A METHOD OF STUDYING THE MODIFICATION BY DRUGS OF THE OXIDATION-REDUCTION SYSTEMS OF THE SKIN

By Geoffrey E. Loxton and David Le Vay

From the Woolwich Group of Hospitals, London

Received December 14, 1953

In 1938 Rötter[1] described a simple test for assessing the degree of ascorbic acid saturation in man. The test consisted of injecting intradermally the oxidation-reduction indicator dye, 2:6-dichlorophenolindophenol, as a solution of 40 mg. in 100 ml. of distilled water, and noting the time taken for the blue colour to disappear. Sufficient dye was injected to make a wheal of approximately 2 mm. diameter. He concluded that a decolorisation time of less than 5 minutes indicated vitamin C saturation, and a time greater than 10 minutes indicated vitamin C deficiency. He also showed that the decolorisation was due to reduction of the dye on the spot and not to its being carried away from the site of injection by diffusion or vascular spread. (This is easily confirmed by the demonstration that the colour of the dye can be restored, after it has vanished, by the injection of potassium ferricyanide solution at the same site.) The usefulness of Rötter's test was confirmed by some workers, Portnoy and Wilkinson[2] and others; but Jetter[3], Poucher and Stubenrauch[4], and Goldsmith, Gow and Ogaard[5] reported it to be entirely unreliable. The subject was more recently reviewed by Slobody[6], who found the test, with certain technical modifications, generally reliable.

Two main criticisms have been levelled at the method. First, the decolorisation time bears no constant relationship to blood ascorbic acid levels; second, the variations in the decolorisation time in a consecutive series of tests made on the same individual are so great as to make the test unreliable. With regard to the first criticism it must be remembered that changes in ascorbic acid content of the blood and other tissues do not necessarily run closely parallel. Again, the dye is altered not only by ascorbic acid, but its decolorisation time will be influenced by other oxidation-reduction systems in the skin, such as those involving glutathione, which rapidly decolorises the dye *in vitro*. Nevertheless, it seemed that the test might be modified to become suitable for studying changes in these systems brought about by the systematic administration of substances such as ascorbic acid, glutathione, adrenocorticotrophic hormone and cortisone, agents whose therapeutic value we had been testing clinically in cases of rheumatoid arthritis. Rötter's technique was therefore considerably modified. To disturb the physiology of the skin as little as possible, normal saline solution replaced distilled water as the dye solvent, and trials were made to determine the most dilute solution which could be seen in the skin while still allowing a sharp end-point. The lowest practical dilution proved to be one of 1 mg./100 ml. which, injected subepithelially, gives a distinct blue colour to the wheal.

A trained observer, with good colour vision, in standard lighting conditions, can determine the time of disappearance of this colour within 2 or 3 seconds. All tests were made on the volar surface of the forearms, using both arms alternately, with a 60-watt daylight bulb six inches above the area of skin under observation. Rötter had emphasised the importance of making uniform wheals of 2 mm. diameter to achieve consistent results, but we found more satisfactory the intradermal wheal resulting from the injection of 0·05 ml. of dye, delivered from a tuberculin syringe, and decolorisation times were recorded only from satisfactory wheals of 4 to 5 mm. diameter. The time was measured with a stop-watch, the dial being turned away from the observer so that the reading was objective.

Coefficient of Variation

Goldsmith *et al.*[35], using Rötter's original method, found in 25 consecutive observations in one individual a range of 4·5 to 11·6 minutes for the decolorisation time, giving a mean of 8·9 minutes, a standard deviation 2·08 minutes and a coefficient of variation of 43 per cent. We determined the coefficient of variation for three different concentrations of dye in normal saline: 20 mg., 4 mg. and 1 mg./100 ml. The results are set out in Table I, which also shows the figures of Goldsmith *et al.*[5] for a solution of 40 mg./100 ml. of distilled water. The coefficient has fallen markedly

TABLE I

COEFFICIENT OF VARIATION

The figures are derived from 25 consecutive observations of decolorisation time in 4 different patients with 4 different concentrations of dye solution. The figures for the 40 mg. per 100 ml. solution are those of Goldsmith *et al.*[5]

Strength of dye solution mg./100 ml.	Mean decolorisation time seconds	Range seconds	Standard deviation seconds	Coefficient of variation per cent
40	534·0	270–696	124·7	43·0
20	320·0	250–340	67·2	21·0
4	59·5	44–74	7·15	12·0
1	34·0	25–40	3·46	10·2

from 43 per cent. to 10·2 per cent., and from this it appears that the variation is due to some factor inherent in the test itself rather than in a timing of the end-point, as, if the latter was the cause of the inaccuracy, the variation would increase with the shorter measurement.

Method of Measuring Change in Decolorisation Time

In view of the results of this experiment, two dilutions of dye were used for subsequent experiments: 4 mg. and 1 mg./100 ml. of normal saline. Because, with a coefficient of variation of the order of 10 per cent., no significance could be attached to the difference between two isolated observations, at least four observations were made, two on each forearm, and the mean of these used in deciding whether a change had occurred after the administration of any agent. The extension of "Student's" treatment of the error of a mean to the comparison of the means of two

samples (Fisher[7]) was then applied, and it was considered that a real change had occurred only when the value of P was less than 0·05. Table VI shows how this analysis is carried out for a patient receiving 300 mg. of cortisone, using a dye solution of 1 mg./100 ml.

With this technique of experimentation, several methods of investigation are possible. The decolorisation time can be measured before, and at varying intervals after, the systemic administration of such agents as ascorbic acid, glutathione, adrenocorticotrophic hormone and cortisone. Small amounts of agents can be added to the dye solution and the decolorisation time compared with that of the unmodified solution. Or these two methods can be combined by noting whether a systemic background of one agent modifies in any way the peripheral action on the decolorisation time of a second agent mixed with the dye solution. Here we report the results of some experiments using the first method of investigation.

Spontaneous Changes in the Decolorisation Time

4 patients, who had been without breakfast, were examined by 10 separate observations at both dilutions of dye on 2 consecutive days at midday, before lunch. In no instance did the means show any significant change with either dilution. In 12 patients who had observations made at varying intervals up to 3 hours, on 24 occasions when the change in mean decolorisation time might have been observed for each dye dilution, a significant change was observed only once with each dilution, and this was less than 20 per cent.—thus the changes recorded in subsequent experiments can safely be considered to be due to the agents administered and not to spontaneous variation in decolorisation times.

The Effect of Intravenous Administration of 1 g. of Ascorbic Acid

This, and the 3 subsequent experiments with glutathione, adrenocorticotrophic hormone and cortisone, were all conducted with patients suffering from active rheumatoid arthritis who had been fasting for at least twelve hours. In 5 patients the decolorisation times were observed before, and at intervals up to 3 hours after, the intravenous injection of 1 g. of ascorbic acid. The figures were analysed, as exemplified in Table VI, and the results summarised in Table II. The figures shown in heavy type are derived from differences of means for which the value of P is less than 0·05 and those in ordinary type are derived from differences of means with a value of P greater than 0·05 and which cannot be regarded as significant. All 5 showed a significant decrease in decolorisation time of one or both dilutions of dye, a maximum reduction being approached within the first 2 hours. This decrease was proportionately greater with the stronger solution.

The Effect of Intravenous Administration of Glutathione

It was originally intended to give each patient 1 g. of glutathione intravenously, but the second to be investigated (No. 7 in Table III) had a severe rigor beginning 15 minutes after injection, so the dose was

TABLE II

ASCORBIC ACID

Shows the change per cent. in the decolorisation time at varying intervals after the injection intravenously of 1 g. of ascorbic acid. The figures in heavy type are statistically significant.

Patient No.	Strength of dye mg./100 ml.	Change per cent. in decolorisation time			
		Intervals in minutes			
		0–30	30–60	60–90	90–120
1	4	—	**−25**	**−16**	—
	1	—	—	**−32**	—
2	4	—	—	**−25**	—
	1	—	—	− 6·5	—
3	4	**−26**	—	**−27**	**−32·5**
	1	−3·5	—	+15	− 4·5
4	4	**−26·5**	—	**−12·5**	—
	1	**−16**	—	+ 6·6	—
5	4	—	**−28·5**	—	**−30**
	1	—	**−28**	—	**−24**

reduced to 0·5 g. in the next 2 patients (Nos. 8 and 9), and the fifth patient (No. 10) was given 0·75 g. All 5 showed a significant decrease in decolorisation time with one or both dilutions of dye, and again this decrease was proportionately greater with the stronger solution. The results are summarised in Table III.

TABLE III

GLUTATHIONE

This table is constructed in a similar way to Table II, glutathione being given intravenously, patients 6 and 7 receiving 1 g. of glutathione, patients 8 and 9 receiving 0·5 g. and patient 10 0·75 g. The figures in heavy type are statistically significant.

Patient No.	Strength of dye mg./100 ml.	Change per cent. in decolorisation time				
		Intervals in minutes				
		20–60	60–80	80–100	100–120	190
6	4	—	**−28**	—	—	—
	1	—	**−27·5**	—	—	—
7	4	**−30**	**−48·5**	—	**−52**	—
	1	**−20**	**−36·5**	—	**−37**	—
8	4	+25	**−12**	**−15**	**−12·5**	—
	1	+25	**−5**	**−5**	**−5**	—
9	4	**−12·5**	—	**−24**	—	—
	1	**−6·4**	—	**−28**	—	—
10	4	**−14**	**−18**	—	**−30·5**	**−38**
	1	+19	+26	—	**−4·3**	**−36**

The Effect of a Single Intravenous Injection of Adrenocorticotrophic Hormone

12 patients with active rheumatoid arthritis were each given a single dose of 25 mg. of adrenocorticotrophic hormone intravenously. Two different batches of the drug were used and, since the clinical effects were not identical, the results of this experiment are tabulated separately for the two batches; Table IV summarises the results obtained in 6 patients

TABLE IV

ADRENOCORTICOTROPHIC HORMONE. BATCH I

The effect of 25 mg. of adrenocorticotrophic hormone given intravenously on the decolorisation time. The figures in heavy type are statistically significant.

Patient No.	Strength of dye mg./100 ml.	Change per cent. in decolorisation time				
		Intervals in minutes				
		20–40	40–80	80–120	120–160	160–200
11	4	—	*19*	—	*10*	—
	1	—	13	—	6	—
12	4	−10·5	—	−8	10·5	—
	1	**56**	—	**43**	19·5	—
13	4	20·5	**39**	**61**	**35**	—
	1	0·0	**24·5**	**30**	**33**	—
14	4	10·5	0·5	0·5	—	**21**
	1	**26·5**	**55**	**21**	—	**27·5**
15	4	−4·5	−13·5	−20	3	**30**
	1	−12·5	−6·5	−3	**36**	**41**
16	4	—	**43**	**43**	—	21·5
	1	—	**26**	**47·5**	—	**45**

receiving batch I, and Table V the results in 6 patients receiving batch II. The batch I patients all showed considerable temporary clinical improvement within 30 to 45 minutes; 3 had transient ill-effects immediately after injection, syncope and abdominal discomfort. Table IV shows that, apart from No. 11, all showed significant prolongation of the decolorisation time, which reached a maximum within 2 hours after injection, and this increase was proportionately greater with the weaker dye. With batch II, clinical improvement was less marked, and absent in No. 17 and 21, and no patients suffered ill-effects. Table V shows that though 5 of these cases had prolonged decolorisation times, this effect was not so marked as with batch I.

It was concluded from this experiment that a single intravenous dose of adrenocorticotrophic hormone can produce a prolongation of decolorisation time, and that this prolongation is more marked with the weaker dye dilution; but, in view of the batch difference, this effect could not be clearly correlated with the clinical improvement and might conceivably be due to impurities.

TABLE V

ADRENOCORTICOTROPHIC HORMONE. BATCH II

Records the effect of 25 mg. of adrenocorticotrophic hormone given intravenously on the decolorisation time. The figures in heavy type are statistically significant.

Patient No.	Strength of dye mg./100 ml.	Change per cent. in decolorisation time				
		Intervals in minutes				
		20–40	40–80	80–120	120–160	160–200
11	4	—	20	—	—	—
	1	—	**82**	—	—	—
17	4	−13·5	−7·5	−8·5	—	−9
	1	0·0	3·2	12	—	5·7
18	4	—	−20·5	**−28**	**−29**	—
	1	—	19	0·5	−10	—
19	4	8·9	5·5	9·1	—	**18**
	1	**30·5**	−17	−1	—	−14
20	4	13·5	12·5	—	**23**	—
	1	3·5	**26·5**	—	12	—
21	4	2·5	—	− 4·5	—	18·5
	1	8·5	—	**23**	—	4

The Effect of Cortisone

4 patients with active rheumatoid arthritis were denied breakfast on 5 consecutive days, and the mean decolorisation times for the 2-dye dilutions were estimated by making at least 10 observations on the fasting patients at midday. Immediately after making these observations

TABLE VI

Showing how the percentage change in the decolorisation times are arrived at, for a person receiving cortisone, for the dye strength of 1 mg./100 ml. The figures in heavy type are statistically significant.

	Days before cortisone		Days after cortisone		
	1	2	1	2	3
Decolorisation times in seconds ..	36 29 29 27 29 28 27 26 30 24	27 26 38 31 20 30 31 26 31 26	40 45 38 43 47 45 35 40 40 31	27 27 25 24 23 27 29 27 25 23	25 30 23 27 27 26 26 28 27 25
Sums	285	286	404	257	264
Means	28·5	28·6	40·4	25·7	26·4
Difference of means	—	0·6	11·8	−2·8	−2·1
t	—	0·055	6·9	2·06	1·56
P	—	<0·9	<0·01	<0·05	<0·2
Per cent. change	—	2	**41·5**	**−10**	−7·5

on the second day of the trial, the patients were given 200 mg. cortisone acetate suspension intramuscularly and, on the third morning at 8 a.m., 100 mg. of cortisone by mouth. Thus, when the tests were made again at midday on the third day, the patients could be considered to be under the influence of cortisone and, in fact, all showed clinical improvement at this time, which began to subside by the fifth day. Table VI analyses the figures obtained with the 1 mg./100 ml. dye dilution in No. 22, and Table VII summarises the changes for all 4 patients. All the patients

TABLE VII

CORTISONE

Shows the change per cent. in the decolorisation time on 3 successive days after the administration of 300 mg. of cortisone. The figures in heavy type are statistically significant.

Patient No.	Strength of dye mg./100 ml.	Change per cent. in decolorisation time		
		Days		
		1	2	3
22	4	**17·8**	**−20·5**	**−29·5**
	1	**41·5**	**−10**	**− 7·5**
9	4	**12·5**	**−24·0**	**−28**
	1	**13**	**−25·5**	**−36**
5	4	**10**	**−5**	**−10**
	1	**32**	**−13**	**−12**
23	4	**8·5**	**−18·2**	**−33·5**
	1	**30**	**−10**	**−19**

had significant prolongation of decolorisation times on the third day while under the influence of cortisone, and a significant shortening, below the starting point, on the 2 subsequent days when the influence of cortisone was fading, presumably a rebound phenomenon although no corresponding clinical exacerbation was observed. This prolongation was proportionately greater with the weaker dye solution.

It can be concluded that cortisone produces a prolongation of the decolorisation time and that this is followed by a rebound decrease in the time at a stage when its effect wears off. This is consistent with the effect observed less constantly with adrenocorticotrophic hormone being a cortisone-like action, and not due to a side effect of the adrenocorticotrophic hormone or to posterior lobe impurities like oxytocin.

Qualitative Observations

Certain qualitative observations were also made. The intradermal decolorisation times were observed of equivalent solutions of 2:6-dichlorophenolindophenol (E_0 at *p*H 7, 0·217 volts), of 2:6-dichlorophenol indo-*o*-cresol (E_0 at *p*H 7, 0·181 volts) and of methylthionine chloride, methylene blue (E_0 at *p*H 7, 0·011 volts). The first two dyes are rapidly decolorised, the times being comparable, and their colours can be restored

by an injection of potassium ferricyanide at the same intradermal site. Methylene blue is only slightly decolorised and its colour cannot be restored by potassium ferricyanide; so that here the eventual disappearance of the colour is probably due to physical carriage from the site of injection. The colour of methylene blue can be made to disappear completely by injecting glutathione at the same site, and partially, by injecting ascorbic acid. After a time the colour reappears in the glutathione-injected wheal, and deepens in the ascorbic-injected wheal. *In vitro*, all 3 dyes are rapidly decolorised by glutathione. Ascorbic acid rapidly decolorised the first 2 completely, but only partly decolorises methylene blue in a short time. Dehydroascorbic acid alters, but does not abolish, the colour of the first two dyes, and does not decolorise methylene blue. Alloxan rapidly decolorises the first two dyes but not methylene blue.

Discussion

We are concerned with an approach to understanding the action of agents at the tissue periphery. Some discussion of what is being measured is worth while, even though it be speculative. It is reasonable to assume that the time taken for an oxidation-reduction indicator dye to be decolorised after introduction to a biological system depends partly on the difference between the E_h of the system and the E_o of the dye* and partly on the difference between the "capacity" of the system and the "capacity" of the dye, the latter varying with the strength of dye solution used. "Many biological systems, although having a clear-cut definite level of potential, may be of small effective oxidation-reduction "capacity" and not be well poised. This is equivalent to a solution being not well buffered in *p*H determination" (Hewitt[8]).

The E_o of 2:6-dichlorophenolindophenol at *p*H 7 is 0·217 volts. The E_h of living human skin (Radaeli[9]), normally lies between 0·115 and 0·181 volts. The skin will probably therefore always attempt to reduce the dye and this will be helped by the circulation, as the flowing blood has an E_h of between 0·030 and 0·080 volts, when measured electrically (Seyderhelm, Mulli and Thyssen[10]). Thus the increase in the coefficient of variation of a series of observations, when more concentrated dye solutions are used, is probably explained by differences in the local circulation at different times in different sites, and the exact depth of the intradermal injection.

The massive doses of ascorbic acid and glutathione we have used might well be expected to alter the capacity of the system, but it seems unlikely

* E_h is the potential set up at an "unattackable electrode" in a reversible oxidation-reduction system, and is measured in volts referred to the hydrogen electrode. Thus a mixture of potassium ferri- and potassium ferro-cyanide will always have the same E_h for the same proportion of constituents. An increase of the oxidised form will produce a rise in E_h, and an increase in the reduced or ferrous form will produce a fall in E_h. Regarding oxidation as a loss of electrons and reduction as an acceptance of electrons, E_h may be considered as a measure of the concentration of available electrons, and so analagous to *p*H—a measure of the concentration of hydrogen ions. E_o is the E_h of a system which has equal concentrations of the two constituents; i.e., the E_o of an oxidation-reduction indicator dye solution is E_h when it is 50 per cent. decolorised.

that a single injection of 25 mg. of adrenocorticotrophic hormone or 300 mg. of cortisone could do so directly. However, it is conceivable that cortisone acts by increasing the E_h of the system and reducing the difference between the E_h of the skin and the E_o of the dye. The fact that the reduction of decolorisation time observed with ascorbic acid and glutathione is more marked with the stronger dye solution, and conversely that its prolongation by adrenocorticotrophic hormone and cortisone is more marked with the weaker solution, lends confirmation to this view. And it would be logical to attempt to measure the E_h of the tissues by direct electrical methods to see whether the hypothesis that cortisone may increase the E_h of the tissues could be confirmed or refuted.*

Disorders of electrolyte metabolism have been classified into two main groups, those of water balance and those characterised by changes in the hydrogen ion concentration (Gastineau[12]). A disturbance resulting in a tendency towards a decrease in *p*H is called an acidosis, and one tending to an increase in *p*H, an alkalosis. It is possible that analagous tendencies towards changes in the E_h of the tissues may occur as the result of disease and the administration of drugs; tendencies towards increase or decrease in the E_h of the tissues might be labelled "oxidosis" and "reductosis" respectively, corresponding to a decrease or increase of available electrons.

SUMMARY

1. A new method is described for studying the modification by drugs of the oxidation-reduction systems of the skin in man.

2. This consists in noting the changes produced in the decolorisation time of a standard intradermal wheal made with 2:6-dichlorophenol indophenol indicator solutions.

3. The decolorisation time is significantly decreased by ascorbic acid and by glutathione, and increased by adrenocorticotrophic hormone and cortisone, when these agents are administered systemically.

4. The significance of the electrode potential (E_h) of the tissues in relation to these findings is discussed.

REFERENCES

1. Rötter, *Wien. klin. Wschr.*, 1938, **51**, 205.
2. Portnoy and Wilkinson, *Brit. med. J.*, 1938, **1**, 38.
3. Jetter, *Proc. Soc. exp. Biol., N.Y.*, 1938, **39**, 169.
4. Poncher and Stubenrauch, *J. Amer. med. Ass.*, 1938, **111**, 303.
5. Goldsmith, Gowe and Ogaard, *Proc. Soc. exp. Biol., N.Y.*, 1939, **41**, 370.
6. Slobody, *J. Lab. clin. Med.*, 1944, **29**, 464.
7. Fisher, *Statistical Methods for Research Workers*, Edinburgh, 1950.
8. Hewitt, *Oxidation-reduction Potentials in Bacteriology and Biochemistry*, Edinburgh, 1950.
9. Radaeli, *Arch. Dermatol. Syphilis*, 1938, **178**, 253–9.
10. Seyderhelm, Mulli and Thyssen, *Munch. med. Workschr.*, 1937, **84**, 620–3.
11. Loxton and Le Vay, *Nature, Lond.*, 1953, **171**, 524.
12. Gastineau, *Proc. Mayo Clin.*, 1950, **25**, 630.

* Since this paper was written we have carried out this suggested investigation using an electrometer-type of potentiometer. The results are not in agreement with the hypothesis suggested above, as cortisone was found uniformly to produce a fall in the E_h of the subcutaneous tissues when measured by this direct method (Loxton and Le Vay[11]).