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Analysis of muscle cell culture medium by size-exclusion chromatography

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Abstract

The application of both low-pressure (preparative) and high-performance (analytical) size-exclusion chromatography to the fingerprinting of muscle cell culture supernatant is reported. The chromatograms showed significant differences between fresh media and muscle cell culture media. In addition, only one fraction derived from muscle culture medium contained factor(s) of proteic nature able to interfere with the cell cycle of a continuous proliferating cell line.

1. Introduction

In many cell types, including muscle cells, proliferation and differentiation are mutually exclusive events which are under the control of opposing cellular signals [1]. Even though the signals that play an important role in timing and regulating myogenesis in vivo are at present unknown, skeletal muscle primary cultures provide a reliable system to approach this problem because differentiation of skeletal myoblasts is accompanied by activation of muscle specific genes [2]. In fact, when skeletal myoblasts are placed in tissue culture, their differentiation is tightly controlled through a repression-type mechanism by serum and exogenous peptide growth factors that prevent entry into the differentiation pathway [2,3]. It has been reported that two of the most potent inhibitors of myoblast differentiation in vitro are fibroblast growth factor (FGF) and transforming growth factor type-β (TGF-β) [4,5]. On the other hand, entry of the myoblasts into the differentiation program depends on the presence of other growth factors, such as insulin like growth factors (IGFs I and II) [6].

In our previous experiments we have observed that supernatant of early muscle culture added to the fresh medium of proliferating cell lines affected their proliferation [7]. Conditioned culture media contain many peptide components, some of which are serum components, while others are secreted by the cells themselves. Purification and isolation of these peptide components might be very difficult since their concentration is usually very low. In addition, another major difficulty is the presence of very high levels of serum proteins (for instance, from fetal calf serum) in the cell culture media.

Size-exclusion chromatography (SEC) and

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high-performance SEC (HPSEC) are well established methods for separating proteins in complex mixtures according to their molecular mass. The aims of this work were to obtain fingerprints of the supernatants of muscle cell cultures by low-pressure (preparative) and high-performance (analytical) size-exclusion chromatography and to isolate factor(s) involved in the regulation of the cell cycle and present in myoblast culture supernatants. Fresh and conditioned myoblast culture media were chromatographed on Sephadex (G-50), Sephacryl (S-200 HR) and Superose 12 HR columns. The various fractions obtained were tested for their regulatory properties by means of an in-vitro bioassay.

2. Experimental

2.1. Cell cultures and media composition

Fresh culture medium (FCM) was composed of DMEM supplied with fetal calf (FCS) or adult horse serum (HS). Myoblast culture medium (MCM) was FCM in which muscle cells were grown. Conditioned medium (CM) consisted of FCM supplied with variable amounts of MCM.

Neonatal rat leg skeletal muscle cells were cultured in FCM according to Ref. [8].

African green monkey kidney VERO cells were cultured in FCM according to standard methods [9].

The effect of muscle culture supernatant on tumor cell proliferation was tested by adding different amounts to growing cellular medium.

2.2. DNA synthesis and cell proliferation assay

DNA synthesis was assayed by [^{3}H]TdR incorporation according to the procedure described in Ref. [10]. VERO cells cultured in microwell plates were pulsed for 6 h with 1 μCi/microwell [^{3}H]TdR, then they were harvested and washed on glass filters with a multiple cell harvester (Skatron, Norway). Results of [^{3}H]TdR incorporation were expressed as a percent of [^{3}H]TdR incorporation (dpm) of the untreated controls. Cell proliferation was estimated by counting the number of cell in several microscopic fields both in control and treated cultures.

2.3. Low-pressure chromatography

Size-exclusion experiments were performed under the following conditions: for Sephadex G-50 resin the column dimensions were 60×1 cm I.D.; the flow-rate was 30 ml/h; the eluent was PBS (phosphate buffered saline); the protein content was determined at 280 nm.

For Sephacryl S-200 HR resin the column dimension were 100×1.5 cm I.D.; the flow-rate was 30 ml/h; the eluent was 10% PBS (v/v in water); the protein content was determined at 280 nm. Sephadex G-50 and Sephacryl S-200 HR were purchased from Pharmacia (Uppsala, Sweden).

2.4. High-performance liquid chromatography

The water used for chromatography was deionized [Milli-Q (Millipore)] and all solutions were degassed and passed through a 0.45-μm Millipore filter prior to chromatography. All reagents used were of analytical-reagent grade.

Superose 12 HR (300×10 mm I.D.) (Pharmacia, Uppsala, Sweden) was connected to a Bio-Rad (Richmond, CA, USA) high-performance liquid chromatographic system, equipped with a Model UV-1806 variable-wavelength detector from Bio-Rad (set at 280 nm) and a Rheodyne Model 7125 sample injector (1-ml loop) [11]. The eluent was 10% PBS (v/v in water).

2.5. Protein assay

Protein concentration was determined by the method of Lowry et al. [12].

3. Results and discussion

We have observed that supernatant of early muscle culture added to the medium of immortalized cell lines inhibited their proliferation [7].

As a first approach towards the identification of the factor(s) involved in this process we have subjected the cell culture medium to gel filtration on a Sephadex G-50 resin. This type of chromatography was used as a relatively rapid method to establish to which molecular mass range the observed inhibitory effect could be assigned. As controls we performed gel-permeation experiments with fresh culture medium (FCM), fetal calf serum (FCS), horse serum (HS) and medium alone (DMEM). As eluent we selected a physiological buffered solution (PBS) that is known to be compatible with biological activities. The results are shown in Fig. 1. The amount of protein loaded was 4.0 mg for each sample. Three peaks were obtained from the supernatant of an early muscle cell culture: one peak was found at 20 ml (peak 1) and two smaller and broader peaks at 50 and 60 ml (peaks 2 and 3), respectively (Fig. 1, panel a). An identical profile was obtained for fresh culture medium (Fig. 1, panel b). In fetal calf serum (FCS) only peak 1 was present (Fig. 1, panel c) while in medium alone (DMEM) only the two smaller peaks 2 and 3 were present (Fig. 1, panel d). The presence of regulatory factor(s) in the

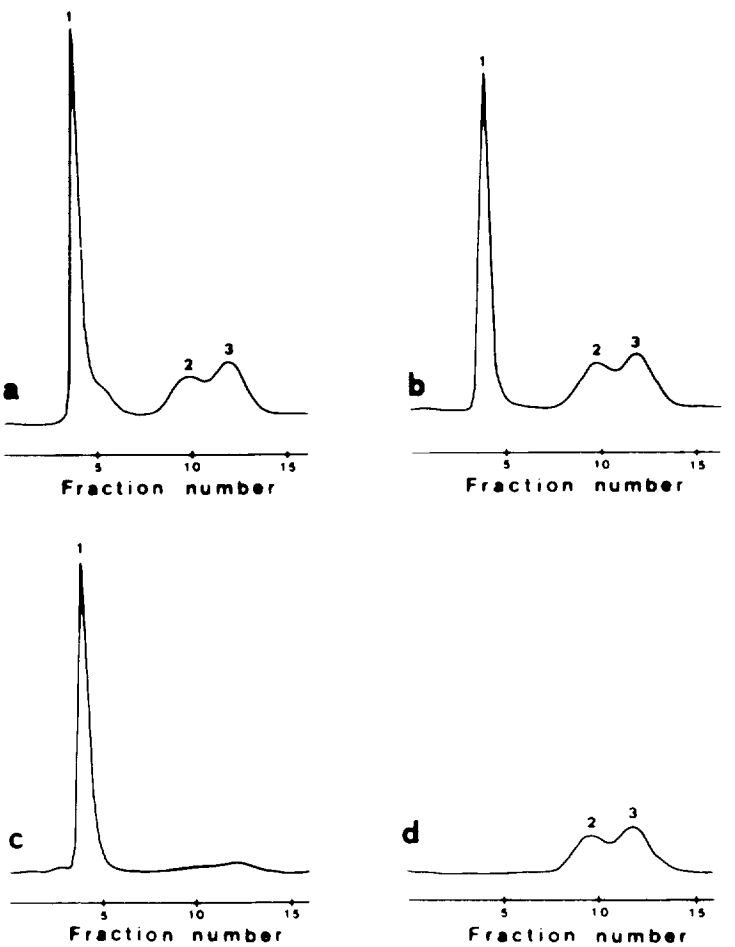

Fig. 1. Fractionation of culture media proteins on Sephadex G-50. Bed dimension: 1 × 60 cm; flow-rate: 30 ml/h; eluent: PBS. The effluents were monitored at 280 nm. Fraction volume: 5 ml. The amount of protein loaded was 4.0 mg. (a) Early muscle cell culture supernatant; (b) fresh cell culture medium; (c) fetal calf serum 10 times diluted in DMEM; (d) DMEM. The molecular size of the material contained in peaks 1, 2 and 3 is 30 kDa, 5 kDa and 1.5 kDa, respectively.

three peaks obtained from early muscle cell culture supernatant was tested with the bioassay described in the Experimental section. The fractions corresponding to each of the three peaks were pooled and used without any previous concentration procedure. The results are shown in Fig. 2. It is evident that only the fractions corresponding to peak 1 were able to inhibit proliferation of the VERO tumor cell line, as judged from reduced DNA synthesis compared with a control culture. The level of inhibition was the same as that displayed by unfractionated early muscle culture supernatant. In a parallel way the number of cells also decreased (results not shown). The decreased DNA synthesis and the lower cell number suggest an inhibitory effect on cell proliferation by peak 1 fractions.

Even though the chromatographic profiles from fresh culture medium and early muscle culture supernatant are identical (see Fig. 1, panels a and b) electrophoretic analysis of peak 1 revealed that the proteins present in fresh culture medium and in early muscle culture supernatant are very similar but not identical. In fact some components in the range of molecular mass 45 to 25 kDa appear to be unique in early culture supernatant and absent in fresh culture medium (see arrows in the electrophoretograms of Fig. 3).

In view of these results we decided to analyse the components of cell culture media in detail by means of a more powerful size-exclusion chromatographic gel. Superose 12 HR, a crosslinked, agarose-based medium is particularly suitable for high-performance gel permeation of biomolecules, characterized by a molecular mass operating range from 1 to 300 kDa. Calibration results were as follows: rabbit IgG with M_r 160 kDa, 19 min; bovine serum albumin with M_r 67 kDa, 24 min; lactalbumin with M_r 35 kDa, 27.5 min; cytochrome c with M_r 12 kDa, 38 min; vitamin B12, with M_r 376 Da, 45 min. Fig. 4 shows the four chromatograms obtained from 350 μg each of fresh culture medium and myoblast culture supernatant, supplied with fetal calf or horse serum. The chromatograms of fresh culture medium and early culture supernatant supplied

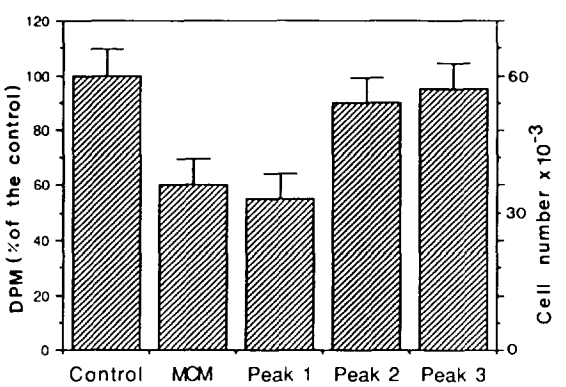

Fig. 2. Effect of Sephadex G-50 peaks on [^{3}H]TdR incorporation in VERO cells. VERO cells were seeded into DMEM medium with 10% heat-inactivated FCS and antibiotics. Unfractionated myoblast culture medium (MCM) and the pools corresponding to peak 1, 2 and 3 obtained from Sephadex G-50 column (see the corresponding chromatogram in Fig. 1, panel a) were added to a final concentration of 20% (v/v). Each experimental point is a mean of three measurements.

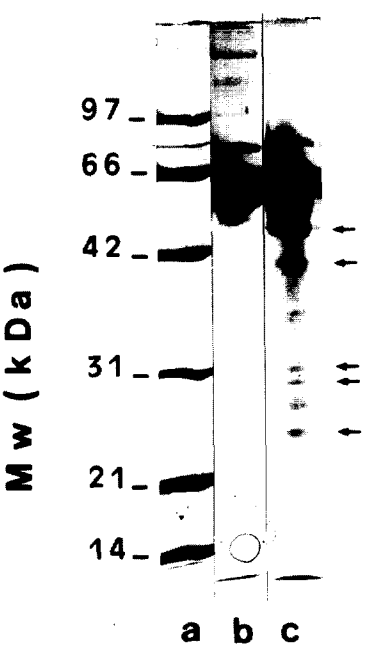

Fig. 3. SDS polyacrylamide gel electrophoretic analysis of effluents of Sephadex G-50 column. (a) Molecular mass standard; (b) peak 1 from fresh medium; (c) peak 1 from early muscle cell culture supernatant. Arrows point to components unique in early culture supernatant. Gel is stained with Coomassie brilliant blue.

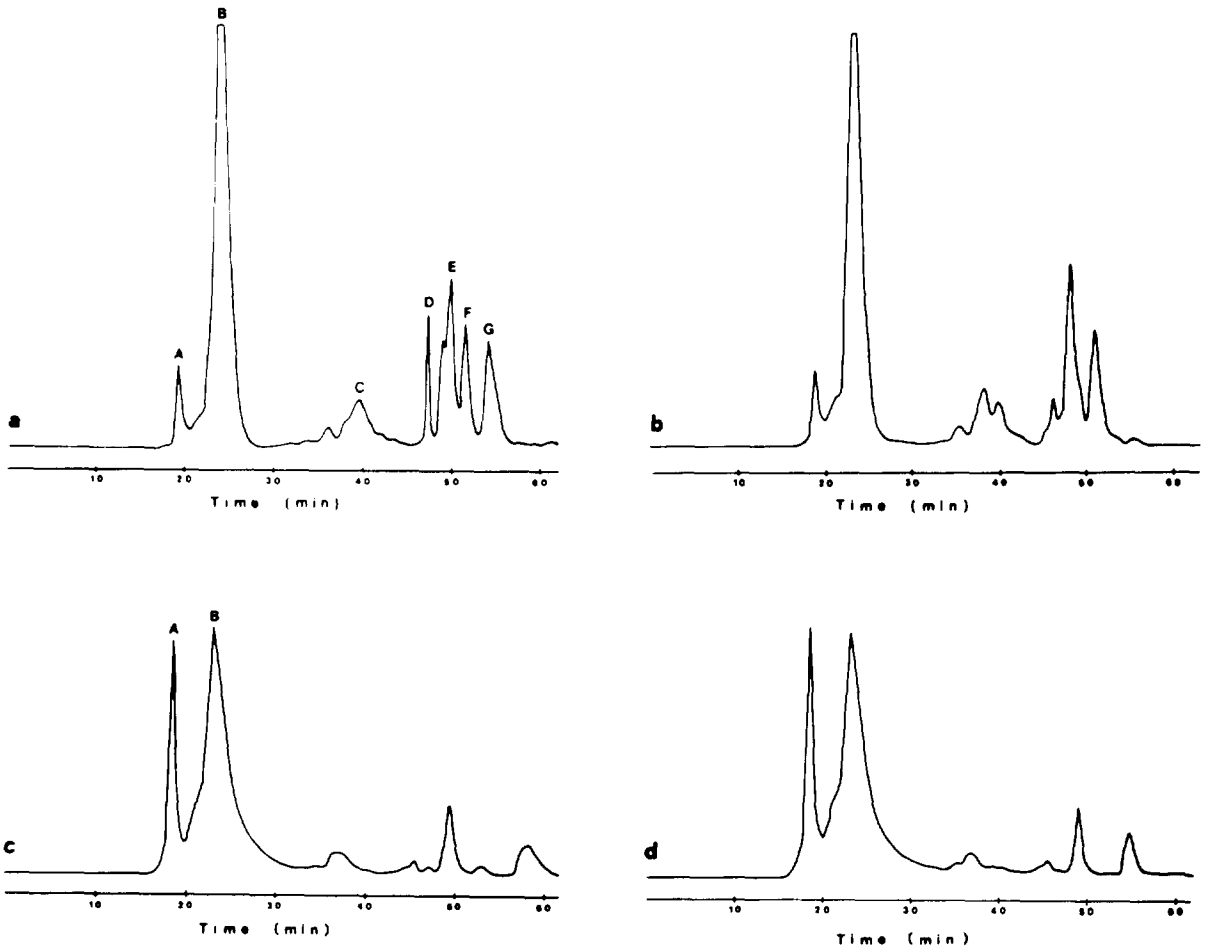

Fig. 4. Chromatograms obtained on a Superose 12 HR column (300 × 10 mm I.D.) with detection at 280 nm. Eluent: 10% PBS (v/v, in water); flow-rate, 0.4 ml/min. (a) Early culture supernatant in fetal calf serum; (b) fresh medium in fetal calf serum; (c) early culture supernatant in horse serum; (d) fresh medium in horse serum.

with fetal calf serum (Fig. 4, panels a and b) are characterized by the presence of several peaks, labelled A–G. The first two eluting peaks, corresponding to 160 kDa (peak A) and 68 kDa (peak B), respectively, are very similar in the two media. In contrast, there are differences in the relative distribution of the molecular masses of the minor compounds, corresponding approximately to the M_r lower than 1000. In the case of culture media supplied with horse serum we still observe peaks A and B, while in the low-molecular-mass area of the chromatograms less peaks are present (Fig. 4, panels c and d).

In order to obtain enough material for determination of the biological activity we used preparative chromatography with an appropriate resin. We selected low-pressure chromatography on Sephacryl S-200 HR, a resin characterized by a fractionation range of 5–250 kDa. The amounts loaded were in the order of 20 mg. The chromatograms obtained are comparable with those obtained on Superose 12 even though, as expected, more time was needed for the analysis and the resolution was not completely satisfactory. Fig. 5 shows, as a typical example of this type of chromatography, the pattern we obtained for myoblast early culture supernatant. The fractions corresponding to each peak (A–G)

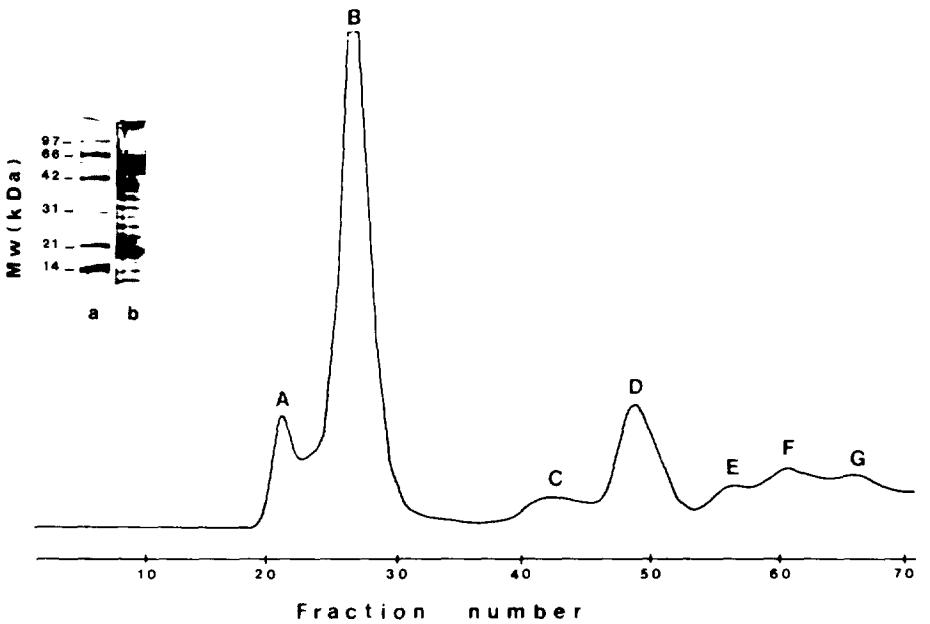

Fig. 5. Fractionation of early culture supernatant in fetal calf serum on Sephacryl S-200. Bed dimension: 15 × 100 cm; flow-rate: 30 ml/h; eluent: 10% PBS (v/v in water). The effluents were monitored at 280 nm. Fraction volume: 4 ml. Inset: electrophoretic analysis by SDS-polyacrylamide gel of proteins present in peak A. (a) Molecular mass standard; (b) peak A. Gels are stained with silver technique.

were pooled, lyophilized and then resuspended in a small volume of water (one tenth of the original volume). The volume of water added was such that the final salt concentration corresponded to 100% PBS. With the bioassay we have tested the presence of inhibitory activity in all the fractions of early myoblast culture supernatants (both fetal calf and horse serum supplied medium). Only the fractions of peak A could inhibit VERO tumor cell proliferation (Fig. 6). Table 1 shows that there was a 3-fold purification of the inhibitory effect. However, when examined in an SDS polyacrylamide gel under denaturing conditions, this column fraction still revealed the presence of several proteins characterized by a molecular mass range of 66 to 10 kDa (inset of Fig. 5). This broad range of molecular masses suggests that either the proteins are associated in the selected mobile phase (10% PBS) and elute as a single peak or that the bands correspond to monomers of large proteins. Since the inhibitory factor was largely impure at this stage, considerable effort is needed to prepare sufficient material for further purification and characterization.

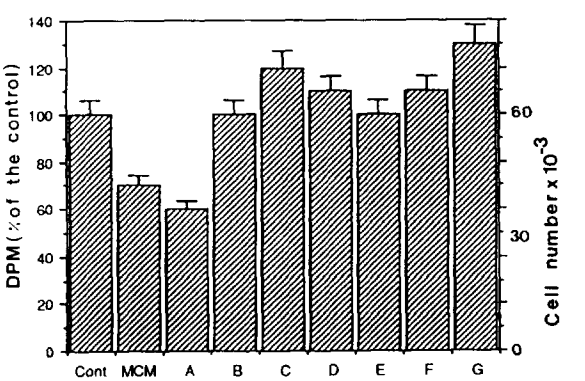

Fig. 6. Effect of S 200 peaks on [^{3}H]TdR incorporation in VERO cells. The experimental conditions are the same as those reported in the legend of Fig. 2, except that the peaks (A–G) are those obtained from S 200 column (see the chromatogram in Fig. 5).

Table 1
Purification of inhibitory factor from myoblast culture medium

	Protein concentration (mg/ml)	Inhibitory effect[a]	Purification factor
Crude	3.6	144	1
SEC (peak A)	1.2	48	3

[a] Amount of protein (μg) necessary to reduce tymidine incorporation in DNA of VERO cells to 50%.

4. Conclusions

In this paper we have demonstrated that SEC can be used for separating the components present in culture media. HPSEC provides a faster separation compared with the classical gels used for low-pressure SEC. Due to the complexity of the media it was not possible to purify the single components. Nevertheless we think it is interesting that a particular component, able to inhibit the growth of a tumor cell line, was present only in one chromatographic fraction.

Acknowledgements

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References

[1] E.N. Olson, *Dev. Biol.*, 154 (1992) 261.
[2] J.R. Florini, D.Z. Ewton and K.A. Magri, *Ann. Rev. Physiol.*, 53 (1991) 201.
[3] E.N. Olson, *Sem. Cell Biol.*, 3 (1992) 127.
[4] C.H. Clegg, T.A. Linkhardt, B.B. Olwin and S.D. Hauschka, *J. Cell Biol.*, 105 (1987) 949.
[5] E.N. Olson, W. Sternberg, J.S. Hu, G. Spizz and C. Wilcox, *J. Cell Biol.*, 103 (1986) 1799.
[6] J.R. Florini, K.A. Magri, D.Z. Ewton, P.L. James, K. Grindstaff and P.S. Rotwein, *J. Biol. Chem.*, 106 (1988) 2127.
[7] P. Arslan, M.L. Massimino and M. Cantini, *Basic Applied Miology*, 3 (1993) 191.
[8] M. Cantini, S. Sartore and S. Schiaffino, *J. Cell Biol.*, 85 (1980) 903.
[9] E. Papini, D. Sandona', R. Rappuoli and C. Montecucco, *EMBO Journal*, 7 (1988) 3353.
[10] M. Cantini, A. Cossarizza, F. Bersani, R. Cadossi, G. Ceccherelli, R. Tenconi, C. Gatti and C. Franceschi, *J. Bioelectricity*, 5 (1986) 91.
[11] L. Dalla Libera and J.H. Collins, *Bioscience Report*, 13 (1993) 289.
[12] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, *J. Biol. Chem.*, 193 (1951) 265.