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Localization of kininogenase in the rat kidney

K. NUSTAD

Institute of Surgical Research, Rikshospitalet, Oslo 1, Norway

Summary

1. The rat kidney kininogenase (KGA) activity was located mainly in the kidney cortex.

2. Differential centrifugation of kidney cortex homogenate revealed that the microsomal fraction contained more KGA activity per unit of protein than the other subcellular fractions.

3. Subfractionation of the microsomal fraction showed that the KGA activity was recovered in a subfraction also containing high specific activity of both alkaline phosphate and glucose-6-phosphatase. It is suggested that the KGA activity is localized in the plasma membrane and/or endoplasmic reticulum membranes of kidney cortical cells.

4. Hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester at pH 8·5 paralleled the KGA activity.

Introduction

Kininogenase (KGA) activity has been found in rat kidney homogenates (Werle & Vogel, 1961 ; Carvalho & Diniz, 1966 ; Nustad, 1970). This activity has been found to be similar to rat urinary KGA activity and, for the most part, to be bound to particles which could be sedimented by centrifugation (Carvalho & Diniz, 1966 ; Nustad, 1970). It has also been shown that the ability of kidney and urinary fractions to hydrolyse *N*-α-benzoyl-L-arginine ethyl ester at pH 8·5 (BAEE esterase activity), paralleled the KGA activity (Nustad, 1970). In this study the localization of KGA and BAEE esterase activities in the kidney have been examined.

Preliminary communications covering part of this work were given at the F.E.B.S., 4th Meeting, Oslo, 1967, and at the Bayer Symposium on Plasma Kinins, Helsinki, 1969.

Methods

Adult male and female rats of a local strain were used in these experiments. Food was withheld for 20 h before the experiment. Water was supplied *ad lib.* In some experiments the kidneys were perfused with saline containing sodium citrate (0·3%) as reported in the preceding paper (Nustad, 1970). In most experiments perfusion was not used.

Preparation of cortical and medullary fractions

In preliminary experiments the kidney was separated into a cortical and a medullary fraction by dissection with curved scissors. Each kidney fraction was

weighed and then homogenized in 0·25 M sucrose containing 0·05 M Tris HCl, pH 7·5, with a Dounce homogenizer (five strokes with a loose fitting pestle and ten strokes with a tight fitting pestle). The homogenate was then adjusted to 1 g of wet weight in 5 ml of the suspension medium (1 : 5 homogenate).

In separate experiments the kidneys were separated into four zones which could be identified macroscopically: cortex, outer and inner stripe of outer medulla and papilla or inner medulla (Peter, 1909 ; Longley, 1969). The dissection was performed on 2 to 3 mm thick transverse sections of the kidneys by means of iris scissors. The homogenates were prepared as described above.

Preparation of subcellular fractions

Differential centrifugation was performed on kidney cortex homogenates. A 1 : 10 homogenate was prepared in 0·25 M sucrose containing 1×10^{-3}M EDTA, pH 7·2. The nuclear fraction (N) and the cytoplasmic extract fraction (E) were isolated as described by Wattiaux-de Coninck, Rutgeerts & Wattiaux (1965). Fractionation of the E fraction into a heavy mitochondrial fraction (M), a light mitochondrial fraction (L), a microsomal fraction (P) and a soluble fraction (S) was performed using centrifugations and washings as described by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). A Spinco Model L2 ultracentrifuge with No. 50 rotor was used. In some experiments the homogenate was separated into three fractions only. The first contained the N + M + L fractions, the second the P fraction and the third the S fraction.

Further fractionation of the microsomal fraction was carried out as described by Dallner (1963). The microsomal fraction in these experiments was either prepared according to Dallner (1963) or as described above. Using the Spinco ultracentrifuge with the SW50 L rotor three subfractions were isolated: a fraction which sedimented in presence of CsCl (1), a second which sedimented in presence of $MgCl_2$ (2), and a nonsedimentable fraction (3).

All procedures were carried out at 4° C.

Enzyme assays

KGA activity. In most experiments KGA activity was bioassayed after a gel filtration procedure which separated the KGA from the kininase activity. In some experiments the KGA activity was assayed after inhibition of the kininase activity as described in the preceding paper (Nustad, 1970).

BAEE esterase (pH 8·5 or pH 6·5 where stated), alkaline phosphatase (Alk. Pase), and kininase activities were determined as reported previously (Nustad, 1970). Glutamic dehydrogenase was assayed according to Beaufay, Bendall, Baudhuin & de Duve (1959), glucose-6-phosphatase (G-6-Pase) by the method of de Duve *et al.* (1955), acid phosphatase (Acid Pase) was determined in 0·25 M sucrose with a 10 min incubation period for free activity and after addition of 0·1% Triton X-100 for total activity as described by Wattiaux & de Duve (1956) and catalase by the method of Baudhuin, Beufay, Rahman-Li, Sellinger, Wattiaux, Jacques & de Duve (1964).

Proteins and ribonucleic acid. Proteins were assayed according to Lowry, Rosebrough, Farr & Randall (1951) using bovine serum albumin as a standard. Ribonucleic acid (RNA) was determined by the method of Mejbaum (1939) using yeast

RNA as a standard. The RNA extract was prepared according to the Schmidt & Thannhauser procedure (1945) as modified by Hutchison & Munro (1961).

Units of enzymatic activities. KGA activity: one unit was defined as the amount of enzyme causing the formation of kinins equivalent to 1 μg of bradykinin in 1 min under the conditions of the assay. Kininase activity: one unit was defined as the amount of enzyme which inactivated 1 μg bradykinin in 1 min under the assay conditions described. BAEE esterase, Alk. Pase, glutamic dehydrogenase, G-6-Pase, and Acid Pase: one unit was defined as the amount of enzyme causing the decomposition of 1 μmol of substrate per min under standard assay conditions. Catalase: one unit corresponded to the amount of enzyme causing the destruction of 90% of the substrate in 1 min in a volume of 50 ml under the assay conditions (Baudhuin *et al.*, 1964).

Reagents

See reagents listed in the preceding paper (Nustad, 1970). In addition: ribonucleic acid (RNA) from yeast, type XI (Sigma Chemical Company, St. Louis, Missouri, U.S.A.).

Results

Localization of activities in macroscopic fractions of the rat kidney

In preliminary experiments the kidneys were separated into a cortical and medullary fraction. The cortical fraction always contained most of the KGA activity. The total amount of this activity determined after the gel filtration procedure was not significantly different in perfused and nonperfused kidneys. The results of all these experiments are given in the first two columns in Fig. 1. The kininase activity (not shown) was about the same in the cortical and medullary fraction, 18·7 (range 12·5–21·1, $n=10$) and 19·8 (range 17·5–23·8, $n=4$) units per g wet weight, respectively. This distribution of kininase activity was most likely the reason why it was almost impossible to detect KGA activity in the medullary fraction before the gel filtration procedure, which separated the kininase activity from the KGA activity.

BAEE esterase, G-6-Pase and Alk. Pase activities were also localized mainly in the cortical fraction, whereas the Acid Pase activity showed less predominance in the cortical fraction (Fig. 1). The amount of Alk. Pase in the medullary fraction was markedly increased when no tissue from the outer medulla was included in the cortical fraction. The distribution of KGA, BAEE esterase and G-6-Pase activities was far less influenced by the separation procedure. This finding suggested that the three latter activities were located essentially in the cortex, whereas the Alk. Pase activity appeared to be present in the outer part of the medulla, too. To test this possibility the cortex, outer and inner stripe of outer medulla and inner medulla were dissected from the kidneys. About 70% of the kidney wet weight was recovered in these four fractions, the distribution being (g wet weight/rat): cortex 0·79, outer stripe 0·25, inner stripe 0·11 and inner medulla 0·04. The corresponding protein content was (mg/g wet weight): 189, 166, 126 and 86, respectively. All the above values are means of at least two experiments.

The distribution of enzymatic activities in the four fractions is shown in Fig. 1. The KGA activity was localized mainly in the cortical zone and so was the BAEE

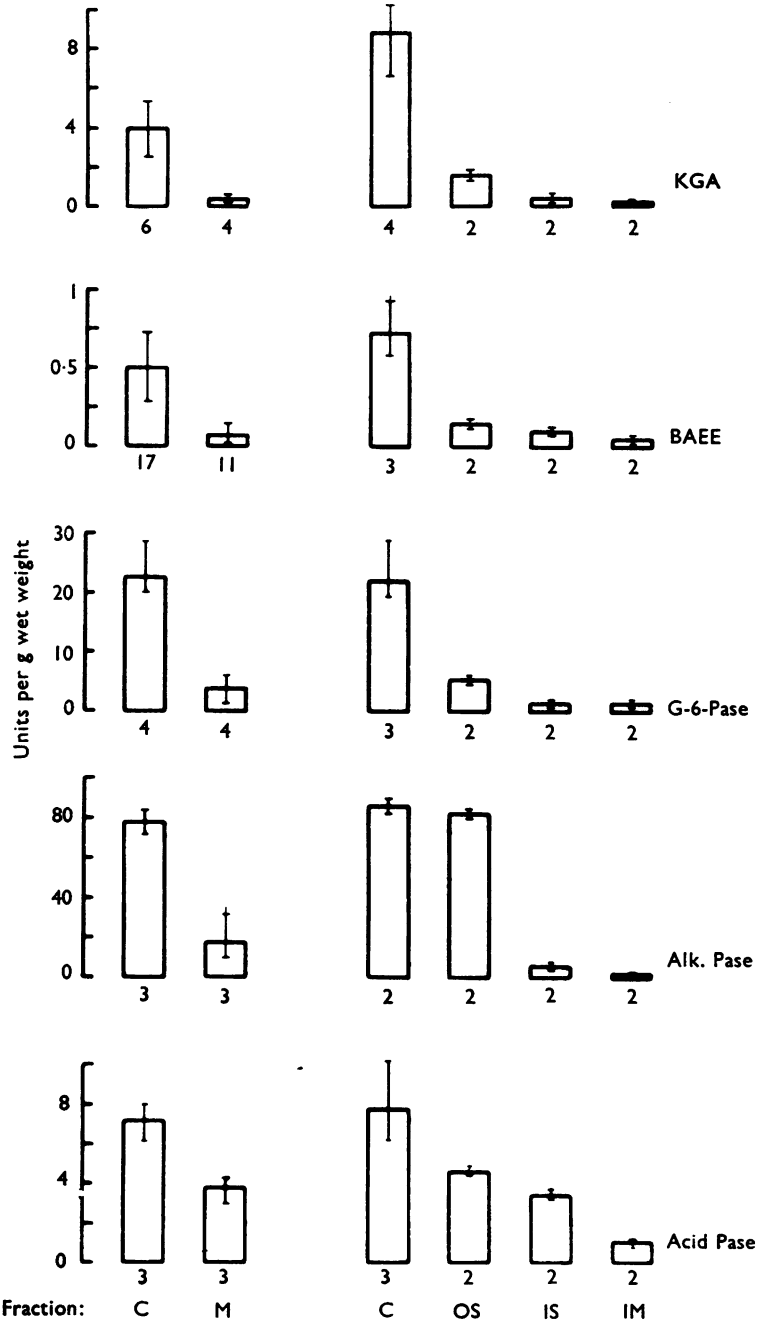

FIG. 1. Localization of activities in macroscopic fractions of the kidneys. The two first columns show the enzymatic activities found in the cortex (C) and medulla (M) after separation of the kidney into two fractions only. The following four columns show activities in the cortex (C), outer stripe (OS) and inner stripe (IS) of outer medulla, and inner medulla (IM), respectively. All values are given as means with absolute range indicated. The number of experiments is given below each column. KGA=kininogenase; BAEE=*N*-α-benzoyl-L-arginine ethyl ester hydrolysis; G-6-Pase=glucose-6-phosphatase; Alk. Pase=alkaline phosphatase; and Acid Pase=acid phosphatase.

esterase and G-6-Pase activities. A similar distribution of G-6-Pase was found by Jörgensen (1968) after removal of samples from the middle of each of the four zones. Alk. Pase activity was distributed equally between the two outer zones. High activity in the outer stripe of the outer medulla has previously been demonstrated for Alk. Pase after microdissection of the kidney by Bonting, Pollak, Muehrcke & Kark (1960). Acid Pase showed a third pattern of distribution between the different zones. A similar distribution has been reported for Acid Pase activity using a different dissection method (Rosen, Coughlan & Barry, 1966).

Localization of activities in subcellular fractions of the kidney

When kidney cortex homogenates were separated into subcellular fractions, the distribution of reference enzyme, glutamic dehydrogenase, catalase, Acid Pase and G-6-Pase, was essentially that reported by Wattiaux-de Coninck *et al.* (1965) (Table 1). On the average 34% of the Acid Pase was recovered as free activity. This indicated that 66% of the particles known as lysosomes were intact after the homogenization and centrifugation procedure described. Less satisfactory results were obtained using a Potter-Elvehjem homogenizer.

KGA activity tested in the presence of kininase inhibitors showed that the microsomal fraction (P) contained more KGA activity per unit protein than did the other subcellular fractions (Table 1). A similar distribution was found for the BAEE esterase activity. Thereafter the fractions isolated before the microsomal fraction (P) were isolated as a combined nuclear-mitochondrial fraction (N+M+L), whereas the microsomal fraction (P) and the soluble fraction (S) were separated as before. KGA activity in these three fractions was then determined after the gel filtration procedure. The extract from the N+M+L fraction and that from the P fraction were gel filtered at the same time on two identical Sephadex G-100 columns and assayed on the same rat uterus preparation. On the average 43% of the KGA activity was found in the microsomal fraction (P), whereas about 10% of the proteins was recovered in this fraction. The relative distribution was the same regardless of whether the KGA activity was determined after the gel filtration procedure or after inhibition of the kininase activity. The total KGA activity, however, was higher in those experiments where the activity was determined after gel filtration of the fractions. The data obtained in these experiments were pooled with those obtained after complete differential centrifugation, Table 1, and are presented as histograms in the manner proposed by de Duve *et al.* (1955) in Fig. 2. The distribution of BAEE esterase, G-6-Pase and Alk. Pase activities resembled that of the KGA activity, whereas the distribution of Acid Pase, glutamic dehydrogenase and catalase activities was different from that of the KGA activity.

Data in the preceding paper indicate that the kidney BAEE esterase activity consisted of at least two enzymes: one potent, but unstable esterase with pH optimum about 6·5, and a less potent, stable esterase with alkaline pH optimum, which could be due to the KGA (Nustad, 1970). BAEE esterase activity (pH 6·5) was therefore assayed in freshly prepared subcellular fractions. In the nuclear-mitochondrial fraction (N+M+L) and in the supernatant fraction (S) the BAEE esterase activity at pH 6·5 was 2 to 6 times higher than the esterase activity at pH 8·5. The microsomal fraction (P), however, showed less BAEE esterase activity at pH 6·5 than at pH 8·5.

TABLE 1. *Localization of activities in subcellular fractions of the kidney*

	No. of experiments	Absolute values E+N	Percentage values						
			E+N	N	M	L	P	S	Recovery
Proteins	4	193±22	100	31·8±7·4	20·5±2·5	2·6±0·6	9·3±1·8	31·2±3·4	95·4±4·0
Kininogenase (KGA)	4	1·8±0·4	100	11·6±7·8	28·3±13·5	3·8±2·0	61·5±24·5	6·4±3·3	112±18
BAEE esterase	3	0·45±0·08	100	15·9±5·1	20·3±6·9	4·5±2·8	40·4±8·2	11·9±0·7	92·9±14·8
Glucose-6-phosphatase	4	20·3±4·4	100	28·3±7·8	14·7±3·6	7·8±4·7	43·2±15·5	2·2 ±0·8	96·2±26
Acid phosphatase (total activity)	3	5·9±0·1	100	38·8±7·0	26·7±4·6	6·4±1·1	16·3±1·3	14·4±4·7	103±5
Acid phosphatase (free activity)	3	2·0±0·2	34						
Glutamic dehydrogenase	3	1·2±0·4	100	36·9±12·1	45·7±5·4	0·7±0·3	1·7±1·5	—	85·0±11·5
Catalase	3	15·5±3·5	100	37·5±9·9	22·3±5·5	3·3±0·2	2·6±0·4	21·8±4·1	87·5±0·4

Absolute values for proteins are given in mg/g wet weight of kidney cortex; for enzymatic activities, in units/g wet weight. The results are given as mean±S.D. In these experiments kininogenase activity was determined after adding kininase inhibitors to the fractions (see **Methods**). E=cytoplasmic extract fraction, N= nuclear fraction, M=heavy mitochondrial fraction, L=light mitochondrial fraction, P=microsomal fraction and S=soluble fraction.

Localization of activities in subfractions of the microsomal fraction

In a separate set of experiments the microsomal fraction (P) was further fractionated, using the method which Dallner (1963) used to separate a liver microsomal fraction into rough and smooth membranes. A similar separation of the kidney microsomal fraction, as judged by the distribution of RNA, was not obtained (Fig. 3). Most of the KGA activity, tested after gel filtration of the fractions as

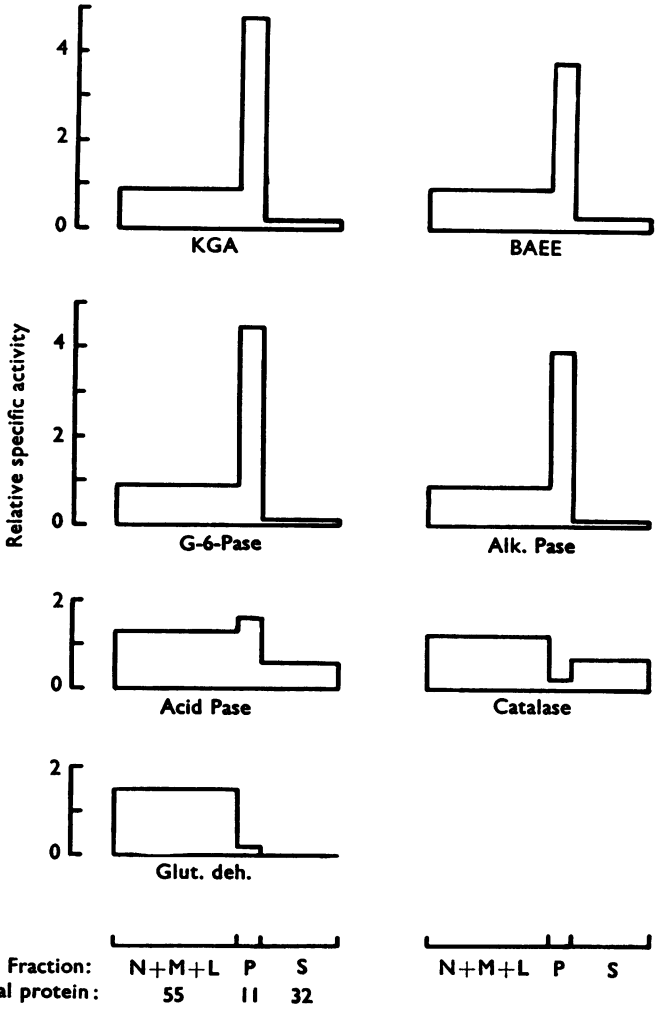

FIG. 2. Distribution pattern of activities in subcellular fractions of the kidney. The results obtained after separation of the kidney homogenate into five fractions (N, M, L, P and S) and those obtained after separation of the homogenate into three fractions (N+M+L, P and S) are pooled. Ordinate: mean relative specific activity of fractions (% of total recovered activity/% of total recovered protein). Abscissa: relative protein content of fractions. Total recovered activities, units/g wet weight of kidney cortex, are given below as mean ± S.D. with number of experiments in parentheses. Proteins are given as mg/g wet weight. Kininogenase (KGA), 2 ± 0·2(4) assayed in the presence of kininase inhibitors, and 6·6 ± 1·2(3) assayed after gel filtration. BAEE esterase (BAEE), 0·45 ± 0·08(5); glucose-6-phosphatase (G-6-Pase), 19·3 ± 3(7); alkaline phosphatase (Alk. Pase), 69·6 ± 14(5); acid phosphatase (Acid Pase), 6·4 ± 1(6); catalase, 13·6 ± 3·1(3); glutamic dehydrogenase (Glut. deh.), 1 ± 0·3(3); and proteins, 179 ± 22(7).

well as with kininase inhibitors, was separated from those membranes which contained the highest specific activity of Acid Pase (subfraction 3, Fig. 3) and from some of the membranes containing high activity of G-6-Pase and Alk. Pase (subfraction 1). The main KGA fraction (subfraction 2), however, also contained high specific activity of both G-6-Pase and Alk. Pase. These results are summarized in Fig. 3. The absolute values found are given in the legend of Fig. 3. The microsomal fraction prepared according to Dallner (1963) contained 12 to 23% less KGA, BAEE esterase, G-6-Pase and Alk. Pase activities than the microsomal fraction obtained by the procedure of de Duve *et al.* (1955), most likely because the latter procedure included washings of the premicrosomal fraction.

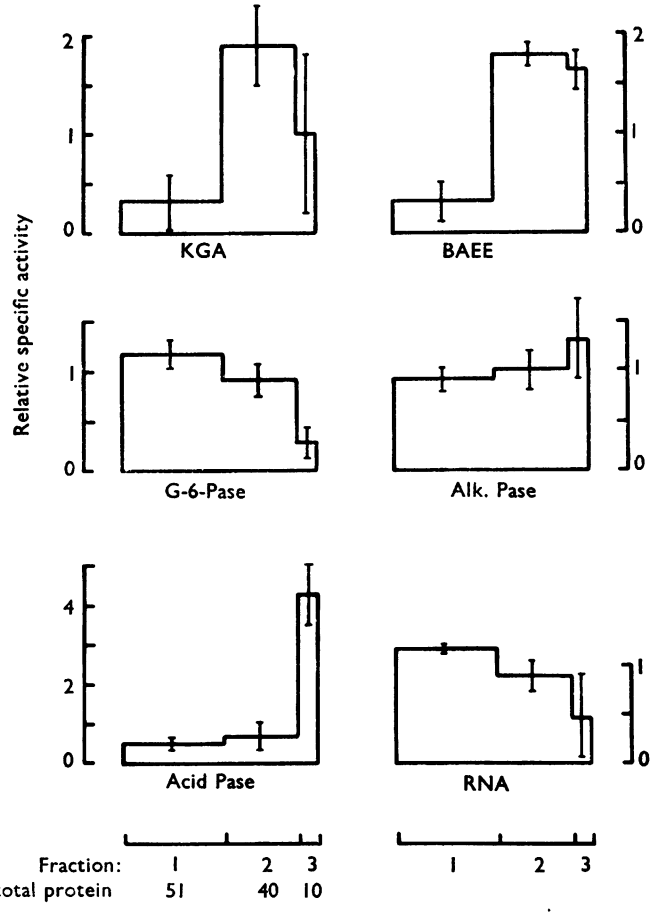

FIG. 3. Distribution pattern of activities in subfractions of the microsomal fraction. Ordinate: mean relative specific activity of fractions ± S.D. (% of total recovered activity/% of total recovered proteins). Abscissa: relative protein content of fractions. Total recovered activities in the microsomal subfractions are given below as units/g wet weight for enzymes and as mg/g wet weight for proteins and ribonucleic acids. Values are given as mean ± S.D. with number of experiments in parentheses. Kininogenase (KGA), 0·8 ± 0·3(3) assayed in the presence of kininase inhibitors, and 4·1 ± 1·1(3) assayed after gel filtration. BAEE esterase (BAEE), 0·19 ± 0·05(4); glucose-6-phosphatase (G-6-Pase), 8·1 ± 3·9(6); alkaline phosphatase (Alk. Pase), 27·7 ± 9·5(3); acid phosphatase (Acid Pase), 0·9 ± 0·5(5); ribonucleic acid (RNA), 1·4 ± 0·1(3); and proteins 14·6 ± 6·3(6).

Discussion

The studies in this paper show that rat kidney kininogenase (KGA) activity is localized primarily in the kidney cortex with little activity in the medulla. Experiments by Abe (1965) and Werle, Leysath & Schmal (1968) have indicated that kinins normally are formed in the kidney. The function of KGA in the kidney might be to form kinins in the kidney cortex and thus participate in local regulation of blood flow or cellular or vascular permeability as suggested by Frey, Kraut, Werle, Vogel, Zickgraf-Rüdel & Trautschold (1968).

The subcellular localization of KGA has been studied and compared with that of marker enzymes. These marker enzymes are found to belong to a single intracellular component in the living cell and are distributed homogeneously through this component. The activity of marker enzymes in a given fraction of a tissue homogenate gives a measurement of the purity or heterogeneity of that fraction (de Duve, 1964). In the present study on kidney subcellular fractions glutamic dehydrogenase serves as a marker of mitochondria ; catalase, as a marker of particles referred to as peroxisomes ; Acid Pase, as a marker of lysosomes ; and G-6-Pase, as a marker of endoplasmic reticulum membranes. All these marker enzymes are used by Wattiaux-de Coninck *et al.* (1965) in a differential and gradient centrifugation study on kidney homogenates. Their homogenization and differential centrifugation procedures were followed in the present studies. After the initial localization of KGA in the microsomal fraction (Table 1), a marker enzyme of the plasma membrane was included. The plasma membrane is one of the major constituents of the microsomal fraction prepared from kidney homogenates as opposed to the exclusive endoplasmic reticulum membrane composition of the liver microsomal fraction (Dallner & Ernester, 1968). Alk. Pase was chosen as a plasma membrane marker enzyme because it is found in plasma membranes in kidney cells (Goldfischer, Essner & Novikoff, 1964) and in purified kidney plasma membrane preparations (Coleman & Finean, 1966). Both G-6-Pase and Alk. Pase were first found to be recovered mainly in the microsomal fraction of kidney homogenates by Hers, Berthet, Berthet & de Duve (1951).

Maximal specific activity of KGA was not found in the microsomal fraction of kidney homogenates by Carvalho & Dinix (1966), who used the same centrifugation scheme as used in the present study. In their study the light mitochondrial fraction (L) had the highest specific activity of KGA, indicating to them a lysosomal localization of the enzyme. However, our studies show that the L fraction of kidney homogenates is a mixed heavy microsomal-light lysosomal fraction. Thus the L fraction of kidney homogenates prepared by Wattiaux-de Coninck *et al.* (1965) and in the present study (Table 1) contains high specific activity of G-6-Pase (marker of endoplasmic reticulum membranes). Unlike the liver L fraction the kidney L fraction shows relatively low specific activity of acid hydrolases (marker of lysosomes). In fact, of six acid hydrolases assayed in kidney fractions by Wattiaux-de Coninck *et al.* (1965) only three showed a small peak of specific activity in the L fraction, whereas the other three showed higher specific activity in the nuclear or heavy mitochondrial fraction. This indicated that most of the kidney lysosomes are heavier and more heterogeneous both in centrifugation behaviour and content of various acid hydrolases than are liver lysosomes (Wattiaux-de Coninck *et al.*, 1965). In their study Carvalho & Diniz (1966) found about the same specific activity of Acid Pase in the heavy (M) and the light (L)

mitochondrial fractions, whereas KGA shows a much higher specific activity in L than in M. Our suggestion would be that the KGA activity in the L fraction is bound to the microsomal component present.

The question remains, however, why Carvalho & Diniz (1966) did not find even higher KGA activity in the microsomal fraction as was found in the present study. Carvalho & Diniz (1966) tested KGA activity in the presence of some kininase activity. The latter is localized in the microsomal fraction (Erdös & Yang, 1966) and would thus primarily affect the KGA value of that fraction. In addition, the total amount of membrane broken up into microsomal size is dependent on the homogenization procedure and the pretreatment of the animals. We have observed that the microsomal amount of KGA could be increased markedly by simply increasing the homogenization force. A concomitant increase in microsomal G-6-Pase and Alk. Pase activities was observed (Nustad & Rubin, to be published). The use of non-starved animals increases the amount of microsomal membranes in the pre-microsomal fractions of liver homogenates (Dallner & Ernester, 1968); Carvalho & Diniz (1966) seem to have used non-starved animals. A combination of the factors mentioned above most likely explains why Carvalho & Diniz (1966) found relatively little KGA activity in the microsomal fraction.

Having established the microsomal localization of the KGA, an attempt was made to determine which of the microsomal components contained the KGA activity. Subfractionation of the microsomal fraction showed that most of the KGA activity was recovered in the second microsomal subfraction, whereas a variable amount of KGA was found in subfraction three and only little activity in subfraction one (Fig. 3). The lower values of subfraction three were found when the KGA activity was determined after gel filtration of the fraction and are therefore considered to be more reliable. In subfraction two KGA showed a specific activity of 9·15 times that of the whole homogenate. The specific activity of G-6-Pase and Alk. Pase in this fraction were 4·10 and 3·96 times that of the whole homogenate, whereas the corresponding value for Acid Pase was 0·96. In the total microsomal fraction the values for catalase and glutamic dehydrogenase were as low as 0·24 and 0·16 respectively. It seems reasonable to assume that KGA is located in the same membranes as G-6-Pase and/or Alk. Pase, that is, in the endoplasmic reticulum membranes and/or the plasma membrane (Goldfischer *et al.*, 1964). The relative absence of KGA from microsomal subfraction one, which is as rich in G-6-Pase and Alk. Pase as subfraction two, does not conform with the homogeneous distribution of enzyme activity throughout a given membrane (de Duve, 1964). A possible explanation would be that KGA is found only in a certain type of those cells which contain G-6-Pase and Alk. Pase in their membranes. By histochemical methods G-6-Pase and Alk. Pase are found to be primarily located in proximal tubular cells of the nephron (Wachstein, 1955), though a much wider distribution is found by microdissection technique (Bonting *et al.*, 1960; Mattenheimer, 1968). There is ample room for speculation as to the cellular localization of the KGA. Both the subcellular as well as the cortical localization of KGA are consistent with a localization of the KGA activity in the proximal convolute part of the nephron because this part makes up 85% of the cortical tissue (Mattenheimer, 1968) and accounts for most of the cortical G-6-Pase and Alk. Pase rich membranes as discussed above. This localization would fit well with the finding of Werle & Vogel

(1960) that injections of drugs which destroy tubular cells reduced the excretion of KGA activity into urine far below the normal level.

In all the fractions assayed, high KGA activity was found in the same fractions which showed high BAEE esterase activity (pH 8·5). This finding suggests that a major part of the BAEE esterase present could be due to KGA activity. It has previously been demonstrated that KGA from human urine retains its esterase activity during a 1,000-fold purification (Moriya, Pierce & Webster, 1963).

Different subcellular localization of BAEE esterase activity at pH 6·5 and KGA activity indicated that this esterase activity was not due to the KGA.

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