes made, with an interval between, and an immediate potentiometer test after the second one. The reactions of the tinted buffer solutions used in the comparisons were determined electrometrically.

Cullen[7] has described the deterioration of electrodes which takes place when they are brought in contact with serum. The difficulty sometimes made itself manifest with our tissue extracts, yet much less often than had been apprehended. A number of electrodes were kept ready and, though occasionally several in succession showed "drifting," one was usually found with which the few observations of the individual experiment could be run off. All readings were at 38°C. All were carried out on the afternoon that the extracts were prepared, except, in some instances, those on the tinted buffers. In these instances the buffer tubes were kept overnight in the ice box prior to the potentiometric determinations.

It is conceivable that there was some small escape of CO_2 from the portions of extract after they had been read colorimetrically, when they were shaken in the hydrogen atmosphere of the potentiometer chamber. But this can scarcely have been an important source of error, since the CO_2 content of the extracts must have been slight, owing to their acid reaction and the frequent aeration involved in the preparation and handling of them.

I. Tests with Phenol Red.—Liver and kidney tissues of the living animal are colored yellow by phenol red, the reaction lying to all appearance outside of the

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range of the indicator on the acid side, even when the latter is present in high concentration, under which circumstances its range may extend as far down as pH 6.10. But as Michaelis and Kramsztyk[11] and others have noted, liver and kidney extracts in water or salt solution have a relatively high pH, as determined electrometrically, usually one between pH 6.40 and pH 6.80. In agreement with this finding our extracts all had a reaction well within the range of phenol red in the concentrations used, and beyond the ranges of brom cresol purple and chlor phenol red on the alkaline side. The extracts made with the lymph, which itself had a reaction close to pH 8.0, were not more alkaline than those with water or salt solution (Table II), a fact which shows that dissolved tissue derivatives were present in sufficient abundance to determine the reaction.

The pronounced buff of the liver extracts rendered color determinations with phenol red relatively difficult, and, in the case of the double strength extracts, far from precise (Table II).

Experiment V.—The liver and kidneys of one guinea pig and the liver of another were washed and extracted with twice-distilled water. The extracts from the two animals were kept separate. Those of the hepatic tissue were made on the basis of 1 cc. of fluid for every gm. of tissue, while twice as much fluid was added in the case of the kidneys. To each 3 cc. portion, 0.025 cc. of 0.2 per cent phenol red was added excepting in the case of Tube *b*, liver, which received, like the appropriate buffers, 0.05 cc.

Experiment VI.—One kidney of a 7 kilo dog was washed out and extracted with salt solution, the other with distilled water; and so also with two lobes of the liver. Extraction was done with the appropriate fluids, using the same proportions as in Experiment V. To the 2.5 cc. specimens 0.02 cc. of 0.2 per cent indicator was added.

In these experiments the extracts were less concentrated and turbid than in the later ones. Colorimetric readings proved relatively difficult in the latter.

Experiment IX.—From the thoracic duct of a dog of 18½ kilos, 130 cc. of lymph was collected. One of the kidneys and the lateral mass of the liver were washed with it, and extracted, in the proportion of 1 cc. lymph to 1 gm. in the case of the renal tissue and most of the hepatic. To part of the latter only half as many cc. lymph were added as there were gm. of tissue. This extract is designated in the table as double strength. Unfortunately not enough phenol red was used to permit of precise readings. The phthalein was mixed with all of the extracts in bulk, 0.1 cc. of 0.1 per cent solution for every 2.5 cc.

Experiment X.—Only 110 cc. of lymph was obtained from this 14½ kilo dog, and there was some blood left in the one kidney that was washed, despite the injection of air after the perfusion. Extraction was made from the renal tissue on the basis of two-thirds as many cc. of lymph as there were gm. of tissue. Two liver extracts were made, one in the cc.-gm. ratio, the other with ½ cc. to a gm.; and to 2.5 cc. portions of these, respectively, 0.2 cc. and 0.1 cc. of a 0.1 per cent

[11] Michaelis, L., and Kramsztyk, A., *Biochem. Z.*, 1914, lxii, 180.

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solution of phenol red were added. The kidney extract received 0.1 cc. The color readings proved exceptionally difficult, and "drifting" necessitated several changes of the electrodes.

Experiment XI.—Since only 60 cc. lymph was obtained from the dog of 16½ kilos it was found necessary to wash out the kidneys and liver with 0.9 per cent salt solution. This was expelled from the vessels as completely as possible with air. The liver material was extracted with half as many cc. of lymph as there were gm. of tissue; and two-thirds as many cc. were employed as gm. of renal tissue. To every 2.5 cc. portion of extract 0.2 cc. of 0.1 per cent phenol red was added.

II. Tests with Brom Cresol Purple and Chlor Phenol Red.—Portions of the same extracts were tinted with these two indicators, so the preparations for both sets of tests can be described together. The virages of the liver parenchyma and of the kidney cortex stained during life are ordinarily well down toward the acid side of the ranges of both brom cresol purple and chlor phenol red.[2] But, as might have been predicted from the readings with phenol red, the extracts had reactions so far toward the alkaline side that the addition of acid was necessary to bring them within the range of the phthaleins. N/4 lactic acid was employed, drop by drop with shaking after each, until the changed hue indicated that readings had become possible. The tubes containing acidified extract tinted with brom cresol purple showed dichromatism, appearing green by ordinary light; but in the colorimeter block there was no suggestion of this color and comparisons with the buffer standards were readily to be made. They proved more difficult with chlor phenol red owing to the fact that the orange hues exhibited by this indicator on the acid side of its range predominated less plainly over the buff natural to the extracts.

Experiment XII.—Lymph to the amount of 120 cc. was collected from a dog of 16 kilos. A kidney and the lobe mass of the liver were successfully washed with some of it, and the perfusion fluid forced out of the vessels with air. Two liver extracts were made, on the gm.-cc. and gm.-½ cc. basis respectively. The kidney extract had the latter basis. To 2.5 cc. portions of each, 0.2 cc. of a 0.1 per cent solution of either chlor phenol red or brom cresol purple was added, followed by lactic acid.

Experiment XV.—From a dog of 10 kilos, 230 cc. of lymph was obtained. Both kidneys and the lobe mass of the liver were perfused with the fluid and further emptied by injections of air. Two extracts were made of each tissue, one with as many cc. of lymph as there were gm. of tissue, the other with half as many. To 2.5 cc. portions of all 0.15 cc. of a 0.1 per cent solution of either brom cresol purple or chlor phenol red was added, followed by enough N/4 lactic acid to bring the color well within the indicator range. A change of electrodes during the potentiometer readings was necessitated by "drifting," and some specimens were lost in consequence.

When the table is consulted (Table II) it will be seen that the colorimetric and potentiometric determinations accorded less closely in

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some experiments than in others, owing, as was evident at the time, to difficulties in reading the color of very turbid solutions naturally buff in hue and containing phthaleins which were themselves orange or orange-red at the reaction studied. All in all though, the readings corresponded far more closely than might *a priori* have been expected. Phenol red gave pH findings that were for the most part slightly higher than those obtained with the potentiometer, though it is questionable whether this was not due to chance. The differences manifested by individual specimens stained with brom cresol purple and chlor phenol red lay now in this direction, now in that, the deviations approximately balancing one another.

The extracts coagulated when they were boiled and they contained a considerable amount of the tissue nitrogen, as the Kjeldahl figures attest (Table II). According to Grund[12] there is about 2.9 per cent of nitrogen in the liver of the dog and 2.1 per cent in the kidney, nearly nine-tenths of these amounts being present as protein. Some of our extracts contained more than 1.6 per cent of nitrogen. Of this as much as 0.7 per cent may have been derived from the lymph.[13]

SUMMARY.

The present paper is the first of two dealing with experiments which were planned to disclose the meaning of the colors assumed by mammalian tissues when vitally stained with some phthalein indicators. Derivatives of the tissues themselves were employed after tests had shown, in agreement with certain facts in the literature, that inferences from model experiments, so called, would be misleading.

Organs were perfused with water, or salt, or lymph of the same creature and extracted with small quantities of the fluid. The turbid extracts were colored with phthalein, the reaction determined colorimetrically with the aid of an arc light, and the findings were compared with potentiometer readings upon the material. The results of the two methods were in close agreement.

The significance of the data will be discussed in our second paper.

[12] Grund, G., *Z. Biol.*, 1910, liv, 173.

[13] Petersen, W. F., and Hughes, T. P., *J. Biol. Chem.*, 1925, lxvi, 229.

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