RAT DIAPHRAGM CARBOHYDRATE METABOLISM IN THE PRESENCE OF THIOLS

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ABSTRACT. — The influence of equivalent concentrations of thioglycolate, β-mercaptoethanesulfonate, α,α′-dimercaptoadipate, cysteine and dithiodiglycolate on carbohydrate metabolism of the rat diaphragm was ascertained. Because of the divergent effects noted with the sulfur compounds tested, the over-all configuration, rather than the sulfhydryl group as such, constitutes a most important factor affecting carbohydrate metabolism.

The rat diaphragm has been used extensively in *in vitro* metabolic studies of the influence of hormones, drugs and metabolites on muscle. Knowledge is lacking on the correlation of resting muscle metabolism with that of the functioning tissue and various facets of this problem have been reviewed by Szent-Györgyi (1951). The rat diaphragm was first employed by Takane (1926) in relation to the *in vivo* effects of insulin on carbohydrate metabolism and later studied in greater detail by Gemmill (1940). It is particularly suitable for immediate use without extensive manipulations because of its inherent structure. The thickness of the diaphragm meets the qualifications necessary for the rapid exchange of gases to and from the cells and can be determined from the surface area and specific gravity (Villee et al., 1949; Umbreit et al., 1951). The *in vitro* results with rat diaphragm might correlate closely with those observed in the *in vivo* response because the number of intact cells remains high and the structural characteristics, such as the vital membrane between the medium and the intracellular fluid, undergo little alteration.

The study of Stadie and Zapp (1947) on the influence of various media on glucose utilization by diaphragm as such and in the presence of insulin is classical. In the pH range of 6.3-7.6, the effect of insulin was constant. With untreated diaphragm, oxygen was utilized at a steady rate for a period of six hours and glycogen synthesis was maximal after about one hour. On introduction of insulin, although the oxygen consumption remained constant, increased glycogen synthesis continued for approximately two hours. The ionic concentration of the medium likewise affects the extent of glycogen formation; magnesium and phosphate ions at high levels depressed glycogenesis in the presence or absence of insulin and glycogen values were lower on deletion of the cation. Glycogen synthesis is affected by diet, sex, insulin and time of fasting, fed rats with an initially high glycogen content displaying the greatest amount of glycogenesis (Kerly and Ottaway, 1954). The metabolic fate of glucose as well as acetate and pyruvate utilized by this muscle has been elucidated (Villee and Hast-

ings, 1949a,b; Walaas and Walaas, 1950; Foster and Villee, 1954). On the basis of experiments in which diaphragm was incubated in the absence of glucose, metabolites other than glycogen were thought to comprise the main source of energy (Haugaard et al., 1951).

Of the various agents investigated in relation to carbohydrate metabolism by the rat diaphragm, insulin is unique in producing an increase in both glucose utilization and glycogen deposition. A direct combination occurs between insulin and the muscle (Stadie et al., 1949a,b). The effect of pituitary hormones, sterols and epinephrine and other amines on diaphragm metabolism has been reported (Verzár and Wenner, 1948; Li et al., 1949; Stadie et al., 1951; Ellis, 1952).

In a previous study (Spencer et al., 1964), the oxygen uptake by rat tissue slices was ascertained in the presence of varying concentrations of the important thiol, thioglycolic acid (TG) and under more limited conditions, with β-mercaptoethanesulfonic acid (MES). As such data reflect over-all tissue response, it was thought that more specific effects attributable to TG might be observed by a study of muscle carbohydrate metabolism. Accordingly, TG and several related thiols were compared by this approach. The additional compounds, included in order to deduce whether the changes, if any, engendered by TG were specific for this molecule alone or would extend to other thiols, comprised MES, cysteine and α,α'-dimercaptoadipic acid (DMA). Dithiodiglycolic acid (DTDG), though a disulfide, was also investigated as it represents the readily obtainable oxidation product from TG. A statistical analysis was carried out on the changes observed in glycogen content, glucose utilization and oxygen uptake by the respective rat hemidiaphragms.

Experimental Procedure

Commercial TG from Evans Chemetics Company was distilled twice under vacuum (b.p. 105-108° C at 20 mm; collected in water). The corresponding disulfide, after two recrystallizations from water, melted at 107-108° C. L-cysteine originated from Nutritional Biochemicals Corporation and MES and DMA which were prepared by the Toni Company, were of high purity. The acids, except for L-cysteine, were introduced into water and the pH adjusted to 7.2 with aqueous sodium hydroxide. The stock solutions contained 200-300 mg per ml as based on the free acid. The medium for the incubation of disphragms comprised the one described by Stadie and Zapp (1947): 0.04 M Na_2HPO_4, 0.005 M $MgCl_2.5H_20$ and 0.08 M NaCl; pH 6.8-7.0. Glucose was used in this mixture at a level of 110 mg %.

Male rats averaging 150 gm in weight at the time of experimentation were of a Wistar and Sprague-Dawley cross. They were starved for 24 hr in order to deplete glycogen and killed by decapitation. The hemidiaphragms were removed quickly with minimum trauma and bleeding, care being exercised to avoid cutting the inferior vena cava and immediately placed in individual tubes each containing 2.0 ml of ice-chilled medium. The trimmed hemidiaphragms were blotted, weighed

placed in the individual vessels containing 1.5 ml test medium and incubated for 90 min under 100% oxygen at 37.5°C. The appropriate solutions without tissue were simultaneously incubated. In the first series of experiments, beakers of 20 ml capacity containing the individual hemidiaphragms with the respective media were incubated in a metabolic shaker (Dubnoff, 1948). The chamber was flushed with 100% oxygen for a 10 min period at a gas flow of 5 cu ft per hr. During incubation, the gas flow was reduced to one cu ft per hr. In the later studies, a similar procedure was carried out in the Warburg apparatus equipped with 18 flasks except that the oxygen uptake was also ascertained.

Dreywood's anthrone reagent was used for the determination of both glucose and glycogen (Morris, 1948). After removal of the hemidiaphragms at the conclusion of the experiment, an aliquot of the mixture was diluted 1:50 with water and the glucose analyzed directly. The hemidiaphragms were rinsed twice with phosphate-saline medium, being blotted before and after each washing to remove adherent solution. They were then immediately placed into tubes containing 0.3 ml of 30% potassium hydroxide and digested for 30 min in a boiling water bath. Glycogen was precipitated in the presence of 0.1 ml of 2% sodium sulfate on addition of 1.2 ml of 90% ethyl alcohol by the procedure of Good, Kramer and Somogyi (1933) as modified by Walaas and Walaas (1950).

In the statistical analysis of data, as the Warburg apparatus imposed a limitation on the number of tissues incubated simultaneously, the balanced incomplete block design as outlined in Figure 1 was applied (Cochran and Cox, 1955).

Results

Metabolic Shaker Series. A total of 50 rats was equally divided between the five compounds at each of the following concentrations. Ten animals were used per day (replication), alloting two rats to each treatment.

In the first experiment, the effect of the thiols at a level of 5×10^{-5} N was ascertained. The two pairs of hemidiaphragms under the same

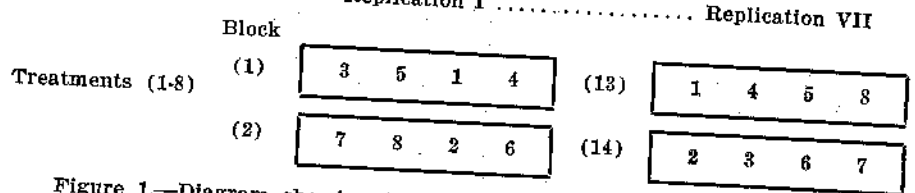

Figure 1.—Diagram showing the incomplete block design employed for the incubation study of hemidiaphragms in the Warburg apparatus. In text and tables, the parts of this design are referred to by the following symbols: R = 7, the number of replications or days; B = 14, the number of blocks or Warburg runs; T = 8, the number of treatments, each being the difference in glucose utilization, total glycogen content, or oxygen uptake between the control and the treated hemidiaphragm of each pair; k = 4, the number of treatments within each block; n = 7, the number of paired hemidiaphragms used for each treatment.

TABLE 1.—Summary of the Average Effects Produced by the Sulfur Compounds on Rat Hemidiaphragms (metabolic shaker series)[a,b]

Treatment	Concentration	Change in glucose uptake[c]	t	Change in glycogen content	t
	N	μg/mg wet tissue/hr		μg/mg wet tissue/90 min	
TG	5×10^{-5}	0.73 ± 0.311	2.35*	0.15 ± 0.059	2.54*
	5×10^{-3}	0.96 ± 0.399	2.41*	0.74 ± 0.252	2.94**
MES	5×10^{-5}	−0.02 ± 0.199	0.10	0.27 ± 0.096	2.81*
	5×10^{-3}	0.63 ± 0.354	1.78	−0.25 ± 0.117	2.14
Cysteine	5×10^{-5}	−0.04 ± 0.346	0.12	−0.19 ± 0.151	1.26
	5×10^{-3}	0.22 ± 0.263	0.84	0.16 ± 0.256	0.62
DTDG	5×10^{-5}	0.09 ± 0.433	0.21	0.23 ± 0.096	2.40
	5×10^{-3}	0.25 ± 0.231	1.08	0.17 ± 0.209	0.81
DMA	5×10^{-5}	0.59 ± 0.346	1.70	−0.23 ± 0.111	1.63
	5×10^{-3}	−0.10 ± 0.137	0.73	0.19 ± 0.240	0.79

[a] The means (± s.e.) are deduced for 10 paired hemidiaphragms.
[b] A positive mean difference indicates a decreased response in the presence of the sulfur compound.
[c] The amount of glucose utilized was based on the final concentration of the respective media incubated without tissue.
* Significant at the 5% level of probability.
** Significant at the 2% level of probability.

treatment within replication were assigned to the media in such a way that if the left hemidiaphragm of one pair were incubated in the control solution and the right in the test medium, the order would then be reversed for the second pair. In this manner, the same number of 'right' and 'left' hemidiaphragms were subjected to treatment and no significant difference was apparent with either tissue. A difference in effect between test compounds on glycogen content is indicated (Table 1). The treatments, MES and DTDG, caused an apparent decrease in the amount of glycogen, while the reverse was observed for DMA and cysteine.

In the second experiment, each of the paired hemidiaphragms was allotted in a random order directly to beakers containing chilled media with or without glucose during the pre-incubation period, the thiols being tested at a level of 5×10^{-3} N. Replications again were found to have a decisive influence upon glucose utilization.

Although no difference in treatment effects was shown from the analysis of variance on glucose uptake, when the average differences for TG at 5×10^{-5} N and 5×10^{-3} N are compared to the mean difference of zero for a pair of hemidiaphragms treated alike, t values significant at the 5% level of probability result. This depression of glucose uptake reflects that of the corresponding final glycogen content.

Warburg Series. For the further study of the contrasting effects between TG, MES and cysteine, the Warburg apparatus was employed, allowing for the determination of oxygen uptake in addition to the glucose and glycogen analyses. Each of the thiols was tested at 5×10^{-3} N and 5×10^{-5} N. As indicated, two Warburg runs were required for each replication due to the limited capacity of the apparatus. Two treatments designated as Controls *1* and *2* were included, both consisting of the differences found between pairs of hemidiaphragms incubated only in glucose-containing medium. One side of each control diaphragm was termed the 'untreated', prior to incubation. The hemidiaphragms of each pair were assigned at random to the respective media (Figure 1). A total of 56 rats was equally divided among the eight treatments. No increase in precision for the evaluation of glucose uptake resulted under these experimental conditions, replications alone being highly variable in agreement with the previous results. In regard to the glycogen content, differences among treatments were substantiated in the analysis of variance (Tables 2 and 3). Unlike glucose and glycogen, analysis of the oxygen uptake data shows neither an influence of replications nor a diversification in treatment response. A marked decrease is noted with TG at 5×10^{-3} N corresponding to the glycogen depression (t test; Table 2).

Discussion

In the present series employing a metabolic shaker, TG and related sulfur compounds were compared at two separate concentrations, 5×10^{-5} N and 5×10^{-3} N, as to their effect on the isolated rat diaphragm. Ten animals were selected for each daily run, thereby allowing for a duplicate comparison of the five

TABLE 2.—Summary of the Average Effects of Sulfur Compounds on the Metabolism of Hemidiaphragms (Warburg series)[a]

Treatment	Concentration	Change in glucose uptake[b]	t	Change in glycogen content[b]	t	Change in oxygen uptake[b]	t
	N	μg/mg wet tissue/hr		μg/mg wet tissue/90 min		μl/mg wet tissue/hr	
TG	5×10^{-5}	0.38(0.48) ± 0.574	0.66	—0.24(—0.20) ± 0.222	1.08	0.02(0.05) ± 0.085	0.24
	5×10^{-3}	0.37(0.32) ± 0.441	0.84	0.50(0.51) ± 0.141	3.55*	0.30(0.22) ± 0.061	4.92**
MES	5×10^{-5}	—0.05(0.04) ± 0.440	0.11	0.32(0.40) ± 0.142	2.25	0.29(0.33) ± 0.154	1.82
	5×10^{-3}	—0.09(—0.11) ± 0.460	0.19	—0.10(—0.19) ± 0.099	1.01	0.17(0.06) ± 0.141	1.20
Cysteine	5×10^{-5}	0.45(0.41) ± 0.543	0.83	0.10(0.12) ± 0.114	0.89	0.00(0.06) ± 0.212	0.00
	5×10^{-3}	0.37(0.36) ± 0.612	0.60	0.05(—0.01) ± 0.160	0.31	0.12(0.10) ± 0.079	1.52
Control (1)	.....	0.26(0.20) ± 0.167	1.56	0.08(0.15) ± 0.055	1.45	0.06(0.00) ± 0.062	0.97
Control (2)	.....	0.31(0.30) ± 0.184	1.68	0.06(0.00) ± 0.043	1.40	—0.06(0.05) ± 0.083	0.72

[a] The averages ± s.e. are based on the results with 7 paired hemidiaphragms. A negative mean difference indicates an increased response in the presence of test compound.

[b] The values in parentheses are the treatment mean differences adjusted for blocks (Warburg runs). For the comparison of two adjusted treatment means by the t test, the standard error in this instance is $\sqrt{2/7\ E_e\ [1 + (T\text{-}k)/T(k\text{-}1)]}$ based on the effective error variance of Table V; cf. Figure 1 for symbols.

* Significant at the 2% level of probability.

** Significant at the 1% level of probability.

TABLE 3.—Analysis of Variance of the Effect of Control, TG, MES and Cysteine, each at 5×10^{-3} N and 5×10^{-5} N on Hemidiaphragms (Warburg series)*

Source of variation	df	Change in oxygen uptake			Change in glucose uptake			Change in glycogen content		
		ss	ms	F	ss	ms	F	ss	ms	F
Replications (R)	6	0.37202	0.06200	0.69	36.55180	6.09196	6.74**	0.41600	0.06933	0.65
Treatments (adjusted)	7	0.51637	0.07377	0.82	1.67798	0.23971	0.26	2.73697	0.39100	3.68**
Blocks within (R)	7	1.73978	0.24854	.....	1.89065	0.27009	.....	1.72379	.....	.....
Intra-block error	35	3.13883	0.08968†	.....	31.61617	0.90332†	.....	3.71594	0.10616†	.....
Total	55	5.76700			71.73660			8.59270		

* The average values for the differences in oxygen uptake, glucose and glycogen appear in Table IV.
** Significant at the 1% level of probability.
† Effective error variance (E_e).

compounds. No difference was observed between the effect of the sulfur compounds within each concentration on the glucose uptake. At 5×10^{-5} N, only a heterogeneous response was noted between treatments on glycogen content only, a slight elevation resulting with cysteine and DMA in contrast to a moderate decrease in the presence of MES, DTDG and TG. A marked depression in glycogen content with the higher concentration, 5×10^{-3} N, was distinct solely for TG.

The 'paired' t test is properly applied in these experiments as it was in the case of the tissue respiration studies (Spencer et al., 1964). Glucose uptake in the presence of 5×10^{-5} N TG was slightly decreased, while at the higher concentration, a more significant depression was observed. The average differences in glycogen content due to the presence of the five sulfur derivatives do not parallel the respective changes in glucose uptake except for TG, where a significant lowering in muscle glycogen was also observed. The two thiols, MES and TG at a level of 5×10^{-3} N, behaved differently as regards glycogen, the former compound increasing the over-all content as shown in Table 1.

This study was further amplified by the use of the Warburg apparatus with three of the agents, TG, MES and cysteine (concentrations: 5×10^{-5} N and 5×10^{-3} N). The general experimental conditions remained the same except that about 20 min were required for additional adjustments. Control solutions included for each test compound served not only as a check on the glucose concentration, but also toward correction of the manometric readings. As more than one Warburg run was necessary to include all test solutions, a balanced incomplete block design was selected as the most suitable for the experiment. An important feature of this procedure provides that each treatment will be tested an equal number of times concurrently with every other one and that an adjustment be applied to the treatment averages to correct for the influence of blocks (runs) in which they appear. It should be pointed out that no comparison could be made in the metabolic shaker series between the varied responses by glycogen to the two levels of MES or TG, both concentrations being studied separately. In the Warburg experiments, this effect was duplicated and the difference between the adjusted treatment averages found to be significant (t - values: 3.14 and 3.77 for MES and TG, respectively; $P < 0.01$).

Control treatments, namely, the differences between paired hemidiaphragms incubated in the absence of any sulfur compound, served to substantiate the reliability of the results. The variance or standard error attached to these treatments in regard to glucose uptake proved smaller than those observed with the test compounds. This finding would indicate that the response to the mercapto-acids is dependent on the individual pairs of hemidiaphragms investigated.

Glucose determinations were consistently dependent upon replications in both series. In the metabolic shaker experiment at 5×10^{-5} N, the mean glucose uptake for the control hemidiaphragms ranged 1.76-2.54 mg

for the five replications, whereas the average of the control hemidiaphragms for each thiol treatment was 2.02-2.59 mg. The variability of glucose estimations affected equally all determinations per day, no interaction with treatments (T x R) being produced. Whether glucose determinations were sensitive to a technical factor or to animal variation (condition) could not be evaluated from these experiments. The corresponding average glucose uptake value was found to be 1.53 mg by Villee and Hastings (1949a) and Krahl and Cori obtained an average of 1.93 mg (1947).

The results of the present investigation possibly confirm one of the published *in vivo* observations. Thus, Freeman and coworkers (1956) demonstrated that the glycogen content of liver was markedly reduced following the injection of toxic doses of TG in mice treated with glucose. However, rat gastrocnemius muscle underwent little alteration but the amount of glucose employed in this instance was much higher. It would be of interest to ascertain whether an elevation in the amount of glucose in the incubation media would alleviate the depression in diaphragm glycogen, a point not investigated in the present study. This is proposed in view of the decreased toxicity noted by the Freeman group when glucose was administered together with TG to intact animals. Certainly, no correlation is justified between the present tissue results and parenteral toxicity data. In fact, MES, which is even more toxic than TG, did not impair glycogenesis but actually increased glycogen formation at the higher concentration.

Although it was thought that a similarity in metabolic action might exist among the four compounds, TG, MES, DMA and cysteine, because of the presence of at least one sulfhydryl group in each, this was not the case when they were compared at equivalent concentrations. Because of their divergent behavior, the over-all structure rather than the presence of the SH group as such must be the responsible factor. Also, no generalization can be advanced as yet relative to thiol configuration and muscle carbohydrate metabolic activity. That the observed effects with TG are also bound up with the SH group is evident from the qualitative differences resulting with the disulfide or oxidized form (DTDG). Of the sulfur compounds investigated, only cysteine is endogenous to the cell. From its innocuous nature at the relatively high level screened in this study, a low order of direct involvement in *in vivo* muscle glycolysis might be implied.

Summary

The influence of equivalent concentrations of thioglycolate, β-mercaptoethanesulfonate, α,α'-dimercaptoadipate, cysteine and dithiodiglycolate on carbohydrate metabolism of the rat diaphragm was ascertained. A phosphate-saline medium containing 110 mg % glucose was employed throughout. Incubation was carried out in a metabolic shaker for the comparison of the sulfur compounds at two levels, 5×10^{-5} N and 5×10^{-4} N. At the former concentration, no decisive difference between the effects of the sulfur compounds resulted. Thioglycolate, in

contrast to the other compounds, produced a definite depression in final glycogen content as well as a lower glucose uptake. β-Mercaptoethanesulfonic acid at the higher level, appeared to stimulate glycogenesis. Neither cysteine, dimercaptoadipate nor dithiodiglycolate at either concentration affected the final glycogen content or glucose utilization.

In a second series, the effect of thioglycolate, β-mercaptoethanesulfonate and cysteine were compared at the two concentrations employing the Warburg apparatus. Two control treatments were included to ascertain the normal variation and average difference between untreated paired hemidiaphragms. Glycogen content and oxygen uptake were depressed only in the presence of thioglycolate at 5×10^{-3} N. A difference in glycogen response to the two levels screened was specific for thioglycolate and β-mercaptoethanesulfonate.

Because of the divergent effects on carbohydrate metabolic activity noted with the sulfur compounds tested, the over-all configuration rather than the sulfhydryl group as such, constitutes a most important factor.

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