Dwek, R. A. (1973), Nuclear Magnetic Resonance in Biochemistry, Oxford, Clarendon Press, p 42.
Eytan, G. D., Carroll, R. C., Schatz, G., and Racker, E. (1975), *J. Biol. Chem. 250,* 8598-8603.
Henderson, R., Capaldi, R. A., and Leigh, J. S. (1977), *J. Mol. Biol. 112,* 631-648.
Hesketh, T. R., Smith, G. A., Houslay, M. D., McGill, K. A., Birdsall, N. J. M., Metcalfe, J. C., and Warren, G. B. (1976), *Biochemistry 15,* 4145-4151.
Hong, K., and Hubbell, W. L. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2617-2621.
Hunig, S., Benzing, E., and Lucke, E. (1957), *Chem. Ber. 90,* 2833-2840.
Jost, P. C., Griffith, O. H., Capaldi, R. A., and Vanderkooi, G. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 480-484.
Lichtenburg, D., Petersen, N. O., Girardet, J.-L., Kainosho, M., Kroon, P. A., Seiter, C. H. A., Feigenson, G. W., and Chan, S. I. (1975), *Biochim. Biophys. Acta 382,* 10-21.
Longmuir, K. J., and Dahlquist, F. W. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 2716-2719.
Lowry, O. H. Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265-275.
Mabrey, S., and Sturtevant, J. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3862-3866.
Mathey, S., and Bensoam, J. (1971), *Tetrahedron 27,* 3965-3969.
Privalov, P. L., Plotnikov, V. V., and Filimonov, V. V. (1975), *J. Chem. Thermodyn. 7,* 41-47.
Robinson, N. C., and Capaldi, R. A. (1977), *Biochemistry 16,* 375-381.
Ruben, G. C., Telford, J. N., and Carroll, R. C. (1976), *J. Cell Biol. 68,* 724-739.
Seelig, A., and Seelig, J. (1974), *Biochemistry 13,* 4839-4845.
Sheetz, M. B., and Chan, S. I. (1972), *Biochemistry 11,* 4573-4581.
Singer, S. J. (1974), *Annu. Rev. Biochem. 43,* 805-833.
Smith, L. (1955), *Methods Enzymol. 2,* 732-739.
Smith, L., and Camerino, P. W. (1963), *Biochemistry 2,* 1432-1439.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., and Smith, I. C. P. (1976), *Biochemistry 15,* 954-966.
Stubbs, G. W., Litman, B. J., and Barenholz, Y. (1976), *Biochemistry 15,* 2766-2772.
Vanneste, W. H., Ysebaert-Vanneste, M., and Mason, H. (1974), *J. Biol. Chem. 249,* 7390-7401.
Vik, S., and Capaldi, R. A. (1977), *Biochemistry* (in press).
Waggoner, A. S., Kingzeth, T. J., Rottschaefer, S., Griffith, O. H., and Keith, A. D. (1969), *Chem. Phys. Lipids 3,* 245-253.
Wells, M. A., and Hanahan, D. J. (1969), *Biochemistry 8,* 414-424.
Yu, C.-A., Yu, L., and King, T. E. (1975), *J. Biol. Chem. 250,* 1383-1392.

Lipid Requirements for Cytochrome *c* Oxidase Activity†

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ABSTRACT: Cytochrome *c* oxidase depleted of endogenous lipid by detergent exchange has been reconstituted into vesicles with synthetic lipids of known head group and fatty acid composition and enzymic activities have been measured. No evidence for head group specificity was found. However, the enzyme does require the fluid environment provided by unsaturated fatty acids. The state of dispersion of the enzyme was found to affect the activities regenerated in reconstitution studies. The highest activities were obtained using lysolecithin containing an oleoyl fatty acid as the lipid component.

Cytochrome *c* oxidase, the terminal member of the respiratory chain, is a multi-subunit enzyme (Briggs et al., 1975; Downer et al., 1976), containing two hemes and two copper atoms (Kuboyama et al., 1972; Yonetani, 1961). The protein spans the mitochondrial inner membrane (Hackenbrock and Hammon, 1975; Schneider et al., 1972; Henderson et al., 1977) and probably exists as a dimer or four-heme complex (Robinson and Capaldi, 1977; Briggs and Capaldi, 1977; Henderson et al., 1977).

After isolation from the membrane, cytochrome *c* oxidase requires phospholipids or certain other amphiphiles for activity. The importance of different lipid head groups for activity has been examined in several laboratories (Brierley and Merola, 1962; Awasthi et al., 1971; Yu et al., 1975) but the results have been variable for reasons which were discussed earlier (Robinson and Capaldi, 1977). Our recent studies on the interaction of detergents with cytochrome *c* oxidase have provided evidence that the activity of this enzyme is sensitive to the fatty acid composition of bound amphiphile (Robinson and Capaldi, 1977). Here, we have measured the activity of cytochrome *c* oxidase in the presence of synthetic phospholipids of known head group and fatty acid composition. Our results indicate that cytochrome *c* oxidase requires phospholipids with unsaturated fatty acids for optimal activity. If a fluid lipid environment is provided, the enzyme is apparently insensitive to the phospholipid head group.

Materials and Methods

Cytochrome *c* oxidase was isolated from beef heart mitochondria as described by Capaldi and Hayashi (1972). Asolectin (95% soy phosphatides) was obtained from Associated Concentrates and used without further purification. Mitochondrial lipids were isolated from beef heart mitochondria as described by Rouser and Fleischer (1967). The following

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synthetic lipids were obtained from Supelco Inc.: DLPC, DSPC, DOPC, POPC, DOPE, lyos-PC-1-stearoyl, and lyso-PC-1-oleoyl.[1] Two phospholipids, DMPC and DOPC, were obtained from Sigma; DPPC, DPPE, and DLPE were the kind gift of Dr. Bruce Gaber, University of Oregon; lyso-PC-1-palmitoyl was generously provided by K. Longmuir, University of Oregon. All these lipids appeared as single spots in thin-layer chromatography.

Cholic acid was obtained from Aldrich Chemical Co. [^{3}H]Cholate was the gift of Dr. P. Jost, University of Oregon. Cytochrome *c* (grade IIA), ascorbic acid, Tween 80, Tween 60, Tween 40, Tween 20, and Triton X-100 were all obtained from Sigma.

Cytochrome *c* oxidase was depleted of lipid by gel filtration in the presence of 1 mM Triton X-100 as described by Robinson and Capaldi (1977). Enzymic activity was measured at 25 °C unless stated using an oxygen electrode (Yellow Springs Co.). The assay medium (2 mL) contained 50 mM sodium phosphate (pH 7.4), 25 μM cytochrome *c*, 30 mM ascorbic acid (previously adjusted to pH 7.4). In some experiments Tween 80 or Tween 60 (0.5%) was included in the assay buffer. Corrections were made for oxygen consumption in the absence of cytochrome *c* oxidase (this value was typically 25 nmol of O min^{-1}).

Lipids were prepared for reconstitution experiments by sonicating with a tip sonicator in the presence of a twofold excess (by weight) of cholate in 50 mM sodium phosphate at pH 7.4. Lipid depleted cytochrome *c* oxidase was mixed with the lipid-cholate mixed micelles and reconstitution was achieved either by diluting the mixture into the assay medium directly or by dialyzing away the cholate and Triton X-100 (in cholate-Triton X-100 mixed micelles) into 1 L of phosphate buffer (pH 7.4).

Protein concentrations were obtained by the method of Lowry et al. (1951). Phospholipid was measured as inorganic phosphate as described by Chen et al. (1956). Head group composition was examined by thin-layer chromatography as described previously (Robinson and Capaldi, 1977). Heme *a* concentrations were measured by the pyridine hemochromogen difference spectral method of Williams (1964).

Results

Phospholipid Depletion and Exchange of Tightly Bound Cardiolipin. Cytochrome *c* oxidase was depleted of phospholipid by detergent exchange using Triton X-100 as the competing amphiphile (Robinson and Capaldi, 1977). The enzyme retained 50–65 μg of phospholipid per mg of protein during this treatment, most of which was cardiolipin, although small amounts of PC and PE were also detected (Table I). The phospholipid-depleted enzyme was essentially inactive but could be activated by the addition of either phospholipids or detergents which contain fatty acids.

The possibility that a synthetic phospholipid would exchange with the detergent nonexchangeable cardiolipin under the conditions of reconstitution was tested. Phospholipid-depleted enzyme (4 mg) was mixed with DMPC (20 mg) added as a cholate-lipid mixture (2 mg of cholate per mg of lipid), and the detergent was removed by dialysis. The vesicles so formed were concentrated by using solid sucrose to remove water and the phospholipid was dissociated from the enzyme by dissolving in Triton X-100 (2 mg of detergent per mg of protein) and eluting this solution through a column of Sepharose 4B equilibrated with the same detergent. Two-dimensional thin-layer chromatography of the small amount of phospholipid which remained associated with the eluted enzyme (60–70 μg of phospholipid per mg of protein) revealed that essentially all of the cardiolipin was retained by the protein and had not exchanged with the nearly 100-fold molar excess of synthetic PC present (Table I).

TABLE I: Phospholipids Extracted from Cytochrome *c* Oxidase after Triton X-100 Exchange.[a]

Phospholipid	μg of phospholipid/mg of protein: Stock cytochrome *c* oxidase	DMPC cytochrome *c* oxidase
Cardiolipin	54	49
PC	13	16
PE	5	5

[a] Stock cytochrome *c* oxidase contained 100–150 μg of phospholipid per mg of protein. DMPC cytochrome *c* oxidase contained 5 mg of DMPC per mg of protein and was reconstituted by the dialysis procedure. Detergent exchangeable lipid was removed by gel filtration in Triton X-100 and the remaining lipid was analyzed.

Reconstitution of Cytochrome c Oxidase with Diacyl Phospholipids. The amount of phospholipid required for optimal regeneration of activity was determined by assaying mixtures containing different ratios of protein to lipid. For all phospholipids tested cytochrome *c* oxidase electron transfer activity increased as more lipid was added. Optimal activity was obtained with DOPC above 1.0 mg of phospholipid/mg of protein and for asolectin above 2.5 mg of phospholipid/mg of protein. Optimal activities were retained at up to 5 mg of phospholipid/mg of protein for each of the lipids tested and this ratio of protein to lipid was used in all experiments unless otherwise stated. Activity was not significantly affected by uncouplers, possibly in part because the vesicles formed at this level of lipid are leaky to protons. When more lipid is used, i.e. at ratios of 1 part of protein to 15 or even 30 parts of lipid, the turnover of the enzyme becomes limited by the availability of protons on the inside of the vesicles and the activity is thus stimulated manyfold by adding uncouplers plus valinomycin (Hinkle et al., 1972; Hunter and Capaldi, 1974; Wrigglesworth and Nicholls, 1975).

As one approach to reassociating phospholipids with the lipid-depleted enzyme, the two were mixed in the presence of cholate and diluted approximately 1:100 for assay with detergent-free buffer. The structure of the protein-phospholipid complex formed by this procedure was examined by electron microscopy. Negatively stained samples showed predominantly small vesicles of 250–1000 Å in diameter along with some nonvesicular material.

Cytochrome *c* oxidase activities obtained in the presence of various phospholipids are compared in Figure 1. These activities were measured during a span of several months and with different batches of enzyme which did not all have the same activity. In order to make meaningful comparisons of different experiments, the enzyme was always assayed in asolectin and the activities in the presence of the other phospholipids were compared to this value. Typically the activity measured in asolectin was 15 μmol of O min^{-1} (mg of protein)$^{-1}$. The results obtained by this dilution procedure were

[1] Abbreviations used are: DLPC, dilaurylphosphatidylcholine; DSPC, distearoylphosphatidylcholine, DOPC, dioleoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPE, dipalmitoylphosphatidylethanolamine; DLPE, dilaurylphosphatidylethanolamine; ML, mitochondrial lipids; PC, phosphatidylcholines; lyso-PC, lysophosphatidylcholine.

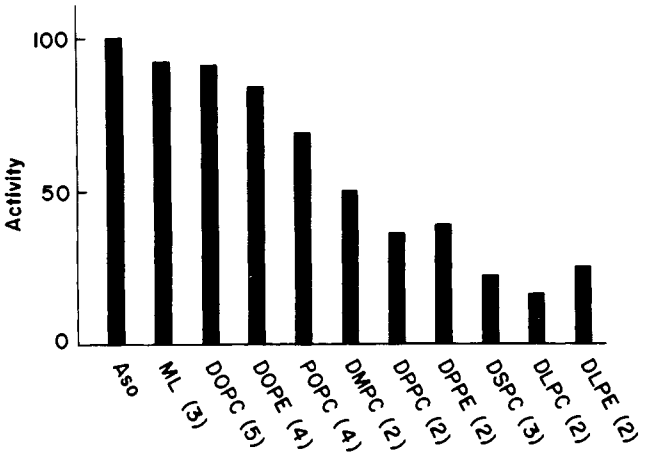

FIGURE 1: Cytochrome *c* oxidase activities reconstituted by dilution. Activities are expressed as a percentage of the activity obtained with asolectin. The numbers in parentheses indicate the number of experiments averaged to give the percentage activity shown in the paragraph.

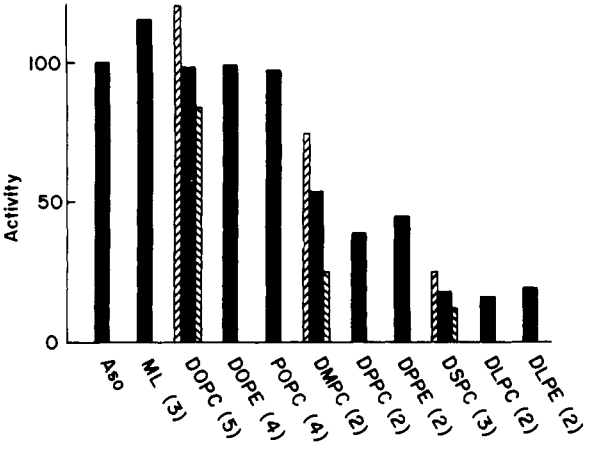

FIGURE 2: Cytochrome *c* oxidase activities reconstituted by dialysis. Activities are expressed as a percentage of the activity obtained with asolectin. The cross-hatched barographs show the activities (average of duplicate experiments) at 30 °C (left side) and 20 °C (right side) for DOPC, DMPC, and DSPC.

reproducible during any one day (±5%), but the reproducibility from one day to the next was not as good (±20%). The activities shown in Figure 1 are averages of all the results obtained. Within experimental error the activities measured in asolectin, ML, DOPC, and DOPE were the same. Phospholipids with only saturated fatty acids were found to regenerate lower enzymic activities than those with one or two unsaturated fatty acids. When two phospholipids differed in head group but not in fatty acid composition, the resulting enzymic activities were essentially the same.

As a second approach to reassociating phospholipids with the lipid-depleted enzyme, the two were mixed in the presence of cholate, as before, but the mixture was dialyzed to remove most of the cholate and some of the Triton X-100 (in mixed micelles of cholate–Triton X-100) before being diluted for assay. The length of time of dialysis was found to affect the activity of the enzyme with any of the phospholipids tested. After 1 h of dialysis the activity with asolectin, DOPC, or DSPC had dropped to about 50% of the activity before dialysis. In this time, 85% of the cholate had been removed as measured by using [^{3}H]cholate. Longer times of dialysis had little further effect on enzymic activity and thus in all experiments the samples were dialyzed for 1 h. The protein–phospholipid complexes obtained by dialysis were also examined by electron microscopy and found to be vesicular, much like those generated by dilution.

Cytochrome *c* oxidase activities obtained in the presence of various phospholipids after dialysis are compared in Figure 2. As before, the activity regenerated by each phospholipid is expressed as a percentage of the activity of the enzyme in asolectin. Typically, the activity measured in asolectin after 1 h of dialysis was 7 μmol of O min^{-1} (mg of protein)$^{-1}$. The results obtained with this reconstitution method were much more reproducible from one day to the next (±10%) than those obtained with the dilution procedure. The activities regenerated by asolectin, ML, DOPC, DOPE, and POPC were the same. The activities obtained with unsaturated phospholipids were significantly higher than those obtained with phospholipids containing only saturated fatty acids. Also, when two phospholipids differed in head group but not in fatty acid composition, the resulting activities were essentially the same, paralleling the results of the dilution procedure.

Cytochrome *c* oxidase reconstituted by the dialysis method with both DOPC and DMPC was assayed at 20 and 30 °C in addition to 25 °C. Figure 2 shows that at 20 °C the activity in DMPC is 30% of the activity in DOPC. At 25 °C this value increases to 50% and at 30 °C it is even higher.

TABLE II: Cytochrome *c* Oxidase Activity in Lysolipids and Tween Detergents.[a]

Lysolipid	Activity	Detergent	Activity
Lyso-PC-1-oleoyl	115	Tween 80	70
Lyso-PC-1-palmitoyl	88	Tween 40	54
Lyso-PC-1-stearoyl	74	Tween 60	48

[a] These activities are expressed as a percentage of the activity obtained for the enzyme in asolectin and reconstituted by the dilution procedure.

Reconstitution of Cytochrome c Oxidase with Lysolipids. Phospholipid-depleted cytochrome *c* oxidase was mixed with lysolipids and the activity regenerated was measured. In each case the enzyme was added to the lysolipid in the ratio of 1 mg of protein per 5 mg of lipid and the protein–lipid micelles were diluted with buffer for assay. Cytochrome *c* oxidase activity was highest in lyso-PC-1-oleoyl, followed by lyso-PC-1-palmitoyl and lyso-PC-1-stearoyl (Table II). For comparison, the lipid-depleted enzyme was also assayed in the presence of detergents of the Tween series. The same ordering in activity according to fatty acid composition was observed, since Tween 80 is on oleate ester, Tween 60 is a stearate ester, and Tween 40 is a palmitate ester. Amphiphiles of each type with an unsaturated fatty acid regenerated higher activities than those with a saturated fatty acid. In each case, lyso-PC's gave a higher enzymic activity than the corresponding Tween detergent and the activity with either lyso-PC's or Tweens was higher than that generated by the corresponding diacyl form of the phospholipid.

Discussion

The role of both polar head groups and fatty acids in the activity of cytochrome *c* oxidase can be examined by reconstitution studies using synthetically derived phospholipids. In this study the enzyme was stripped of lipid (except for the tightly bound cardiolipins) by detergent exchange, using Triton X-100 as the competing amphiphile. This provided a water-soluble and lipid-depleted enzyme which was inactive in the absence of any amphiphile and which was inactive in the presence of high concentrations of Triton X-100 or cholate

used in our reconstitution procedures. Detergent-solubilized protein was mixed with phospholipid, added in soluble form as mixed cholate–lipid micelles in the case of diacyl lipids, or as lysolipid micelles in the case of the monoacyl lipids. Protein and lipid were reacted by lowering the concentration of detergents below their critical micelle concentration and this led to regeneration of enzymic activity. The detergent was removed by dialysis or by diluting the mixture into detergent-free buffer. The two different approaches were adopted in order to check that differences in activity seen in the presence of different lipids were not an artifact of a particular reconstitution procedure. The same conclusions about head group and fatty acid specificity could in fact be drawn from either of the reconstitution procedures used.

Cytochrome *c* oxidase was found to have no phospholipid polar head group specificity. The activities regenerated by PC and PE containing the same unsaturated fatty acids were identical and were as high as those obtained with soybean lipid and beef heart mitochondrial lipids, both of which contain cardiolipin in addition to PC and PE.

With respect to fatty acid requirements, optimal cytochrome *c* oxidase activity was obtained with unsaturated fatty acids whether diacyl lipids, lysolipids, or Tween detergents were used. The effect of fatty acids on activity was most dramatic with diacyl lipids. Cytochrome *c* oxidase in vesicles of DOPC was 5 times as active as in vesicles of DSPC and three times as active as in vesicles of DPPC. The mixed lipid, 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), activated the enzyme to the same extent as DOPC. These results indicate a correlation of bilayer fluidity with enzymic activity. The temperature of the assay (25 °C) is well above the phase transition temperature of DOPC (−22 °C) and POPC (3 °C) (Phillips et al., 1972; op den Kamp et al., 1975; de Kruyff et al., 1973) but is below the phase transition temperature of DPPC (42 °C) and DSPC (54 °C) (Hinz and Sturtevant, 1972). The activity obtained in DLPC was not as high as might be expected given that the transition temperature of this lipid is −1 °C (Mabrey and Sturtevant, 1976) but this may be a consequence of the short chain length of this fatty acid.

The importance of fluidity to cytochrome *c* oxidase activity is further emphasized by results with DMPC. This phospholipid undergoes a phase transition near 23 °C (Hinz and Sturtevant, 1972). At 20 °C with DMPC in a crystalline state, enzymic activity was only 30% of that in DOPC. Just above the phase transition temperature (at 25 °C), with the lipids in a fluid state, enzymic activity was much higher and at 30 °C had risen to more than 60% of that regenerated by DOPC at this temperature.

The relationship between the fluidity of the lipid and cytochrome *c* oxidase activity is unclear. It could be that the enzyme molecules become aggregated together when lipids are in a "crystalline state", as seen when sarcoplasmic reticulum Ca^{2+} ATPase is reconstituted with saturated lipids (Kleemann and McConnell, 1976). Such an effect might drastically affect activity. Alternatively, a crystalline lipid phase may impose conformational constraints on cytochrome *c* oxidase which would presumably lower the turnover rate of the enzyme. Further experiments are needed to determine between these and other possibilities.

Cytochrome *c* oxidase activity was less sensitive to fatty acid composition when assayed in lysolipids and detergents of the Tween series. The activity obtained with lyso-PC-1-oleoyl is less than twice as high as that obtained with the saturated fatty acid containing lyso-PC-1-palmitoyl or lyso-PC-1-stearoyl. The activity with Tween 80, which contains an oleoyl fatty acid, is less than twice as high as that obtained with other Tweens, including Tween 20 with its lauryl fatty acid. These findings are consistent with the generally more fluid interior of micelles made from lysolipids or detergents containing a saturated fatty acid. These structures do not show the phase properties seen in bilayers.

The different activities obtained when protein was reassociated under different conditions and the enhanced activity of the enzyme in the presence of monoacyl lipid indicate that the state of dispersion of cytochrome *c* oxidase affects the activity that can be obtained in reconstitution studies. When protein and lipid were reassociated relatively slowly, i.e., by dialyzing away the detergent, a lower activity was regenerated than when the two were interacted rapidly, i.e., by diluting out the detergent. It may be that dialysis produces vesicles with more of the cytochrome *c* oxidase molecules oriented with their cytochrome *c* binding site inward, or this procedure may produce more concentric vesicles than the dilution procedure. Either of these effects would shield cytochrome *c* oxidase from substrate cytochrome *c*. The highest electron-transfer activities were obtained when cytochrome *c* oxidase was mixed with lyso-PC-1-oleoyl. The micelles so formed not only provided the optimal environment for enzyme function, but the protein was fully dispersed under these conditions. Lyso-PC-1-oleoyl has been shown to act as an activating detergent for sarcoplasmic reticulum Ca^{2+} ATPase (Peterson and Deamer, 1977), and this monoacyl lipid may be a generally useful detergent for membrane studies.

In summary, cytochrome *c* oxidase functions optimally in a fluid environment of fairly long fatty acid chains. There appears to be no head group specificity, other than a possible requirement for the tightly bound cardiolipin identified earlier (Awasthi et al., 1971; Robinson and Capaldi, 1977). These lipid molecules did not exchange for added synthetic PC (in a 100-fold excess) during the course of reconstitution. Their importance to the structure and functioning of cytochrome *c* oxidase cannot be assessed definitively until methods are devised to remove them without denaturing the enzyme.

References

Awasthi, Y. C., Chuang, T. Y., Keenan, T. W., and Crane, F. L. (1971), *Biochim. Biophys. Acta 226,* 42.

Brierley, G., and Merola, A. (1962), *Biochim. Biophys. Acta 64,* 205.

Briggs, M. M., and Capaldi, R. A. (1977), *Biochemistry 16,* 73.

Briggs, M., Kamp, P.-F., Robinson, N. C., and Capaldi, R. A. (1975), *Biochemistry 14,* 5123.

Capaldi, R. A., and Hayashi, H. (1972), *FEBS Lett. 26,* 261.

Chen, P. S., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28,* 1756.

de Kruyff, B., Demel, R. A., Stotboom, A. J., van Deenan, L. L. M., and Rosenthal, A. F. (1973), *Biochim. Biophys. Acta 307,* 1.

Downer, N. W., Robinson, N. C., and Capaldi, R. A. (1976), *Biochemistry 15,* 2930.

Hackenbrock, C. R., and Hammon, K. M. (1975), *J. Biol. Chem. 250,* 9185.

Henderson, R., Capaldi, R. A., and Leigh J. (1977), *J. Mol. Biol.* (in press).

Hinkle, P. C., Kim, J. J., and Racker, E. (1972), *J. Biol. Chem. 247,* 1338.

Hinz, H. J., and Sturtevant, J. M. (1972), *J. Biol. Chem. 247,* 6071.

Hunter, D. R., and Capaldi R. A. (1974), *Biochem. Biophys. Res. Commun. 56,* 623.

Kleemann, W., and McConnell, H. M. (1976), *Biochim. Biophys. Acta 419,* 206.
Kuboyama, M., Yong, F. C., and King, T. E. (1972), *J. Biol. Chem. 247,* 6375.
Lowry, O. H., Roseborough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Mabrey, S., and Sturtevant, J. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 3862.
op den Kamp, J. A. F., Kauerz, M. Th., and van Deenen, L. L. M. (1975), *Biochim. Biophys. Acta 406,* 169.
Peterson, S. W., and Deamer, D. W. (1977), *Arch. Biochem, Biophys. 179,* 218.
Phillips, M. C., Hauser, H., and Paltauf, F. (1972), *Chem. Phys. Lipid 8,* 127.
Robinson, N. C., and Capaldi, R. A. (1977), *Biochemistry 16,* 375.
Rouser, G., and Fleischer, S. (1967), *Methods Enzymol. 10,* 385.
Schneider, D. L., Kagawa, Y., and Racker, E. (1972), *J. Biol. Chem. 247,* 4074.
Williams, J. N. (1964), *Arch. Biochem. Biophys. 107,* 537.
Wrigglesworth, J. M., and Nicholls, P. (1975), *Biochem. Soc. Trans. 3,* 168.
Yonetani, T. (1961), *J. Biol. Chem. 236,* 1680.
Yu, C., Yu, L., and King, T. E. (1975), *J. Biol. Chem. 250,* 1383.

Amino Acid Dependent and Independent Insulin Stimulation of Cartilage Metabolism†

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ABSTRACT: The effects of insulin on embryonic chicken cartilage in organ culture and the dependence of these effects on essential amino acids have been studied. In the presence of all essential amino acids, insulin: (1) increases 2-deoxy-D-glucose and α-aminoisobutyric acid uptake; (2) increases [5-^{3}H]uridine flux into uridine metabolites and the intracellular UTP pool; (3) expands the size of the intracellular UTP pool; (4) does not change the specific activity of the UTP pool; and (5) stimulates RNA, proteoglycan, and total protein synthesis. In lysine (or other essential amino acid)-deficient medium, the effects of insulin are different. While insulin stimulates incorporation of [5-^{3}H]uridine into RNA, it does so by increasing the specific activity of the UTP pool without increasing RNA synthesis. Insulin stimulates 2-deoxy-D-glucose and α-aminoisobutyric acid uptake but no longer stimulates proteoglycan, total protein, or RNA synthesis or expands the size of the UTP pool. These data indicate that there are amino acid dependent and independent effects of insulin on cartilage. Transport processes are amino acid independent, while synthetic processes are amino acid dependent.

Insulin actions on cartilage are of considerable interest because of the relationship between insulin and a variety of insulin-like serum factors (somatomedins and NSILAs)[1] that stimulate anabolic processes in cartilage (Lebovitz and Eisenbarth, 1975; Van Wyk et al., 1975). High concentrations of insulin stimulate the incorporation of radiolabeled precursers into DNA, RNA, and total proteins of costal cartilage removed from hypophysectomized rats (Salmon et al., 1968) and $^{35}SO_4$ incorporation into the proteins of embryonic chicken cartilage (Hall and Uthne, 1971). Some evidence suggests that insulin and the somatomedins interact with the same receptor on embryonic chicken cartilage (Hintz et al., 1972).

The dependence of RNA synthesis upon simultaneous protein synthesis is a significant regulatory mechanism in both prokaryotes and eukaryotes. Deprivation of an essential amino acid during incubation of eukaryotic cells markedly reduces protein synthesis by interfering with initiation of translation and causes stringent control of RNA synthesis (Juergen and Pogo, 1974).

The present study was undertaken to characterize the effects of insulin on transport processes and macromolecule synthesis in embryonic chicken chondrocytes in organ culture and to determine whether amino acid deprivation alters these effects.

Materials and Methods

Incubation Techniques. The incubation procedures were similar in all studies. Pelvic cartilages from 11 or 12 day chicken embryos were removed, cleaned, weighed, and distributed randomly into flasks containing 2 mL of incubation medium. The complete incubation medium was pH 7.45 50 mM Tris buffer which contained all essential amino acids, electrolytes, glucose, and penicillin as previously described (Drezner et al., 1975). In some experiments the essential amino acids lysine, arginine, phenylalanine, or valine were omitted from the incubation medium. All incubations were carried out in an atmosphere of 95% O_2–5% CO_2 (v/v) in a gyrorotary shaker bath at 37 °C.

Sulfate Incorporation into Cartilage Proteoglycans. Carrier-free $Na_2{}^{35}SO_4$ (1.0 μCi) was added to each flask and the cartilages were incubated for 12 to 14 h. At the conclusion of the incubation, the cartilages were boiled for 8 min and soaked for an additional 2 h in 4 mL of saturated Na_2SO_4. The cartilages were rinsed, digested in Pirie's reagent, and $^{35}SO_4$ incorporation determined (Jeffay et al., 1960).

Uridine Incorporation into Cartilage RNA. [5-^{3}H]Uridine

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[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; AIB, α-aminoisobutyric acid; NSILAs, nonsuppressible insulin-like activities.