THE EFFECTS OF SULPHYDRYL REAGENTS ON GLYCOLYSIS IN MUSCLE HOMOGENATES

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It is a common observation that freshly excised muscle gives rise on mincing to a viscous paste from which no juice can be immediately expressed, but after a brief interval fluid can be removed by simple hand pressure. The change is not due entirely to a fall in pH, which may be quite small. The phenomenon bears a resemblance to changes occurring in intact muscle, which becomes moist and in some cases exudes fluid during the rapid phase of the onset of rigor mortis[1].

These changes are also of great interest in relation to experiments on "model" fibres — the actomyosin thread (SZENT-GYÖRGYI[2]) and the glycerol-treated intact fibre. It is difficult, however suggestive the behaviour of these systems, to accept them unreservedly as true models as they lack many of the components of muscle and much of its fibrillar organisation. The homogenate, on the other hand, consisting of short pieces of highly organised fibre, allows physical and chemical changes to be followed simultaneously, permits the introduction and removal of components, and is amenable to qualitative and semi-quantitative measurements of fibre volume, fibre length, and synaeresis.

This investigation was begun with the main object of relating volume changes in the fibres to changes in the adenosine triphosphate (ATP) content of the system, since all current research emphasises the importance of ATP in modifying the state of the contractile substance. It was hoped, when the work was commenced, to extend the time-scale of the changes by arresting the enzymic breakdown of ATP with –SH reagents, which inhibit the ATP-ase activity of isolated myosin (SINGER AND BARRON[4]). The same reagents were shown by BAILEY AND PERRY[5] to affect the ATP-ase activity and the actomyosin-forming ability of myosin in parallel fashion. Our object was defeated, however, partly because the reagents were not altogether effective in inhibiting the ATP-ase of the whole homogenate, and partly because the glycolytic resynthesis of ATP was also arrested, the overall effect being to shorten rather than extend the interval during which ATP was present in appreciable concentration. However, the effects of the inhibitors on various enzymes and on the accumulation of certain phosphate esters

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seemed a study of sufficient interest in itself. The original aims of the investigation were pursued by a different technique in the absence of –SH reagents, and are reported in the paper which follows[6].

EXPERIMENTAL

Materials

The psoas major muscles of the rabbit were used. To obtain material of fairly uniform phosphate ester content, glycogen content, and initial pH from one animal to another, the rabbits were well fed on oats and cabbage for some days before death, and were killed by decapitation after about thirty minutes' complete relaxation of the musculature had been secured by intravenous or intraperitoneal injections of myanesin (BATE-SMITH AND BENDALL[7]).

p-Chloromercuribenzoic acid and chloroacetophenone were prepared by the methods described by GILMAN[8], *o*-iodosobenzoic acid by the method of ASKENASY AND MEYER[9], and chloroiodosofumaric acid as described by THIELE AND PETER[10]. Solutions of the reagents in water were adjusted before use to pH 7.0 with NaOH.

Technique

Five samples of muscle, each of 1–2 g, were taken within a few minutes of the death of the animal. One was homogenized immediately in ice-cold 10% (w/v) trichloroacetic acid (TCA), using the apparatus of MARSH AND SNOW[11], while the remaining four were homogenized for exactly one minute each in 10 ml of the inhibitor solution. TCA was added to these after intervals of 3, 10, 30 and 60 minutes. The extracts, after removal of the precipitated proteins by filtration, were neutralised to pH 7 with 2 *N* NaOH and kept at 0°.

pH measurements (glass electrode) were made at intervals on that portion of the homogenate which was to be deproteinized after 60 minutes, commencing immediately after preparation of the brei and ending just prior to addition to TCA. The ultimate pH (the pH in full rigor) was determined on another sample of muscle left at 0° for 24 hours. All experiments were performed at room temperature.

Phosphate ester analysis

Inorganic phosphate was determined by the method of ALLEN[12]. Phosphate esters were hydrolysed by the procedures described below and estimated by the same method.

Creatine phosphate was determined as inorganic P after standing for 36 minutes at 17° in the perchloric acid-ammonium molybdate reagent (method of EGGLETON AND EGGLETON[13] as modified by J. R. BENDALL (private communication)).

Acid-labile P was estimated by hydrolysing for 10 minutes in *N* HCl at 100°. The inorganic P thus obtained includes the acid-labile P of ATP, the P of creatine phosphate, and part of that of hexose diphosphate and triose phosphate. Although the amounts of these two latter esters in muscle are normally small, their concentrations may be greatly increased in the presence of inhibitors, and accurate methods for their estimation are essential. Their determination by alkaline hydrolysis was found to be both quicker and more accurate than by barium salt separation.

The triose phosphates undergo complete hydrolysis in *N* NaOH at 17° in twenty minutes, and 57% of the P is mineralized in ten minutes in *N* HCl at 100° (MEYERHOF AND LOHMANN[14]). Thus a correction may be applied to the acid-labile P to allow for partial hydrolysis of triose phosphate.

Hexose diphosphate is completely hydrolysed, together with the triose phosphates, by 0.2 *N* NaOH in three minutes at 100°, and 37% of the ester is hydrolysed in ten minutes in *N* HCl at 100° (MACLEOD AND ROBISON[15]). A further correction may therefore be applied to the acid-labile P content to allow for partial mineralization of this ester during acid treatment. Alkaline hydrolysis does not convert either ATP or creatine phosphate to inorganic orthophosphate.

Total acid-soluble phosphate was estimated as inorganic phosphorus after three hours' digestion in perchloric acid.

The difference between total phosphate and the sum of the five fractions estimated ("unidentified P") presumably consists largely of the phosphorus of adenylic acid or its de-aminated derivative inosinic acid, and also of such difficultly-hydrolysed esters as glucose-6-phosphate and fructose-6-phosphate.

Phosphate ester analyses are quoted as percent of total acid-soluble phosphorus, which for rabbit muscle is always about 2.0 mg/g (BENDALL[16]).

RESULTS

Standardization of material

BATE-SMITH AND BENDALL[17] have drawn attention to the high initial pH values of muscles from myanesin-treated rabbits. Table I extends their results by illustrating the

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remarkably consistent material which may be obtained by ample feeding and relaxation before death.

TABLE I

INITIAL PHOSPHATE ESTER CONTENT AND pH OF THE PSOAS MUSCLES OF WELL-FED, MYANESIN-TREATED RABBITS

(P fractions as % of the total acid-soluble phosphorus; mean of seven animals in each case)

*Initial pH**	7.02 ± 0.07
*Ultimate pH***	6.01 ± 0.15
Phosphorus of	
Inorganic phosphate	14 ± 4
Creatine phosphate	32 ± 5
ATP	21 ± 2
Hexose diphosphate	6 ± 2
Triose phosphate	2 ± 1
Unidentified phosphate	25 ± 4

* The pH within a few minutes of death.
** The pH in full rigor, 24 hours post mortem.

Control

It proved impracticable to perform complete control experiments at the same time as the inhibition experiments, but the small variation from one animal to another as shown in Table I, and the uniform degree of homogenizing produced in each sample, made this unnecessary. Table II, showing the effect of homogenizing fresh muscle in water, may be considered as a control for inhibition experiments. The temporary accu-

TABLE II

GLYCOLYSIS IN HOMOGENATES OF MUSCLE IN WATER

(P fractions as % of total acid-soluble phosphorus)

Min after preparation	0	3	10	30	60
pH	7.08	6.80	6.32	6.05	6.04
Phosphorus of					
Inorganic phosphate	18	26	42	69	75
Creatine phosphate	29	2	0	0	0
ATP	23	9	4	0	0
Hexose diphosphate	5	32	15	3	2
Triose phosphate	2	5	6	5	5
Unidentified phosphate	23	26	33	23	18

mulation of hexose diphosphate could always be confirmed; its concentration attained a maximum about 3–4 minutes after preparation of the brei and was not accompanied by a comparable rise in triose phosphate. It seems likely that the aldolase is temporarily saturated, and must therefore control the rate of acid production. This, nevertheless, is very high. The initial rate of decrease of pH is about 0.1 unit/min, compared with that of 0.007 unit/min when muscle is homogenized in 0.16 *M* KCl (unpublished observation), and 0.002 unit/min in intact, excised muscle at 17° (BATE-SMITH AND BENDALL[17]).

o-Iodosobenzoate

This oxidant was found by SINGER AND BARRON[4] to be an effective inhibitor of myosin ATP-ase: an inhibition of 80% may be caused by a concentration of $8 \cdot 10^{-5}$

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mmol./mg myosin (BAILEY AND PERRY[5]). Its effect on the muscle homogenate is given in Table III. Acid production is prevented after a delay of at least 30 minutes and the decomposition of creatine phosphate is arrested in the same period. The accumulation of hexose diphosphate and triose phosphate indicates that some enzyme component, possibly triose phosphate dehydrogenase, has been inactivated, though this is a relatively slow process as indicated by the delayed arrest in acid production.

Assuming that between 30 and 60 minutes the aldolase-isomerase system is in

TABLE III

EFFECT OF *o*-IODOSOBENZOATE ON GLYCOLYSIS IN A MUSCLE HOMOGENATE
(P fractions as % of total acid-soluble phosphorus)

Min in 0,004 M reagent	0	3	10	30	60
pH	7.02	6.88	6.76	6.67	6.64
Phosphorus of					
Inorganic phosphate	12	15	15	18	17
Creatine phosphate	33	31	28	23	22
ATP	20	1	0	0	0
Hexose diphosphate	6	15	19	19	19
Triose phosphate	2	7	9	9	9
Unidentified phosphate	27	31	29	31	33

equilibrium, the amount of triose phosphate relative to hexose diphosphate can be calculated. The equilibrium constant for aldolase at 18°, extrapolated from the data of HERBERT, GORDON, SUBRAHMANYAN AND GREEN[18], is $2.7 \cdot 10^{-5}$; that for isomerase ([dihydroxyacetone phosphate]/[glyceraldehyde phosphate]) is 20–25 (MEYERHOF AND JUNOWICZ-KOCHOLATY[19]). For a hexose diphosphate concentration corresponding to 19% of the total acid-soluble P (Table III), the amount of triose phosphate P should amount to 7% against 9% found. For lower concentrations of hexose diphosphate (*e.g.* Table V), the triose found is lower than that calculated, while in Table II (30 and 60 minutes analyses) it is higher; but with such low contents of these esters, the estimations of which depend on difference methods, it is hardly possible to expect the experimental results always to satisfy the stern test imposed by application of equilibrium constants.

Chloroiodosofumarate

First synthesised by THIELE AND PETER[10], this compound has not so far been used as an inhibitor, and was tested in the hope that it would penetrate into the fibres more quickly than *o*-iodosobenzoate. It was found to be more efficient in arresting the fall in pH, but less effective as an inhibitor of the Lohmann enzyme (Table IV).

TABLE IV

EFFECT OF CHLOROIODOSOFUMARATE ON GLYCOLYSIS IN A MUSCLE HOMOGENATE
(P fractions as % of total acid-soluble phosphorus)

Min in 0.004 *M reagent*	0	3	10	30	60
pH	6.93	6.70	6.63	6.63	6.63
Phosphorus of					
Inorganic phosphate	13	20	21	24	24
Creatine phosphate	39	30	24	18	12
ATP	19	0	0	0	0
Hexose diphosphate	6	15	20	26	24
Triose phosphate	3	4	5	5	8
Unidentified phosphate	20	31	30	27	32

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Chloroacetophenone

This lachrymator is a powerful inhibitor of several enzymes (MACKWORTH[20]), but like other alkylating reagents[5,21] it reacts only slowly with myosin –SH groups. Although

TABLE V

EFFECT OF CHLOROACETOPHENONE ON GLYCOLYSIS IN A MUSCLE HOMOGENATE

(P fractions as % of total acid-soluble phosphorus)

Min in 0.003 M reagent	0	3	10	30	60
pH	7.06	6.93	6.65	6.40	6.38
Phosphorus of					
Inorganic phosphate	16	35	46	53	57
Creatine phosphate	29	9	9	8	7
ATP	21	22	9	2	0
Hexose diphosphate	9	8	7	10	7
Triose phosphate	2	1	2	2	2
Unidentified phosphate	23	25	27	25	27

in the present system the LOHMANN enzyme was inactivated after a slight delay (Table V), ATP could still be detected after 30 minutes; the appreciable decrease in pH during this interval, however, indicates that glycolytic resynthesis of ATP was proceeding rapidly.

p-*Chloromercuribenzoate*

This reagent, which functions by virtue of the great affinity of thiol groups for heavy metals, has been used in the characterisation of –SH enzymes (BARRON AND SINGER[22]), and reversibly inhibits myosin ATP-ase by at least 95% at a concentration of $2 \cdot 10^{-4}$ *M*[5]. On the homogenate it inhibits the Lohmann enzyme immediately and completely (Table VI), and arrests the fall in pH within ten minutes; this latter effect is due primarily to its action on aldolase, the hexose diphosphate remaining high and the

TABLE VI

EFFECT OF *p*-CHLOROMERCURIBENZOATE ON GLYCOLYSIS IN A MUSCLE HOMOGENATE

(P fractions as % of total acid-soluble phosphorus)

Min in 0.004 M reagent	0	3	10	30	60
pH	6.93	6.85	6.78	6.78	6.78
Phosphorus of					
Inorganic phosphate	11	12	16	20	18
Creatine phosphate	27	26	26	26	25
ATP	21	16	3	0	0
Hexose diphosphate	7	13	19	19	19
Triose phosphate	3	2	2	2	3
Unidentified phosphate	31	31	34	33	35

triose phosphate low. HERBERT *et al.*[18] noted the inhibitory effect of heavy metals on aldolase.

A reagent which inhibits both the LOHMANN enzyme and aldolase (or triose phosphate dehydrogenase) in whole muscle would be of particular interest, since it could prevent completely the resynthesis of ATP; changes of ATP content during activity of

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the intact excised muscle might then be correlated directly with mechanical events. Perfusions of the psoas muscle immediately post-mortem were carried out by Prof. R. GARCIA without achieving the desired result. It is quite probable that the reagent does not pass into the intact fibre.

DISCUSSION

It is possible to calculate indirectly how much ATP is decomposed by enzymic hydrolysis in the systems studied above, and thus to gain information on the effects of the inhibitors on ATP-ase activity. ATP is removed from the system both by direct enzymic dephosphorylation and by phosphate transfer to hexose monophosphate, and is added from creatine phosphate and by glycolytic resynthesis. The contribution of the latter can be calculated from the observed fall in pH in the following manner.

The buffering power of muscle over the pH range 7–6 is about $6 \cdot 10^{-5}$ equivalents acid per g muscle (BATE-SMITH[13]), and the formation of 1 mol lactic acid is accompanied by the production of 1½–2 mols labile phosphate (LIPMANN[24]). From these data it may be calculated that a pH decrease of one unit is accompanied by the resynthesis of about 3.2 mg ATP-P. Only one phosphate of hexose diphosphate is derived directly from ATP, and thus only one half of the increase of this ester and of triose phosphate is derived from direct transfer. We thus obtain the following relation:

[ATP decomposed by ATP-ase] = [ATP present at commencement]
+ [ATP synthesised by glycolysis]
+ [ATP derived from creatine phosphate]
— ½[increase of hexose diphosphate + triose phosphate]
— [ATP undecomposed]

In Table VII are collected the changes occurring in the first three minutes after homogenizing, taken from Tables II–VI inclusive; the results provide a measure of the relative rates of ATP decomposition by ATP-ase action. The most effective inhibitor of ATP-ase in the system is *p*-chloromercuribenzoate (almost 80% inhibition), followed by iodoso-

TABLE VII

ATP REMOVAL FROM HOMOGENATES IN PRESENCE OF INHIBITORS

(Differences during first 3 min as % of total acid-soluble phosphorus)

	No inhibitor	*0,004 M iodoso-benzoate*	*0,004 M chloroiodoso-fumarate*	*0,004 M chloromercuri-benzoate*	*0,003 M chloroaceto-phenone*
pH decrease	0.28	0.14	0.23	0.08	0.13
a. ATP–P resynthesis	45	22	37	13	21
b. ATP–P decrease	14	19	19	5	—1
c. Creatine phosphate P decrease	27	2	9	1	20
d. Hexose diphosphate P increase	27	9	9	6	—1
e. Triose phosphate P increase	3	5	1	—1	—1
ATP breakdown by ATP-ase (*i.e.* $a+b+c-\frac{1}{2}(d+e)$)	71	36	60	16½	41
Inhibition of ATP-ase (%)	0	49	15	77	42

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benzoate (50%) chloroacetophenone (40%) and chloroiodosofumarate (15%). The inhibitor concentrations used here were much higher than those required for the inhibition of isolated myosin ATP-ase (see BAILEY AND PERRY[5]); this is not surprising since many proteins are competing for the reagents. Moreover, the inhibitions are recorded immediately after addition of reagent, and not after previous incubation in absence of substrate.

The complete and immediate inhibition of creatine phosphokinase, the LOHMANN enzyme, by *p*-chloromercuribenzoate confirms earlier suggestions (LEHMANN AND POLLAK[25]; BAILEY AND PERRY[5]) that this enzyme is of -SH character. Neither this reagent nor chloroacetophenone inhibited triose phosphate dehydrogenase as effectively as iodoacetate, which was found (unpublished observations) to inactivate this enzyme completely in 0.001 *M* concentration. In the case of aldolase, oxidants and alkylating reagents have little effect (see also HERBERT *et al.* [18]; MACKWORTH[20]), but *p*-chloromercuribenzoate, in common with other heavy metal inhibitors, causes complete inactivation.

The authors are grateful to Mr J. R. BENDALL, who injected with myanesin the rabbits used in this work, and to Prof. R. GARCIA, of Instituto de Terapeutica, Montevideo, who carried out the perfusions.

So far as one of us (B.B.M.) is concerned, the work described in this paper was carried out as part of the programme of the Food Investigation Organisation of the Department of Scientific and Industrial Research.

SUMMARY

1. The effects of several enzyme inhibitors on glycogenolysis in homogenates of whole muscle have been examined by studying the accumulation of phosphate esters.

2. Of the four inhibitors used—*o*-iodosobenzoate, chloroiodosofumarate, chloroacetophenone and *p*-chloromercuribenzoate—the last-named caused a complete and immediate inhibition of the LOHMANN enzyme; it also inhibited aldolase. The other inhibitors were less effective on the LOHMANN enzyme and in arrest ing production of lactic acid.

3. The hydrolysis of ATP by ATP-ase was assessed indirectly. All reagents produced some inhibition, and *p*-chloromercuribenzoate most.

RÉSUMÉ

1. En étudiant l'accumulation des esters phosphatiques on a examiné les effets de quelques inhibiteurs des enzymes glycolytiques dans le muscle homogénéisé.

2. Le *p*-chloromercuribenzoate accomplit l'inhibition immédiate et complète de l'enzyme de LOHMANN et de l'aldolase. Les autres réactifs —l'*o*-iodosobenzoate, le chloroiodosofumarate et la chloroacétophenone — inhibent moins fortement ces deux enzymes.

3. L'hydrolyse de l'ATP par l'ATP-ase, évaluée indirectement, est inhibée par tous les réactifs, parmi lesquels le *p*-chloromercuribenzoate est le plus efficace.

ZUSAMMENFASSUNG

1. Die Wirkung verschiedener Hemmstoffe der glycolytischen Enzyme in Muskelhomogenaten wurde durch Bestimmung der Anhäufung von Phosphatestern untersucht.

2. Von den vier angewendeten Hemmstoffen — *o*-Jodosobenzoat, Chlorjodosofumarat, Chloracetophenon und *p*-Chlormercuribenzoat — bewirkte letzterer eine vollständige und sofortige Hemmung des Lohmann-Enzyms; er hemmte auch die Aldolase. Die anderen Hemmstoffe waren weniger wirksam und zwar sowohl gegenüber dem LOHMANN-Enzym als gegenüber der Milchsäurebildung.

3. Die Hydrolyse von ATP durch ATP-ase wurde indirekt bestimmt. Alle Reagentien bewirkten eine gewisse Hemmung, *p*-Chlormercuribenzoat die stärkste.

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Received November 22nd, 1951