Fractionation of Transfer Ribonucleic Acid by Counter Double Current Distribution*

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ABSTRACT: Counter double current distribution, in which upper and lower phase move simultaneously in opposite directions after each equilibration, has been used to purify alanine transfer ribonucleic acid and valine transfer ribonucleic acid from bulk yeast transfer ribonucleic acid. The method compares favorably in terms of yield and efficiency with countercurrent distribution, in which only uppers move. In addition, purification is considerably faster, at least for alanine transfer ribonucleic acid, by counter double current distribution than by countercurrent distribution. The major disadvantages of the counter double current distribution procedure are the sensitivity of the solvent system to unidirectional temperature changes and the requirement for special equipment.

Of the various techniques that have been used for large-scale fractionation of tRNA, countercurrent distribution has, until very recently, been the most successful. The countercurrent distribution method, developed extensively by Craig (Craig and Craig, 1956), was first applied to the fractionation of RNA by Warner and Vaimberg (1958). The method was extended to the fractionation of tRNA by Holley and his collaborators (Holley and Merrill, 1959) and by Zachau *et al.* (1961). It has now been used successfully in several laboratories, including ours, for the fractionation of tRNA from rat liver, yeast, and *Escherchia coli*.

In the conventional countercurrent distribution machine, upper phases move over stationary lower phases after each equilibration as shown in Figure 1. A modification of this technique called counter double current distribution has been developed by Post and Craig (1963). In counter double current distribution, both upper and lower phases are transferred simultaneously in opposite directions after each equilibration as shown in Figure 2. The material to be fractionated is placed in the middle tubes of the machine and the component to be isolated from the mixture is held in the machine by adjusting the ratio of the upper volume to the lower volume so that the *effective partition coefficient*, K_{eff}, is 1.

In principle, counter double current distribution appears to have certain advantages over the conventional countercurrent distribution procedure for the isolation of a minor component from a mixture. First, the sample moves with each transfer in countercurrent distribution. This places a theoretical limitation on the number of transfers that can be performed before the compound of interest emerges from the machine.[1] This problem can, in some cases, be solved by using a machine with more tubes or by an automatic recycling procedure. In counter double current distribution, however, the component of interest does not move[2] except through band broadening and the only practical limit on the number of transfers that can be performed is the rate at which this band broadening occurs with each successive transfer. It should be noted, however, that the band spreads twice as rapidly in counter double current distribution as in countercurrent distribution and this can be a serious disadvantage.

Counter double current distribution offers a further theoretical advantage over countercurrent distribution. The greatest efficiency in separating binary mixtures is obtained when the geometric mean of the effective partition coefficients is 1 (Craig and Craig, 1956). In counter double current distribution, the effective partition coefficient of the component of interest is adjusted to unity so that the geometric mean of this partition coefficient and its closest impurity is fairly close to 1. In the countercurrent distribution procedure, on the other hand, the partition coefficients are not adjusted and the geometric mean of the partition coefficients will often differ significantly from unity. The increased efficiency in counter double current distribution means that fewer transfers are required for a purification than in countercurrent distribution.

In order to compare countercurrent distribution and counter double current distribution from a practical standpoint, we have investigated the fractionation of yeast tRNA by these two procedures. This paper is concerned mainly with the purification of $tRNA^{Ala}$, but some preliminary results on the fractionation of $tRNA^{Val}$ by counter double current distribution are also reported.

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[1] In countercurrent distribution, the position of the sample band after n transfers is defined by the equation, $N = nK_{eff}/(K_{eff} + 1)$, where N is the position of the peak tube, n is the number of transfers and K_{eff} is the effective partition coefficient (Craig and Craig, 1956). N must remain less than the number of tubes in the train, T, and the number of transfers is thereby limited. If $K_{eff} = 1$, 2T transfers could not be done.

[2] In counter double current distribution, the position of the peak tube, N, is given by the equation $N = N_0 + n(K_{eff} - 1)/(K_{eff} + 1)$, where N_0 = tube to which sample is added initially. It can be seen that $N = N_0$ if $K_{eff} = 1$. Thus, the band will be stationary and a large number of transfers can be done.

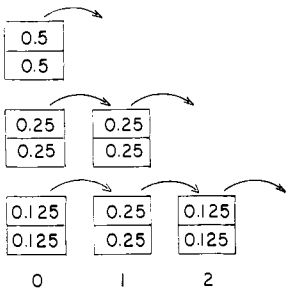

FIGURE 1: Scheme for countercurrent distribution when $K_{eff} = 1$.

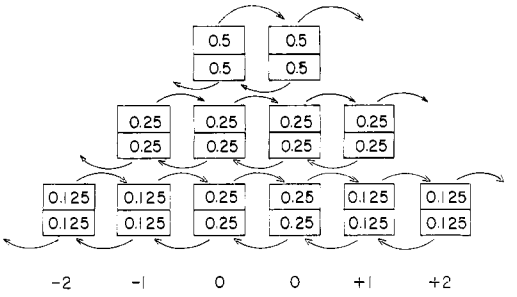

FIGURE 2: Scheme for counter double current distribution when $tRNA^{Ala}$ $K_{eff} = 1$.

Experimental Section

Countercurrent Procedures. All runs were carried out in a constant-temperature room, usually at 24 ± 0.5°. Countercurrent distribution was carried out in a 200 tube, 10 ml/phase machine (H. O. Post Scientific Instrument Co., New York). Counter double current distribution was carried out in a 100-tube machine (H. O. Post Scientific Instrument Co., New York) using a 10-ml lower phase volume and varying the upper phase volume as necessary to give an effective partition ratio of 1. The solvent system of Apgar *et al.* (1962) for redistribution was used for both procedures. A typical preparation of the solvent for a counter double current distribution run is as follows: Phosphate buffer, approximately 1.9 M (pH 6), was made by dissolving 3850 g of dipotassium hydrogen phosphate (Mallinckrodt, analytical reagent grade) and 5950 g of sodium dihydrogen phosphate monohydrate (Mallinckrodt, analytical reagent grade) and 28 l. of distilled water. The solution was filtered by gravity through a cotton plug to remove some insoluble impurities. After removing 700 ml of this solution for preparation of the sample, 2050 ml of formamide (Fisher Scientific Co.) and 8890 ml of isopropyl alcohol (Fisher Scientific Co.) were added and the mixture was stirred overnight in a constant temperature room to ensure equilibration.

Six grams of baker's yeast tRNA (Schwarz BioResearch, Inc., New York) was dissolved in 318 ml of the 1.9 M phosphate buffer. To the clear solution, 21.4 ml of formamide was added followed by 92.6 ml of isopropyl alcohol added dropwise with stirring. Considerable turbidity develops during this step and some tRNA precipitates from solution. The entire mixture was poured into a graduated cylinder and adjusted with lower phase (approximately 23 ml) so that the volumes of upper and lower phases were 205 ml each. Aliquots of both the upper and lower phase were removed, assayed for the component of interest (as described below), and the actual partition coefficient, K, was determined. Using the relationship, $K_{eff} = K \times$ upper volume/lower volume, additional upper phase was added in order to adjust the effective partition coefficient to 1. The mixture was shaken in a separatory funnel and allowed to separate. Approximately 1 g of tRNA that separated at the interface was removed during the separation of upper and lower phase.

The counter double current distribution machine was filled automatically with 10 ml of the lower phase. The amount of upper phase required to give $K_{eff} = 1$ was determined for the component of interest (as described later) and the machine was filled with upper phase automatically. Tubes 41–60 were emptied. To each of these tubes was added 10 ml of lower *sample* phase and the predetermined amount of upper *sample* phase. The settling time was set for 6 min to allow the emulsion that forms in the sample tubes to break completely. The remainder of the procedure was carried out automatically. A 300-transfer distribution was completed in 39 hr.

Assays. Assays were performed using a modification of the procedure of Cherayil and Bock (1965). Aliquots (~0.1 A_{260} units) from both the upper and lower sample layers were applied to 2.5-cm Whatman No. 3MM filter disks held on needles. The disks were transferred to Millipore funnels and washed successively, under gentle suction, with 5-ml portions of cold 10% trichloroacetic acid, cold 5% trichloroacetic acid, ethanol–ether–0.5 M Tris·HCl (pH 7) (100:100:4, v/v/v), and ether. This procedure removes the formamide and isopropyl alcohol and fixes the tRNA to the disks. A reaction mixture consisting of 2.5 μmol of Tris·HCl (pH 7), 0.5 μmol of sodium EDTA, 2.6 μmol of $MgCl_2$, 24.5 nmol of sodium ATP, and 1 nmol of L-[^{14}C]amino acid (90 cpm/pmol) in a total volume of 0.1 ml was spread on each disk. Aliquots (0.1 ml) of activating enzyme prepared as described previously (Reeves *et al.*, 1968) containing about 0.3–0.4 mg of protein were added immediately to each disk. The disks were kept moist during a 20-min incubation at room temperature by supporting them on needles over wet paper towels and covering them with small, inverted jars containing wet filter paper liners. After incubation, the disks were dried for 3 min under infrared lamps and then washed and counted in the usual manner (Reeves *et al.*, 1968). This procedure does not give accurate specific activities, but it is adequate for the determination of the partition coefficient and the location of the tRNA of interest after a number of transfers.

In order to obtain accurate specific activities, tRNA was assayed as described previously (Reeves *et al.*, 1968) after isolation by the hexadecyltrimethylammonium bromide flotation method of Mirzabekov *et al.* (1964) as modified by Zachau *et al.* (1966).

The conditions used for RNase T1 digestion and DEAE-cellulose chromatography have been reported previously (Reeves *et al.*, 1968).

Results

Purification of $tRNA^{Ala}$ by Counter Double Current Distribution. The partition coefficient of $tRNA^{Ala}$ is 0.2 at 24°. In counter double current distribution, K_{eff} is adjusted to

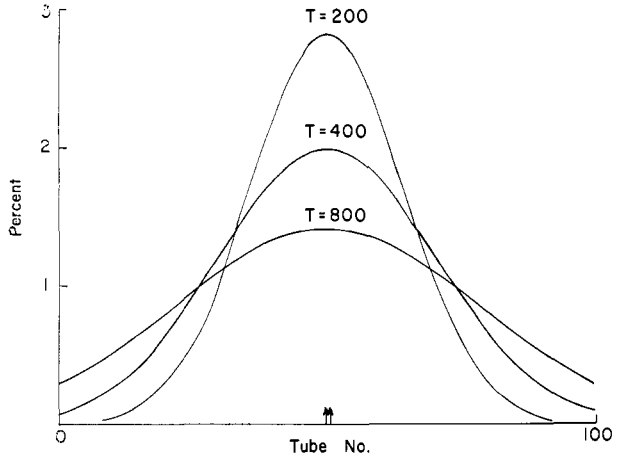

FIGURE 3: Theoretical counter double current distribution curves for $K_{eff} = 1$ after 200, 400, and 800 transfers when the starting material is placed in the pair of tubes, 50 and 51, corresponding to the two 0 tubes in Figure 2. The amount of material in each tube after the run is shown as a per cent of the total starting material. The curves were calculated from the equation

$$y = \frac{1}{\sqrt{8\pi\, n\, K_{eff}/(K_{eff}+1)^2}} e^{-x^2/[8nK_{eff}/(K_{eff}+1)^2]}$$

where y is the fraction of substance in a given tube, K_{eff} is the effective partition coefficient, n is the number of transfers, and x is the distance from the maximum of the tube in question.

unity by using an upper to lower volume ratio of 5. When crude tRNA is placed in the center tubes of a counter double current distribution machine, those components of the mixture with partition coefficients less than 0.2 will move with the lower phase while those components with partition coefficients greater than 0.2 will move with the upper phase. The $tRNA^{Ala}$ will remain stationary in the machine. The

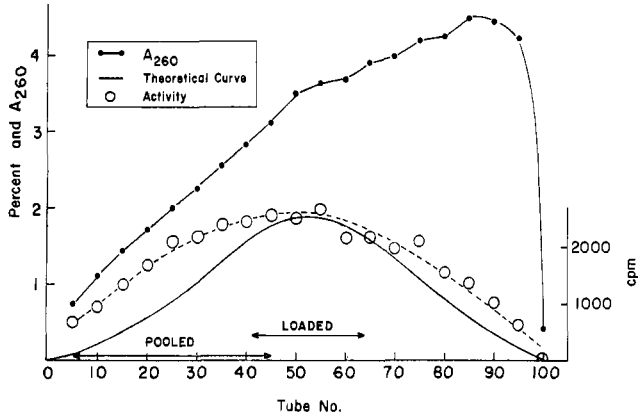

FIGURE 4: Counter double current distribution purification of yeast $tRNA^{Ala}$. The sample was prepared from 6 g of crude yeast tRNA and K_{eff} was adjusted to 1.0 as described in the Experimental Section. The sample was loaded into tubes 41–60, and a 300-transfer distribution was performed. At this point, the A_{260} of the lower phase and the biological activity of 100-μl aliquots from each lower phase were measured. Tubes 5–45 were pooled and isolated. The theoretical curve (as a per cent of total starting material in each tube) for 300 transfers of counter double current distribution with $K = 1$ is shown normalized to the experimental activity.

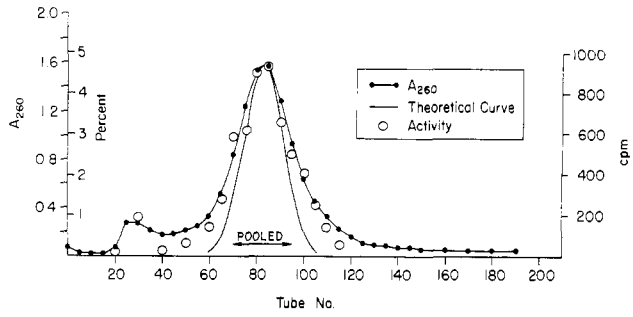

FIGURE 5: Redistribution by countercurrent distribution of partially purified $tRNA^{Ala}$ from counter double current distribution. The material isolated from the pool in Figure 3 was loaded into tube 0, in the usual manner, after tubes 1–200 had been filled with 10 ml of lower phase per tube; 10 ml of upper solvent was added to tubes 1–12 and a 180-transfer countercurrent distribution was performed with a settling time of 12.5–13 min. At this point, tubes 0–9 were emptied to remove material that causes an emulsion and refilled with fresh solvents. A further 420 transfers with a settling time of 10 min was performed bringing the total transfers to 600. The A_{260} of the lower phase and the biological activity of 100-μl aliquots of the lower phase were measured. Tubes 70–95 were pooled and isolated. A theoretical curve for $K = 0.16$ is shown.

distribution scheme of $tRNA^{Ala}$ under these conditions is shown in Figure 2.

It is obvious from this discussion that K_{eff} must be as close to unity as possible. Once the ratio of upper to lower has been established, it should remain constant as long as the temperature does not change. Unfortunately, the solvent system used in these studies is extremely sensitive to temperature fluctuation. The temperature must be controlled during solvent preparation as well as during the run. A unidirectional change of 1° causes a significant change in K, and the tRNA of interest moves from its center position.

When $K_{eff} = 1.0$, band spreading is the only practical limitation on the number of transfers that can be performed. The theoretical curves for 200, 400, and 800 transfers are shown in Figure 3. Significant amounts of material begin to be lost from 100-tube machine after about 400 transfers.

To compare the results of an actual run with those predicted on the basis of these considerations, $tRNA^{Ala}$ was purified from 6 g of crude tRNA by a 300-transfer counter double current distribution. The distribution is shown in Figure 4. It is obvious from the A_{260} pattern that only a partial purification was achieved. The *activity* curve, however, indicated that the $tRNA^{Ala}$ had remained in the machine and that its distribution had approximated the theoretical curve. The tubes were pooled as indicated in Figure 4, and the tRNA was isolated. It had a specific activity of 765 pmol/A_{260} (40% of theory). This represents a 17-fold purification and a 22% recovery of the $tRNA^{Ala}$ in the starting material. Other runs have given specific activities from 600 to 930 pmol per A_{260}. Specific activity and yield are both functions of the pool chosen.

The material remaining in the machine was rich in $tRNA^{Ala}_{II}$ and is suitable for further purification of this material as described previously (Reeves *et al.*, 1968). Thus, the over-all yield of $tRNA^{Ala}$ is considerably higher than 22% although it is difficult to give exact numbers from our data.

Purity. The purity of pooled material (Figure 4) was examined by redistribution in a conventional countercurrent distribution machine. The results of a 600-transfer distri-

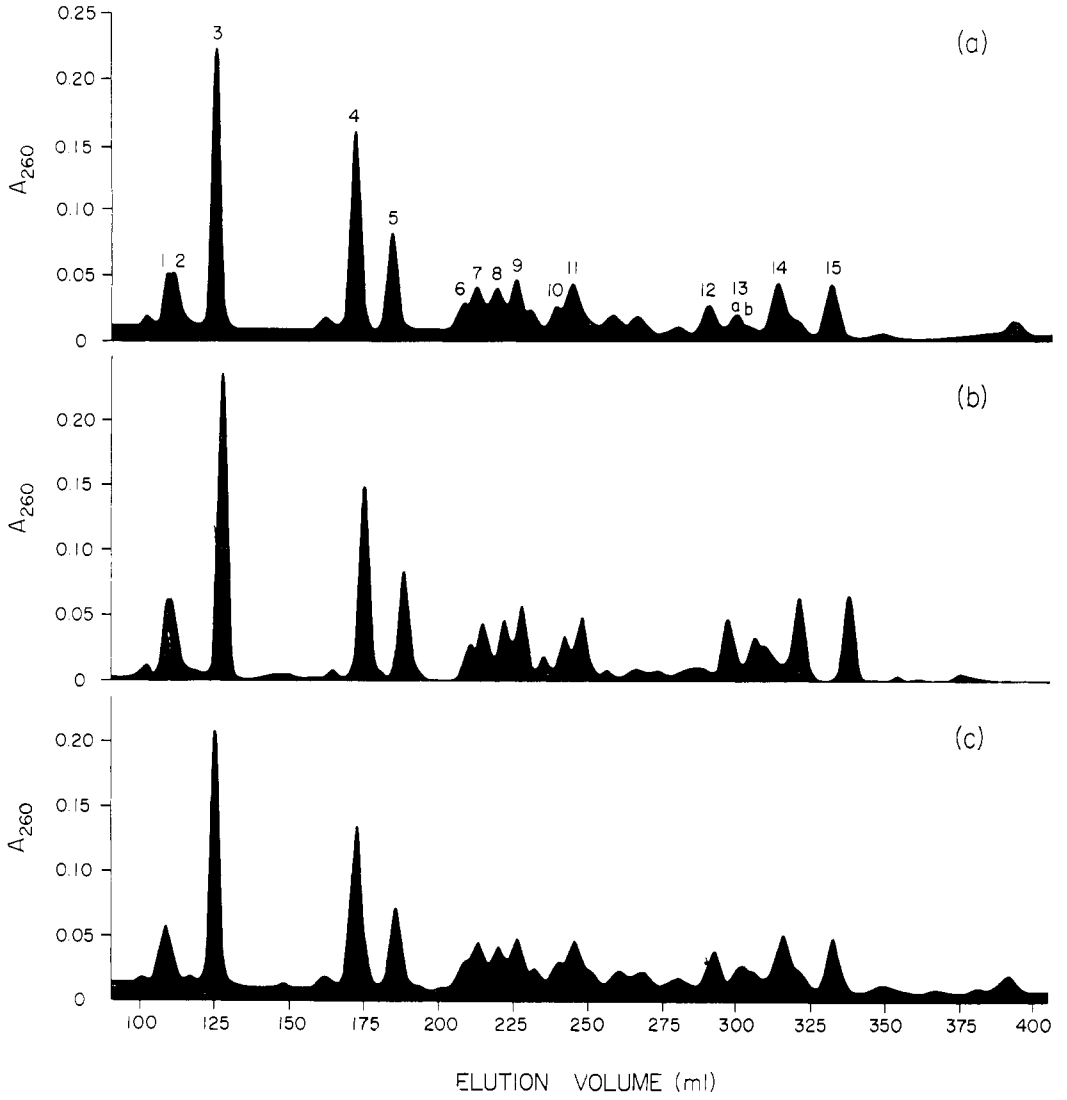

FIGURE 6: Fractionation of RNase T1 digests of $tRNA^{Ala}$ by chromatography on DEAE-cellulose. (a) Material obtained from a 300-transfer counter double current distribution; (b) material obtained from a redistribution of counter double current distribution material; (c) material obtained from the 1000-transfer countercurrent distribution pool shown in Figure 7.

bution are shown in Figure 5. Two peaks, both having biological activity, were observed. Both the absorbance and the alanine acceptor activity followed the theoretical curve for $K = 0.16$ fairly well. This low K is probably due to the temperature being slightly higher than 24°. Heterogeneity, however, is indicated in both the leading and trailing portions of the main peak. The nature of the small, slow-moving component is unknown. It appears to be an artifact of the Cetavlon (hexadecyltrimethylammonium bromide) isolation procedure since it was not observed in redistribution of material isolated by the procedure of Apgar *et al.* (1962).

The specific activity of the material isolated from tubes 70 to 95 of the redistribution (Figure 5) was 1060 pmol/A_{260}. This is only 62% of theory. As the specific activity of the counter double current distribution purified material and redistributed material is less than theoretical, the purity was investigated further by digesting them with RNase T1 and examining their oligonucleotide pattern after fractionation by DEAE-cellulose. A typical T1-produced oligonucleotide pattern obtained from counter double current distribution material is shown in Figure 6a. Evidence of impurities is seen in a number of small peaks, *e.g.*, just before peak 4, between peaks 11 and 12, and between peaks 14 and 15. The amount of $tRNA_{II}^{Ala}$ in this sample is small as indicated by the small peak just beyond 15 (Reeves *et al.*, 1968).

The oligonucleotide patterns obtained from redistributed material is shown in Figure 6b. The impurities evident in the counter double current distribution material have been reduced by a suitable pooling of fractions from the redistribution. The sample is now mainly $tRNA_{Iab}^{Ala}$ containing a small amount of $tRNA_{II}^{Ala}$. Once again, the discrepancy between purity based on specific activity (62%) and that estimated from the oligonucleotide pattern (90%) is evident. The redistributed material must contain a considerable quantity of denatured $tRNA_{Iab}^{Ala}$ or some other RNA that is very closely related to $tRNA^{Ala}$ both with respect to its

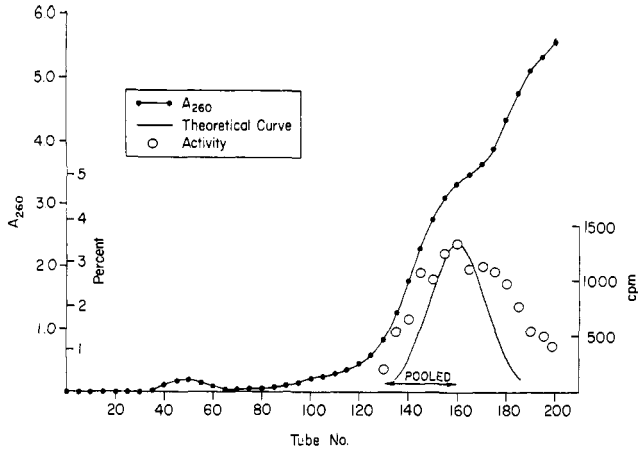

FIGURE 7: Countercurrent distribution purification of yeast $tRNA^{Ala}$. The sample was prepared from 3 g of crude yeast tRNA, as described by Apgar *et al.* (1962), and loaded into tubes 0–18 after filling 19–200 with 10 ml of lower phase/tube. Ten ml of upper solvent was added to tubes 19–29 and 169 transfers were performed with a 12 min settling time. The settling time was then reduced to 10 min and 831 further transfers were performed. At this point, the A_{260} of the lower phase and the biological activity of 20-μl aliquots of the lower phase was measured. Tubes 130–160 were pooled and isolated. A theoretical curve for $K = 0.19$ is shown.

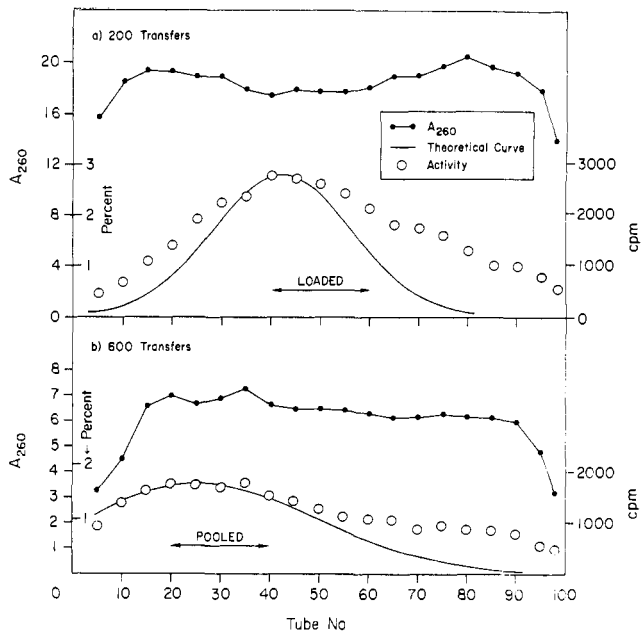

FIGURE 8: Counter double current distribution purification of yeast $tRNA^{Val}$. (a) Two hundred transfers. The sample was prepared from 6 g of crude, yeast tRNA as described in the Experimental Section, and K_{eff} was adjusted to one by using an upper to lower volume ratio of 1.55. The sample was loaded into tubes 41–60. After 200 transfers the A_{260} of the lower phase and the biological activity of 25-μl aliquots of the lower phase were measured. A theoretical curve for $K_{eff} = 0.925$ is shown. The low value of K_{eff}, and the deviation of the activity curve from the theoretical curve at the right may all be due to the presence of more than one species of $tRNA^{Val}$ (Mirzabekov *et al.*, 1965). (b) Six-hundred transfers. A_{260} and biological activity was measured as before. Tubes 20–40 were pooled and isolated. The theoretical curve for $K = 0.925$ is shown.

partition coefficient and its RNase T1-produced oligonucleotides. Since $tRNA^{Ala}_{Iab}$ does denature (Reeves, 1969), the first possibility is most likely.

Comparison of Counter Double Current Distribution and Countercurrent Distribution. In order to compare the counter double current distribution and the countercurrent distribution procedures, a 3-g sample of unfractionated tRNA was distributed for 1000 transfers in a conventional countercurrent distribution machine. The pattern is shown in Figure 7. The material obtained from the pool shown in Figure 7 had a specific activity of 810 pmol/A_{260} (48% of theory) and represented 23% recovery of the tRNA present in the unfractionated mixture. The purity of this material was examined by digestion with RNase T1 in the usual way. The pattern (shown in Figure 6c) is almost identical with that obtained with material which had been obtained with 300 transfers of counter double current distribution (Figure 6a).

Other tRNAs. Purification of some other tRNAs by counter double current distribution has been investigated in a preliminary manner. For example, the counter double current distribution patterns of $tRNA^{Val}$ after 200 and 600 transfers are shown in Figure 8. The specific activity of material isolated from the pool shown in Figure 8b after 600 transfers was 230 pmol of $tRNA^{Val}/A_{260}$. This represents only a 5-fold purification compared with 17-fold for $tRNA^{Ala}$ in 300 transfers. This result is expected, of course, because $tRNA^{Val}$ must be separated from tRNAs with partition coefficients both higher and lower than its own. On the other hand, $tRNA^{Ala}$ has the lowest partition coefficient in the distribution and, therefore, must only be separated from tRNAs with higher partition coefficients.

Discussion

On the basis of these data, a direct comparison between the countercurrent distribution and the counter double current distribution procedures can be made. Counter double current distribution is clearly superior to countercurrent distribution for partial purification of $tRNA^{Ala}$ since it takes three times as many transfers with countercurrent distribution to achieve the same degree of purity as with counter double current distribution. Furthermore, the separation of phases is faster in our counter double current distribution machine than in our countercurrent distribution machine. This, plus the fewer transfers required in the counter double current distribution procedure, add up to a sizeable time factor. In fact, $tRNA^{Ala}$ required almost 10 days in our countercurrent distribution equipment to bring it to the same purity as could be achieved in 3 days with our counter double current distribution machine. It must be emphasized, however, that some of this time difference may be unique to the particular equipment that we have used, since Apgar *et al.* (1962), using a machine of different design, reported a much shorter settling time than we found necessary in our countercurrent distribution machine.

The advantage of the counter double current distribution procedure over countercurrent distribution disappears in the fractionation of $tRNA^{Val}$. The increase in specific activity obtained after 600 transfers by counter double current distribution is actually slightly less than that reported by Apgar *et al.* (1962) after 200 transfers by the conventional countercurrent distribution procedure. It should be noted, however,

that the specific activity of $tRNA^{Val}$ after 200 transfers of counter double current distribution was not measured. It is possible that the band broadening prevents the separation of $tRNA^{Val}$ from its close impurities and the last several hundred transfers were unnecessary. If this is the case, then counter double current distribution may still offer some advantage over countercurrent distribution from the standpoint of operating time.

The necessity for close temperature control during counter double current distribution represents a major disadvantage of this procedure. This, of course, is not an inherent difficulty in counter double current distribution, but a specific problem of the solvent system employed for this particular fractionation. The partition coefficient is often sensitive to temperature-induced phase shifts in multicomponent solvent systems when the phases are miscible in each other to a considerable extent (Craig and Craig, 1956). With the Warner–Vaimberg–Holley solvent, an unidirectional change in temperature of only 1.0° causes a significant change in K_{eff} and this, in turn, causes the material of interest to move toward one end of the machine. Such a shift quickly destroys the major advantage of the counter double current distribution procedure. Temperature fluctuations also effect countercurrent distribution, but in general the effect is less serious because the method does not depend upon immobilization of the component of interest. Therefore, temperature fluctuations that completely ruin a counter double current distribution run are usually not disastrous in countercurrent distribution. The temperature problem could be solved by a new solvent system, but so far all the solvents developed for fractionation of tRNA are either temperature sensitive (Holley *et al.*, 1961) or sensitive to changes in solute concentration (Tada *et al.*, 1962).

A second modification of the countercurrent distribution method has been developed recently. In this procedure, called *steady-state distribution, either* upper or lower phase, but not both, can be transferred after each equilibration. The machine can be programmed for any sequence of upper and lower transfers. Thus, by choosing an appropriate program the component of interest can be "held" in the machine. For example, if the actual partition coefficient is 0.2, setting the upper to lower transfer ratio at 5 would hold the material in the center of the machine. The overall effect is similar to that accomplished with counter double current distribution procedure. Since the program can be changed during the run, the steady-state distribution machine may be particularly useful in correcting for small fluctuations in room temperature. This, plus the fact that band spreading occurs less rapidly in steady-state distribution than in counter double current distribution, may more than offset the fact that steady-state distribution is inherently a slower procedure than counter double current distribution. Steady-state distribution has been used to partially purify yeast $tRNA^{Ala}$ (Fleetwood, 1966).

In our hands, the counter double current distribution procedure has proven extremely valuable in preparing reasonably large amounts of partially purified $tRNA^{Ala}$. In addition, it led to the discovery of a new alanine tRNA. Because of the manner in which fractions were pooled for redistribution, $tRNA^{Ala}_{II}$ was missed in the early work on $tRNA^{Ala}$ purification by countercurrent distribution (Apgar *et al.*, 1962). In our laboratory, further examination of counter double current distribution fractions with K values slightly higher than that of $tRNA^{Ala}_{Iab}$ led to the discovery of $tRNA^{Ala}_{II}$ (Reeves *et al.*, 1968).

We cannot, however, recommend counter double current distribution in its present stage of development as a *general procedure* for the fractionation of tRNA. Some of the recently developed chromatographic procedures are capable of fractionating tRNA on a scale as large (Gillam *et al.*, 1967) or larger (Kelmers *et al.*, 1965) than that possible by counter current procedures. The column procedures will appeal to most laboratories since they do not require special fractionating equipment, and they require less operating space. It should be recognized, however, that we have explored only one of the three capabilities of the counter double current distribution procedure, and it is unfair to judge the procedure on the basis of these results alone. Other potential uses of the counter double current distribution equipment for fractionation of complex mixtures are discussed by Post and Craig (1963) and are beyond the scope of this paper.

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