method used in calculating the results for the heart preparations, though offering uncertainties of its own, is probably nevertheless the more general and desirable procedure.

SUMMARY

1. The greatest activity of the pyruvate oxidation system by finely divided dialyzed ox heart suspensions is observed in the presence of inorganic phosphate, adenine nucleotide and fumarate added simultaneously.

2. The presence of Mg^{++} is necessary to prevent irreversible inactivation of the enzyme during dialysis.

3. Addition of catalytic amounts of fumarate (0.0005M) greatly increases both the O_2 uptake and pyruvate disappearance.

4. The pyruvate is completely oxidized to CO_2 and water, the molecular ratio O_2 /pyruvate deduced by extrapolation from the experimental data being 2.5 and the R.Q. 1.2.

5. A comparison is made between the course of pyruvate oxidation in ox heart and pigeon brain suspensions.

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Haemolysis and Fixation of Erythrocytes by X-Rays

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It has previously been reported (Halberstaedter & Goldhaber, 1943; Halberstaedter, 1944) that following irradiation with an X-ray dose of 2×10^6 r. at high intensity, erythrocytes *in vitro* undergo either haemolysis or fixation. The type of the response depends on the dose of X-rays, on the concentration of the erythrocyte suspension and on the medium in which they are suspended during irradiation. At high cell concentrations ($> 0.5\%$) the effect of the irradiation is always haemolysis independently of the nature of the medium. At low concentrations ($< 0.5\%$), on the other hand, the type of the response obtained depends on the medium in which the cells are suspended. At low concentration, the erythrocytes are resistant to haemolysis if irradiated in a medium of 0.9% sodium chloride solution, but undergo complete haemolysis if irradiated in a medium of isotonic glucose or 1 vol. serum : 3 vol. of 0.9% sodium chloride solution.

Continuing this investigation, the influence of different media on the response of erythrocytes to X-ray irradiation has been studied. Experiments

were made to determine what serum constituent is responsible for the haemolytic reaction of erythrocytes which are irradiated at low concentration with an X-ray dose of 2×10^6 r.

EXPERIMENTAL

Blood. Fresh rabbit or human blood was defibrinated, the erythrocytes washed twice with 0.9% NaCl and suspended in concentrations of 0.1% and 1% (v/v) in the different media. Small hanging drops under mica slips were irradiated and the effects observed with the microscope.

X-rays. Irradiation was carried out by means of a demountable X-ray tube with copper anode at a tension of 35 kV. and a current of 40 ma. The window of aluminium foil was 30μ , and the mica slip for the hanging drop 0.02–0.025 mm. in thickness. The X-ray intensity at the distance of the hanging drop (3.5 cm.) was about 95,000 r./min. The dose was always the same, namely 2×10^6 r.

Irradiation media. The effect of irradiation under the above-mentioned circumstances on 0.1% erythrocyte suspensions in saline (0.9% NaCl solution) was always fixation ('saline effect', s.e.), but in a medium of 1 vol.

serum: 3 vol. of saline complete haemolysis always took place. Therefore 0.9% NaCl as well as a mixture of 3 vol. 0.9% NaCl: 1 vol. serum were employed as standard control media. For the experiments, different isotonic (i.e. iso-osmotic with normal saline) salt solutions mixed with 3 vol. saline were used as media. Graded dilutions of these mixtures as well as of the standard serum-saline were prepared for comparison. Comparisons were also made in some cases between the effect of the isotonic salt solutions as such and that of the undiluted serum. The pH was controlled by potentiometric measurements.

All experiments were carried out at room temperature (19–25°), and the controls were always observed simultaneously with the experiments and under the same conditions.

RESULTS

Range of saline effect

Isotonic solutions of many alkali and alkali-earth salts (CaCl_2 , KCl , MgSO_4) resemble saline in their effect on the X-ray response of erythrocytes. Isotonic NaNO_3 and $(\text{NH}_4)_2\text{SO}_4$ likewise produce the s.e. but diverge slightly from the behaviour of the other salts in some other respects. In NaNO_3 solution the X-ray haemolysis of 1% erythrocyte suspension proceeds much more slowly than in NaCl and the haemolysis is not complete. In

$(\text{NH}_4)_2\text{SO}_4$ solutions spontaneous haemolysis of non-irradiated 0.1% suspensions occurs. The failure of haemolysis in that medium after irradiation shows that the effect of X-rays in this case is in fact a kind of fixation.

Definite evidence of the state of fixation, i.e. of the total inability to become haemolyzed, of erythrocytes irradiated at 0.1% cell concentration in any of the above salt media was obtained from experiments with saponin and eosin. Saponin, a powerful haemolytic agent, produced immediate haemolysis of non-irradiated suspensions of erythrocytes at 0.1% concentration but failed to dissolve the same suspensions after they were irradiated with 2×10^6 r. *in vitro*. The irradiated cells absorbed eosin readily in the manner typical of fixed cell preparations.

Among non-electrolytes investigated, glycerol in isotonic solution diluted with 3 vol. of isotonic NaCl produced s.e. Considering the chemical relationship of glycerol and glucose this finding is somewhat surprising.

A totally different type of influence is exerted by irradiation in isotonic solutions of alkali phosphates, alkali bicarbonates, glycine and urea. In both KH_2PO_4 (pH 4.5) and Na_2HPO_4 (pH 9.1) as well as in a mixture of the two at the physiological pH (7.3),

Table 1. *Effect of X-ray irradiation (2×10^6 r.) on erythrocytes in different media with and without addition of serum*

(Media: 1 vol. isotonic salt solution + 3 vol. 0.9% NaCl. Concentration of erythrocytes 0.1 and 1.0%.)

Medium	Erythrocytes irradiated						Erythrocytes non-irradiated	
	Media (medium + saline)			Media + $\frac{1}{3}$ vol. serum				
	pH	(0.1%)	(1.0%)	(0.1%)	(1.0%)	pH	(0.1%)	(1.0%)
NaCl	6.95	0 + Sap. 0	c	c	c	7.35	0	0
KCl	6.95	0 + Sap. 0	c	c	c	7.35	0	0
CaCl_2	6.9	0 + Sap. 0	c	c	c	7.25	0	0
$(\text{NH}_4)_2\text{SO}_4$	6.9	0 + Sap. 0	Not c + Sap. c	c	c	7.3	c	0
MgSO_4	6.9	0 + Sap. 0	24 hr. 0 + Sap. c	c	c	7.25	0	0
NaNO_3	6.95	0 + Sap. 0	Fading + Sap. not c	Fading	c	7.35	0	0
Glycerol	6.9	0 + Sap. 0	c	c	c	7.3	0	0
Glucose	6.9	c	c	—	—	7.3	0	0
KH_2PO_4	4.5	c	c	—	—	—	0	0
Na_2HPO_4	9.1	c	c	—	—	—	0	0
Phosphate mixture	7.3	c	c	—	—	—	0	0
Glycine	—	c	c	—	—	—	0	0
Bicarbonate	7.35	c	c	—	—	—	0	0

0 = No haemolysis, c = complete haemolysis, Sap. = saponin solution.

Saponin added to the non-irradiated controls always gives complete haemolysis.

suspensions of erythrocytes at all cell concentrations were susceptible to X-ray haemolysis exactly as in a medium of isotonic glucose or serum-saline mixture. Glycine, like serum or phosphate, prevented fixation. Isotonic urea solution caused haemolysis of erythrocytes, and therefore experiments on the effect of X-rays in this medium could not be performed. Erythrocytes also underwent haemolysis when suspended in isotonic bicarbonate; no haemolysis occurred, however, when a mixture of 1 vol. isotonic bicarbonate and 3 vol. saline served as medium. The response of the erythrocytes in the latter case resembled their behaviour in either serum, glucose, phosphate or glycine medium.

Among the substances which have been discussed, three—glucose, phosphate and bicarbonate—are constant and physiologically active components of normal serum. Their proportions/100 mol. NaCl are, approximately, 5 mol. glucose, 1 mol. phosphate and about 20 mol. bicarbonate (the amount of the latter depending on the varying CO_2 saturation of arterial and venous blood). The effect of separate addition of each of these three substances on the response of erythrocytes in saline to X-ray irradiation was studied.

Addition of glucose to saline up to the normal blood sugar concentration did not prevent s.e. Phosphate prevented s.e. only when added in overwhelming excess. In mixtures of phosphate and NaCl containing less than 80 % (v/v) phosphate, s.e. persisted.

Table 2. *Rabbit erythrocytes (0.1 and 1.0 % suspensions) irradiated with 2×10^6 r. in phosphate solution*

(30.5 ml. 2.11 % (M/6.4) KH_2PO_4 + 69.5 ml. 1.84 % (M/9.6) $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (isotonic) diluted with saline, pH 7.3.)

Vol. concentration of phosphate solution in saline (%)	Erythrocyte concentration	
	(0.1 %) Haemolysis	(1 %) Haemolysis
0	None	Complete
20	None	Complete
40	None	Complete
60	None	Complete
80	Marked	Complete
100	Complete	Complete

Table 4. *Influence of undialyzed and dialyzed serum with or without addition of NaHCO_3 on the effect of X-rays on erythrocytes*

(0.1 % suspensions of human erythrocytes in different dilutions of undialyzed serum, dialyzed serum, and dialyzed serum plus 2.5 % of its vol. of isotonic NaHCO_3 , irradiated *in vitro* with 2×10^6 r.)

Dilution in saline	Undialyzed serum	Dialyzed serum	Dialyzed serum plus NaHCO_3
1: 4	Complete haemolysis	No haemolysis	—
1: 10	Complete haemolysis	No haemolysis	Very strong haemolysis
1: 25	Almost complete haemolysis	No haemolysis	Very strong haemolysis
1: 50	No haemolysis	No haemolysis	No haemolysis
1: 75	No haemolysis	No haemolysis	No haemolysis

Bicarbonate, on the other hand, prevented s.e. even when minute quantities of it were added to saline.

Table 3. *Human erythrocytes (0.1 % suspensions) irradiated with 2×10^6 r.*

(Medium: different dilutions in saline of an isotonic mixture of 2.5 ml. NaHCO_3 and 97.5 ml. saline.)

Dilutions	
1: 3	Immediate complete haemolysis
1: 10	Complete after 40 min.
1: 20	Marked haemolysis
1: 50	Slight haemolysis
1: 100	No haemolysis

Identification of the s.e. antagonist of serum

Both the dialyzable constituents of serum and the serum protein (freed from salts other than NaCl by dialysis against isotonic saline) were tested.

In a mixture of 100 vol. NaCl, 2 vol. KCl, 2 vol. CaCl_2 , 1 vol. phosphate, 2 vol. glucose and 10 vol. NaHCO_3 (all isotonic), erythrocytes in a suspension of 0.1 % cell concentration behaved under irradiation exactly as in serum (haemolysis). Omission from this mixture separately or jointly of CaCl_2 , KCl, PO_4 and glucose did not affect the response obtained. On the other hand, omission of bicarbonate from the mixture caused the appearance of s.e. (fixation) both in absence or presence of CaCl_2 , KCl, PO_4 , or glucose.

Serum was dialyzed for 24–48 hr. in an ice-box against a large excess of repeatedly renewed saline through a cellophan membrane. At the end of this time micro-determinations for sugars were invariably negative, and the dialyzed serum was presumed to contain practically no dialyzable constituent except NaCl. During dialysis, the surface of the external solution was kept carefully below the surface of the internal one, the hydrostatic pressure preventing entry of water and even causing some loss of water through filtration. After dialysis the vol. of the serum could be made up to the original vol. with saline.

The dialyzed serum showed the s.e. (fixation) and its behaviour in this respect was indistinguishable from that of protein-free saline solution. In dialyzed serum to which bicarbonate was added in an amount barely corresponding to the minimum

occurring in normal serum (20 % v/v of an isotonic NaHCO_3 solution), the erythrocytes were haemolyzed by X-rays at all cell concentrations. Addition to dialyzed serum of bicarbonate alone thus regenerates the original quality of serum as an irradiation medium.

For quantitative evaluation of the anti-s.e. potency of genuine and of bicarbonate-reconstituted dialyzed serum respectively, the two media were added in varying concentrations to saline and the dilution in saline to which the anti-s.e. persisted was then determined.

As Table 4 shows, haemolysis does not occur in dialyzed serum. Undialyzed serum and dialyzed serum reconstituted with sodium bicarbonate are similar in their behaviour as media at all dilutions with saline. Haemolysis still occurs in both cases at a dilution of 1 : 25, but at 1 : 50 fixation takes place.

DISCUSSION

The experiments presented show that X-rays can act on erythrocytes in two opposing and mutually excluding ways: (a) haemolysis and (b) fixation. The particular effect produced by a given X-ray irradiation, i.e. the alternative which takes precedence and excludes the other, depends on three factors: intensity and dose of the irradiation, the cell concentration of the erythrocyte suspension, and the nature of the medium. The three factors are interdependent. The dependence of the nature of the response (haemolysis or fixation) on the cell concentration exists only in certain media (saline type), while in others (serum type) haemolysis is produced by an effective X-ray dose independently of the cell concentration. The dependence of the nature of the response on the composition of the medium exists only at low cell concentrations (< 0.5 %), whereas at high cell concentrations haemolysis is induced by an effective dose independently of the composition of the medium.

The conditions determining haemolysis and fixation following irradiation of erythrocytes with X-rays are thus quite different. Fixation only occurs if three conditions are fulfilled: (1) the cell concentration of the erythrocyte suspension is less than 0.5 %; (2) the X-ray dose is 2×10^6 r. or more; (3) the medium is of the saline type. On the other hand, haemolysis occurs provided that only one of the following conditions is fulfilled: the erythrocyte concentration is 0.5 % or more, the X-ray dose is smaller than 2×10^6 r., or the medium is of the serum type. Haemolysis is therefore the ordinary, i.e. the most general, response to X-ray influence, and fixation is only produced in exceptional circumstances. The following further important difference should also be noted: the haemolysis elicited by irradiation can continue for many hours or days, the

time required for complete haemolysis being dependent on the dose employed. In fixation, however, the complete effect is produced within the time of the irradiation. When at the completion of the irradiation fixation has not occurred the response later obtained is always haemolysis.

Haemolysis caused by X-rays is considered by Ting & Zirkle (1940) and by Liechti & Wilbrandt (1941) to be an osmotic effect. According to Ponder (1936) and Davson (1943) haemolysis is not a simple permeability process. The escape of haemoglobin 'results from the formation of holes in the membrane, which are large compared with the haemoglobin molecule, so that the escape is probably determined by simple laws of diffusion in an aqueous medium'. The membrane alterations which occur in X-ray haemolysis can probably be attributed to a direct action of the X-rays on the membrane. The known facts do not in any case contradict the assumption that the mechanism of the action is the production within the membrane of ionization centres which bring about an alteration whereby the escape of haemoglobin becomes possible.

On the other hand, we have to admit that the interpretation of the mechanism of the fixation of erythrocytes by X-rays (s.e.) is obscure. s.e. is produced by X-rays in many neutral salt solutions, but cannot be characterized as an electrolyte effect since it is also produced in glycerol and since it is opposed by specific electrolytes, like phosphate and bicarbonate, as well as by glucose and glycine. The explanation in terms of pH is ruled out by the fact that primary and secondary phosphates in a wide range of pH values (4.5-9.0) show no difference in behaviour and that bicarbonate and phosphate of the same pH behave quite differently.

The experimental findings with regard to fixation of erythrocytes by X-rays cannot be explained on the assumption that the effect of the irradiation in this case is direct. Since fixation must be brought to completion during the irradiation itself it is comprehensible that a much larger dose is required for fixation than for haemolysis. If fixation were brought about by the direct action of the X-rays, i.e. on the basis of the direct hit mechanism, it would be incomprehensible that it depends at a given dose and in a given medium (saline type) on the erythrocyte concentration. The observation that fixation following irradiation with doses greater than 2×10^6 r. is prevented by the presence in the medium of serum and certain other substances is likewise difficult to reconcile with the view that the effect of the X-rays on the erythrocytes is direct.

The theory of indirect action through 'activation' of accompanying substances, on the other hand, seems *prima facie* in good accord with the observed dependence of the irradiation effect on the composition of the irradiation medium. But it suggests no ready explanation for the effect of the cell concentration. It might be argued that a regular dependence of radiation effect on substrate concentration is to be expected on the basis of the indirect action theory, as the ratio: substrate concentration (number of cells)/density of 'activation centres' for a given radiation intensity and a given medium naturally depends on the substrate concentration. In our case, however, the change of the nature of response to irradiation with increasing cell dilution is not gradual, but assumes the form of an appearance of a qualitatively different effect (s.e.) at

some specific cell concentration level (<0.5%). Moreover, 'activation centres' of 'indirect action' theories have mostly been regarded as 'activated water molecules' involving production of H_2O_2 and/or H^+ (Fricke, 1934, 1935). In our experiments there is nothing which suggests oxidation as an explanation for the different behaviour of media of saline and serum type, as both groups are not defined by different oxido-reduction properties. The irrelevance of an H^+ effect has already been pointed out.

The influence of the concentration and of the nature of the medium in our experiments on erythrocytes has an analogy in results obtained with other objects. Dale (1940) first demonstrated the dependence of the X-ray effect on enzymes on the substrate concentration and the medium. Similar results have been reported for virus by Lacassagne & Nyka (1938), Friedewald & Anderson (1941), Luria & Exner (1941). Evans, Slaughter, Little & Failla (1942) showed that the effect of X-ray irradiation on *Arbacia* sperm depends on the medium and on the concentration of the irradiated sperm suspension. A growing susceptibility to irradiation with diminishing concentration of the irradiation substrate has always been described. Dale (1943) and other investigators consider these phenomena to be indirect effects mediated by ionization of the water molecules.

In the case of the effect of ultra-violet irradiation on taka-carboxylases it has been shown (Goldhaber & Leibowitz, 1943) that the alleged dependence of the inactivation effect on the concentration of the target is really a dependence not on the concentration of the active enzyme molecules as such but rather a dependence on the concentration of substances (proteins?) which accompany the enzyme, but which do not participate in its activities. The same applies also in the case of certain proteolytic enzymes of pancreatin (Goldhaber, Leibowitz & Feigenbaum, unpublished results). In the case of living cells, however, it is clearly hazardous to venture any statement on what constituents are active in the mechanisms of haemolysis and fixation and what are merely accompanying substances.

Haemolysis and fixation have been observed in experiments with photodynamic substances on erythrocytes. Blum (1930) found that haemolysis and fixation are

brought about by non-irradiated eosin at defined hydrogen ion and dye concentrations. Irradiation shifts the region of occurrence of these phenomena into lower concentrations of the dye. Blum (1935) also found that H_2O_2 and eosin may reinforce each other in producing haemolysis and fixation. In our experiments no dependence on hydrogen ion concentration or on oxidation processes was observed.

SUMMARY

1. Suspensions of erythrocytes in a concentration range from 5% (v/v) down to about 0.5% respond to irradiation with an effective X-ray dose by haemolysis independently of the nature of the medium in which they are suspended.

2. At cell concentrations below 0.5%, the response of erythrocytes to irradiation depends on the medium in which they are suspended: (a) In isotonic glucose, KH_2PO_4 , Na_2HPO_4 , $NaHCO_3$, and glycine the response to an effective X-ray dose is haemolysis. (b) In isotonic NaCl, KCl, $CaCl_2$, $NaNO_3$, $(NH_4)_2SO_4$, $MgSO_4$, and glycerol the effect of an X-ray dose of 2×10^6 r. and more is not haemolysis but cell fixation, the total loss of the ability to haemolyze.

3. Addition to media listed in 2 (b) of serum, phosphate or bicarbonate prevents fixation. The fixation-opposing activity of phosphate is considerably below that of serum or bicarbonate. The bicarbonate content of serum accounts completely for its high activity in preventing fixation.

4. The relevance of the nature of the medium and of the cell concentration of the erythrocyte suspension for the occurrence of the fixation effect points to some 'indirect action' mechanism of the irradiation.

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