Biochem. J. (1960) **75**, 557

Carcinogenesis: Changes in the Properties of some Rat-Liver Proteins after Administration of 4-Dimethylamino-3′-methylazobenzene

By J. M. WHITCUTT, D. A. SUTTON and J. R. NUNN
National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, South Africa

(*Received* 25 *August* 1959)

Many changes occur in rat-liver cells during the long period of induction between the first treatment with a carcinogenic azo dye and the appearance of actual cancer cells; these are described in an extensive literature which has been well reviewed. Histological differences are noticeable at an early stage (Chang, Spain & Griffin, 1958), and changes in the chemical composition of the cells have been reported (see Miller & Miller, 1953).

A significant development in recent years has been the attempt by Miller and co-workers to correlate carcinogenicity with the covalent binding of a small proportion of the carcinogen to certain liver proteins. It has been reported that: (i) appreciable binding of azo dye occurs only in the liver of the rat, i.e. the susceptible organ of the susceptible species (Miller & Miller, 1947; Salzberg, 1958); (ii) the rapidity with which maximum binding is reached is proportional to the potency of the carcinogen (Miller, Miller, Sapp & Weber, 1949); (iii) conditions which inhibit carcinogenesis also inhibit dye-binding (Miller & Miller, 1955; Gelboin, Miller & Miller, 1958). Azo carcinogens are not bound to the proteins of liver tumours (Miller & Miller, 1947), and the particular group of proteins to which they are bound in normal cells is largely absent from tumour cells (Sorof & Cohen, 1951). These results are in harmony with the theory that malignant change occurs through the gradual deletion of key proteins essential for growth control (Miller & Miller, 1953, 1955).

Recent work on azo-dye carcinogenesis has described the distribution of bound dye among the various cell components (Price, Miller, Miller & Weber, 1949; Hultin, 1955, 1957; Hughes, 1956; Salzberg, 1958; Hultin & Decken, 1958), the effect of diet upon dye-binding (Conney, Miller & Miller, 1956; Gelboin *et al.* 1958), distribution of dye in the soluble liver proteins (Sorof, Golder & Ott, 1954; Sorof, Young & Ott, 1958) and degradative examination of the proteins binding the dye (Wirtz & Arcos, 1958; Kusama & Terayama, 1957).

The present investigation is an attempt to study some of the changes occurring in the soluble liver proteins of rats a few hours after oral administration of the carcinogenic azo dye 4-dimethylamino-3′-methylazobenzene.

EXPERIMENTAL AND RESULTS

Ultraviolet spectra were measured on a Unicam model SP. 500.

Phosphate buffers were prepared by mixing stock solutions of disodium hydrogen phosphate (0·1 M) and potassium dihydrogen phosphate (0·1 M), and adding the calculated amount of sodium chloride and diluting. The borate buffer was prepared by diluting a stock solution containing boric acid (0·3 M) and sodium hydroxide (0·06 M) and adding the calculated amount of sodium chloride.

Protein solutions were stored at −20°; operations were carried out below 5° wherever possible, and were usually completed within 14 days of isolating the original material. Solutions of large volume were concentrated by standing overnight over calcium chloride at 2 mm. mercury pressure; those of small volume were concentrated and dialysed by means of the apparatus shown in Fig. 1.

Synthesis of 4-dimethylamino-3′-methylazobenzene. (Based on the synthesis of methyl red given in *Organic Syntheses*, Collective Volume 1.) *m*-Toluidine (20 ml.) in hydrochloric acid solution (150 ml. of 3·5 N) was diazotized by slowly adding a solution of sodium nitrite (15·2 g. in 36 ml. of water). After 1 hr. at 0° a solution of dimethylaniline (34 ml.) in dilute hydrochloric acid (75 ml. of water and enough conc. hydrochloric acid to dissolve) was added slowly with stirring. The mixture was allowed to stand for 2 hr. at 0° before adding a solution of sodium acetate (150 ml. of 4 M), and standing for a further 3 hr. with occasional stirring. The product was filtered off, recrystallized several times from ethanol and from benzene, and finally purified by chromatographing it on neutral alumina (E. Merck, Darmstadt, Germany) with *iso*hexane as solvent. M.p. 119·5–120·5° (Found: C, 75·5; H, 7·0; N, 17·1. Calc. for $C_{15}H_{17}N_3$: C, 75·28; H, 7·16; N, 17·56%).

Preparation of livers. The animals were provided by the National Nutrition Research Institute, Pretoria. Young male albino rats (wt. 200–250 g.) of the same strain were housed in wire cages at 20° and fed *ad lib.* on the Institute's stock diet (protein, 20%; ash, 7·4%; main component: maize meal, 56%) and water. A solution of 4-dimethylamino-3′-methylazobenzene (10 mg.) in olive oil (0·5 ml.) was administered orally in one dose and the animals were starved thereafter; in control experiments the same pro-

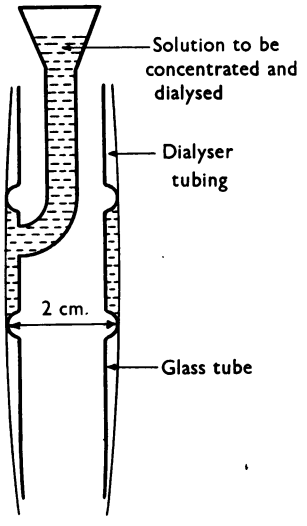

Fig. 1. Sectional view of apparatus for concentrating and dialysing small volumes of protein solutions. The dialyser tubing (visking) seals off the space between the ridges on the outside of the glass tube (0·5–2 ml., depending on the height of the ridges). Solutions are concentrated by standing the apparatus in front of a fan and dialysed by standing it in a beaker of dialysing solution; equilibrium is reached in 2–3 hr. without agitation. Solutions are expelled through the funnel by gently screwing the dialyser tubing at one of the ridges.

cedure was followed, olive oil alone being used. The animals were anaesthetized with ether 13–15 hr. after dosing, the vena cava was opened between the liver and the diaphragm, and the livers were perfused via the right ventricle with warm (40°) sodium chloride solution (0·15 M) for 20 min. or until no red colour remained. In spite of this a thin band due to red blood cells was always obtained in the tissue fractionations carried out immediately afterwards.

Tissue fractionation (see Hogeboom, Schneider & Striebich, 1952). The following operations were carried out below 5°. Perfused rat livers were washed in 0·25 M-sucrose solution, minced with scissors after removal of the larger blood vessels and homogenized one or two at a time for 90 sec. in a Potter–Elvehjem-type homogenizer in the presence of sucrose solution (30 ml. of 0·25 M). The homogenate was centrifuged for 8 min. at 800 *g* and the precipitate was resuspended in sucrose solution (30 ml. of 0·25 M), homogenized and centrifuged once more for 8 min. at 800 *g*. The two supernatant fractions were then combined and centrifuged for 4 hr. at 22 000 *g*. All products other than the final supernatant fraction were discarded.

Chromatography on ion-exchange celluloses. Diethylaminoethyl- (DEAE) and carboxymethyl- (CM) celluloses were prepared as described by Peterson & Sober (1956), highly purified cellulose fibres being used as the base.

The ion-exchange celluloses were thoroughly equilibrated against the starting buffer by suspending them in 0·1 M-buffer of the same pH as the starting buffer and then repeatedly filtering and resuspending them in the starting buffer. The final suspension was evacuated at a water pump to remove air bubbles, poured into the column and allowed to settle by gravity and filtration.

The final supernatant fraction prepared from two livers was dialysed against 2 l. of buffer solution (5 mM-phosphate, 5 mM-chloride, pH 7·9), concentrated to 20 ml. and dialysed once more against the same (used) buffer solution to reduce the volume to 20 ml. and bring it into equilibrium with 2 l. of buffer (5 mM-phosphate, 5 mM-chloride, pH 7·9). It was then chromatographed on DEAE cellulose as shown in Fig. 2 and the fractions shown in solid line were pooled, concentrated to 20 ml. and equilibrated by dialysis against about 200 ml. of 0·01 M-$H_2PO_4^-$, the volume of the latter being varied (depending on the volume of the protein solution before concentration) so as to give a final pH of 6·0. The product, which was yellow from treated rats and faint brown from controls, now contained a faint precipitate and was chromatographed on CM cellulose as shown in Figs. 3 and 4. The fractions shown in Figs. 3 and 4 were collected separately, concentrated to small volume (0·5–1·5 ml., depending on the size of the peak) and dialysed against the electrophoresis buffer (0·025 M-borate, 5 mM-chloride, pH 8·5). The DEAE first-peak proteins from one treated and one control liver were also concentrated to small volume and dialysed against the electrophoresis buffer.

Fractions C, D and E (Fig. 3) and c, d and e (Fig. 4) denatured rapidly and it was not possible to prepare concentrates of these without losing some material in this way.

Starch electrophoresis. The procedure has been fully described (Smithies, 1955). Gels were prepared from potato starch (12 g.; British Drug Houses Ltd.) that had been partially hydrolysed in the manner described by Smithies, and a buffer solution (100 ml. of 0·025 M-borate, 5 mM-chloride, pH 8·5) and were cast in Perspex trays with dimensions 23 cm. × 2 cm. × 0·6 cm. Slots 1–2 mm. wide were cut across the gels and filled with a supporting medium of dry starch grains previously floated in acetone to remove

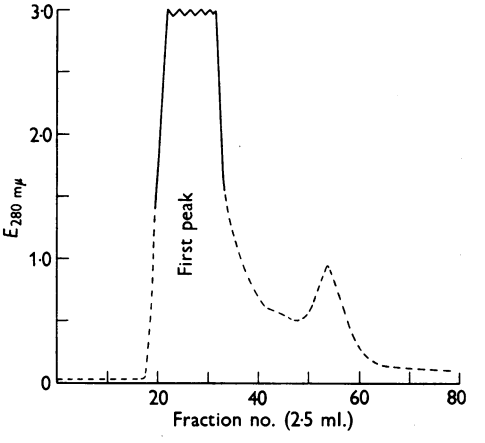

Fig. 2. Incomplete DEAE cellulose chromatogram of the supernatant fraction from two treated rat livers (20 ml. equilibrated against the starting buffer). Column, 20 cm. × 2·4 cm. (12 g.). Temperature, 2°. Flow rate, 30 ml./hr. Solvent: 5 mM-PO_4^{3-}, 5 mM-Cl^-, pH 7·9. Fractions under the solid line were combined to give the DEAE first peak. Untreated livers give the same graph.

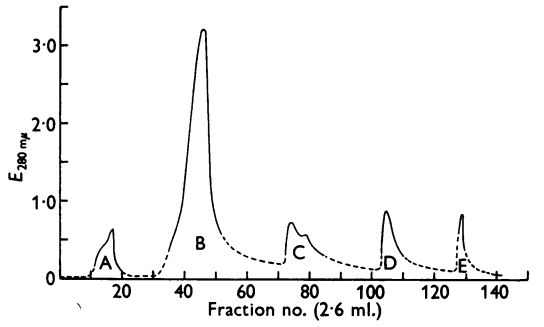

Fig. 3. CM cellulose chromatogram of the DEAE first-peak fraction (Fig. 2) from two untreated livers (15 ml. equilibrated against the starting buffer). Column, 13 cm. × 1·4 cm. (3 g.). Temperature, 2°. Flow rate, 30 ml./hr. Solvents: 0·01 M-PO_4^{3-}, 0·01 M-Cl^-, to fraction 23; 0·02 M-PO_4^{3-}, 0·02 M-Cl^-, to fraction 63; 0·03 M-PO_4^{3-}, 0·03 M-Cl^-, to fraction 95; 0·05 M-PO_4^{3-}, 0·05 M-Cl^-, to fraction 120; 0·1 M-PO_4^{3-}, 1·0 M-Cl^-, to fraction 143. All solvents were at pH 6·0. No gradients. Fractions under the solid lines were combined to give CM peaks A–E.

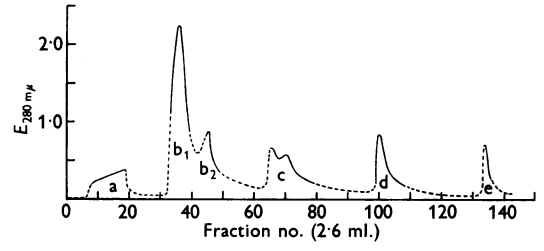

Fig. 4. CM cellulose chromatogram of the DEAE first-peak fraction (Fig. 2) from two treated livers (20 ml. equilibrated against the starting buffer). Column, 13 cm. × 1·4 cm. (3 g.). Temperature, 2°. Flow rate, 30 ml./hr. Solvents: 0·01 M-PO_4^{3-}, 0·01 M-Cl^-, to fraction 24; 0·02 M-PO_4^{3-}, 0·02 M-Cl^-, to fraction 56; 0·03 M-PO_4^{3-}, 0·03 M-Cl^-, to fraction 94; 0·05 M-PO_4^{3-}, 0·05 M-Cl^-, to fraction 127; 0·1 M-PO_4^{3-}, 1·0 M-Cl^-, to fraction 143. All solvents were at pH 6·0. No gradients. Fractions under the solid lines were combined to give CM peaks a–e.

the smaller particles. Concentrated protein solutions were then applied to the surface of the supporting medium by means of a capillary pipette and allowed to soak in until all the air had been displaced from the spaces between the grains. A potential of 15 v/cm., giving a current of 3–5 mA, was applied for 5 hr. during which time the whole system was kept at a temperature of 5°. As there was sometimes considerable smearing on the upper and lower surfaces of the gels, the latter were sliced in half horizontally before staining with Amido-Black 10 B, and, after excess of staining reagent had been washed out, the gels were soaked in water until they returned to their original size.

Protein bands containing azo dye were detected by soaking one strip in 15% trichloroacetic acid solution for 1 hr. to bring out the pink colour of the dye, and afterwards comparing it with the other half of the gel which had been stained with Amido-Black 10 B.

Diagrams showing the patterns of bands obtained when various fractions were subjected to starch-gel electrophoresis are given in Fig. 5.

Estimation of azo dye bound to protein. Rapid qualitative estimations were made by heating 1 ml. of protein solution over a water bath until precipitation was complete. Trichloroacetic acid solution (1 ml. of 15%, w/v) was then added and the precipitate developed a pink colour if dye was present.

For quantitative determinations, the dried protein sample (50–80 mg.) was extracted with ethanol (50 ml.) for 15 min. at 75°. After centrifuging and decantation, the precipitate was re-extracted twice with a boiling mixture of ethanol (30 ml.) and diethyl ether (10 ml.). The extracts were combined, concentrated to 5 ml., chilled, 5 ml. of chilled 7 N-hydrochloric acid solution was added and the extinction measured after 15 min. at 505 mμ in a 4 cm. cell. The blank correction was obtained after addition of 0·45 ml. of a saturated solution of stannous chloride in conc. hydrochloric acid to reduce the azo dye, and the difference between the two readings gives the amount of free dye present. The above method is that of J. Dijkstra & F. J. Joubert (in preparation).

The residue from the above-described extractions was dried under vacuum, a weighed amount was hydrolysed with potassium hydroxide solution and the hydrolysate was extracted with *n*-hexane to obtain non-polar bound dye and with diethyl ether to obtain polar bound dye. The procedure has been fully described by Miller & Miller (1947) and the only modification introduced was the addition of two drops of the stannous chloride solution at the end of each determination to get the blank correction. Results are given in Table 1.

DISCUSSION

Chromatography of soluble rat-liver proteins on DEAE cellulose (Fig. 2) gave a first-peak concentrate of neutral and basic proteins comprising about 30% of the total soluble protein. This product was separated into five components by rechromatographing on CM cellulose (Fig. 3). A small amount

Table 1. *Azo-dye analysis of protein fractions from livers of rats treated with 4-dimethylamino-3′-methylazobenzene*

The DEAE first peak (see Fig. 2) contains all the supernatant liver proteins with which azo dye is associated as 'free' dye, 'polar bound' dye or 'non-polar bound' dye. Peaks a–e (see Fig. 4) are subfractions of this material.

Fraction		10^9 × concn. of dye (moles/100 mg. of protein)
DEAE first peak		20 (free dye)
		2 (non-polar bound dye)
		60 (polar bound dye)
Peak	a	+ (slight)
	b_1	−
	b_2	−
	c	−
	d	+
	e	+

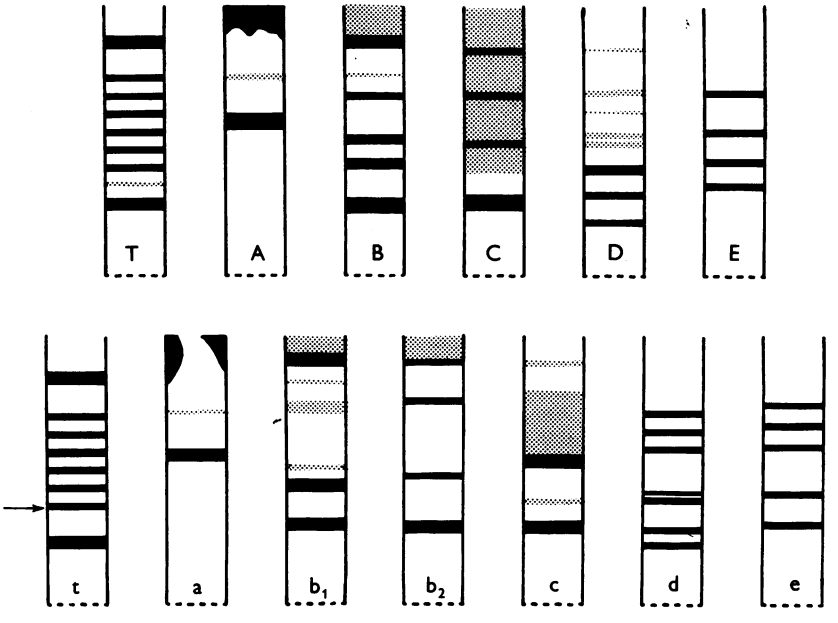

Fig. 5. Diagram showing the patterns produced by starch-gel electrophoresis of protein fractions from treated and untreated rat livers after staining the gels with Amido-Black 10B. Only that section of the gel between the sample slot (top) and the cathode (broken line) is shown, although components which moved slowly in the direction of the anode were also present in some cases. Stippling denotes fainter bands and areas of stippling indicate background absorption strong enough to obliterate such bands. Gels t and T were obtained from the DEAE first peaks of treated and untreated livers respectively (see Fig. 2); the band bearing the azo dye is marked with an arrow. Gels A–E were obtained from the corresponding untreated CM peaks A–E (Fig. 3) and gels a–e from the corresponding treated CM peaks a–e (Fig. 4).

of material was always irreversibly adsorbed on to CM cellulose columns and peaks showed a tendency to trail, with the result that some of the later peaks contain material also present in earlier ones. Use of a cation-exchange resin having groups less acidic than carboxyl may enable these difficulties to be overcome.

The preparative procedures described above gave little indication of the degree of separation actually achieved, and a sensitive method was required for the qualitative analysis of complex mixtures of proteins. Starch-gel electrophoresis was found to be very satisfactory for this purpose and gave reproducible results on small amounts of material. All the starch-gel bands discussed in this paper migrate in the direction of the cathode.

Starch-gel electrophoresis of the DEAE first peak (Fig. 2) gave a pattern of eight distinct bands (Fig. 5), the slowest of which is probably an artifact produced by fractionation on DEAE cellulose, since it does not appear in starch-gel patterns of the total soluble liver proteins. When the DEAE first-peak proteins were rechromatographed on CM cellulose, starch-gel electrophoresis of the resulting peaks showed that the material has been separated into several different groups of proteins. However, the bands given by peaks A–E cannot all be correlated with the bands given by the DEAE first peak from which they were obtained.

The authors consider that use of the separative procedures in sequence gives a greater degree of resolution than any previously obtained. It is necessary to emphasize that the DEAE first-peak proteins to which this investigation was confined are only a small group of the total liver proteins.

Soluble liver proteins from rats which had been fed with the carcinogenic azo dye 4-dimethylamino-3′-methylazobenzene a few hours previously were fractionated by the procedures discussed above. Chromatography on DEAE cellulose gave the same separation as with the untreated liver proteins; protein-bound dye was found only in the first peak. A complete DEAE chromatogram of the soluble liver proteins from a treated liver is shown in Fig. 6. In Fig. 6 the two peaks of Fig. 2 were eluted as a single first peak, and this was the only peak to contain protein-bound dye.

Starch-gel electrophoresis of the DEAE first-peak proteins from treated livers gave a pattern in no way significantly different from that given by

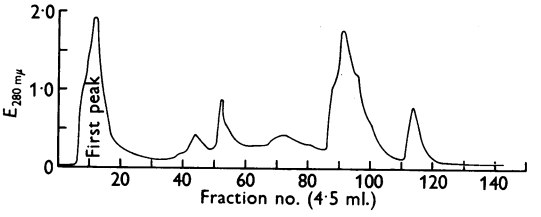

Fig. 6. Complete DEAE cellulose chromatogram of the supernatant fraction from one treated rat liver (20 ml. equilibrated against the starting buffer by dialysis). Column, 13 cm. × 1·4 cm. (3 g.). Temperature, 2°. Flow rate, 54 ml./hr. Solvents: 5 mM-PO_4^{3-}, 5 mM-Cl^-, pH 7·9, to fraction 30; 0·05 M-PO_4^{3-}, 0·05 M-Cl^-, pH 5·9, to fraction 80; 0·1 M-PO_4^{3-}, 1·0 M-Cl^-, pH 4·5, to fraction 145. Solvents were run through a mixing chamber (hold-up volume 150 ml.) to provide a gradient, so the final concentrations were never reached.

untreated livers (see Fig. 5). The band marked with the arrow was the only band in which azo dye could be detected.

When the DEAE first-peak proteins from a treated liver were rechromatographed on CM cellulose, a separation was obtained identical with that obtained with the corresponding untreated material, except that the single peak B was now replaced by the two peaks b_1 and b_2 (compare Figs. 3 and 4). Neither b_1 nor b_2 contained any bound azo dye, nearly all of which was found in peaks d and e.

The patterns obtained after starch-gel electrophoresis of the various CM peaks are shown in Fig. 5. Peaks b_1 and b_2 appear to contain the same components present in the corresponding peak B from the untreated liver, but one of these is present only in b_1 and others are largely concentrated in b_2. Peaks d and e from the treated livers were extremely unstable and attempts to develop azo-dye bands after starch-gel electrophoresis were unsuccessful; the only colour obtained was in the vicinity of the slot, indicating that the dye protein had denatured in the early stages of electrophoresis.

Since in most other respects the DEAE first-peak proteins from treated and untreated livers had almost identical behaviour, it seems that the majority of these proteins were not affected by administration of the carcinogen. It is significant that, in spite of the extensive fractionation carried out, there was no evidence at any stage to show that the dye was bound to more than one component, and this supports the contention that binding of azo dye to liver protein is highly specific.

Sorof *et al.* (1958), who carried out a very efficient fractionation of the so-called 'h' proteins from treated rat livers and obtained six sub-components, did in fact find that two of these contained bound azo dye. We hestitate to draw any comparisons between our results and those of Sorof *et al.* because we do not know how far our DEAE peak corresponds to their 'h' proteins. Furthermore, we have confined ourselves to the effects of a single 10 mg. dose of carcinogen over a few hours, whereas Sorof *et al.* used a continuous dose (about 5 mg./day) over a period of 2½ weeks, and it is possible that other changes occur in the protein composition of the cell during this time. As the half-life of the protein-bound dye is only 4 days (Gelboin *et al.* 1958) a considerable amount of dye already bound to protein must have been metabolized during this period.

Our results show that in addition to dye-binding there is an immediate qualitative change in a group of soluble liver proteins that do not bind the dye. This has not been shown before.

SUMMARY

1. Soluble rat-liver proteins were fractionated in sequence by anion- and cation-exchange chromatography and by starch-gel electrophoresis.

2. A fraction corresponding to the so-called 'slow-moving proteins' was resolved into eight components.

3. Only one of these components contained covalently bound azo dye when the carcinogen 4-dimethylamino-3'-methylazobenzene was fed to the animals shortly before they were killed.

4. The properties of another component were found to have changed as a result of feeding with the dye.

The authors wish to thank Dr W. S. Rapson for his interest and encouragement throughout this work. We are greatly indebted to Dr A. W. Lategan for making certain facilities in the South African Bureau of Standards available to us, and to Dr F. W. Quass for providing all the animals used in these experiments.

This project was sponsored by the National Cancer Association of South Africa.

REFERENCES

Chang, J. P., Spain, J. D. & Griffin, A. C. (1958). *Cancer Res.* **18**, 670.

Conney, A. H., Miller, E. C. & Miller, J. A. (1956). *Cancer Res.* **16**, 450.

Gelboin, H. V., Miller, J. A. & Miller, E. C. (1958). *Cancer Res.* **18**, 608.

Hogeboom, G. H., Schneider, W. C. & Striebich, M. J. (1952). *J. biol. Chem.* **196**, 111.

Hughes, P. E. (1956). *Aust. J. exp. Biol. med. Sci.* **35**, 173.

Hultin, T. (1955). *Exp. Cell Res.* **10**, 71.

Hultin, T. (1957). *Exp. Cell Res.* **13**, 47.

Hultin, T. & Decken, A. von der (1958). *Acta chem. scand.* **12**, 596.

Kusama, K. & Terayama, H. (1957). *Gann*, 48/2, 151.

Miller, E. C. & Miller, J. A. (1947). *Cancer Res.* **7**, 468.

Miller, E. C. & Miller, J. A. (1955). *J. nat. Cancer Inst.* **15**, 1571.

Miller, E. C., Miller, J. A., Sapp, R. W. & Weber, G. M. (1949). *Cancer Res.* **9**, 336.
Miller, J. A. *Org. Synth.* (*Coll.*) (1958). 2nd ed., **1**, 374.
Miller, J. A. & Miller, E. C. (1953). *Advanc. Cancer Res.* **1**, 339.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Price, J. M., Miller, E. C., Miller, J. A. & Weber, G. M. (1949). *Cancer Res.* **9**, 398.
Salzberg, D. A. (1958). *Cancer Res.* **18**, 768.
Smithies, O. (1955). *Biochem. J.* **61**, 629.
Sorof, S. & Cohen, P. P. (1951). *Cancer Res.* **11**, 376.
Sorof, S., Golder, R. H. & Ott, M. G. (1954). *Cancer Res.* **14**, 190.
Sorof, S., Young, E. M. & Ott, M. G. (1958). *Cancer Res.* **18**, 33.
Wirtz, G. H. & Arcos, J. C. (1958). *Experientia*, **14**, 177.

Biochem. J. (1960) **75**, 562

Carbon Dioxide Fixation in *Trichomonas vaginalis*

BY R. WELLERSON, G. E. DOSCHER AND A. B. KUPFERBERG
Ortho Research Foundation, Raritan, New Jersey, U.S.A.

(*Received* 18 *December* 1959)

Reports of carbon dioxide fixation by non-chlorophyll-containing protozoa are rarely encountered in the literature. With the exception of a report by Kupferberg, Singher, Lampson, Levy & Romano (1953) on *Trichomonas vaginalis*, all other studies have been confined to *Tetrahymena* (van Niel, Thomas, Ruben & Kamen, 1942; Ryley, 1952; Lynch & Calvin, 1952) and *Trypanosoma* (Searle & Reiner, 1940; Moulder, 1948).

Kupferberg *et al.* (1953) demonstrated that *T. vaginalis* fixed carbon dioxide during growth and that the product was almost entirely outside the cell. This material isolated from the culture medium served as the starting material for this study. The ^{14}C-labelled product has been isolated and identified and the site of the radioactive carbon determined.

METHODS

Fixation material. The entire investigation was conducted with the $^{14}CO_2$-fixation product isolated by Kupferberg *et al.* (1953). This material had been freeze-dried and stored in a closed container at 5°.

Chromatographic procedures. The freeze-dried material was reconstituted with water, passed through an Amberlite IR-120 cation-exchange column (1·1 cm. × 16 cm.) in the hydrogen form, and washed from the column with water at the rate of 14–16 drops/min. The material was prepared for chromatography by concentration of the eluate *in vacuo*. The dried material was resuspended in water (0·5–1·0 ml.) and was spotted or streaked onto Whatman no. 1 filter paper. The chromatograms were run ascending at 26°, either the ethyl ether–acetic acid–water (13:3:1, by vol.) system of Denison & Phares (1952) or a butanol–88% formic acid (95:5, v/v) system saturated with water, being used. The former system was used initially for a rapid differentiation of organic acids whereas the latter was employed when better definition of lactic acid and malic acid was required. The chromatograms were air-dried and then steamed gently while being exposed to the heat of an infra-red lamp, as described by Denison & Phares. The acids were identified on the paper chromatogram as yellow spots on a purple background after spraying with bromophenol blue (0·045 g./100 ml. of ethanol, pH 7·0). The location of the radioactivity on the paper chromatograms was determined either by placing the chromatogram in direct contact with X-ray film (Kodak Ltd. No-Screen) for 31 days, or by dividing it into small segments (5 mm. × 25 mm.) and making direct counts on each section with a windowless Geiger–Müller counter.

Preparation for infrared spectrophotometry. To accumulate sufficient material for the infrared studies, large sheets (50 cm. × 50 cm.) of Whatman no. 1 filter paper were streaked with the concentrated material along the length of the origin. The chromatograms were run ascending at 26° with the butanol–formic acid system. The location of the acids was established by cutting a thin vertical strip from each edge and a third from the centre and spraying them with the indicator. The acids were eluted from the unsprayed chromatogram with water, each zone being eluted several times with a total volume of 100 ml. Each eluate was filtered through Whatman no. 2 filter paper, concentrated *in vacuo* and the residue treated several times with 95% ethanol. The ethanol solutions were pooled and concentrated and the insoluble residue was discarded. The concentrated material was placed between KCl crystals and the infrared spectrum was determined in a Baird infrared spectrophotometer.

Degradations studies. Material to be degraded was obtained in the same manner as that for the infrared determinations. Lactic acid, with and without carrier, was degraded according to the procedure of Elsden & Gibson (1954), and the aeration technique was used. The aldehyde was trapped as the bisulphite complex and the carbon dioxide was trapped in NaOH. The carbonate was precipitated as $BaCO_3$ by the addition of a saturated solution of $BaCl_2$. Self-absorption corrections were made as described by Schweitzer & Stein (1950).