PATHWAYS FROM GLUCONIC ACID TO GLUCOSE IN VIVO

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Using gluconic acid uniformly labeled with C^{14} and deuterium, we have previously studied the oxidation, excretion, and conversion of gluconic acid to glucose in rats (1). The C^{14} concentration and its approximately even distribution in glucose derived from liver glycogen or excreted in the urine suggested that gluconic acid was converted to glucose predominantly by pathways more direct than *via* CO_2. The alternative pathways suggested were direct reduction of the acid to the aldehyde, or degradation or fragmentation of gluconic acid to smaller molecules and subsequent resynthesis of hexose. The present study, involving parallel administrations of gluconate-1-C^{14} and uniformly labeled gluconate-C^{14}, was undertaken in order to evaluate the relative importance *in vivo* of the over-all pathways by which gluconic acid carbon may be utilized and converted to glucose.

EXPERIMENTAL

Preparation of Isotopic Sodium Gluconates—Uniformly labeled sodium gluconate-C^{14} was prepared by I_2 oxidation of uniformly labeled glucose derived from bean leaf starch, as previously described (1).

Sodium gluconate-1-C^{14} was synthesized by condensing 1 gm. of D-arabinose with $HC^{14}N$ by the method of Koshland and Westheimer (2). After hydrolysis of the mixture of gluconamide and mannonamide with $Ba(OH)_2$ the solution was neutralized with CO_2, clarified with charcoal, and filtered. The barium salts were treated with cation exchange resin (Amberlite IR-100-H) and the free hexonic acids so generated were neutralized with $KHCO_3$. 3 gm. of non-isotopic potassium gluconate were then added and the solution was evaporated to dryness *in vacuo*. The potassium mannonate was extracted from the residue with an excess of boiling methanol. The residual solid was crystallized from aqueous methanol-ether. The potassium gluconate was converted to sodium gluconate by removal of K ions with cation exchange resin and neutralization of the free gluconic acid in the eluate with NaOH. After concentration of the solution *in vacuo*, the sodium gluconate obtained was recrystallized from aqueous methanol-ether.

The C^{14} specific activities of the several preparations of the isotopic gluconate ranged between 31,800 and 450,000 c.p.m. per milliatom of C.

All tabulated specific activities have been expressed relative to 100,000 c.p.m. per milliatom of C in the injected sodium gluconate. In all preparations used a single radioactive component was found when chromatographed on paper with collidine (3).

Biological Experiments—Experiments were conducted on normal (Experiments A, B, C, D, and E) and alloxan-diabetic (Experiments F, G, and H) male rats of the Sherman strain. The methods of collection of urine and of CO_2 and the analytical procedures for total C and C^{14} have been described (1). Sodium gluconate-1'-C^{14} and uniformly labeled sodium gluconate-C^{14} were injected intraperitoneally in parallel experiments conducted either on pairs of similar rats (Experiments A, B, D, and E) or on the same rat after intervention of a suitable time interval (Experiments C, F, and G). The diabetic rat of Experiment F was used in three such pairs of studies at different dosage levels (Experiments F_1, F_2, and F_3). Experiments F_2 and F_3 were carried out with the same diabetic rat about 5 months after Experiment F_1. The diabetic rat of Experiment H received only sodium gluconate-1-C^{14}. Dosage levels and times of CO_2 and urine collections are given in Tables I and II. The rats were fed either a synthetic diet (4) (Experiments A, B, and H) or pellets (Experiments C, F_2, F_3, and G). In Experiment F_1 no food was given. In the short term Experiments D and E, designed to obtain maximal radioactivity in the liver glycogen, the rats were fasted for 24 hours and then given 2 mM (Experiment D) or 4 mM (Experiment E) of glucose per 100 gm. of rat by stomach tube at the same time that the radioactive gluconate was injected. The normal rats were killed for liver glycogen isolation after 24 hours (Experiments A and B) or 3 hours (Experiments D and E).

Diabetic Rats—Rats were made diabetic by the intraperitoneal injection of 150 mg. of alloxan monohydrate per kilo of rat. Three severely diabetic rats were selected for use. The rat used in Experiment H had been diabetic for 6 weeks and, when fed the synthetic diet, was excreting about 20 gm. of glucose in a volume of about 200 ml. of urine per day. The same rat excreted 12 gm. of glucose per day on a diet of pellets. Another rat, used in Experiment G, was excreting about 10 gm. of glucose in 180 ml. of urine each day while on a diet of pellets and was used about 4 weeks after alloxan administration. A third diabetic rat was used for Experiment F_1, 3 weeks after receiving alloxan, while excreting about 3 gm. of glucose in 75 ml. of urine per day. This same rat was employed 5 months later in Experiments F_2 and F_3, at which time it was excreting about 12 gm. of glucose in 200 ml. of urine on a diet of pellets.

Glucose Degradation Studies—From part of the urine of each diabetic rat glucose was isolated as glucosazone, which was recrystallized to constant radioactivity. Samples of glucosazone were oxidized with periodate (5, 6),

the osazone derived from C-(1 + 2 + 3) was removed by filtration, and the formaldehyde from C-6 isolated as its dimedon derivative. In contrast to all other samples, dimedon formaldehyde was plated and counted directly without prior combustion. An experimentally determined correction factor of 1.15 was applied to these counts to make them directly comparable with those done on $BaCO_3$. C-(4 + 5) was obtained by HgO oxidation of the formic acid in the residual solution. Glucose pentaacetate was isolated from the remainder of the urine in each case. Recrystallized glucose pentaacetate was hydrolyzed by refluxing with 0.3 N H_2SO_4, acetate and sulfate were removed with anion exchange resin (Amberlite IR-45), and the free glucose in the eluate was used for determination of radioactivity in C-1. C-1 was obtained in some cases by a Wohl degradation (7) and in others by oxidation of glucose in a bomb tube with HBr (8). Analyses for the average of C-(1 through 6) were carried out on both the free glucose and the glucosazone samples, the results obtained agreeing well in all cases.

Glycogen—Glycogen was isolated from the cold trichloroacetic acid extracts of the livers of normal rats killed either 3 or 24 hours after injection of isotopic sodium gluconate. Part of each purified glycogen sample was subjected to wet combustion to obtain the average C^{14} concentration of C-(1 through 6). Pooled samples of liver glycogen were hydrolyzed, C-1 was isolated as $BaCO_3$ after treatment with HBr, and C-6 as dimedon formaldehyde after a periodate oxidation.

DISCUSSION

The purpose of the present study has been to evaluate, in so far as possible, the extent to which gluconic acid is metabolized and the general routes of its metabolism in the intact rat. It is altogether likely that an initial phosphorylation must precede the further transformations of gluconic acid, as suggested by the finding of a gluconokinase in various microbial cells (9, 10), and it is possible that in effect the present results represent the fates of 6-phosphogluconic acid in the animal body. The techniques here employed do not yield any direct evidence relating to the occurrence or non-occurrence of such a phosphorylating step.

Urinary Excretion of Gluconate—Tables I and II give a summary of the per cent of injected dose of radioactive material recovered in the urines and expired CO_2 of a number of normal and alloxan-diabetic rats. The first facts to be noted from these figures are the magnitude, speed, and variability of the urinary excretion of gluconic acid. A very large part of the injected gluconate, regardless of the size of the injected dose, is excreted unchanged. We have repeatedly isolated gluconic acid of undiluted isotope composition from the urines of rats receiving isotopic gluconic acid (1). The per cent of dose found in the urine varied greatly, ranging, in

most experiments, from 60 to 85 *per cent* in 24 hours. Most of this excretion occurred in the first few hours after injection. For example in Table I, Experiment C, and in Table II, Experiments F_1, F_2, and G, it can be seen that nearly as much C^{14} was recovered in the urine after 6 or 7 hours

TABLE I

Per Cent of C^{14} Excreted by Normal Rats after Intraperitoneal Injection of Na Gluconate-C^{14}

Experiment	No. of rats*	Quantity injected	Time	Per cent of dose in CO_2		Per cent of dose in urine C	
				Gluconate-1-C^{14} administered	Uniform gluconate-C^{14} administered	Gluconate-1-C^{14} administered	Uniform gluconate-C^{14} administered
		mM per 100 gm. rat	*hrs.*				
A	2	2	24	12.7	8.3	66.7	70.4
B	2	1	24	9.0	4.9	82.4	81.5
C	1	1	7	9.4	3.8	32.6	81.8
			24	15.1	5.2	33.2	82.6
D	2	1	3	2.7	2.7	71	67
E	2	1	3	4.4	1.9	46	53

* In Experiments A, B, D, and E pairs of rats were used, one rat for gluconate-1-C^{14} and the other for uniform gluconate-C^{14}. In Experiment C the same individual rat was used for both studies.

TABLE II

Per Cent of C^{14} Excreted by Alloxan-Diabetic Rats after Intraperitoneal Injection of Na Gluconate-C^{14}

Experiment (1 rat in each)*	Quantity injected	Time	Per cent of dose in CO_2		Per cent of dose in urine C	
			Gluconate-1-C^{14} administered	Uniform gluconate-C^{14} administered	Gluconate-1-C^{14} administered	Uniform gluconate-C^{14} administered
	mM per 100 gm. rat	*hrs.*				
F_1	2	6	4.8	2.2	76.4	73.5
		24	6.7	4.7	77.2	74.8
F_2	1	7	4.6	27.6	79.0	13.9
		24			79.5	16.6
F_3	0.2	7	3.1	10.7		
		24	3.7	14.0	85.0	64.4
G	1	7	6.8	7.3	62.3	66.6
		24	7.5	8.9	63.0	68.0
H	2	6	33.5		26.9	

* Experiments F_1, F_2, and F_3 were all carried out with the same individual diabetic rat.

as after 24 hours. Even in Experiments D and E (Table I), in which normal rats were killed only 3 hours after receiving isotopic sodium gluconate intraperitoneally, up to 70 *per cent* of the C^{14} had already been eliminated in the urine. Thus it would seem that gluconic acid is readily absorbed from the peritoneum and rapidly enters the blood stream from which it is efficiently removed by the kidney. Occasionally, as in Experiment C (Table I) and again in Experiment F_2 (Table II), the same rat, under apparently identical conditions, showed great variability in urinary excretion of the injected gluconate. In Experiments F_1, F_2, and F_3, in

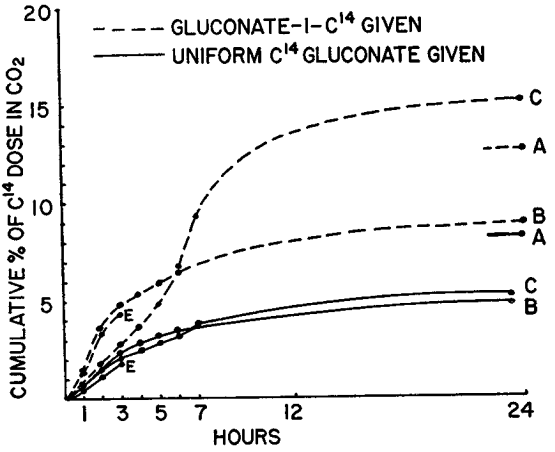

FIG. 1. Production of $C^{14}O_2$ by normal rats. The per cent of injected C^{14} excreted as $C^{14}O_2$ has been plotted cumulatively against time. Comparable experiments in which gluconate-1-C^{14} and uniform gluconate-C^{14} were given are indicated by letter. The differences between the various experiments are indicated in the text and in Table I.

which the same alloxan-diabetic rat was used for all six experiments, no systematic effect of quantity injected upon urinary excretion of gluconate was observed over a dosage range of 2 to 0.2 mM per 100 gm. of rat. Thus it would seem that the major fate of injected gluconate in both the normal and the diabetic rat is elimination in the urine, leaving available for utilization only such as is not rapidly excreted.

Gluconate → CO_2—A comparison of the per cent of dose in the CO_2 from the two differently labeled gluconates is somewhat complicated by the variability of the urinary excretions under apparently identical conditions. The results in normal rats (Table I, Fig. 1), however, are sufficiently consistent to indicate that C-1 of gluconate contributes to the expired CO_2 at a more rapid rate and to a greater extent than does the average of the 6 carbon atoms of gluconic acid. Thus a major metabolic fate of retained

gluconic acid in the rat involves the preferential contribution of the carboxyl carbon to CO_2, possibly via 6-phosphogluconic acid over steps delineated by Horecker *et al.* (11). It is also clear that this is not the sole contribution of gluconic acid to CO_2, since, if this were the case, the anticipated radiochemical yield of CO_2 from gluconate-1-C^{14} would be 6 times that from the uniformly labeled material. Carbon atoms 2 through 6 also

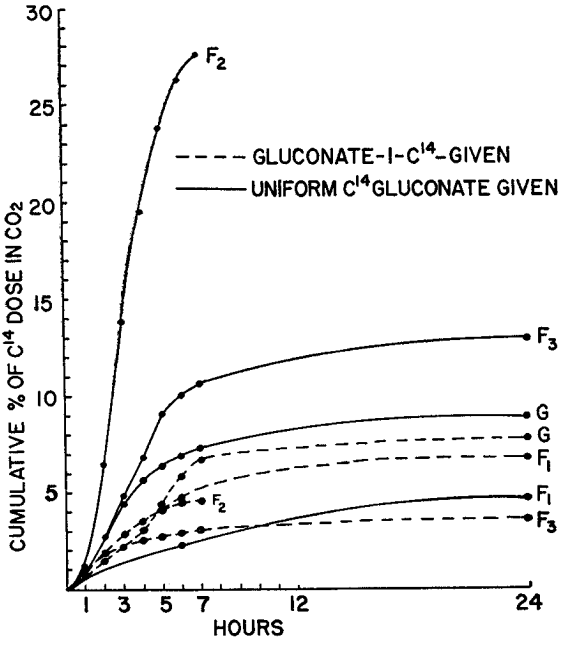

FIG. 2. Production of $C^{14}O_2$ by alloxan-diabetic rats. The per cent of injected C^{14} excreted as $C^{14}O_2$ has been plotted cumulatively against time. Comparable experiments in which gluconate-1-C^{14} and uniform gluconate-C^{14} were given are indicated. The differences between the various experiments are indicated in the text and in Table II.

make a contribution to the CO_2, but in the normal rats combustion of the remainder of the molecule lags behind the decarboxylation.

When alloxan-diabetic rats were similarly studied (Table II, Fig. 2), this relationship of radiochemical yields of $C^{14}O_2$ was in most instances reversed (Experiments F_2, F_3, and G). More C^{14} was contributed to CO_2 from uniformly labeled gluconate than from gluconate-1-C^{14}. Although no definitive explanation for this can be given, it may be suggested that a metabolic fragment from the upper part of the gluconic acid molecule may accumulate and be less readily converted to CO_2 in the diabetic than in the normal rat. Alternatively, the effect might be due to depressed decarboxy-

lation of gluconate in diabetic rats. The simpler explanation involving reduction of gluconate to glucose prior to oxidation may be ruled out on the basis of the low concentrations of isotope found in glucose after gluconate-1-C^{14} administration (see below).

Gluconate → Glucose—Isotope evidence of the glucogenic nature of gluconic acid has been presented in an earlier communication (1). The extent of utilization of gluconic acid carbon in glucogenesis may be seen from the

TABLE III

*C^{14} in Glycogen and Glucose after Administration of Gluconate-1-C^{14} and Uniform Gluconate-C^{14}**

Experiment	Quantity injected	Time	Gluconate-1-C^{14} administered		Uniform gluconate-C^{14} administered		Ratio, $\frac{(B)}{(A)}$
			C^{14} specific activity (A)	Per cent of C^{14} dose	C^{14} specific activity (B)	Per cent of C^{14} dose	
			Liver glycogen of normal rats				
	mM per 100 gm. rat	*hrs.*	*c.p.m. per milliatom C*		*c.p.m. per milliatom C*		
A	2	24	52	0.03	482	0.30	9.3
B	1	24	0		0		
D	1	3	91	0.02	1050	0.33	11.5
E	1	3	146	0.02	1480	0.55	10.1
			Urine glucose of alloxan-diabetic rats				
F_1	2	6	429	0.27	3300	1.7	7.7
F_2	1	7	79	0.18	1750	5.1	22.2
F_3	0.2	7	26	0.53	450	7.1	17.3
G	1	7	71	0.27	684	3.0	9.6

* All specific activities are expressed relative to 100,000 c.p.m. per milliatom of C in injected gluconate.

data given in Table III. In normal animals it was found that relatively small amounts of gluconate had been used in glycogen synthesis. After administration of 2 mM of labeled gluconate per 100 gm. of rat to well fed normal rats, only 0.3 *per cent* of the administered total gluconate carbon or 0.03 *per cent* of C-1 was recovered in liver glycogen after 24 hours. In similar experiments, when 1 mM of labeled gluconate was given, only traces of C^{14} were found in liver glycogen. In experiments designed to obtain higher concentrations of C^{14} in glycogen for degradation studies (Experiments D and E), gluconate-C^{14} was injected simultaneously with tube feeding of glucose to previously fasted rats. Liver glycogen, isolated after 3 hours, had a higher C^{14} concentration, but the per cent of dose incor-

porated was only 0.3 to 0.6 *per cent* of total gluconate carbon or 0.02 *per cent* of C-1. In diabetic rats 2 to 7 *per cent* of total gluconate carbon administered or 0.2 to 0.5 *per cent* of C-1 was recovered in urinary glucose.

Whether glycogen from the livers of normal rats or glucose from the urine of diabetic rats was studied, the striking finding was that the specific activity of the product ranged from 8 to 22 times as high (B:A, Table III) when uniformly labeled rather than when gluconate-1-C^{14} served as substrate. This finding requires that the bulk of the glucose which is formed from gluconic acid carbon arise by reactions which involve the loss of carbon 1 of gluconic acid. If direct conversion by reduction of gluconic or 6-phosphogluconic acid were the major pathway, this ratio would approximate 1.

Chemical Degradation Studies and Routes of Glucogenesis from Gluconate—Three possible routes whereby gluconate carbon atoms can be delivered to glucose have been considered and the resultant distributions of isotope derived from gluconate-1-C^{14} are indicated in Fig. 3.

1. Gluconic acid, with or without preliminary phosphorylation, may be reduced to the level of aldohexose without splitting of the carbon chain. The resulting glucose would contain isotope in C-1 after gluconate-1-C^{14} administration (Fig. 3 (*c*)). It would be uniformly labeled after administration of uniform gluconate-C^{14}.

2. CO_2 arising from any of the carbon atoms of gluconate in the course of its metabolism may be utilized in glucose synthesis. Gluconate-1-C^{14} can contribute $C^{14}O_2$ by any series of steps resulting in decarboxylation at C-1. Uniform gluconate-C^{14} can contribute $C^{14}O_2$ from any of its carbon atoms. In either case, through operation of the "succinate shuttle," the C^{14} would be located predominantly in C-3 and C-4 of glucose formed (Fig. 3 (*b*)) (12). The same distribution of isotope might arise if gluconate underwent a cleavage yielding a glucogenic 3-carbon acid in which the carboxyl carbon arose from C-1 of gluconate-1-C^{14} (13).

3. Gluconic acid, probably as its 6-phosphate, may be split and compounds derived from the lower portion of the molecule, C-(2 through 6), such as 3-phosphoglyceraldehyde from C-(4 + 5 + 6) may be used for glucose synthesis. By reversal of glycolysis such compounds would give glucose containing no C^{14} when gluconate-1-C^{14} was given (Fig. 3 (*a*)). Uniformly labeled gluconate would give uniformly labeled glucose by this pathway. The hypothetical 3-carbon fragment might arise by direct cleavage between carbon atoms 3 and 4 (13) or by a secondary split of pentose-5-phosphate formed from the gluconate (14).

Partial degradations designed to study the distribution of C^{14} in the glucose excreted by the diabetic rats have been carried out and the analytical data are presented in Table IV. As in the earlier study (1) isotope

was found to be approximately uniformly distributed throughout the 6 carbon atoms of glucose when uniformly labeled gluconate served as precursor. From this finding, considered together with the observation (Table

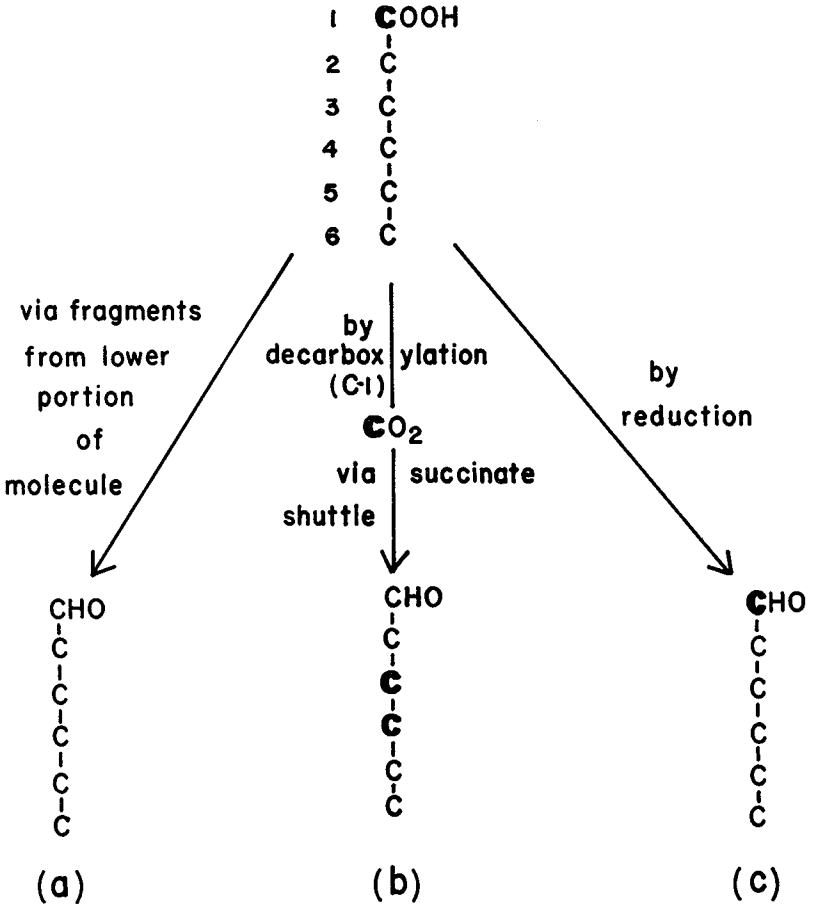

FIG. 3. Postulated pathways of carbon from gluconate to glucose. The heavier lettering indicates the major distribution of the carboxyl carbon of gluconic acid resulting from each of these pathways.

III and Experiment G, Table IV) that about 10 times as much isotope entered glucose from uniformly labeled gluconate as from gluconate-1-C^{14}, it is concluded that about 90 *per cent* of the glucose carbon derived from gluconic acid comes from carbon atoms 2, 3, 4, 5, or 6. In terms of the three pathways postulated in Fig. 3, it follows that pathway (*a*), via fragments from the lower portion of the molecule, is the major one in the generation of glucose from gluconic acid.

To evaluate the pathways whereby the residual 10 *per cent* of gluconic acid carbon enters glucose, it is necessary to consider the distribution of isotope in glucose following administration of gluconate-1-C^{14}. In order to clarify the discussion, the data of Table IV have been recalculated (Table V) with specific activities assigned to each of the 6 carbon atoms of glucose, on the assumptions that carbon atoms 3 and 4 have identical specific activities and that the slight "smear" of C^{14} found in C-6 is the same as that in C-2 and C-5. Whereas it is recognized that these assumptions can not be rigorously defended, it is believed that they are valid as a first approximation.

TABLE IV

Degradation of Urinary Glucose from Diabetic Rats Given Na Gluconate Intraperitoneally

Glucose C	C^{14} specific activity, c.p.m. per milliatom C			
	Gluconate-1-C^{14} given			Uniform gluconate-C^{14} given
	Experiment H	Experiments $F_1 + F_2$*	Experiment G	Experiment G
C-(1 through 6)	1280	80	71	665
C-1	369	33	20	661
C-(4 + 5)	1790	98	82	588
C-6	150	9	6	591

* Glucose samples pooled for degradation.

TABLE V

*C^{14} Concentrations in Individual Carbon Atoms of Glucose from Diabetic Rats Given Gluconate-1-C^{14}**

Glucose C	C^{14} specific activity, c.p.m. per milliatom C		
	Experiment H	Experiments $F_1 + F_2$	Experiment G
C-1	369	33	20
C-2	150	9	6
C-3	3430	187	158
C-4	3430	187	158
C-5	150	9	6
C-6	150	9	6

* Numbers were obtained by calculation from values given in Table IV, assuming C-3 = C-4 and C-2 = C-5 = C-6.

Study of the values in Table V reveals that in each case, after injection of gluconate-1-C^{14}, the major portion of the isotope in glucose resides in

positions 3 and 4. However, there is also specific isotopic enrichment of C-1 as is shown by the finding of about 3 times as much C^{14} in C-1 as in C-6. From these observations it would appear that both pathways (*b*) and (*c*) (Fig. 3) are operative. From the finding (Table V) that the difference in specific activities between C-1 and C-6 is about one-tenth or less of the specific activity in positions 3 or 4, it is concluded that far more, possibly 20 times as much, carbon of glucose is derived from position 1 of gluconate via CO_2 (*b*) as by the pathway of direct reduction (*c*).

Liver Glycogen from Normal Rats—The quantity of glycogen available for degradation studies was very small and, in the case of that from the gluconate-1-C^{14} experiments, so low in C^{14} concentration that only an incomplete degradation could be carried out. Approximately uniformly labeled glycogen followed administration of uniform gluconate-C^{14} to normal rats, while, after administration of gluconate-1-C^{14}, C-1 and C-6 contained very little C^{14}. A pooled sample of liver glycogen from several gluconate-1-C^{14} experiments had a specific activity of 396 c.p.m. per milliatom of C in C-(1 through 6) and only 50 in C-1 and 10 c.p.m. per milliatom of C in C-6, suggesting relatively much higher activity in C-(3 + 4). The results obtained from liver glycogen in normal rats are thus in accord with those from urinary glucose of diabetic rats.

Evaluation of Pathways from Gluconate to Glucose—A major metabolic fate of intraperitoneally injected gluconic acid in the rat appears to involve the loss of carbon atom 1 as carbon dioxide and the formation of glucogenic fragments from the residue of the molecule. This view is supported both by the finding that the carboxyl carbon of gluconic acid contributes preferentially to CO_2 and by the fact that the major contribution to glucose stems from carbon atoms 2, 3, 4, 5, or 6. In the present study, roughly 90 per cent of glucose carbon derived from gluconate came over this or some similar pathway.

Carbon atom 1 of gluconic acid also enters glucose in the body of the rat. The present results indicate that the major share of this contribution stems from fixation of CO_2 derived from the decarboxylation step mentioned above. This process may account for about 10 per cent of all of the carbon from gluconate which is introduced into glucose.

The consistent finding of greater isotope enrichment at C-1 than at C-6 of glucose after injection of gluconate-1-C^{14} indicates that the carboxyl group of gluconic acid is subject to reduction to the carbonyl level but only to a very minor extent. Whereas the direct reversal of either the TPN oxidation of glucose-6-phosphate or the DPN oxidation of glucose seems unlikely on thermodynamic grounds, evidence has been adduced recently favoring the δ-lactone as an intermediate in the oxidation and it has been suggested that reduction of the lactone might be more readily effected than reduction of the carboxylic acid (15, 16). It is concluded from the present

experiments that in the intact rat less than 1 per cent of all of the glucose carbon derived from gluconic acid comes via direct reduction without rearrangement of the carbon skeleton.

SUMMARY

Gluconate-1-C^{14} and uniform gluconate-C^{14} acid have been prepared and administered intraperitoneally in parallel experiments to normal and alloxan-diabetic rats.

Gluconic acid carbon was found to be rapidly and extensively excreted in the urine. In normal rats CO_2 was produced more rapidly from C-1 of gluconate than from the average of C-(1 through 6). In most experiments with diabetic rats, on the other hand, less CO_2 came from C-1 than from C-(1 through 6).

Gluconic acid carbon was found to have been utilized for glucose synthesis. The relative isotope concentrations and the distribution of C^{14} found in glycogen and in urinary glucose after administration of gluconate-1-C^{14} and uniform gluconate-C^{14} permit an evaluation of the pathways by which gluconate carbon gets to glucose.

Of the gluconate carbon which goes to glucose it has been estimated that (1) a significant but small amount, probably less than 1 *per cent*, goes by pathways involving a direct reduction of the carboxyl group without a split of the carbon chain; (2) roughly 10 *per cent* can be accounted for as coming from CO_2 derived from C-1 of gluconic acid; (3) the major fraction, approximately 90 *per cent*, of such gluconate carbon as gets to glucose, goes by steps involving loss of C-1 and utilization of glucogenic compounds derived from C-(2 through 6) of gluconate.

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