(Hartley & Kilby, 1952) or cholinesterase by DFP (Aldridge, 1950). If reaction (2) is the rate-governing step, the inhibition will be gradual, partly reversible, and of zero order with respect to inhibitor.

When k_4 is not zero, A-B is a substrate for the enzyme. However, if reaction (3) is the rate-governing step, the kinetics will be similar to those observed in the reaction of NPA and NPC with chymotrypsin. These esters might, therefore, be looked upon as intermediate between substrates and inhibitors of the enzyme, and their structural analogy with the inhibitor E 600 is reflected in the kinetics of their reaction with chymotrypsin.

SUMMARY

1. The rate of inhibition of the nitrophenyl esterase activity of chymotrypsin by diethyl *p*-nitrophenyl phosphate (E 600) does not correspond with the rate of reaction of enzyme with inhibitor. After complete reaction with E 600, chymotrypsin still reacts with *p*-nitrophenyl acetate (NPA) and *p*-nitrophenyl ethyl carbonate (NPC) to liberate nitrophenol.

2. NPA and NPC react with amino and phenolic groups in derivatives and analogues of tyrosine.

3. NPA and NPC also react with insulin and protamine to liberate nitrophenol. The reaction with insulin is a catalysed hydrolysis of the esters. A mechanism is proposed.

4. A similar reaction of NPA or NPC in proteins would explain the observed reaction with inhibited chymotrypsin.

5. Chymotrypsin catalyses the hydrolysis of these esters at the same active centre as is responsible for the other enzymic activities. The kinetics of this reaction are discussed, and a mechanism is proposed.

6. The catalytic activities of insulin and chymotrypsin are compared.

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In vitro Demonstration of Metabolic Changes During Heat Regulation in Rats

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In a preliminary note (Wertheimer & Bentor, 1950) we described the fact that the diaphragms of rats maintained for 5 hr. at 5–7° required more glucose and synthesized greater quantities of glycogen from glucose when incubated in their own serum than did the diaphragms of rats maintained for the same period at 28–30°. These changes in organ metabolism were already demonstrable at about 30 min.

after the start of cooling, and disappeared a few hours after the temperature stimulus had been discontinued. If such diaphragms were incubated in Krebs-Ringer solution, rather than in homologous serum, the increased glucose requirement of the 'cold' diaphragms could no longer be demonstrated, and the glycogen synthesis was only slightly augmented. These experiments were repeated at

different seasons and the effect of different ranges of temperature was studied, including ranges over which the body temperature of the rat either rose or fell, i.e. where temperature regulation no longer functioned. In addition, the effect of cold and heat on the metabolism of organs other than muscle was studied.

METHODS

The basic experiment. The experiments were carried out on 3- to 3.5-month-old male rats weighing 100–120 g., from a strain which attains a mature weight of about 250 g. in 6–7 months. The animals were maintained under identical conditions of nutrition (wheat and vegetables) and temperature for at least 1 month before the experiment. Food was withheld on the evening before the experiment. After 17 hr., two rats ('cold') were placed in the refrigerator at 6° ($\pm 1^\circ$), while two other rats ('warm') were maintained at 30° (28–30°). Unless specifically mentioned, no changes were observed in the temperature of the rats. After exposure for 4 hr. to the experimental temperature, one each of the 'cold' and the 'warm' rats was killed and bled, and their excised diaphragms were placed into Krebs-Ringer-phosphate solution. The blood of the two rats was collected and centrifuged. The remaining two rats were then killed and their diaphragms were placed into homologous serum and glycogen synthesis, and glucose utilization were measured as described below.

Preparation of the diaphragms. This was carried out according to the method of Gemmill (1940).

Incubation medium. The Krebs-Ringer-phosphate solution (pH 7.4) contained 0.9% NaCl, 100 parts; 1.15% KCl, 4 parts; 1.22% CaCl_2 , 3 parts; 3.8% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 part; 40 ml. of 0.25M- Na_2HPO_4 + approx. 2 ml. of N-HCl brought to a volume of 100 ml., 21 parts; glucose content, 0.2% (all w/v).

The serum was used immediately after centrifugation. Glucose (1 mg.) was added to 1 ml. serum, so that the total glucose concentration was about 0.2% (w/v).

The diaphragms were suspended in 1 ml. of medium and gassed for 30 sec. with O_2 in 10 ml. Erlenmeyer flasks, which were then placed in a water bath at 37° and shaken at a rate of 120 excursions/min. for 2.5 hr.

Measurement of glycogen synthesis. The method of Tuerkischer & Wertheimer (1948) was used.

Measurement of glucose utilization. This was by the method of Krah & Cori (1947).

Experiments on glycogenolysis. These were carried out in Krebs-Ringer-phosphate solution without the addition of glucose. Serum was freed of sugar by incubating 1 ml. with about 0.5 g. freshly washed yeast at 37° for 10 min., then testing for reducing substances. Glycogenolysis is expressed as decrease in the glycogen content.

Liver slices. Experiments on glycogen synthesis in liver slices were carried out in both the 'intracellular solution' described by Buchanan, Hastings & Nesbett (1949), and in normal serum. The incubation time was 4 hr. and the initial glucose concentration was 1% (w/v).

Abdominal muscle. The excised abdominal muscle of young mice was trimmed free of connective tissue and cut into three equal parts. The media used were the 'cold' and 'warm' rat sera, as in the basic experiment.

Measurements of respiration. Determination of oxygen uptake in Krebs-Ringer-phosphate solution was carried out by the Warburg direct method (Dixon, 1951). Determinations of O_2 uptake in serum, with glucose content adjusted to 0.2% (w/v) were carried out by the Dixon-Keilin method (Dixon, 1951). Measurements were continued for 2 hr. at 37°.

Chemical procedures. Determinations of glycogen in the diaphragm were carried out by the method of Good, Kramer & Somogyi (1933); glucose was estimated according to the method of Somogyi (1937). In all cases the values were related to wet tissue weight. Determination of acetoacetic acid was by the method of Engfeld as described by Rappaport (1949). The values were expressed in mg. acetoacetic acid/100 mg. wet tissue.

RESULTS

Glycogen synthesis and glucose utilization by diaphragms, incubated in serum, of rats maintained at 6 or 30°. These experiments were carried out over the period of an entire year. Except on a few particularly hot days (khamsin) no significant seasonal variations were obtained in the results of this investigation. The results of 48 experiments done to date are summarized in Table 1. This is the basic experiment for all further studies. It shows that diaphragms from 'cold' rats synthesize more glycogen from glucose and utilize more glucose than do diaphragms from 'warm' rats. In one section of the study, Krebs-Ringer-phosphate as well as serum was employed as the medium. As in former studies (Wertheimer & Bentor, 1950) the difference between diaphragms of 'cold' and 'warm' rats in glycogen synthesis from glucose in Krebs-Ringer-phosphate was less marked, and no difference in glucose utilization was noted.

The initial glycogen content of diaphragms ($\pm$ s.e.) in the 48 experiments was: at 6°, 0.226 ± 0.035 mg./100 mg. tissue; at 30°, 0.190 ± 0.034 mg./100 mg. tissue.

In a previous paper (Wertheimer & Bentor, 1950) we found no difference in the glycogen synthesis of

Table 1. *Glycogen synthesis and glucose utilization by the diaphragms of rats maintained at 6 or 30°*

No. of measurements	(Diaphragms incubated in serum. Results $\pm$ s.e.)			
	Glycogen synthesis (mg./100 mg. tissue/2.5 hr.)		Glucose utilization (mg./100 mg. tissue/2.5 hr.)	
	6°	30°	6°	30°
48	0.104 ± 0.017	0.040 ± 0.009	0.90 ± 0.03	0.56 ± 0.07

the 'warm' diaphragm incubated with 'cold' serum as compared with 'cold' diaphragm incubated with 'warm' serum; however, glucose consumption was found to be greater in the 'warm' diaphragm incubated in 'cold' serum.

Glycogenolysis by diaphragms from 'cold' and 'warm' rats in homologous, glucose-free serum. Glycogenolysis was usually more marked in diaphragms from 'cold' rats than in those from 'warm' rats, but the differences were not significant. In five additional experiments, 0.1 % (w/v) acetoacetate was added to glucose-free serum as substrate. Under these conditions, glycogenolysis was significantly higher in diaphragms from 'cold' rats (Table 2).

Oxygen consumption by diaphragms from 'cold' and 'warm' rats in homologous sera. No difference in oxygen uptake was observed between the two types of diaphragms. Eight experiments with 'cold' rats showed an oxygen uptake of $246 \pm 6 \mu\text{l./100 mg. tissue/2 hr.}$; with diaphragms from 'warm' rats the uptake was $254 \pm 8 \mu\text{l./100 mg. tissue/2 hr.}$ Furthermore, no difference was seen when Krebs-Ringer-phosphate was substituted for serum as medium.

Acetoacetate consumption by diaphragms from 'cold' and 'warm' rats in homologous, glucose-free serum. In order to determine whether the rise in metabolism of diaphragms from 'cold' and 'warm' rats is limited to glucose consumption, studies were made on the acetoacetate uptake of diaphragms under identical conditions. No differences were observed. In five experiments with 'cold' rats, $0.54 \pm 0.02 \text{ mg. acetoacetic acid/100 mg. tissue}$ was utilized; with diaphragms from 'warm' rats the utilization was $0.57 \pm 0.04 \text{ mg. acetoacetic acid/100 mg. tissue}$.

Glycogen synthesis and glucose utilization by the diaphragms of rats maintained at different temper-

atures. In this series of experiments, studies were carried out between 6 and 30°. The results are given in Table 3.

Glycogen synthesis and glucose utilization by the diaphragms of rats maintained under such external conditions of temperature that the body temperature rose or fell. Up to this point, experiments were so planned that the body temperature of the rat remained unchanged under the influence of either heat or cold. The following experiments were so arranged that the heat regulation of the rat was inadequate, with the result that the body temperature rose or fell. A rise in the body temperature of the rats was obtained by keeping them at a temperature of 37° for a given period. The carbohydrate metabolism of the diaphragms of such rats is compared in Table 4A with that of rats maintained at 30°. The initial glycogen content of the diaphragms in the nine experiments was $0.179 \pm 0.013 \text{ mg./100 mg. tissue}$ (37°) and $0.160 \pm 0.013 \text{ mg./100 mg. tissue}$ (30°). In further experiments, an average rise in body temperature of 1.6° was obtained by the intraperitoneal injection of 2 mg. of amphetamine sulphate. The synthesis of glycogen from glucose and the glucose utilization of the diaphragms of such rats are given in Table 4B.

A reduction in the body temperature of rats to about 28° was obtained by keeping them in a water bath at 25° for 30 min. In eight experiments with the diaphragms of these rats incubated in Krebs-Ringer-phosphate, the glycogen synthesis was $0.175 \pm 0.020 \text{ mg./100 mg. tissue}$; in the controls this was $0.091 \pm 0.014 \text{ mg./100 mg. tissue}$ ($P=0.01$). Glucose utilization was $0.68 \pm 0.03 \text{ mg./100 mg. tissue}$ for the experimental diaphragms, and $0.60 \pm 0.04 \text{ mg./100 mg. tissue}$ for the controls. The initial glycogen content of diaphragms in the eight experiments was $0.068 \pm 0.009 \text{ mg./100 mg. tissue}$

Table 2. Glycogenolysis in the diaphragms of rats maintained at 6 or 30°

(Diaphragms incubated in glucose-free serum. Results $\pm$ s.e.)

No. of measurements	Medium	Glycogen decrease (mg./100 mg. tissue/2.5 hr.)	
		6°	30°
10	Serum without substrate	0.055 $\pm$ 0.008	0.041 $\pm$ 0.006
3	Serum with addition of 0.1 % (w/v) sodium acetoacetate	0.079 $\pm$ 0.020	0.049 $\pm$ 0.002

Table 3. Glycogen synthesis and glucose utilization by the diaphragms of rats maintained at different temperatures

(Diaphragms incubated in serum. Results $\pm$ s.e.)

Environmental temperature ...	30°	20°	15°	6°
No. of measurements ...	17	11	6	14
Glycogen synthesis (mg./100 mg. tissue/2.5 hr.)	0.050 $\pm$ 0.004	0.075 $\pm$ 0.006	0.126 $\pm$ 0.006	0.100 $\pm$ 0.006
Glucose utilization (mg./100 mg. tissue/2.5 hr.)	0.64 $\pm$ 0.16	0.77 $\pm$ 0.07	0.90 $\pm$ 0.06	0.94 $\pm$ 0.03

Table 4. *Effect of increase in body temperature on glycogen synthesis and glucose utilization by rat diaphragms incubated in serum*

(Results $\pm$ s.e.)						
A						
No. of measurements	Glycogen synthesis (mg./100 mg. tissue/2.5 hr.)		Glucose utilization (mg./100 mg. tissue/2.5 hr.)		Increase in body temperature at	
	37°	30°	37°	30°	37°	30°
9	0.000 $\pm$ 0.018	0.080 $\pm$ 0.011	0.65 $\pm$ 0.06	0.53 $\pm$ 0.07	1.5°	0°
B. Rats injected intraperitoneally with 2 mg. amphetamine sulphate and killed after 1 hr.						
No. of measurements	Glycogen synthesis (mg./100 mg. tissue/2.5 hr.)		Glucose utilization (mg./100 mg. tissue/2.5 hr.)		Increase in body temperature produced by amphetamine sulphate	
	Rats given amphetamine sulphate	Control	Rats given amphetamine sulphate	Control		
8	-0.023 $\pm$ 0.007	0.052 $\pm$ 0.002	0.98 $\pm$ 0.06	0.72 $\pm$ 0.06	1.6°	

Table 5. *Glycogen synthesis and glucose utilization by liver slices from rats maintained at 6 or 30°*

(Liver slices incubated in serum. Results $\pm$ S.E.)				
Medium	No. of measurements	Glycogen synthesis (mg./100 mg. tissue/2.5 hr.)		
		6°	30°	
Serum	21	0.302 $\pm$ 0.031	0.240 $\pm$ 0.045	($P=0.10$)
Hastings's medium	18	0.378 $\pm$ 0.055	0.261 $\pm$ 0.043	($P=0.05$)

for the experimental rats and 0.234 ± 0.024 mg./100 mg. tissue for the controls.

Glycogen synthesis and glucose consumption in serum by abdominal muscle from mice maintained at 15 or 30°. Since diaphragm is a special type of striated muscle, another type of muscle was studied in the basic experiment. For technical reasons, only a few muscles could be used, and the abdominal muscle of the young mouse (10–15 g. body weight) was chosen. In six experiments, glycogen synthesis in muscle from mice maintained at 15° was 0.059 ± 0.017 mg./100 mg. tissue; in muscle from mice maintained at 30°, glycogen synthesis was 0.024 ± 0.003 ($P=0.03$). Glucose utilization in muscle from mice maintained at 15° was 0.82 ± 0.02 mg./100 mg. tissue, whilst in muscle from mice maintained at 30°, glucose utilization was 0.35 ± 0.02 mg./100 mg. tissue ($P=0.01$). It is clear that the results obtained with the abdominal muscle of the mouse are similar to those observed using rat diaphragm.

Glycogen synthesis from glucose in homologous serum or in Hastings's solution by liver slices of rats maintained at 6 or 30°. In this series of experiments changes in the carbohydrate metabolism of the livers of rats were studied. The glucose utilization of liver slices cannot be determined by our technique. On the other hand, glycogen synthesis by liver slices in a medium described by Buchanan *et al.* (1949) is easily determined. Since our studies showed that glycogen synthesis by rat-liver slices can be demonstrated in serum as well, we were able to carry out

similar experiments on rats, using a serum medium. The results of this investigation are summarized in Table 5.

Oxygen uptake by liver slices, incubated in serum, from rats at 6 and 30°. No difference was found between 'cold' and 'warm' rats. In seven experiments, the rate of respiration of liver slices from 'cold' rats was 513 ± 9 μ l. O₂/100 mg. tissue/2 hr. The rate of respiration of liver slices from 'warm' rats was 499 ± 26 μ l. O₂/100 mg. tissue/2 hr.

Acetoacetic acid formation by liver slices, incubated in serum, from rats maintained at 6 and 30°. The serum contained 0.2% glucose and 0.0075M sodium octanoate. No difference was found. In five experiments, the rate of production in liver from 'cold' rats was 0.600 ± 0.039 mg. acetoacetic acid/100 mg. tissue. The rate of production in liver slices from 'warm' rats was 0.624 ± 0.036 mg. acetoacetic acid/100 mg. tissue.

DISCUSSION

Metabolic changes in the muscle of rats exposed for short periods to heat or cold. All of the metabolic changes that have been found are fully demonstrable only when the serum of homologous rats is employed as the incubation medium for the diaphragm. It must therefore be assumed that substances are present in the sera of 'cold' rats which either determine, or else augment, the metabolic changes in the muscle. No evidence was found of

increased ketolysis in the muscle of 'cold' animals; the acetoacetate utilization by the diaphragms of 'cold' and 'warm' rats was identical. Negative results do not prove that no changes occur in this process during heat regulation *in vivo*, since such changes may possibly be no longer demonstrable *in vitro*. On the other hand, the positive observations contribute to our understanding of the localization and the type of metabolic change which occur during heat regulation. These experiments demonstrated that certain changes in carbohydrate metabolism are associated with heat regulation in rat diaphragms as well as in the abdominal muscles of mice, and probably in muscle in general.

In most studies, changes in muscle metabolism under the influence of cold are associated with shivering. No information has hitherto been available to show any changes during heat regulation in muscle metabolism which was not associated with shivering. If cooling is carried so far that the rat is overchilled, shivering also occurs, and in such a case the same changes in carbohydrate metabolism are observed as in the basic experiment. The *in vitro* metabolic changes in the diaphragms of shivering rats whose body temperature has fallen to 28° are certainly no greater, and may be less, than in the basic experiment; since the glycogen content of the diaphragms and other muscles falls sharply under these special conditions, the observed increases in glycogen synthesis and glucose utilization prove nothing, inasmuch as these values are always increased when the initial content of glycogen in the diaphragm is low (Hansen, Rutter & Samuels, 1951).

If the body temperature of the rats is experimentally increased at high environmental temperatures (37°), markedly low rates of glycogen synthesis, and frequently even glycogenolysis, are observed in their diaphragms (see also Fishgold, Grant, Field & Hall, 1951; Grant & Hirsch, 1950). Since this tendency to glycogenolysis can be demonstrated only when serum from homologous rats, and not Krebs-Ringer-phosphate, is used as the medium in which the diaphragms are incubated, it is probable that during the fever something appears in the blood which inhibits glycogen synthesis or favours glycogenolysis. We do not yet possess any information as to the nature or origin of this material.

Metabolic changes in liver slices of rats exposed to different temperatures. The medium capable of inducing optimum glycogen synthesis in rat-liver slices is the so-called intracellular medium, rich in potassium, described by Buchanan *et al.* (1949). Liver slices from rats maintained for a short time at 6° tend to synthesize more glycogen than do those from rats maintained at 30°, but the difference is not significant (Table 5). It is noteworthy that glycogen synthesis in liver slices may also be carried out in serum. Further studies on this serum effect are in

progress, but it seems likely that the serum does not affect potassium metabolism in liver slices but has a specific effect. In any case, the glycogen synthesis of liver slices from 'cold' rats maintained in a serum medium was somewhat higher than that of 'warm' rats, but in this case also the differences were not significant. According to these experiments, metabolic changes induced by heat regulation in muscle are far more marked than those in liver. Furthermore, no differences in oxygen uptake or in the production of acetoacetate were observed between the livers of 'warm' and 'cold' rats.

The cold stimulus which induces metabolic changes in the diaphragm. Our experiments show that metabolic changes in the diaphragm *in vitro* were not proportional to the ranges of difference in temperature to which the rats were exposed. Changes in the diaphragms of rats exposed to a range of from 6 to 30° were no greater than in those exposed to a range of from 15 to 30°. Every deviation in the temperature of the room in which the rats were normally kept is apparently capable of inducing the described metabolic changes. For example, if the room temperature is 20° and the experimental rat is exposed to an environmental temperature of 15° for a short time, this temperature difference is already sufficient to induce a small, but significant, metabolic change in the diaphragm *in vitro*.

The use of serum in in vitro studies. The metabolic changes in the diaphragm following exposure to cold were clearly demonstrable only when serum from homologous rats was used as the medium, and not, or to a much slighter extent, when Krebs-Ringer-phosphate was used. It is known that salt solutions with certain additions may be substituted for serum as a medium in the study of cell respiration (Krebs, 1950). However, the present studies have demonstrated that under some conditions substances may appear in the serum which are the cause of changes in cellular metabolism. It follows that homologous serum should be used as a medium parallel to the customary salt solutions in experiments in which physiological regulations or pathological changes in cellular metabolism are to be investigated *in vitro*.

SUMMARY

1. Metabolic changes are described in the diaphragms of rats previously maintained for a short time in cold or warm environments. The homologous serum of such rats was employed as medium.

2. Glycogen synthesis from glucose and glucose utilization were increased in the diaphragms of 'cold' rats. Glycogenolysis in glucose-free serum was also augmented. No differences in oxygen

utilization were observed between the diaphragms of 'cold' and 'warm' rats. Acetoacetate utilization was identical in the two types of rat.

3. Glycogen synthesis and glucose utilization by the abdominal muscle of mice previously exposed to cold were also higher than those of mice maintained at a higher temperature.

4. If Krebs-Ringer-phosphate was substituted for homologous serum as medium, no difference in glucose utilization was observed between the diaphragms of 'cold' and 'warm' rats, and differences in glycogen synthesis were smaller under these conditions.

5. The extent of the temperature variations to which the rats were exposed was not proportional to the metabolic effect in the diaphragm. The effect was still observable when rats were maintained at 15 or 20°, respectively.

6. Glycogen synthesis was much reduced in the diaphragms of rats maintained at 37° compared with that in the diaphragms of rats maintained at 30°, and glycogenolysis was often observed. This effect was demonstrable only when homologous serum and not Krebs-Ringer-phosphate was used as medium. Glycogenolysis was also observed in diaphragms of rats in which fever had been induced by amphetamine sulphate.

7. Rat-liver slices were capable of synthesizing almost as much glycogen from glucose in serum as in Hastings's medium. No differences in oxygen uptake nor in the production of acetoacetate were observed between liver slices from 'cold' and 'warm' rats.

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An Investigation into the Turnover Rates of Organophosphates

1. EXTRACELLULAR SPACE AND INTRACELLULAR INORGANIC PHOSPHATE IN SKELETAL MUSCLE

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In experiments involving the determination of the turnover rates of those organophosphates for which inorganic phosphate (inorganic P) is thought to be the immediate source of phosphorus, it is necessary to determine the specific radioactivity (counts/min./ μ g.) of the inorganic P. Furthermore, since these compounds are metabolized within the cell, interest centres upon the intracellular inorganic P. The inorganic P contained in a trichloroacetic acid extract of tissue is the sum of the extracellular and the intracellular inorganic P, and it is necessary to differentiate between these moieties if the turnover rate is to be determined. Considerable emphasis has been placed upon the necessity for such a differentiation by Furchgott & Shorr (1943) and by

Kalckar, Dehlinger & Mehler (1944). The problem has been investigated by these two groups of workers in different ways. Furchgott & Shorr (1943), using slices of heart muscle, have shown that inorganic P may, by a washing technique, be separated into two well defined fractions. One fraction, which was readily diffusible and constituted 30–40% of the total inorganic P of the tissue, was regarded as the extracellular inorganic P. The other fraction was retained in the tissue and was regarded as being of intracellular origin. Kalckar *et al.* (1944), in *in vivo* experiments, employed a perfusion technique and claimed to have achieved a similar separation. Hevesy & Hahn (1940), on the other hand, have calculated the specific radioactivity of the intra-