DISCUSSION

Precipitation with ethanol was used to obtain fractions by a mild method. With hot-water extracts a large amount of material, containing much mineral matter and two-thirds of the total N, was precipitated by ethanol. From cold-water extracts ethanol precipitated much less material. The precipitated material contains carbohydrate, ether-soluble compounds, N-containing compounds and mineral constituents.

The carbohydrate consists of starch and a carbohydrate apparently containing galactose. The presence of galactose in hemicellulose obtained from ungerminated peas was shown by Schulze, Steiger & Maxwell (1890) and Schulze (1892). The ether-soluble matter is present in large amount. The presence of lecithin in the pea has been demonstrated by Winterstein & Hiesstand (1908). The distribution of N shows that a large proportion of it is present in compounds of a basic nature. The trace elements of the large mineral portion of the fraction are of interest, particularly the Mo, previously demonstrated by Ter Meulen (1932) and by Bertrand (1939).

In the ethanol-soluble fraction carbohydrate forms a large proportion of the dry weight but the ether-soluble portion is small in amount. The N is apparently present mainly in compounds of a basic nature. The inorganic moiety appears to consist mainly of potassium phosphate and is not important in penicillin production.

Although both the ethanol-soluble and ethanol-insoluble fractions support growth of the mould, it is the ethanol-precipitable fraction which stimulates penicillin production. There are, however, com-

ponents in the ethanol-soluble fraction which increase its activity, particularly if the extracts have been prepared with cold water or if germinated or fresh pea extracts are used.

Routine determinations of pH showed that although an increase in pH of the medium was often associated with increased penicillin production this is not always so. A marked increase in pH was always shown early in media in which the protein and starch contents were high, as for example, in unclarified extracts.

SUMMARY

1. A scheme of preliminary fractionation of aqueous extracts of ground dried peas to separate the constituents responsible for penicillin production is described.

2. 80% ethanol precipitates a fraction which is very active. The ethanol-soluble portion, although it supports growth, has only slight activity in affecting penicillin production, but in the presence of the precipitable fraction increases the latter's activity.

3. Preliminary analyses of the 80% ethanol-soluble and ethanol-insoluble fractions are given.

4. The inorganic constituents of the precipitable fraction are important. The migration of certain trace elements from the medium to the mycelium has been demonstrated.

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The Glucose Metabolism *in vitro* of *Trypanosoma rhodesiense*

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It was first shown by Biot, Biot & Richard (1911) that glucose is favourable to the continued life and motility *in vitro* of the rat-trypanosome *T. lewisi*; this effect of glucose has been confirmed by many authors using different species of trypanosomes.

Kudicke & Evers (1924) showed that other sugars and glycerol could be used to maintain *T. brucei* in culture media. The rates of glucose decomposition, oxygen consumption, and acid production by certain trypanosomes have been determined (Nauss &

Yorke, 1911; Fennyvessy & Reiner, 1924, 1928; Fennyvessy, 1926; Reiner, Smythe & Pedlow, 1936; Christophers & Fulton, 1938). The mechanism of breakdown of glucose and glycerol was studied by Reiner *et al.* (1936) in the case of *T. equiperdum* and *T. lewisi*, and the metabolic products described; the role of CO_2 in the glucose metabolism of *T. lewisi* has been investigated more recently by Searle & Reiner (1940, 1941).

In the case of a normal and an arsenic-resistant strain of *T. rhodesiense* kept in buffered medium at 37°, Christophers & Fulton (1938) found that the ratio of molecules of glucose decomposed to oxygen consumed approached unity; the ratio of equivalents of acid formed to oxygen consumed was approximately 2, so that for one molecule of glucose used, two equivalents of acid were formed. In the present experiments, the nature of the glucose metabolic products of the normal strain of *T. rhodesiense* has been determined. The quantitative aspect of this investigation suffered through enemy action.

MATERIALS AND METHODS

The strain of *T. rhodesiense* studied was that used in earlier work (Christophers & Fulton, 1938). To obtain live trypanosomes in quantity, mice were infected intraperitoneally; when their blood was swarming with trypanosomes, cardiac puncture was performed aseptically under anaesthesia and as much blood as possible withdrawn. The blood was mixed in centrifuge tubes with about one-third of its volume of 2% sodium citrate containing 0.1% glucose. The pooled blood from a number of animals was centrifuged for 10 min. at 1700 r.p.m. The plasma layer was removed, and then the layer of trypanosomes was pipetted off from above the red cell layer. After suspension of the trypanosome mass in glucose-Ringer solution, the whole was recentrifuged at the same speed for 5 min. and the process of washing repeated. The washed trypanosomes, now free from red cells, were removed in volumes of 1 ml. at a time, and added to 100 ml. of a mixture of 1 volume of $\text{m}/15$ phosphate buffer (pH 7.4) and 1 volume of Ringer's solution containing 0.6% glucose. This investigation was carried out on a very large scale and many hundreds of mice were used.

In one series of experiments 3% (v/v) of horse serum was added to the medium, since it, like rabbit serum, appeared to exercise a favourable influence on the survival of the trypanosomes as judged by the results of respiration and the yield of metabolic products, which was much larger when serum was present. In another series of experiments, serum was not added.

RESULTS

The dilute suspensions of trypanosomes were kept in sterile flasks at room temperature with frequent shaking. Trypanosomes can be kept alive for long periods under these conditions, but at the end of 6 hr., when the medium had become strongly acid, proteins were precipitated by the addition of $\frac{1}{2}\text{N}$ - H_2SO_4 and an equal volume of 10% sodium tungstate. The precipitate was removed by centrifuga-

tion and examined for the presence of metabolic products, with negative results.

Detection of pyruvic acid. No aldehyde was detected in the medium before or after precipitation of protein. Tests were made for the presence of ketones by adding 2:4-dinitrophenylhydrazine in 2N-HCl to the protein-free metabolic solution, when a yellow amorphous precipitate appeared after a short time; it was collected by centrifugation. The product separated in microcrystalline form on solution in a large volume of chloroform followed by concentration, after filtration from a little insoluble material. The product was then dissolved in ethyl acetate, extracted with sodium carbonate solution, and recovered by acidification. The deep yellow leaflets (found: C, 40.3; H, 3.0; N, 20.3%. Calc. for $\text{C}_9\text{H}_8\text{O}_6\text{N}_4$: C, 40.3; H, 3.0; N, 20.8%) were identical (m.p. and mixed m.p. 213° decomp.; cf. Allen, 1930) with authentic pyruvic acid dinitrophenylhydrazone.

Detection of ethanol. Four lots of 600 ml. of the protein-free metabolic solution were slowly distilled using a very efficient fractionation column; 50 ml. of distillate were collected from each lot up to 100° and gave a positive iodoform reaction. The resulting 200 ml. of distillate were redistilled, and 2 ml. of a liquid boiling at approximately 78° collected. It was identified as ethanol by various tests; the *p*-nitrobenzoate melted at 56.5° alone or mixed with an authentic specimen.

Detection of acetic and formic acids. The remainder of the protein-free solution was concentrated at 16–20 mm. Hg pressure at a temperature not exceeding 40°. A trap containing 0.1N-NaOH was inserted to catch any volatile acid which distilled. The contents of the trap were allowed to evaporate in air, yielding c. 4.5 g. of mixed salts, including some carbonate. Part of this material was dissolved in dilute sulphuric acid and fractionally extracted, first with ether-light petroleum and then with ether. The first small fraction (containing 3–4% of the total organic acid) gave no evidently homogeneous *p*-phenylphenacyl ester; the next (like the original mixture of salts) readily yielded the acetate (m.p. and mixed m.p. 111°; cf. Drake & Bronitsky, 1930. Found: C, 75.7; H, 5.5%. Calc. for $\text{C}_{16}\text{H}_{14}\text{O}_3$: C, 75.6; H, 5.5%). The last fractions were rich in formic acid, its presence being confirmed by reducing another portion with magnesium and dilute sulphuric acid; and distilling; the distillate afforded methylene-bis-dimedone, m.p. and mixed m.p. 184–188° (cf. Klein & Linser, 1929. Found: C, 69.8; H, 8.0%. Calc. for $\text{C}_{17}\text{H}_{24}\text{O}_4$: C, 69.9; H, 8.2%). A further portion of the salts was steam-distilled from dilute sulphuric acid, and the total acidity and the formic acid content (Auerbach & Zeglin, 1923) determined in the distillate; the formic acid accounted for 50.0% of the acidity.

Detection of succinic acid. The bulk of the metabolic solution was reduced to 700–800 ml., then exhaustively extracted with ether, and the ether extract, after drying with anhyd. sodium sulphate, was allowed to evaporate in air in the cold room. A rapid separation of crystals followed. The crystals (1.61 g.), from about 4 g. of total extract, proved to be succinic acid as shown by m.p. and mixed m.p. with an authentic specimen. (Found: C, 40.6; H, 5.2%. Calc. for $C_4H_6O_4$: C, 40.7; H, 5.1%.)

Detection of lactic acid. The syrupy residue from which succinic acid had crystallized gave negative results when tested for oxalic, malic, tartaric, and citric acids; it gave no coloration with ferric chloride solution. No appreciable precipitate was produced on treatment first with neutral and then with basic lead acetate. Lead was removed by hydrogen sulphide, the filtrate evaporated below 70° and boiled with water and excess of zinc carbonate. Concentration of the filtered solution yielded zinc lactate, characterized by conversion, via the sodium salt, into *p*-phenylphenacyl lactate, m.p. and mixed m.p. 141° (cf. Drake & Bronitsky, 1930. Found: C, 72.0; H, 5.4%. Calc. for $C_{17}H_{16}O_4$: C, 71.8; H, 5.6%). The total yield corresponded to approximately 2.4 g. lactic acid.

Detection of glycerol. The mother liquors from the zinc lactate were freed from a little zinc by sodium carbonate, and treated with benzoyl chloride and sodium hydroxide (Lipp & Miller, 1913), yielding 0.2–0.3 g. tribenzoylglycerol, m.p. and mixed m.p. 71°. (Found: C, 71.1; H, 5.2%. Calc. for $C_{24}H_{20}O_6$: C, 71.3; H, 5.0%.)

Absence of gluconic acid. No gluconic acid was found by the application of the method of Birkinshaw & Raistrick (1931) to the residue of the metabolic solution after ether extraction.

Metabolic products in absence of serum

As stated earlier, trypanosomes were also kept in a medium from which serum was absent, and the products of metabolism isolated as described above. The yields were, however, much smaller; the trypanosomes do not survive so well in absence of serum. From the salts of the volatile acids, acetic and formic acids were recognized. Of the volatile acids, 49.7 equivalents % were due to formic acid. Succinic acid and lactic acid were also identified, but the suspected presence of glycerol could not be established.

DISCUSSION

The main series of experiments on the glucose metabolism of *T. rhodesiense* was carried out with a medium which contained small amounts of serum in order to aid the survival of the parasites, thus increasing the yield of products. Brand (1933) concluded that protein breakdown by suspensions of

T. brucei was negligible. Reiner *et al.* (1936) also believed that the course of decomposition of glucose by *T. lewisi* and *T. equiperdum* was uninfluenced by the presence of serum. They found, however, that the mechanism of glucose decomposition was very different in the two species of trypanosome. The products obtained in their experiments have all been encountered in the present investigation, in which succinic, pyruvic, lactic, acetic and formic acids, glycerol and ethanol have been identified. Previously, in respiration experiments, the production of small amounts of CO_2 was also established.

The formation of large amounts of succinic acid (approx. 40% of the acids extracted) is of great interest. Pasteur (1860) noted that it was formed in small amounts during alcoholic fermentation of sugar by yeast. Ehrlich (1909) suggested that it arose from glutamic acid formed by autolyzed yeast cells, but Kleinzeller (1941) showed that this view was incorrect and discussed the mechanism of the formation of succinic acid from glucose. Wood & Werkman (1940), in their study of the utilization of CO_2 by bacteria, noted that inhibition of its use caused a decrease in the amount of succinic acid formed. This fact was considered as evidence that succinic acid might be formed by the union of 1- and 3-carbon compounds, but the condensation of acetic or pyruvic acid was suggested as an alternative method of formation. They drew attention to the possibility that fixation of CO_2 with formation of succinic acid may be a general biological phenomenon. Searle & Reiner (1941) showed that *T. lewisi* also assimilated CO_2 , but such fixation has not so far been demonstrated for *T. rhodesiense*. Krebs (1943) pointed out that most micro-organisms produce CO_2 from added substrate, and thus mask a simultaneous uptake. He considered that carboxylation of pyruvic acid is the chief mechanism whereby the 4-carbon chain arises. The observation of Searle & Reiner (1941) that pyruvic acid is not metabolized by *T. lewisi* however suggests some other method of formation.

Lactic and pyruvic acid formation by glycolysis and oxidation is a familiar reaction of many types of cell, and by further oxidation acetic acid may be produced. The formation of formic acid has, in the case of other organisms, been explained by the oxidation of formaldehyde resulting from the reduction of CO_2 . At present we have no proof that this occurs in the case of *T. rhodesiense*, and aldehyde was never detected in the metabolic solutions. When Reiner & Smythe (1934) investigated the glucose metabolism of *T. equiperdum*, they suggested that the main path of glucose utilization by this organism *in vitro* was as follows:

Both pyruvic acid and glycerol have been isolated as metabolic products of *T. rhodesiense* by us. The mode of formation of the ethanol is less clear.

SUMMARY

1. Products of the glucose metabolism *in vitro* by suspensions of *T. rhodesiense* have been isolated

and characterized. They were: succinic, pyruvic, lactic, acetic and formic acids, glycerol, ethanol and carbon dioxide.

2. The possible mode of formation of some of these substances is discussed.

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On the Distribution of Glyoxalase and Glutathione

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The experiments to be described were directed to determine the presence or absence of glyoxalase and glutathione in such living organisms as have not been previously examined from this point of view. Our results added to those published indicate an exceedingly wide distribution of these cell constituents, especially of the enzyme. On the animal side we have dealt chiefly with invertebrates, which have been but little explored. Dakin & Dudley (1913) stated that glyoxalase is present in the oyster, and Lohmann (1936) found it in the muscle of the octopus. We have been unable to find in the extensive literature on the subject any other similar reference to invertebrates. We have also proved the presence of glyoxalase in several plant species in which its presence has not been previously shown.

SECTION I. GLYOXALASE

Method. Our method has been that used by Lohmann (1932), Jowett & Quastel (1933), and Platt & Schroeder (1934), namely, the determination of the rate of CO₂ evolu-

tion, from sodium bicarbonate solution, due to the lactic acid production from methylglyoxal. We employed Barcroft differential manometers provided with standardized flasks. Each flask contained 1.0 ml. of the tissue extract, 0.6 ml. of 0.2M-sodium bicarbonate, with 2 mg. of glutathione in 0.3 ml. water in a Keilin cup. To one flask, 2 mg. of methylglyoxal were added and the total contents of each flask made up to 3 ml. with distilled water. The apparatus was filled with N₂ + 5% CO₂ gas mixture, the glutathione tipped in and the manometer shaken in a bath at 25°. The stopcocks were left open for 4 min. and then closed.

Invertebrates

In each case the organisms were ground up with sand in a mortar with five times their weight of distilled water. After 30 min. the material was centrifuged, 10 ml. of the supernatant fluid were tied in a short length of cellophan tubing, dialyzed for 24 hr. against distilled water, with frequent changes and then made up to 12 ml. The 1.0 ml. of extract used in the Barcroft apparatus corresponded to 0.166 g. of tissue.