13. NATH, R. B. B. V. Scientific Reports of the Imp. Inst. of Agr. Research Pusa, 1934–1935, p. 116. Report of the Imp. Agr. Chemist, Delhi 1936.
14. NATH, R. B. B. V. Scientific Reports of the Imp. Inst. of Agr. Research New Delhi, for year ending 30 June '37, p. 143. Report of the Imp. Agr. Chemist, Delhi 1937.
15. SCHMID, H. and SERRANO, M. Untersuchungen über die Nikotinbildung des Tabaks. Experientia 6: 311–316. 1948.
16. TSO, T. C. and JEFFREY, R. N. Paper chromatography of alkaloids and their transformation products in Maryland tobacco. Arch. Biochem. Biophys. 43: 269–285. 1953.
17. TSO, T. C. and JEFFREY, R. N. Studies on tobacco alkaloids. II. The formation of nicotine and nornicotine in tobacco supplied with N^{15}. Plant Physiol. (In press).
18. WOLF, F. A. Statement at Tobacco Chemists Research Conference at Richmond, Virginia. 1954.

UTILIZATION OF GLYCEROL IN THE TISSUES OF THE CASTOR BEAN SEEDLING [1, 2]

HARRY BEEVERS

DEPARTMENT OF BIOLOGICAL SCIENCES, PURDUE UNIVERSITY, LAFAYETTE, INDIANA

Although it has been recognized for some time that glycerol, as one of the products of lipase action, is produced during the germination of fatty seeds, no definitive work seems to have been done on its subsequent metabolism. The present experiments with glycerol-1-C^{14} were begun in the hope that the use of labeled material might allow a successful approach to this problem.

While this work was in progress Stumpf reported an investigation of glycerol oxidation by cell-free preparations from peanut seedlings (11). He succeeded in providing evidence for the existence of enzyme systems which cooperated in converting glycerol to CO_2 by a pathway including α-glycerol phosphate and pyruvate and subsequent oxidation in the Krebs cycle. Although the amounts of glycerol oxidized by the cell free system were very small, the results gave firm support for the conclusion that an oxidation pathway which might have appeared the most likely from a knowledge of comparative biochemistry did in fact exist in the tissue concerned.

All of the experiments in the present report were carried out on intact tissues. It will be shown, however, that there is evidence for the participation of a sequence of reactions, analogous to that described by Stumpf, in the very active oxidation of glycerol by castor bean cotyledons. In addition, it becomes clear that oxidation to CO_2 represents only one of the metabolic fates of glycerol in the tissues; a considerably larger fraction of the supplied glycerol is rapidly incorporated into sucrose. Preliminary reports of this work have appeared elsewhere (2, 4).

MATERIALS AND METHODS

Castor bean seeds (*Ricinus communis* L.) var. U.S. 70 were germinated as described elsewhere (5). Under these conditions the seedling produces a primary root of about 8 cm in 4 days, when the hypocotyl is just beginning to elongate. After 7 to 8 days the hypocotyl has reached a height of about 10 cm and the endosperm tissue has by then been almost completely absorbed by the enlarging cotyledons. It has been shown (4, 10) that active hydrolysis of the fats in the seed begins after 3 to 4 days of germination and free sugars, particularly sucrose appear in quantity in the seedling. Most of the experimental work in the present paper was done with cotyledons taken from 5- to 6-day seedlings; i.e., at a stage when an active breakdown and utilization of fats in the endosperm was occurring. Some experiments with other tissues of the seedling are also included, and for comparison the ability of some other tissues to oxidize glycerol was also examined.

Glycerol-1-C^{14} with a rated specific activity of 1 millicurie per millimole was supplied by California Foundation for Biochemical Research. For use it was diluted with an unlabeled glycerol solution so that 0.1 ml contained 10 micromoles and from 1,000 to 6,000 cpm (counts per minute). The actual activities of the glycerol solutions used in the individual experiments were determined by oxidation in the apparatus of Stutz and Burris (12).

The methods used are similar to those which have been described previously in another connection (6). The intact cotyledons (or thin slices of the other tissues) were placed in 100-ml Warburg flasks with 3 to 4 ml solution containing 0.5 ml 0.1 M potassium phosphate at pH 5.0. The CO_2 was collected in KOH, converted to $BaCO_3$ and assayed for radioactivity in a windowless gas flow counter. The figures for radioactivity are recorded in cpm, corrected for background and self-absorption.

At the end of the experimental period the tissues were extracted in hot 80% ethanol. After evaporation of the water and alcohol on a steam bath the residue was washed with ether and then dissolved in water. The solution was treated with Amberlite MB-1 exchange resin (Rohm and Haas) to remove ionic materials, and concentrated to a small volume for application as a band on Whatman No. 3 paper. It was chromatographed for 24 hours with *n*-butanol-ethanol-water (52.5:32:15.5 v/v) as the solvent. The solvent was allowed to drip off the paper. After drying, the paper was cut crosswise into ½″ strips.

[1] Received May 8, 1956.

[2] This paper is based on research supported by a grant (NSF-G 265) from the National Science Foundation.

The sugars were eluted by suspending the individual strips on hooked "cold fingers" in test tubes containing about 3 ml of water and refluxing for 30 to 40 minutes. The eluates were made to 5 ml and 2 ml samples were taken from each for sucrose analysis by the orcinol method (7). A further 2-ml sample from each tube was dried down on a steel planchet and counted directly. Hydrolysis of the sucrose was accomplished with a commercial invertase preparation (Nutritional Biochemicals Corporation) and the separation of glucose from the products carried out as in table IV.

Degradation of the glucose was carried out by the method of Gunsalus and Gibbs (8).

Results

Respiration of Glycerol by Plant Tissues: The results in table I show that glycerol is converted to CO_2 by each of the materials tested, but at widely different rates; several tissues without fatty food reserves nevertheless attack glycerol actively. For a given weight of tissue, the castor bean cotyledon was the most efficient in its ability to oxidize glycerol but this is also the tissue with the highest metabolic rate as shown by the rate of endogenous CO_2 release. The addition of small amounts of glycerol did not lead to increases in the already high endogenous rates of O_2 uptake and CO_2 output. It will be noticed that the castor bean endosperm tissue, which may be regarded as the source of glycerol, was much less efficient in oxidizing it than the neighboring tissues (cotyledon and hypocotyl) of the seedling proper.

Course of $C^{14}O_2$ Release and $C^{14}O_2$ Yield During Glycerol Oxidation by Castor Bean Cotyledons: Figure 1 shows that, within the range 10 to 30 micromoles, the total yield of $C^{14}O_2$ depends on the amount of substrate supplied. At each substrate level, the initially high rate of $C^{14}O_2$ evolution declined rapidly, even though the rate of endogenous CO_2 output was maintained. The progress curves for percentage yield follow a closely similar course throughout, and show that at each level of glycerol roughly 25 % of the substrate provided had appeared

Table I

The Relative Abilities of Tissues to Oxidize Glycerol

Tissue	Mg $BaCO_3$ produced	Percent of supplied radioactivity in respired CO_2
	Castor bean, 6-day	
Endosperm	6.0	0.2
Petiole	8.2	1.7
Hypocotyl	14.4	6.0
Cotyledon	29.4	14.4
	Tomato	
Internode	6.9	0.7
	Corn	
Stem	5.2	1.5
Root	6.9	1.7
	Pea	
Internode	6.0	4.1
	Red kidney bean	
Petiole	8.1	7.7

One gm samples of the various tissues were allowed to respire for 4 hrs in a medium containing 20 micromoles glycerol-1-C^{14}. The respired CO_2 was collected in KOH and converted to $BaCO_3$.

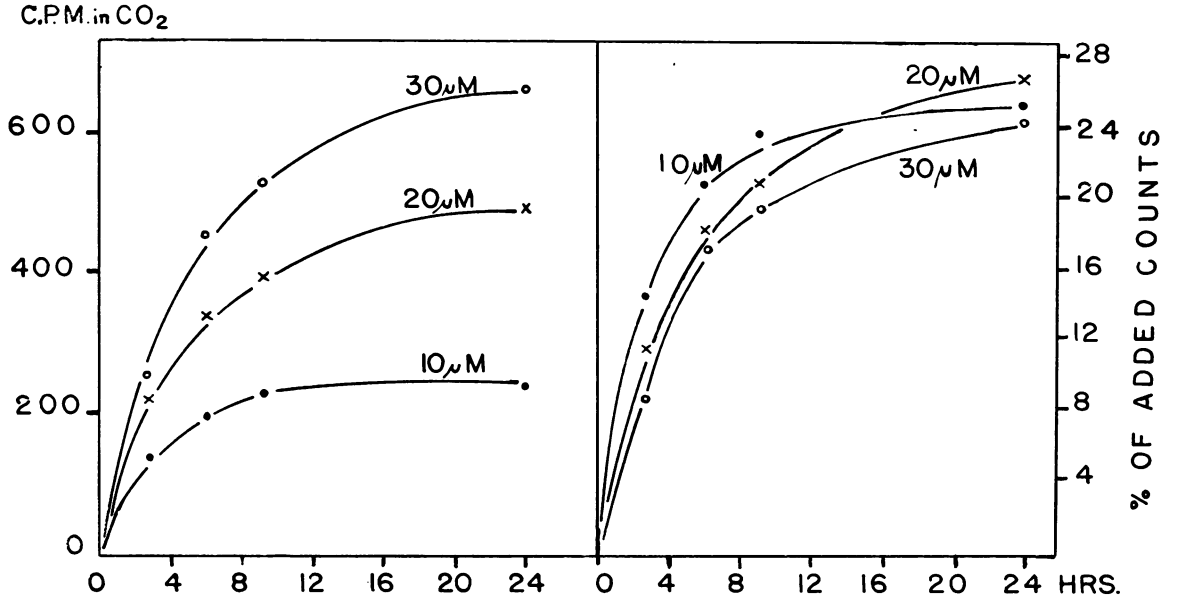

Fig. 1. Time course of glycerol oxidation by castor bean cotyledons. One gm samples were incubated with the amounts of glycerol indicated, in 4.0 ml solution containing 1.0 ml 0.1 M KH_2PO_4, pH 5.0.

as CO_2 at the end of 23 hours, when the rates of $C^{14}O_2$ release had slowed down to a low value.

This evidence might be taken to indicate that a major fraction of the supplied glycerol suffers an alternative fate and subsequent experiments have shown this to be so. Most of these experiments have been of 4- to 6-hr duration and the amount of glycerol oxidized during this period was usually in the range of 15 to 20 % of that added ("control" values in table V).

PATHWAY OF GLYCEROL OXIDATION: Some clues as to the mechanism of the glycerol-CO_2 conversion in vivo are provided by the following considerations.

(a) Anaerobic conditions almost completely prevent the breakdown of glycerol to CO_2. Whereas in air, in a particular experiment, 20.4 % of the added glycerol-C^{14} had appeared as CO_2 after 4.75 hours, the percentage yields from duplicate samples in nitrogen were 1.68 and 1.43; a reduction of some 92 % due to removal of O_2 from the system. Fermentation of endogenous substrates was, nevertheless, quite high (CO_2 output 64 % of that in air). It thus appears likely that the hindrance to glycerol breakdown imposed by anaerobic conditions is not due to any attendent deficiency in phosphorylating capacity of the tissue. One might, rather, suppose that some oxidative step in glycerol utilization is obligatorily linked to atmospheric O_2 and thus cannot be maintained by the acetaldehyde to alcohol reduction which is recognized as the major internal H-accepting system under these conditions. Although these considerations do not rule out the possible participation of a DPN-linked α-glycerophosphate dehydrogenase they would be neatly accommodated, if, as in Stumpf's system, the dehydrogenation of α-glycerophosphate by the cytochrome-linked enzyme (which would require O_2 for its continued operation) afforded the main pathway for the introduction of glycerol into the glycolytic sequence.

(b) Fluoride slows down the endogenous O_2-uptake of the cotyledons and inhibits the $C^{14}O_2$ release from glycerol-C^{14} to a similar degree (table II).

TABLE II

THE EFFECTS OF FLUORIDE AND MALONATE ON O_2 UPTAKE AND $C^{14}O_2$ OUTPUT IN THE PRESENCE OF GLYCEROL-1-C^{14}

INHIBITOR		O_2 UPTAKE	% OF CONTROL	$C^{14}O_2$ OUTPUT	% OF CONTROL
TYPE	CONC.				
	$M \times 10^{-2}$	μl		cpm	
0	0	2150; 2300	100	1065; 1087	100
NaF	5	1013	46	477	44
"	13	175	8	54	5
"	25	16	1	8	1
Malonic acid	13	895	40	1550	143
" "	25	458	21	697	65
" "	50	326	15	525	49

Samples (500 mg) of cotyledons were incubated for 6.75 hrs with 20 micromoles glycerol-1-C^{14} (10,800 cpm).

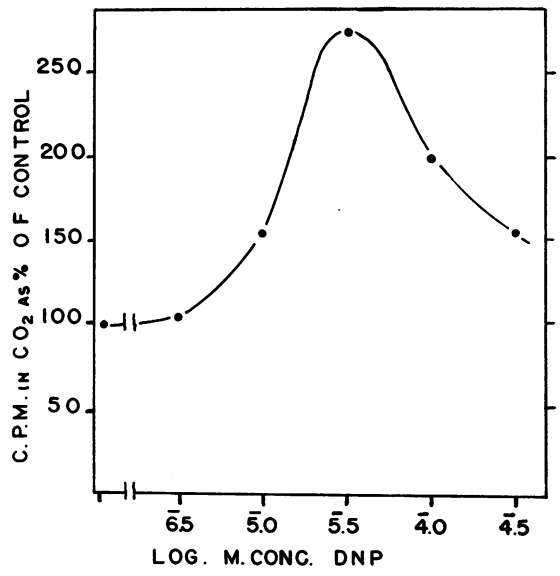

FIG. 2. The effect of 2,4-dinitrophenol on $C^{14}O_2$ release from glycerol. Samples of 800 mg cotyledon tissue were incubated for 5 hrs in the presence of 20 micromoles (4470 cpm) of glycerol-C^{14}.

(c) Malonate also reduces $C^{14}O_2$ output, but only in concentrations greater than 0.02 M. However, O_2-uptake was much more strikingly depressed, and at the lowest malonate level, which actually stimulated $C^{14}O_2$ output, O_2 uptake was reduced by more than half. From other experiments with malonate it has been concluded that when the operation of the Krebs cycle is slowed down by malonate, pyruvate is diverted to CO_2 and alcohol (1) and the CO_2 output (which under these conditions results from the residual Krebs cycle activity and the decarboxylation of pyruvate to acetaldehyde) may be much less inhibited than the O_2 uptake. If this were so in the present case then a fraction of the $C^{14}O_2$ produced from glycerol in the presence of malonate might have arisen from direct pyruvate decarboxylation. The fact that radioactive alcohol, with 97 % of its activity in the CH_3 group, has now been isolated from cotyledons respiring glycerol-1-C^{14} in the presence of malonate is obviously in accord with this supposition, and may, in fact be considered as evidence for the participation of pyruvate as an intermediate in glycerol oxidation.

THE EFFECT OF 2,4-DINITROPHENOL (DNP) ON GLYCEROL OXIDATION: The addition of DNP brought about effects of the kind shown in figure 2. At concentrations between 6×10^{-6} and 1×10^{-3} M, $C^{14}O_2$ output from glycerol-C^{14} was strikingly stimulated, and at about 4×10^{-5} M the yield of $C^{14}O_2$ was some 2.5 times that of the control. In the absence of DNP, as pointed out earlier, the $C^{14}O_2$ yield from glycerol amounts to about 20 % of that supplied; at the most effective DNP levels, yields of up to 73 % of the supplied glycerol have been obtained.

IDENTIFICATION OF SUCROSE AS A MAJOR PRODUCT OF GLYCEROL UTILIZATION: When extracts from untreated cotyledons were chromatographed and the papers sprayed for reducing sugars before and after hydrolysis with invertase, it was at once clear that sucrose comprised by far the greatest proportion of free sugars. Only faint traces of glucose and fructose were observed. Extracts from tissues which had been incubated for several hours with glycerol-C^{14} contained similarly large amounts of sucrose (up to about 100 micromoles/gm fresh weight in 7-day cotyledons) and direct counting on the paper showed that the sucrose area was radioactive. An autoradiograph showed that other areas of the chromatogram were virtually without activity.

At the end of 6- to 7-hr experiments in which 1 gm of cotyledons had been incubated with 20 micromoles glycerol-C^{14}, it was found that the cotyledon residues, after 80 % alcohol extraction, contained only small amounts of activity as judged by a thin window counter. The ether washings contained less than 10 % of the added C^{14}. Thus the material placed on the chromatogram contained the bulk of the C^{14} which had not been respired as CO_2, and since this appeared to be concentrated in the sucrose area it was clear that a substantial fraction of the glycerol had been incorporated into this product. Quantitative estimation was therefore undertaken by the methods outlined above.

The results obtained from a typical chromatogram are shown in figure 3. The coincidence of the sucrose and radioactivity peaks is striking. The smaller peak in radioactivity in samples 11 and 12 corresponds to unreacted glycerol. Any radioactive glucose or fructose would have given rise to peaks in the region of samples 6 to 10. Estimates of total sucrose and radioactivity were obtained by summing the contributions of samples comprising the sucrose peak of the curve; in the example shown these would be samples 3 and 4.

The "control" values in table V are examples of such estimates and these show that as much as 60 % of the glycerol carbon supplied may be incorporated into this sucrose component, and at the same time somewhat less than 20 % of the C^{14} was appearing as CO_2.

After chromatographing again in the same solvent and eluting, a sample of the radioactive sucrose was partially hydrolyzed with invertase and the products separated chromatographically in phenol-water. The following amounts of radioactivity were observed in the products: unhydrolyzed sucrose, 420 cpm; glucose, 968 cpm and fructose, 843 cpm. It is clear therefore that the glucose and fructose moieties of the sucrose are fairly equally labeled.

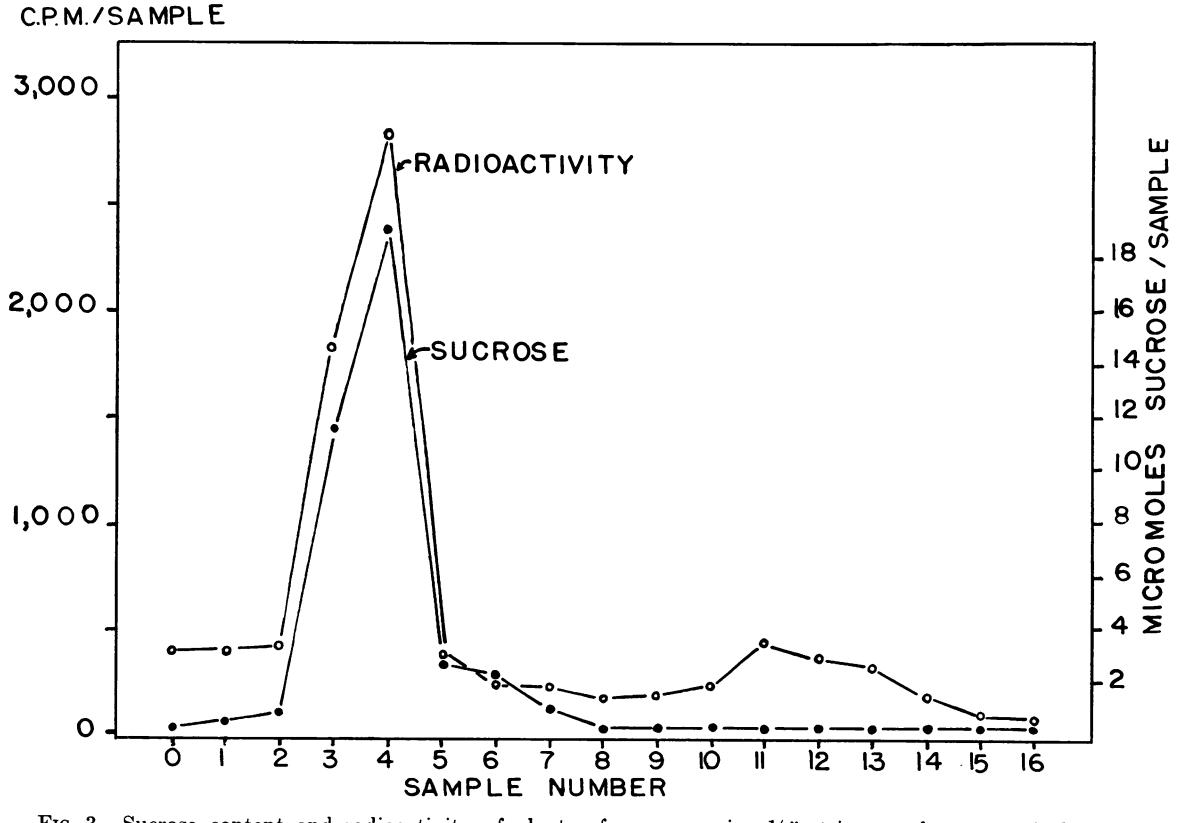

FIG. 3. Sucrose content and radioactivity of eluates from successive ½" strips cut from a typical chromatogram (see text).

TABLE III

THE EFFECT OF ADDED GLYCEROL ON THE SUCROSE CONTENT OF 7-DAY COTYLEDONS

CONDITIONS	GLYCEROL ADDED	SUCROSE/GM FRESH WT OF TISSUE
	micromoles	*micromoles*
Beginning of experiment	0	95.2
After 6-hr incubation	0	87.0
" " "	50	91.0
" " "	400	96.4

As shown in table III there is a considerable utilization of native sucrose in the cotyledon during the experimental period. The provision of the small amounts (20 micromoles) of glycerol used in the above experiments does not lead to any net synthesis of sucrose, although a sparing effect on the utilization of sucrose is evident. In the presence of larger amounts of glycerol, however, the sucrose deficit may be completely offset and even a small amount of net synthesis obtained (table III). Although the endosperm, in contrast to the cotyledon, was found to oxidize glycerol only very slowly, a single experiment showed that there is a rapid assimilation of glycerol into sucrose in this tissue also.

The large percentage incorporation of glycerol carbon into sucrose would indicate that the intact C-3 skeleton, rather than any smaller breakdown product, was being assimilated. That this is, in fact, so was shown by degrading stepwise a glucose sample obtained from hydrolysis of the radioactive sucrose by the procedure outlined in table IV. The last row of figures shows that 97 % of the C^{14} was confined to carbons 1, 3, 4 and 6 of the glucose, and distributed fairly evenly between these positions. Such a distribution of activity would be predicted only if the terminally labeled glycerol skeleton was incorporated intact into the glucose.

TABLE IV

ISOLATION AND DEGRADATION OF GLUCOSE

CARBON ATOM OF GLUCOSE	SPECIFIC ACTIVITY MμC/MG C	ACTIVITY AS % OF TOTAL
1	1.66	26.8
2	0.09	1.5
3	1.58	25.3
4	1.51	24.4
5	0.11	1.8
6	1.23	20.0

Castor bean cotyledons were incubated with glycerol-1-C^{14} and extracted with hot 80 % alcohol. The extract was evaporated to dryness, washed with ether and dissolved in water. After treatment with Amberlite MB 1, it was chromatographed on Whatman No. 3 paper in *n*-butanol-ethanol-H_2O (solvent I). The sucrose area was eluted and chromatographed again in the same solvent. After elution the sucrose was hydrolyzed with invertase and the products separated chromatographically using solvent I. The glucose area was eluted and chromatographed in phenol-H_2O. The glucose (80 micromoles, 12,500 cpm) was eluted and 400 micromoles carrier glucose added before degradation by the Leuconostoc procedure (8).

EFFECTS OF INTERFERING SUBSTANCES ON THE INCORPORATION OF GLYCEROL INTO SUCROSE, *Dinitrophenol:* DNP in the same concentrations which stimulate glycerol oxidation produces equally striking and opposite effects on the conversion of glycerol to sucrose. As the data in table V show, the reduction in assimilation is offset in each case by a corresponding increase in CO_2 output from the glycerol and the total C^{14} accounted for in the two fractions (60 to 80 % of that added) was essentially the same whether DNP was present or not.

Malonate and Fluoride: Malonate and fluoride in concentrations which strongly inhibited glycerol oxidation, also slowed down its incorporation into sucrose, but to a somewhat lesser degree. Thus when the fluoride (0.07 M) and malonate (0.07 M) inhibitions of $C^{14}O_2$ output were 97 % and 84 %, respectively, the incorporation of glycerol-C^{14} into sucrose was reduced by 82 % and 69 %.

TABLE V

THE EFFECT OF DINITROPHENOL ON THE FATE OF GLYCEROL SUPPLIED TO CASTOR BEAN COTYLEDONS

CONC DNP $M \times 10^{-5}$	CPM IN CO_2	% OF ADDED ACTIVITY	CPM IN SUCROSE	% OF ADDED ACTIVITY
	Expt I—4 hrs			
0	1800	15.3	5086	43.0
1.5	4040	34.3	3230	27.7
3.0	6450	54.7	1766	15.0
5.0	5250	44.5	1658	14.1
	Expt II—6 hrs			
0	2152	18.1	7177	60.4
2.9	5920	49.7	3644	30.6
	Expt III—5 hrs			
0	890	19.9	2030	45.6
2.9	2360	52.7	734	16.4

To each sample 20 micromoles of glycerol-1-C^{14} were added. Sucrose isolated from the tissue and the respired CO_2 were assayed for radioactivity.

DISCUSSION

The results show that, at least in the excised castor bean cotyledon, the bulk of the glycerol supplied to it suffers one of two fates. One of these, incorporation into sucrose accounted for about three times as much of the glycerol carbon as did the other, conversion to CO_2. These changes are accomplished in a few hours and after this there is a slow release of $C^{14}O_2$, which presumably originates from the radioactive sucrose pool in the tissues.

The effects of anaerobiosis and of the inhibitors malonate and fluoride were shown to be those expected if the glycerol were being oxidized by a sequence involving introduction of a phosphorylated

glycerol derivative into the glycolytic sequence and oxidation of the resulting pyruvate through the Krebs cycle. Such a sequence which had seemed likely from considerations of comparative biochemistry has in fact recently been demonstrated in a cell-free system from peanut seedlings by Stumpf (11). The present demonstration that the pattern of labeling in the glucose (derived from the sucrose by hydrolysis) is such as to indicate that two glycerol skeletons have condensed together is strong presumptive evidence that triose phosphates are also intermediates in the synthesis of sucrose from glycerol.

It is therefore supposed that the metabolism of glycerol in the cotyledon involves conversion to triose phosphate and that this may be the point at which the two major pathways of its utilization diverge. Since each molecule of sucrose synthesized represents four glycerol residues, and the incorporation of glycerol carbon into sucrose was about 3 to 4 times that of its appearance in the CO_2 it is clear that under the conditions of experimental glycerol supply, the rates of the reactions which competed for the triose were fairly similar. In the presence of DNP, it will be recalled, there were striking changes in the relative contributions of the synthetic and oxidative pathways of glycerol utilization (table V). The increased oxidation of the glycerol is thought to be due to the indirect induction of a higher rate of glycolysis by the DNP (3). Under these conditions glycolysis competes on superior terms for the triose intermediate and thus a much smaller fraction of the glycerol is converted into sucrose. This interpretation would give a further point of emphasis to the importance of the control exerted over the glycolysis rate by the "aerobic" phosphorylative events which are subject to uncoupling by DNP.

Finally, it should be mentioned that the ability of the castor bean tissues to convert smaller molecules into sucrose is not confined to glycerol, since a large fraction of supplied acetate carbon may also be so incorporated (unpublished results). These reactions whereby each of the presumptive breakdown products of fat may be converted to sugars are apparently of some importance in the economy of the castor bean seedling, since it has been shown that conversion of fatty reserves into carbohydrate takes place in this material (10) and the respiratory quotients of those parts of the embryo other than the cotyledons indicate that carbohydrates alone are being respired (9).

Summary

Non-green cotyledons excised from young germinating seedlings of castor bean have been shown to metabolize glycerol-C^{14} actively under aerobic conditions. About one quarter of the supplied glycerol was oxidized to $C^{14}O_2$ by a sequence which apparently involved glycolytic intermediates, pyruvic acid and the Krebs cycle. A large fraction of the remainder of the glycerol was found in sucrose isolated from the tissue, and degradation showed that the glycerol carbon skeleton had been incorporated intact, as would be required if the glycerol were first converted to triosephosphate and then subject to the action of aldolase.

The effects of 2,4-dinitrophenol on this system are described, and the interpretation put on these results is that the extent to which synthesis of sucrose from glycerol occurs is governed by the rate of glycolytic utilization of triosephosphate, which is itself regulated by the aerobic phosphorylations in the tissue.

I am indebted to Dr. Martin Gibbs for instructing me in the use of the Leuconostoc degradative procedure.

Literature Cited

1. Beevers, H. Malonic acid as an inhibitor of maize root respiration. Plant Physiol. 27: 725–735. 1952.
2. Beevers, H. Glycerol metabolism in the germinating castor bean. Plant Physiol. 30: *xxxviii*. 1955.
3. Beevers, H. 2,4-Dinitrophenol and plant respiration. Amer. Jour. Bot. 40: 91–96. 1953.
4. Beevers, H. and Walker, D. A. Oxidative abilities of germinating castor beans and derived particulate enzymes. Abstract 39 Amer. Soc. Plant Physiol. Ann. Meeting, Gainesville, Florida, 1954.
5. Beevers, H. and Walker, D. A. The oxidative activity of particulate fractions from germinating castor beans. Biochem. Jour. 62: 114–120. 1956.
6. Beevers, H. and Gibbs, H. Position of C^{14} in alcohol and carbon dioxide formed from labeled glucose by corn root tips. Plant Physiol. 29: 318–321. 1954.
7. Brown, A. H. Determination of pentose in the presence of large quantities of glucose. Arch. Biochem. 11: 269–278. 1946.
8. Gunsalus, I. C. and Gibbs, M. The heterolactic fermentation. II. Position of C^{14} in the products of glucose dissimilation by *Leuconostoc mesenteroides*. Jour. Biol. Chem. 194: 871–875. 1952.
9. Murlin, J. R. The conversion of fat to carbohydrate in the germinating castor bean, I. Jour. Gen. Physiol. 17: 283–302. 1933.
10. Pierce, H. B., Sheldon, D. E. and Murlin, J. R. The conversion of fat to carbohydrate in the germinating castor bean, III. Jour. Gen. Physiol. 17: 311–325. 1933.
11. Stumpf, P. K. Fat metabolism in higher plants. III. Enzymatic oxidation of glycerol. Plant Physiol. 30: 55–58. 1955.
12. Stutz, R. E. and Burris, R. H. Photosynthesis and metabolism of organic acids in higher plants. Plant Physiol. 26: 226–243. 1951.