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 stop-cocks 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.

Table 1. *Glyoxalase activity of invertebrates*

Phylum (and class)	Species	Name	Part used	$\mu\text{l. CO}_2$ evolved		
				20 min.	60 min.	
Coelenterata (Anthozoa)	<i>Actinia equina</i>	Anemone	Whole	129.2	327.2	
Nematoda	<i>Ascaris Lumbricoides</i>	Round worm (pig)	"	405.0	—	
Annelida (Polychaeta)	<i>Aphrodite aculeata</i>	Sea mouse*	"	84.0	240.0	
Annelida (Oligochaeta)	<i>Lumbricus</i> sp.	Earthworm	"	21.0	21.0	
Arthropoda (Crustacea)	<i>Carcinus maenas</i>	Shore crab	Hepatopancreas	204.0	558.0	
Arthropoda " (Insecta)	<i>Bombyx " mori</i>	"	Muscle	82.5	244.7	
		Silk worm (larva)	Whole	96.0	261.0	
	<i>"</i>	Silk worm (pupa)	"	132.0	342.0	
		<i>Auriflua</i>	Caterpillar (larva)	"	45.0	99.0
	<i>"</i>	Caterpillar (pupa)	"	42.0	132.0	
		<i>Locusta</i>	Locust*	"	321.7	—
	<i>"</i>	<i>Athous haemorrhoides</i>	Wireworm	"	60.0	300.0
Mollusca (Gastropoda)		<i>Helix aspersa</i>	Garden snail	"	88.0	217.2
Mollusca " (Lamellibranchiata)	<i>Patella vulgata</i>	Limpet	"	121.0	324.5	
	<i>Mytilus edulis</i>	Mussel	"	150.0	390.0	
	<i>"</i>	<i>Paphia (Tapes)</i> sp.	—	"	189.0	513.0
	<i>"</i>	<i>Chione (Venus)</i> sp.	—	"	93.8	239.2
	<i>"</i>	<i>Ostrea edulis</i>	Oyster	"	63.2	178.7
Protochordata (Tunicata)	<i>Ascidella aspersa</i>	Sea squirt*	"	39.0	102.0	

* In the case of the sea mouse the cuticle was removed, the test from the sea squirt, and the legs and wings from the locusts.

It will be seen from the results (Table 1) that the concentration of the enzyme (or at least its activity) is considerably less than that recorded in the literature for mammalian organs. It must be remembered, however, that the enzyme is likely to be confined to some special tissue or tissues, so that in the extracts from the whole organism its concentration must be greatly reduced. Again for a similar reason the results do not indicate the relative physiological importance of the enzyme among the different species. Also the temperature employed in the experiments was 25° instead of 37° as used by some authors. Girsavicius, Efendi & Ryzhova (1936) have shown that the temperature coefficient of glyoxalase in this region is from 1.5 to 1.6. Even for the lowest results given in Table 1 there is evidence that they are due to glyoxalase activity.

Whenever the preparation has been completely freed from glutathione by dialysis the evolution of CO₂ by the enzyme and methylglyoxal alone is negative or negligible. From the standpoint of present knowledge, at any rate, this would seem to give proof that the specific enzyme is responsible.

Plants

The plants (Table 2) were ground up with sand in a mortar with an equal weight of distilled water, squeezed through linen, and centrifuged. 10 ml. were dialyzed as before. 1 ml. of this was used in the Barcroft apparatus, other details being the same as for the invertebrates.

It will be seen from the above data that the red seaweeds contain a considerable concentration of the enzyme; this also applies to the green varieties we have tested, although here the activity of the

Table 2. *Glyoxalase activity of algae, fungi and conifers*

Order	Species	Name	Colour	$\mu\text{l. CO}_2$ evolved	
				20 min.	60 min.
Algae	<i>Cystocodium</i> sp.	Seaweed	Red	84.0	203.0
	<i>Calliblepharis</i> sp.	"	"	69.0	183.0
	<i>Chondrus crispus</i>	"	"	81.0	198.0
	<i>Rhodomenia palmata</i>	"	"	55.0	143.0
	<i>Ulva latissima</i>	"	Green	51.0	151.0
	<i>Enteromorpha</i> sp.	"	"	60.5	162.2
	<i>Cladophora</i> sp.	"	"	55.0	99.0
	<i>Fucus spiralis</i>	"	Brown	36.0	87.0
	<i>Fucus serratus</i>	"	"	18.0	39.0
	<i>Ascophyllum nodosum</i>	"	"	20.5	33.4
	<i>Laminaria digitata</i>	"	"	21.0	33.0
	<i>Laminaria saccharina</i>	"	"	21.0	42.0
	<i>Penicillium notatum</i>	Mould	—	36.0	102.0
	<i>Psalliota campestris</i>	Mushroom	—	156.0	375.0
Coniferae	<i>Cypress</i> sp.	Cypress	—	27.0	39.0
	<i>Taxus baccata</i>	Yew	—	15.0	18.0
	<i>Pinus</i> sp.	Young pine cones	—	15.0	18.0

enzyme is somewhat less. In the brown seaweeds its presence is doubtful except perhaps in *Fucus spiralis*. In the case of the mould studied, although the activity of the enzyme is relatively low, there is no doubt of its presence. In the mushroom its activity is considerable. In those cases where the evolution of CO_2 is very low and tends to become very slow after a short period, we feel that the presence of glyoxalase is not proved and our experience leads us to the conclusion that some chemical reaction not due to the enzyme is responsible. When the enzyme is present in adequate amount we have come to believe that this chemical action is so accelerated that the CO_2 is evolved during the 4 min. when the manometer stopcocks are open. In the absence of the enzyme results like those seen in the case of the brown seaweeds and the conifers (of which we used young spring growth) are obtained. We believe that glyoxalase is absent from these.

The higher plants (Table 3) were put through a mincer, squeezed through linen, and centrifuged. 10 ml. of the juice were dialyzed as before, and then made up to 20 ml. 1 ml. of this was used in the Barcroft apparatus, other details being the same as in the case of the invertebrates.

found in the petals of certain flowers, e.g. *dianthus* (Table 3). Dakin & Dudley (1913) state that they did not find it in young potatoes; it is nevertheless clearly present in mature tubers.

SECTION II. GLUTATHIONE

Note on the separation of glutathione. We have previously shown (Hopkins & Morgan, 1943) that a pure product could be obtained from peas by a relatively simple method. The peas were first ground up in a mortar with 0.1N- H_2SO_4 and a little washed sand, the extract centrifuged and the solution half saturated by the addition of solid ammonium sulphate; the filtrate from the precipitate was then brought to 0.5N with respect to H_2SO_4 . The addition of Cu_2O yielded a completely pure product. It is to be noted, however, that precipitation is delayed in the presence of the ammonium sulphate, and only after standing overnight does a precipitate form. We have since found that the presence of proteins and protein products does not interfere with the precipitation by Cu_2O so that the use of ammonium sulphate may not be necessary. 2 ml. of serum diluted to 50 ml., and 1 g. of proteose preparations in the same volume, gave no trace of precipitate with Cu_2O , but when to similar solutions 6 mg. of glutathione were first added precipitation of the cuprous compound was immediate. We have, however, found that the purine bases, but not the pyrimidines, are

Table 3. *Glyoxalase activity of the higher plants*

Order	Species	Name	Part of plant	$\mu\text{l. CO}_2$ evolved	
				20 min.	60 min.
Compositae	<i>Helianthus tuberosus</i>	Artichoke	Tubers	46.7	118.2
"	<i>Lactuca sativus</i>	Lettuce	Leaves	63.2	178.7
Cruciferae	<i>Brassica oleracea</i>	Cauliflower	Florets	96.0	288.0
"	"	Cabbage	Leaves	93.5	225.5
"	<i>Nasturtium officinale</i>	Watercress	"	96.0	228.0
"	<i>Raphanus sativus</i>	Radish	Root	54.0	154.0
"	"	"	Leaves	68.7	167.7
Chenopodiaceae	<i>Beta vulgaris</i>	Beetroot	Root	35.7	101.7
Liliaceae	<i>Allium cepa</i>	Onion	Bulb	99.0	255.0
"	"	"	Tops	118.2	283.2
Solanaceae	<i>Solanum tuberosum</i>	Potato	Mature tubers	110.0	288.7
"	"	"	Leaves	84.0	222.0
Tropaeolaceae	<i>Tropaeolum majus</i>	Nasturtium	"	143.0	349.0
Urticaceae	<i>Urtica dioica</i>	Nettle	"	115.5	264.0
Umbelliferae	<i>Petroselinum sativum</i>	Parasley	"	255.7	649.0
Ranunculaceae	<i>Delphinium</i> sp.	Delphinium (blue)	"	48.0	138.0
Scrophulariaceae	<i>Antirrhinum majus</i>	Antirrhinum (white)	"	30.0	78.0
Oleaceae	<i>Syringa vulgaris</i>	Lilac (white)	"	21.0	30.0
Polemoniaceae	<i>Phlox</i>	Phlox (white)	"	39.0	102.0
Rosaceae	<i>Rosa</i>	Rose (red)	"	33.0	39.0
"	"	"	Petals	15.0	21.0
"	"	Rose (white)	"	21.0	51.0
Caryophyllaceae	<i>Dianthus</i> sp.	Pink (double white)	"	144.0	429.0
Labiatae	<i>Salvia officinalis</i>	Sage	Leaves	22.0	27.5

To judge from our own observations, and from the results reported in the literature, glyoxalase is widely distributed in the higher plants. Most of the plants which we have examined in fresh condition have contained it. It is perhaps noteworthy that it should be present in green leaves, although long ago Neuberg & Kobel (1926) were the first to show this. It is certainly remarkable that it should be

readily precipitated by Cu_2O from 0.5N- H_2SO_4 solutions, and this is important with respect to what follows. Isolation of the tripeptide from the invertebrates presents serious difficulties; the concentrations are in any case relatively low and it has proved difficult in most cases to obtain sufficient of a species at any time, while to accumulate extracts from successive supplies at somewhat long intervals involves risk of removal of the loosely bound glutamic acid from the compound. Moreover, a difficulty presents itself

which is not found in the isolation of the tripeptide from mammalian or from plant tissues, namely, that acid extracts from many invertebrates contain purine bases in considerable quantity, which as stated above are precipitated by Cu_2O .

Invertebrates

Earthworms. From 1700 g. of earthworms we obtained 120 mg. of crystalline glutathione, m.p. 187° . Found: C, 38.63; H, 5.54; N, 13.1; S, 10.19%. Calc.: C, 39.09; H, 5.54; N, 13.68; S, 10.42%. The product, though not quite pure, is nearly so; it had not been recrystallized. **Locusts.** In the case of these insects we were able to take advantage of the circumstance that the purine bases contained in the acid extract are precipitated by Cu_2O immediately, and can then be centrifuged off, while owing to the influence of the ammonium sulphate precipitation of glutathione is delayed for several hours. From 461 g. of whole locusts we obtained 20 mg. of crystalline glutathione (N, 13.4; S, 10.40%).

It is unfortunate that the species of which we could obtain a good supply contained relatively little glutathione and offered special difficulties in attempts at isolation. This is true of the marine mussel, of the garden snail and, when all its soft parts are extracted, of the shore crab. The latter shows some local concentration as the hepatopancreas gives a good nitroprusside reaction, undoubtedly due to glutathione. The extracts of all these species reacted with the enzyme and methylglyoxal in the manometer. When dealing with these species we had not yet observed the interesting circumstance which was afterwards applied to the locusts for the separation of the glutathione from the bases (see above). Owing to the exceedingly large amount of glycogen contained in *Ascaris* we saturated the acid extract from 182 g. with ammonium sulphate and then diluted it with an equal volume of water; after making 0.5N with respect to H_2SO_4 , Cu_2O gave a small precipitate which was undoubtedly pure glutathione. We obtained later supplies at somewhat long intervals, but in the extract from these the glutathione had decomposed before we attempted isolation. With the anemone, sea-squirt and *Ascaris* we had to rely upon the circumstance that undialyzed aqueous extracts showed a definite glyoxalase activity, which in the current view should constitute indirect proof that glutathione is present in these invertebrates. The amount of extract in the Barcroft flasks corresponded to 2 g. of anemone and sea-squirt tissue, and to 0.5 g. of *Ascaris*. In 1 hr. the amount of CO_2 evolved was in the case of the anemone 84, in that of the sea squirt 42, and of *Ascaris* 222 μl . It is clear that in the two former the concentration of glutathione must be very low.

Plants

Broad bean (*Vicia Faba*). From 1400 g., soaked in water for 24 hr. and the skins removed, we obtained 0.38 g. of crystalline glutathione, m.p. 190° . Found: C, 38.93; H, 5.50; N, 13.90; S, 10.33%. **Vegetable marrow** (*Cucurbita pepo*). We obtained a few mg. of crystalline glutathione, m.p. 188° , from a young vegetable marrow. This was not analyzed, but compared with pure glutathione in the Barcroft apparatus it gave an identical evolution of CO_2 with the enzyme and methylglyoxal.

DISCUSSION

The results of the experiments described add considerably to the knowledge of the distribution of glyoxalase. We have given special attention to invertebrates, which have been very little studied from this point of view. We have found that the enzyme is present in all but one of the sixteen different species from eight different orders examined. It proved to be present in a mould (*Penicillium notatum*) and among algae, in red and green seaweeds, and also in fungi. In addition we have shown its presence in nineteen of the higher plants not previously examined. These results added to the cases described in the literature show how extremely widely distributed is this enzyme in living systems.

The effect must here be considered of the varying concentration of glutathione, the apparently essential concomitant for enzyme activity. In the case of the invertebrates there is no constancy in the relative proportion of the two components present in different species. The earthworm is an extreme but exceptional case of this, in which it is at least doubtful whether the enzyme is present, whereas glutathione is present in considerable amount; but in most species the reverse is the case and the deficiency is in the tripeptide. Glutathione itself is certainly present in the invertebrate order; we have, for instance, isolated it from the earthworm and from the locust, the products proving on analysis to be practically pure. In the case of plants we have not obtained such satisfactory evidence for a relative deficiency in glutathione. We have not attempted to isolate it from the plants in Table 3, and the nitroprusside reaction was obscured owing to the fact that addition of ammonia alone generally produced coloration.

It is quite certain that a deficiency in glutathione may become a limiting factor in the activity of the enzyme. Our measurements of this activity in the various species were made upon extracts which had been dialyzed until the naturally occurring glutathione was removed; an adequate amount of glutathione was then added before the solution was tested in the manometer. The results in Table 1 were thus

obtained. In certain cases, when the extract was not dialyzed and no glutathione was added, the activity proved to be much less than that of dialyzed extract plus glutathione. While the weak activity of the undialyzed extracts might conceivably be due to the presence of some inhibitory material, we found that weak activity goes hand in hand with a weak nitroprusside reaction. Moreover, when to the undialyzed extract 2 mg. of glutathione were added, the activity rose and approximated to that of the dialyzed extract plus glutathione. Thus it seems clear that in the untreated extracts the amount of glutathione present was not sufficient to develop the full activity of the enzyme.

With present knowledge it is, of course, generally assumed that the sole activity of this enzyme is to convert substituted glyoxals into their corresponding hydroxy acids. The absence of satisfactory evidence for the occurrence of such a substrate in the cells and tissues studied has involved disappointment to those interested in the enzyme; this will not be lessened by knowing that the enzyme may exist in certain cells and yet be unable to exert its full activity owing to a deficiency of glutathione.

This failure to gain reliable evidence for any definite function for glyoxalase has now lasted for the thirty years which have elapsed since the discovery of the enzyme by Dakin & Dudley (1913). The realization of this may at the present time be

discouraging further work on the subject. We find that some few still hold that in the case of an agent separated from a living system and studied *in vitro* the assumption that it has a function in life partakes of the teleological. It seems more logical to believe that during the processes of development and evolution a functionless agent would have been eliminated, whereas the glyoxalase system remains with its very wide distribution. We feel that our experiments extending the evidence for this should encourage further work in the field. It is possible, though perhaps unlikely, that the enzyme may prove to have a function of a kind not yet recognized, or that its activity has interrelationships with that of other enzymes. We know that this is true in the case of glutathione, especially in the higher animals and plants.

SUMMARY

1. The results of the experiments described have added considerably to the evidence for the wide distribution of the enzyme glyoxalase in living organisms.

2. While glutathione is also widely distributed, in some cases, especially among the invertebrates, its concentration may be very low and limits the activity of the enzyme actually present.

3. We have isolated glutathione in crystalline form from the earthworm, locust, broad bean and vegetable marrow.

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The Biological Assay of Vitamin D₃. The Effect of the Calcium and Phosphorus Content of the Diet

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That the content and proportion of calcium and phosphorus have a marked effect on the efficacy of rachitogenic diets used in the biological assay of vitamin D on rats and on the responses of the animals to doses of the vitamin, is well known. Investigations of the effect of the same factors in the assay of vitamin D₃ on chicks have been relatively few and in this paper are presented the results

of an extended series of investigations of their effect on the growth, tarso-metatarsal distance, and bone-ash of chicks receiving graded doses of vitamin D₃ and, in some cases, on serum Ca, P and phosphatase.

In rats it has been shown (Bethke, Kick & Wilder, 1932; Brown, Shohl, Chapman, Rose & Saurwein, 1932; Shohl, 1935, 1936) that growth and production of rickets depend on the level of Ca and P as well