1973. Moreover, since the kinetics of the heat production following the initial transient heat production after the mixing of nucleotide and HMM were very reliable in our experiments, we maintain that our previous conclusion (Yamada et al., 1973) is valid that a large amount of heat is produced on the decomposition of M·ADP·P_i to M·ADP + P_i. These conclusions have been well substantiated recently by Kodama and his associates as described above.

Acknowledgments

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Relationship between Negative Cooperativity and Insulin Action†

Jerrold M. Olefsky,* Allan Green, Theodore P. Ciaraldi,‡ Mark Saekow, Arthur H. Rubenstein,§ and Howard S. Tager‖

ABSTRACT: We have compared the respective abilities of porcine insulin and [Leu^{B25}]insulin to enhance the rate of dissociation of receptor-bound [^{125}I]insulin from IM-9 lymphocytes and isolated rat adipocytes and to increase the rate of adipocyte glucose transport and oxidation. Although porcine insulin (100 ng/mL) greatly enhanced the dissociation rate of previously bound [^{125}I]insulin, [Leu^{B25}]insulin (at a concentration yielding equivalent receptor occupancy) had no effect. Nevertheless, the analogue fully stimulated adipocyte glucose transport and oxidation at concentrations consistent with its reduced (1.7% of normal) intrinsic binding affinity. Activation of glucose transport by the analogue was rapid, and the corresponding rate of activation was indistinguishable from that produced by native insulin. The increased dissociation rate observed with increasing receptor occupancy by native porcine insulin has been interpreted as evidence for negative cooperative site–site interactions between occupied receptors. According to this formulation, [Leu^{B25}]insulin is a "noncooperative" insulin analogue. Since this [Leu^{B25}]insulin retains full biologic activity, the enhancement of the insulin dissociation rate at high levels of receptor occupancy does not reflect a phenomenon inherent in insulin's action to augment glucose metabolism.

Most workers find that when insulin binding to receptors is studied over a wide range of insulin concentrations, Scatchard analysis of the resulting data yields curvilinear plots (DeMeyts et al., 1976a; Ginsberg, 1977). DeMeyts et al. (1973) have also demonstrated that when receptor occupancy is increased with unlabeled insulin the dissociation rate of previously bound [^{125}I]insulin is greatly increased and have suggested that this provides evidence for negatively cooperative receptor interactions. This interpretation could at least partly explain the characteristic curvilinear Scatchard plots of insulin binding. While some workers have failed to detect this phenomenon (Gliemann et al., 1975), and others have questioned its interpretation (Pollet et al., 1977), numerous studies have confirmed the original observations of DeMeyts et al. (1973, 1976a) in a variety of receptor systems (DeMeyts et al., 1976b).

Studies of chemically and enzymatically modified insulins have suggested that residues important for enhancing the rate of receptor-bound [^{125}I]insulin dissociation (notably A21 and

† From the Department of Medicine, University of Colorado School of Medicine, Denver, Colorado 80262, and the Departments of Medicine and Biochemistry, University of Chicago School of Medicine, Chicago, Illinois 60637. *Received December 29*, 1980. This work was supported by Grants AM 25241, AM 25242, AM 18347, AM 13941, and AM 20595 from the National Institute of Arthritis, Metabolism and Digestive Diseases of the National Institutes of Health and by funds from the Veterans Administration.

* Correspondence should be addressed to this author at the Department of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262.
‡ During the course of this work, T.P.C. was a recipient of a National Institutes of Health postdoctoral fellowship award.
§ A.H.R. is an Established Investigator of the American Diabetes Association.
‖ H.S.T. is the recipient of Research Career Development Award AM 00145 from the National Institutes of Health.

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B23–B26) represent a subset of those required for insulin binding and activity (DeMeyts et al., 1978). In addition, it has been proposed that insulin-induced negative cooperative interactions are involved in the initiation of insulin action (DeMeyts et al., 1976a, 1980). We have used the insulin analogue [Leu^{B25}]insulin (Tager et al., 1980) to examine the relationship between insulin's ability to augment the receptor-bound [^{125}I]insulin dissociation rate and insulin's action on glucose metabolism in rat adipocytes. The results demonstrate that the analogue retains full activity as an insulin agonist but has lost the ability to enhance insulin dissociation.

Experimental Procedures

Materials. Porcine insulin was a generous gift of Dr. Ronald Chance of Eli Lilly and Co. (Indianapolis, IN). Bovine serum albumin (BSA) (fraction V) was purchased from Armour Pharmaceutical Co. (Phoenix, AZ), collagenase was from Worthington Biochemical Corp. (Freehold, NJ), Na^{125}I, L-[^{14}C]glucose, L-[1-^{3}H]glucose, and 3-*O*-methyl[U-^{14}C]glucose were from New England Nuclear Corp. (Boston, MA), and 2-deoxy-D-[1-^{3}H]glucose and D-[1-^{14}C]glucose were from Amersham Corp. (Arlington Heights, IL).

Preparation of Isolated Adipocytes. Animals were stunned by a blow to the head and decapitated, and epididymal fat pads were removed. Isolated fat cells were prepared by shaking at 37 °C for 60 min in Krebs–Ringer bicarbonate buffer containing collagenase (3 mg/mL) and BSA (40 mg/mL) according to the method of Rodbell (1964). Cells were filtered through 250-μm nylon mesh, centrifuged at 400 rpm for 3 min, and washed 3 times in the appropriate buffer.

2-Deoxyglucose Uptake Studies. 2-Deoxyglucose uptake was measured by using the same cell centrifugation technique as described for the binding studies, and the details of this method have been previously reported (Olefsky, 1978). Cells were preincubated with or without insulin in a buffer consisting of 120 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.4 mM sodium acetate, 35 mM Tris, and 10 mg/mL BSA, pH 7.4, for 60 min at 24 °C. At the end of this period, 2-deoxy-D-[1-^{3}H]glucose (specific activity 1.6 mCi/mmol) was added at a concentration of 0.1 mM. The assays were terminated 3 min later by transferring 200-μL samples from the assay mixture to plastic microtubes containing 100 μL of silicone oil. The tubes were centrifuged for 30 s in a Beckman microfuge, and the assay was considered terminated when centrifugation began. The amount of sugar trapped in the extracellular water space of the cell layers was determined in each experiment with L-[1-^{3}H]glucose, and all data of sugar uptake are corrected for this factor. The oxidation of D-[1^{14}C]glucose to $^{14}CO_2$ was measured by the method of Rodbell (1964).

3-O-Methylglucose Transport. 3-*O*-Methylglucose transport was assayed by a previously described method (Ciaraldi & Olefsky, 1979; Siegel & Olefsky, 1980). The substrate [3-*O*-methyl[U-^{14}C]glucose (0.4 μCi)] was incubated in a 20-μL volume in 17 × 100 mm plastic test tubes. The reaction was started by the rapid addition of 50 μL of a concentrated cell suspension containing (1–2) × 10^5 cells in a modified Krebs–Ringer phosphate buffer (KRP) consisting of 128 mM NaCl, 5.2 mM KCl, 0.15 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.3 mM KH_2PO_4, 10.0 mM Na_2HPO_4, and 50 mg/mL BSA, pH 7.4. The transport reaction was terminated after 4 s by the addition of 11 mL of iced buffer containing 0.3 mM phloretin. Control studies demonstrated that phloretin completely inhibits the transport process for at least 8 min. Approximately 2.0 mL of silicone oil was layered over the diluted cell suspension, and the tubes were rapidly centrifuged (2000*g* for 15 s) in a Hereaus labofuge. The cells coalesce on the surace of the oil and may be collected by sweeping the oil surface with absorptive material (such as a small piece of pipe cleaner). The adsorptive material and collected cells are then added to ACS (aqueous liquid scintillant, Amersham Corp.) liquid scintillation cocktail for determination of radioactivity. For the correction of extracellular trapping of water, parallel reactions were carried out in which L-[^{14}C]glucose uptake was determined; L-glucose uptake was subtracted from the value for 3-*O*-methylglucose uptake. The extracellular water space in the cell pellet was always less than 5% of the intracellular water space.

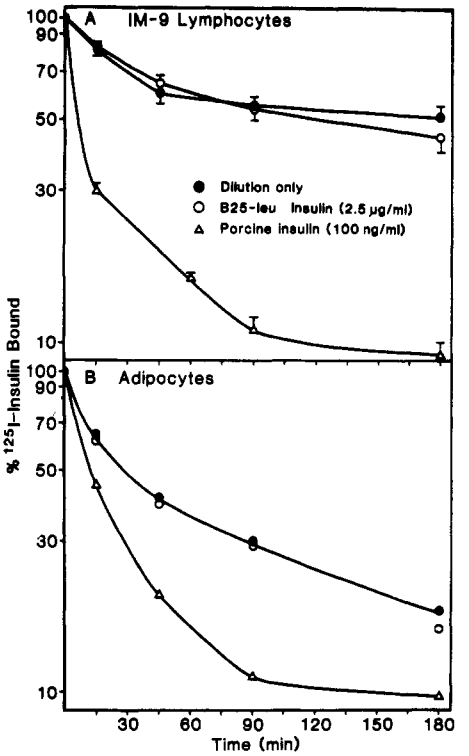

FIGURE 1: Dissociation of [^{125}I]insulin at 24 °C into insulin-free buffer (●), buffer containing 100 ng/mL unlabeled porcine insulin (△), or buffer containing 2.5 μg/mL [Leu^{B25}]insulin (○). Studies were performed with cultured IM-9 lymphocytes (A) or isolated rat adipocytes (B).

Dissociation Studies. The technique for assessing insulin dissociation has been previously described in detail (Olefsky & Chang, 1978). Adipocytes were allowed to associate with [^{125}I]insulin for 90 min in a total volume of 3 mL at 24 °C. The cells were then centrifuged (2000 rpm for 1 min at 4 °C), and the buffer was removed and replaced with an equal amount of iced, insulin-free buffer. The cells were then distributed in 100-μL aliquots into tubes containing 1.9 mL of buffer at 24 °C. The cells in these tubes were then centrifuged through 0.5 mL of silicone oil at the indicated time points, and the infranates were removed. The radioactivity remaining in the cell layer was determined directly in these tubes. The amount bound at time zero is the amount of specific binding in a 100-μL aliquot of the cell suspension obtained just as the aliquots were distributed into the dissociation tubes. Degradation of insulin by adipocytes is minimal (<5% degraded at 90 min) under these conditions. Dissociation studies using IM-9 cultured lymphocytes were performed according to previously described methods (DeMeyts et al., 1973, 1976a).

Semisynthesis of [Leu^{B25}]Insulin. The insulin analogue was prepared by the trypsin-catalyzed coupling of bis(*tert*-butyloxycarbonyl)desoctapeptide insulin to the synthetic protected octapeptide Gly-Phe-Leu-Tyr-Thr-Pro-trifluoroacetyl-Lys-Ala. Details of the semisynthesis and characterization of the product

Table I: Comparative Binding and Biologic Potencies of Porcine and [Leu^{B25}]Insulin

	% bound[a]	relative binding affinity (%)	half-maximal concn (ng/mL)[b]	relative biologic potency (%)
porcine insulin	41 ± 5	100	0.20	100
[Leu^{B25}]-insulin	1.2 ± 0.09	1.7 ± 0.14	10.1	2.0 ± 0.21

[a] Binding studies were conducted with ^{125}I-labeled preparations of both insulins at 10^{-10} M using IM-9 cultured lymphocytes (2×10^6 cells/mL). Under these conditions, the mean (±SE) of ^{125}I-labeled porcine insulin bound was 41 ± 5%. Although the absolute affinity constant cannot be determined by this approach, the relative binding affinities of different insulin species can be assessed. [b] Biologic potency was determined by comparing the hormone concentrations which elicit half-maximal stimulation of glucose oxidation (Figure 2).

have been described (Tager et al., 1980).

Results

Comparisons of the ability of native porcine insulin and [Leu^{B25}]insulin to enhance the dissociation rate of previously bound [^{125}I]insulin were performed with both IM-9 lymphocytes and isolated adipocytes. Cultured IM-9 lymphocytes were allowed to reach steady-state binding (90 min) at 24 °C with a tracer concentration (10^{-10} M) of ^{125}I-labeled porcine insulin. At this point, the cells were washed, and dissociation of the previously bound [^{125}I]insulin was studied in insulin-free buffer, or in buffer containing unlabeled porcine insulin or [Leu^{B25}]insulin (Figure 1A). Dissociation of [^{125}I]insulin was faster in the presence of 100 ng/mL porcine insulin as compared to insulin-free buffer. Thus, the rate of insulin dissociation is enhanced when fractional receptor occupancy is high, a finding consistent with negatively cooperative receptor interactions and with the reports by DeMeyts et al. (1973, 1976a).

The synthetic insulin analogue [Leu^{B25}]insulin has a binding potency of 1.7% as determined by the relative binding potency of ^{125}I-labeled porcine vs. ^{125}I-labeled [Leu^{B25}]insulin. Thus, at tracer hormone concentrations, $B/F = K_A \times$ receptor number,[1] and under these conditions, [Leu^{B25}]insulin binding to IM-9 lymphocytes demonstrated a relative binding affinity of 1.7 ± 0.14% (Table I). Consequently, in order to study ^{125}I-labeled porcine insulin dissociation under conditions in which a high proportion of the receptors were occupied by the analogue, the rate of dissociation of receptor-bound [^{125}I]-insulin was determined in medium containing 2.5 μg/mL [Leu^{B25}]insulin. At these concentrations, the average fractional receptor occupancy for pork insulin was 75% and for [Leu^{B25}]insulin 61% as calculated from Scatchard plots using mixtures of iodinated and unlabeled pork and [Leu^{B25}]insulin, respectively. It should be noted that the negative cooperative effect is maximal at a fractional receptor occupancy of 10–20% (DeMeyts et al., 1976a; Olefsky & Chang, 1978). The results shown in Figure 1A indicate that the analogue fails to enhance the rate of dissociation of [^{125}I]insulin. Identical results were obtained when dissociation experiments were conducted with isolated rat adipocytes (Figure 1B).

An assessment of the ability of [Leu^{B25}]insulin to stimulate 2-deoxyglucose uptake by rat adipocytes showed that the analogue has 2% of the biologic activity of porcine insulin (Figure 2A). Nevertheless, at high concentrations (≥100 ng/mL), [Leu^{B25}]insulin is fully able to maximally stimulate the transport process in a manner identical with that of native insulin. Comparable findings, as they relate to both half-maximal and maximal stimulation of the insulin-sensitive process, were obtained when measurements of [1-^{14}C]glucose oxidation by isolated adipocytes were made (Figure 2B and Table I).

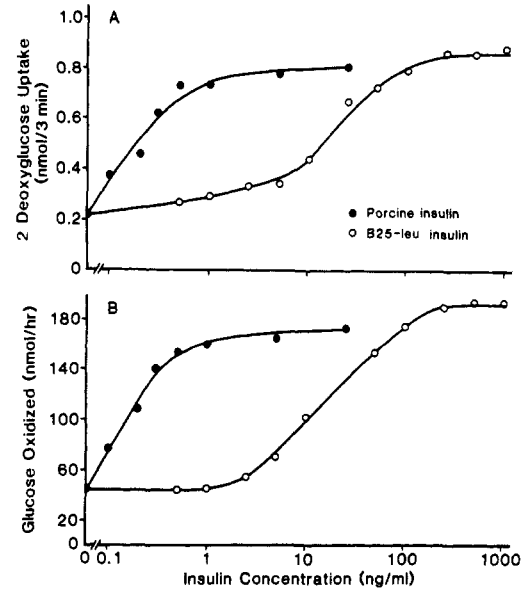

FIGURE 2: Ability of porcine insulin (●) and [Leu^{B25}]insulin (○) to stimulate 2-deoxyglucose uptake (A) or [1-^{14}C]glucose oxidation (B). In the glucose oxidation studies, incubations were performed at 37 °C for 60 min at a glucose concentration of 2 mM. For 2-deoxyglucose uptake, cells were incubated with or without insulin for 60 min at 24 °C; following this, 2-deoxyglucose was added (0.1 mM), and uptake was measured at the end of a 3-min incubation.

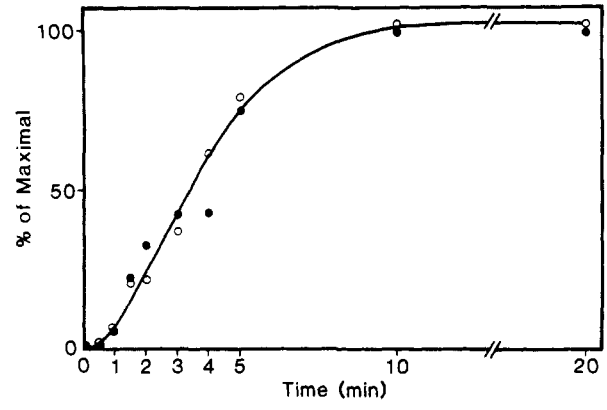

FIGURE 3: Time course of the effect of porcine insulin (●) or [Leu^{B25}]insulin (○) on the rate of 3-*O*-methylglucose transport. Insulin was added in a 10-μL volume at time zero to achieve a final insulin concentration of 100 ng/mL porcine insulin or 2.5 μg/mL [Leu^{B25}]insulin. After the indicated periods, aliquots of the cells were removed and transferred to separate tubes for measurement of the rate of 3-*O*-methylglucose transport over a 4-s assay period as described under Experimental Procedures. The 3-*O*-methylglucose concentration was 14 μM, and studies were performed at 37 °C. Data are expressed as the percentage of the maximal insulin effect, and in these experiments, the maximal effect represented an increase of 830 ± 56% above the basal transport rate, which was 0.12 pmol (2×10^5 cells)$^{-1}$ 4 s^{-1}.

[1] For the reaction

$$[H] + [R] \underset{k_d}{\overset{k_a}{\rightleftharpoons}} [HR]$$

[H] equals the free insulin concentration, [R] equals the free receptor concentration, and [HR] equals the concentration of occupied insulin receptors. Then the equilibrium affinity constant $K_A = [HR]/([H][R])$ and $K_A[R] = [HR]/[H]$. At tracer hormone concentration, fractional receptor occupancy is negligible, [R] can be taken as total receptor concentration, and [HR]/[H], or the bound/free ratio, is equal to the $K_A \times$ receptor number.

The data in Figure 2 demonstrate that [Leu^{B25}]insulin ultimately expresses full agonist properties. However, these results do not elucidate the time course of activation of glucose metabolism induced by the analogue. Therefore, to assess the rapidity at which [Leu^{B25}]insulin stimulates glucose transport and to compare the activation kinetics of this analogue to that induced by native porcine insulin, we performed the experiments in Figure 3. Following an initial 30–45-s lag phase, insulin activates the glucose transport system in a gradual manner, reaching the fully activated state by 5 min at 37 °C (Ciaraldi & Olefsky, 1979). Under these conditions, the coupling events between occupied insulin receptors and the glucose transport system are rate determining. Figure 3 demonstrates that the kinetics of activation of 3-*O*-methylglucose transport by the two insulins are indistinguishable. In both cases, the activation proceeds through an initial lag period and through a short but continuous period of activation. Thus, [Leu^{B25}]insulin, an analogue which fails to increase the rate of receptor-bound insulin dissociation under conditions of high receptor occupancy, retains the full complement of activities displayed by native insulin.

Discussion

DeMeyts et al. (1973, 1976a) previously demonstrated that the rate of dissociation of cell-bound [^{125}I]insulin is increased in the presence of high concentrations of unlabeled insulin. This observation has subsequently been confirmed by many (DeMeyts et al., 1976b; Ginsberg, 1977; Olefsky & Chang, 1978) but not all (Gliemann et al., 1975; Pollet et al., 1977) investigators, and DeMeyts et al. have interpreted this finding as evidence for negatively cooperative interactions between insulin receptors. The onset of this effect is extremely rapid (Olefsky & Chang, 1978), and it has been suggested that the information transfer leading to negative cooperativity is responsible for the initiation of insulin's biologic action (DeMeyts et al., 1976a, 1980). To test this idea, we utilized a recently synthesized insulin analogue which contains a leucine for phenylalanine substitution at position 25 of the insulin B chain, [Leu^{B25}]insulin (Tager et al., 1980). Because of the placement of residue B25 within the negatively cooperative site of the insulin molecule (DeMeyts et al., 1978), we reasoned that the insulin analogue might have an impaired ability to increase the rate of insulin dissociation and that a comparison of this property with the biologic activity of the analogue might help clarify the overall relationship between negative cooperativity and insulin action.

The results demonstrate that at concentrations which produce a high level of fractional receptor occupancy (Tager et al., 1980), [Leu^{B25}]insulin does not increase the rate of dissociation of previously bound ^{125}I-labeled porcine insulin. Assessment of the ability of this insulin analogue to stimulate glucose transport or oxidation showed that [Leu^{B25}]insulin displays 2% of normal biologic potency relative to that of pork insulin, and this value coincides with its relative binding affinity of 1.7% (Table I). However, provided high enough concentrations are used, the analogue expresses full agonist properties and maximally stimulates glucose metabolism. It should be noted that we (Olefsky & Chang, 1979) and others (DeMeyts et al., 1978) have previously found that desoctapeptide and desalanine-desasparagine-insulin fail to enhance the rate of dissociation of receptor-bound [^{125}I]insulin, and although their rate of onset of action and activation kinetics are unknown, it has been reported that these analogues retain biologic activity (Freychet et al., 1973). It has recently been demonstrated that the biologic activity of desoctapeptide insulin (0.1% of porcine insulin) is not due to residual contamination with native insulin (Kikuchi et al., 1980), and for [Leu^{B25}]insulin, we can also exclude the possibility that the reported biologic activity is due to residual native insulin. Thus, since our preparation of desoctapeptide insulin (the staring material for synthesis of the analogue) has only 0.1% of the biologic activity of the hormone (Tager et al., 1980; unpublished experiments), contamination cannot account for the 2% biologic potency of the product.

When cells are exposed to very high hormone concentrations (as employed in the experiment of Figure 3), the fraction of insulin receptors (<10%) necessary to generate maximal insulin stimulation of glucose transport will be filled rapidly (<2 s), and binding will no longer be rate limiting for insulin action (Ciaraldi & Olefsky, 1979). Under these conditions, the rate of glucose transport activation represents the time course of the coupling events between occupied insulin receptors and the glucose transport system. With this approach, we have previously shown that at 37 °C porcine insulin rapidly activates the glucose transport system such that following an initial 30–45-s time lag the transport system is activated in a gradual fashion and full stimulation is achieved by 5 min (Ciaraldi & Olefsky, 1979). Determination of the kinetics of glucose transport activation by [Leu^{B25}]insulin demonstrated that the rate at which the analogue activates transport is identical with that of porcine insulin. This is a critical finding since it demonstrates that an insulin analogue with low affinity for the insulin receptor can reproduce insulin's effects at the normally rapid rate and with the ultimate full magnitude. Furthermore, it shows that the dissociation rate enhancement produced at high fractional receptor occupancy is not involved in the immediate effects of insulin on glucose transport, nor is it necessary for the final expression of full biologic activity.

Our results demonstrate that the mechanism underlying the ability of unlabeled insulin to increase the dissociation rate of receptor-bound insulin is not a prerequisite for the expression of insulin's biological effects. Nevertheless, these studies by no means exclude receptor–recptor interactions or conformational changes as mediators of the insulin-induced biologic response. Such changes, however, clearly need not have the character or magnitude required for increasing the apparent rate constant for the dissociation of the receptor-bound hormone.

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Enzymatic Synthesis of Selenocysteine in Rat Liver†

Nobuyoshi Esaki, Takeshi Nakamura, Hidehiko Tanaka, Tetsuya Suzuki, Yoshimasa Morino, and Kenji Soda*

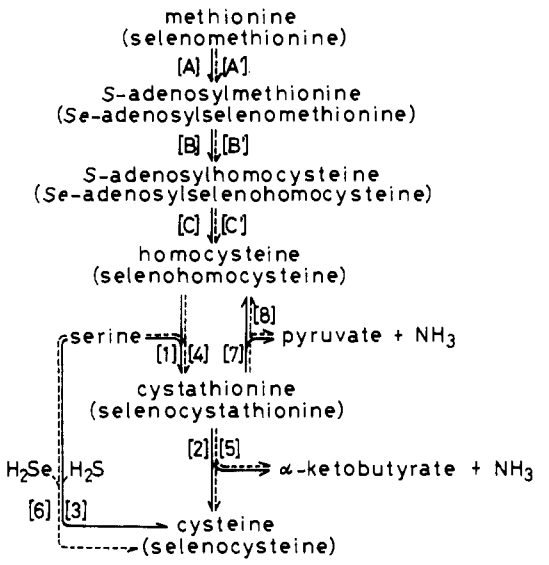
ABSTRACT: We have investigated selenocysteine (2-amino-3-hydroselenopropionic acid) synthesis with cystathionine β-synthase (EC 4.2.1.22) and cystathionine γ-lyase (EC 4.4.1.1) of rat liver. When selenohomocysteine and serine were incubated with cystathionine β-synthase, selenocystathionine was formed at a rate of 69% of that of cystathionine synthesis. Cystathionine γ-lyase catalyzed α,γ elimination of selenocystathionine to yield α-ketobutyrate, selenocysteine, and NH_3. The reaction rate was about 3 times higher than that of cystathionine elimination. Cystathionine β-synthase, however, did not catalyze direct formation of selenocysteine from serine and H_2Se. Thus, selenocysteine is synthesized from selenohomocysteine and serine through selenocystathionine by coupling of cystathionine β-synthase and cystathionine γ-lyase reactions. We confirmed this synthetic pathway also with a mixture of both enzymes and with a homogenate of rat liver.

Various selenium-containing amino acids occur in nature and play important physiological roles (Shrift, 1973; Stadtman, 1980). Several microbial and mammalian proteins, in particular enzymes, contain selenium as an essential component (Stadtman, 1980). Three of them have been shown to contain a selenocysteine (2-amino-3-hydroselenopropionic acid) residue in their polypeptide chains: selenoprotein A of glycine reductase complex from *Clostridium sticklandii* (Cone et al., 1976), formate dehydrogenase of *Methanococcus vannielii* (Jones et al., 1979), and glutathione peroxidase of rat liver (Forstrom et al., 1978) and bovine erythrocyte (Ladenstein et al., 1979). Evidence has been obtained for the participation of a selenocysteine residue in the catalytic processes (Forstrom et al., 1978; Ladenstein et al., 1979). There are two possible pathways for synthesis of the selenocysteine residue of glutathione peroxidase, posttranslational incorporation of selenium to the residue, as suggested by Sunde & Hoekstra (1980), and direct incorporation of selenocysteine (Hawkes et al., 1979). However, little attention has been paid to the biosynthesis of selenocysteine.

Selenocysteine can be formed from *O*-acetylserine and H_2Se by *O*-acetylserine (thiol)-lyase (EC 4.2.99.8) of selenium accumulator and nonaccumulator plants (Ng & Anderson, 1978) and of *Paracoccus denitrificans* (Burnell & Whatley, 1977). Selenomethionine has been demonstrated in wheat and some other grains, but there are few reports of the occurrence of selenocysteine and selenocystine (Olson et al., 1970). In mammalian tissues, selenocysteine synthesis has been assumed to be analogous to that of cysteine (Scheme I), but no evidence has been obtained.

We here present the evidence that selenocysteine is produced enzymatically by coupling of reactions 4 and 5, but not by reaction 6, with cystathionine β-synthase (EC 4.2.1.22) and cystathionine γ-lyase (EC 4.4.1.1) of rat liver.

Scheme I: Biosynthetic Pathways of Cysteine (–) and Selenocysteine (---)[a]



[a] Cystathionine β-synthase catalyzes reactions 1, 3, 4, and 6; cystathionine γ-lyase catalyzes reactions 2, 5, 7, and 8. Reaction A or A' is catalyzed by ATP:L-methionine *S*-adenosyltransferase of rabbit liver (Greene, 1969). Reaction B or B' is catalyzed by various transmethylation systems (Mudd & Cantoni, 1957; Bremer & Natori, 1960; Pan & Tarver, 1967). Reaction C is catalyzed by *S*-adenosylhomocysteine hydrolase. No evidence has been obtained for reaction C'.

Experimental Procedures

Materials. DL-Selenomethionine and DL-selenocystine were purchased from Sigma, L-homocysteine, L-cystathionine, dithiothreitol, and heptafluorobutyric acid were from Nakarai Chemicals, Kyoto, Japan, and aluminum selenide was from Alfa Division—Ventron. *Se*-Benzyl-L-selenohomocysteine was synthesized from dibenzyl diselenide and L-α-amino-γ-

† From the Laboratory of Microbial Biochemistry, Institute for Chemical Research, Kyoto University, Uji, Kyoto-Fu 611 (N.E., T.N., and K.S.), Laboratory of Biochemistry, Kyoto College of Pharmacy, Kyoto 607 (H.T.), Research Institute for Food Science, Kyoto University, Uji, Kyoto-Fu 611 (T.S.), and Department of Biochemistry, Faculty of Medicine, Kumamoto University, Kumamoto 860, Japan (Y.M.). *Received December 31, 1980.*

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