changed by storage for 7 days under the conditions of Fig. 1. The ease with which apparently low-molecular-weight fragments are split off cod myosin, e.g. by urea concentrations as low as 0·3 M, may point to a relatively loose internal cohesion of the molecule.

The results of Lowey & Holtzer (1959) and the present ones have given little help in discovering the nature of the bonds holding myosin aggregates together. There is no evidence for sulphydryl interactions being involved. The slight inhibition or reversal of aggregation in concentrated urea solutions suggests that hydrogen bonds might be concerned. The negative results given by the ultraviolet-difference spectra of aggregated versus monomeric cod myosin suggest that tyrosyl groups do not participate in any hydrogen bonding which may occur.

SUMMARY

1. The aggregation of cod-myosin monomer at concentrations of 0·2–1·5% has been studied by following changes in weight-average molecular weight, sedimentation, diffusion, viscosity and optical rotation.

2. The kinetics of the disappearance of monomer have been analysed and can be plausibly accounted for by assuming that the reaction involves two steps: a denaturation stage followed by a relatively rapid stepwise side-to-side aggregation.

3. Additional evidence for a denaturation stage has come from a study of the reaction at different temperatures, conditions of pH and ionic strength, from the effects of added substances and from an examination of the concurrent enzymic inactivation.

4. Aggregation, which under extreme conditions is often accompanied by a dissociation of the molecule, cannot be explained on the basis of sulphydryl–disulphide interactions.

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Intracellular Distribution of Carbonic Anhydrase

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The presence of carbonic anhydrase in various mammalian tissues has been recognized for many years. The only known function of the enzyme is catalysis of the reaction

$$CO_2 + H_2O \rightleftharpoons H_2CO_3.$$

In certain organs, such as stomach, kidney and pancreas, it appears that carbonic anhydrase is involved in adjusting the rate of a steady state of production of carbon dioxide or carbonic acid to specific functional demands. The necessity for an increased rate of the above reaction is most clearly understood for the example of elimination of

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carbon dioxide. The relatively short period of time in which blood remains in capillaries, both in systemic and in the lung, would not be adequate for elimination of carbon dioxide in the absence of carbonic anhydrase. Hence, in this particular example, the function of the enzyme has been clearly demonstrated to be an integral part of the more general function of respiration.

The role of carbonic anhydrase in tissues such as liver and brain, however, which generally are not associated with recognizable functions involving carbon dioxide or acid secretion is unknown. In view of this fact a study of the intracellular distribution of the enzyme in several different tissues was conducted in order to obtain additional descriptive information, which in turn might be useful in developing an understanding of the role of the enzyme.

Available data on the intracellular locus of carbonic anhydrase are limited. Datta & Shepard (1959) reported that the distribution of the enzyme in rat liver and kidney was restricted to the soluble fraction, whereas Karler & Woodbury (1959) presented preliminary evidence which indicated that the intracellular distribution was more complicated. Our studies indicated that carbonic anhydrase was present, not only in the soluble fraction, but also in particulate matter which corresponded to mitochondria.

The work described here represents continued investigation of the intracellular distribution of the enzyme in the rat tissues, cerebral cortex, kidney, liver, salivary glands and thyroid. The results support the previous report that generally carbonic anhydrase is distributed intracellularly between soluble and particulate fractions.

METHODS

The various tissues studied were taken from male, Sprague–Dawley rats which weighed approx. 250 g. Tissues were perfused *in vivo* by the following procedure: animals were anaesthetized with ether and a needle and syringe were used to withdraw as much blood as possible from the dorsal aorta at the junction of the common iliacs. The needle was held in position by a haemostat. After the withdrawal of blood, 10 ml. of 0·25 M-sucrose solution was injected and 10 ml. of diluted blood removed. This perfusion procedure was repeated three times. With cerebral cortex, kidney, liver and salivary glands approx. 1–2 g. of tissue was taken for homogenization. Thyroid determinations, however, involved about 100 mg. of tissue, which was collected by pooling glands from 10 rats. Perfused tissues were homogenized in 10 vol. of 0·25 M-sucrose solution with a glass–Teflon type of tissue grinder (A. H. Thomas Co.), 15–20 strokes at 350 rev./min. for about 3 min. being used. The homogenization was performed in an ice bath and all subsequent manipulation of tissue fractions was in a cold room (5°).

The differential-centrifuging procedure used for the determination of the intracellular distribution of the enzyme corresponded in general to that proposed by Hogeboom & Schneider (1955). Tissue homogenates were fractionated into total particulate matter and soluble fraction by centrifuging at 100 000 *g* for 30 min. The particulate fraction was washed once by resuspension and centrifuging again in 0·25 M-sucrose solution. The homogenization and centrifuging procedures were repeated with different suspending media. In addition to 0·25 M-sucrose solution, 0·88 M-sucrose solution (Hogeboom & Schneider, 1948) and 0·25 M-sucrose–7·3 % polyvinylpyrrolidone (General Aniline and Film Corp.) solution (Novikoff, 1957) were also employed.

Homogenates of cerebral cortex were fractionated in more detail by the following procedure. A nuclear fraction was collected by centrifuging at 600 *g* for 10 min.; a mitochondrial fraction was obtained by continuing the centrifuging at 10 000 *g* for 25 min.; the final particulate fraction, the microsomes, consisted of particles removed by centrifuging the remaining suspension at 100 000 *g* for 30 min. Each particulate fraction was subjected to one washing. The soluble fraction represented the supernatant obtained from the differential centrifuging of the particulate fractions. Kidney homogenates were also fractionated but the mitochondrial fraction was collected by centrifuging the nuclear-free homogenate at 20 000 *g* for 10 min.

Carbonic-anhydrase activity was measured by the manometric method described by Roughton & Clark (1951). Enzymic activities were determined in samples that were frozen for storage and subsequently thawed for assay, and in fresh samples, which were assayed as soon as possible after isolation. Except for initial experiments, all assays were performed on fresh samples.

Enzymic activities of tissue homogenates were expressed in terms of units as defined by Meldrum & Roughton (1934). Units are calculated from the expression $(t_u - t_c)/t_c$, in which t_u represents the time required for the uncatalysed reaction to produce the second quarter of the total manometric displacement and t_c represents the time required for the catalysed reaction to produce the same quarter of the reaction.

The enzymic activities of subcellular fractions were expressed as a percentage of the total enzymic activity of the original homogenate by determining a standard curve of homogenate activity (in terms of rates) at various concentrations. The expression of recoveries for the fractionation studies represents the sum of the activities of the fractions in terms of homogenate activity.

RESULTS

Carbonic-anhydrase activity in particulate and soluble fractions

Preliminary attempts to determine the intracellular distribution in the two major fractions were quantitatively unsuccessful because of an inability to obtain adequate enzymic recoveries. Initially, only 60–70 % of the total activity was recoverable. At this time samples were frozen for storage before they were assayed, but it was subsequently observed that freezing and thawing significantly affected the activity of the soluble fraction. The data are given in Table 1.

As is indicated, the total activities in the homogenate and particulate fractions were not strikingly affected by freezing. However, the enzymic activity in the soluble fraction, which represented 67 % of the total activity before freezing and only 46 % after freezing, was greatly decreased by the freezing and thawing procedure.

The results obtained when the fractions were assayed immediately after preparation are given in Table 2. With the exception of the thyroid, the soluble fraction from the other tissues contained about 75–85 % of the total enzymic activity. The remaining 15–25 % of the activity was recovered in the particulate fraction.

The results for the thyroid indicated that, under the conditions of these experiments, most if not all of the activity appeared in the soluble fraction. No activity was detected at any time in the particulate fraction.

The distribution results obtained with 0·88 M-sucrose and 0·25 M-sucrose–7·3 % polyvinylpyrrolidone media as compared with the conventional 0·25 M-sucrose solution are cited in Table 3. It can be seen from this table that the distribution of carbonic-anhydrase activity between total particulate and soluble fractions remains practically the same in the three different fractionation media.

The results in Table 3, however, raise an interesting problem. From comparison of the total enzymic activity recovered with comparable deter-

Table 1. *Effect of freezing on the recovery of carbonic-anhydrase activity from cellular fractions of cerebral cortex*

Cortical tissue was homogenized in 10 vol. of 0·25 M-sucrose soln. and the particulate fraction was separated from the soluble fraction by centrifuging at 100 000 *g* for 30 min. Samples were first assayed immediately after preparation and then stored at −20° for 24 hr. Samples were subsequently thawed at room temperature and assayed again. Duplicate values given represent the results obtained from two experiments.

	Percentage of activity of homogenate	
	Before freezing	After freezing
Total homogenate activity	100 (280, 290 units)	100 (296, 312 units)
Particulate fraction	21, 25	21, 19
Soluble fraction	63, 71	41, 51
Total recovery	84, 96	62, 70

Table 2. *Approximate intracellular distribution of carbonic-anhydrase activity between particulate and soluble fractions*

Tissues were homogenized in 10 vol. of 0·25 M-sucrose soln. and the particulate fraction was separated from the soluble fraction by centrifuging at 100 000 *g* for 30 min. Samples were assayed immediately after preparation. The number of experiments on each tissue is given in parentheses; the enzymic activities represent average values. Values for total enzymic activities are to the nearest hundred, except for the thyroid, which is to the nearest ten. The percentage total activity recovered in soluble and particulate fractions is to the nearest 5 %. The percentage recovered values represents recoveries for the individual experiments.

Tissue	Total enzymic activity (units/g. wet wt.)	Recovered enzymic activity (%)		Total enzymic activity recovered (%)
		Soluble fraction	Particulate fraction	
Submaxillary gland (2)	2400	75	25	88, 100
Kidney (2)	1000	80	20	77, 87
Cerebral cortex (6)	300	75	25	86, 90, 94, 96, 85, 94
Liver (2)	300	85	15	84, 96
Thyroid (3)	60	100	—	76, 80, 94

Table 3. *Influence of various homogenization media upon the intracellular distribution of carbonic-anhydrase activity in cerebral cortex*

Fractionation procedures are described in the Methods section. Duplicate values given represent the results obtained from two experiments with each medium.

Medium	Total activity (units)	Percentage of total enzymic activity		Percentage recovery
		Particulate fraction	Soluble fraction	
0·25 M-Sucrose	70, 73	37, 45	49, 54	86, 99
0·25 M-Sucrose–7·3 % polyvinylpyrrolidone	72, 78	39, 42	49, 57	88, 99
0·88 M-Sucrose	98, 111	40, 45	55, 68	95, 113

minations reported in Tables 1 and 2, it can be seen that the total enzymic activity (units/g.) decreased from about 300 to less than 100. Further comparison of these tables indicates that relative changes in the intracellular distribution also occurred. As shown in Table 3, about 40 % of the enzymic activity is associated with the particulate fraction, whereas the corresponding fraction in the previous tables contained only 25 % of the total activity. At the present time these changes in total activity and distribution are not understood.

Intracellular distribution of carbonic anhydrase in rat cerebral cortex and kidney

The results obtained by differential centrifuging are given in Table 4. With cerebral cortex, the experimental results indicated that the soluble fraction contained approximately 75 % of the total enzymic activity, whereas the mitochondrial fraction itself possessed 20 % of the activity. The remaining 5 % of activity in cortical homogenates was recovered in the nuclear fraction.

The intracellular distribution of carbonic anhydrase in the various kidney fractions appeared similar to that obtained for cerebral cortex. The majority of the total activity (80 %) again appeared associated with the soluble fraction, and the activity in the particulate fractions was also greatest in the mitochondria (10 %). The kidney nuclei and microsomes each contained approximately 5 % of the total activity.

Effect of solubilization procedures upon the carbonic-anhydrase activity associated with mitochondria of cerebral cortex

The data in Table 5 represent the effects upon the insoluble carbonic anhydrase observed after exposing mitochondria to water, to repeated freezing and thawing and to digitonin. The results demonstrated that the enzymic activity associated with mitochondria isolated in a sucrose medium (control value) was not solubilized by any of the experimental procedures employed, that is, the enzymic activities of the mitochondrial washes remained zero even after the various treatments.

Such observations tend to indicate that the enzyme is not merely contained in solution within mitochondria, but that the enzyme is intimately associated with the mitochondrial membrane.

DISCUSSION

The data presented on the intracellular distribution of carbonic anhydrase provide evidence for the existence of the enzyme in both the soluble and particulate components of cells. The more detailed studies which were performed on cerebral cortex and kidney indicate that the enzymic activity of the particulate matter is generally associated with the mitochondrial fraction.

Any intracellular-distribution study involves several technical problems. The fact that red blood cells contain carbonic anhydrase and hence are capable of contaminating the true tissue activity is in itself an additional complication. Some studies in the past on tissue carbonic anhydrase have taken this into account by correcting levels of enzymic activity for the presence of blood. This type of correction was not felt to be adequate for intracellular-distribution studies because rat blood contains, in addition to significant amounts of carbonic anhydrase in red blood cells, a potent, carbonic-anhydrase inhibitor in serum or plasma,

Table 4. *Intracellular distribution of carbonic anhydrase in rat cerebral cortex and kidney*

Fractionation procedures are described in the Methods section. The activities in cerebral-cortex fractions represent the average of four experiments; the activities in kidney fractions represent the average of two experiments. The averages (%) for all fractions are expressed to the nearest 5 %.

	Percentage of total activity recovered	
Cellular fraction	Cerebral cortex	Kidney
Nuclei	5	5
Mitochondria	20	10
Microsomes	—	5
Soluble fraction	75	80
Percentage recovery	95	90

Table 5. *Effect of solubilization procedures upon carbonic-anhydrase activity of mitochondria of cerebral cortex*

Control indicates a mitochondrial sample suspended in 0·25 M-sucrose solution for 1 hr. at 5°; water indicates that a sample was suspended in water for 1 hr. at 5°; water, frozen and thawed, indicates that a sample was suspended in water and frozen and thawed three times; digitonin indicates that a sample was suspended in 1 % digitonin (Cooper & Lehninger, 1956) for 1 hr. at 5°. After these treatments the suspensions were centrifuged at 100 000 *g* for 30 min. and the carbonic-anhydrase activity was determined in the pellet and supernatant. Results represent values obtained from two experiments for each treatment. The activity of mitochondria after treatment represents the average of the duplicate experiments.

	Activity of supernatant (units)		Activity of mitochondria after
Treatment	Before treatment	After treatment	treatment (units)
Control	0	0	40
Water	0	0	40
Water, frozen and thawed	0	0	50
Digitonin	0	0	65

as reported by Booth (1938). In view of the complexity of rat blood with respect to carbonic-anhydrase activity, the technique of repeated perfusions through the dorsal aorta was introduced in an attempt to remove as completely as possible the contaminating blood. The perfusion procedure as described in the Methods section reduced tissue-carbonic-anhydrase activity to a constant level as determined by the observation that additional perfusions did not lower the enzymic activity in the various tissues. Furthermore, the report by Datta & Shepard (1959) that contaminating red cells survived the homogenization and were isolated with the particulate fraction of cells was confirmed. In our studies the few red blood cells which remained after perfusion were isolated with the nuclear fraction, as could be determined by observation of this pellet. The fact that the nuclear fraction contained only a small percentage of the total enzymic activity was taken as additional evidence for the completeness of the perfusion.

In addition to the problem of blood contamination, other technical problems common to all fractionation studies exist. The presence of carbonic anhydrase in both particulate and soluble fractions immediately raises two general considerations about the validity of such a distribution pattern. It is conceivable that the activity of the soluble fraction is a consequence of a leaching out or an elution of the enzyme from subcellular particles, and also the possibility exists that the activity associated with the particulate fraction is due to contamination by the soluble fraction.

Experiments were performed to test the possibility that the activity in the soluble fraction may be derived from particles. Such an elution phenomenon was reported for catalase, whose intracellular locus was ultimately restricted to particles, as shown by Greenfield & Price (1954) with the use of a sucrose–polyvinylpyrrolidone suspending medium. Various media have been employed by many investigators in attempts to preserve the morphological and biochemical integrity of isolated mitochondria.

The experiments cited in this paper include the fractionation of tissue in three different suspending media: (i) 0·25M-sucrose solution; (ii) 0·25M-sucrose solution–7·3% polyvinylpyrrolidone; (iii) 0·88M-sucrose solution. The results from such studies indicated that the distribution of enzymic activity between soluble and particulate fractions remained constant, regardless of the suspending medium. Therefore on the basis of these experiments it does not appear that the carbonic-anhydrase activity in the soluble fraction is derived from morphological or biochemical disturbances of isolated mitochondria. This conclusion was also reached by Datta & Shepard (1959).

The evidence which argues against the possibility that the enzymic activity associated with particulate matter is due to contamination rests on the observations that repeated washings of the particulate matter yielded decreasing activity in the washes. For practical purposes one washing yielded a particulate fraction with a constant activity. This fact tends to indicate that such contamination as exists is distinct from a more fixed enzymic activity. The same observation was reported by Datta & Shepard (1959), who maintained that 20–30% of the total activity in their preparations was associated with particulate matter. However, they attributed the presence of activity in particulate fractions to contamination, despite the fact that activity persisted even after repeated washings. No explanation was offered for the inability to wash out the activity.

Additional support for the presence of carbonic anhydrase in particles is provided by procedures designed to disrupt mitochondria and then to attempt physically to separate enzymic activity from mitochondrial residue. Such experiments demonstrated that the insoluble nature of the enzyme was not affected by treatment with water, by repeated freezing and thawing and by exposure to digitonin. Hence it is concluded that the enzyme is bound inextricably to the mitochondrial membrane, as has been reported for certain insoluble enzymes of the Krebs cycle (Hogeboom, 1955).

Another means of contaminating particles must also be considered; namely, the activity associated with the particulate fraction may be the result of an adsorption arising from a juxtaposition with the soluble enzyme as a consequence of homogenization. In this manner, microsomes have been shown to be capable of adsorbing ribonuclease (Schneider & Hogeboom, 1952), fumarase (Kuff, 1954) and haemoglobin (Paigen, 1956). Attempts to associate adsorption characteristics with mitochondria have not been successful (Schneider & Hogeboom, 1952; Beinert, 1951). From the experiments cited on carbonic anhydrase such a phenomenon cannot be ruled out.

The results of the experiments reported above corroborate our earlier observation of a bimodal intracellular distribution for carbonic anhydrase. The problem of the physiological role of this enzyme in such tissues as the brain still exists.

SUMMARY

1. Various tissues of the rat were studied by differential centrifuging for their intracellular distribution of carbonic anhydrase.

2. Cerebral cortex, kidney, liver and salivary glands appeared to contain enzymic activity in both particulate and soluble fractions. A majority

of the activity was associated with the soluble fraction.

3. The activity of the thyroid gland, however, was restricted to the soluble fraction.

4. More detailed fractionation studies of cerebral cortex and kidney indicated that the particulate matter associated with enzymic activity corresponded in general to the mitochondrial fraction.

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The Metabolism of Phenolic Antioxidants

2. THE METABOLISM OF BUTYLATED HYDROXYANISOLE IN THE RAT*

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Commercial 'butylated hydroxyanisole' consists of a mixture of 15% or less of 2-*tert.*-butyl-4-hydroxyanisole (isomer A) and 85% or more of 3-*tert.*-butyl-4-hydroxyanisole (isomer B). The introduction of butylated hydroxyanisole as a fat-stabilizer about 10 years ago, and its subsequent extensive use in edible fats, has been accompanied by studies of its acute and chronic toxicity (e.g. Graham, Teed & Grice, 1954). Wilder & Kraybill (1948), found none of the substance, other than questionable trace amounts in depot and carcass fat of rats receiving 3% of butylated hydroxyanisole in the diet over a prolonged period. Studies on the storage of the compound in dogs given daily doses of 0·3, 30 and 100 mg./kg. for more than a year (Hodge & Fassett, unpublished work) also failed to reveal any butylated hydroxyanisole in perirenal, omental or subcutaneous fat or in the brain, liver and kidney.

The metabolism of butylated hydroxyanisole has been studied in the rabbit by Dacre, Denz & Kennedy (1956), who found that an oral 0·5 g. dose was excreted as glucuronide (60%), ethereal sulphate (12%) and free phenols (4% of the dose). This dosage is very large compared with human intake. The ingestion of phenolic antioxidants as a result of their use in fat stabilization is probably less than 0·1 mg./kg. It is known that variations in dose may alter the proportions of metabolites excreted, where more than one metabolic pathway is available (cf. phenol; Bray, Thorpe & White, 1952). To justify extrapolation from experimental doses to the usage dose, it is desirable to study the pattern of metabolism with a variety of doses, including the lowest possible so that any unsuspected changes in metabolic pattern may be detected. This paper deals in this way with the metabolism in the rat of butylated hydroxyanisole and its component isomers.

* The paper by Astill, Fassett & Roudabush (1959) is to be regarded as Part 1.

METHODS AND MATERIALS

Animals, diets and dosage. Male and female albino rats of Sprague–Dawley strain, average wt. of 128 rats 321 g. (224–443 g.), were fed with Purina Chow and kept singly or