Amino Acid Transfer Factors from Yeast. Isolation and Properties of Three Different Transfer-Active Protein Fractions*

D. Richter and F. Klink

ABSTRACT: A cell-free system for testing amino acid transfer enzymes was developed from yeast. Three fractions (1-P, 2-P, and 3-P) were separated from the 105,000*g* cell supernatant by TEAE-cellulose chromatography. Fractions 1-P and 2-P, showing transfer activity only when combined, were precipitated at nearly the same ammonium sulfate concentration, but possessed different heat-lability properties. Fraction 3-P represented an association of at least two transfer factors. As gel filtration indicated, one of them (FI) was identical with the transfer factor of fraction 2-P, the other (FIIb) showed striking differences in molecular weight and stability, compared with the main component FIIa of fraction 1-P.

Separation and partial purification of two complementary transfer factors has been achieved up to now from the soluble cell supernatant of *Escherichia coli* (Allende *et al.*, 1964; Nishizuka and Lipmann, 1966a), reticulocytes (Arlinghaus *et al.*, 1964), and liver (Fessenden and Moldave, 1963; Gasior and Moldave, 1965; Klink *et al.*, 1963, 1967a,b) with various methods. In a previous brief note we communicated the isolation of two corresponding enzymes from yeast (Klink and Richter, 1966). From *Pseudomonas fluorescens* Lucas-Lenard and Lipmann (1966) isolated three complementary transfer factors. In all these systems the messenger-directed formation of peptide chains from tRNA-linked amino acids required at least two enzyme factors. Enzymatically catalyzed binding of aminoacyl-tRNA to ribosomes without simultaneous peptide synthesis could not be observed in *E. coli* (Nakamoto *et al.*, 1963) or liver (Klink *et al.*, 1967b) systems. On the other hand, Arlinghaus *et al.* (1964) noted tRNA adaption to appear in the presence of an enzymic factor (TFI) isolated from reticulocytes. The enzymatic binding of aminoacyl-tRNA to ribosomes has recently been reported in *E. coli* (Ravel, 1967).

These results rather suggested the "binding enzyme" to be necessary for some kind of ribosomal translocation process involving messenger and/or aminoacyl-tRNA transport under GTP[1] hydrolysis (Schweet and Heintz, 1966). Similar functions were presumed for the ribosome-dependent GTPases FI from liver (Klink *et al.*, 1967b) and G from *E. coli* (Nishizuka and Lipmann, 1966b); the complementary enzymes (TF-II, F-II, and T, respectively) may be responsible for the peptide synthesis step. Similar studies in cell-free systems from yeast had been handicapped by difficulties in isolating and purifying the transfer active enzymes (Heredia and Halvorson, 1966).

This paper details the separation of three protein fractions involved in amino acid polymerization from yeast. Striking differences in chemical behavior suggest these fractions to contain three different transfer enzymes, though only one of them could be proved to be complementary to each of the others.

Materials and Methods

Preparation of the Cell-Free Extracts. The yeast cultures were grown at 30° with vigorous aeration in a peptone–glucose–salt solution (Halvorson and Spiegelmann, 1952) and harvested in the middle of the logarithmic phase (MSE Ltd., London, high-speed 18; continuous-flow rotor). The cells were washed four times with buffer I (0.02 M Tris-HCl, pH 7.6, and 0.001 M Mg $(CH_3COO)_2$) and either immediately disrupted or lyophilized, and stored at −25° up to 3 weeks. The yield was 12–15 g of fresh or 3 g of dry yeast (lyophilized) per 1 l. of culture.

The following steps were carried out at 2°. For the isolation of ribosomes the cells were disrupted by grinding with twice the weight of acid-washed sand for 10 min in an ice-cold mortar. For preparation of enzymes a cell homogenizer from "Apparatebau Braun" (Melsungen, Germany) was used. Fresh yeast (15 g) and 50 g of acid-washed glass beads (diameter, 0.45–0.50 mm) were suspended in 15 ml of buffer I and homogenized for 40 sec at a frequence of 4000 rpm under cooling by CO_2. The homogenized mixtures were extracted with 2.5 volumes of buffer I or, when preparing ribosomes, with buffer II (0.05 M Tris-HCl (pH 7.6), 0.008 M $Mg(CH_3COO)_2$, 0.25 M sucrose, and 0.1 M KCl). After removing large debris and glass beads or sand (5 min at 12,000*g*) the pH was adjusted to 7.6 with 1 M Tris-HCl. After further cen-

* From the Institute of Physiological Chemistry, University of Kiel, Kiel, Germany. *Received June 6, 1967.* A preliminary note of this work has been published (Richter and Klink, 1967b). This investigation was supported in part by Research Grants No. K1 151/5–7 from the Deutsche Forschungsgemeinschaft.

[1] Abbreviations: GTP, guanosine triphosphate; ATP, adenosine triphosphate; CTP, cytidine triphosphate; DOC, sodium deoxycholate; TCA, trichloroacetic acid.

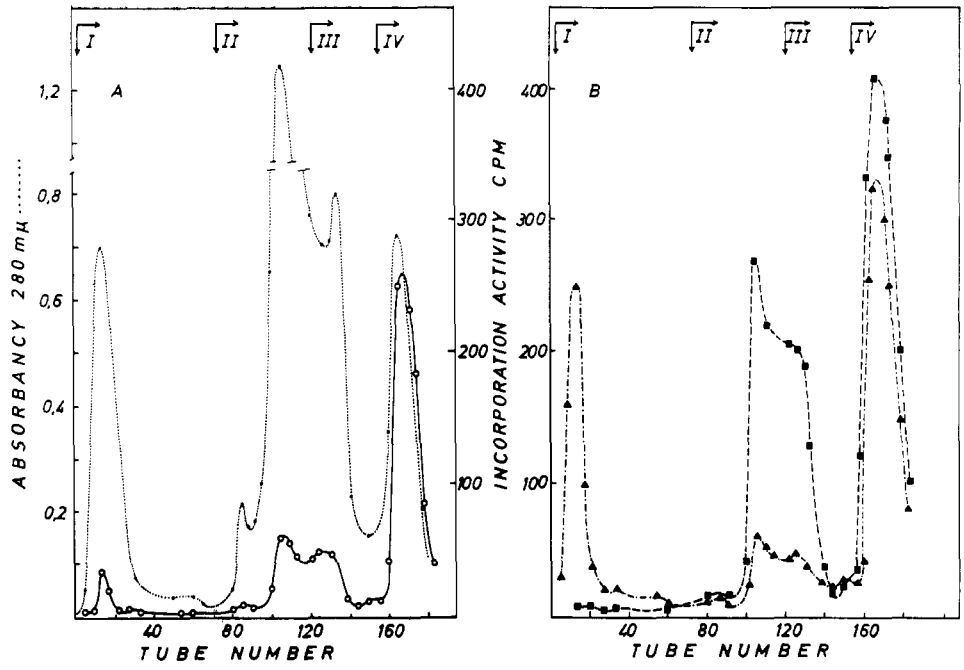

FIGURE 1: Separation of 1-P, 2-P, and 3-P by TEAE column chromatography of an ammonium sulfate fraction (40–70% saturation) from yeast S-2. Elution steps (all buffers contained 0.02 M Tris-HCl (pH 7.4), 0.001 M $Mg(CH_3COO)_2$, 0.005 M cysteamine, and KCl as indicated): (I) washing without KCl, (II) gradient (closed mixing chamber with 150 ml of 0.01 M KCl, stock with 0.2 M KCl), (III) 0.2 M KCl, and (IV) 0.35 M KCl. Fraction volume, 5 ml. The appearance of the buffers in effluent has been marked. (A) (· · · · · ·) $A_{280\ nm}$; (O——O) incorporation activity of 0.3 ml of the fractions alone. (B) (■- - - -■) Incorporation activity of the eluate fractions in the presence of 0.3 ml of the fractions 14:15:16 (minus a blank of 25 cpm); (▲- · -▲) activity in the presence of 0.3 ml of the fractions 99:100:101 (minus a blank of 15 cpm). Test in the standard polymerization system with polysomes from liver.

trifugation at 18,000*g* for 25 min, the supernatant fluid (S-1) was carefully decanted.

Isolation of the Ribosomes. METHOD I (STANDARD RIBOSOMES). The supernatant fluid S-1 from mortared cells was centrifugated at 78,000*g* for 2 hr, the upper four-fifths of the supernatant was removed, and the ribosome pellets were dissolved in the remaining supernatant. The ribosome solution was diluted with 2.5 volumes of buffer III (0.05 M Tris-HCl (pH 7.6), 0.25 M sucrose, 0.008 M $Mg(CH_3COO)_2$, and 0.6 M KCl). After treatment with one-tenth of an 8% DOC solution, 18 ml of the ribosome suspension was put on a discontinuous density gradient consisting of two sucrose layers (7 ml of 1.5 M sucrose and 5 ml of 0.7 M sucrose, in buffer III), and spun down in 4 hr at 78,000*g*. The ribosome pellets were dissolved and dialyzed against buffer II. After removing undissolved aggregates by centrifugation (10 min at 10,000*g*) the concentration of the ribosome solution was adjusted to 45 absorbancy units (A(260 nm, 1%, 1 cm)). The preparations were stored frozen at −30° up to 4 weeks.

METHOD II (DOC-FREE RIBOSOMES). (IIa) Method I was modified by omitting the DOC treatment. (IIb) The density gradient centrifugation of the first ribosome pellets (see method I) was replaced by washing them twice with buffer II. The concentration of this ribosome preparation was that of method I.

Preparation of the Enzyme Fractions. Supernatant S-1 was recentrifugated for 2 hr at 78,000*g*. After removing of the fat layer, three-fourths of the remaining supernatant was collected, adjusted to pH 6.6–6.9 with 1.8 N acetic acid, and fractionated by adding solid ammonium sulfate. The protein fraction precipitated between 40 and 70% salt saturation was redissolved in buffer I and reprecipitated in the same range of salt concentration. The final solution (S-2) was clarified by centrifugation (1.5 hr at 105,000*g*) and adjusted to pH 7.4 with 1 M Tris-HCl. S-2 containing about 10 mg of protein/ml was dialyzed against buffer IV (0.02 M Tris-HCl (pH 7.4), 0.001 M $Mg(CH_3COO)_2$, 0.005 M cysteamine, and 0.25 M sucrose) and filtered through Sephadex G-25 to remove traces of ammonium sulfate.

Chromatography of the Enzyme Fractions. S-2 solution (60–70 ml) was applied to a TEAE-cellulose column (3.0 × 65.0 cm), equilibrated with buffer V (buffer IV but without sucrose), and eluted as indicated in Figure 1. The protein peaks were pooled, precipitated with ammonium sulfate, and dialyzed against buffer II with 0.01 M cysteamine (two buffer changes). The protein fractions were stored frozen or lyophilized at −30°.

Gel Filtration. All experiments were performed with columns of 410-ml gel volume and 572-mm bed height.

The dextran (Sephadex G-200) was equilibrated with buffer VI (0.05 M Tris-HCl (pH 7.4), 0.008 M Mg-$(CH_3COO)_2$, 0.1 M KCl, and 0.005 M cysteamine). The protein fractions to be analyzed were concentrated by lyophilization and dialyzed against buffer II with 0.01 M cysteamine. The protein solution (2 ml) containing 0.5 mg of adenosine as a marker for the imbibed volume (V_i) was applied to the top of the column by layering them under the buffer already present. The fractions were eluted with sucrose-free buffer V. Molecular weight estimation was carried out as described (Klink *et al.*, 1967b).

Preparation of the [^{14}C]*Aminoacyl-tRNA.* Aminoacyl-tRNA was prepared from purchasable yeast tRNA. Esterification with [^{14}C]amino acids was performed according to Klink *et al.* (1967a).

Reaction Mixture for Amino Acid Incorporation into Ribosomal Protein. The transfer activity of the enzyme fractions was examined at 37°, in an incubation mixture containing in 1 ml: 0.1 ml of ribosome solution (A(260 nm, 1%, 1 cm) 4.5), 25 μg of [^{14}C]aminoacyl-tRNA (2500–3500 cpm), 1 μmole of ATP, 0.33 μmole of GTP, 4.0 μmoles of phosphoenolpyruvate, 25 μg of pyruvate kinase, 30 μmoles of cysteamine, 6 μmoles of $Mg(CH_3COO)_2$, 100 μmoles of KCl, and 50 μmoles of Tris-HCl (pH 7.6). In tests with standard ribosomes 0.2 μmole/ml of spermine was added. Experiments with ribosomes from rat liver contained 8 μmoles of magnesium acetate. Liver ribosomes were isolated as described (Klink *et al.*, 1967b). Before adding the transfer enzyme fractions to the incubation mixture, they were kept for 30 min at 4° in buffer II containing 0.04 M cysteamine. After incubation for 15 min the reaction was stopped by 1 ml of an ice-cold 0.8% casein solution, followed by 2 ml of 10% TCA. Washing and isolation of the ^{14}C proteins was modified (Klink *et al.*, 1967a) according to Siekevitz (1952). Protein determination was carried out by the method of Lowry *et al.* (1951).

Materials. The yeast culture, *Saccharomyces cerevisiae* strain Kaneka, was obtained by the Centraelbureau voor Schimmelcultures (The Netherlands). Algal or yeast ^{14}C protein hydrolysate (specific activity, approximately 300 μc/mmole) was supplied by the Radiochemical Centre, Amersham, England; RNA was a product of Boehringer (Mannheim, Germany) or Nutritional Biochemical Corp., Cleveland, Ohio; other biochemicals (ATP, GTP, CTP, phosphoenolpyruvate, and pyruvate kinase) were purchased from Boehringer; cysteamine from Serva Ltd. (Heidelberg, Germany) or from Nutritional Biochemical Corp.; dextran gels from Deutsche Pharmacia GmbH, Frankfurt, Germany; anion-exchange cellulose (capacity 0.7 mequiv/g) from Schleicher & Schüll, Dassel, Germany.

Results

Activity of Different Ribosome Preparations in Amino Acid Transfer. Biologically active ribosome preparations almost completely dependent on cell sap are required to study functions and properties of transfer enzymes. Ribosomes washed twice with buffer II (method IIb) showed maximum transfer activity in the absence of supernatant (Table I); the addition of S-2 even inhibited the amino acid polymerization. Similar observations were reported by Downey *et al.* (1965). Gradient centrifugation (see method IIa) caused the ribosomes to exhibit a partial dependence on S-2. An absolute requirement of S-2 was found when the ribosome suspension was additionally treated with DOC. A considerable decrease of activity, which may be owing to DOC treatment, could be almost completely compensated by addition of 0.2 μmole/ml of spermine. An increase of the Mg^{2+} concentration in the incorporation system diminished the amount of spermine required. The methods of cell disruption and storage greatly influenced the activity of the ribosomes, but not of the cell supernatant. Ribosomes from freshly prepared cells and from those being lyophilized and stored for 3 weeks at −25° showed the same activity, whereas ribosomes from cells stored frozen and thawed, were about 30% less active. Amino acid incorporation by ribosomes obtained from mortared cells was about 1.5 times higher than by an equal amount of ribosomes from cells disrupted in a cell disintegrator.

TABLE I: Influence of Yeast Ribosome Preparation Method on Amino Acid Polymerization.

Ribosome Preparation	[^{14}C]Amino Acid Incorp (cpm) 105,000g Supernatant S-2	
	Minus	Plus
(1) Washed with buffer II (method IIb)		
One time	1547	1026
Two times	1398	820
(2) Sucrose gradient centrifugation + 0.6 M KCl (method IIa)	198	890
(3) Standard ribosomes (method I)	6	226
Standard ribosomes + 0.2 μM spermine/ml of test volume	8	731

Though the properties of the standard yeast ribosome preparations seemed suitable for testing transfer enzymes, most of the chromatographic studies were analyzed using liver polysomes, the preparation of which was less complicated. As recently reported (Klink and Richter, 1966), ribosomes and transfer factors of both species were freely exchangeable without decrease in transfer effects. No polyamine was necessary in liver ribosome systems.

Chromatographic Separation of the Transfer Factors.

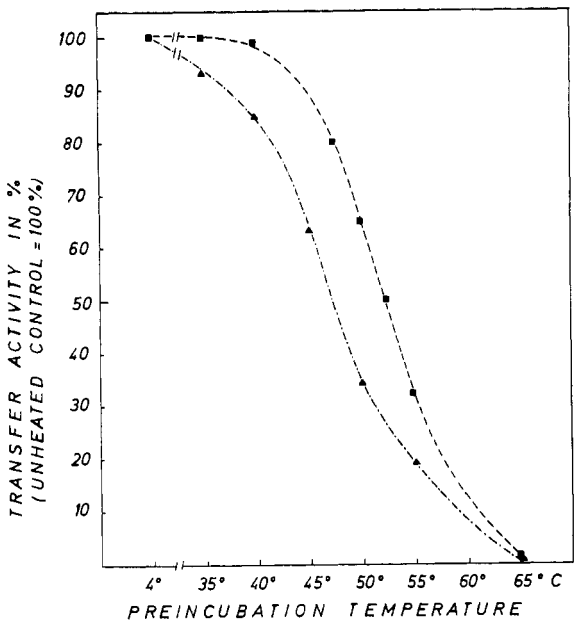

FIGURE 2: Inactivation of 1-P and 2-P by heating for 5 min at the temperatures indicated in the absence of cysteamine. The heated fractions were cooled and incubated at 32° for 15 min in the standard test system with liver polyribosomes and saturating amounts of the complementary factor. Transfer activity in per cent of the unheated control: (▲·····▲) 1-P and (■- - -■) 2-P.

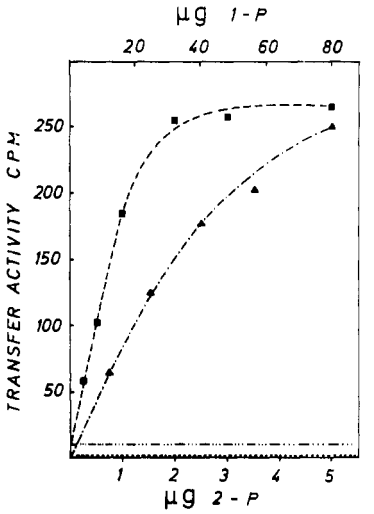

FIGURE 3: Amino acid incorporation by various amounts of 1-P (▲·····▲) and 2-P (■- - -■) in the presence of the other fraction (15 µg of 2-P and 80 µg 1-P, respectively). (·····) 1-P blank and (· · · ·) 2-P blank. Incorporation system see Figure 1.

Amino acid transfer activity was precipitated almost completely between 40 and 70% ammonium sulfate saturation. Chromatographic analyses of this protein fraction on TEAE-cellulose yielded three main peaks. When being tested separately, the first peak (1-P) obtained with KCl-free buffer, and the second one (2-P) eluted between 0.15 and 0.2 M KCl, showed only low transfer effects (see Figure 1A), whereas a combination of 1-P and 2-P exhibited high transfer activity (see Figure 1B). The fractions of the third peak (3-P) eluted with 0.35 M KCl, possessed a considerable high transfer activity, which did not increase appreciably by addition of 1-P or 2-P. Therefore we supposed that 3-P included a combination of the factors present in 1-P and 2-P. Rechromatography on TEAE-cellulose, however, did not result in splitting the fraction 3-P into 1-P and 2-P. The bulk of the 3-P transfer activity was recovered at 0.35 M KCl; only small quantities of 2-P were obtained, but no 1-P activity was found in the KCl-free eluate.

Ammonium Sulfate Precipitation of the Transfer Factors. Table II shows ammonium sulfate fractionations of 1-P and 2-P. The bulk of transfer activity was precipitated between 50 and 60% salt saturation from 1-P and between 50 and 70% saturation from 2-P. The specific activities indicated that 2-P contained considerably less concomitant proteins than 1-P. The precipitation areas of 1-P and 2-P overlapped almost completely, thus preventing even partial separation of the components from cell supernatant, as performed in the liver system to enrich the individual enzymes before the chromatographic step (Klink *et al.*, 1967a).

Heat Lability of the Transfer Factors. The fractions 1-P and 2-P were heated for 5 min at the temperatures indicated (Figure 2). Fraction 1-P was somewhat easier to be inactivated than 2-P; the difference was

TABLE II: Salt Fractionation of the Yeast Fractions 1-P and 2-P.[a]

Ammonium Sulfate Satn (%)	[^{14}C]Amino Acid Incorp (cpm)		Incorp Act./µg of Protein (cpm)	
	2-P	1-P	2-P	1-P
0–40	8	62	18	1.5
40–50	40	80	31	2.3
50–60	135	180	38	4.3
60–70	144	52	31	1.1
>70	10	5	15	0.4

[a] The supernatant S-2 was precipitated with solid ammonium sulfate at 0–75% saturation. The fractions 1-P and 2-P were obtained by chromatography on TEAE-cellulose, concentrated by lyophilization, dialyzed against buffer V, and fractionated at pH 6.6 with ammonium sulfate as indicated. The fractions were brought to equal volumes; the pH of these solutions was adjusted to 7.6 with 1 M Tris-HCl. Equal volumes of the salt fractions were tested in the presence of the complementary enzymes (1-P = 105 µg and 2-P = 45 µg, respectively). Incubation time was 12 min.

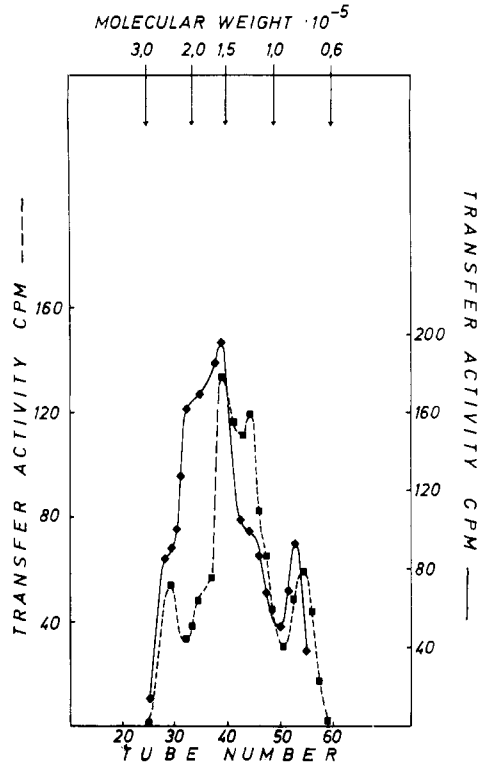

FIGURE 4: Gel filtration on Sephadex G-200 of yeast 2-P (100 mg of protein, ■- - - -■) and 3-P (80 mg of protein, ◆——◆), respectively. Transfer activity of the fractions has been measured in the standard incorporation system (see Methods) with liver polysomes in the presence of 1-P.

too small, however, to employ this method for isolating one of the enzymes. In the presence of cysteamine the heat lability of 2-P was reduced; no effect was observed in the case of 1-P.

Complementarity of the Fractions 1-P and 2-P. Figure 3 shows the transfer effects of varied concentrations of 1-P or 2-P in the presence of saturating amounts of the complementary protein fractions indicating incorporation rates and enzyme concentrations to be proportional over a wide range. A total of 60–80 μg of 1-P protein, but only 1–2 μg of 2-P protein was needed to saturate the test system. The small blanks of the two fractions alone demonstrated their complementary nature and the partial contamination by each another.

Gel Filtration of the Fractions 1-P, 2-P, and 3-P. Attempts were made to find out further characterization of the two complementary fractions 1-P and 2-P by gel filtration on Sephadex G-200 (Dimigen *et al.*, 1965). The same method was used to decide whether the fraction 3-P, actually, was an association of 1-P and 2-P. Moreover, this method made it possible to estimate approximate values of molecular weight. For this purpose the dextran columns were calibrated

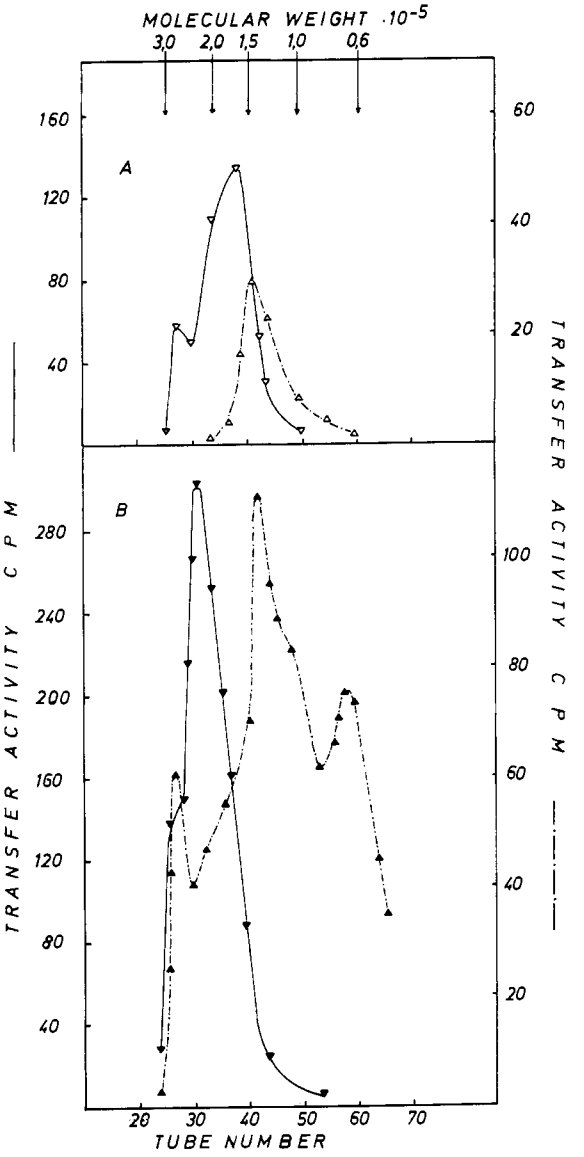

FIGURE 5: Gel filtration on Sephadex G-200 of yeast 1-P (150 mg of protein) and 3-P (80 mg of protein), respectively. Transfer activity has been measured in the standard system (see Figure 4). Amino acid polymerization. (a) Eluate fractions alone: (△- · · · -△) 1-P and (▽——▽) 3-P. (b) Eluate fractions tested with addition of 12 μg of 2-P: (▲- · · · -▲) 1-P and (▼——▼) 3-P.

with standard proteins as described (Klink *et al.*, 1967b).

In Figures 4 and 5 gel filtration runs of the 3-P fraction are combined with those of 2-P and 1-P, respectively. In Figure 4, the transfer activity of the eluate fractions is shown in the presence of saturating amounts of 1-P; when measuring this activity complemented with 1-P, we obtained similarly shaped elution diagrams for 2-P and 3-P. Thus, both fractions seemed to contain the same transfer factor. With 2-P

as the complementary factor, however, we achieved quite different pictures, when comparing the transfer activity of gel filtration experiments with 1-P and 3-P (see Figure 5a,b). The position of the single 3-P peak suggested a molecular weight greater than 200,000, whereas fraction 1-P containing only small amounts of this heavier component yielded two additional peaks of activity corresponding to molecular weights of about 130,000 and 65,000, respectively. Though being stimulated by 2-P, these transfer factors present in 1-P and 3-P did not appear to be identical. Furthermore both factors differed in stability. Fraction 1-P was kept lyophilized at −25° for 10 weeks without loss of activity, and frozen preparations lost only 25% of activity under the same conditions; the corresponding factor present in 3-P, however, was inactivated at least within 2 weeks when stored frozen. The crude 3-P fraction before gel filtration and fraction 2-P were quite stable. Further investigations will have to prove the question, whether the two peaks of 1-P (molecular weights approximately 130,000 and 65,000) are representing monomer and dimer forms of the enzyme.

TABLE III: Interchangeability of Yeast and Liver Transfer Factors.

	[^{14}C]Amino Acid Incorpn (cpm)		
	Transfer Factors Alone	Transfer Factors in Presence of	
		Yeast 1-P	Liver F-II[a]
Yeast F-I from 2-P	19	107	196
Yeast F-I from 3-P	21	96	236
Liver F-I[a]	0	92	204
Liver F-II[a]	75		
Yeast 1-P	17		74

[a] The isolation of liver F-I and F-II has been published (Klink *et al.*, 1967a). No activity was measured by the combination of yeast F-I from 2-P or 3-P with liver F-I. Test in the standard polymerization system with polysomes from liver.

Discussion

From reticulocytes as well as from liver two complementary transfer factors were obtained, whereas from *E. coli* and yeast an additional fraction could be isolated, showing high activity by itself. The third fraction from *E. coli* (T + G), being eluted between T and G from anion-exchange columns, represented an association of these two factors (Nishizuka and Lipmann, 1966a). The third enzyme fraction (3-P) from yeast exhibited a stronger affinity to TEAE resins than 1-P and 2-P, however, and contained two main components, one of them showing an elution profile similar to fraction 2-P. This component as well represented by 2-P as isolated from 3-P possessed the same properties in heat lability and could be precipitated by an equal amount of ammonium sulfate. The interchangeability of this yeast fraction with the transfer factor L-FI from calf liver suggested the peaks 2-P and 3-P to contain yeast FI (see Table III). The FI factors from yeast and calf liver were found to be quite similar in all properties investigated as yet. Liver FI was shown to possess a ribosome-dependent GTPase activity (Klink *et al.*, 1966); recent results confirmed yeast FI to have a similar function (H. Hameister and D. Richter, unpublished data). Therefore we suppose that yeast FI is comparable to the GTP-splitting factors G from bacteria (Nishizuka and Lipmann, 1966a,b; Lucas-Lenard and Lipmann, 1966), transferase II from rat liver (Sutter and Moldave, 1966), and perhaps to factor TF-I from reticulocytes (Arlinghaus *et al.*, 1964), too. The behavior of yeast FI on Sephadex G-200 forming three peaks of various activity (see Figure 4) suggested FI to be dependent on free SH groups. Preliminary results gave evidence that the component with the lowest molecular weight increased in the absence of cysteamine (Richter and Klink, 1967a); similar findings were reported from calf liver FI (Klink *et al.*, 1967b).

In spite of striking differences in molecular weight and stability to each other the second constituent of fraction 3-P and the transfer factor present in 1-P both showed a complementary function to yeast or liver FI. Therefore we distinguish them as FIIa (from 1-P) and FIIb (from 3-P). The properties of FIIa and FIIb raised the question whether there exist two different physicochemical states of the same enzyme, or two enzymes with different functions. Considering the resolution of the bacterial fraction T into two factors T_s and T_u, different in stability and molecular weight (Lucas-Lenard and Lipmann, 1966), one may compare yeast FIIa to T_s and yeast FIIb to T_u, the latter being less stable and higher in molecular weight than T_s.

Attempts are in progress to change the apparent complementarity between FI and FIIa or FIIb, respectively, into a complementarity of all three factors as demonstrated in the *Pseudomonas fluorescens* system. For this purpose further purification of the yeast factors is necessary assuming that our enzyme preparations are partially contaminated by each other.

Acknowledgments

The authors are grateful for the expert technical assistance of Mrs. Ilse M. Struck.

References

Allende, J. E., Monro, R., and Lipmann, F. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 1211.

Arlinghaus, R., Shaeffer, J., and Schweet, R. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 1291.
Dimigen, J., Klink, F., and Richter, D. (1965), *Z. Naturforsch. 20b*, 924.
Downey, K. M., So, A. G., and Davie, E. W. (1965), *Biochemistry 4*, 1702.
Fessenden, J., and Moldave, K. (1963), *J. Biol. Chem. 238*, 1497.
Gasior, E., and Moldave, K. (1965), *J. Biol. Chem. 240*, 3346.
Halvorson, H. O., and Spiegelmann, S. (1952), *J. Bacteriol. 64*, 207.
Heredia, C. F., and Halvorson, H. O. (1966), *Biochemistry 5*, 946.
Klink, F., Kloppstech, K., Kramer, G., and Dimigen, J. (1967b), *Biochim. Biophys. Acta 134*, 373.
Klink, F., Kloppstech, K., and Netter, H. (1966), *Z. Naturforsch. 21b*, 497.
Klink, F., Kramer, G., Nour, A. M., and Petersen, K. G. (1967a), *Biochim. Biophys. Acta 134*, 360.
Klink, F., Nour, A. M., and Aepinus, K. F. (1963), *Biochim. Biophys. Acta 72*, 654.
Klink, F., and Richter, D. (1966), *Biochim. Biophys. Acta 114*, 431.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. C. (1951), *J. Biol. Chem. 193*, 265.
Lucas-Lenard, J., and Lipmann, F. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1562.
Nakamoto, T., Conway, T. W., Allende, J. E., Spyrides, G. J., and Lipmann, F. (1963), *Cold Spring Harbor Symp. Quant. Biol. 28*, 227.
Nishizuka, Y., and Lipmann, F. (1966a), *Proc. Natl. Acad. Sci. U. S. 55*, 212.
Nishizuka, Y., and Lipmann, F. (1966b), *Arch. Biochem. Biophys. 116*, 344.
Ravel, J. M. (1967), *Proc. Natl. Acad. Sci. U. S. 57*, 1811.
Richter, D., and Klink, F. (1967a), *Z. Naturforsch. 22b*, 355.
Richter, D., and Klink, F. (1967b), *Naturwissenschaften 54*, 117.
Schweet, R., and Heintz, R. (1966), *Ann. Rev. Biochem. 35*, 734.
Siekevitz, P. (1952), *J. Biol. Chem. 195*, 549.
Sutter, R. P., and Moldave, K. (1966), *J. Biol. Chem. 241*, 1698.