# ON GLUTATHIONE.

## II. A THERMOSTABLE OXIDATION-REDUCTION SYSTEM.

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(Received for publication, September 12, 1922.)

### INTRODUCTION.

In a previous paper (Hopkins, 1921) an account was given of the isolation from yeast and from animal tissues of a dipeptide containing sulfur. The constituent amino-acids were shown to be glutamic acid and cysteine, and *glutathione* was suggested as a convenient name for the substance. The hydrogen of the $-SH$ group in the cysteine moiety is, as might be expected from many analogies, capable of easy oxidation. Since such oxidation results in the formation of a disulfide group, $-S-S-$, and involves the linkage of 2 molecules, the substance in its oxidized form is no longer a dipeptide in a strict sense, but an association of four amino-acid residues. It would be consistent with the analogy of the cysteine-cystine nomenclature to give a separate name to each form; but it will be convenient, at any rate until the constitution of the substance is completely established, to use the name glutathione alone, and to speak respectively of its reduced and oxidized form. Equilibrium in the living cell would seem to be such that the greater part of the substance present exists in the reduced condition; but oxidation and reduction of the constituent sulfur groups are reversible processes in the tissues, and both forms may at any moment be present.

A full study of the general chemical properties of the substance is in progress. The present communication is concerned with observations meant to elucidate further its relations and functions in the living cell and for the most part only with experimental results which have shown that its activities are associated in an

unexpected manner and to a remarkable extent with tissue agencies which are thermostable.

A word may be said as to the terminology used in the discussion of the facts. In the case of a system containing a tissue catalyst, together with substances susceptible to oxidation under the influence of such a catalyst, it is difficult without detailed knowledge to find criteria concerning the nature of the system which will fully distinguish between different possibilties suggested in views that are at present held. If, indeed, the oxidation occurs only upon access of free oxygen it is legitimate in the present state of knowledge to assume that an "oxidase" system is involved, of the type pictured by Engler, Bach, Chodat, and others. If, however, (a reducible substance being present or artificially provided) oxidation occurs also indirectly under strictly anaerobic conditions, the suggestion then is that the catalyst acts either as one determining simultaneous oxidation and reduction by means of the elements of water, as suggested by Bach; or it may act rather (the distinction, in certain cases at least, being real) by determining the transfer of hydrogen from an oxidizable substance to a reducible substance on the lines suggested by Wieland (1914). It is impossible without knowing full chemical details concerning the reaction or reactions involved to decide between the last two possibilities. For the purpose of the following discussion it is extremely convenient to assume the view of Wieland and to employ the related terminology of Thunberg (1920); to speak, that is, of the substance oxidized as the "hydrogen donator" and of the substance reduced as the "hydrogen acceptor." Under aerobic conditions the acceptor, of course, may be oxygen itself. When, however, the term hydrogen donator is used it should be remembered that in any particular case under consideration the term oxygen acceptor might better correspond with the facts. No essential confusion, as a matter of fact, arises, when only general aspects of the phenomena are under discussion, if these two terms be taken as equivalent.

Into Wieland's conception of hydrogen transport the properties of the sulfur group of glutathione fit with special readiness. It will be found, however, that the facts now to be described suggest that the activities of the dipeptide seem but little concerned with those of any recognized type of tissue catalyst.

The experiments upon which this paper is based have dealt chiefly with amphibian and mammalian muscle; but less complete observations upon other animal tissues have indicated that the essential statements hold true for these also.

Reduced glutathione, at suitable hydrogen ion concentrations, is oxidized by molecular oxygen, and under similar conditions it freely reduces methylene blue. On the other hand, as was shown in the previous communication, factors are present in the tissues which promptly reduce the oxidized product whenever its concentration is raised above an equilibrium value.

While, therefore, the sulfydryl group of the dipeptide constitutes one at least of the reducing agents of the tissues, its special significance depends upon the circumstance that when it transfers its hydrogen to molecular oxygen, or to such an acceptor as methylene blue, it is itself reduced afresh by normal tissue agencies. A continuous transfer of hydrogen is thus established and, as should be noted, at a velocity much greater than that with which the hydrogen is directly transferred—if it be transferred at all—to the acceptor from the particular sources concerned.

In so far as they relate to the reduction of methylene blue these facts were established by observations described in the first paper. Further experiments have shown that they hold true when the final acceptor is atmospheric oxygen.

An experimental analysis of vital oxidations must involve more than the isolation of intermediary oxidation products. It is clear from what is known that some analysis of the oxidative mechanisms is possible as well as desirable.

The acquisition of interesting facts by the study of tissues before and after they have been merely extracted with water has justified some attempt at analysis on these simple lines. Just as it helped Harden and Young to a recognition of the coferment of alcoholic fermentation, so has this method enabled Meyerhof (1918), to show that two factors, at least, are concerned in the maintenance of respiratory oxidations, and Batelli and Stern (1914) to demonstrate a distinction between soluble and insoluble catalysts in the tissues. Further, by the use of the same simple procedure, involving the removal by washing of the soluble hydrogen donators present, and the restoration of the lost reducing power by the addition of substances of known constitution,

Thunberg has thrown light upon intermediary products as well as upon certain aspects of the oxidative mechanism.

A fresh tissue, when very thoroughly washed, fails to reduce methylene blue or reduces it very slowly. The washing removes *inter alia* the greater part of its content of glutathione. When, under suitable conditions the dipeptide is alone restored to the system, a considerable part of the original reducing power is also restored, just as completely—it should be noted—when the added dipeptide is in the oxidized form (containing, therefore, no mobile hydrogen) as when it is added already reduced.[1] This fundamental fact, demonstrated by observations described in the first paper, has been confirmed by a great number of later experiments. Evidence is supplied in the present paper to show that the dipeptide under these circumstances does not itself act as a primary hydrogen donator. The changes its molecule undergoes are strictly reversible and involve the oxidation and reduction of the sulfur group alone.

Since a preparation of fresh tissue rendered almost incapable of reducing methylene blue by thorough washing with cold water still reduces the oxidized form of the dipeptide and in conjunction with the latter actively reduces the dye, it must presumably still contain sources of labile hydrogen.

It was during an endeavor to ascertain the nature of these by the use of various solvents—among them boiling water and alcohol—that the remarkable stability of a residual tissue system capable of acting in conjunction with the sulfur groups of the dipeptide was first observed.

### *A Thermostable Reducing System in Tissues.*

A mass of chopped muscle after it has been very thoroughly washed with cold water may be heated for hours in water at 100°C. or repeatedly extracted with boiling water and finally dehydrated with alcohol, dried *in vacuo*, and ground to a fine powder without losing more than a fraction of that part of its

[1] In all our experiments the substance has been used in the oxidized form. Its effect in accelerating reduction or oxygen uptake must then be due to its special relations with the tissue or tissue residue. If added in the reduced form its own reducing power must be allowed for.

reducing power which depends upon the presence of glutathione. Especially is this true if the treatment just described is carried out without contact with oxygen.

A preparation from muscle after undergoing such treatment may fail by itself to reduce methylene blue under any circumstances. But if it be placed in a dilute solution of glutathione and especially if, as in our experiments, it is suspended in a phosphate buffer solution of which the hydrogen ion concentration is around about 7 to 8, and to which glutathione in its oxidized form has been added in amount equal to, say, 10 mg. per gm. of tissue used, a system is established which reduces with rapidity.

The results of an actual experiment may be quoted here; others of a similar nature are more fully described in a later section.

The muscles from two freshly killed rats were passed through a mincing machine and the finely divided tissue was washed ten times with distilled water by shaking in a stoppered cylinder in which the air above the fluid was replaced by nitrogen each time the water was renewed. A portion of the tissue was at this stage reserved, (a), and the rest was heated for 2 hours anaerobically in twice its own bulk of water at 100°C. A portion being again reserved, (b), the rest was boiled out six times with successive quantities of water, (c). At each of the three stages mentioned the muscle residue after the treatment with water was washed with 95 per cent alcohol and dried *in vacuo* over sulfuric acid. All three preparations were then finely powdered and sampled.

With equal quantities of dry tissue and of methylene blue in each case (for details see the Experimental section) the reducing time of (a) by itself was $3\frac{1}{4}$ hours and of (b), $5\frac{1}{4}$ hours; (c) showed no observable reducing power.

Under precisely similar conditions but with the addition of 10 mg. of oxidized glutathione per gm. of dried tissue, (a) reduced in 28 minutes, (b) in 24 minutes, and (c) in 24 minutes (see also Series 2 a). The reducing system which works in conjunction with, and only in conjunction with, the sulfur groupings could have suffered scarcely at all from the severe treatment described, as in each case the velocity of reduction remained practically the same when glutathione was restored to it. The thermostability of a catalytic—or quasi-catalytic—system in the tissue is indeed remarkable.

Whatever the nature of the factors comprised in the system it would, we think, be wholly wrong to look upon them as other than physiological.

It is possible even with fresh unwashed muscle to show that raising the concentration of glutathione accelerates reduction by the tissue, but in this case a relatively large amount should be added. Otherwise, the mechanism with which it is especially concerned is merged in the general reducing power of the tissue and the effect may not be pronounced. After slight washing, sufficient to remove only a part of the dipeptide, the effect of restoring it comes immediately to light. Thorough washing at ordinary temperature removes nearly the whole, and then the effect of supplying it is nearly at a maximum. As we have just seen, the effect is the same after the tissue has been finally submitted to boiling water. At each stage of the treatment factors are shown to be present which reduce only with the coagency of glutathione and by the time the latter has itself been washed away the only remaining factors of this sort left in the tissue prove to be entirely insoluble and thermostable. It does not follow, of course, that these are the only factors having this special dynamic association with the sulfur groupings of the dipeptide. Other hydrogen donators may be soluble in cold water and so be washed away during the removal of the dipeptide itself. But the thermostable factors are quantitatively the more important and once the tissue has been extracted with cold water they seem to be the only ones left. All the evidence points to the circumstance that these thermostable factors act with glutathione in the intact tissue. It is highly noteworthy in this connection that such tissue preparations after thorough extraction with boiling water, can, in the presence (though only in the presence) of glutathione and under controlled conditions, continuously absorb oxygen and yield carbon dioxide. They, in a limited sense at any rate, "respire."

The thermostable tissue residues actively reduce the oxidized form of the dipeptide (as can easily be shown, especially in anaerobic experiments, by the development of the nitroprusside reaction which is given by the reduced form alone). Otherwise the system which "respires," or reduces methylene blue, could not be established. Such residues it would seem must contain hydrogen donators and, presumably, some form of primary catalytic

system for which the glutathione is a coagent. If so, the catalytic system is remarkably stable and the hydrogen donators exceedingly insoluble in water. The factors present in the tissue residue together with the glutathione constitute what may be called the thermostable reducing system of the tissue. It should be understood that this contributes not a negligible fraction, but a quite noteworthy amount to the total reducing power and "respiration" of the tissue.

*Effects of Oxygen on the Thermostable System.*

The residual tissue system which shows itself to be so highly thermostable, and is activated by glutathione, is sensitive to oxidation. The dry powder when kept in stoppered bottles only very slowly loses its power of transferring hydrogen to the sulfur groups of the dipeptide. On exposure to the air in thin layers this property is lost with somewhat greater rapidity. It is natural to suppose that the change is due to the slow oxidation of the relatively labile hydrogen atoms which in the presence of glutathione are so rapidly activated and transferred to other acceptors.

To hydrogen peroxide the thermostable tissue preparations are exceedingly sensitive. A single observation may be quoted in support of this statement.

A dry muscle powder (2 gm.) which in the presence of glutathione originally reduced methylene blue with high velocity was placed for 15 minutes in 50 cc. of a 0.5 per cent solution of the peroxide and occasionally shaken. It was filtered off and very thoroughly washed with water until neither the washings nor the powder itself showed any trace of peroxide or of active oxygen by the use of the most delicate tests. It was then dried *in vacuo*. All capacity to reduce methylene blue in the presence of glutathione was found to be lost, though a control specimen of the powder, after equally thorough washing with water, was found to have lost none of its reducing power.

Related to the above facts are others which bear directly on the properties of the dipeptide. A thermostable preparation from muscle made on the lines already described and in the form of a dry powder, although not, as already stated, entirely resistant to the influence of atmospheric oxygen, may yet be suspended in, say,

a phosphate buffer solution of pH 7.4, and aerated for some hours at room temperature with a stream of air, without losing more than a small fraction of its powers to reduce methylene blue anaerobically, when subsequently supplied with oxidized glutathione. But suppose a few mg. per gm. of tissue of glutathione (wholly absent from the original thoroughly extracted tissue) be added to the buffer solution *before* the aeration. It will now be found that, after even relatively brief exposure to an air stream at room temperature, the tissue will have wholly lost its power to reduce under anaerobic conditions. This is found to be the case if it be tested in the presence of the glutathione as originally added before the aeration, or on the other hand, if in order to make the conditions comparable with those of other experiments, it be first washed, dried, and supplied with fresh dipeptide.

Such results indicate clearly that the thermostable extracted residues from muscle contain oxidizable material, or, keeping to the point of view and terminology chosen for the purposes of our discussion, such residues still retain transponible hydrogen for which molecular oxygen, no less than methylene blue, can act as acceptor. The results show also that transport to oxygen is as greatly accelerated by the presence of the sulfur group of glutathione as is transport to methylene blue.

It is clearly desirable that in such a connection quantitative data should be obtained, especially because with oxygen the changes involved can be made to proceed to completion instead of ceasing at an unknown equilibrium point, as in the case of the anaerobic observations made with methylene blue in closed tubes.

For this purpose we employed the well known differential apparatus of Barcroft (see Series 6 and 7 in the next section).

Suspended in phosphate buffer solutions of pH 7 to 8 and shaken with air, such thermostable preparations as we have described are found by themselves to take up oxygen with extreme slowness or not at all. If, however, oxidized glutathione, showing by itself, of course, no oxygen uptake, be added to the suspension fluid in amounts equal to, say, 10 mg. per gm. of dried tissue, a brisk uptake of oxygen occurs. Varying with the amount of glutathione added, and, of course, with the temperature, the time required for the oxidation to reach completion is in the case of 0.5 gm. of tissue from 1 to 5 hours. The total uptake is of the order of

400 c.mm. of oxygen per gm. of dried muscle residue. In a later discussion it will be shown that this represents a by no means unimportant fraction of the normal respiratory uptake of the tissue from which the preparation was made. Significant is the fact that the uptake of oxygen is associated with the production of carbon dioxide in definite amount.

It is difficult to decide what type of material, oxidizable by atmospheric oxygen in the absence of an enzyme, but requiring for its oxidation the aid of glutathione, can thus remain associated with a tissue residue after very thorough extraction with hot water and alcohol. Until this is decided, the full significance of the phenomena described in this paper will not be understood. It is remarkable that the respiratory quotient (if in such a connection the term may be allowed) of the extracted muscle tissue is not constant during the course of the oxygen uptake, but gradually falls from high to lower values. An endeavor to determine what is oxidized in and by the thermostable system is in progress.

In the particular instance of muscle, one point in connection with the oxidations which occur in the thermostable residue, though it is probably a minor one, is worthy of mention.

In the case of yeast, and, almost certainly, in the case of cellular animal tissues such as hepatic and renal tissue, the substance responsible for the nitroprusside reaction given by the fresh tissue, is wholly removable by extraction with water. The reaction is apparently wholly due to the reduced glutathione present. In muscle fiber this is not the case. After the dipeptide has been completely removed from muscle the fibers still yield a strong nitroprusside reaction. The character of the reaction leaves little doubt that it is due to a sulfydryl group, and the suggestion is that one, at least, of the muscle proteins contains cysteine instead of cystine in peptide association. If so, the case is exceptional. As is well known, the proteins of the blood and connective tissue give no reaction, and though it is difficult perhaps so to wash a cellular organ as to obtain an absolutely negative result, the residual reaction is so slight that it may well be due to remaining traces of adsorbed glutathione. This is not so with muscle.

In any case the fixed $-SH$ group in the muscle fiber can exert none of the functions subserved by that present in the soluble dipeptide. The extracted residues which give in all cases a

strong nitroprusside reaction are, as we have seen, by themselves quite incapable of reducing methylene blue or of taking up oxygen. An interesting phenomenon in which both the "fixed" sulfur group and the sulfur group of glutathione are concerned may be easily observed. The sulfydryl as it exists in the tissue residue is itself scarcely at all autooxidizable. When the muscle residue is suspended in water or buffer solutions and the suspension thoroughy aerated, or when it is shaken in a Barcroft apparatus, as in the experiments just described, the nitroprusside reaction survives almost indefinitely. If, however, glutathione be added to the solution before the aeration, the fixed —SH is oxidized and the strong nitroprusside reaction originally given by the tissue residue rapidly disappears. One of the tissue constituents of which the oxidation is catalyzed by the sulfur group of glutathione is, therefore, itself a sulfur group.[2] It is clearly not the only constituent so oxidized because the "respiration" of the thermostable residue involves always the production of carbon dioxide.

*Oxygen Uptake by Washed but Unheated Tissue.*

In discussing the influence of glutathione in promoting oxygen uptake we have hitherto, for convenience in discussion, referred chiefly to its effect upon heated tissue residues. Since it acts with these it would be expected to act with tissues simply washed. It undoubtedly does so; but for the clear display of this property certain experimental conditions must be secured. If muscle, after thorough washing with cold water, but without further treatment, be supplied with glutathione and shaken in a Barcroft apparatus under conditions similar to those already described, an uptake of oxygen is observed; but it is slow and irregular. If, however, the washed residue be first extracted with alcohol, or if it receive only a very brief preliminary treatment with alcohol—the alcohol being then completely removed by further washing with water—the moist residue will now, in the presence of glutathione show a steady uptake of oxygen, which if due allowance be made for any variation in the water content,

[2] A paper by Dixon and Tunnicliffe on the conditions controlling the oxidation of -SH groups, which will shortly be published from this laboratory, contains results which bear upon the above phenomenon.

will be found equal to the uptake of a heated preparation. This correspondence is easily shown if the unheated and heated preparations are both employed in the form of a dry powder. There seems to be little doubt that the effect of the preliminary treatment is on permeability. Heat and treatment with alcohol seem to influence this on similar lines. It is noteworthy that although, as we have repeatedly stated, a washed residue without treatment establishes with the dipeptide a system which actively reduces methylene blue yet even in this case preliminary washing with alcohol or heating to 100°C. increases the velocity with which the system reduces.

*The Stability of Glutathione.*

It is important for proper appreciation of the part played by the dipeptide in the phenomena we have described, to realize that when its addition evokes activity in inactivated tissue preparaparations, this is not because it, itself, acts as a primary hydrogen donator or oxygen acceptor. Its molecule is not oxidized in the sense that, say, succinic acid is oxidized by isolated tissues, or in the sense that the various substances studied by Thunberg are oxidized anaerobically by washed tissue in the presence of methylene blue. As Thunberg found that glutamic acid, one of its constituent amino-acids, is so oxidized, it might be expected that the dipeptide would suffer the same fate. This is not so. To judge, at least from experiments with excised tissue, its molecule as a whole is stable. In its case hydrogen attached to carbon is not mobilized, but only that attached to sulfur. The reversible change from the sulfydryl to the disulfide grouping is the essential happening.

In proof of this statement we rely in particular upon the study of equilibria. When in anaerobic experiments with washed tissue and methylene blue a substance which acts as a primary hydrogen donator is added to the system (succinic acid, for instance, as a precursor of fumaric acid) the amount of methylene blue reduced when equilibrium is reached will naturally be proportionate to the amount of donator supplied. This is not the case when glutathione is in question. The final equilibrium in the system is quite independent of the amount present. This is clearly seen from the results of a series of observations (Series 3) in the next

section. An 8-fold increase in the concentration (the weight of tissue being the same) is seen to have no effect whatever upon the amount of methylene blue finally reduced. On the other hand the velocity of reduction increases with the concentration of the dipeptide. Such results are consistent with the properties of a substance concerned with hydrogen transport on the lines assumed in the course of this discussion, but the facts could not apply to one acting as the primary hydrogen donator.

Equally definite evidence is obtained from a study of the effects of varying the concentration upon the oxygen uptake of a tissue residue. The results of Series 7, for instance, show that a 4-fold increase in the concentration of glutathione, while increasing the velocity of uptake, made no difference to the amount of oxygen ultimately taken up.

It may be well to point out that tissue preparations have no power to oxidize glutathione as the result of such surface activity as that involved in the oxidation of amino-acids by charcoal.

Warburg[3] has recently demonstrated that various substances, including certain amino-acids, such as leucine, tyrosine, and cystine, are completely oxidized when their solutions are shaken with blood charcoal in the presence of air. No such property as this belongs to the tissue residues either when moist or when they have been dried and ground to a fine powder. We could discover at least no trace of ammonia or carbon dioxide production when leucine or tyrosine solutions were shaken for long periods in the presence, *e.g.* of a dried muscle powder supended in the fluid. Warburg obtained striking results with cystine. In this case the behavior of a tissue residue differs entirely from that of charcoal. It reduces the cystine to cysteine and then follow effects qualitatively similar to those observed with glutathione.

*The Relation of Glutathione to other Respiratory Systems.*

The total reducing power of any tissue represents doubtless the sum of the activities of several different types of chemical mechanism. This is equally true of its total capacity for oxygen consumption. It is becoming evident, indeed, in a greater number of cases than was previously recognized, that a catalytic agency which

[3] Warburg, O., and Negelein, E., *Biochem. Z.*, 1921, cxiii, 257.

promotes a respiratory process may be one which under other conditions is responsible for reductions.

A cold water extract of a tissue contains those oxidizing agencies which are at present known more particularly as "oxidases"—the alcoholase of Batelli and Stern, catalysts of the tyrosinase type, purine oxidases, and the like. To these, or to some of them, and to the simultaneous presence of substances upon which they act (individually present in minute amounts) a cold water extract of a tissue owes part of its reducing power. Morgan, Stewart, and Hopkins (1922), for instance, have shown that tissue extracts oxidize xanthine and hypoxanthine anaerobically in the presence of methylene blue, the latter acting as the hydrogen acceptor in what is probably a hydrolytic oxidation-reduction process. There is no good reason to suppose that the catalyst here concerned is distinct from the xanthine "oxidase" of other authors. Its action has usually been studied under aerobic conditions when oxygen instead of methylene blue becomes the hydrogen acceptor.

The point, however, that we wish to emphasize here is that so far as we have been able to discover, glutathione exercises no functions in connection with oxidizing agencies of this type. If, at any rate, it be added to a cold water extract of muscle the velocity with which such an extract reduces methylene blue is, at most, but slightly accelerated. The sulfur group seems to have no relation with the soluble tissue enzymes.

As is well known, Batelli and Sterns have distinguished from the soluble oxidases certain insoluble agencies in the tissues which they have called oxydones. The activities of these are, so far as we know at present, associated with the tissue structure as left after extraction with cold water. Such agencies may represent something more than the equivalent of specific enzymes; they may comprise surface effects or other such factors. It is abundantly certain, however, that animal tissues after the most complete extraction with cold water retain unstable oxidation-reduction mechanisms possessed of marked activity. Their activity can be demonstrated either by the occurrence of direct oxidation in the presence of air or by indirect anaerobic oxidations brought about in the presence of such a substance as methylene blue. The systems in question are thermolabile and characteristically unstable. It is by means of mechanisms of this class that excised tissues so

readily oxidize succinic and citric acids (Thunberg, and Batelli and Stern) and probably by similar means many, if not all, of the substances in the extended list recently studied by Thunberg are oxidized.

With these insoluble but unstable catalytic mechanisms, as with the soluble catalysts, the sulfur constituent of the cell would again seem to have no concern. Studying at any rate the typical cases of the oxidation of succinic and citric acids, or rather, the power of these substances to act as oxygen acceptors (or hydrogen donators) in the process of methylene blue reduction, we have found that glutathione exerts in these connections no influence. The washed tissue with little or no reducing power of its own reduces actively when succinic acid is supplied. If to this combination glutathione is also supplied the effects observed are merely additive (Series 4). It would seem indeed that the power to reduce the disulfide (−S−S−) group to the sulfydryl group (−SH) and thus to establish with glutathione a system which can transport hydrogen to methylene blue, or on the other hand a system which is autooxidizable, is to a peculiar degree attached to agencies which are not enzymes and are neither extracted nor destroyed by boiling water. Furthermore, we have so far been unable to prove with certainty that any known soluble tissue constituent or metabolite is oxidized under the influence of the system constituted by the thermostable residue plus glutathione. No pure substance yet tried has by its addition to the system increased the amount of methylene blue it can reduce or added to its total oxygen uptake. Nevertheless, we have so fractionated and concentrated aqueous muscle extracts as to obtain preparations which do affect equilibrium in the system to a marked degree.

*The Relation of Glutathione to the Atmungskörper of Meyerhof.*

There seemed from the first to be grounds for supposing that glutathione with its active sulfur group might be identical with the "respiratory substance" (Atmungskörper) of Otto Meyerhof (1918), or, at least, form part of a chemical system described under that name. Since the first brief account of the properties of the dipeptide was published this possibility has occurred to others.[4]

[4] Compare Dakin, H. D., *Physiol. Rev.*, 1921, i, 403.

3 years, indeed, before glutathione was isolated, Meyerhof had fully considered the possibility that his Atmungskörper might be a substance carrying the —SH group, and he endeavored to test the matter experimentally. He points out in a highly interesting paper that, broadly speaking, there is a parallelism between the intensity of the nitroprusside reaction, as given by a yeast extract or "Kochsaft" and the activity of such an extract in restoring respiratory activity to washed acetone yeast. In his experiments cysteine appeared to have no such effect, but in order to test that matter further he studied the influence of thioglycollic and thiolactic acids. He found as a matter of fact that when a yeast residue, inactivated by washing, was suspended in a neutral or slightly acid solution of either of these substances, an uptake of oxygen occurred, considerably in excess of what would be required to oxidize the added thio-acid to the disulfide form.

The properties of the substances employed by Meyerhof were, however, such as to make the precise meaning of the experimental results somewhat obscure. The author found, for instance, that if thioglycollic or thiolactic acid be added to the tissue in the form of the corresponding disulfide derivative, neither substance promotes oxygen transport. The reason must be that the tissue does not reduce the —S—S— group of these compounds.[5] The processes involved do not seem to be in any obvious sense reversible, therefore, and it is difficult under the circumstances to understand how the —SH group of these thio-acids can promote continuous oxygen transport.

On the other hand, as we have many times pointed out, the disulfide form of glutathione is freely reduced by tissues, or washed tissue preparations, a characteristic which in this connection seems to be highly important.

Meyerhof noted another quality in the sulfur group of thioglycollic acid (the substance most fully studied) which renders remarkable its ability to promote under the conditions of his experiments the uptake of oxygen by a washed tissue.

In neutral or slightly acid solutions (pH 6 to 7) the —SH group of this acid is stable and not autooxidizable, yet it is only in solu-

[5] Since the above was written we have ourselves found that dithiodiglycollic acid is, as a matter of fact, reduced by washed muscle and washed acetone yeast. The addition of these substances does promote oxygen transport, though much less efficiently than glutathione.

The above remarks, based upon Meyerhof's own findings, therefore lose their point.

tions with this range of pH that the substance promotes respiration of the washed tissue. In more alkaline solutions it is itself autooxidizable, but then does not promote respiration, because as soon as it is oxidized to the disulfide form its relations with the tissue cease. Here again the properties of the natural constituents differ in a significant sense. The —SH group of glutathione is autooxidizable in neutral solution and it promotes the uptake of oxygen by a washed tissue within just that range of pH in which it is itself autooxidizable, and also capable of reduction by the tissue. It would seem at first sight as though the sulfur group of the natural constituent is more likely to possess the functions generally pictured for the Atmungskörper than that of thioglycollic acid. Nevertheless, the facts brought forward in previous sections of this paper show that it is impossible to identify the dipeptide with any substance supposed to stimulate respiratory processes in general. Meyerhof, having observed that the influence of the sulfur group of thioglycollic acid was but little affected when the tissue residue (washed yeast) had undergone heating to 100°C.,[6] pointed out that this must constitute the most important distinction between the thio-compound and an agent of the coferment class, to which, *ex hypothesi*, the Atmungskörper belongs.

From our own standpoint, as maintained throughout the present paper, the fact that the activities of the sulfur group seem to be unrelated to enzyme activities does not remove biological significance from the former; but enough has been said in previous sections to show that there are processes involved in the sum total of respiratory activity with which it has no direct concern.

Meyerhof's fundamental observation which led to the conception of the Atmungskörper was the activation of washed tissues by a tissue "Kochsaft." It is not unimportant to consider all that is involved in this phenomenon. A considerable part at least of the oxygen uptake which occurs is due to the fact that relatively insoluble catalysts present in the washed tissue are again supplied with soluble oxygen acceptors from which the original washing divorced them.

[6] When we first observed that glutathione worked with heated tissue residues we were unaware that Meyerhof had 3 years before (1918) made the above observation with thioglycollic acid.

Any of the numerous substances proved by Thunberg, by Batelli and Stern, and by Meyerhof to be oxidized by a washed tissue, or shown in the later work of Thunberg to be capable of acting as hydrogen donators, may constitute oxidizable material in a Kochsaft. The concentration of any one of them will admittedly be very low, but now that we know they may be numerous it must be recognized that, collectively, their oxidation may account for a considerable oxygen uptake. As already stated the oxidation of such substances—as represented by succinic, citric, and certain amino-acids—by thermolabile catalysts in the tissue is unaffected by the presence or absence of glutathione. There is, on the other hand, no evidence to show that these particular oxidations require the presence of a soluble coagent of any sort.

Some observations of our own, concerned with animal tissues only, have a bearing on these considerations. We have prepared on Meyerhof's lines from perfectly fresh tissues very active samples of Kochsaft and have found that such preparations, if not exposed to the air, retain their activity for long periods. On the other hand we found that if the tissues from which they were prepared were allowed to stand before extraction, the extracts made from them grew progressively less active as the time allowed for survival or postmortem changes in the tissue was made longer. Such results suggest the disappearance of oxidizable material during the course of survival events rather than the destruction of a coferment. We are, of course, not suggesting that a Kochsaft contains no substance of such a type. Meyerhof's classical studies have shown that it does. We find, however, one difficulty in connection with the present discussion. It seems to be commonly assumed, though not by Meyerhof himself, that the Atmungskörper is a single substance stimulating respiratory processes as a whole. If that were so it would certainly be an agent quite distinct from glutathione. If, on the other hand, the name may be taken to connote the activities of two or more substances each related to some particular aspect of respiratory oxidations, then glutathione shows itself to be one of these; its activity being more particularly, though not exclusively, related to respiratory factors (whatever they may prove to be) which remain intact in a tissue when it has been heated.

A direct experimental demonstration, however, even of this established fact is not easy by the use of Kochsaft preparations. The concentration of glutathione in a Kochsaft may be considerably increased with relatively little effect upon its activity when added to a washed tissue. This, we think, is partly because the effect is at first swamped in the progress of rapid oxidations which, as we have just pointed out, proceed without the influence of any soluble coagent. There is, however, a further circumstance of importance. The physical condition of a washed tissue is such that part of its uptake of oxygen which depends specifically upon the presence of glutathione is slow (see above). On the other hand, the preliminary treatment with alcohol which, as we have seen, so efficiently promotes the combined activity of the tissue and the sulfur group renders the former entirely inactive towards the other constituents of a Kochsaft. Such facts as these require further study for their elucidation.

## EXPERIMENTAL.

### *Section 1. Anaerobic Experiments.*

All our measurements of reduction velocity were made under anaerobic conditions. The tissue preparations were suspended in a phosphate buffer solution contained in test-tubes which could be evacuated. The tubes employed (somewhat similar to those used by Thunberg) are $4\frac{1}{2}$ inches long with a diameter of $\frac{3}{5}$ inch. A short side tube projects from a tubular neck which is ground to receive a hollow glass stopper perforated by a hole at the level of the side tube. The tubes are evacuated when the hole has been made to correspond with the opening of the side tube, and if a suitable stopper grease be used a vacuum is maintained almost indefinitely after the tube has been closed by turning the stopper. For the purpose of our experiments it was found sufficient to evacuate the tubes by means of a good filter pump. In order, however, that the small residual oxygen tension should be identical in any series involving a comparison, branch connection tubes were provided so that six or more of the test-tubes could be connected at once with the same pump, and so exhausted to exactly the same degree.

All the determinations of reduction time recorded in the following tables were made in a phosphate buffer solution containing

primary potassium phosphate and secondary sodium phosphate (Sörensen). Almost invariably the pH of the system was 7.6. The glutathione, which is itself markedly acid, was dissolved in a somewhat more alkaline phosphate mixture (*e.g.*, of pH 8) and the solution then adjusted to 7.6, the volume being also adjusted to yield a known concentration of the dipeptide. The required amount of a tissue preparation was first weighed into a reduction tube. It was then covered with a known volume of the buffer solution, either plain or containing glutathione (or other substance to be tested) in known amount. The final volume and the pH were always made the same in each tube of a series under comparison. The methylene blue solution was added last and the tube or tubes immediately evacuated. After exposure to the tension of the pump for a standard time (usually 3 minutes after the manometer registered a steady pressure) the tubes were closed and transferred to a bath at 37°C. In most of the experiments which were concerned with a comparison of reduction velocities very small amounts of methylene blue were employed (0.2 to 0.3 cc. of 1:5,000). The observations were thus shortened without any loss of significance in the results. When final equilibria were to be determined stronger solutions of the dye were employed. The end-point taken coincided with complete decolorization of the tissue mass as well as of the supernatant fluid.

The observations recorded in the tables which follow represent only a small proportion of the consistent series which have been made. The records occupy much space. We have selected, therefore, results which are typical of the average.

*Series 1. Tissue Extracted with Cold Water Only.*

The muscle (or other tissue) after excision was finely chopped with sharp scissors until a homogeneous almost pasty mass resulted. It was then transferred to a stoppered cylinder and well shaken with successive quantities of distilled water. The ease with which the glutathione contained in the original tissue, together with other reducing factors, is removed varies somewhat with the tissue employed. In the case of frog muscle six successive washings with 50 to 60 times its weight of distilled water will usually yield a preparation with very small residual reducing power. In the case of fresh muscle from the rabbit or rat we have usually em-

ployed ten washings. After each successive washing the tissue mass was squeezed in a linen cloth. If its reducing power was to be studied in the moist condition the mass was finally pressed between filter papers and the amount required for an experiment

TABLE I.

| Washed tissue preparation. | Buffer solution (pH 7.6). | Oxidized glutathione. | Methylene blue 1:5,000. | Reduction time. | |
|---|---|---|---|---|---|
| *gm.* | *cc.* | *mg.* | *cc.* | *hrs.* | *min.* |
| (a) Frog muscle.....0.5 | 5 | 0 | 0.3 | 6+ | |
| 0.5 | 5 | 2 | 0.3 | 0 | 55 |
| 0.25 | 5 | 2 | 0.3 | 1 | 35 |
| 0.5 | 5 | 4 | 0.3 | 0 | 31 |
| (b) Rat muscle.....0.6 | 3 | 0 | 0.2 | 0 | 50 |
| 0.6 | 3 | 2 | 0.2 | 0 | 25 |
| 0.3 | 3 | 0 | 0.2 | 1 | 40 |
| 0.3 | 3 | 2 | 0.2 | 0 | 59 |
| 0.6 | 3 | 4 | 0.2 | 0 | 14 |
| (c) Rabbit muscle.....0.5 | 3 | 0 | 0.3 | 2 | 40 |
| 0.5 | 3 | 4 | 0.3 | 0 | 18 |
| (d) Ox muscle.....0.5 | 3 | 0 | 0.3 | 5+ | |
| 0.5 | 3 | 4 | 0.3 | 0 | 21 |
| (e) Ox muscle as dry powder.....0.2 | 3 | 0 | 0.3 | 6 | 20 |
| 0.2 | 3 | 2 | 0.3 | 0 | 40 |
| 0.2 | 3 | 4 | 0.3 | 0 | 18 |
| (f) Rabbit kidney.....0.5 | 3 | 0 | 0.3 | 5 | 30 |
| 0.5 | 3 | 4 | 0.3 | 0 | 16 |
| 0.5 | 3 | 8 | 0.3 | 0 | 9 |

The few results recorded in Table I are sufficient to show the restoration of reducing power to washed tissue which results when glutathione is supplied. The latter, which was always employed in the oxidized form, has itself no reducing power. In all cases except (e), the tissue preparation was weighed moist. Prepared as described above the tissue residues have a water content which is somewhat higher than that of the original tissue. The preparations were made immediately after the death of the animal except in the case of ox muscle, (d) and (e), which was prepared from butchers' meat (rump steak).

weighed out as quickly as possible into the tubes. Occasionally the washed tissue was dried *in vacuo* over sulfuric acid and finally ground to powder.

*Series 2. Tissue Residues after Extraction with Boiling Water.*

After the activity (in conjunction with glutathione) of thermostable tissue residues was first observed we found it desirable for the purpose of comparisons to deal with dry powdered preparations prepared on standard lines. When, for instance, the activity of a preparation obtained by simply washing the original tissue is to be compared with that of the same tissue after heating to 100°C., or after extraction with hot water, a change in the physical condition of the preparation may somewhat affect the velocity with which it reduces methylene blue or takes up oxygen. If both preparations are dried, reduced to powder, and properly sampled—treatment which does not at all reduce the capacity to work with glutathione—the comparison is more accurate.

The tissue may be first thoroughly extracted with cold water and subsequently heated to 100°C. and dried. It is best, however, to arrest survival processes by heating immediately after removal from the animal. Our standard preparations which in the case of muscle show remarkable uniformity of behavior were made as follows.

The tissue is put through a mincing machine and immediately thrown into about thrice its bulk of boiling water. After cooling it is squeezed in a linen cloth and then quickly extracted six times with successive small quantities of boiling water. It is finally washed with a small quantity of alcohol, dried over sulfuric acid *in vacuo*, powdered, and sampled.

All the tissue preparations in Table II were used in this form.

*Series 2a.*—The two experiments described under this head illustrate how small is the effect upon the activity of a tissue with glutathione when simple washing is followed by extraction with hot water.

In each case the muscle was first washed ten times with 60 times its weight of distilled water under anaerobic conditions (nitrogen). A portion of this washed, but unheated, preparation was reserved for experiment. The remainder was placed in a flask just covered with water and then heated on the water bath

for 2 hours in an atmosphere of nitrogen. A portion was again reserved at this stage. The rest was finally extracted six times with boiling distilled water. A portion of the residue at each stage was washed once with 96 per cent alcohol, dried in a vacuum desiccator, powdered, and sampled. Preparation A was from ox muscle (beef); Preparation B was from the muscle of a recently killed rabbit.

TABLE II.

| Tissue preparation weighed in form of dry powder. | Buffer solution (pH 7.6). | Oxidized glutathione added. | Methylene blue 1:5,000. | Reduction time. | |
|---|---|---|---|---|---|
| *gm.* | *cc.* | *mg.* | *cc.* | *hrs.* | *min.* |
| (a) Frog muscle....0.2 | 3 | 0 | 0.2 | 24+ | |
| 0.2 | 3 | 4 | | 0 | 22 |
| (b) Rat muscle....0.2 | 3 | 0 | 0.2 | 12+ | |
| 0.2 | 3 | 2 | 0.2 | 0 | 17 |
| (c) Rabbit muscle....0.2 | 3 | 0 | 0.2 | 24+ | |
| 0.2 | 3 | 4 | 0.2 | 0 | 18 |
| (d) Rabbit muscle, another preparation....0.2 | 3 | 0 | 0.2 | 20 | |
| 0.2 | 3 | 4 | 0.2 | 0 | 12 |
| (e) Ox muscle....0.2 | 3 | 0 | 0.2 | 5 | 36 |
| 0.2 | 3 | 2 | 0.2 | 0 | 30 |
| Same preparation heated further....0.2 | 3 | 0 | 0.2 | 9+ | |
| 0.2 | 3 | 2 | 0.2 | 0 | 28 |
| (f) Rabbit muscle....0.5 | 3 | 0 | 0.2 | 7 | 20 |
| 0.5 | 3 | 2 | 0.2 | 0 | 15 |
| 0.5 | 3 | 8 | 0.2 | 0 | 4 |

In each observation the tissue was suspended in a phosphate buffer solution of pH 7.6. The dipeptide added was dissolved in a similar fluid with pH adjusted to the same figure. The total volume in each tube was the same ($2\frac{1}{2}$ cc.). Dried tissue 0.2 gm., glutathione 4 mg., methylene blue 0.2 cc. (1:5,000). The figures are the times taken for complete reduction.

Although the residual reducing power of the tissue by itself became zero after final treatment the reduction with glutathione remained as active as after simple washing. When the washed tissue is tested in its original moist condition heating to 100°C. always markedly increases the velocity with which it reduces in the presence of the dipeptide.

*Series 3.*—Experiments were carried out to determine the effect of varying the concentration of glutathione upon the total amount of methylene blue reduced when equilibrium is attained. The observations were made in evacuated test-tubes on the lines already described. The tissue employed was muscle in the form of the thermostable preparation, dried and powdered. The dipeptide was dissolved in a phosphate buffer and the solution finally made to contain 8 mg. in 1 cc. at pH 7.6.

TABLE III.

| | Washed anaerobically. | | Heated 2 hrs. | | Extracted with hot water. | |
|---|---|---|---|---|---|---|
| **Tissue alone.** | | | | | | |
| | *hrs.* | *min.* | *hrs.* | *min.* | *hrs.* | *min.* |
| Preparation A..... | 3 | 16 | 5 | 16 | 20+ | |
| " B..... | 2 | 8 | 2 | 30 | 20+ | |
| **Tissue plus glutathione.** | | | | | | |
| Preparation A..... | 0 | 30 | 0 | 25 | 0 | 26 |
| " B..... | 0 | 21 | 0 | 19 | 0 | 19 |

For each experiment four series of tubes were prepared, every tube containing 0.2 gm. of dried tissue. The first series contained in each tube 2 mg. of glutathione, the second series 4 mg., the third 8 mg., and the fourth 16 mg. In each series the amount of methylene blue (1:1,000) was increased in successive tubes by increments of 0.1 cc. The total volume of fluid was made in every tube the same, variations in the quantity of glutathione solution being balanced by suitable additions of buffer solution, and variations in the methylene blue solution by additions of distilled water. To each tube 1 drop of chloroform was added. All, after evacuation, were placed in a bath at 35°C. and left over night. When equilibrium was reached the point of transition from a tube in which the contents still showed a faint blue color to one which was colorless was noted in each series.

The wide variations in the concentration of the dipeptide had little or no effect upon the equilibrium point.

In one experiment with rabbit muscle the contents of the tube in the first series containing 1.2 cc. of methylene blue were faintly but definitely blue. The next tube (1.1 cc.) was colorless. This point of transition was identical in each of the four series; with 2 mg. of glutathione it was exactly the same as with 16 mg., though in the latter case equilibrium was reached much sooner. The tissue preparation by itself showed no reducing power.

In a second experiment carried out on similar lines with a preparation of rabbit muscle, made with special precautions to avoid oxidation during the earlier stages of washing and extraction, the point lay between 1.7 and 1.8 cc. of methylene blue and was again identical in each series.

In an experiment with frog muscle it lay in each series between 0.8 and 1.0 cc. Sometimes in such experiments the results were not quite so sharp, it being difficult to distinguish with certainty between two adjacent tubes in a series. Even an 8-fold increase in the concentration of the dipeptide was never observed, however, to make an appreciable difference in the equilibrium point, whereas, no matter what the concentration of the associated dipeptide, an increase in the amount of tissue produced always a proportionate increase in the amount of methylene blue finally reduced.

Such results show that glutathione cannot itself be a primary hydrogen donator in the reducing system which it establishes with the tissue residue (see also Series 8).

*Series 4.*—A number of experiments have been carried out to discover whether known metabolites can act as primary hydrogen donators when added to the system constituted by a washed tissue residue and glutathione; in other words to decide whether the anaerobic oxidation of such substances by a tissue residue in the presence of methylene blue is accelerated by the sulfur group of the dipeptide. In general, negative results have been obtained; but the enquiry is by no means complete. It will be sufficient for the purposes of the present paper to give certain illustrative cases.

The oxidation of succinic and citric acids by tissue catalysts has been much studied, and Thunberg has shown that these acids

act typically as hydrogen donators in the reduction of methylene blue by washed muscle.

The following observations show that the presence of glutathione is without effect upon the velocity of this reduction.

Preparation A was one of rat muscle washed ten times with distilled water under conditions made as anaerobic as possible. It was not heated and was weighed moist. Preparation B was rabbit muscle extracted six times with boiling water, washed with alcohol, dried, and powdered. The succinic and citric acids were dissolved in phosphate buffer solution adjusted to pH 7.6. The final solution was one-tenth molar, and 1 cc. was used for each observation. The glutathione (oxidized form) was also in 7.6 buffer solution, 1 cc., containing 4 mg., being employed. Adjustments to secure equal volumes were made with plain buffer solution also at pH 7.6. Methylene blue 0.2 cc. (1:5,000). Observations in evacuated test-tubes as before. The figures in Table IV are times taken for complete reduction at 35°C.

TABLE IV.

| | Preparation A 0.5 gm. | | Preparation B 0.2 gm. | |
|---|---|---|---|---|
| | *hrs.* | *min.* | *hrs.* | *min.* |
| Tissue alone | 5 | 26 | 24+ | |
| " with glutathione | 0 | 34 | 0 | 26 |
| " " succinic acid | 0 | 33 | 24+ | |
| " " glutathione and succinic acid | 0 | 16 | 0 | 28 |
| " " citric acid | 0 | 55 | 24+ | |
| " " glutathione and citric acid | 0 | 21 | 0 | 28 |

In the unheated Preparation A the catalysts responsible for the oxidation of succinic and citric acids (the oxydones of Batelli and Stern) are, of course, intact. It will be seen from the table that glutathione has no apparent effect upon the anaerobic activity of these. The reduction velocity induced when glutathione is added to either acid represents only the combined effect of each constituent acting with its own proper mechanism. In the case of the heated Preparation B the oxydones are destroyed. The presence of succinic or citric acid has in this case no effect upon the reduction velocity induced by glutathione alone.

Many experiments of the above type have been made, using a variety of substances in the place of the above acids. Only in the case of lactic acid has evidence so far been obtained to show that the anaerobic oxidation of the substance is influenced by the

presence of the dipeptide. Even in this case the requisite conditions are somewhat difficult to define. The experiments which bear upon the matter will form the subject of a separate paper.

*Section 2. Observations on Oxygen Uptake.*

*Series 5.*—The following observations show on other lines that what we have called thermostable tissue residues contain oxidizable materials. The oxidation of these by atmospheric oxygen is however, exceedingly slow, except when glutathione is present. It then becomes characteristically rapid.

A thermostable preparation of rabbit muscle made on the lines already described and in the form of a powder was found, as in all cases, to show by itself little or no power of reducing methylene blue. In the presence of glutathione it reduced freely.

TABLE V.

| | Reduction time. | |
|---|---|---|
| | *hrs.* | *min.* |
| Original preparation | 0 | 18 |
| Aerated alone (6 hrs.) | 0 | 22 |
| " in the presence of glutathione (3 hrs.) | 5 | 14 |

1 gm. of the powder was suspended in a phosphate buffer solution (pH 7.6) and the suspension briskly aerated at room temperature for 6 hours. A second gm. was similarly suspended but in the presence of 10 mg. of glutathione. This was aerated also at room temperature and side by side with the above, but for 3 hours only. Both samples were filtered off, thoroughly washed side by side until the second sample was again free from dipeptide, and then dried *in vacuo* so as to be comparable with the original powder. The portions thus treated were then compared in respect of their residual reducing power with the original preparation. The figures given in Table V are for 0.2 gm. of the preparation, 4 mg. of oxidized glutathione being supplied in each case. Conditions as in previous experiments with methylene blue.

The results in Table VI were obtained in the case of rat muscle, prepared and treated on lines similar to the above. The aeration was carried out at 35°C.

Such experiments show that the thermostable preparations contain hydrogen donators which are quickly oxidized by molecular oxygen in the presence of the sulfur group of the dipeptide, but which show considerable resistance to oxidation in its absence.

*Series 6.*—The oxygen uptake of thermostable tissue residue in the presence of glutathione was determined quantitatively in the familiar differential apparatus of Barcroft. A weighed quantity of the preparation under study, suspended in a measured quantity of a buffer solution containing a known amount of glutathione (pH usually 7.6), was placed in one flask of the apparatus. KOH for the absorption of $CO_2$ was present in the chamber provided. In the second flask was placed a quantity of distilled water or buffer solution equal to that in the first. The apparatus after proper adjustment was then shaken in a water bath at known temperature until cessation of movement in the manometric fluid showed that the oxygen uptake was complete. Control observations (*e.g.* with the tissue preparation in the absence of glutathione) were usually made in a second apparatus shaken side by side with the first. Apart from the ordinary careful

TABLE VI.

| | Reduction time. | |
|---|---|---|
| | *hrs.* | *min.* |
| Original preparation | 0 | 17 |
| Aerated alone (1½ hrs.) | 0 | 24 |
| " in the presence of glutathione (1½ hrs.) | ∞ | |

calibration of the instruments it is then important if velocity of uptake is under study, to make sure that they yield strictly comparable results. In the case of a system such as that constituted by a suspension of a tissue a relatively small difference in the shape of the flasks may appreciably affect the rate of uptake. It is in any case best to use flat bottomed flasks shaped so as to secure a relatively large surface for the suspension fluid.

The observations recorded in Table VII show the total oxygen uptake of various thermostable preparations in the form of a dry powder suspended in a buffer solution. It should be observed that as the dipeptide was always added in the oxidized form there was no need to allow for oxygen taken up by preexisting —SH groups. If any such experiment be stopped before the uptake of oxygen by the system is complete a nitroprusside reaction can always be observed in the suspension fluid, showing that the tissue preparation has reduced the disulfide group. When the oxygen

uptake has ceased no reaction is obtained. The hydrogen donators in the tissue are then exhausted and the reduced dipeptide itself reoxidized.

*Series 7.*—Experiments were done to determine the carbon dioxide production which is associated with the oxygen uptake of thermostable tissue preparations. It is easy to demonstrate that $CO_2$ is actually produced and that its production with any appreciable velocity is dependent upon coagency of glutathione. If a gram or two of a dried preparation be suspended in water, or in a buffer solution, and if the suspension be then briskly aerated with a stream of air which is subsequently led through a solution of barium hydroxide, scarcely any visible clouding will be seen

TABLE VII.

| Tissue preparation. | | Glutathione present. | Temperature. | Total oxygen uptake. | Time for complete uptake. | | Uptake per 1 gm. tissue preparation. |
|---|---|---|---|---|---|---|---|
| | *gm.* | *mg.* | *°C.* | *c.mm.* | *hrs.* | *min.* | *c.mm.* |
| Rabbit muscle | 0.5 | 0 | 20 | 6 | 4 | | 12 |
| | 0.5 | 4 | 20 | 209 | 4 | | 418 |
| | 0.2 | 8 | 20 | 82 | 1½ | | 410 |
| Rabbit muscle. Another preparation | 0.5 | 0 | 20 | 8 | 3 | 10 | 16 |
| | 0.5 | 8 | 20 | 205 | 3 | 10 | 410 |
| Rat muscle | 0.5 | 0 | 20 | 0 | 3 | 20 | 0 |
| | 0.5 | 4 | 20 | 211 | 3 | 20 | 422 |
| | 0.5 | 8 | 35 | 201 | 1 | 50 | 404 |

even in the course of several hours. If, however, a few milligrams of glutathione be added to the suspension the baryta solution begins to cloud almost at once. This, as other experiments show, is not due to the oxidation of the dipeptide itself.

To measure the $CO_2$ production two separate sets of the Barcroft apparatus, known to give corresponding results, were employed simultaneously. In one of these potash was placed in the flask containing the tissue, whereas in the other apparatus no potash was present. Exactly similar quantities of tissue preparation, glutathione, and buffer solution were placed in each and the two were shaken side by side. The difference shown in the respective manometer readings measures the $CO_2$ production.

To save space the results of one experiment only will be given (Table VIII). They are typical of others obtained except that in different experiments the actual rate with which the ratio $\frac{CO_2}{O_2}$ falls off may vary somewhat.

*Series 8.*—Several experiments were made to determine the effect of varying the concentration of glutathione upon the total uptake of oxygen by the system constituted by a thermostable tissue residue and the dipeptide. The results were entirely consistent in showing that wide variations in the concentration, while of course, influencing the velocity of uptake, have no effect upon the total amount of oxygen finally absorbed. Such results confirm those obtained with the methylene blue technique (Series 3) in showing that the dipeptide is not itself oxidized.

TABLE VIII.

Rabbit muscle, dry preparation, 0.2 gm., glutathione 8 mg., buffer solution 3 cc., bath at 35°C.

| | Oxygen absorbed. | $CO_2$ produced. | $\frac{CO_2}{O_2}$ |
|---|---|---|---|
| | *c.mm.* | *c.mm.* | |
| First 20 minutes..... .. | 27.2 | 27.2 | 1.0 |
| Next 30 " ..... | 33.2 | 20.0 | 0.6 |
| Final 35 " ..... | 12.1 | 4.0 | 0.33 |

We give, in the form of curves of oxygen uptake, the results of a typical observation of this kind. It will be seen that a 4-fold increase in the concentration of the dipeptide (the amount of tissue remaining the same) while it about doubled the initial velocity of uptake had no effect upon the total amount of oxygen absorbed.

## DISCUSSION OF RESULTS.

Considered from any standpoint that is familiar, the circumstance that a not inconsiderable part of the respiratory activity of a tissue can depend upon factors that are completely thermostable must appear surprising. The conclusion that such factors function in life is one which seems, at first sight, difficult to accept. We think, nevertheless, that if the evidence be duly considered this conclusion becomes inevitable.

With regard to the substance glutathione it would seem that the properties of its sulfur group are such that once the presence of the dipeptide in a tissue has been shown, a recognition of the fact that it exerts a real influence in the processes of reduction and oxidation as they occur in that tissue follows almost as a corollary.

The $-SH$ group under the physical and chemical conditions which exist in living tissue is certainly autooxidizable. It is equally certain that once the $-SH$ group has given place to the $-S-S-$

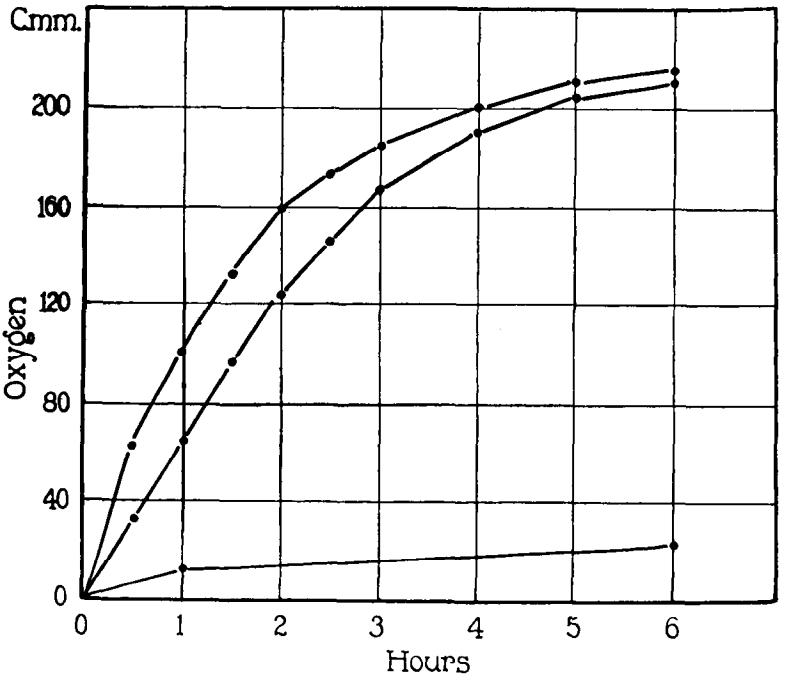


FIG. 1. Curves showing uptake of oxygen by 0.5 gm. of thermostable muscle residue (rabbit) in phosphate buffer solution at pH 7.6 and 20°C. (a) In the presence of 16 mg. of glutathione (upper curve); (b) in the presence of 4 mg. of glutathione (middle curve); and (c) by tissue residue alone without dipeptide (lower curve). The glutathione was added in the oxidized form which absorbs no oxygen until reduced by the tissue.

It is seen that a 4-fold increase in the concentration of the dipeptide, while increasing the initial velocity of uptake, had no effect upon the total amount of oxygen absorbed. The curves indicate that the extracted residue contains a definite limited amount of material readily oxidizable in the presence of glutathione, but oxidized only very slowly in its absence.

group as the result of such oxidation there are factors in the tissues which promptly restore equilibrium by fresh reduction of the latter. This ability to reduce the sulfur groupings is displayed in high degree by a tissue during the first stages of its survival processes; that is, immediately after its removal from the body, though the intact tissue loses it in process of time. It follows that when the —SH group is undergoing oxidation, or (in a possible case) when its hydrogen is being transferred to some other acceptor, a certain concentration of this oxidizable group is maintained so long as the tissue contains transponible hydrogen for the purpose. Whether, therefore, the quantitative importance of this process in the tissues be small or large it seems to follow from the properties of the sulfur group and from the particular oxidation and reduction potentials which exist in the tissues that glutathione *must* play a part in certain respiratory activities. What is remarkable, however, is the fact that its part in the tissues should apparently be played, not in connection with processes of a familiar type; not, that is to say, with chemical reactions controlled by enzymes or unstable catalysts, but in relation with tissue elements which are heat-stable and, directly, at least, with materials which are insoluble in aqueous media. It should be understood that although little has been said of the matter in the present communication there is evidence to show that soluble metabolites do, when proper conditions are secured, come under the influence of what we have called the thermostable system. The facts which bear upon this statement will be given in a later paper.

It is scarcely possible to deny that the thermostable residue described in this paper as reducing and "respiring" in the presence of the sulfur group of glutathione (but effectively, only in its presence) really represents—doubtless imperfectly—a system with actual functions in the living cell.

A freshly excised normal tissue freely reduces the disulfide group in neutral solution and at the temperature of the body (a property which chemically speaking, is itself exceptional); the same tissue after thorough washing with water or normal saline solution exhibits still the same property, and no one probably will deny that this is due to factors surviving from the original tissue. When, however, the washed tissue residue is subsequently repeatedly extracted with hot water the final residue retains

almost undiminished the reducing power in question. Whatever factors are then responsible it is scarcely logical to suppose that essentially they are other than those which confer the same property on the original tissue. Yet it is precisely this power of reducing the disulfide group to the autooxidizable sulfydryl group that makes any system containing glutathione an active mechanism.

It is noteworthy that the activities which are established when glutathione is brought into relation with a tissue which has been simply well washed with cold water show little if any quantitative difference if the same tissue has been first completely extracted with boiling water. They are scarcely affected if the tissue be finally dried and powdered. In this particular relation the properties of what we have called the thermostable residue are identical with those of a tissue simply washed.

It is certainly not easy to picture what precisely can be the factors of constitution in the thermostable residue which determine its peculiar relations with sulfur groupings. It is sure that it does not act exclusively in a physical sense as a colloidal system. The fact that the uptake of oxygen, which it exhibits in the presence of glutathione is associated with a definite output of $CO_2$ shows that materials are actually oxidized in the system. The dipeptide in conferring activity upon it undergoes itself no change other than reversible changes which are confined to the sulfur group. The materials oxidized are, therefore, certainly present in the tissue residue itself, and are apparently very closely associated with the remains of structural elements. They do not represent merely a residuum from soluble metabolites imperfectly removed during the treatment. When a tissue has been well washed and then twice or thrice extracted with boiling water no amount of subsequent extraction affects the extent of oxygen uptake which occurs when the preparation is afterwards aerated in the presence of glutathione. In the case of muscle this uptake seems indeed to have a definite and characteristic value, being nearly the same in preparations made from the muscle of different individuals, and, to judge from a few observations, even of different species.

The nature of the materials thus closely associated with the solid elements of a tissue, and displaying towards oxygen the peculiar relations described, is as yet obscure. They are not apparently

of a lipoid nature because extraction of a dried preparation with alcohol and ether does not diminish its activity. It is remarkable that during the course of oxygen uptake the respiratory quotient of the thermostable system characteristically falls from a high initial value to one which is near zero.

It is clear that in the oxidations (anaerobic or aerobic) involved the dipeptide accelerates transport and acts as a "carrier." In this sense it is a catalyst. It would seem that catalytic functions of a kind must also be ascribed to the residual tissue structures in spite of the fact that their influence withstands the effects of heat. We have at least been quite unable to find any substance physiological or other which by itself in neutral aqueous solution and at ordinary temperatures is capable of reducing the disulfide group, or upon the oxidation of which under such circumstances the dipeptide exerts any influence. It is possible that the molecules which suffer oxidation are in some way orientated as the result of their association with tissue structures. If so, the configuration must be very stable.

While there is much that is obscure in the phenomena involved the facts in our opinion fully justify the claim that a non-enzymic oxidation-reduction system represented by the thermostable residue *plus* the sulfur grouping of glutathione actually functions in the cell.

It is, of course, quite another matter to decide what actual share of the normal respiratory activities of a living tissue can be attributed to the chemical mechanism under consideration. It is, indeed, scarcely possible to obtain real information of any such kind from experiments such as ours, or from those of others using a similar technique. When the tissues are chemically dissected and partially reconstituted, the quantitative relationships among interacting factors and no less their spatial relations are necessarily greatly disturbed.

Such technique can satisfactorily and conclusively demonstrate the existence of a particular chemical mechanism in a tissue; quite other evidence must decide what precise quantitative part it plays in support of normal tissue activities as a whole.

Apart from reference to tissues normally metabolizing it is not even a simple matter to decide what proportion of the whole oxidation-reduction capacity possessed by a freshly excised tissue

is reproduced when glutathione alone is added to a residue which has been rendered inactive by washing.

It should be fully understood that in our experiments the reduction velocities recorded, and no less the rates of oxygen uptake, were for the most part obtained by supplying to the system constituted by a washed, or by a washed and heated, tissue suspended in buffer solution a quantity of glutathione much greater than what would be contained in a corresponding weight of the original tissue. We have usually supplied from 2 to 8 mg. per gm. of tissue, whereas 1 gm. of fresh muscle contains probably no more than 0.25 mg. It is difficult to determine what may be the potentiality of this lower concentration when it exists in proper relations with the tissue structure.

As the result for instance of heating fresh intact muscle to 100°C. the velocity with which it reduces methylene blue is very greatly reduced; so much so that a superficial observation might decide that the reducing power had disappeared, though this is by no means the case. The reduction proceeds very slowly to an equilibrium which may be reached very rapidly when sufficient glutathione is supplied. In the latter case the relative importance of the thermostable system (compared with that of other reducing systems present in the original tissue but destroyed by heat) may seem to be exaggerated by an artificial increase in one of its constituent factors. It is equally sure on the other hand, that relying on the velocity of change, its importance would be much underestimated if the behavior of the heated fresh tissue without addition were alone considered. It would be entirely wrong to assume from this that with the relatively low concentration of glutathione present in fresh muscle the reducing mechanism described in this paper is under normal circumstances of quite small quantitative importance. A cell constituent possessing functions such as those claimed for glutathione can scarcely fail to have in the intact tissue an unequal distribution. It will be locally concentrated in correlation with its functions. The disintegration due to such treatment as heating to 100°C. must, especially in the case of a very soluble and diffusible substance, gravely disturb such efficient distribution. Moreover, in experiments upon methylene blue reduction, or upon oxygen uptake, the tissue must almost necessarily be suspended in a fluid,

and into this soluble constituents diffuse. The effective local concentration becomes, therefore, greatly reduced. If it is to be restored by increasing the concentration in the fluid this increase may need to be considerable.

In our experiments with preparations deprived altogether of their stock of glutathione by extraction with water, cold or hot, the dipeptide was restored by adding it to the fluid in which they were suspended to the extent of about 1 to 2 parts per 1,000 (about 0.002 M). Although as already stated the artificial system (tissue preparation *plus* fluid) contained, as a whole, much more of the substance than would appertain to a corresponding weight of the original tissue, it is unlikely that the concentration maintained at the actual locus of change was above normal. It was quite probably much less. The reduction, it should be understood, proceeds within, or at the surface of, a solid phase (the tissue residue), and not in solution.

For any attempted appraisement of the relative quantitative importance of the phenomena involved, comparisons of final equilibria are, owing to limitations in the available experimental methods, perhaps more significant than comparisons of velocity.

Even from these, however, it is difficult to draw satisfactory conclusions. A dry preparation from muscle, representing what we have called throughout this discussion the thermostable residue when "respiring" under the influence of glutathione, takes up in all about 400 c.mm. of oxygen per gm. This amount appears to be characteristic and constant for preparations made on the lines described in the last section. Calculated for moist tissue it means an uptake of rather more than 100 c.mm. per gm. If we take Thunberg's figures for the respiration of excised frog muscle at 20°C. this corresponds with the consumption of the intact tissue for a period of $1\frac{1}{2}$ hours. If, again, we take Verzar's figure for the respiration of mammalian muscle *in situ* the above figure corresponds to the consumption of something less than $\frac{1}{2}$ hour. It is not easy to determine satisfactorily what is the total capacity for oxygen uptake possessed by a given weight of fresh excised muscle. To judge from approximate data obtained by ourselves the uptake of the thermostable system is about one-tenth of the total possible uptake of the tissue from which it is prepared.

Such figures, however, have but a limited application. They give no conclusive information as to the relative importance of events in a tissue normally metabolizing. What is sure (as it seems to us) is that in the living cell catalysis by enzymes is associated with quite another type of catalysis. How they are related in the chemical organization of the cell as a whole can only be established by much further study.

SUMMARY.

When a tissue is washed until it has lost its power of reducing methylene blue the subsequent addition of glutathione to a buffer solution in which the tissue residue is suspended restores reducing power.

This is the case when the dipeptide is added in its oxidized (disulfide) form. The tissue residue first reduces the sulfur group and a system is thus established which under anaerobic conditions continuously reduces methylene blue until an equilibrium is reached.

A tissue washed until it no longer "respires" will, when suitably treated and supplied with glutathione, again take up oxygen and yield carbon dioxide.

Such part of its reducing power and respiratory activity as is regained by a washed tissue on the restoration of glutathione remains almost unaffected when the tissue is heated to 100°C. or even thoroughly extracted with boiling water.

The residue from muscular tissue so heated and extracted will in the presence of glutathione take up about 400 c.mm. of oxygen per gm. of dry material. During the earlier stages of oxygen uptake the quotient $\frac{CO_2}{O_2}$ is usually about unity; later it falls to lower values.

Glutathione does not appear to be a coagent in any known enzymic system.

The facts suggest that coexisting in living tissues with the specialized enzymic mechanisms is a thermostable mechanism for oxidations and reductions. Materials in some close association with structural elements are oxidized, anaerobically or aerobically, with the coagency of the sulfur group in glutathione.

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# ON GLUTATHIONE: II. A THERMOSTABLE OXIDATION-REDUCTION SYSTEM

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*J. Biol. Chem. 1922, 54:527-563.*

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