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A Study of the Irradiation of Catalase by Ionizing Radiations in the Presence of Cysteine, Cystine and Glutathione

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Any claim that H atoms generated by ionizing radiations in aqueous solutions of biochemical substances are the active agent responsible for the radiation effects observed deserves special attention, since oxidative reactions via OH and O₂H radicals are by far more common. Such claims are of particular importance in the field of enzymes for which no example of such a mechanism was known until it was found that catalase was inactivated by H atoms produced by X-irradiation of an aqueous solution of the enzyme (Forssberg, 1947). This conclusion was based on the experimental observation that cysteine, present in solutions of catalase during irradiation, did not protect the enzyme from inactivation, but on the contrary increased the inactivation. It was suggested that cysteine was scavenging the OH radicals, thus leaving the H atoms to act upon the enzyme. On the other hand, cystine was assumed to react with H atoms, because it protected the enzyme. A similar situation obtained with reduced and oxidized glutathione.

It was also found that keto acids and unsaturated acids such as pyruvic and fumaric acid protected catalase, but that saturated acids, such as succinic acid, did not.

Evidence against Forssberg's conclusions was put forward by Sutton (1952) and Bella (1949). Sutton found that H atoms produced by means other than irradiation did not inactivate catalase, and that irradiation of the enzyme in an atmosphere of oxygen was much more damaging than irradiation in an atmosphere of hydrogen. Bella

irradiated pyruvic and lactic acid, and found that no lactic acid was produced from pyruvic acid, but that pyruvic acid was formed from lactic acid. Thus, the work of Sutton and Bella seemed to indicate that H atoms played no part in the inactivation of catalase by irradiation.

It was therefore decided to re-investigate the original problem, that is, whether cysteine and glutathione decrease the radiation effect on the enzyme, i.e. protect, or whether, on the contrary, they increase this effect. Further, does cystine protect the enzyme?

MATERIALS AND METHODS

Crystalline catalase was prepared from ox liver by the method of Tauber & Petit (1952) and Tauber (1953). This was found to be the simplest and quickest method. The preparation was electrophoretically homogeneous and had Kat. F. 39000.

Cysteine hydrochloride (British Drug Houses, Ltd.) was recrystallized and freed from iron by the method of Warburg (1927).

Electrophoresis. The electrophoretic properties of the enzyme were studied by the technique of paper electrophoresis, using barbitone-sodium barbitone buffer (pH 8.6, *I*=0.1). Paper strips (Whatman no. 1) 38 × 7 cm. were used in a Shandon Vertical Electrophoresis tank (Shandon Scientific Co., Cromwell Place, London, S.W. 7). Catalase solutions (12.5 μl.; catalase content 2.2–4.5 mg./ml.) were applied at a spot 5 cm. from the centre line towards the cathode. Electrophoresis (150 V, ca. 4 v/cm.) was allowed to proceed at room temp. for about 20 hr., during which catalase migrated towards the anode. The strips were air-dried and developed with methyl orange-eosin Y in aqueous

acetone (Tauber, Vannier, Petit & Magnuson, 1953) followed by washing with very dilute H_2SO_4 (1 drop conc. H_2SO_4 in 1 l. water) at 60°. Catalase stained orange-red under these conditions. Cysteine, cystine, and glutathione did not stain.

Chromatography. Chromatographic analysis was carried out using the method of Tauber *et al.* (1953) in which the solvent is trisodium citrate-NaCl buffer brought to pH 6.0 with 2N-HCl. To paper strips of 40 × 7 cm. catalase was applied 3 cm. from the end dipped into the solvent. The solvent was allowed to ascend for 3.5 hr., after which the papers were dried and stained as after the electrophoresis.

Absorption spectroscopy. The absorption spectra of catalase and of haematin hydrochloride were examined in a Hilger Uvispek spectrophotometer, in the wavelength range 210–600 mμ. The catalase concentration was 0.445 mg./ml. and that of haematin hydrochloride 0.010 mg./ml. Absorption was measured in 5 mm. cells. The haematin hydrochloride (British Drug Houses, Ltd.) had Fe, 8.56%. It was dissolved in 0.06N-NaOH and diluted 40 times with M/15 sodium phosphate buffer, pH 7.4, i.e. the same buffer as used in the examination of catalase itself.

Catalase assay. The procedure adopted for the assay of catalase activity was a combination of two methods described by Bonnichsen, Chance & Theorell (1947) in which (a) samples of reaction mixture (catalase + hydrogen peroxide) were added to sulphuric acid, and (b) acid was added to the entire reaction mixture, three separate reaction mixtures being necessary. The present method facilitated accurate control of the time for which the catalase was allowed to react with the hydrogen peroxide (as in method (b) of Bonnichsen *et al.*), and also enabled three estimations to be made using one reaction mixture (as in method (a) of Bonnichsen *et al.*).

The assay was carried out as follows. Catalase solution (0.03 ml.) was pipetted on to a watch glass (17 mm. diam.). This was dropped at zero time into 25 ml. of H_2O_2 (approx. 0.005M in 0.01M sodium phosphate buffer, pH 7.4), continuously stirred magnetically. Samples (2 ml.) were transferred from the reaction mixture at intervals of about 15 sec. into three 50 ml. flasks where the reaction proceeded until stopped at 20, 40 and 55 sec. respectively by the addition of 25 ml. 2% (w/v) H_2SO_4 , containing titanium sulphate, from automatic pipettes. The important accurate timing of the short reaction periods of 20, 40 and 55 sec. was conveniently achieved by using a slave clock operated by electric impulses every second from a master clock. The same impulses were supplied to a condenser-loud-speaker system giving a series of crisp clicks.

The tenth second before the desired stopping of the reaction was noted from the clock hand and the remaining second-intervals were counted in time with the clicks, thus avoiding observation of the clock diverting attention from the pipetting procedure.

The hydrogen peroxide concentration was determined colorimetrically with a Hilger Spekker absorptiometer (4 cm. cells and filters Violet 601). A reference curve was constructed of optical density against H_2O_2 concentration at 21.3°. It was necessary to refer all readings to this temperature, as the optical density of the H_2O_2 -titanium complex was found to be very temperature-sensitive (see p. 54).

Irradiation. Three types of ionizing radiations were used: (1) γ-rays from a ^{60}Co source (approx. 350 r./min.); (2) high-energy electrons from a 4 Mev travelling-wave linear accelerator (Orthotron) (2 μsec. pulse duration; 300 pulses/sec.); dose rate equivalent to approx. 10^6 r./min.; (3) X-rays from a 350 kv Resomax X-ray tube (approx. 350–1400 r./min.) (filter, 0.63 mm. Cu and 1 mm. Al). (Newton Victor Ltd., 132, Longacre, London, W.C. 2).

RESULTS

Electrophoresis

After irradiation catalase gave not a single spot, but a streak, when subjected to electrophoresis. The length of the streak varied with the radiation dose, being shorter the greater the dose. The presence of cysteine or glutathione during irradiation decreased the effect of irradiation so that the electrophoretic pattern remained unaltered if the concentration of added substances was great enough for full protection. Fig. 1 shows the effect of 10^6 r. (electron beam) in air on a solution of catalase with or without cysteine hydrochloride. It can be seen from the controls that cysteine itself has no effect on the electrophoretic pattern and, from the irradiated solution, that it strongly protects catalase against destruction by the irradiation. Parallel measurement of the catalatic activity showed that it was decreased to 15% of the control value in the absence of cysteine, but only to 95% when cysteine was present. Fig. 2 shows a similar behaviour in nitrogen. There is less destruction of the catalase (lower radiation dose), since the streak reaches the same point as the spot of unirradiated enzyme. Cysteine again protects strongly.

Fig. 3 illustrates the effect of cystine on the electrophoretic behaviour of catalase irradiated in nitrogen. As can be seen, a streak is still produced, but its front reaches the same point as that of the unirradiated solution. Parallel activity estimates showed a decrease to 25% and to 64% of the control, in the absence and presence of cystine, respectively.

The effect of glutathione is seen in Fig. 4. Catalase was irradiated with and without glutathione in nitrogen. There is very little difference in the patterns produced by irradiated and unirradiated solutions of catalase containing glutathione, thus indicating strong protection. Parallel activity estimations showed a decrease to 25% of the control in the case of catalase alone, and to only 93% when glutathione was present.

The protection of catalase by cysteine is dependent on their relative concentrations (Fig. 5). Here the catalase concentration was 2.22 mg./ml. and the cysteine hydrochloride concentration varied as shown. The presence of a well-pronounced

spot in *A* indicates the strong protection afforded by cysteine at a concentration only one-twentieth of that in Fig. 2. In *B* there is still a faint spot (difficult to reproduce photographically) parallel to that in *A*, whereas in *C*, with the lowest concentration of cysteine, no spot appears. The effect of decreasing the concentration of cysteine shows it-

self in the decreasing length of the streak. This further proves the dependence of protection upon the relative concentrations of catalase and cysteine as the variation in their proportions does not affect the electrophoretic behaviour of unirradiated catalase. It should be noted that the cysteine hydrochloride concentration in *B* (0.044 mg./ml.) is

Fig. 1

Fig. 3

Fig. 2

Fig. 4

Fig. 1. Electrophoresis: effect of 10^6 r. (electron beam 1 min.). Air; catalase, 4.45 mg./ml.; cysteine HCl, 4.45 mg./ml.

Fig. 2. Electrophoresis: effect of 5×10^5 r. (electron beam 30 sec.). Nitrogen; catalase, 4.45 mg./ml.; cysteine hydrochloride, 4.45 mg./ml.

Fig. 3. Electrophoresis: effect of 3.5×10^5 r. (γ -rays approx. 16.5 hr.). Nitrogen; catalase, 2.22 mg./ml.; cysteine, 0.05 mg./ml.

Fig. 4. Electrophoresis: effect of 3.5×10^5 r. (γ -rays approx. 16.5 hr.). Nitrogen; catalase, 2.22 mg./ml.; glutathione, 5.55 mg./ml.

comparable to the cystine concentration in Fig. 3 (0.05 mg./ml.). The effect of the two substances at similar concentration is much the same. This is borne out by activity experiments (see Table 1).

Fig. 5. Dependence of protection upon concentration of cysteine hydrochloride. Radiation dose, 3.5×10^5 r. (γ -rays approx. 16.5 hr.); nitrogen; electrophoresis; catalase, 2.22 mg./ml. Cysteine HCl (mg./ml.): A, 0.22; B, 0.044; C, 0.022; D, zero.

Fig. 6. Dependence of length of chromatogram streak upon radiation dose. A, 5×10^5 r. (30 sec.); B, 7.5×10^5 r. (45 sec.); C, 10^6 r. (60 sec.) (electron beam). Nitrogen; catalase, 4.45 mg./ml. The photograph was overexposed in order to show the faint streaks at the higher doses.

Chromatography

Catalase gives a streak, as is common in chromatography of proteins. The length of this streak is much affected by the radiation dose, as shown in Fig. 6. However, on adding cysteine hydrochloride (4.45 mg./ml.) all the streaks were of the same length as the control, when subjected to the same radiation doses as in Fig. 6.

Glutathione behaved similarly. Catalase (2.22 mg./ml.) was irradiated (3.5×10^5 r., γ -rays) in nitrogen in the absence and presence of glutathione (5.55 mg./ml.). The protective effect was clearly seen.

Absorption spectroscopy

It was convenient to use weaker solutions for examining the ultraviolet spectrum of catalase. Fig. 7 shows the normal curve obtained with

Fig. 7. Ultraviolet absorption spectra of catalase: 1, unirradiated; 2, irradiated; 3, irradiated in presence of cysteine hydrochloride; 4, irradiated in presence of reduced glutathione. Catalase, 0.445 mg./ml.; radiation dose, 3.5×10^5 r. (γ -rays approx. 16.5 hr.); nitrogen; 5 mm. quartz cells.

Fig. 8. Absorption spectra of haematin hydrochloride (10 μ g./ml.) before and after irradiation (γ -rays). Air. 1, control; 2, 6×10^8 r. (18.2 min.); 3, 20×10^8 r. (60.6 min.); 4, 40×10^8 r. (121.2 min.).

catalase and the effect of irradiation, i.e. a marked rise in absorption at $275\text{ m}\mu$, and a decrease in the Soret band at $405\text{ m}\mu$, the ratio D_{405}/D_{275} changing from 0.66 to 0.27. However, in the presence of cysteine this ratio became 0.62 and in the presence of glutathione, 0.66. Activity estimations showed that catalase activity was decreased to approx. 2.7 % of the control when the catalase was irradiated alone, but only to 76 % when cysteine was present.

The pronounced drop in absorption at $405\text{ m}\mu$ is indicative of damage to the haem group in catalase. The effect of irradiation on haematin hydrochloride is shown in Fig. 8. It can be seen that this substance is quite sensitive to radiation and that the disappearance of the absorption at $390\text{ m}\mu$ is not accompanied by any increase in absorption at other wavelengths. An interesting feature of the spectrum of irradiated haematin is the appearance of another peak at $350\text{ m}\mu$ in addition to the one at $390\text{ m}\mu$.

Activity measurements

During colorimetric estimations of hydrogen peroxide it was found that the optical density of a solution of hydrogen peroxide-titanium complex varied inversely with temperature. Fig. 9 shows

Fig. 9. Variation of optical density of $\text{H}_2\text{O}_2\text{-Ti}_2(\text{SO}_4)_3$ complex with temperature. H_2O_2 concentration, $10.5\text{ }\mu\text{g./ml.}$ 4 cm. cells.

that over the range $18\text{--}32^\circ$ the optical density falls by 0.01 for each 1° rise, a marked temperature effect. All readings were, therefore, corrected by this factor in measurements of catalatic activity.

Activity was measured during experiments on electrophoretic and other properties, but in addition a particular study was made of the effects of irradiation on activity. The catalase

concentration was approx. 0.1 mg./ml. , cysteine hydrochloride 0.5 mg./ml. , glutathione 1 mg./ml. and cystine 0.05 mg./ml. This concentration of catalase was chosen so that the 'rapid assay method' described above could be used. The ratio cysteine:catalase of 5:1 (w/w) was the same as in Forssberg's experiments. It was found that glutathione and cysteine markedly depressed the activity of catalase. The extent of the depression varied with the ratio of cysteine (glutathione) to catalase, with the time of contact between these and with the pH of the solution.

Ratio of SH compound to catalase. As shown in Fig. 10, the higher the ratio of cysteine to catalase,

Fig. 10. Effect of concentration on depression of catalatic activity by cysteine hydrochloride (O) and glutathione (x). Catalase, 0.112 mg./ml. Substances in contact for 35 min. in air before estimation of activity.

the lower is the catalatic activity. At a ratio 5:1 (w/w) the activity was depressed to approx. 50 % (after contact for 35 min.). This holds whether the solution contains oxygen or not. The effect of glutathione is less marked, 50 % activity being reached at a ratio of 15:1 (w/w), i.e. the depressing effect of cysteine and glutathione appears to depend on their sulphhydryl content.

Time of contact of SH compound with catalase. Fig. 11 illustrates the effect of the time of contact between catalase and cysteine and catalase and glutathione on the subsequent catalatic activity. The depression at first increases rapidly with time, 50 % activity being left after 40 min., but then the curve flattens out. It seems that the formation of the inactive complex is more rapid with cysteine than with glutathione, but that they are equally inhibitory after about 40 min.

Fig. 11. Effect of time on depression of catalatic activity by cysteine hydrochloride (x) and glutathione (o). Catalase, 0.112 mg./ml.; cysteine hydrochloride, 0.56 mg./ml.; glutathione, 1.69 mg./ml. Air.

Effect of pH. At present, preliminary work suggests that the depression of activity of catalase noted above is pH sensitive, decreasing with decreasing pH. All the experiments described in this communication were carried out at pH 7.4 (0.066 M sodium phosphate buffer).

Cystine had little effect on catalase activity. At the ratio 0.4:1 (w/w), catalase activity was depressed to about 88 % of the control after contact for 40 min. Similarly, at the same ratio cysteine depressed catalatic activity to about 78 % (see Fig. 10).

The fact that cysteine and glutathione themselves depressed catalatic activity made it necessary to

make allowance for this effect when calculating the radiation effect on the enzymic activity in experiments with catalase solutions containing these substances. This was done by using a formula derived as follows:

Let E = enzyme activity, unirradiated.

E_i = enzyme activity, irradiated.

U = enzyme activity in presence of cysteine, etc., unirradiated.

I = enzyme activity in presence of cysteine, etc., irradiated.

D = depression in activity caused by cysteine, etc.

Then

$$D = E - U.$$

The fraction of activity left after irradiation of the enzyme alone is

$$E_i/E.$$

The fraction of activity left after irradiation of the enzyme in the presence of cysteine, etc., is

$$(I + D)/(U + D) = (I + E - U)/E.$$

Therefore, the effect of the added substances may be represented as

$$\frac{I + E - U}{E} \bigg/ \frac{E_i}{E} = \frac{I + E - U}{E_i} = Q.$$

If $Q > 1$, there is protection. If $Q < 1$, there is increased inactivation. In the present work, Q was never < 1 . Typical experimental values for the calculation of Q are given in Table 1. Very large destruction of the enzyme outweighs the depressive effect of the added substances. Hence, protection is evident even without correction in this case. It will be noted that I/E , which does not take the depression into account, would lead to the result of an increased inactivation in the case of cysteine and glutathione.

In the derivation of the formula for Q it has been assumed that the depression of the activity caused by cysteine etc. is not affected by irradiation. This is very probably so, as only a slight oxidation of cysteine and glutathione would be caused by irradiation with 12 500 r. (Rotheram, Todd & Whitcher, 1951). However, there is a small

Table 1. *Effect of radiation on catalatic activity*

Catalase, 0.11 mg./ml. Cysteine hydrochloride, (a) 0.56 mg./ml.; (b) 0.06 mg./ml. Cystine, 0.05 mg./ml. Glutathione, 1.12 mg./ml. X-ray dose, 12.5×10^3 r. (approx. 40 min.). Nitrogen. Activity constant k_1 calculated according to Bonnichsen, Chance & Theorell (1947).

	$10^{-7} k_1$ unirrad.	$10^{-7} k_1$ irrad.	E_i/E	I/E	Q
Catalase	1.80	1.07	0.59	—	—
Catalase + cysteine (a)	1.18	0.81	—	0.45	1.34
Catalase + cysteine (b)	1.65	1.15	—	0.64	1.23
Catalase + cystine	1.64	1.08	—	0.60	1.16
Catalase + glutathione	1.23	0.92	—	0.51	1.39

amount of hydrogen sulphide formed (Dale & Davies, 1951) which will have a further depressing effect on catalase. It has in fact been found that the activity of catalase solutions containing air and irradiated in the presence of cysteine may be increased, if the hydrogen sulphide is removed before assay. Application of a further correction for this inhibition would increase the value of Q by about 10%.

DISCUSSION

Barron & Flood (1952) have shown that the Soret band produced by the haem group of ferrocytochrome *c* is decreased by X-radiation and suggested that this was due to oxidation of the double bond of the pyrrole groups. Dr H. C. Sutton (personal communication) has independently confirmed the finding (Dale & Russell, 1955) of an increase in absorption at 275 $m\mu$. and a decrease at 405 $m\mu$. in the spectrum of irradiated catalase. Dr Sutton believes, and we agree, that the decrease at 405 $m\mu$. is the important radiation effect, as the absorption at 275 $m\mu$. may be increased by a number of reagents and is not directly related to inactivation of the enzyme. It will be noted that cysteine and glutathione almost completely prevent the decrease in the Soret band, i.e. protect that part which is the active centre of catalase.

Catalase may be inhibited by a number of compounds, including hydrogen sulphide, which combine with the haem group (Keilin & Hartree, 1951). The work reported here suggests that cysteine and glutathione inhibit catalase by virtue of their sulphydryl content, and that inactive complexes are formed between catalase and these substances. The relationship between inactivation and the time of contact of the enzyme and the inhibitor seen in Fig. 1 indicates a comparatively slow rate of formation of this complex. Inhibition of catalase by cysteine has been previously noticed by Stern (1932), Waldschmidt-Leitz, Scharikova & Schäffner (1933) and Boeri & Bonnichsen (1952). The last-named found that catalase could bring about the oxidation of cysteine in the presence, but not in the absence, of oxygen. Oxidation proceeded best when the ratio of cysteine to catalase was low. This, combined with the fact that the inhibition noted in the present paper occurred in nitrogen as well as in air, indicates that the inhibitory mechanism is not linked to any inactivation of catalase during its action upon cysteine, in contrast to the inactivation of catalase during its action on hydrogen peroxide (Bonnichsen *et al.* 1947)). Dr R. K. Bonnichsen (personal communication) says that an inactive complex is probably formed, and that glutathione and thioglycollic acid inhibit catalase. Work in this laboratory (Dale & Davies, unpublished) has shown that thiourea is also inhibitory.

The electrophoretic properties of proteins before and after irradiation have been studied by Kepp & Michel (1953), using serum albumin and globulin. They found that irradiation markedly affected the electrophoretic pattern and that cysteine prevented this change, i.e. protected the protein from the effects of irradiation. The fact that glutathione protects various enzymes against inactivation by irradiation has also been known for some time (Barron & Flood, 1952). Thus the present findings that cysteine and glutathione are protective substances is in accord with the general evidence available.

The results of Forssberg (1947), that cysteine and glutathione increased the radiation effect on catalase, led him to believe that H atoms were the active agents responsible for the inactivation. It has been shown in the present paper that these substances do not increase the radiation effect on catalase, but on the contrary always protect against radiation. There is, therefore, no reason to assume that H atoms are the inactivating agents. The probable mechanism of protection, as with other enzymes, is the competition of catalase with cysteine, glutathione and also cystine for oxidizing radicals (Rotherham *et al.* 1951).

SUMMARY

1. Catalase has been irradiated with X-rays, γ -rays and electrons from a linear accelerator and the effects on the activity, electrophoretic and chromatographic behaviour and the ultraviolet spectrum of the enzyme were examined.
2. It has been found that irradiation causes a profound change in these properties, but that these changes are decreased by the presence of cysteine, cystine and glutathione during irradiation.
3. In the estimation of the catalytic activity in the presence of these substances allowance must be made for the depressive effect they have on the enzymic activity. Some aspects of this depression have been investigated.

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The Oxygen Uptake, Glucose Utilization and Lactic Acid Production of Guinea-pig Skin in Relation to Oxygen Tension

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In spite of the many disorders of unknown aetiology to which the skin is subject, and which may be possibly caused by metabolic abnormalities, accounts of the metabolic activity of skin *in vitro* are relatively scanty as compared with those of internal organs or tumours. Among these reports are the investigations of Peters & Wakelin (1946) and Peters, Sinclair & Thompson (1946) leading to the development of 2:3-dimercaptopropanol (BAL), the work of Dixon & Needham (1946) providing evidence in favour of the enzyme theory of vesication, that of Barron, Meyer & Miller (1948) and of Thompson (1946) on the metabolism of skin and the effect of vesicant agents, the work of Berenblum, Chain & Heatley (1940) on the metabolism of skin and neoplastic tissue, that of Bullough & Johnson (1951) on respiration and mitotic activity, and that of Griesemer & Gould (1954) on the citric acid cycle in epidermal tissues.

Medawar (1948) first demonstrated that skin slices could be maintained *in vitro* for several days, and in a previous paper (Cruickshank, 1954) a differential micro-respirometer was described for making continuous observations of the oxygen uptake of skin slices under similar conditions of cultivation. Some of the factors affecting the respiration of skin slices were discussed in that study. The present work is concerned with the metabolism of glucose by skin under conditions of tissue culture and thus differs from previous reports in that serum was present in the medium and that observations were made over periods of 24 hr. as well as for the usual short initial periods of up to 5 hr. In earlier experiments it had been shown that the oxygen tension affected the respiratory rate, and it seemed desirable to establish the role of oxygen tension in the survival of

skin *in vitro*, and to determine its effect upon glucose consumption and upon lactic acid production.

MATERIALS AND METHODS

The respirometer consists essentially of two chambers in a nickel-plated brass block which are connected by a graduated capillary containing an indicator droplet. A special tap permits the addition of any desired gas mixture to the chambers by means of metal tubes projecting above the level of the water bath. This tap was slightly modified for the present experiments by increasing the size of the Perspex base-plate which was cut in the form of a circle. This was done to permit observations at gas pressures greater than atmospheric without leakage.

The skin slices float on the surface of a culture medium in a glass cup in one of the chambers, a cup containing only medium being placed in the control chamber. CO₂ is absorbed by filter paper soaked with 2% KOH in the floor of each chamber.

The medium consisted of serum 5 parts, Krebs-Ringer phosphate 3 parts, 5% glucose 1 part and streptomycin 500 µg./ml. 1 part. Amounts of 0.5–0.7 ml. were accurately measured into each cup and the utilization of glucose and the production of lactic acid were measured by comparing the final concentrations of these substances in the 'culture' and 'control' cups.

Glucose was estimated by the method of King, Haslewood & Delory (1937*a*). Portions of 0.2 ml. of medium were diluted to volume with isotonic sodium sulphate and deproteinized by precipitation with copper tungstate (King *et al.* 1937*b*).

Lactic acid was estimated by the method of Barker & Summerson (1941) as adapted for blood analysis (Hawk, Oser & Summerson, 1947).

Skin was obtained from the shaved guinea-pig ear. This was prepared the previous day and anointed with a fungicide, 'Mycostatin', to prevent the occasional contamination of 24 hr. cultures with a mould frequently found on guinea-