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USE OF SIMPLE CONCENTRATION GRADIENTS FOR THE FRACTIONATION OF HUMAN SERUM PROTEINS ON DEAE-CELLULOSE, WITH ESPECIAL REFERENCE TO THE ISOLATION OF ALBUMIN, PREALBUMIN, HAEMOPEXIN AND TRANSFERRIN

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SUMMARY

The possibility of using simple concentration gradients, formed by an automatic gradient former, was explored for the fractionation of human serum. Especial reference was paid to the isolation of albumin, prealbumin, haemopexin and transferrin. Prealbumin was most clearly resolved from albumin by a linear gradient whilst haemopexin and transferrin were partially resolved using a non-linear gradient with a very shallow initial rise in concentration. The disadvantages of the system and how these may be minimized using gamma emitters were pointed out.

INTRODUCTION

Since the introduction of celluloses[1,2] containing substituted functional groups, ion-exchange chromatography has become one of the most popular tools for the fractionation of proteins. Apart from its operational simplicity, reproducibility and high capacity, one other feature which recommends itself to those interested in the fractionation of serum proteins is the fact that the technique causes little or no denaturation[3].

A variety of methods have been used to elute proteins; these include: (a) single eluants; (b) stepwise changes in pH or buffer concentration; (c) gradient elution using continuously varying pH, buffer concentration or salt concentration superimposed upon a buffer of constant composition.

Of these, the last method is probably the most commonly used. Although in theory the concentration profile of the gradient might possess an infinite number of shapes, the fact that linear or near linear gradients are the most easily produced has led to them being most frequently employed.

The availability of an LKB Ultragrad capable of producing highly complex gradients led us to take a simple system which has been well-tried in the author's laboratory to see if the use of non-linear gradients possesses any real advantage for relative large-scale fractionations. This report is of a preliminary investigation using quite simple non-linear gradients and by no means represents what might ultimately

be achieved by such a system. It deals principally with the behaviour of four proteins, haemopexin, transferrin, albumin and prealbumin, the first two of which are moderately difficult to resolve from one another.

EXPERIMENTAL

Materials

The ion-exchange cellulose (DE-32) was obtained from Whatman Ltd., Springfield Mill, Maidstone, Kent, and was pre-cycled according to the manufacturers' instructions before use and each successive re-use. All other chemicals were of analytical grade (BDH Ltd.).

Serum was obtained from outdated plasma containing acid, citrate, dextrose which was recalcified using a 40% calcium chloride solution (calcium chloride–plasma, 1:100 v/v). After standing for 17 h at 4 ° the formed elements of the clot were spun off and the serum dialysed against the starting buffer for the ion-exchange chromatography.

Method

The equipment was set up as shown in Fig. 1. Elution was effected by concentration gradients formed by mixing two solutions: (a) starting buffer, 0.05 *M* Tris titrated to pH 8 using concentrated hydrochloric acid; (b) strong buffer, 0.5 *M* Tris titrated to the same pH with hydrochloric acid.

In Expt. 1, after the sample was added the column was washed for a short time with starting buffer followed by a linear gradient and finally a short period with strong buffer. The total period for the cycle was four days. The level sensor was omitted.

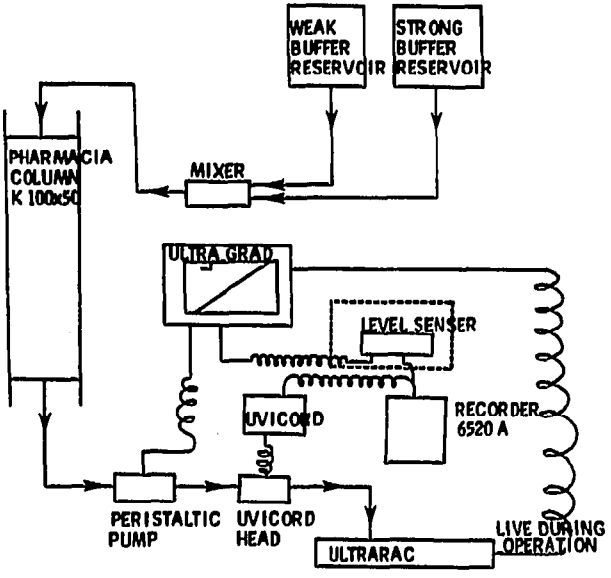


Fig. 1. Flow diagram of apparatus.

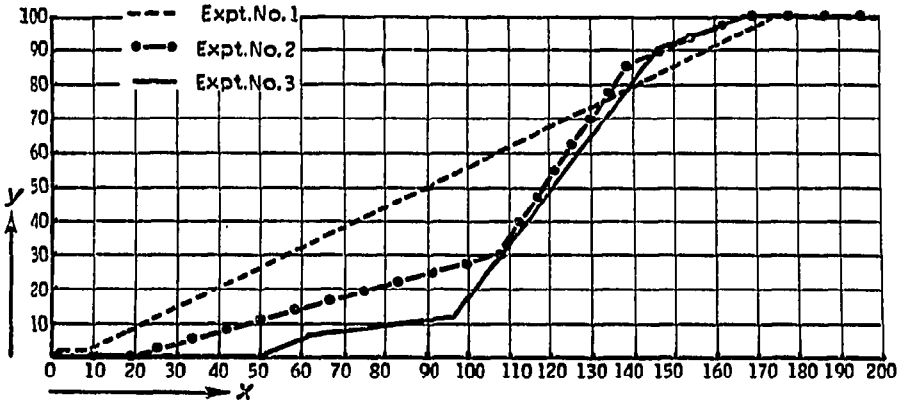


Fig. 2. Profile of gradients. *y*-Axis: salt concentration, in arbitrary units; *x*-axis: time, in arbitrary units.

In succeeding experiments (2 and 3) the gradient was progressively modified and the level sensor incorporated into the system. These modifications, with the original linear gradient, are shown in Fig. 2. The set time for the completion of the gradients was four days; however, the holding action of the level sensor prolonged this to approximately seven days. The set percentage transmittance at which the gradient was stabilised was 80%, *i.e.* a decrease in transmittance of 20% caused the gradient to be held.

In Expt. 4 the same gradient was used as in Expt. 3 but the set time for completion was two days and the action of the level sensor brought this to almost six days; the set transmittance for stabilisation of the gradient was the same as in Expts. 2 and 3.

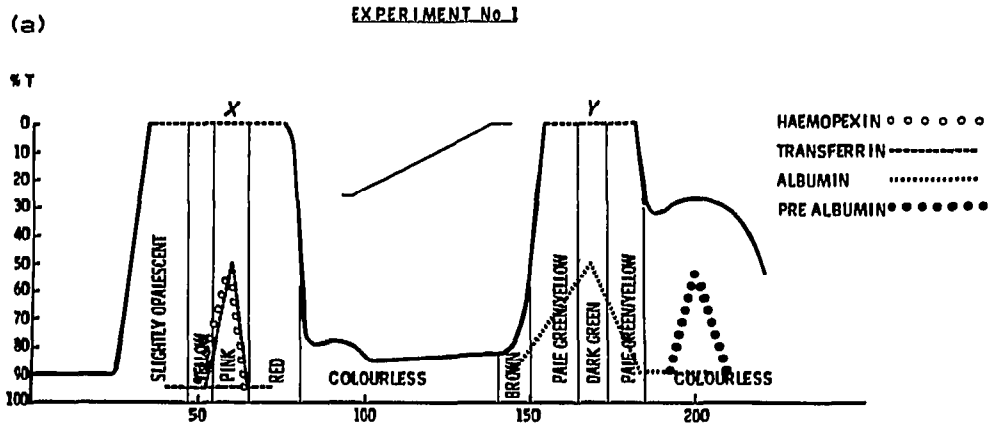
RESULTS

The UV absorption traces of the effluent obtained using the differential experimental conditions are shown in Figs. 3a,b,c and d. The profile of the gradient is shown as an insert in the centre of each diagram. The position of the individual proteins of especial interest are shown as an insert in the centre of each diagram. The position of the individual proteins of especial interest are shown as a series of peaks under the UV trace.

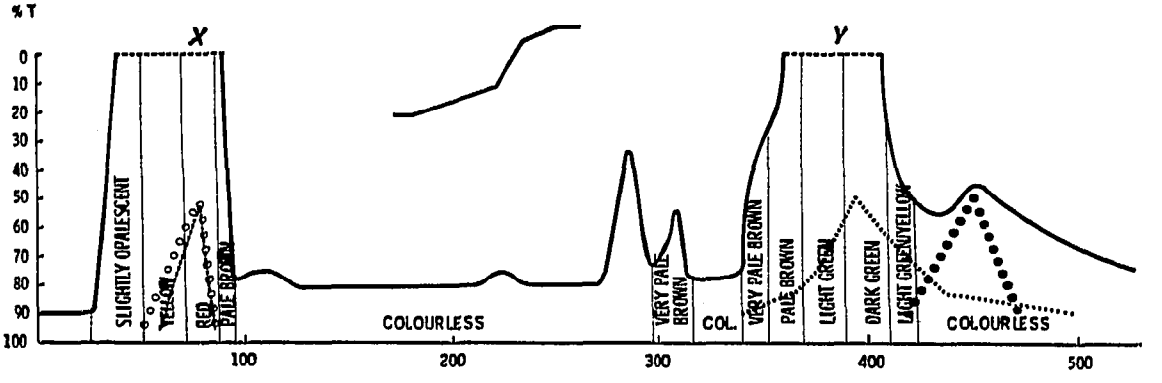
Figs. 4a,b, 5a,b, 6a,b and 7a,b show Laurell immunoelectropherograms of two corresponding zones on each UV absorption trace. Zone X was rich in haemopexin and/or transferrin and zone Y in albumin.

It can be seen that using a linear gradient, no effective separation of haemopexin or transferrin was achieved although pre-albumin and albumin were widely separated. In fact the pre-albumin obtained was free of most other serum proteins (Fig. 8), as shown by Laurell immuoelectrophoresis. In Expt. 2, the washing time with starting buffer was prolonged and the steepness of the initial part of the gradient diminished as compared to Expt. 1, achieving only a marginal improvement in the resolution in transferrin and haemopexin; slightly more haemopexin was eluted in

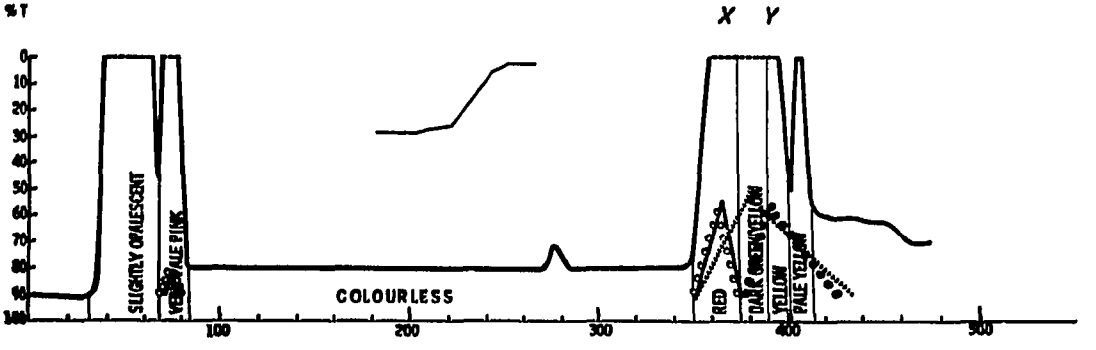
(a)


EXPERIMENT No 1
%T
X
Y
HAEMOPEXIN
TRANSFERRIN
ALBUMIN
PRE ALBUMIN
SLIGHTLY OPALESCENT
YELLOW
PINK
RED
COLOURLESS
BROWN
PALE GREEN/YELLOW
DARK GREEN
PALE GREEN/YELLOW
COLOURLESS
0
10
20
30
40
50
60
70
80
90
100
50
100
150
200

(b)


EXPERIMENT No 2
%T
X
Y
SLIGHTLY OPALESCENT
YELLOW
RED
PALE BROWN
COLOURLESS
VERY PALE BROWN
COL.
VERY PALE BROWN
PALE BROWN
LIGHT GREEN
DARK GREEN
LIGHT GREEN/YELLOW
COLOURLESS
0
10
20
30
40
50
60
70
80
90
100
100
200
300
400
500

(c)


EXPERIMENT No 3
%T
X
Y
SLIGHTLY OPALESCENT
PALE PINK
COLOURLESS
RED
DARK GREEN/YELLOW
YELLOW
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30
40
50
60
70
80
90
100
100
200
300
400
500

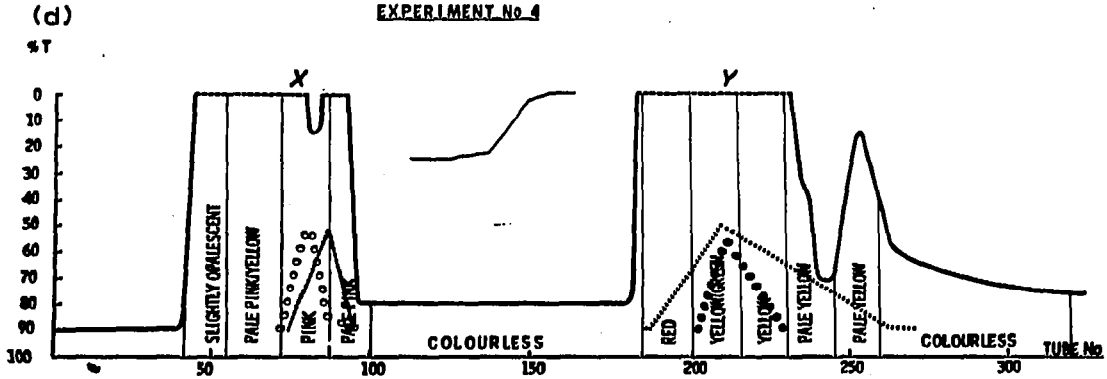

Fig. 3 (a) Elution profile obtained using gradient No. 1; (b) elution profile obtained using gradient No. 2 combined with level sensor; (c) elution profile obtained using gradient No. 3 combined with level sensor on 4-day setting; (d) elution profile obtained using gradient No. 3 combined with level sensor on 2-day setting.

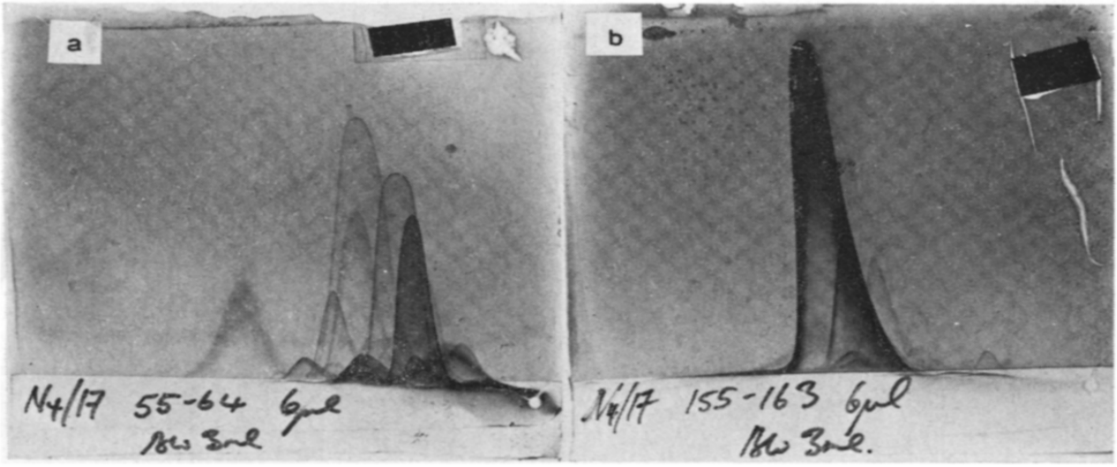

Fig. 4. (a) Laurell immunoelectropherogram of zone X from Expt. No. 1; (b) zone Y from Expt. No. 1.

the zone immediately preceding zone X. Protein occupying the front part of the second major UV absorbent peak was more clearly resolved.

In an attempt to optimise the resolution of haemopexin and transferrin, in Expt. 3 the initial washing with starting buffer was increased and the steepness of the first part of the gradient still further diminished. As can be seen from Fig. 3c, this was an over-correction and zones X and Y almost merged. To redress this situation and also improve resolution of the haemopexin and transferrin, the set time for completion of the gradient was reduced from four to two days while retaining the same gradient profile as in Expt. 3. As can be seen from Fig. 3d, zones X and Y were widely separate again and haemopexin was eluted significantly earlier than transferrin.

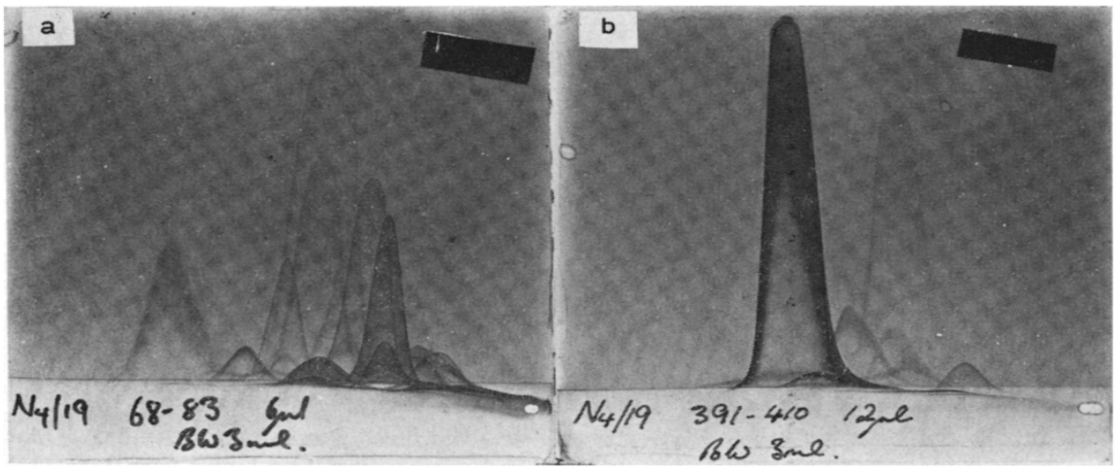

Fig. 5. (a) Laurell immunoelectropherogram of zone X from Expt. No. 2; (b) zone Y from Expt. No. 2.

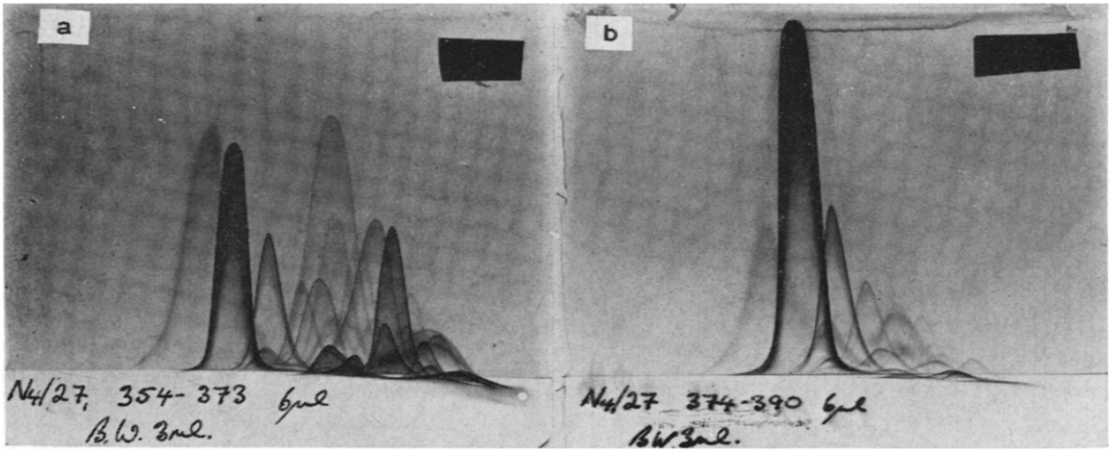

Fig. 6. (a) Laurell immunoelectropherogram of zone X from Expt. No. 3; (b) zone Y from Expt. No. 3.

The resolution of albumin and pre-albumin decreased progressively with deviation from a strictly linear gradient. Indeed the behaviour of albumin was not entirely predictable as it was split into a variety of subfractions. In Expt. 2 a small percentage was eluted some two hundred tubes earlier and in Expts. 3 and 4 the final absorption peak was almost exclusively albumin.

DISCUSSION

It can be seen from the preceding section that variations in the shape and/or time taken to complete a gradient produced significant alterations in the order in

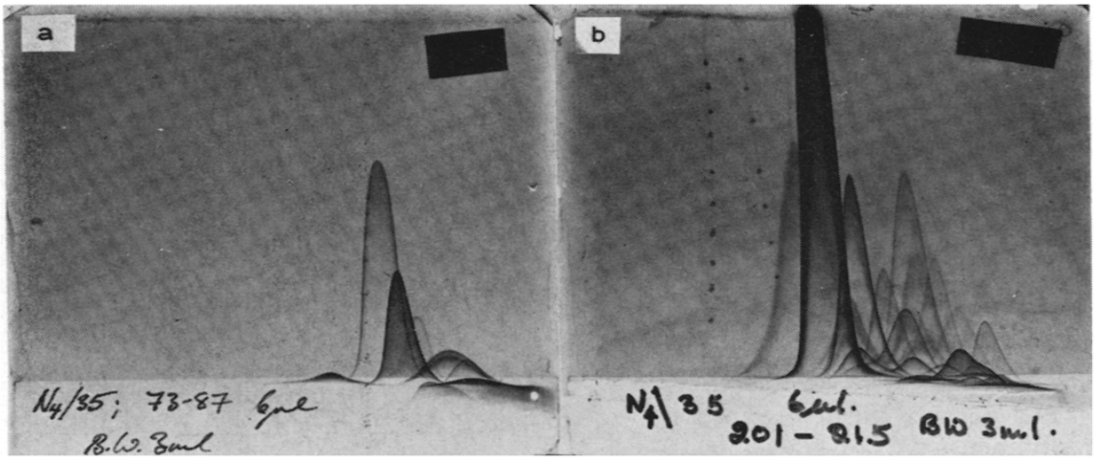

Fig. 7. (a) Laurell immunoelectropherogram of zone X from Expt. No. 4; (b) zone Y from Expt. No. 4.

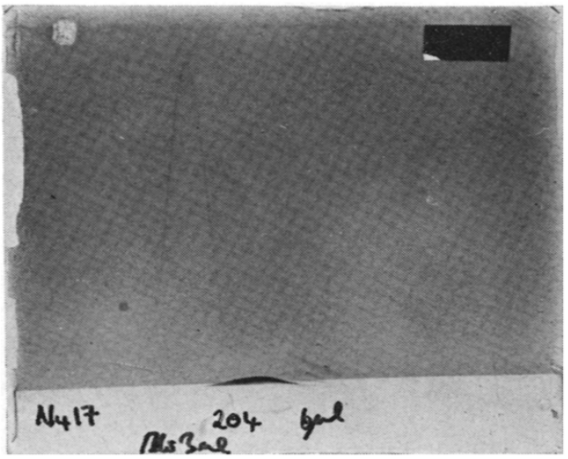

Fig. 8. Laurell immunoelectropherogram of the prealbumin band from Expt. No. 1.

which proteins were eluted from the DEAE-cellulose. Further, these variations did not have to be particularly gross to do this. The combination of the ultrograd, with its ability to form precisely controlled gradients, and the level sensor form a highly sensitive means of exploiting small differences in the ion-exchange characteristics of proteins. However, the level sensor did not make the shape of the gradient employed redundant as might have been suspected, but merely enhanced the basic resolution achieved using any of the gradient profiles. The fundamental disadvantage of the system as shown here was the non-selectivity of the property used to regulate the gradient former via the level sensor, *i.e.* UV absorption at 254 nm. This disadvantage is further increased because the monitoring device (Uvicord) was necessarily situated at the end of the colum. A time lag occurs between the

exceeding of the required counter ion concentration, the protein travelling down the column and emerging to trigger the level sensor which stabilises the gradient. During this lag, many more proteins may also be eluted off the column than the one desired. This will occur irrespective of the length of the column although it is a function of the actual duration of the time lag.

For a number of carrier proteins which transport ligands into which gamma emitters can be introduced (*e.g.* ^{59}Fe for transferrin, and in haem and haemoglobin for haemopexin and haptoglobin, respectively, [^{131}I]thyroxine for thyroxine binding globulin and prealbumin), delay could be minimised by triggering the level sensor via a collimated X-ray detector situated on the column itself.

ACKNOWLEDGEMENT

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